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Preface

The number of human gene therapy clinical trials employing adenoviral vectors is expanding at an unprecedented rate. This increased use of adenoviral vectors has both fueled, and has in turn been fueled by, a parallel explosion in our knowledge of the biology of adenoviruses and their vectors. Moreover, there have been concomitant advances in associated technologies. It is therefore timely to review both basic and applied aspects of adenoviruses and adenoviral vectors in a single, comprehensive, multi-author volume.

The first few chapters focus on basic virology—the structure of adenoviruses and the biology of adenoviral infection and replication. Advances in our understanding of the parental virus have facilitated the rational design of adenoviral vectors for gene therapy. The construction, propagation, and purification of adenoviral vectors have benefited from a number of technological advances, as discussed in the next series of chapters.

In addition to the underlying biological features that favor their use for gene therapy, it is recognized that adenoviral vectors have suffered from a number of limitations. These limitations, together with strategies by which they might be overcome, are considered. Thus, separate contributions discuss approaches to target adenoviral vectors to specific cell types, as well as strategies to circumvent the host immune response. Replication-competent adenoviruses, which are increasingly being used as oncolytic agents for the treatment of cancer, are described. Other vectorological advances covered in this section include high capacity adenoviral vectors, xenogenic adenoviral vectors, and

hybrid adenoviral vectors, which combine the advantages of adenoviral vectors with beneficial features derived from other vector systems.

The next group of contributors describes the use of adenoviral vectors in animal models of human disease—cancer, genetic disease, and acquired diseases. These chapters discuss the lessons that have been learned from these model systems and their implications for the employment of adenoviral vectors in humans. Specific approval from the regulatory bodies must be obtained prior to the implementation of human trials, as detailed in the following chapter. Finally, the recognition of the need for noninvasive methods to monitor adenovirus-mediated gene transfer in human patients has predicated the development of novel imaging technologies.

In the aggregate, we have provided herein a comprehensive overview of adenoviral technology, both classical and novel. This update should provide an entrée into the field for the neophyte as well as a reference source for the practitioner.

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CHAPTER 1

Adenovirus Structure

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I. Introduction

The world got its first look at the icosahedral symmetry of adenovirus (Ad) in 1959 with published electron micrographs of negatively stained Ad5 [1]. In this classic work, Horne *et al.* were able to resolve the basic subunits and thus determine that the adenovirus capsid is composed of 252 subunits, 12 of which have five neighbors (pentons) and 240 of which have six neighbors (hexons). A few years later, Valentine and Pereira [2] published a striking electron micrograph of a single Ad5 particle, revealing the long protruding fibers that are characteristic of adenovirus. In analogy to what was known at the time about the role of phage tails, the authors correctly deduced that the adenovirus fiber might be involved in adsorption to the host cell surface. Since then electron microscopy has continued to play a role in our understanding of the structure of adenovirus and its interaction with αv integrins [3, 4]. In recent years X-ray crystallography has contributed atomic structures for the capsid proteins hexon [5, 6], fiber knob [7–9], and shaft [10], the fiber knob complexed with a receptor domain[11], and the virally encoded protease [12].

Our growing knowledge of adenovirus structure has already contributed to the field of vector design [13]. For example, initial attempts at modifying the C-terminal end of the fiber protein gave suboptimal results for gene delivery [14], while subsequent efforts utilizing knowledge of the fiber knob structure produced vectors with enhanced performance [15, 16]. Strategies for improving adenoviral vectors by making genetic modifications to capsid proteins and by designing hybrid vectors are discussed in later chapters. An understanding of adenovirus structure will be essential for these endeavors.

ADENOVIRAL VECTORS FOR GENE THERAPY

II. Molecular Composition

The approximately 50 known human adenovirus serotypes are classified into six subgroups, A–F, and all share a similar structure and genomic organization [17]. Adenovirus is an nonenveloped virus of ~150 MDa, composed of multiple copies of 11 different structural proteins, 7 of which form the icosahedral capsid (II, III, IIIa, IV, VI, VIII, IX) and 4 of which are packaged with the linear double-stranded DNA in the core of the particle (V, VII, mu, and terminal protein). For clarification of the nomenclature, note that most of the Ad polypeptides were named based on their position on a polyacrylamide gel. The highest molecular mass protein band turned out to be a complex of components, and consequently there is no polypeptide I in adenovirus. Also note that polypeptide IIIa was not originally resolved as a separate band; however, it is a distinct structural protein. In addition to the capsid and core components, approximately 10 copies of the adenovirus protease are incorporated into each virion [18].

For many icosahedral viruses, determination of a crystal structure has resolved outstanding molecular composition issues. In the case of adenovirus, there is as yet no atomic structure for the intact virion. In 1985, a preliminary X-ray crystallographic density map of the Ad2 hexon showed that the capsomer was a trimer of polypeptide II with a triangular top and a pseudohexagonal base [19]. Together with the early electron microscopy of the intact virion [1], the crystallographically observed hexon symmetry fixed the copy number of polypeptide II at 720 in the Ad virion. The stoichiometry of eight other structural proteins (III, IIIa, IV, V, VI, VII, VIII, and IX) was inferred by careful sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analyses of radiolabeled virions ([^{35}S] methionine) using hexon as the standard [20]. After adenovirus protease cleavage sites were found in the sequences of polypeptides IIIa, VI, and VIII [18], changing the number of methionines in the mature proteins, their predicted copy numbers were revised [21].

The molecular stoichiometry indicated that there is symmetry mismatch in the Ad penton [20]. Symmetry mismatches are not unheard of in icosahedral viruses. One example is SV40, which has pentamers of VP1 at sites of both local fivefold and sixfold symmetry in the crystal structure [22]. The conformationally flexible C-termini of VP1 are able to adapt to the position of the pentamer within the SV40 capsid. In the case of adenovirus, three copies of polypeptide IV form the fiber and five copies of polypeptide III form the penton base. The fiber and penton base together compose the penton, which sits at the fivefold symmetry axes of the icosahedral capsid. Microheterogeneity in the Ad penton base has been offered as an explanation for the symmetry mismatch [20].

More recently a reversed-phase high-performance liquid chromatographic (RP-HPLC) assay was developed in order to more fully characterize the Ad5 proteome [23]. N-terminal protein sequencing and matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectroscopy were used to identify each component protein contributing at least 2% to the total protein mass of the virus. Peaks for the fiber protein, which contributes only 1.8% of the total protein mass, as well as the terminal protein and the protease, were not identified. The mass of the remaining structural proteins was determined to within ±0.1%. Their copy numbers were estimated using hexon as the standard and with the exception of the copy number for the core polypeptide VII, which was significantly reduced, the new copy numbers are in good agreement with the SDS-PAGE numbers [20, 21]. The precise mass measurements confirmed the proteolytic processing of polypeptides IIIa, VI, VII, VIII, and mu and interestingly cleaved precursor products of all but polypeptide IIIa were found to be present in the purified Ad5 virions.

III. Structure of the Intact Virion

In 1991 the first structure of an intact Ad particle was determined by cryo-electron microscopy (cryo-EM) and three-dimensional image reconstruction methods [24]. The technique of cryo-EM was developed in the mid-1980s by Dubochet and colleagues [25] for imaging viruses and other macromolecular assemblies in a native-like, frozen–hydrated state. Since then it has proven to be a powerful approach for studying icosahedral viruses and it has been applied to numerous members of over 20 different viral families [26]. The method involves placing a droplet of concentrated virus on an EM grid layered with a holey carbon film (carbon with holes 1–10 μm in diameter), blotting with a piece of filter paper to leave a thin (~1000 Å) layer of water and sample stretched across the holes of the grid, and then plunge freezing into a cryogen such as ethane slush chilled by liquid nitrogen. This rapid freezing causes formation of vitreous (amorphous) ice rather than crystalline ice. Formation of normal crystalline (hexagonal) ice would be harmful to the biological sample because of its expansion relative to liquid water. After cryo-freezing the sample grids are maintained at liquid nitrogen temperature to preserve the vitreous state. Transmission electron micrographs are collected using a low dose of electrons to avoid significant radiation damage to the frozen, unstained sample. The real power of the technique lies in the fact that many particle images can be computationally combined to generate a three-dimensional density map [26–28].

In the early 35-Å-resolution reconstruction of Ad2, the features of the icosahedral protein capsid were clear and its dimensions without the fiber were measured as 914, 884, and 870 Å along the five-, two-, and threefold symmetry

axes, respectively [24]. The reconstruction showed the trimeric shape of the hexon, the pentameric shape of the penton base, and a short portion (~88 Å) of the fiber shaft. The full-length fiber, ~300 Å long including the knob at the distal end, was occasionally visible in cryo-electron micrographs. Comparison of these particle images with projections of modeled full-length fibers indicated that the knobs were not positioned as would be expected if the fibers were straight. This suggested that the Ad fibers in the intact Ad2 particle are bent or flexible. Electron micrographs of negatively stained Ad2 fibers show a bend close to the N-terminal end, which binds the penton base [29]. A pseudo repeat of 15 residues was noted in the central section of the Ad2 fiber sequence [30] and later analysis of the fiber sequences from a variety of Ad serotypes revealed a range of 6–23 pseudorepeats in the shaft [31]. A long, nonconsensus repeat at motif 3 was proposed to induce a bend in the shaft of many Ad serotypes [31]. The idea that the fiber is bent for many Ad serotypes is consistent with both negative-stain electron micrographs [29] and the fact that only a short rigid portion of the Ad2 fiber shaft was reconstructed [24].

A more recent cryo-EM reconstruction of Ad2 [3] is shown in Fig. 1 (see color insert) with modeled full-length fibers. Reconstructions have now been published of Ad2 at 17-Å resolution [32], Ad5 [33], Ad12 [3], Ad2 complexed with a Fab fragment from a monoclonal antibody directed against the integrin-binding region of the penton base [34], both Ad2 and Ad12 complexed with a soluble form of αvβ5 integrin, the internalization receptor for many Ad serotypes [3], and a fiberless Ad5 vector [33]. The capsids of these Ad serotypes appear quite similar, with only subtle differences observed in the size and flexibility of the surface protrusions of the hexon and penton base [3].

IV. Structure of the Capsid Components

A. Hexon, Polypeptide II

Crystal structures have been published for hexon of serotype Ad2 [5] and Ad5 [6], two members of subgroup C. The sequences of these hexons (967 amino acids for Ad2, 951 for Ad5) are closely related with 86% amino acid identity. Both structures show that the monomer has two eight-stranded β-barrels at the base and long loops that intertwine in the trimer to form a triangularly shaped top (Fig. 2). The high degree of interlocking observed between the monomers might explain why an adenovirus-encoded 100-kDa protein is required for trimer assembly [35]. In the trimer the six β-barrels, two from each monomer, form a ring with pseudohexagonal symmetry that allows for close packing with six neighboring capsomers in the icosahedral capsid. Regions of the electron density for the Ad2 hexon, refined to 2.9-Å resolution,

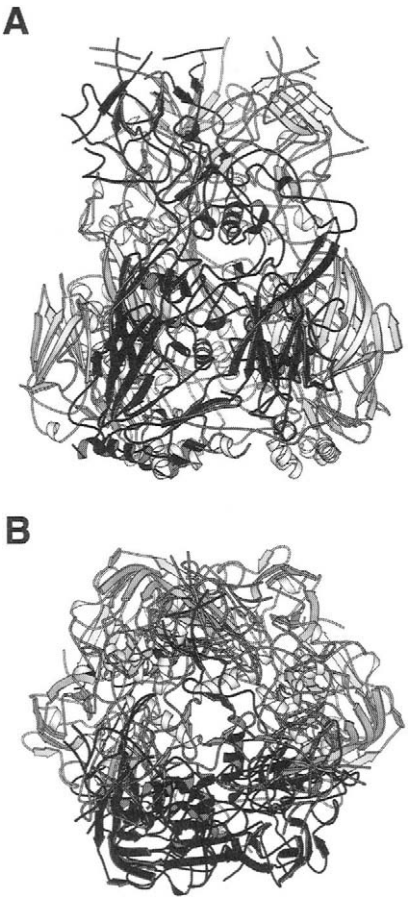

Figure 2 The crystallographic structure of the Ad5 hexon trimer [6] with one monomer shown in black (PDB ID: 1RUX [90]). (A) A side view showing the two β-barrels near the bottom of the black monomer. Note that there are several gaps in the atomic model at the top of the molecule. (B) A top view revealing the pseudohexagonal shape of the bottom of the trimer. This figure was generated with the program MOLSCRIPT [91].

were unclear and gaps were left in the atomic model. During refinement of the Ad5 hexon to 2.5-Å resolution, significant changes were made in the atomic model involving reassignment of greater than 25% of the sequence. In light of this result and the high homology between the two hexons, it has been suggested that the Ad2 atomic model should be revised [6]. The most significant change was a shift of the first 130 amino acids leaving a gap of just four residues at the N-terminus of the Ad5 structure vs an N-terminal gap of 43 residues in the initial Ad2 model.

Revision of the hexon structure has cleared up several mysteries in the literature. First, a comprehensive comparison of hexon sequences from serotypes in all six human subgroups as well as bovine and mouse serotypes found seven hypervariable regions [36]. Alignment with the Ad2 hexon structure indicated that five regions were in exposed loops as expected, while two regions were buried. The Ad5 hexon structure now shows all seven hypervariable loops exposed on the top of the molecule [6]. Second, trypsin cleavage sites were identified at Arg-142 and Arg-165 in Ad2 [37] and these are now located in the exposed top of the hexon molecule [6]. Similarly a pH-dependent cleavage site for the proteolytic enzyme dispase was found somewhere between residues 135 and 150 of the Ad2 hexon [38]. In the original Ad2 hexon structure this stretch was buried and far from the top of the molecule. In the Ad5 hexon structure this region is likely exposed on the molecule, although it is in an unmodeled region of the structure [6].

The Ad5 structure places a previously buried highly acidic stretch of residues, 133–161 for Ad2, at the top of the molecule and accessible to solvent [6]. The acidic region is also found in the Ad5 hexon sequence, but not in those of Ad9, Ad12, or Ad37. In the Ad8 hexon sequence there is a longer, slightly basic insertion at this position [36]. It has been suggested that the acidic stretch may create an electrostatic repulsion between the exterior of the Ad2 or Ad5 virion and acidic cell surface proteins [39]. Others have proposed that perhaps the acidic region plays a role in tissue tropism for the subgroup C viruses [40].

B. Penton Base, Polypeptide III

In the absence of a crystal structure for the penton base, structural information on this protein comes mainly from cryo-EM reconstructions of the dodecahedron formed by Ad3 pentons [41] and intact Ad virions of various serotypes [3, 32–34]. Alignment of the known penton base sequences from subgroups A, B, C, and E shows high homology throughout the protein except for a central variable length region that contains the nearly always conserved Arg-Gly-Asp (RGD) sequence, residues 340–342 for Ad2 [4,42]. The Ad2 and Ad5 penton bases (571 residues each) have among the longest variable RGD regions [4,43,44]. The RGD sequence, utilized for interaction with cellular αv integrins [4,45], is lacking from the enteric Ad40 and Ad41 serotypes of subgroup F [46]. Presumably these two serotypes don't interact with αv integrins during viral cell entry.

Site-directed mutagenesis of the Ad2 penton base has indicated particular residues that are important for various functions including pentamerization and stable fiber-penton base interaction [47]. While recombinantly expressed Ad2 penton base is known to self-assemble into homo-pentamers, two mutations in the N-terminal portion of penton base, R254E and W119 H, and several

in the C-terminal region, W439 H, Y553F, and K556E, reduce or abolish pentamerization. Several mutations, C432S, W439 H, RRR(547-549)EQQ, and K556E, completely abolish the association of fiber with penton base. Other mutations throughout the penton base (W119 H, W165 H, R245E, R340E, and W406H) reduce the penton base interaction with fiber. Screening with a filamentous phage-display library indicated that the Ad2 penton base sequence RLSNLLG, residues 254–260, is important for fiber binding [48]. One of these residues, R245E, was also identified by mutagenesis, but clearly residues throughout the penton base play a role in fiber association.

Electron micrographs of negatively stained penton base, fiber, and the penton complex indicate that the isolated fiber is ~40 Å longer than the fiber that extends from the penton base [29]. It is not clear whether or not the N-terminal end of the fiber inserts into a central cavity of the penton base or merely attaches to the outer surface. Cryo-EM reconstructions of the Ad3 penton dodecahedron both with and without the fiber reveal a subtle shift of the RGD protrusions outward by ~15 Å when the fiber is present, but no open hole in the fiberless complex [41]. A similar observation was made for an Ad5 vector both with and without the fiber [33]. These results indicate a subtle conformational change of the penton base during fiber binding and possible expansion of the penton base to allow insertion of the N-terminal end of the fiber.

Numerous Ad serotypes are known to utilize the RGD residues for infection via interaction with cell surface αv integrins [4,45]. The position of RGD on the penton base has been determined by a cryo-EM reconstruction of Ad2 complexed with Fab fragments of the DAV-1 monoclonal antibody [34]. Curiously, Fab fragments of DAV-1 are capable of neutralizing Ad2 infection, but the biologically relevant DAV-1 IgG molecules are not. MALDI mass spectroscopy identified the DAV-1 binding site as containing a linear epitope of nine residues including RGD [34]. The cryo-EM structure of the Ad2/DAV-1 complex localized the RGD residues to the top of five 22-Å protrusions on the penton base. The observation of weak density at the top of the protrusions in the control uncomplexed Ad2 reconstruction, as well as the diffuse nature of the bound Fab density, indicated that the RGD residues are in a highly mobile surface loop [34]. Perhaps the mobility of the RGD loops, as well as their relatively close spacing around the central protruding fiber (Fig. 3, see color insert), contributes to the ability of the virus to evade antibody neutralization at this exposed receptor binding site [34]. The combined steric hindrance of the fiber and a few bulky IgG molecules bound to flexible epitopes effectively shields the remaining RGD sites from saturation by IgG, while the less bulky Fab fragments can bind to all five protrusions.

Prior to complexing adenovirus with a soluble form of αvβ5 integrin, a comparison between the known penton base sequences indicated that Ad12 has the conserved RGD residues within a much shorter variable region than

Ad2 [3]. Cryo-EM reconstructions of both Ad2 and Ad12 complexed with αvβ5 revealed better defined integrin density in the Ad12 reconstruction, suggestive of a less mobile RGD loop for Ad12. A careful analysis of the penton base density in the control Ad2 and Ad12 reconstructions also supported this idea with a smaller region of weak, diffuse density over the Ad12 protrusions than the Ad2 protrusions (Fig. 4, see color insert). The spacing of the RGD protrusions on the penton base is thought to be important for the clustering of integrin molecules, thus triggering signaling events required for virus internalization [3]. Notably a monomeric RGD peptide (50-mer) derived from the penton base is unable to activate p72 Syk kinase or promote adhesion of B lymphoblastoid cells, two demonstrated functions of the pentameric penton base [49]. Structural support for the importance of the RGD spacing comes from the crystal structure of foot-and-mouth disease virus, which also utilizes αv integrins for cell entry [50, 51]. The RGD loops of both the Ad penton base and foot-and-mouth disease virus have the same spacing, 60 Å, around the fivefold symmetry axes despite these two viruses being structurally and evolutionarily unrelated [3].

C. Fiber, Polypeptide IV

All human Ad serotypes have 12 fibers, one protruding from each penton base at the vertices of the icosahedral capsid. The length of the fiber protein varies from 320 to 587 residues and the sequence can be broken down into three segments: an N-terminal tail, a central shaft of variable length, and the C-terminal domain, which forms the distal knob of the fiber [31]. Biopanning of a phage-library peptide library has shown that the conserved N-terminal motif (FNPVYP, residues 11–16 in Ad2) interacts with the penton base [48]. The knob of most, but not all, serotypes [52] has a high affinity for the cellular receptor known as Coxsackie and adenovirus receptor (CAR) [53, 54]. Although a single fiber gene is the norm, Ad40 and Ad41 have two fiber genes of different length[31,55]. Perhaps the expression of two different fibers enables the virus to interact with a wider array of cell receptors as the knobs are quite different [55] and only one fiber type binds CAR [11]. Although there are two fiber genes in Ad40 and Ad41, only one fiber is found per penton. Notably, avian adenoviruses have two fibers per penton and they may have evolved distinct cell-entry strategies [56–58].

The first atomic resolution information for the fiber was a crystal structure of the Ad5 knob domain (residues 386–581 of the intact fiber protein) [7, 8]. The structure revealed a trimer with an eight-stranded antiparallel β-sandwich in each monomer (Fig. 5). More recently, crystal structures have been published of the Ad2 knob [9], and the Ad12 knob both alone and complexed with the D1 domain of CAR [11]. The main differences between the knob structures are found in the N-terminal region and the loops. The Ad12 knob/CAR-D1

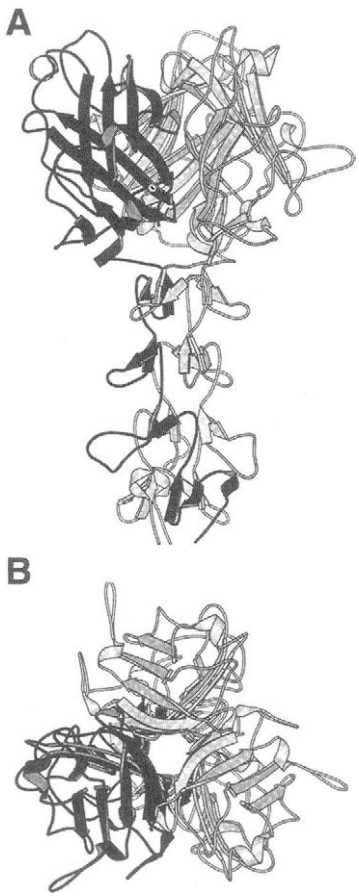

Figure 5 The crystallographic structure of the Ad2 fiber knob and a portion of the fiber shaft [10] (PDB ID: 1QIU [90]). The trimeric molecule is shown with one monomer in black and two in gray. (A) A side view oriented to show the eight-stranded β sandwich in the knob domain of the black monomer and four repeats of the triple β-spiral fold in the shaft. (B) A top view looking along the molecular threefold axis in the direction of the virus. This figure was generated with the program MOLSCRIPT [91].

complex reveals that the CAR binding site is on the side of the knob and involves primarily the AB-loop [11].

Two models, both high in β-strand content, were predicted for the fiber shaft [30, 59] before a crystal structure was published for a portion of the Ad2 shaft in 1999 [10]. The structure shows a novel triple β-spiral motif (Fig. 5) that is different from either model in that the β-strands lie more along the fiber axis. The hydrogen bonding pattern observed in the structure suggests that

the basic repeating structural motif should be redefined. Also a linker region was observed between the shaft and the knob, indicating that Ad2 has only 21 repeats, as opposed to 22 suggested by the earlier sequence analysis [30].

As noted in section III, fiber shafts of various serotypes appear by both negative stain electron microscopy [29] and cryo-EM [24] to be bent near the N-terminus, presumably in the region of the third fiber shaft repeat [31]. Some of the fibers with short shafts may, however, be relatively straight. The cryo-EM reconstruction of the Ad3 penton dodecahedron showed the full-length straight fiber including the knob extending 136 Å from the penton base [41].

D. Polypeptide IIIa

Polypeptide IIIa plays an important role in the assembly of adenovirus, as a temperature-sensitive mutation in polypeptide IIIa produces only empty capsids [60]. The full-length polypeptide IIIa, prior to proteolytic cleavage, is 585 residues for Ad2 [43]. A protease cleavage site was predicted after residue 570 [18] and the MALDI-TOF mass spectroscopic analysis of Ad5 confirms that this cleavage does occur [23]. According to both the SDS-PAGE analysis [20, 21] and the RP-HPLC analysis [23] there are approximately 60 copies of polypeptide IIIa in one adenovirus virion.

The position of polypeptide IIIa within the capsid has been tentatively assigned in a cryo-EM difference map [61]. The difference map was generated by positioning 240 copies of the crystallographic Ad2 hexon [5] within the cryo-EM reconstruction of Ad2 [24] and then subtracting the hexon density. The hexon positions in the calculated capsid, published in [21], were optimized for their agreement with the cryo-EM reconstruction rather than for optimum contacts between hexons and as such represent only a crude pseudoatomic model for the hexon portion of the capsid. Approximately 65% of the density assigned to polypeptide IIIa was observed on the external surface of the capsid and the other 35% on the inner surface [21,61]. Contradictory biochemical information indicated that polypeptide IIIa is exposed on both the inner and outer capsid surfaces and thus it had been suggested that this protein might span the capsid [62]. The external density assigned to polypeptide IIIa is clearly visible without difference mapping in the recent 17-Å resolution of Ad2 [32] (Fig. 6, see color insert). Two elongated density regions are observed along each of the 30 edges of the icosahedral capsid.

E. Polypeptide VI

The full-length precursor form of the Ad2 polypeptide VI has 250 residues [63], but 33 residues are cleaved by the protease from the N-terminus and 11 residues from the C-terminus [18, 23]. Interestingly the cleaved C-terminal peptide functions as a cofactor for the protease [64, 65]. Analysis of

the Ad2 cryo-EM difference map led to an assignment for polypeptide VI on the inner capsid surface [61]. Trimeric density regions were observed spanning the bottoms of the five hexons around each penton, often called the peripentonal hexons on the basis of their location in the capsid. The assigned positions are indicated on the outer surface of the 17 Å Ad2 reconstruction [32] (Fig. 7, see color insert). In order to account for ~360 copies of polypeptide VI in the virion [20, 21, 23], each trimeric density region was suggested to be a trimer of dimers [61]. In other words, six copies of polypeptide VI might form each observed trimeric density region. Five trimeric regions found in one vertex would contain 30 copies of polypeptide VI, and all 12 vertices would have a total of 360 copies. The observed volume of one trimeric region is too low to account for six copies of polypeptide VI, but a large portion of the protein may be loosely ordered and interacting with the viral core. It has been known for some time that polypeptide VI can bind nonspecifically to DNA [66] and thus the proposed location on the inner capsid surface seems logical.

F. Polypeptide VIII

The sequence of polypeptide VIII in both Ad2 [67, 68] and Ad5 [44] indicates an uncleaved precursor molecule of 227 amino acid residues. Protease cleavage sites are predicted for molecules of both serotypes following Gly-111, which implies a much smaller mature protein [18]. MALDI-TOF mass spectroscopy confirms this cleavage site for Ad5 and also indicates a second cleavage site after Ala-157, as the fragment from Gly-158 through the C-terminus is found in the virion [23]. Polypeptide VIII is present in roughly 127 copies per virion [20, 21], but little is known about its structure or its position within the virion other than the general observation that it is associated with hexons [69].

G. Polypeptide IX

Polypeptide IX is thought to help stabilize adenovirus as mutant virions lacking this protein are less stable than wild type [70]. In Ad2, polypeptide IX is 140 residues [71] and it is not cleaved by the viral protease. This capsid component can be isolated from both intact virions and from the viral dissociation product known as the group-of-nine hexons [69]. Scanning transmission electron microscopy (STEM) analysis of the group-of-nine hexons indicated that there are 12 copies of polypeptide IX arranged as four trimers within this capsid fragment [72]. The Ad2 cryo-EM difference map confirmed this arrangement and showed that polypeptide IX is on the outer surface of the capsid [61]. The locations of the polypeptide IX trimers are indicated on the 17-Å resolution Ad2 cryo-EM reconstruction [32] (Fig. 8, see color insert). The copy number for polypeptide IX has been measured as approximately

240 [20, 21, 23] and this is consistent with four trimers in each of the 20 triangular facets of the icosahedral capsid.

V. Core Structure

The first cryo-EM reconstruction of Ad2 showed that the DNA/protein core does not follow icosahedral symmetry throughout, although the outer surface of the core does interact with the capsid and may be partially ordered [24]. There is presently no atomic structure known for any of the core proteins (V, VII, mu, and terminal protein). Ad2 was the first serotype to be completely sequenced and its DNA genome has 35,937 base pairs [43]. The two 5′ ends of the DNA genome are covalently linked to the terminal protein [73] via Ser-562 [74]. Terminal protein (488 residues in Ad2) is the proteolytically cleaved form of the preterminal protein (653 amino acids in Ad2) [74–76]. It has been proposed that the terminal protein–DNA complex, present in the mature virion, serves as a template for early transcription and the first round of DNA replication, while the preterminal protein–DNA complex formed after DNA replication serves only for subsequent rounds of DNA replication [77].

In the Ad core the terminal protein–DNA complex is associated with ~160 copies of polypeptide V [20, 23], ~633 copies of polypeptide VII [23], and ~104 copies of late L2 mu, also known as polypeptide X [78]. Little is known about polypeptide V (369 amino acids in Ad2) [79]other than the fact that it is moderately basic [69]. The polypeptide VII precursor (198 amino acids in Ad2) [44]and mu precursor (79 amino acids in Ad2) [80]are both cleaved by the viral protease. Of the three core proteins that are noncovalently linked to the viral DNA, polypeptide VII is most tightly bound [81] and it is sometimes referred to as the major core protein since it contributes the most protein mass to the core.

VI. Adenovirus Protease

The adenovirus protease plays a role in maturation of the virus, cleaving six virion precursors (IIIa, VI, VII, VIII, mu, and terminal protein) [18]. Analysis of the temperature-sensitive mutant virion, *ts* 1 [82], indicates that the protease also plays a role during Ad cell entry [83, 84]. The observation that the cleavage products of polypeptides VI, VII, VIII, and mu are present in the mature Ad5 virion [23] is consistent with the idea that the adenovirus protease is incorporated inside of the viral capsid and that peptide cleavage takes place either on the inner surface of the capsid or in the core of the virion [18]. The cleaved C-terminal tail of polypeptide VI serves as a cofactor for the protease [64, 65] increasing its catalytic rate constant (k_{cat}) by 300-fold [85]. It has also been reported that viral DNA is a cofactor [64, 85]

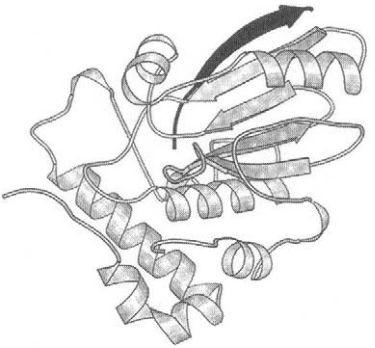

Figure 9 The crystallographic structure of the Ad2 protease (gray) with its 11-amino-acid cofactor (black), a proteolytic cleavage product of polypeptide VI [12] (PDB ID: 1AVP [90]). Note that the cofactor extends a β-sheet in the enzyme. This figure was generated with the program MOLSCRIPT [91].

although this is disputed in the literature. Other studies suggest that DNA may not be necessary for catalysis, but rather might enhance the interaction of protease and substrates *in vivo* [86]. Either way, the apoenzyme is relatively inactive and thus the cofactor(s) may help to control the activity of the enzyme so that the virion proteins are cleaved at the appropriate time during the viral life cycle.

A crystal structure has been determined for the protease of Ad2 (204 amino acids) complexed with its 11-amino-acid cofactor (Fig. 9) [12]. The structure reveals that the peptide cofactor becomes the sixth β-strand in a β-sheet and forms a disulfide bond and numerous hydrogen bonds with the protease. The hydrophobic pockets observed in the structure help to explain the known consensus sequences for cleavage, which are (M,L,I)XGX/G or (M,L,I)XGG/X where X is any residues and "/" indicates the cleavage site [87, 88]. The active site contains a Cys-His-Glu triplet and an oxyanion hole similar to papain and the Ad protease probably has a similar catalytic mechanism to papain. However, the fold as well as the order of the catalytic residues in the sequence is different in the two enzymes. The Ad protease is considered to be the first member of a fifth group of cysteine proteases [12].

VII. Summary

Adenovirus is a complex human virus whose structure still holds many mysteries. The synthesis of results from a diverse array of experimental techniques has led to our current level of understanding. MALDI-TOF mass spectroscopy [23] has confirmed the predicted protease cleavage sites [18] of

several structural proteins. The use of phage-display libraries has pinpointed the residues involved in the interaction between penton base and fiber [48], which was first observed by negative stain electron microscopy [2]. X-ray crystallography [6] and sequence analysis [36] together reveal the hypervariable regions of hexon at the top of the molecule where the most variation is tolerated. The early biochemical characterization of the component molecules [69] was advantageous for interpreting the first cryo-EM reconstruction [24] and difference map [61]. Identification of the CAR receptor [53, 54] and the crystal structure of a fiber knob complexed with one domain of CAR [11] have advanced our understanding of cell attachment. The finding that αv integrins are utilized as internalization receptors by many serotypes [89] led to the observations by cryo-EM that the penton base RGD protrusions are located appropriately to both evade antibody neutralization [34] and facilitate receptor clustering [3]. Clearly the more we learn about adenovirus structure, assembly, and cell entry, the better our position will be for designing the adenoviral vectors of the future.

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CHAPTER 2

Biology of Adenovirus Cell Entry

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I. Pathway of Adenovirus Cell Entry

Adenoviruses cause a significant number of acute respiratory, gastrointestinal, and ocular infections in man. While these infections are usually self-limiting they can result in significant morbidity and in immunocompromised individuals are capable of causing fatal disseminated infections [1]. Among the ~50 different adenovirus (Ad) serotypes, representing six different subgroups (A–F) [2, 3], the majority of information on the molecular basis of host cell interactions is derived from studies on the closely related types 2 and 5 (subgroup C) [4]. It is, therefore, not surprising that replication-defective forms of Ad5 are currently being used for most *in vitro* and *in vivo* gene delivery applications [5, 6]. Despite some reported successes, adenovirus-mediated gene delivery remains hampered due in large part to the host immune response to viral proteins [7, 8]. Increased knowledge of Ad structure [9, 10] and host cell interactions [11] may allow redesigning of viral vectors in order to avoid some of the major problems in this area.

Ad types 2/5 bind to cells via their fiber protein [12], which recognizes a 46-kDa cell receptor known as CAR (Coxsackie and adenovirus receptor) [13, 14]. However, this high-affinity receptor interaction is unable to promote efficient virus uptake into the host cell. Instead, secondary interactions of the virus penton base protein with $\alpha v\beta 3$ or $\alpha v\beta 5$ integrins facilitates virus internalization [15] (Fig. 1). Adenovirus particles enter cells via ~120 nM clathrin-coated pits and vesicles [16]. Hela cells expressing a mutant form of dynamin; a large GTPase associated with endosome formation, fail to support efficient virus uptake or infection, indicating that clathrin/receptor-mediated endocytosis is the primary pathway of Ad2/5 infection of host cells [17]. Adenovirus internalization also requires the participation of cell signaling

ADENOVIRAL VECTORS FOR GENE THERAPY

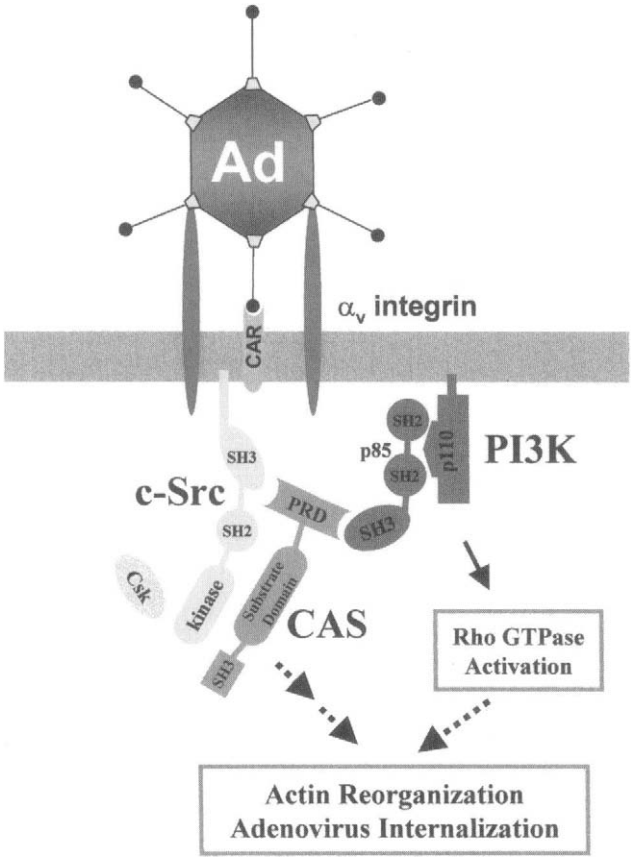

Figure 1 Schematic diagram of adenovirus cell entry events. Virus attachment is mediated by fiber protein (black) association with CAR. Subsequent interaction of the penton base (light gray) with αv integrins (dark gray) promotes Ad internalization. Integrin-mediated Ad internalization also requires the participation of several signaling molecules (c-Src, p130CAS, P13K, and Rho GTPAses) that mediate actin polymerization.

molecules including phosphatidylinositol 3-OH kinase [18], a lipid kinase that regulates a number of important host cell functions. These signaling proteins form a complex that promotes the polymerization of cortical actin filaments needed to efficiently internalize virus particles [16, 19]. Similar processes are used for cell invasion by a number of pathogenic bacteria [20, 21]. While the role of actin in virus or bacteria cell entry has not been clearly defined, polymerized actin filaments may serve as a scaffold to prolong the half-life of signaling complexes or they may provide the mechanical force necessary for the formation of endocytic vacuoles [22–24].

An important step of Ad entry postinternalization involves disruption of the early endosome allowing escape of virions into the cytoplasm prior to degradation by lysosomal proteases [25–27]. As is the case for many nonenveloped viruses, the precise mechanisms involved in Ad-mediated endosome penetration remain poorly defined. The majority of studies indicate that exposure of the virus to mildly acidic conditions (~pH 6.0) are sufficient to initiate the loss of key virus coat proteins as well as activate the viral encoded cysteine protease. However, there is not complete agreement on the requirement for a proton gradient in the early endosome to initiate its disruption [28]. Following endosome disruption, adenovirus particles are rapidly (30–60 min) translocated from the cytoplasm to the nucleus. Transmission electron micrographs obtained by several investigators have shown that viral capsids are docked at the nuclear pore complex [29, 30]. Current information indicates that virus association with microtubules [31] may play a key role in nuclear transport. Biochemical analyses have indicated that the majority of the viral capsid remains in the cytoplasm during transport of the viral genome into the nucleus [32]. This latter process appears to require the major host cell factors involved in nuclear import, the heat-shock p70 protein (Hsp70) and perhaps other cellular factors [33]. Viral gene expression and/or viral replication takes place once nuclear transport has occurred and ultimately results in the generation of transgene products in the case of viral vectors or in progeny virions in the case of wild-type particles. As is the case with many human pathogens, important questions remain to be answered regarding the precise mechanisms involved in each step of adenovirus cell entry.

II. Cell Receptors Involved in Attachment

A. CAR

Studies carried out by Lonberg-Holm *et al.* [34] first demonstrated that adenovirus and Coxsackie B viruses share the same receptor. A function-blocking monoclonal antibody was subsequently raised against the adenovirus receptor [35]; however, it was only recently that this antibody was used to identify the attachment receptor, CAR [13]. A murine homolog (MCAR) of human CAR (HCAR) is also capable of serving as an Ad attachment receptor [14]. The mechanisms by which CAR expression is regulated in different cell types as well its role in normal host cell functions have not yet been determined. The gene encoding HCAR was recently localized to the short arm of chromosome 21 [36], a finding that may provide further clues to the function and/or regulation of this receptor. The extracellular domain of CAR contains two Ig-like domains but only the most N-terminal domain is necessary for virus interaction [37]. HCAR is anchored in the cell membrane

by a single transmembrane domain followed by a relatively long cytoplasmic tail. Previous studies have indicated that only the extracellular domain of CAR is required for adenovirus-mediated gene delivery since recombinant forms of CAR lacking the cytoplasmic tail are fully capable of supporting virus infection [38]. These studies indicate that signaling events are probably not involved in CAR-mediated virus attachment.

Several lines of evidence indicate that CAR is a major determinant of Ad infection *in vivo*. For example, CAR expression is particularly high in cardiac tissue [14] and this correlates with efficient Ad-transgene delivery to the heart *in vivo* [39, 40]. In contrast, CAR expression is low or absent in primary human fibroblast [41] as well as most hematopoietic cells [42, 43] and these cell types are also difficult to infect with Ad5-based vectors. CAR expression is also limited to the basolateral surface of ciliated airway epithelial cells [44] and this has hampered efficient Ad-mediated gene delivery to the apical surface of these cells [45, 46]. Other investigators have reported that certain cell culturing conditions may also alter CAR expression and, thereby, influence Ad-mediated gene delivery [47]. The recent generation of transgenic mice expressing high levels of HCAR on peripheral blood lymphocytes has allowed efficient transduction of these cells by Ad5 vectors [48].

Ad binding to CAR is mediated by a high-affinity interaction with the fiber knob domain (K_d ~1 nM). There are approximately 30–50,000 CAR molecules per epithelial cell depending upon the tissue type [15]. Recent high-resolution structure studies and mutagenesis experiments have shed considerable light on the molecular basis for fiber-CAR association. Bewley and coworkers have solved the crystal structure of the Ad12 fiber knob domain in a complex with the first Ig-domain of CAR [49]. The cocrystal structure revealed that CAR interacts with the lateral surface of the fiber knob rather than on top of the fiber as had been predicted in earlier structure studies of the Ad5 fiber [50]. The CAR binding sites, which are composed of multiple regions on extended loop structures, are situated at the interface between individual fiber monomers. As many as three CAR molecules could bind to each fiber knob domain; however, this has not been formally demonstrated. Roelvink and colleagues reported that highly conserved amino acid residues located in the AB loop of the fiber knob domain of adenovirus types are involved in CAR binding as well as in the cocrystal contacts [49]. In contrast, divergent sequences are present in the same region of Ad types (i.e., subgroup B) which do not use CAR [51, 52]. They also showed that site-specific mutations in the AB loop significantly reduced virus binding and infection.

The identification of the precise regions in the fiber involved in CAR association has provided an opportunity to generate novel Ad vectors in which the CAR binding sites have been deleted and new receptor binding epitopes inserted (i.e. HI loop). Roelvink *et al.* have demonstrated the feasibility of this approach by redirecting an Ad5 vector containing a CAR-binding mutation to

a novel host cell molecule [52]. Thus, the development of new Ad vectors with increased host cell specificity may be on the horizon.

B. Other Adenovirus Receptors

Although CAR represents the major host cell receptor for Ad binding and infection, recent studies have suggested that other host cell molecules may also serve as attachment receptors. The α2 domain of MHC class I molecules has been reported to serve as a receptor for Ad5 particles based on competition experiments with phage-display peptides [53]. At the present time, these findings have yet to be confirmed by other investigators and thus it remains uncertain as to whether MHC class 1 molecules are specific Ad receptors. Recently Dechecchi *et al.* provided data suggesting that heparin sulfate proteoglycans (HSPGs) may also promote cell attachment of subgroup C (Ad2/5) but not subgroup B (Ad3) virus particles [54]. These findings suggest that HSPG may work in concert with CAR to facilitate high-affinity subgroup C virus binding to cells. These investigators also suggested that heparin sulfate proteoglycan interactions may occur via a site(s) located in the fiber shaft rather in the knob domain.

Belin and Boulanger have also analyzed host cell proteins capable of interacting with virus particles by cross-linked Ad2 to Hela cells. They showed that cross-linked virus was bound to three major host cell proteins with molecular weights of 130, 60, and 44 kDa [55]. They concluded that the 130-kDa protein was a β1 integrin subunit; however, they did not identify the lower molecular weight proteins. Based on its apparent mobility on SDS gels, the 44-kDa protein likely is CAR.

Ad types belonging to subgroup B that lack the conserved CAR binding residues noted above are very likely to use alternative cell receptors; however, these molecules have yet to be fully characterized. For example, serotypes 3 and 7 have been shown to bind to cells in a CAR-independent manner since the fiber proteins from these types fail to compete Ad5 fiber binding to cells [56]. While the receptor for subgroup B adenoviruses have not been identified, a partial characterization of a candidate receptor has been reported [57].

Additional investigations have indicated that Ad serotypes belonging to other subgroups may also use distinct cell receptors for virus attachment. Using virus protein blot assays, Roelvink *et al.* demonstrated that Ad serotypes belonging to subgroups A, C, D, E, and F were capable of binding to CAR [51]; however, these investigators did not establish that different virus types were actually capable of associating with CAR on intact cells. This distinction may be important given the fact that there are structural differences in the fiber proteins of different Ad serotypes. For example, adenoviruses from subgroup B and D fibers have relatively short and inflexible fiber shaft domains. These structural features could restrict interaction with CAR on the lateral surface

of a short-shafted fiber. In support of this, Shayakhmetov and Lieber found that truncated Ad5 fiber molecules have reduced binding capacity [58]. Huang *et al.* previously showed that Ad37 (a subgroup D serotype) contains a short-shafted fiber protein displaying the conserved CAR binding residues in its AB loop, but fails to efficiently infect CAR-expressing epithelial cells. Instead, a critical lysine residue at position 240 in the CD loop of the Ad37 fiber knob, mediates association with a cell receptor, expressed on conjunctiva epithelial cells [59]. Arnberg and coworkers also reported that Ad37 does not use CAR but instead recognize sialic acid residues present on one or more unidentified cell membrane proteins [60]. In recent studies, Wu *et al.* discovered that a 60-kDa protein expressed on diverse cell types is recognized by Ad37 particles and that this association is dependent upon sialic acid [61]. These authors also found that a 50-kDa membrane protein that is preferentially expressed on conjunctiva and supports Ad37 binding in a sialic acid-independent manner. They concluded that the 50-kDa putative receptor represents a portal of entry for pathogenic strains of adenovirus that are associated with severe ocular infections. Further biochemical and molecular biological studies are needed to identify different Ad receptors and determine their precise role in tissue tropism and disease.

Despite the fact that alternative Ad receptors have yet to be identified, new viral vectors with altered cell tropism have been generated. For example, several investigators have replaced (pseudotyped) the fiber protein in a first-generation Ad5 vector with an Ad3 or Ad7 fiber [56, 62–64]. The Ad3 pseudotyped vectors were shown to improve gene delivery to several different cell types. Thus, human lymphocytes which are very poorly transduced by Ad5 vectors supported substantially higher levels of infection with vectors equipped with the Ad3 fiber [64], presumably because of higher level of expression of the Ad3 receptor on these cells compared to CAR. Chillon et al also showed that an Ad5 vector pseudotyped with an Ad17 (subgroup D) fiber showed enhanced gene delivery to neuronal cells [65] while vectors retargeted with an Ad35 fiber improve gene delivery to stem cells [66]. It is likely that as new Ad receptors are identified, further knowledge of their tissue expression and structure should lead to improved modifications of standard E1Δ - Ad5 vectors in order to increase host cell specificity.

III. Adenovirus Internalization Receptors

A. Role of αv Integrins as Coreceptors

In studies conducted over 40 years ago, Pereira [67] and Everitt *et al.* [68] described a soluble toxic factor produced during adenovirus infection that caused cell rounding. This toxic factor was later identified as the penton base

protein [69]. Wickham *et al.* subsequently demonstrated that the penton base protein was not actually toxic, although it was capable of inducing epithelial cell detachment from plastic tissue culture surfaces. Cell detachment is due to the presence of an integrin-binding motif (RGD) in the penton base [70] that is able to compete for vitronectin, an extracellular matrix protein. In further studies, Wickham showed that penton base association with the vitronectin binding integrins αvβ3 and αvβ5 promotes adenovirus internalization rather than virus attachment [15]. While the overall contribution of αv integrins in adenovirus infection *in vivo* has not been firmly established, several lines of evidence suggest that integrins play a significant role. The penton base proteins of most adenoviruses representing different subgroups contain a conserved RGD motif and these viruses also use αv integrins for infection [71, 72]. Interestingly, adenoviruses belonging to subgroup F (types 40, 41) lack an RGD motif and show delayed uptake into cells [73]. Bai *et al.* also showed that mutations in the penton base RGD motif reduce the kinetics of Ad2 infection *in vitro* [74]. Huang *et al.* demonstrated that human B lymphocytes lack αv integrins and are not susceptible to infection with Ad5 vectors [43]. In contrast, transformation of B cells with Epstein–Barr virus upregulates αv integrin expression and allows infection with Ad5-based vectors. Von Seggern *et al.* has produced a fiberless adenovirus vector that fails to bind to CAR [75]. These particles are significantly less infectious that wildtype Ad particles; however, they can infect human monocytic cells in an integrin-dependent manner.

Recently, mice genetically deficient (knockouts) in cell integrins have been generated that may allow further investigation of the role of αv integrins in adenovirus infection *in vivo*. Bader and coworkers described the generation of mice genetically deficient in the αv integrin subunit which, therefore, lack both αvβ3 and αvβ5 [76]. Unfortunately, the majority of these animals die early during development, thus precluding analyses of adenovirus infection. Huang *et al.* have reported the generation of β5-integrin-deficient mice and fortunately these animals do not show enhanced developmental lethality [77]. Interestingly, β5-deficient mice did not show decreased susceptibility to adenovirus infection suggesting that expression of this coreceptor is not an absolute requirement for virus infection. However, compensatory cell entry pathways mediated by integrin αvβ3 or perhaps other as yet unidentified receptors may confound interpretations of these findings.

While the precise contribution of integrins to adenovirus infection *in vivo* remains to be determined, knowledge of integrin interactions has allowed further modification of Ad vectors to take advantage of the integrin/coreceptor pathway to improve gene delivery. For example, Vigne and coworkers showed that a recombinant adenovirus containing RGD motifs inserted into the hexon protein could infect vascular smooth muscle cells in CAR independent manner [78]. Wickham and colleagues have also replaced the penton base RGD motif

with a β1-integin binding motif (LDV) [79] and suggested that this might be advantageous for expanding the cell tropism of modified Ad vectors since this receptor is broadly distributed on most cell types.

B. Structural Features of Penton Base-αv Integrin Association

A monoclonal antibody (DAV-1) was used to localize the integrin binding sites on the penton base protein using cryoelectron microscopy and image reconstruction [80]. This antibody recognizes the Ad2 penton base RGD motif as well as several flanking residues (IRGDTFATR). In more recent studies, Mathias *et al.* have produced a soluble form of αvβ5 integrin containing the entire ectodomain of the receptor [81]. This recombinant protein retained ligand-binding activity and was subsequently used to examine the complex of Ad particle and soluble αvβ5 by cryo-EM [82] (Fig. 2, see color insert). The integrin ectodomain consists of an N-terminal globular (proximal) region, which is attached to slender stalk-like segments that are intertwined in the cryo-EM images. Approximately four to five soluble integrin molecules were capable of binding to each penton base protein as assessed by surface plasmon resonance analyses (BIAcore), consistent with density measurements obtained in the cryo-EM studies. The integrins form a ring-like structure above the virus surface. Each integrin molecule binds at an approximately 45° angle relative to the fiber shaft, a feature that may allow multimeric receptor association. A small cleft at the base of the integrin proximal domain, which interacts with the 20 Å RGD protrusion, could also be visualized (Fig. 3, see color insert). The five RGD protrusions on the penton base are spaced approximately 60 Å apart. It is interesting to note that foot-and-mouth disease virus (FMDV), a nonenveloped RNA virus that also uses integrins for infection, has a similar geometrical arrangement of its RGD motifs [83]. This observation suggests that the precise display of RGD sites on a nanoscale level plays a key role in promoting integrin clustering at the cell surface. In support of this concept, Stupack *et al.* demonstrated that the multimeric penton base protein but not a monomeric RGD peptide could stimulate B cell signaling and cell adhesion [84]. Maheshawri *et al.* have also shown that conjugation of RGD peptides on a synthetic substrate with an average spacing of 50 Å allows efficient integrin-mediated cell motility [85]. Integrin clustering is intimately associated with signaling processes and actin rearrangement required for efficient virus entry (discussed below).

C. Signaling Events Associated with Adenovirus Internalization

Association of cell integrins such as αvβ3 with the extracellular matrix induces the formation of focal adhesion complexes. These integrin complexes contain a number of cytoskeletal associated proteins that recognize specific amino acid sequences located in β integrin cytoplasmic domains as well

as tyrosine and mitogen-activated kinases, lipid kinases, and various other adapter molecules [86, 87]. Integrin-mediated signaling events play a crucial role in several important cell processes including cell motility, tumor cell metastasis, wound healing, and cell growth and differentiation [88, 89]. Integrin-mediated signaling events also facilitate host cell invasion by a number of pathogenic bacteria [90] as well as other viruses [91]. A general feature observed in integrin signaling is the rearrangement of actin filaments underlying the plasma membrane. Recent studies have indicated that actin assembly may play a significant role in receptor-mediated endocytosis in mammalian cells [22]. Filamentous actin could provide additional mechanical force necessary for endosome formation [24] or it may serve as a platform to stabilize the half-life of signaling complexes needed to induce receptor internalization [92].

Cytochalasin D, an agent that disrupts the actin cytoskeleton also inhibits adenovirus entry and infection [16]. Li and colleagues therefore investigated whether specific signaling events leading to actin reorganization were also involved in adenovirus internalization [19]. They found that adenovirus interaction with cells altered the cell membrane shape, induced polymerized cortical actin filaments as well as activated phosphatidylinositol-3-OH kinase (PI3K). PIP3, a major product of PI3K, acts as a second messenger in many different cell signaling processes, including those regulating cytoskeletal function [93] and bacterial cell invasion [94]. Li *et al.* found that activation of PI3K was also required for efficient Ad internalization but not virus attachment [18]. PI3K is also capable of activating Rab5, a GTPase associated with early endosome formation. Overexpression of a dominant negative Rab5 in host cells significantly inhibits adenovirus endocytosis and infection [95]. Several lines of evidence suggest that it is the penton base interaction with integrin coreceptors that initiates the key signaling events for virus entry and infection. First, recombinant penton base but not fiber protein is capable of activating PI3K [18]. Second, fiberless adenovirus particles induce similar levels of cell signaling as wild-type fiber-containing virions [96]. Finally, Bergelson *et al.* have shown that mutant forms of CAR that lack a normal transmembrane anchor and cytoplasmic domain support normal levels of adenovirus-mediated gene delivery [38].

In addition to PI3K, the Rho family of small GTPases including Rac1, CDC42 and RhoA also are involved in adenovirus cell entry. These small GTPases are tightly regulated molecular switches that control changes in cell shape as well as actin reorganization [97] via interaction with additional downstream effector molecules such as WASP and PAK1 [98]. Expression of dominant-negative forms of Rac or CDC42 reduce virus entry and infection [19]. Recently, Li *et al.* found that p130CAS is also required for efficient adenovirus entry [96]. This large adapter molecule provides an important functional link between the tyrosine kinase c-SRC [99] and the p85 catalytic

subunit of PI3K [96]. The downstream effect molecules downstream of PI3K and CAS have yet to be fully characterized.

It is interesting to note that other signaling molecules may become activated upon adenovirus interaction with host cells; however, they may not actually contribute to virus entry. For example, p125FAK (focal adhesion kinase) becomes tyrosine phosphorylated during adenovirus entry [19], but cells expressing dominant negative forms of FAK exhibit normal levels of Ad uptake [19]. Moreover, mouse embryonic fibroblasts genetically deficient in FAK support very similar levels of Ad infection as expressing cells. Bruder *et al.* [100] also reported that MAP kinases are activated during Ad infection, whereas inhibitors of ERK1/ERK2 MAP kinases have little if any effect on virus entry [18].

Despite recent progress, the precise mechanisms by which signaling processes regulate virus entry have not been elucidated. Impediments to further advances include the difficulty of studying complex signaling events in live (unfixed) cells. Moreover, signaling processes may vary among different cell types and thus the overall role of a given signaling pathway may differ in different cell types. Finally, the involvement of a specific signaling molecule may be difficult to assess if related molecules (functional homologs) perform similar functions.

While further research is needed to fully characterize Ad cell entry mechanisms, the identification of specific signaling molecules involved in adenovirus cell entry may allow improvements in Ad-mediated gene delivery to cells which lack CAR and/or αv integrins. For example, ligation of certain growth factor receptors (i.e., epithelial growth factor (EGF)) or cytokines (i.e., tumor necrosis factor alpha (TNFα)) results in activation of remarkably similar signaling pathways as those induced by integrin clustering [101–103]. Li *et al.* recently investigated whether activation of growth factor receptors could circumvent the need for αv integrins/CAR in adenovirus-mediated gene delivery [104]. They generated a bifunctional antibody that recognizes the penton base RGD motif (DAV-1) as well as one of several different cytokine or growth factor receptors. Ad vectors complexed with these bifunctional molecules significantly increased PI3K activation in host cells and improved gene delivery to human melanoma cells that lack αvβ3 and αvβ5 integrins. The bifunctional antibody also increased gene delivery by a fiberless adenovirus vector.

In addition to having a direct role in adenovirus cell entry, signaling events may also contribute to host immune responses to viral vectors. For example, Bruder and Kovesdi previously reported that adenovirus infection triggers expression of interleukin-8 [100], a response that may enhance the inflammatory reactions associated with *in vivo* delivery of viral vectors for gene therapy. Zsengeller and coworkers also reported that adenovirus internalization into macrophages involves PI3K-mediated signaling and this is associated with the production of inflammatory cytokines *in vivo* [105]. Further studies

are therefore needed to determine the extent to which the signaling events elicited during cell entry influence host immune responses to the virus. These processes are likely to have an impact on vector toxicity as well as the duration of transgene expression.

IV. Virus-Mediated Endosome Disruption and Uncoating

In contrast to enveloped viruses, much less is known about how nonenveloped viruses traverse cell membranes during the infectious process. Early electron microscopic studies by Chardonnet and Dales [29] and subsequently by Patterson *et al.* [16] showed that Ad5 particles are rapidly internalized into clathrin-coated vesicles and shortly thereafter are found free in the cytoplasm. The ability of endocytosed Ad particles to escape the early endosome prior to degradation in lysosomes is a key feature of Ad-mediated gene delivery. Although the precise details of adenovirus-mediated endosome penetration remains a mystery; prior studies have provided a few clues that may ultimately lead to further advances in our understanding of Ad entry.

A. Role of Penton Base and αv Integrins

Seth and coworkers first showed that adenovirus interaction with cells alters membrane permeability [25] and that this depends upon association of the penton base with αv integrins [15, 26, 106, 107]. Ad-mediated membrane permeabilization occurs at a pH that is very similar to the environment of the early endosome (pH 6.1) [27, 108–110]. The exact nature of the membrane lesion has not yet been revealed; however, it does not appear to be the result of ion channel formation. Further studies indicated that of all the major Ad capsid proteins, the penton base plays a key role in facilitating membrane permeabilization. Interestingly, the penton base of different adenovirus serotypes exhibit different levels of membrane permeabilizing activity. For example, type 3 but not type 2/5 penton base is capable of forming a dodecahedron [111] and Ad3 dodecahedra also directly transduce cDNA into host cells [111], whereas Ad2 penton base monomers do not [107].

Wickham *et al.* previously demonstrated that although integrins αvβ3 and αvβ5 both support adenovirus internalization, αvβ5 plays a preferential role in membrane permeabilization and infection [15]. Wang *et al.* subsequently showed that the cytoplasmic tail of the β5 integrin subunit regulates Ad escape from early endosomes [30]. In these studies, they identified multiple TVD motifs, present in the β5 cytoplasmic tail but not in other integrin subunits, that promote membrane permeabilization. These findings suggest that other as

yet unidentified host cell molecules may interact with β5 integrin cytoplasmic tail to promote virus penetration.

B. Role of the Adenovirus Cysteine Protease

The Ad2 penton base, either in its native form or presented in a multivalent form on latex particles, is unable to directly mediate membrane permeabilization [15]. This suggests that other virus/host cell factors are required for efficient virus penetration. Hannan *et al.* first described a temperature-sensitive Ad particle, designated *ts*1, which failed to cleave five precursor viral proteins at the nonpermissive temperature as well as lacked infectivity and uncoating activities associated with wild-type virions [112]. *ts*1 particles can bind and enter host cells but remain trapped inside cell vesicles and eventually undergo lysosomal degradation. Cotton and Weber subsequently showed that *ts*1 particles fail to incorporate the adenovirus-encoded 23K cysteine protease which is normally present in approximately 10 copies per virion particle and as a consequence, fail to mediate efficient gene delivery of membrane permeabilization [113]. Further biochemical studies by Greber *et al.* showed that *ts*1 virions also lack the ability to cleave the capsid-stabilizing protein VI [114], a molecule associated with virus uncoating and endosome escape. Interestingly, these investigators reported that interaction of Ad particles with cell integrins was required for activation of the cysteine protease based on competition studies with RGD peptides. While these and other studies have provided some clues as to the events associated with virus penetration and uncoating, further studies are needed to determine the precise mechanisms underlying these events.

V. Beyond the Endosome: Trafficking of Viral Capsids and Import of Viral DNA into the Nucleus

A. Intracytoplasmic Transport of Viral Capsids

An important step in adenovirus cell entry is the transport of viral capsids to the nucleus following their escape from the early endosome. Early electron microscopic studies by Chardonnet and Dales had suggested that adenovirus particles associate with microtubules during nuclear transport [29]. Unfortunately, it was difficult to discern from these early investigations whether Ad particles were nonspecifically associated with these structure elements during sample preparation. Several investigators have therefore sought to test the validity of these early findings. Using fluorescence-tagged viruses, Greber and colleagues found that adenovirus particles fail to traffic to the

nucleus in nocodazole-treated cells, consistent with a role for microtubules [31]. Furthermore, overexpression of p50/dynamitin, a molecule which is known to regulate microtubule motor (dynein)-mediated transport, altered adenovirus movement [31]. Thus, as is the case for some other large DNA viruses, [115] adenovirus appears to use the microtubule apparatus to achieve vectorial movement through the host cell. Matthews and Russell also reported that a cellular protein, p32, may also participate in vectorial transport of Ad capsids [116].

B. Docking at the Nuclear Pore and Translocation of Viral DNA

Since adenovirus replicates in the nucleus, it must deliver its genome into this cellular compartment to complete the infectious cycle. Consistent with this concept, electron microscopic studies have revealed partially uncoated adenovirus capsids docked at nuclear pore complexes of infected cells within 1–2 h postinfection. The relatively limited size of the nuclear pore complex, approximately 25 nM in diameter, also indicates that Ad capsids (approx. 90 nM) do not directly enter into the nucleus. Moreover, proteins of greater than 20–40 kDa cannot passively diffuse through the nuclear pore complex and thus the classical nuclear import machinery must then be used to facilitate translocation of the viral genome and associated proteins through pore complexes. Previous studies have indicated that after exposure of viral particles to low pH, the hexon protein is the major capsid protein that docks at the nuclear pore complex [33]. Using a permeabilized cell system, Saphire *et al.* showed that purified nuclear transport factors such as importin-α and -β, as well as heat shock 70 (hsp70), are required to facilitate nuclear import of purified hexon proteins but these factors cannot promote import of adenovirus DNA [33]. These findings indicated that other as yet unidentified cellular factors may also be required for DNA translocation.

One major question that remains to be addressed is whether nuclear import of the Ad genome requires a protein chaperone(s). In this regard, Greber and coworkers previously showed that protein VII, a protein that is associated with the viral DNA inside the capsid also enters the nucleus along with the viral DNA [114]. In contrast, the vast majority of the hexon outer coat protein remains in the cytoplasm [32]. Further studies are needed to directly demonstrate a role for protein VII or other molecules in facilitating DNA import.

VI. Conclusions

Adenovirus cell entry requires interactions of multiple host cell receptors with distinct virus capsid proteins. Adenovirus associations with different

receptors influences cell tropism and undoubtedly plays an important role in determining the efficiency of Ad-mediated gene delivery *in vivo*. The Ad capsid structure is particularly well designed to mediate multiple receptor events. The elongated and flexible fiber protein of most Ad serotypes mediates high-affinity binding with a receptor (CAR) that is broadly distributed on different host cells. Further studies are needed to determine how the structure of the fiber shaft influences receptor usage and to uncover other host cell receptors that can serve as receptors for different Ad types. The Ad penton base displays five RGD integrin binding motifs and the precise geometrical arrangement of these motifs likely facilitates integrin clustering and subsequent signaling events. In particular, integrin coreceptors induce activation of P13K and Rho GTPases that promote virus entry and endosome penetration. Events occurring subsequent to internalization including endosome disruption remain obscure. Other host cell molecules interacting with integrin $\alpha v\beta 5$ may play a key role in this process. Finally, adenovirus may provide important clues as to the mechanisms by which nucleic acids are transported into the nucleus. Increased knowledge of virus structure and host cell interactions has led to reengineering of first-generation Ad vectors to improve tissue targeting, and this may improve transgene expression as well as reduce vector toxicity.

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CHAPTER 3

Adenovirus Replication

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I. Introduction

Since their discovery, adenoviruses have served the scientific community as a powerful tool for research of important virological as well as cellular events. Adenoviruses were first isolated as a result of researchers pursuing the causative agent of the common cold. Rowe and colleagues, in 1953, observed cytopathic effect in primary cell cultures derived from human adenoids [1]. The following year, the same effect was seen in cells exposed to respiratory secretions by Hilleman and Werner, who were trying to uncover the cause of acute respiratory disease in Army recruits [2]. It was later shown that adenoviruses, so named after its source of origin, were not the etiologic agent of the common cold, since they cause practically no respiratory morbidity among the general population. However, adenovirus (Ad) has been shown to cause severe respiratory distress in immunocompromised individuals [3]. Ad infection can also result in epidemic conjunctivitis [4] as well as a number of other syndromes, including gastroenteritis [5]. These infections are usually resolved quickly, resulting in lifelong immunity to the virus.

The adenovirus family is a large one, containing members that can infect a wide range of animals, including monkeys, livestock, mice, and birds as well as humans. All of these viruses consist of a naked icosahedral protein shell (70–100 nm in diameter) that encapsidates a linear, double-stranded DNA molecule. The exact dimension of the virion particle and size of the Ad genome can differ quite greatly between adenoviruses that infect different species.

Less than 10 years after their initial discovery, it was seen that adenovirus serotype 12 (Ad12) could cause malignant tumors in infected newborn hamsters [6]. This seminal finding by Trentin and colleagues was the first evidence

that a human virus could induce cellular transformation. The fact that the transformation occurred *in vivo* and produced disease made the finding even more profound. However, to date, there has been no significant evidence that would implicate adenovirus in oncogenesis in human beings. Trentin's discovery thrust adenovirus into the forefront of model systems in the study of cancer as well as basic cellular processes. Adenovirus proved a worthy experimental system due to the ability to grow the virus to high titers *in vitro* as well as infect a wide variety of cell types. The relative safety and ease with which adenovirus and its genome can be manipulated also make Ad an attractive tool to study basic virology as well as cellular responses to viral infection. Discoveries in adenovirus research has provided a greater understanding of viral and cellular gene expression, DNA replication, cell cycle control, and cellular transformation.

A notable example of the impact that the study of adenovirus has had on the scientific field is the discovery of mRNA splicing. It was shown that adenovirus produces a number of mRNAs from a single large transcript [7]. The analysis of the structure of mRNAs by Sharp and colleagues effectively revealed the existence of introns. The existence of splicing sites was then observed. From this finding, it was possible to dissect cellular mRNAs to show the presence of introns and the function of splicing in eukaryotes.

II. Classification

The family to which adenoviruses belong, *Adenoviridiae*, is divided into two genera: *Mastadenovirus* and *Aviadenovirus*. The *Mastadenovirus* genus contains viruses that infect a wide range of mammalian species, including human, simian, bovine, ovine, equine, porcine, and opposum. The *Aviadenovirus* group infects only bird species (i.e., chicken and turkey). The viruses are classified into six subgroups based on two different criteria: percentage of guanine–cytosine in the DNA molecule and the ability to agglutinate red blood cells [8]. Within these groups are the serotypes of adenovirus. To date, human adenoviruses have been further subdivided into >50 specific types, primarily on the basis of neutralization assays. Type-specific neutralization occurs by antibodies binding the virion capsid hexon protein and, to a much lesser extent, the capsid fiber and penton proteins.

III. Genome Organization

The human Ad genome is present as a linear double-stranded DNA molecule approximately 35–36 kbp in length. The genome is contained within the capsid in a highly condensed form, associated with viral proteins V and

VII. These proteins organize the DNA into a nucleosome-like structure known as the core. The core is tethered to the capsid through the interaction of protein V with protein VI, a protein associated with internal facets of the capsid. The Ad replication origins are present in the first 50 base pairs of the ~100-bp inverted terminal repeats (ITRs) located at each end of the viral genome. The inverted nature of the ITRs plays a functional role in viral DNA replication (discussed below). A terminal protein is covalently attached to each 5′ terminus of the viral genome. This protein, along with the Ad DNA polymerase, Ad single-strand DNA binding protein, and cellular factors, are essential for viral DNA replication. A *cis*-acting packaging sequence is located at one end of Ad genome, conventionally called the left end, which directs the polar encapsidation of the viral DNA into the capsid.

The Ad chromosome contains one immediate–early region (E1A), four early transcription units (E1B, E2, E3, and E4), two "delayed" early units (IX and IVa2), and one late unit (major late) that produces five families of mRNAs (L1 to L5) (Fig. 1). All of the viral transcription units utilize cellular RNA polymerase II for their transcription. Ad-encoded regulatory proteins participate in the specificity of the transcription program. The viral genome also contains at least one gene that codes for VA RNA (some Ad serotypes have two) which is transcribed by RNA polymerase III. The schematic representation of the genome (Fig. 1) is conventionally drawn with the E1A transcription unit at the left end, adjacent to the packaging sequences (ψ). The transcription units of the Ad genome are transcribed from both strands of the chromosome: E1A, E1B, pIX, the major late transcription unit, VA RNA, and E3 are transcribed using the rightward reading strand and E4, E2, and IVa2 are transcribed using the leftward reading strand.

The Ad genome is an excellent example of the need for viruses to efficiently use limited genetic space and information to produce the maximum number of proteins necessary for virus propagation. In the case of adenovirus, the host cell's RNA producing/processing machinery is manipulated to the advantage of the virus. It would appear that the viral genome is organized in

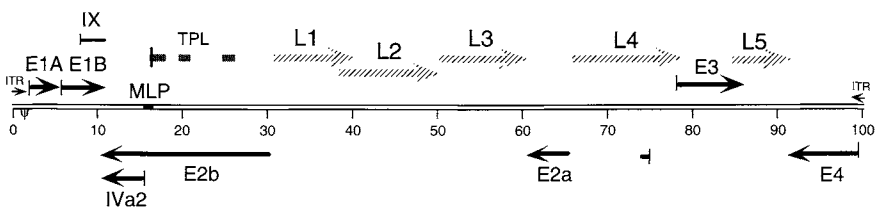


Figure 1 Schematic representation of the human adenovirus genome. Black arrows depict immediate–early, early, and delayed–early genes and hatched arrows depict the late genes. The inverted terminal repeats are labeled ITR and the packaging sequence is denoted as ψ. MLP corresponds to the major late promoter.

its current fashion as a result of evolution determining the most functionally prudent structure and order. It would also seem obvious that evolution has selected for particular grouping of RNAs, since the majority of Ad transcription units produce proteins with related functions. The grouping of related proteins within the same transcription unit might indicate that replication of the virus requires a logical, stepwise progression of gene expression in order to usurp control of the cellular machinery to direct the efficient production of virus.

IV. Virus Infection

The primary targets of adenovirus infection are the terminally differentiated epithelial cells of the upper respiratory tract, gut, and eye. However, it has been shown that adenovirus can infect almost any cell type. Adenovirus entry into a cell is discussed in detail in chapter 2 of this volume. Briefly, adenovirus binds to a cell via a cell surface receptor known as CAR (Coxsackie and adenovirus receptor) through interaction of CAR with the fiber protein [9]. The penton base portion of the fiber structure contains an RGD amino acid sequence that binds integrins on the cell surface. The integrins act as coreceptors for viral entry. Integrins are not essential for attachment of the virus to the cell, but are necessary for gaining access to the interior of the cell [10]. Virus infection can be blocked by the presence of excess RGD peptides. Recently, it was shown that at least one adenovirus serotype, Ad37, can utilize sialic acid to enter cells [11]. Both CAR and sialic acid are expressed on most, if not all, cell types, which may explain the ability of adenovirus to infect a wide variety of cells.

Once bound to its receptor, adenovirus is internalized via receptor mediated endocytosis in clathrin coated pits. The adenovirus can be visualized in endosomes shortly after infection. The low pH of the endosome facilitates release of the Ad particles that move to the nucleus, apparently via microtubule transport. During transport, the viral capsid is partially degraded, allowing the genome to be inserted into the nucleus through the nuclear pore complexes. Inside the nucleus, the genome positions itself adjacent to particular nuclear organelles through attachment of the terminal protein to the nuclear matrix. This attachment is believed to position the genome in a manner that makes it available for early gene expression and viral DNA replication.

V. Early Gene Expression

Once the viral genome has entered the nucleus, Ad early gene expression is directed toward achieving three main objectives. First, the host cell must be stimulated to enter S phase of the cell cycle to provide the correct intracellular

environment for viral replication. Second, the infected cell must be protected from the anti-viral host response to virus infection both from within the cell and due to the extracellular immune response. Third, viral gene products are produced to be used in concert with cellular proteins to carry out the viral DNA replication program.

VI. Early Region 1A (E1A)

Adenovirus encodes over 25 individual early gene products. The early genes are expressed in a temporal and coordinated manner. The first early region expressed after Ad infection is the immediate–early transcription unit E1A since it requires only cellular proteins for its expression. The E1A gene products in turn activate transcription from the other early promoter regions. The E1A gene is composed of two exons and several E1A polypeptides are produced following alternative splicing of a primary RNA transcript (Fig. 2). The most abundant of the E1A proteins early after infection are referred to as the E1A 12S (243 amino acids) and 13S (289 amino acids) gene products based on the mRNAs that encode them. The E1A 12S and 13S proteins act as major regulators of early viral transcription as well as important modulators of host cell gene expression and proliferation (reviewed in [12–14]. The E1A 12S and 13S proteins share two conserved regions within the 5′ exon referred to as CR1 and CR2. The two proteins differ only in a 46-residue internal exon segment present in the 13S protein. This region, referred to as conserved region 3 (CR3), is important for the transcriptional transactivation properties of the E1A 13S protein. Both proteins are localized to the nucleus due to a carboxy-terminal

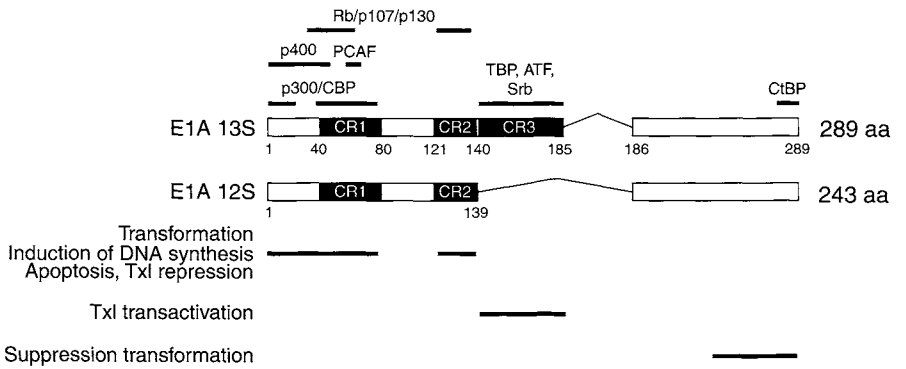


Figure 2 Functional map of E1A proteins. The coding sequences of the 12S and 13S E1A proteins are shown with conserved regions depicted (CR1, CR2, and CR3). Binding sites for cellular proteins are indicated by bars along with E1A functional activities.

nuclear localization sequence (NLS). The E1A gene products exert their effects by interactions with numerous cellular proteins, most of which are involved in transcriptional regulation [12, 14]. The E1A products interact with a number of important cellular proteins (Fig. 2), including: (1) the retinoblastoma tumor suppressor, pRb, and related family members p107 and p130 via CR1 and CR2; (2) transcriptional coactivators p300 and CBP via CR1 and amino terminal sequences; (3) a number of transcription factors such as TATA-binding protein (TBP), members of the ATF family (e.g., ATF-2, Sp1, and c-Jun), and the Srb/mediator complex via CR3 and CtBP via the C-terminus.

Rb family members repress the activity of the E2F family of transcription factors, among numerous binding partners (reviewed in [13]). p300/CBP have histone acetyl transferase (HAT) activity and play a role in chromatin remodeling (reviewed in [15]). The E1A 13S product is the major transcriptional activator of viral early gene expression and mediates its function principally through the CR3 domain that acts as a powerful modulator of other proteins involved in transcription [12, 14]. The E1A 13S protein may increase transcription through stabilization of the transcription factor complex TFIID (via interaction with TBP) at viral and cellular promoter regions. E1A also may increase transcription through stimulation of specific transcription factors. For example, E1A binding to ATF-2 may result in a conformational change resulting in transcriptional activation (reviewed in [16]). Finally, E1A binds to CtBP, a transcriptional corepressor; CtBP binding correlates with E1A suppression of transformation [17].

The most well characterized instance of E1A activation of gene expression involves the E2F family of transcription factors. The E2F family of proteins was initially discovered through studies of Ad E2 promoter regulation [18]. The Ad E2 early promoter contains binding sites for both ATF and E2F transcription factors. E2F transcription factors play a major role in the expression of cellular genes important for the regulation of cell cycle progression [13]. E2Fs exist in the cell as heterodimers containing one of six identified E2F proteins with one of two DP molecules. E2Fs both positively and negatively regulate gene expression. As repressors, E2Fs are bound to DNA at specific sites in complexes with members of the Rb tumor suppressor family (pRb, p107, p130). Rb family members interact with histone deacetylase complexes (HDACs), which repress the activity of promoter regions via deacetylation of histones and other promoter-bound transcription factors [19, 20]. Specific members of the Rb family bind to different E2F complexes, determined by the member of the E2F present. E2F binding to Rb members involves the large binding pocket domain of Rb family members which is also the target for E1A protein binding. In uninfected cells, E2F is negatively regulated by binding to Rb family members. Rb family binding to E2Fs is controlled through phosphorylation by cyclin-dependent kinases (cdks) [13]. The hyperphosphorylation of Rb family proteins by Cdks in G1 phase of the cell cycle results in dissociation of Rb from

the E2F complexes, and derepression of E2F responsive genes. The activation of E2F complexes results in the promotion of G1 and S phase progression. E1A acts to subvert the tight control of E2F by binding directly to Rb proteins and sequestering them, freeing E2F heterodimers to activate viral and cellular gene expression [13]. E2Fs activate transcription by the recruitment of HATs to promoter regions [21, 22]. Both the E1A 12S and 13S products direct the release of Rbs from E2Fs, and both E1A proteins are capable of stimulating Ad E2a transcription [12, 14].

As stated, the primary targets of adenoviruses are terminally differentiated epithelial cells. As such, these cells are generally quiescent with low metabolic activity. It is for these reasons that the virus must pressure the infected cell into S phase of the cell cycle in order to augment viral macromolecular synthesis. The interaction of E1A with p300/CBP or Rb family proteins is sufficient to stimulate cellular DNA synthesis (reviewed in [23]). It appears that the increase in DNA synthesis may be due, in part, to the activation of E2F transcription through E1A–Rb interactions. The ability of E1A to foster unscheduled DNA synthesis also contributes to its oncogenic potential. Almost all adenoviruses are capable of transforming cells in culture and this ability is primarily attributed to E1A. The regions of E1A responsible for transformation and its oncogenic potential are involved in the binding of p300 and Rb family of tumor suppressor proteins. It appears as though E1A's ability to induce S phase is directly responsible for its ability to cause transformation [23].

E1A also plays a role in the induction of apoptosis in infected cells (reviewed in [24, 25]). It has been shown that E1A causes an increase in the level of the tumor suppressor p53. The rise in p53 levels is a result of the stabilization of this usually labile protein by E1A. The presence of p53 is a major obstacle to efficient lytic infection by adenovirus. One function of p53 is to protect the genomic integrity of the cell. Unscheduled DNA synthesis, such as adenovirus DNA production, causes activation of p53. Activated p53 induces gene expression by binding specific promoter sequences, which activates genes that are involved in a number of cellular processes. The presence of p53 can affect cells in primarily two ways [24, 25]. First, p53 can induce G1 arrest, thus inhibiting progression of the cell cycle. This arrest can be facilitated by the transactivation of a gene encoding an inhibitor of Cdks, termed $p21^{WAF-1/Cip-1}$, which prevents the phosphorylation of Rb family members. p53 also can induce cell death by apoptosis. It does so by inducing the activation of degradative enzymes, caspases, which generate the classic apoptotic pathway. This proteolytic cascade results in a characteristic apoptotic phenotype of shrinkage and rounding of the cell due to breakdown of the cytoskeleton, cleavage of cellular DNA and condensation of the chromatin, cytoplasmic vacuolization and membrane blebbing, and in the final stages, fragmentation of the cell membrane into vesicles or apoptotic bodies that can be taken up by neighboring cells. The activation of p53 and induction

of cellular apoptosis at this stage of infection would be quite deleterious to the virus replication program. Therefore, adenovirus has evolved several mechanisms to decrease or inhibit p53 activity (discussed below). Recently, E1A has been shown to suppress p53 transactivation [26]. E1A causes the activation of $p19^{ARF}$, which leads to the upregulation and stabilization of p53 [27]. E1A represses p53 transcriptional activation through the binding and sequestration of p300/CBP, coactivators required for p53-dependent gene expression [28].

VII. Early Region 1B (E1B)

The second E1 gene expressed is early region 1B that leads to the production of two major species of mRNAs. One mRNA codes for a 19-kDa polypeptide (E1B 19K) and the other codes for a 55-kDa protein (E1B 55K). The two proteins are encoded by alternative reading frames and share no sequence homology. The major roles of these proteins in Ad infection are to inhibit apoptosis and further modify the intracellular environment in order to make the cell more hospitable to viral protein production and viral DNA replication [24, 25]. Viruses with mutations in either or both E1B proteins are significantly reduced in virus yield due to cell death by apoptosis prior to the completion of the replicative cycle.

The E1B 55K protein is essential for a variety of important functions in the viral life cycle. One important function is inhibition of the p53 tumor suppressor and inhibition of the induction of p53-dependent apoptosis [24, 25]. The E1B 55K protein binds to the acidic transactivation domain of p53, thus inhibiting p53-induced transcription [29]. However, the binding of E1B 55K to p53 alone cannot inhibit p53 functions. It is theorized that E1B 55K directs repression of promoters when held in a complex with p53 due to strong transcriptional repression by E1B 55K [30]. By doing so, E1B 55K inhibits the activation of p53-responsive promoter regions and blocks cycle arrest and apoptosis programs before they get underway. It is not clear if the E1B 55K protein itself is a transcriptional repressor or it recruits a repressor to the p53-bound complex on DNA.

E1B 55K acts in a complex with another early protein, E4 ORF6, which leads to the proteasome-dependent degradation of p53, further decreasing p53 effects on the infected cell [31]. E1B 55K also plays a very important role in producing a cellular environment conducive to viral protein production. A complex containing the E1B 55K protein and the E4 ORF6 product contributes to host cell protein synthesis shutoff by selectively stabilizing and transporting viral mRNAs from the nucleus to the cytoplasm while inhibiting the transport of host cell mRNAs. This topic will be discussed further in the section on early region 4 (E4).

The E1B 19K protein is also involved in the inhibition of apoptotis. E1B 19K acts to block apoptotic pathways that do not rely on p53, such as the TNF and Fas ligand cell death pathways [24, 25]. E1B 19K is a functional homolog of a cellular suppressor of apoptosis, Bcl-2. Homodimers of a pro-apoptotic protein, such as Bax, result in the activation of caspases, leading to cell death. Bcl-2 heterodimerizes with Bax and inhibits its function, preventing the induction of apoptosis. The dimerization occurs through interaction of specific binding regions, Bcl-2 homology or BH domains. E1B 19K acts in the same manner as Bcl-2 by binding Bax and other apoptosis inducers. E1B 19K shares sequence similarity with Bcl-2 in two BH domains present in E1B 19K that are necessary to bind Bax. The binding of Bax by E1B 19K leads to inhibition of apoptosis [24, 25].

E1B 19K also plays a role in inhibition of TNF-induced apoptosis by blocking the oligomerization of death-inducing complexes involving FADD [32]. FADD is a protein that is activated by binding Fas via death domains, thus its name (Fas-associated death domain). The exact function of E1B 19K in FADD regulation is not well understood.

VIII. Early Region 2 (E2)

The E2 transcription unit encodes the viral proteins involved in adenovirus DNA replication: Ad DNA polymerase (Ad Pol), preterminal protein (pTP), and DNA binding protein (DBP). The E2 transcription unit is transcribed from the E2 early promoter (E2A at genome coordinate 76), which is activated by E1A at early times after infection, and the E2 late promoter (E2B at genome coordinate 72), which is activated at intermediate times after infection through an unknown mechanism. DBP is expressed by the E2A region (Fig. 1), which shares common RNA leader sequences near genome coordinates 75 and 68 with mRNAs for pTP and Ad Pol. Ad Pol and pTP are encoded by the E2B region of the viral genome (Fig. 1) and their mRNAs share a common exon at genome coordinate 39. These short exons are spliced to the main body of the open reading frames (ORFs) for pTP and Ad Pol at genome coordinates 28.9 and 24.1, respectively.

The E2 early promoter was shown to possess four *cis*-acting elements that upregulated transcription of the gene: a TBP binding site, two E2F binding sites, and an ATF binding site (reviewed in [33]). The efficient transcription of the E2 early promoter is dependent on the E1As via Rb binding and E2F derepression and by transactivation via TBP and ATFs. The binding of E2F/DP heterodimers to the E2 early promoter is stabilized by a product of the E4 transcription unit, E4-ORF6/7 (discussed below). The mechanism by which the E2 late promoter is delayed or transactivated is not known.

DBP is a nuclear phosphoprotein of apparent molecular weight of 72 kDa that is produced in large quantities in an infected cell. The protein is from

473 to 529 amino acids in length, according to Ad serotype, and is expressed throughout the infectious cycle. DBP is involved in a number of functions including viral DNA replication, early and late gene expression, host range, transformation, virion assembly, and possibly DNA recombination (reviewed in [34–36]. The N-terminal portion of DBP is highly phosphorylated and contains the NLS. The C-terminal domain is not phosphorylated, but it binds to DNA and is involved in viral DNA replication.

DBP binds cooperatively to single-stranded DNA with high affinity and acts to protect the DNA from nuclease digestion. DBP possesses a helix-destabilizing property that is required for unwinding double-stranded DNA in an ATP-independent manner during the elongation phase of viral DNA replication by strand displacement [37]. DBP enhances renaturation of displaced complementary strands [38]. DBP is also responsible for enhancement of the initiation of DNA replication by facilitating formation of the initiation complex pTP-dCMP as well as increasing NF-I/CTF binding to its recognition site in the auxiliary origin (see below). Finally, DBP increases the processivity of Ad Pol [39].

The pTP protein exists as a stable heterodimer with Ad-Pol and is critical for the initiation of viral DNA replication [34–36]. Ad-Pol catalyzes the covalent linkage of dCMP to serine 580 of pTP. The pTP–dCMP complex functions as the protein primer for Ad DNA synthesis. In adenovirus-infected cells, pTP in a 1:1 stoichiometric ratio with Ad Pol; both of these proteins are expressed at significantly lower levels than DBP. During the initiation of viral DNA replication, pTP binds to the core origin sequences in a phosphorylation-dependent manner. At late times in infection, the 80-kDa pTP is processed to the 55-kDa TP via cleavage by the virus-encoded protease [40]. The 55-kDa TP protein is covalently linked at the 5′ ends of the genome in the mature virion. The processing of pTP–DNA to TP–DNA is not required for viral DNA replication or virion assembly, but it is necessary for full infectivity of mature virus particles. The presence of pTP or TP at the 5′ termini may protect the viral genome from exonuclease digestion. Also, covalent attachment of pTP/TP to the genome has been shown to facilitate unwinding of the DNA duplex at the origin of replication. pTP (and TP) is responsible for the attachment of the adenovirus genome to the nuclear matrix [41, 42]. The interaction of pTP and a protein complex that directs pyrimidine biosynthesis known as CAD (carbamyl phosphate synthetase, aspartate transcarbamylase, and dihydroorotase) at active sites of viral DNA replication might suggest that the area in which the genome is deposited is predetermined due to the presence of proteins necessary for DNA synthesis [43].

Ad Pol is a 140-kDa phosphoprotein that is responsible for both the initiation and elongation steps of adenovirus DNA replication (reviewed in [34–36]). Ad Pol is localized to the nucleus via its association with pTP. Ad Pol is a member of a family of proteins known as the alpha DNA polymerases

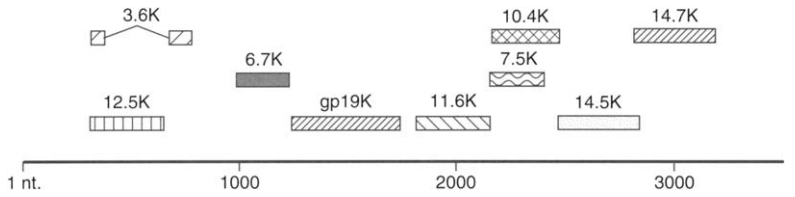
and possesses five of the six regions conserved among other members. However, the regions necessary for Ad Pol activity are distributed over the entire length of the molecule and are not limited to the five regions of homology with the other DNA polymerases [44]. Ad Pol possesses an intrinsic $3' \rightarrow 5'$ proofreading exonuclease activity as well as two potential zinc finger motifs important for its DNA binding and viral DNA replication initiation functions. Along with forming a stable heterodimer with pTP that is crucial for viral DNA replication, Ad Pol physically interacts with NF-I/CTF (a cellular factor involved in Ad replication, see below), and this interaction directs the Ad Pol–pTP complex to the origin of replication. Ad Pol is phosphorylated on serine residues, with serine 67 being the major site of phosphorylation. Phosphorylation appears to be important for Ad Pol to initiate replication [45].

The molecular and physical mechanism of Ad replication and the roles of viral and cellular proteins described in this section will be discussed below in the section on viral DNA replication.

IX. Early Region 3 (E3)

In order to conduct and successfully complete the infectious cycle, adenoviruses have evolved a number of mechanisms to evade the host antiviral defense array. Many of the proteins responsible for counteraction of the host immune response are encoded within the E3 region. The primary host defense against Ad infection is to eliminate the infected cell. The E3 region encodes multiple proteins that function to inhibit multiple pathways of cell death induced by the host innate and cellular immune responses to the infected cell (Fig. 3) (reviewed in [46, 47]). The E3 transcription unit is an early region located at genome position 76–86 whose transcription is induced by the E1A 13S protein. The E3 promoter has a TATA box as well as upstream binding sites for the ATF, AP1, NF1 transcription factors as well as NFκB [46].

It is believed that adenovirus-specific cytotoxic T lymphocytes (CTLs) are the major mechanism by which adenovirus-infected cells are eliminated. E3 encodes at least four proteins that are capable of inhibiting CTL killing [46, 47]. For CTL to destroy a virus-infected cell, the T-cell receptor must first recognize viral peptides presented on the cell surface in association with major histocompatibility complex (MHC) class I antigens. Adenovirus encodes a protein, E3 gp19K, which prevents the transport of MHC I to the cell surface. E3 gp19K is a membrane glycoprotein that localizes to the endoplasmic reticulum (ER), where it forms a complex with newly synthesized class I antigens, thus preventing their transport to the cell surface. When this protein is expressed, CTL killing of Ad-infected cells is greatly reduced. E3 gp19K, and subsequently MHC I, are retained in the ER by an ER-retention signal



gp19K: Integral membrane protein, inhibits kill by CTLs (blocks MHCI presentation)

10.4K (RIDα) and 14.5K (RIDβ): Integral membrane protein, together form the RID complex to block FasL and TNF mediated apoptosis by degrading Fas and internalizing TNFR1

14.7K: Inhibits FasL and TNF-mediated apoptosis

11.6K (aka ADP): Integral membrane protein, promotes host cell death and virus release

12.5K, 6.7K and 3.6K: Functions unknown

Figure 3 Schematic of the E3 transcription unit. The different proteins encoded by the E3 region are indicated by bars. The functions ascribed to different E3 proteins are listed below the diagram.

(KKXX) found at the extreme C-terminus of E3 gp19K [48]. E3 gp19K binds to all MHC class I antigens, but with different affinities to which a hierarchy can be assigned [46, 47].

The E3 region also produces proteins that inhibit defenses involving ligand–receptor interactions and activation of cell death pathways [46, 47]. On the surface of most cells, receptors containing death domains (DDs) are expressed. Once CTL are activated, they kill via three main pathways. The primary mechanism of cell killing involves perforin and granzymes that act in concert: perforin forms holes in the target cell and granzymes are then introduced into the cytoplasm of the target cell. One of these enzymes, granzyme B, activates caspases to induce apoptosis. The second pathway involves the receptor Fas expressed on the surface of the infected target cell. The third pathway is mediated through the tumor necrosis factor (TNF) receptor type 1 (TNFR1). The ligands to these receptors are found on the surface of activated CTL: Fas ligand (FasL) and TNF, respectively.

The interaction of ligand with receptor triggers a series of protein–protein interactions in the target cell that results in the induction of apoptosis (reviewed in [49]). Upon binding of FasL to Fas, the latter trimerizes and then binds a protein named FADD. This binding is facilitated though the "death domain" (DD), present in both Fas and FADD. FADD also has a "death effector domain" (DED) through which it associates with procaspase 8, thus causing autocleavage and activation of caspase 8. Activated caspase 8 cleaves and activates downstream caspases—a cascade that results in apoptosis. TNF binding to TNFR follows a similar pathway except TNFR binds the DD of TRADD (TNF-receptor-associated death domain) which then binds FADD

and caspase 8 as well as another DD-containing protein named RIP [50]. RIP is a serine/threonine kinase whose exact function is unclear.

E3 RID (for receptor internalization and degradation) is an integral membrane protein composed of two E3 products RIDα (E3 10.4K) and RIDβ (E3 14.5K). The RID complex localizes to the plasma membrane, Golgi apparatus, and ER. RID inhibits apoptosis through the TNFR1 and Fas pathways [51, 52]. Expression of RID leads to the clearance of Fas from the cell surface, which results in degradation in lysosomes. RID-mediated elimination of TNFR1 is less efficient, and it is not known if TNFR1 is degraded in lysosomes. RID also stimulates internalization of certain other receptors whose activation may result in a less direct inflammatory response. Once RID has deposited the receptor in the lysosome for destruction, RID is recycled back to the cell surface to repeat the internalization process.

E3 14.7K also inhibits apoptosis induced by the cytokine activation of receptors. Its effects on TNF pathway is more dramatic than on Fas [53, 54]. Unlike many of the other E3 proteins, E3 14.7K is not associated with a membrane structure, but is present in the cytosol and nucleus. E3 14.7K acts by binding proteins involved in the apoptotic pathway. One protein is FIP-3, which also binds RIP, a component of TNF-induced activation of NFκB [55]. NFκB activation appears to inhibit apoptosis; FIP3 may activate an apoptotic pathway in conjunction with inhibition of NFκB transactivation. Therefore, the binding of E3 14.7K to FIP-3 allows NFκB to induce transcription of genes that defend against the TNF signal. Also, the presence of NFκB sites in the E3 promoter may lead to the increased expression of E3 proteins [56]. It has also been reported that E3 14.7K may bind caspase 8 directly to inhibit the caspase/protease cascade.

Both RID and E3 14.7K prevent TNF-induced release of arachidonic acid (AA). Cytosolic phospholipase A_2 (cPLA2) is activated by TNF signaling which causes it to translocate to membranes and cleave phospholipids, producing AA. RID inhibits the translocation of cPLA2 to membranes. This action occurs prior to RID clearing TNFR from the cell surface [54]. The mechanism of E3 14.7K inhibition of AA release is not known. Both RID and E3 14.7K are required to inhibit inflammation and pathology in infected mouse lung. These Ad-encoded E3 proteins inhibit two of the three mechanisms utilized by CTL for cell killing. If Ad also inhibits perforin/granzyme lysis of cells is presently unknown.

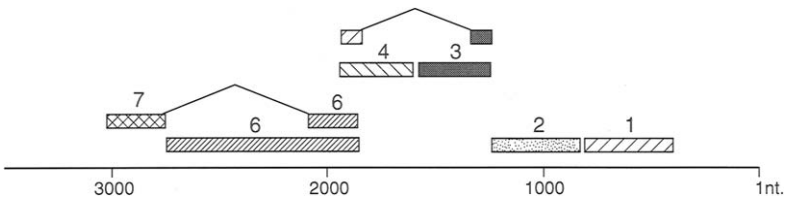
The E3 11.6K protein, also known as the adenovirus death protein (ADP), is an integral membrane protein localized to the Golgi and ER that is modified with complex oligosaccharides at a single N-linked site. This protein promotes cell death very late in the infectious cycle in order to release mature virions into the surrounding environment. Cells infected with an Ad mutant that does not express the E3 11.6K protein remain viable much longer than cells infected with wild-type adenovirus [57]. This action may seem contradictory to the functions of the other E3 proteins, but E3 11.6K is not produced in significant

amounts until very late stages of infection when virions accumulate awaiting release [58].

X. Early Region 4 (E4)

Whether they are early or late, a common theme among the transcription units of adenovirus is that they encode multiple proteins of related functions. However, early region 4 (E4) is the only transcription unit that produces proteins of relatively disparate functions. E4 encodes at least seven proteins according to analysis of ORFs and spliced mRNAs. The gene products exhibit a wide range of activities (Fig. 4). Proteins expressed from the E4 region have been shown to be important for viral DNA replication, viral mRNA transport and splicing, shutoff of host cell protein synthesis, and regulation of apoptosis. Viruses lacking the entire E4 region are extremely compromised for growth, decreased >5 logs in virus growth compared to wild-type Ad [59, 60]. Several of the proteins produced by E4 appear to be cytotoxic to cells. The E4 products cytotoxicity may influence the virus life cycle as well as the decision to include them in gene therapy vectors. The E4 region is transcribed in response to induction by E1A. The E4 promoter is regulated to a certain extent by ATF sites, but expression depends more on two sites that bind a transcription factor termed E4F [12]. If E1A is not present, the E4 transcription unit is still expressed, but to a much lower extent.

The E4 ORF1 14.3-kDa protein is relatively uncharacterized. The E4 ORF1 proteins of a number of Ad serotypes are capable of transforming



ORF 1: Transformation, mammary tumors, binds PDZ proteins
ORF 2: Function unknown
ORF 3: Redundant with ORF6, virus growth, disrupts PODS, persistence of transgene expression *in vivo*
ORF 4: Stimulates PP2A, induces apoptosis
ORF 6: Redundant with ORF3, host cell shutoff, virus growth
ORF 6/7: E2F induction

Figure 4 Schematic of the E4 transcription unit. The different proteins encoded by the E4 region are indicated by bars. The functions ascribed to different E4 proteins are listed below the diagram.

primary rat cells in culture. In addition, the E4 ORF1 protein of Ad9 induces mammary carcinomas in rats, independent of E1A [61]. The E4 ORF1 protein binds to a number of cellular proteins that possess a motif referred to as the PDZ domain, including the cellular *dlg* tumor suppressor protein, and the binding of E4 ORF1 to PDZ-containing proteins appears to mediate the oncogenic nature of this viral gene product [62]. The exact role that E4 ORF1 plays in the viral replication cycle is currently being analyzed.

Nothing is known about the role of the E4 ORF2 14.6-kDa protein in viral replication. This is also true of the E4 ORF3/4 7.1-kDa protein that is the product of a spliced mRNA that fuses the amino terminus of E4 ORF3 to the carboxy terminus of E4 ORF4.

The E4 ORF3 11- to 14-kDa protein is expressed early in infection. E4 ORF3 is very tightly associated with the nuclear matrix. E4 ORF3 has been shown to have redundant function(s) with another E4 protein, E4 ORF6, with respect to virus growth and splicing of viral mRNAs [59, 60, 63]. A profound defect in Ad growth is observed with mutants that lack all of E4 coding sequences. However, if either E4 ORF3 or E4 ORF6 is expressed with an otherwise E4-deleted virus, growth capacity is restored to within 10-fold of wild type. Further, individual mutation of either the E4 ORF3 or E4 ORF6 proteins has only a modest impact on viral growth, whereas mutation of both protein reading frames results in a significant reduction in virus yield. Thus, either the E4 ORF3 or E4 ORF6 proteins are sufficient to confer the majority of E4 function in an Ad lytic infection in cultured cells. The E4 ORF3 and E4 ORF6 proteins both bind to the E1B 55K product, although to different ends. E4 ORF6 enhances the inhibition of p53 by E1B 55K, whereas E4 ORF3 transiently relieves the repression of p53 by E1B 55K [31, 64, 65]. Yet another function that E4 ORF3 has been proposed to have in common with E4 ORF6 is the ability to bind and inhibit the activity of DNA-protein kinase (DNA-PK), thus resulting in an inhibition of double strand break repair (DSBR) mechanism [66]. Ad DNA replication is likely to induce cellular DSBR. The binding of E4 ORF3 or E4 ORF6 proteins to DNA-PK appears to inhibit DSBR and block the formation of viral DNA concatamers that occurs in the absence of E4 expression. The formation of Ad DNA concatamers would block viral DNA replication and packaging of the genome into the capsid.

E4 ORF3 has been shown to localize with discrete nuclear structures known as PODs, PML oncogenic domains, or ND10s [67]. PODs exist as multiprotein complexes that exhibit a discreet, punctate appearance in the nucleus of an uninfected cell. E4 ORF3 is necessary and sufficient to cause redistribution of these protein complexes into long, track-like structures. PODs have been implicated in a number of cellular processes ranging from transcriptional regulation to the regulation of apoptosis (reviewed in [68]). PODs have also been shown to react to stresses such as heat shock and heavy metals as well as interferon, suggesting a role in cellular defense mechanisms.

A number of DNA viruses express proteins that function to disrupt PODs, i.e., herpesviruses, cytomegalovirus, and papillomavirus [69]. The exact function of PODs is still unknown, as is the purpose for POD reorganization by E4 ORF3, although it has been linked to adenovirus replication [67]. E4 ORF3 is also capable of binding a number of other proteins, some of which are involved in transcriptional regulation such as p300 and CBP (Evans and Hearing, unpublished results). Despite considerable research, the exact function(s) of E4 ORF3 in the viral replication cycle is still unclear.

E4 ORF4 is a 14-kDa protein that plays a role in several different processes during Ad infection. First, E4 ORF4 binds to the Bα subunit of the serine/threonine phosphatase PP2A [70]. By binding this subunit, the trimeric form of PP2A is activated to dephosphorylate targets such as mitogen-activated protein (MAP) kinases that are important in signal transduction pathways. Increased PP2A activity leads to decreased phosphorylation and inactivation of certain transcription factors, such as E4F, through direct interaction or through the inactivation of MAP kinases. E4 ORF4 expression also results in decreased E1A phosphorylation at MAP kinase consensus sites that are important for E4 transactivation [71]. Through decreasing the activity of E1A and E4F, E4 ORF4 regulates the expression of the E4 region itself through its interaction with PP2A, perhaps to reduce the amount of potentially toxic E4 products [72]. Second, E4 ORF4 plays a role in the regulation of mRNA splicing. Third, much attention has been paid recently to the ability of E4 ORF4 to induce p53-independent apoptosis. The binding to and regulation of PP2A by E4 ORF4 is essential for the induction of cell death. E4 ORF4-dependent apoptosis also requires modulation of Src-family kinases [73].

E4 ORF6/7 is a 17-kDa protein produced from a spliced mRNA that encodes the amino terminus of E4 ORF6 linked to the unique E4-ORF7 sequence. E4 ORF6/7 molecules form stable homodimers that contribute to viral DNA synthesis by enhancing the production of E2 products. E4 ORF6/7 binds free E2F and induces cooperative and stable binding of E2F/DP heterodimers to inverted E2F binding sites in the Ad E2 early promoter [74]. Recently, it was shown that E4 ORF6/7 induces expression from the cellular E2F-1 promoter and is able to functionally compensate for E1A in adenovirus infection [75, 76].

The E4 ORF6 34-kDa protein provides a number of functions that are important in Ad infection. As stated above, E4 ORF6 has been shown to be redundant with E4 ORF3 for a number of roles in the Ad replication cycle. However, E4 ORF6 has a set of unique functions that have led to it being studied more intensively than its counterpart. E4 ORF6 binds to and inhibits p53, providing adenovirus yet another defense for p53 effects within the cell [64]. E4 ORF6 enhances E1A-dependent cellular transformation, possibly through the inhibition of p53 [77]. E4 ORF6 also directly binds E1B 55K, and this complex leads to the proteasome-dependent degradation of p53, counteracting

the induction of p53 stability provided by E1A [31]. The E4 ORF6–E1B 55K complex is also important in the replication cycle of adenovirus. These proteins lead to host protein synthesis shutoff by selectively transporting viral mRNAs from the nucleus to the cytoplasm and inhibiting the transport of host mRNAs. E4 ORF6 possesses three targeting signals with its amino acid sequence: an arginine/lysine-rich NLS in its amino terminus, a nuclear export signal in its central region where it also binds p53, and a nuclear retention signal (NRS) toward its carboxy terminus. The association of E4 ORF6 causes the relocalization of E1B 55K from the perinuclear region to the interior of the nucleus via the NLS and NRS [78]. E1B 55K was also shown to have a shuttling capability independent of its binding to E4 ORF6 [79]. E1B 55K is capable of binding mRNAs in a sequence-independent manner [80]. E1B 55K also has been shown to bind a cellular protein that binds to RNA [81]. The localization of the E1B 55K–E4 ORF6 complex to the viral transcription centers in the nucleus ensures that primarily only adenovirus late mRNA transcripts are bound and selectively transported from the nucleus. The NES of E4 ORF6 mediates the transport the RNA–protein complex out of the nucleus to the cytoplasm for translation.

Early gene expression sets the stage for the replication of the viral genome. The accumulation of the replication proteins encoded in the E2 region is necessary to provide the machinery capable of carrying out viral DNA replication while a variety of early proteins attempt to stimulate the cell into S phase or negate the defense systems of the host. If the virus is successful in these pursuits, the virus will replicate very efficiently.

XI. Viral DNA Replication

Replication of the adenovirus DNA genome has been intensively studied over the past two decades. An *in vitro* Ad replication system was the first example of a mammalian cell-free DNA replication system which led to a number of discoveries on the mechanics of DNA replication, on the function of nucleoprotein complexes, and on the intricacies of virus–host interactions [34, 36, 82]. Ad DNA replication is the result of an organized interplay between viral proteins, cellular factors, and viral template DNA at distinct sites within the nucleus termed replication factories. DNA synthesis requires three viral proteins (Ad Pol, pTP, and DBP) encoded by the E2 region. Ad replication is significantly stimulated by three cellular proteins (NFI/CTF, NFII, and NFIII/Oct-1) [34, 36, 82]. These cellular factors increase replication initiation up to 200-fold. Ad replication is initiated by a protein priming event, followed by a "jumping back" mechanism, and completion by strand elongation to termination (Fig. 5).

The defined origin of Ad DNA replication is located within the first 50 bp of the ITR (Fig. 5A). The terminal 18 bp of the viral genome contains

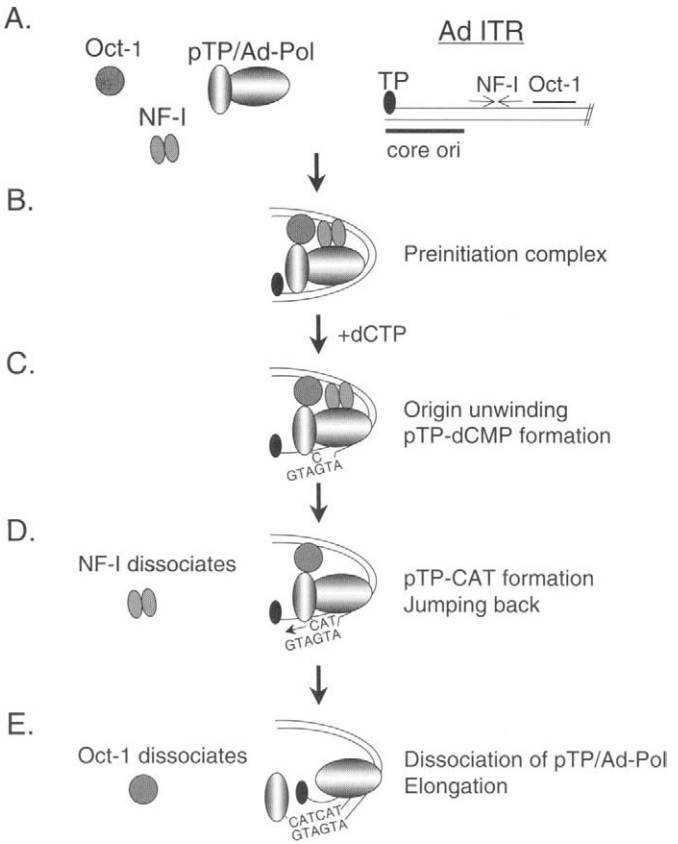


Figure 5 Model of adenovirus DNA replication. See text for details.

the minimal replication origin (core origin) with an essential triplet repeat at the molecular ends (5′-CATCAT in Ad2/5). Although this region contains the core origin, alone it can support only very limited levels of replication initiation. Immediately adjacent to the core origin is an auxiliary region that contains binding sites for NFI/CTF and NFIII/Oct-1. Binding of these cellular factors to the Ad ITR increases the efficiency with which initiation and elongation are undertaken.

Nuclear factor I (NFI), a cellular transcription factor also known as CTF, was purified from HeLa cells as a protein that could enhance Ad DNA replication *in vitro* [34, 36, 82]. NFI/CTF binds as a dimer to the auxiliary origin of replication. This binding is enhanced by DBP, probably via changes in the DNA structure. NFI/CTF interacts with the Ad Pol–pTP complex and recruits this complex to the core origin [83]. The position of the NFI/CTF site

with relation to the core origin is critical and suggests the spatial distance is necessary for NFI/CTF to position Ad Pol–pTP correctly at the terminus of the genome. The interaction of NFI/CTF with Ad Pol–pTP leads to increased stability of the Ad Pol–pTP complex at the origin, thus increasing stimulation of initiation up to 60-fold.

Oct-1, originally identified in adenovirus replication as NFIII, is an extremely well studied transcription factor, which binds to the octamer element present in a variety of promoter and enhancer regions [34, 36, 82]. The only portion of Oct-1 that is required for stimulation of Ad DNA replication is the DNA-binding POU domain [84]. The POU domain is a bipartite sequence of two conserved subdomains separated by a nonconserved or variable linker region. The presence of both subdomains is required for high-affinity DNA binding. In the adenovirus origin, the Oct-1 POU domain binds to a recognition site next to the NFI/CTF site in the auxiliary region and stimulates initiation six- to eightfold, depending on the Ad Pol–pTP concentration. The POU domain contacts the pTP protein in the Ad Pol–pTP complex, whereas NFI/CTF contacts Ad Pol, suggesting a cooperative effect of these proteins to enhance the binding of the initiation complex to the origin. Also, as with NFI/CTF, the spatial relation of the Oct-1 binding site to the core origin is important for stimulation of DNA replication.

In order for efficient replication of the entire genome, a third cellular factor is required. NFII is necessary if replication is to proceed beyond 30% of the genome. This protein is a type I DNA topoisomerase and is required for elongation *in vitro* [85]. The reason for the need for topoisomerase function *in vitro* is currently unknown. Interestingly, both type I and II topoisomerase activities are required for effective Ad replication *in vivo* [86]. Inhibition of type I topoisomerase activity leads to an immediate block of all adenovirus replication, while inhibition of type II topoisomerase activity blocks replication after completion of the first round of synthesis.

The model of the dynamics of adenovirus DNA replication involves the cooperative efforts of a number of proteins during initiation, jumping back, and elongation (Fig. 5B) [34, 36, 82]. These events need to be carefully orchestrated and organized, in order to be carried out in an efficient manner. Preceding the initiation event, the preinitiation complex composed of Ad Pol–pTP, DBP, NFI/CTF, and Oct-1 is formed at the origin. The assembly of this complex can occur in the absence of nucleotides. Binding of NFI/CTF to its site in the auxiliary origin region is facilitated by DBP [87]. Specific interactions of NFI/CTF and Oct-1 in the auxiliary region recruit and stabilize the interaction of the Ad Pol–pTP complex with the core origin [88]. The binding of the Ad Pol–pTP complex to the core origin is further enhanced by the TP linked to the genome. The phosphorylation state of Ad Pol and pTP is likely to influence the interactions of these proteins with DNA and with other proteins. After recruitment of the Ad Pol–pTP complex to the origin, DNA replication

initiates with the covalent coupling of the first dCTP to pTP, resulting in the formation of a pTP–dCMP complex necessary for the protein priming function. The initiation reaction is stimulated by DBP. It is also believed that DBP may be responsible for the unwinding of the origin.

After the unwinding, the Ad Pol–pTP complex then forms a pTP-trinucleotide intermediate, pTP–CAT, by Ad Pol using the complementary sequence 3′-GTA located at nucleotides 4–6 from the genomic terminus. The presence of nucleotides in the complex causes the dissociation of NFI/CTF from its binding site. This trinucleotide–protein complex then jumps back from positions 4–6 by base pairing with template strand nucleotides 1–3 [89]. Following the jumping back event, Ad Pol dissociates from pTP linked to the end of the viral genome and elongation ensues. As Ad Pol replicates the viral DNA, it displaces the Oct-1 from its binding site. Ad Pol carries out DNA replication by displacing the nontemplate strand, with DBP assisting in the unwinding of downstream duplex DNA [37]. DBP also coats the single-stranded DNA resulting from elongation in order to protect it from nuclease digestion. The single-stranded DNA is then available to act as a template for a new round of pTP-primed initiation. However, in order for the Ad Pol–pTP to recognize and bind DNA, the template must be double-stranded. A double-stranded DNA template may be achieved by the annealing of the left and right ITRs on one DNA strand to one another to form a loop or panhandle structure, the end of which looks like the end of an intact genome and contains covalently attached TP. Alternatively, two single-stranded DNA molecules could anneal to form duplex DNA. The pTP attached to the 5′ end of newly synthesized may protect the genome from nuclease digestion as well as assist in loading of an initiation complex in subsequent rounds of replication.

The adenovirus E4 region produces several proteins that are required for efficient DNA replication in cell culture. As described above, the E4 ORF3 and E4 ORF6 proteins display redundant functions in Ad infection. A virus that lacks both of these two proteins is severely delayed in the onset of viral DNA replication, whereas viruses that express either one of these E4 gene products exhibit only a modest delay in the onset of DNA replication [59, 60]. Since neither of these proteins is required for DNA replication *in vitro*, they probably play a regulatory role in the process rather than have a direct effect in DNA synthesis. The exact nature of their participation in viral DNA replication is still unclear. Another E4 product that affects viral DNA replication is E4 ORF4. E4 ORF4 was shown to have an inhibitory effect on viral DNA replication in the absence of the E4 ORF3 and E4 ORF6 proteins [90]. E4 ORF4 may downregulate DNA synthesis through its interaction with PP2A. Dephosphorylation of E1A may affect the accumulation of E2 products, which would then decrease viral DNA synthesis. Also, PP2a activated by E4 ORF4 may dephosphorylate the viral phosphoproteins involved in DNA replication.

XII. VA RNA Genes

Adenovirus encodes one or two VA (virus-associated) RNAs, depending on serotype, of about 160 nucleotides that are transcribed by host cell RNA polymerase III. VA-RNA$_I$ targets the protein kinase named PKR (reviewed in [91]). PKR is activated by the presence of low levels of double-stranded RNA, a likely product from symmetrical transcription of the Ad genome. Upon activation, PKR phosphorylates and inactivates eukaryotic initiation factor 2 alpha (eIF-2α), thus inhibiting protein synthesis in general. VA RNA is produced in large quantities in Ad-infected cells. PKR binds to the significant secondary structure found in VA-RNA$_I$, and the high level of VA-RNA$_I$ interferes with PKR activation. This allows for the maintenance of efficient translation in Ad-infected cells [91].

XIII. Late Gene Expression and Virus Assembly

Efficient late gene expression commences with the onset of Ad DNA replication. Late transcripts are initiated from the major late promoter (MLP) located at 16 map units (Fig. 1) [92]. Activation of the major late promoter appears to be mediated by both *cis*-acting changes in the viral genome as well as *trans*-acting factors. Both cellular and viral *trans*-acting components have been identified that bind sequences within the major late promoter. Cellular transcription factors TBP/TF-IID, USF/MLTF, and CAT box factor interact with *cis*-acting elements upstream of the major late promoter initiation site and are important activators of MLP expression [93]. Activation of the MLP also is specified by protein binding sites located downstream from the transcriptional start site [94]. The *trans*-acting components binding these sites are not fully characterized, but constitute multiprotein complexes containing the virally encoded IVa2 protein [95]. Through a mechanism(s) that is not understood, the Ad replication process significantly stimulates the activity of the MLP.

The primary transcript from the major late promoter extends to the right end of the viral genome and is ~30,000 nucleotides (nt) in length. This primary transcript is polyadenylated at one of five sites and undergoes multiple splicing events to generate five families of late mRNAs (L1 to L5; Fig. 1) [92]. At least 18 distinct late mRNAs are produced by alternative polyadenylation and splicing of the primary major late transcript. The 5′ ends of all Ad late mRNAs contain an ~200-nt leader sequence referred to as the tripartite leader (due to the joining of three short exons in the primary late transcript). The tripartite leader sequence directs efficient translation of Ad late mRNAs independent of the host cell initiation factor eIF4F [96, 97]. eIF4F

is a protein complex composed of phosphorylated cap-binding protein eIF4E, eIF4E kinase Mnk1, eIF4A, poly(A)-binding protein, and eIF4 G. Adenovirus infection blocks cellular translation by displacing Mnk1 from eIF4F, thereby blocking phosphorylation of eIF4E [98]. The Ad-encoded 100K late protein binds to eIF4 G in the same region bound by Mnk1 and displaces Mnk1 from the eIF4F complex. This results in the shut off of translation of host mRNAs and Ad mRNAs that lack the tripartite leader sequence. The translation of Ad late mRNAs that carry the tripartite leader sequence continues, effectively shutting down host mRNA translation while permitting viral late mRNA translation. The mechanism by which translation of Ad late mRNAs continues despite inactivation of eIF4F is not fully understood. Ad late mRNAs encode proteins that are part of the Ad capsid structure (discussed in Chapter 1 of this volume), that are involved in the virus assembly process, or that play other regulatory roles during the late phase of virus infection.

Adenovirus DNA packaging into virus particles occurs in a polar manner from left to right and relies on a *cis*-acting packaging domain located between approximately nt 200 and 380 nt (Fig. 6) (reviewed in [99]). It is thought that *cis*-acting packaging sequences and *trans*-acting protein components act in conjunction to mediate DNA packaging, similar to a number of bacteriophages like lambda or ϕ29. The formation of Ad particles proceeds through an

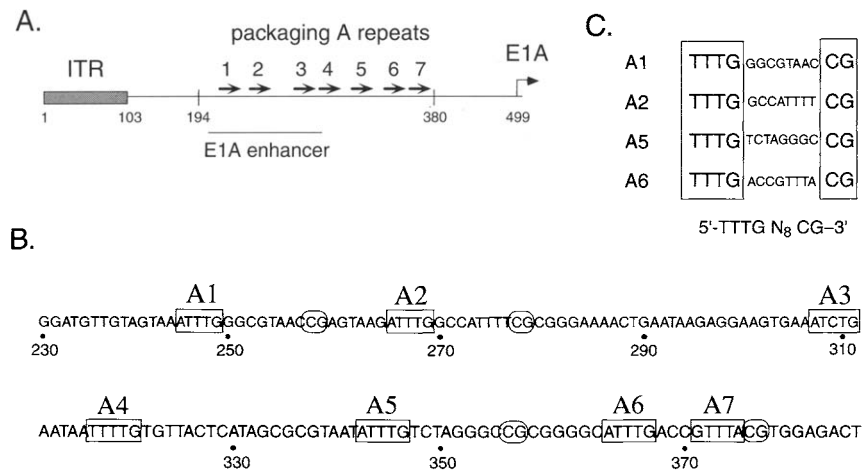


Figure 6 Schematic of the adenovirus DNA packaging domain. (A) A schematic diagram of the left end of the adenovirus genome including the inverted terminal repeat (ITR), the packaging/enhancer region (nts. 194 to 380), and the E1A 5′ flanking region. The packaging repeats (A1 through A7) are represented by arrows. The E1A transcriptional start site is indicated by an arrow at nt 499. (B) The nucleotide sequence of the Ad5 packaging domain is shown. Numbers at the top correspond to nucleotides relative to the left end terminus. A repeats 1 through 7 are encircled. (C) The A repeat consensus sequence is shown.

ordered series of assembly events. The first virus assembly intermediate is the light particle that appears to be equivalent to a bacteriophage prohead. Light intermediate particles contain all of the major capsid proteins, lack viral DNA, and contain additional proteins that exit the particle during maturation and that may act as scaffolding proteins. Light particles mature to heavy intermediate particles upon the packaging of viral DNA and associated core proteins. As the final maturation step, activation of the virus-encoded and packaged protease results in numerous protein cleavages within the virus particle that result in maturation into the infectious virus [100]. These proteolytic cleavages are absolutely essential for the formation of highly infectious Ad particles. Further, Ad protease plays an important role in the infection process for proper release of the viral core particle from endosomes following initial infection (reviewed in Chapter 2 of this volume).

The Ad5 packaging domain is depicted in Fig. 6. The Ad5 packaging domain consists of at least seven redundant, although not functionally equivalent, elements termed A repeats 1 through 7 [101, 102]. The functionally most important A repeats (A1, A2, A5, and A6) share a bipartite consensus motif 5′-TTTGN$_8$CG-3′, which is conserved among different Ad serotypes [103]. There are spacing constraints between the two conserved parts of the bipartite consensus motif rather than between different A repeats. Multimerized copies of individual packaging repeats can restore viability to a mutant virus lacking a packaging domain. The Ad5 packaging domain displays considerable flexibility in its position and orientation, but it must be located within ~600 bp of a genomic terminus [104].

Very little is known about the identity of *trans*-acting packaging components involved in the packaging process. The packaging repeats very likely are binding sites for a *trans*-acting factor(s) that directs the packaging process. Several cellular DNA binding activities including OCT-1, COUP-TF1, and an unknown activity termed P complex interact with the TTTG half site of the packaging consensus sequence [105]. The functional significance of the binding of these cellular factors is presently under evaluation (Erturk and Hearing, unpublished results). The Ad IVa2 protein interacts with sequences that overlap the CG half of the Ad5 packaging repeat consensus [106]. It is not known if IVa2 is involved in Ad packaging, but the Ad IVa2 protein forms a protein complex with an Ad late gene product, L1 52/55K, that has a clear link to the Ad assembly process [107]. L1 52/55K mutants produce empty Ad particles or particles that are only partly packaged [108, 109]. It is easy to imagine that viral and cellular proteins form a multiprotein complex on Ad packaging signals that direct the encapsidation of the viral genome into virions. The identity and specificity of Ad packaging elements has been used to engineer Ad helper viruses whose packaging may be suppressed and to optimize yields of gutted Ad vectors. These approaches are reviewed in Chapter 15 of this volume.

XIV. Vector Design

The ability of adenoviruses to infect a wide range of cell types as well as the ease with which their genomes can be manipulated has made Ad extremely attractive as a gene therapy vector. In order to obtain viral vectors for gene therapy, the viral genome carrying the transgene must be replicated and packaged to relatively high titers. Therefore, the replication events described in this chapter must be carried out to fruition in order for progeny virus to be produced, whether it is through traditional or alternative means.

The first generation of Ad vectors lacked functional E1 and E3 regions, thus making them replication deficient due to the absence of E1A expression and greatly reduced transactivation of other early genes including the E2 region. These viruses were propagated in cell lines that provided E1 proteins in *trans* allowing for efficient replication. The lack of E3 expression did not affect virus production in culture. These viruses were very effective in introducing a transgene to cells in culture and in the animal. However, the first-generation viruses elicited a significant host immune response including innate, cell-mediated, and humoral responses, preventing prolonged therapy and efficient reintroduction of the viral vector (reviewed in [110, 111]). Also, the production of these E1-deficient viruses in complementing cell lines often resulted in E1-positive, replication-competent adenovirus (RCA) due to recombination with endogenous viral DNA sequences present in the complementing cell line. The contamination of gene therapy vector stocks with essentially wild-type adenovirus is unacceptable due to the outcome of lytic virus infection. Production of first generation vectors in complementing cell lines with integrated; nonoverlapping sequences will reduce the instances of recombinant RCAs in virus stocks.

It is believed that a low level of expression of Ad proteins using first generation Ad vectors resulted in an immune response to viral infection. This may in part reflect low levels of replication of the viral genome despite the lack of E1 gene products. Second-generation Ad vectors were developed with additional deletions in genes involved in the replication cycle [110, 111]. The development of E1-complementing cells lines that express additional Ad gene products from early region 2 or early region 4 greatly facilitated the development of the second generation Ad vectors. The inclusion of the E2 region proteins required for Ad DNA replication (Ad Pol, DBP, and pTP) in a gene therapy virus was primarily for ease of propagation of the virus due to the lack of complementing cell lines. Recently, several cell lines were described that constitutively or inducibly produce one or more of the E2 proteins, allowing for vectors lacking these sequences [112–114]. Similar lines were developed that express E4 gene products [115]. The removal of the E2 or E4 genes would provide more genetic space for the insertion of larger transgene sequences as well as allow the production of Ad vectors with greatly

reduced replication potential. The net effect of second-generation Ad vectors was the development of vectors with greater safety and significantly reduced host inflammatory responses and CTL responses to infected cells. However, the deletion of additional Ad gene products may not be without consequence to the utility of the vector *in vivo*. For example, the presence of the E4 ORF3 gene in Ad vectors that utilize the CMV promoter has been found to significantly contribute to sustained transgene expression both *in vitro* and *in vivo* [116, 117]. Thus, Ad vectors that express certain early gene products may be most useful under certain circumstances.

An intriguing new approach in the development of adenovirus vectors is the design of oncolytic vectors that replicate in selected cells or types of cells (reviewed in [111]). This approach involves conditionally replicating viruses that undergo lytic infection in tumor cells. An example of conditionally replicating viruses is ONYX-015 [118]. This virus has been shown to replicate more efficiently in cells lacking p53. ONYX-015 is deleted for E1B 55K and it cannot replicate well in p53-positive cells, but is capable of productive infection in cells lacking active p53, such as tumor cells. The ONYX virus is discussed in great detail in Chapter 11 of this volume. Other conditionally replicative cells could be produced with E1 genes under the control of cell specific promoters, which are discussed in Chapters 9 and 10 of this volume. Other approaches to attack and eliminate p53 mutant cell refractory to other treatments could include vectors possessing E4 ORF4 or the E3 ADP proteins, which induce cell death independent of p53 status.

The strategies mentioned above all result in the death of a target cell, such as a tumor cell, which would generally benefit from an inflammatory response and CTL infiltrate. This response would result in clearance of virus-infected cells. However, an immune response would not be beneficial while attempting to treat other diseases, such as metabolic disorders, that may require more than one treatment or prolonged presence of the viral genome. One approach that may delay or evade the immune system would be to include genes from the Ad E3 region that are involved in evasion of host immune responses during viral infection [119]. Additionally or alternatively, other viral or cellular immunomodulatory genes may be incorporated into Ad vectors toward the same goal. These types of approaches are discussed in Chapter 14 of this volume.

XV. Conclusion

The life cycle of adenovirus represents a complex series of events that must occur in a temporally and stoichiometrically appropriate fashion in order for efficient production of progeny virus. The virus must usurp control of the cellular machinery while controlling the expression and functions of its own

proteins. The manner with which adenovirus exerts these controls is through a myriad of protein–protein interactions. The use of adenovirus as a model system has led to a wealth of information regarding many aspects of viral as well as cellular processes, including transcriptional and posttranscriptional regulation, oncogenic transformation, virus entry into host cells, virus–host immunological interactions, translational regulation, and DNA replication. The future of adenovirus research is still fruitful with respect to both basic as well as clinical studies. A greater understanding of adenovirus infection as a whole will allow for better development and applications of gene therapy vectors through rational design. The result of which would be more effective and safer vectors, including those that are targeted to specific cell types and conditionally replicate.

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CHAPTER 4

Adenoviral Vector Construction I: Mammalian Systems

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I. Introduction

Adenoviruses (Ads) are excellent gene transfer vectors and are extensively used for high level expression of transgene products in cultured cells, as potential recombinant viral vaccines and for gene therapy. Ads are particularly well suited for these applications because their genome is relatively easy to manipulated, they grow to high titers, they are stable and easy to purify, and they can transduce many cell types from numerous mammalian species including both dividing and nondividing cells *in vitro* and *in vivo* [1–4].

A. Adenovirus Biology

The adenovirion is a nonenveloped icosohedral capsid containing a linear double-stranded DNA genome of ~30–40 kb. Of the ~50 serotypes of human Ad, the most extensively characterized are serotypes 2 (Ad2) and 5 (Ad5) of subgroup C (reviewed in [5]). The 36-kb genomes of Ad2 and Ad5 are flanked by inverted terminal repeats (ITRs) which are the only sequences required in *cis* for viral DNA replication. A *cis*-acting packaging signal, required for encapsidation of the genome, is located near the left ITR (relative to the conventional map of Ad). The Ad genome can be roughly divided into two sets of genes (Fig. 1): the early region genes, E1A, E1B, E2, E3, and E4, are expressed before DNA replication and the late region genes, L1 to L5 are expressed to

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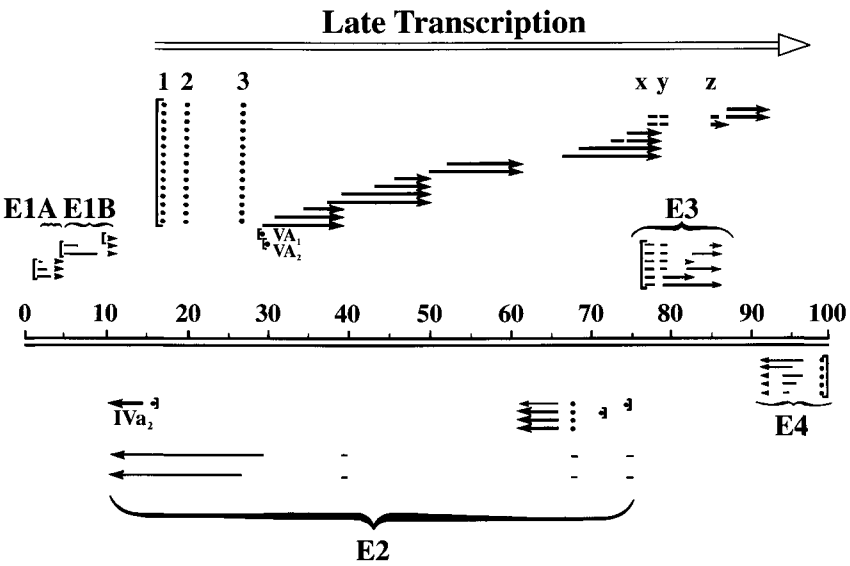


Figure 1 Transcription map of human adenovirus serotype 5. The 100 map unit (~36 kb) genome is divided into four early region transcription units, E1–E4, and five families of late mRNA, L1–L5, which are alternative splice products of a common late transcript expressed from the major late promoter (MPL) located at 16 μ. Four smaller transcripts, pIX, IVa, and VA RNAs I and II, are also produced. Not shown are the 103-bp inverted terminal repeats located at the termini of the genome involved in viral DNA replication and the packaging signal located from nucleotides 190 to 380 at the left end of the genome involved in encapsidation.

high levels after initiation of DNA replication. The E1A transcription unit encodes two major E1A proteins that are involved in transcriptional regulation of the virus and stimulation of the host cell to enter an S phase-like state and is the first early region to be expressed during viral infection. The two major E1B proteins are necessary for blocking host mRNA transport, stimulating viral mRNA transport and blocking E1A-induced apoptosis. The E2 region encodes proteins required for viral DNA replication and can be divided into two subregions; E2a encodes the 72-kDa DNA-binding protein and E2b encodes the viral DNA polymerase and terminal protein precursor (pTP). The E3 region, which is dispensable for virus growth in cell culture, encodes at least seven proteins, most of which are involved in host immune evasion. The E4 region encodes at least six proteins, some functioning to facilitate DNA replication, enhance late gene expression and decrease host protein synthesis. The late region genes are expressed from a common major late promoter (MLP) and are generated by alternative splicing of a single transcript. Most of the late mRNAs encode virion structural proteins. In addition to the early and late region genes, four other small transcripts are also produced. The

gene encoding protein IX (pIX) is colinear with E1B but utilizes a different promoter and is expressed at an intermediate time, as is the pIVa2 gene. Other late transcripts include the RNA polymerase III transcribed VA RNA I and II.

Virus infection is initiated through the Ad fiber protein binding to specific primary receptors on the cell surface [6, 7] followed by a secondary interaction between the virion penton base and $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins [8]. The efficiency with which Ad binds and enters the cell is directly related to the level of primary and secondary receptors present on the cell surface [9, 10]. Penton–integrin interaction triggers Ad internalization by endocytosis where it escapes from the early endosome into the cytosol prior to lysosome formation [11, 12]. The virion is sequentially disassembled during translocation along the microtubule network toward the nucleus where the viral DNA is released into the nucleus [13] where viral DNA replication, beginning 6 to 8 h postinfection, and assembly of progeny virions occur. The entire life cycle takes about 24–36 h, generating about 10^4 virions per infected cell. Ads have never been implicated as a cause of malignant disease in their natural host and, in immunocompetent humans, they generally cause only relatively mild, self-limiting illness. The reader is referred to an excellent review by Shenk [5] for a more comprehensive discussion of adenoviruses.

B. Adenovirus Vectors

Typically, Ads are converted into mammalian gene transfer vectors by replacing the E1 region with the foreign DNA of interest. This serves two important purposes. First, since the packaging constraint of Ad is 105% of wild type [14], deletion of E1 increases the cloning capacity to ~5 kb. Second, it renders the vectors replication-deficient, which is important with respect to safety for human gene therapy and other applications. These replication-deficient vectors must be propagated in E1-complementing cell lines such as 293 [15]. The E3 region can also be deleted from the vector since it is not required for virus propagation in culture. The combination of E1 and E3 deletions results in a cloning capacity of ~8 kb, a size that is more than adequate for most expression cassettes.

C. Early Methods of Constructing Recombinant Adenoviruses

All methods for manipulating Ad genomes for construction of vectors rely on the observation that purified viral DNA is infectious [16]. Early methods for generating recombinant Ad involved direct manipulation of viral DNA extracted from virions. These methods included *in vivo* homologous recombination between viral DNAs cotransfected into cells [17] and *in vitro* ligation of viral DNAs cleaved by restriction enzymes [18, 19]. However, a major limitation of these methods was that precisely defined alterations

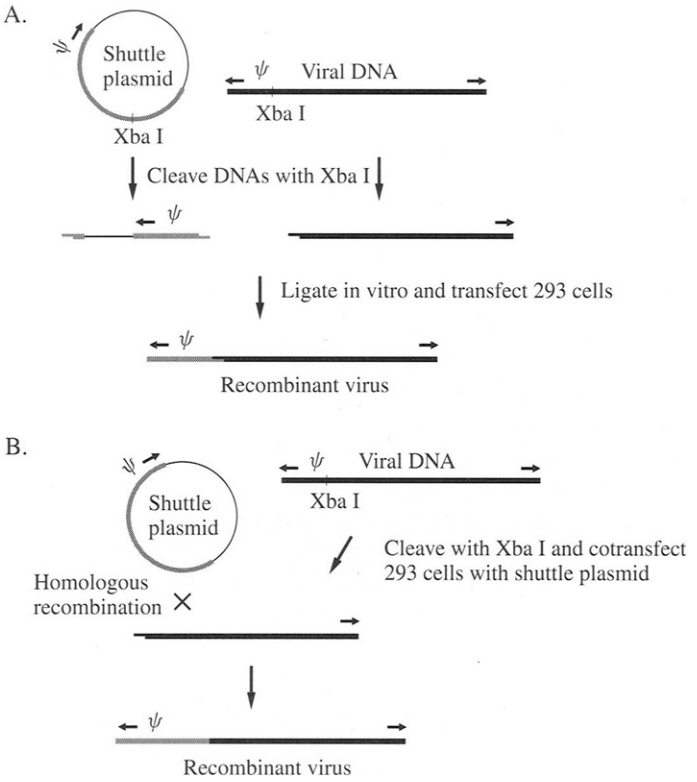


Figure 2 Early methods for constructing recombinant Ad vectors. In the method depicted in (A), shuttle plasmids bearing the modified left end of the Ad genome and purified viral DNA are cleaved with a restriction enzyme. The recombinant Ad genome is generated by direct *in vitro* ligation between the shuttle plasmid and the viral DNA and infectious recombinant viruses are generated by transfecting the ligation product into 293 cells. In the method depicted in (B), shuttle plasmids bearing the modified left end of the Ad genome and purified viral DNA are cotransfected into 293 cells. Recombinant viruses are generated as a result of *in vivo* homologous recombination between their overlapping region of homology. To minimize production of nonrecombinant parent virus, the viral DNA is cleaved with a restriction enzyme at the left end prior to cotransfection. Thick gray lines represent cloned Ad DNA, thick black lines represent Ad viral DNA, thin black lines represent bacterial plasmid sequences and small arrows represent ITR. ψ, packaging signal.

could not be introduced into the genome owing to the difficulty inherent in manipulating the large linear viral DNA. In 1981, Stow [20] devised a method to overcome this limitation, at least for modifications of the left end of the genome, that employed *in vitro* ligation between a cloned subgenomic Ad fragment and viral DNA (Fig. 2A). In this study, the left end *HpaI* E fragment (0 to 4.5 mu) bearing the E1A region of Ad2 was inserted into pBR322, thus

permitting the plasmid-borne E1A sequences to be easily modified. The plasmid and Ad5 viral DNA (from variant dl309 which has a unique *XbaI* site [21]) were cleaved with *XbaI* at 3.7 mu and ligated *in vitro*. Recombinant Ads bearing the modified E1A region were generated by transfecting the ligation product into 293 cells. The significance of this method lay in the ability to reconstruct an infectious viral genome by using a cloned Ad subgenomic fragment as one of the substrates, thus allowing modifications engineered into the cloned sequence to be readily introduced into the viral genome. However, despite this advance, the approach was limited because few unique restriction enzyme sites were available due to the relatively large size of the Ad genome. A method that overcame these limitations was developed by Kapoor and Chinnadurai [22], who demonstrated that recombinant Ads could be generated by *in vivo* homologous recombination between a cloned Ad subgenomic fragment and viral DNAs (Fig. 2B). As in the method developed by Stow [20], a shuttle plasmid bearing a left end subgenomic Ad fragment was first constructed to permit easy modification of E1 sequences. The shuttle plasmid along with viral DNA were cotransfected into 293 cells and *in vivo* homologous recombination between their overlapping sequences resulted in the generation of recombinant Ad bearing the modified E1. While this method does not rely on ligation of two restriction enzyme sites, the viral DNA is still cleaved prior to cotransfection to reduce the background of nonrecombinant parental virus. Currently, the unique *XbaI* site at 3.7 mu in Ad5 variant dl309 and the unique *ClaI* site at 2.5 mu in wt Ad5 are the most useful for manipulation of the left end.

While both of these early methods have proven useful, a major limitation is the requirement for viral DNA as a substrate for vector construction. Purification of viral DNA is time consuming and laborious and its use leads to a background of parental nonrecombinant viruses resulting in the need to screen a large number of viruses to isolate the desired recombinant. This can prove especially problematic when the parental virus has a growth advantage over the recombinant vector. Considering the importance and utility of Ad vectors as a tool for mammalian gene transfer, development of improved systems for their efficient and reliable construction was clearly imperative.

II. The Two-Plasmid Rescue System

One of the first methods that was developed to overcome the limitations of the earlier approaches was the two-plasmid rescue system (Fig. 3A). In this method, recombinant Ad vectors are generated by *in vivo* homologous recombination between two noninfectious plasmids cotransfected into 293 cells. Since its development, the two-plasmid rescue method has been widely used due to its simplicity. Other methods of constructing Ad vectors have

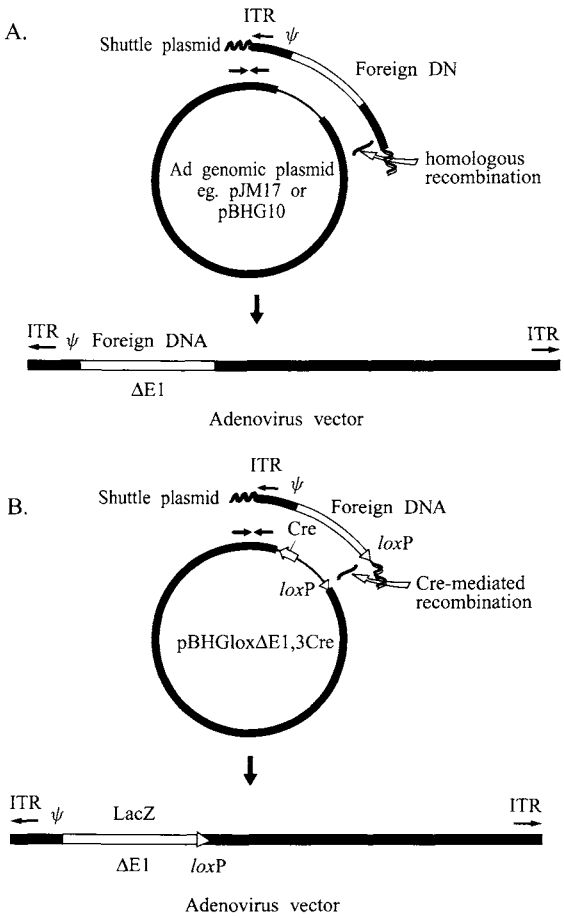


Figure 3 Construction of Ad vectors by (A) *in vivo* homologous recombination following cotransfection of 293 cells with a shuttle plasmid and an Ad genomic plasmid and (B) Cre-mediated site-specific recombination following cotransfection of 293Cre4 cells with a shuttle plasmid bearing a lox P site and pBHGloxΔE1,3. Ad sequences are represented by thick black lines, bacterial plasmid sequences are represented by thin black lines and the position and orientation of the *lox*P site is represented by a white triangle. Only the relevant portions of the shuttle plasmids are shown.

also been developed; however, discussion of these is beyond the scope of this chapter. The reader is encouraged to consult the other chapters in this book for further details regarding these other methods. The remainder of this section will focus on the development of the two-plasmid rescue method and the recent improvements that have been made to increase the method's efficiency and expand its versatility. The final section provides detailed protocols for

the construction, using the two plasmid rescue method, and propagation of recombinant Ad vectors.

A. Development of the Two-Plasmid Rescue System

In developing the two-plasmid rescue method, advantage was taken of observations made in early studies of Ad. One such observation was made in 1983 by Berkner and Sharp [23], who demonstrated that infectious recombinant Ads could be generated by cotransfecting 293 cells with cloned, noninfectious subgenomic Ad fragments. In this study, subgenomic fragments of Ad were cloned into the *EcoRI* site of pBR322. Infectious recombinant virus could be generated following cotransfection of 293 cells with these plasmids as a result of *in vivo* homologous recombination between their overlapping Ad sequences. Generation of infectious virus by this method was dependent on releasing the Ad ITR from at least one of the plasmids by *EcoRI* cleavage. The significance of this study lies in using only noninfectious plasmids, instead of viral DNA, as the substrates for vector construction, thus avoiding the need to isolate viral DNA and the problem of contaminating nonrecombinant virus.

Another key finding, made in 1983 by Ruben *et al.* [24], was the discovery that up to 10% of Ad viral DNA molecules become circularized following infection of mammalian cells. This permitted cloning of the entire Ad genome as an infectious bacterial plasmid. One such Ad genomic plasmid, pFG140, consisted of a circularized dl309 Ad genome [21] with a 2.2-kb insert in the *XbaI* site at 3.7 mu containing the ampicillin resistance marker and a bacterial origin of DNA replication [25]. This Ad genomic plasmid could be stably propagated in *Escherichia coli* and was capable of generating infectious virus following transfection into mammalian cells at efficiencies comparable to purified virion DNA.

In 1987, Ghosh-Choudhury *et al.* [26] observed that an Ad genomic plasmid of wild-type size but, unlike pFG140, bearing a deletion of the Ad protein IX gene was noninfectious. Based on this observation, they hypothesized that pIX was essential for the packaging of full-length Ad genomes. According to this hypothesis, reintroduction of the pIX gene into the noninfectious Ad genomic plasmid should restore its infectivity. To test this, 293 cells were cotransfected with the noninfectious pIX-deleted Ad genomic plasmid and a plasmid bearing the left end of the Ad genome including the pIX gene. Infectious viruses, all bearing the pIX gene, were generated as a result of *in vivo* homologous recombination between the two cotransfected plasmids thus demonstrating that pIX is essential for packaging full-length genomes.

Based on the early studies of Ad described above, McGrory *et al.* [27] developed the first two-plasmid rescue system designed specifically for constructing recombinant, replication-defective Ad vectors in which the E1 region was substituted with a foreign transgene as depicted in Fig. 3A. To accomplish

this the infectious Ad genomic plasmid pFG140 was modified by replacing the 2.2-kb insert with a 4.4-kb segment to generate the 40-kb plasmid pJM17. The resulting genome exceeded the packaging constraint of Ad and was noninfectious but could replicate following transfection into 293 cells. To generate infectious recombinant Ads bearing foreign DNA of up to 5.4 kb in place of the E1 region, 293 cells were cotransfected with pJM17 and a shuttle plasmid bearing the left end of the Ad genome with the desired E1 region substitution. Since, in principle, neither plasmid was infectious only the desired recombinant E1 substituted vector should be generated as a result of *in vivo* homologous recombination between the overlapping Ad sequences in the shuttle plasmid and pJM17. While this system proved to be useful and highly successful, it was observed that pJM17 was not absolutely noninfectious, being able to generate infectious virus, albeit at low frequencies, following single transfection into 293 cells. It was discovered that the restoration of infectivity of pJM17 was due to deletions of the bacterial plasmid sequences following transfection into 293 cells resulting in size reduction to within the packaging constraints of Ad.

The two plasmid rescue method was refined in 1994 by Bett *et al.* [28]. In this iteration of the system, an improved Ad genomic plasmid, pBHG10, was constructed to address the shortcoming of pJM17 and to introduce additional flexibility into the system. The plasmid pBHG10 contains essentially the entire Ad5 genome joined at the ITRs with two modifications. The first modification is a deletion from 0.5 to 3.7 mu, which removes the E1A region as well as the packaging signal required for encapsidation of the adenoviral genome thus rendering the plasmid noninfectious. The second modification was removal of ~2.7 kb from the nonessential E3 region, from 78.3 to 85.8 mu, and introduction of a *PacI* restriction enzyme site. A series of shuttle plasmids were also developed to be used in conjunction with pBHG10. These shuttle plasmids contained the left 15.8 mu of the Ad5 genome including the left end ITR and the packaging signal but with a 3181-bp deletion in E1 from 0.9 to 9.8 mu into which a polylinker was introduced for transgene insertion. This version of the two-plasmid rescue system offered several improvements over the original system of McGrory *et al.* [27]. First, the combination of the E1 region deletion in the shuttle plasmid and the E3 region deletion in the Ad genomic plasmid increased the cloning capacity of the recombinant vector, permitting rescue of up to ~8 kb of foreign DNA. Second, recombinant vectors bearing foreign DNA insertions in the E3 region could be easily constructed by utilizing the unique *PacI* site in the Ad genomic plasmid. To simplify cloning into the large pBHG plasmids, insertion of foreign DNA into the *PacI* site is facilitated by using the kanamycin resistant pABS series of small shuttle plasmids (www.microbix.com). The pABS plasmids bear two *PacI* sites which flank a polylinker and the kanamycin resistance gene. The kanamycin resistance gene is flanked by *SwaI* sites. The foreign DNA is first inserted into the polylinker of the pABS plasmid. The *PacI* fragment bearing

the foreign DNA and the kanamycin resistant gene is then cloned into the *PacI* site of the pBHG plasmid. Following transformation of *E. coli*, positive clones bearing the E3 insertion are easily identified by their resistance to both ampicillin and kanamycin. Finally, the kanamycin resistance gene is collapsed out of the pBHG plasmid by *SwaI* digestion and religation, leaving behind the foreign DNA in the E3 region. Thus, by using this system, a total of ~8 kb of foreign sequence could be easily rescued into the E1 and/or E3 regions of the recombinant vector. Third, the deletion of the packaging signal rendered pBHG10 absolutely noninfectious and, thus, all progeny virus generated following cotransfection were the desired recombinant. This version of the two-plasmid rescue system has become very popular for construction of E1 replacement vectors due to its versatility and simplicity; one need only clone the foreign DNA into the small shuttle plasmid and cotransfect it along with pBHG10 into 293 cells to generated recombinant vectors. However, one limitation of the two plasmid rescue method, especially for those not experienced in adenovirology, was the low efficiency of vector rescue if cells or transfection parameters were suboptimal.

B. Refinements to the Two-Plasmid Rescue Method

In 1999, Ng *et al.* [29] hypothesized that the low efficiency of vector rescue was due, in part, to the inefficiency of *in vivo* homologous recombination. Consistent with this hypothesis was the observation that the plaque-forming efficiency of the infectious Ad genomic plasmid pFG140 was ~100-fold higher than that achieved by a typical cotransfection for vector rescue. To address this limitation the two-plasmid rescue system was modified to make use of high efficiency site-specific recombination catalyzed by bacteriophage P1 recombinase Cre instead of homologous recombination (Fig. 3B). To accomplish this, a loxP site was inserted into pBHG10, 5′ of the pIX gene and into the shuttle plasmid, 3′ of the foreign transgene. Thus, vector rescue could be achieved by high efficiency Cre-mediated recombination between the two modified plasmids following their cotransfection into 293 cells expressing Cre recombinase (293Cre4 [30]). Ng *et al.* [29] demonstrated that the efficiency of vector rescue by Cre-mediated recombination was ~30-fold higher than by homologous recombination.

Further improvements were subsequently introduced when Ng *et al.* [31] demonstrated that replacement of the single ITR in the shuttle plasmid with a head to head ITR junction resulted in a 14-fold increase in the efficiency of homologous recombination mediated vector rescue. Combining Cre-mediated recombination and shuttle plasmids bearing an ITR junction increased the efficiency of vector rescue by ~100-fold over the earlier methods of McGrory *et al.* [27] and Bett *et al.* [28]. A number of nonmutually exclusive explanations were postulated to account for the effect of ITR junctions on vector rescue

efficiency (Fig. 4). Based on the fact that ITR junctions serve as an efficient origin of viral DNA replication [25] in contrast to a single ITR linked to plasmid DNA and that the ITRs are the only *cis*-acting Ad sequences required for viral DNA replication, it was postulated that shuttle plasmids bearing an ITR junction, in contrast to shuttle plasmids having only a single ITR, were capable of virus-mediated DNA replication following cotransfection of 293 cells with the Ad genomic plasmid which would supply all the *trans*-acting factors required for viral DNA replication. Thus, the increased vector rescue efficiency may reflect an increase in the substrate pool for recombination. In addition, since Ad DNA replicates as a linear molecule (reviewed in [32]), it is also possible that linearization of the shuttle plasmid by ITR junction-mediated DNA replication may produce a preferred substrate for recombination in contrast to shuttle plasmids bearing a single ITR which remains circular. Also, generation of an infectious genome may be more complex following recombination between a circular and a linear DNA molecule (single ITR shuttle plasmid and replicating Ad genomic plasmid) (Fig. 4A) than between two linear DNA molecules (replicating shuttle and Ad genomic plasmid) (Fig. 4B). In the former case (Fig. 4A), recombination would first result in integration of the circular substrate into the linear substrate. This intermediate is not packagable owing to its size [14] and the distance between the packaging signal and the genome terminus [33]. An infectious packagable genome could be generated from this intermediate by a second step following DNA replication in which the internal ITR is utilized as an origin of replication through the formation of a panhandle structure and repair, a process that is known to occur [34] but which might be less efficient than utilization of the terminal ITRs. In contrast, in the latter case (Fig. 4B), a packagable, infectious genome is generated immediately following recombination between two linear substrates.

To further expand the versatility of this method, the system was modified to permit high-efficiency Cre-mediated vector rescue to be achieved using the ubiquitous 293 cells (or any other E1-complementing cell line) thus abrogating the need for Cre-expressing cell lines which are not as widely available as the parental 293 cell line. To accomplish this, a Cre expression cassette was inserted into a region of the Ad genomic plasmid which would not contribute to the final recombinant vector genome but permitted transient Cre expression following cotransfection. The vector rescue efficiency following cotransfection of 293 cells using this Ad genomic plasmid was found to be nearly as high as with 293Cre4 cells [31].

One limitation of Cre-mediated vector rescue is that it would be unsuitable for constructing vectors bearing loxP sites elsewhere in the genome designed, for example, to regulate transgene expression [35–37] or to inhibit vector packagability [38] since it would lead to undesired Cre-mediated vector rearrangements. This was addressed by modifying the two plasmid rescue

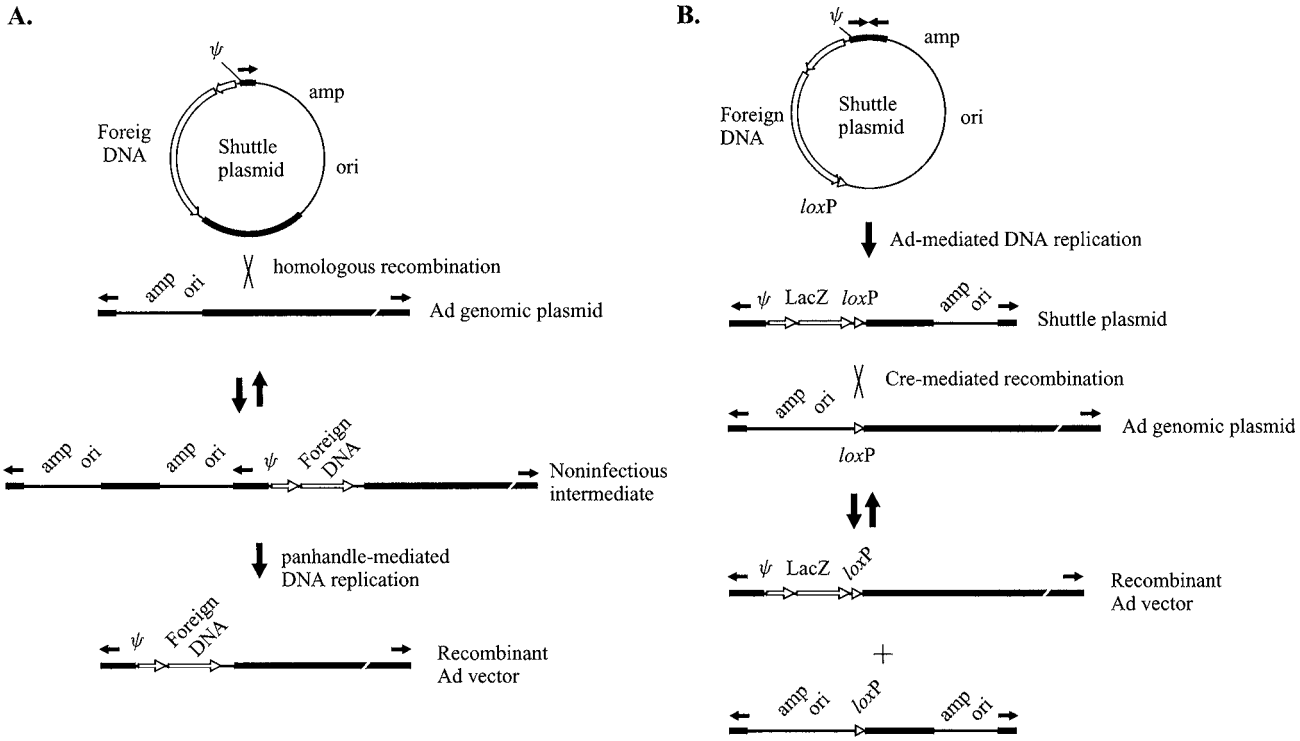


Figure 4 Postulated mechanisms of vector construction by (A) homologous recombination between a shuttle plasmid bearing a single ITR and an Ad genomic plasmid and (B) Cre-mediated recombination between a shuttle plasmid bearing an ITR junction and an Ad genomic plasmid.

method to utilize the yeast FLP-mediated site-specific recombination system [39]. With loxP sites in the Ad genomic and shuttle plasmids replaced with frt sites and the Cre-expression cassette replaced with a FLP-expression cassette in the Ad genomic plasmid the efficiency of FLP-mediated vector rescue was comparable to that mediated by Cre. The choice of either Cre- or FLP-mediated recombination further expanded the versatility of the two plasmid rescue method by permitting high-efficiency vector rescue in cases where one of the recombinases is unsuitable or undesirable for vector construction.

C. The Ad Genomic Plasmid

A variety of Ad genomic plasmids are available for construction of vectors by site-specific recombination (Fig. 5). The plasmids pBHGloxE3Cre and pBHGfrtE3FLP bear a wild-type E3 region and are used to generate vectors by Cre-mediated and FLP-mediated recombination, respectively. Owing to the size constraints of Ad [19], the maximum foreign DNA insert that can be rescued into an E1-deleted vector with a wild-type E3 region is ~5 kb. The plasmids pBHGloxΔE3(X1)Cre and pBHGfrtΔE3(X1)FLP have a 1864-bp deletion in the E3 region and thus permit foreign sequences up to ~7.2 kb to be rescued into vectors, whereas the plasmids pBHGloxΔE1,3Cre and pBHGfrtΔE1,3FLP have a 2653-bp deletion in the E3 region and permit rescue of up to ~8. While these latter two plasmids offer maximum cloning capacity, vectors bearing this larger E3 deletion may grow slightly slower and result in lower yields (~2-fold) than vectors bearing the wild-type E3 or smaller E3 deletion (F. L. Graham; unpublished results). As with the earlier pBHG10 based methods, the unique *PacI* sites in pBHGfrtΔE1,3FLP, pBHGfrtΔE1,3FLP, pBHGloxΔE3(X1)Cre, and pBHGfrtΔE3(X1)FLP permit insertion of foreign sequences into the E3 deletion for rescue into virus if desired. The choice of these Ad genomic plasmids is dictated by the size of the foreign sequence to be rescued, whether a wild-type or a deleted E3 region is desired and which site-specific recombination system is preferred/necessitated.

D. The Shuttle Plasmid

A variety of shuttle plasmids are available for insertion and rescue of foreign sequences into Ad vectors by Cre or FLP-mediated recombination (Fig. 6). The shuttle plasmids pDC311 and pDC312 are designed for rescue of expression cassettes into E1 by Cre-mediated recombination and pDC511 and pDC512 for rescue by FLP-mediated recombination. The shuttle plasmids pDC315, pDC316, pDC515, and pDC516 carry promoters and poly(A) sequences and are designed for insertion of coding sequences. PDC315 and pDC316 use Cre-mediated recombination and pDC515 and pDC516 use FLP-mediated recombination. The polycloning site in these plasmids is flanked by

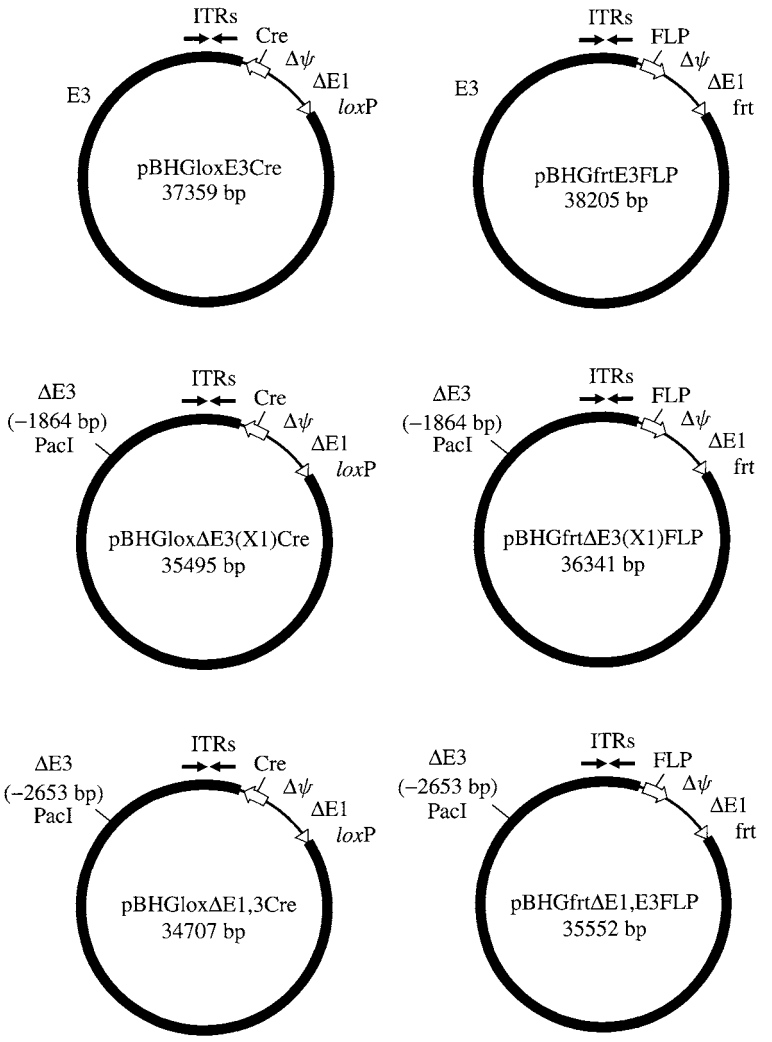
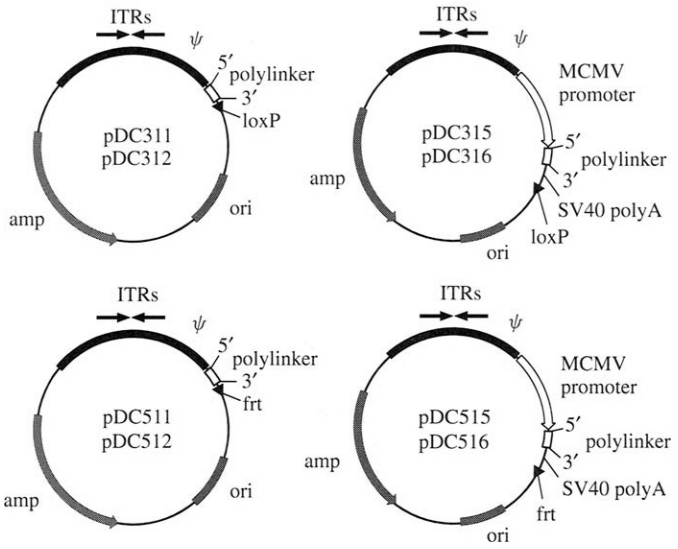


Figure 5 Ad genomic plasmids used for vector rescue by *in vivo* site-specific recombination. The plasmids pBHGloxE3Cre, pBHGloxΔE3(X1)Cre, and pBHGloxΔE1,3Cre are used to rescue vectors by Cre-mediated recombination bearing a wildtype E3 region, a 1864 bp deletion or a 2653 bp deletion of E3, respectively. Analogous plasmids pBHGfrtE3FLP, pBHGfrtΔE3(X1)FLP and pBHGfrtΔE1,3FLP are used to rescue vectors by FLP-mediated recombination. The unique *PacI* restriction enzyme site in pBHGloxΔE1,3Cre, pBHGloxΔE3(X1)Cre, pBHGfrtΔE3(X1)FLP, and pBHGfrtΔE1,3FLP permit insertion of foreign sequences into the E3 deletion. Ad and bacterial plasmid sequences are represented by thick and thin lines, respectively, and *lox*P or frt sites are represented by "▷".

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                    XbaI  EcoRI      StuI      NheI      BglII     HindIII  Ecl136II  SalI
                                                                              SacI      AccI
pDC311  5′ TCTAGAGAATTCAGGCCTGCTAGCAGATCTAAGCTTGAGCTCGTCGAC  3′

                    XbaI  EcoRI      StuI      NheI      BglII     HindIII  Ecl136II  SalI
                                                                              SacI      AccI      BamHI
pDC511  5′ TCTAGAGAATTCAGGCCTGCTAGCAGATCTAAGCTTGAGCTCGTCGACGGATCC  3′

                          SalI
                    XbaI  AccI  Ecl136II  HindIII  BglII     NheI      StuI      EcoRI     BamHI
pDC312                            SacI
pDC512  5′ TCTAGAGTCGACGAGCTCAAGCTTAGATCTGCTAGCAGGCCTGAATTCGGATCC  3′

                                                                        SalI
pDC315          EcoRI             NheI      BamHI                     AccI
pDC515  5′ GAATTCAAGCTGCTAGCAAGGATCCAGCTTGTCGAC  3′

                          SmaI
                          XmaI                          HindIII  Ecl136II  SalI
pDC316          EcoRI     ——      BglII                          SacI      AccI
pDC516  5′ GAATTCCCCGGGAGATCTAAGCTTGAGCTCGTCGAC  3′
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Figure 6 Shuttle plasmids and their polylinker sequences used for vector rescue by *in vivo* site-specific recombination. The shuttle plasmids pDC311 (3276 bp), pDC312 (3288 bp), pDC315 (3913 bp), and pDC316 (3913 bp) are used to rescue vectors by Cre-mediated recombination. The shuttle plasmids pDC511 (3277 bp), pDC512 (3277 bp), pDC515 (3957 bp), and pDC516 (3957 bp) are used to rescue vectors by FLP-mediated recombination. The plasmids pDC311, pDC312, pDC511, and pDC512 are used for insertion of expression cassettes (inserts with a promoter/enhancer and polyadenylation signal as well as coding sequence). The plasmids pDC315, pDC316, pDC515, and pDC516 bear a polylinker flanked by the murine cytomegalovirus immediate–early promoter/enhancer and SV40 polyadenylation signal and are used for insertion of coding sequences (e.g., cDNAs).

a murine cytomegalovirus (MCMV) immediate–early promoter and the SV40 polyadenylation signal for high level transgene expression in most cell types. The choice of shuttle plasmid is dictated by whether expression from a strong viral promoter is desired, by the orientation of the polylinker and by the site-specific recombination system desired for vector rescue. It has been observed that higher expression levels are obtained when the transcription orientation of the transgene is in the same direction as E1 and that the MCMV immediate early promoter is stronger in most cell lines than its more commonly used human counterpart [40].

III. Protocols for the Two-Plasmid Rescue System

The remainder of this chapter provides detailed protocols for each of the steps involved in the rescue and propagation of recombinant Ad vectors. A flow chart of these steps is presented in Fig. 7. Briefly, 293 cells are cotransfected with the Ad genomic plasmid and the shuttle plasmid. The recombinant vector is generated by *in vivo* site-specific recombination between the two plasmids and forms a plaque in the cell monolayer. The plaques are isolated, the virus expanded, and the vector DNA is extracted for confirmation by restriction enzyme digestion. The vector is plaque purified by titration and a high titer stock is generated which is then purified by CsCl banding and characterized with respect to concentration, DNA structure, level of RCA contamination and transgene expression. It is recommended that all the steps outlined in Fig. 7 be followed. However, since all infectious viruses generated after cotransfection are the desired recombinant [29, 31, 39], vector production can be expedited if necessary by following one or more shortcuts indicated in Fig. 7 and described in section III.I.

A. Preparation of Plasmid DNA

The foreign DNA is inserted into an appropriate shuttle plasmid and transformed into *E. coli* by conventional molecular biology techniques. This section describes the preparation of high-quality plasmid DNA for cotransfection.

MATERIALS

1. Plasmid DNA: All plasmids described in this chapter and their sequences can be obtained from Microbix Biosystems Inc. (www.microbix.com).
2. Sterile LB broth (Lennox) (Difco) and LB–agar plates supplemented with 50 μg/mL ampicillin. *Optional:* Sterile Super Broth; LB broth

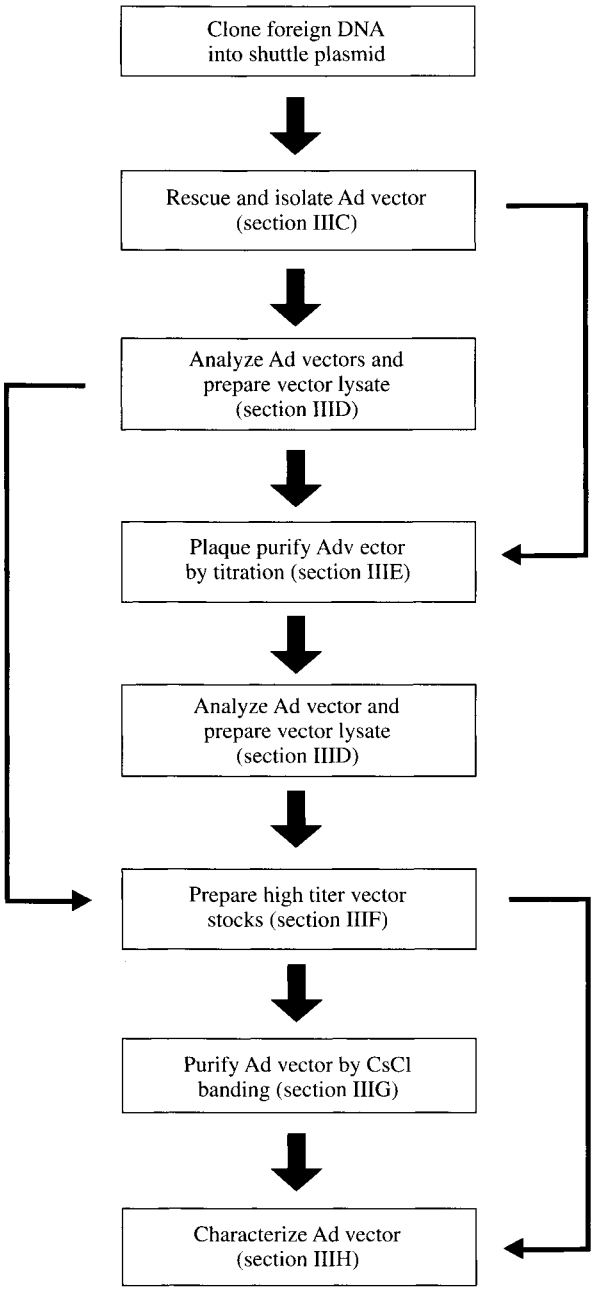

Figure 7 Overview of the steps involved in rescue, propagation, purification, and characterization of Ad vectors. The recommended steps are indicated by thick arrows. Acceptable alternatives to expedite vector production are indicated by thin arrows (see section III.I).

supplemented with 22 g/mL peptone, 15 g/mL yeast extract, 1 g/mL D-glucose, 0.005 N NaOH, and 50 μg/mL ampicillin.
3. Solution I: 10 mM EDTA, pH 8.0, 50 mM glucose, 25 mM Tris, pH 8.0, prepared from sterile stock solutions.
4. Solution II:1% SDS, 0.2 N NaOH, freshly prepared.
5. Solution III: 3 M potassium acetate, 11.5% glacial acetic acid, autoclave sterilized.
6. Isopropanol.
7. TE: 10 mM Tris, pH 8.0, 1 mM EDTA, pH 8.0, autoclave sterilized.
8. Pronase stock solution: 20 mg/mL pronase in 10 mM Tris, pH 7.5; preincubate at 56°C for 15 min, followed by 37°C for 1 h. Aliquot and store at −20°C.
9. Pronase–SDS solution: 0.5 mg/mL pronase (above) in 0.5% SDS, 10 mM Tris, pH 7.4, 10 mM EDTA pH 8.0.
10. CsCl (biotechnology grade).
11. 10 mg/mL ethidium bromide.

METHOD

1. Inoculate 5 mL of LB supplemented with 50 μg/mL ampicillin with bacteria bearing the desired plasmid in the morning. Incubate culture at 37°C with shaking. For the large Ad genomic plasmid, bacterial cultures should be started from well-isolated colonies picked from a bacterial plate less than 1 week old.
2. Inoculate 500 mL of LB supplemented with 50 μg/mL ampicillin with the above culture in the late afternoon. Incubate overnight at 37°C with shaking. *Optional:* For higher yields of plasmid DNA use richer medium such as Super Broth.
3. Transfer culture to a centrifuge bottle and pellet bacteria by spinning at 6000 *g* for 10 min at 4°C. Resuspend bacterial pellet in 40 mL of cold solution I so that no cell clumps are visible.
4. Add 80 mL of freshly prepared solution II, mix thoroughly but gently by swirling to produce a relatively clear, viscous lysate.
5. Add 40 mL of cold solution III, mix thoroughly but gently by swirling and incubate for 20 min on ice. The viscosity should be greatly reduced and a white precipitate should form.
6. Add 10 mL of dH_2O and centrifuge at 4°C for 10 min at 6000 *g*.
7. Collect the supernatant by filtering it through two to three layers of cheesecloth into a centrifuge bottle.
8. Add 100 mL (0.6 vol) of isopropanol, mix well, and incubate for 30 min at room temperature to precipitate plasmid DNA, centrifuging at 4°C for 10 min at 6000 *g* to pellet plasmid DNA.
9. Discard the supernatant and air dry the pellets for 15 min. Wipe inside the rim with a clean KimWipe to remove all residual isopropanol.

10. Dissolve plasmid DNA pellet in 5 mL TE and transfer to a 50-mL conical tube.
11. Add 2 mL pronase–SDS solution. Mix well and incubate for 30 min at 37°C.
12. Add 8.6 g CsCl, mix to dissolve completely, and incubate on ice for 30 min.
13. Centrifuge at 3000 *g* for 30 min at 5°C. Slowly collect the supernatant using a 10 cc syringe and 16-gauge needle, avoiding as much of the pellicle as possible.
14. Transfer to a VTi65 ultracentrifuge tube. Add 25 μL of 10 mg/mL ethidium bromide and fill the tube with light parafin oil.
15. Seal the tube and mix by inversion. Centrifuge in a Beckman VTi 65.1 rotor at 55,000 rpm for 10–14 h at 14°C.
16. Remove tube and support it with a stand. The supercoiled plasmid DNA band should be the thick red band in the gradient. Puncture the top of the tube to allow entry of air and collect the plasmid DNA through the side of the tube with a 3 cc syringe and 18-gauge needle by puncturing the side of tube just below the band. Except when recovering plasmid DNA bands, keep the tubes in the dark or covered with foil to avoid unnecessary exposure to fluorescent or UV light.
17. Transfer plasmid DNA to a 15-mL polypropylene tube containing 5-mL isopropanol which has been saturated with CsCl in TE. Mix immediately to extract the ethidium bromide into the solvent layer. Allow the phases to separate and discard the ethidium bromide-solvent (pink) layer. Repeat extraction until the solvent layer is colorless.
18. Add TE to bring the volume up to 4 mL, add 8 mL cold 95% ethanol, and mix by inversion to precipitate the DNA.
19. Spin at 3000 *g* in a table-top centrifuge at room temperature for 15 min to pellet DNA. Wash pellet twice with 5 mL 70% ethanol.
20. Remove as much of the 70% ethanol as possible, allow the pellet to dry, and resuspend with an appropriate volume of TE. Ideally, the concentration should be 1 to 2 μg/μL.
21. Determine the plasmid DNA concentration by OD_{260} and digest a sample with appropriate diagnostic restriction enzymes and confirm the structure by agarose gel electrophoresis.

B. Cell Culture

Low-passage 293 cells are maintained in 150-mm dishes and are split 1 to 2 or 1 to 3 when they reach confluency (every 2 to 3 days). Generally, a ~90% confluent 150-mm dish of 293 cells is split into 10 60-mm dishes for use the next day for cotransfections. Never allow the cells to become overconfluent or to be seeded too thinly. Change the medium regularly between splits (twice weekly

if they are not growing rapidly enough to permit splitting every 2–4 days). A sufficient number of ampoules of the cells should be frozen and stored in liquid N_2 to permit initiation of new cultures when the passage number of the lab stocks has reached 40–45 passages or when the cells are no longer behaving well under agar overlays (see sections III.C and III.E). Higher passage or poorly adherent cells which are unsuitable for cotransfections or titrations may still be adequate for virus propagation. 293N3S are suspension-adapted 293 cells and can be used for large-scale vector production instead of 293 cells due to greater ease of handling.

MATERIALS

1. Low-passage 293 and 293N3S cells (Microbix Biosystems Inc.).
2. Complete medium: MEM (Gibco BRL 61100) containing 10% fetal bovine serum (FBS) (heat inactivated), 100 units/mL penicillin/streptomycin, 2 mM L-glutamine, and 2.5 μg/mL fungizone.
3. Joklik's modified-MEM (Gibco BRL 22300) supplemented with 10% horse serum (heat inactivated).
4. Citric saline: 135 mM KCl, 15 mM sodium citrate, autoclave sterilized.
5. Spinner flasks (Bellco).

Prewarm all cell culture reagents to 37°C prior to use.

METHOD

1. Remove medium from 150-mm dish of 293 cells and rinse monolayer twice with 5 mL citric saline.
2. Remove citric saline from step 1, add 0.5 mL citric saline, and leave the dish at room temperature until cells start to round up and detach from the dish (no more than 15 min).
3. Tap the side of the dishes to detach all cells.
4. Resuspend cells with complete medium and distribute into new dishes.

293N3S cells are grown at 37°C in spinner flasks in Joklik's modified MEM supplemented with 10% horse serum (heat inactivated) and should be diluted 1 to 2 or 1 to 3 when the density reaches 5×10^5 cells/mL.

C. Cotransfection

Under optimal conditions, large numbers of plaques are typically generated by cotransfecting a single 60-mm dish of 293 cells with 2 μg of the shuttle plasmid and 2 μg of the Ad genomic plasmid by site-specific recombination (Table I). However, many factors can influence the efficiency of vector rescue including the quality of the DNA, the efficiency of transfection and especially the state of the 293 cells. Another important consideration is that the plaques

Table I
Vector Rescue Efficiency by *in Vivo* Site-Specific Recombination[a]

Shuttle plasmid	Ad genomic plasmid	Average plaques/60-mm dish
pCA35loxΔITR	pBHGloxΔE1,3Cre	43
	pBHGloxΔE3(X1)Cre	63
	pBHGloxE3Cre	48
pCA35frtΔITR	pBHGfrtΔE1,3FLP	41
	pBHGfrtΔE3(X1)FLP	27
	pBHGfrtE3FLP	25
	pFG140[b]	103

[a]60-mm Dishes of 293 cells were cotransfected with 2 μg of each plasmid and plaques were counted 10 days postcotransfection.
[b]60-mm Dishes of 293 cells were transfected with 0.5 μg of pFG140 and plaques were counted 10 days postcotransfection.

be well isolated. Thus, it is recommended that a range of DNA amounts be cotransfected to ensure that plaques are obtained and that they are well isolated. The infectious Ad genomic plasmid pFG140 [18] provides a control for transfection efficiency and under optimal conditions should yield up to ~100 plaques per 0.5 μg. The following is a protocol in which four 60-mm dishes of 293 cells are cotransfected with 0.5, 2, and 5 μg of each plasmid (Fig. 8).

MATERIALS

1. Monolayers of low passage 293 cells at ~80 to 90% confluency in 60-mm dishes.
2. Hepes-buffered saline (HBS): 21 mM Hepes, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 5.5 mM glucose, pH 7.1 (adjusted with NaOH), filter sterilized. Store at 4°C in small aliquots in tightly sealed plastic conical tubes.
3. Salmon sperm DNA (2 μg/μL in TE).
4. 2.5 M $CaCl_2$, filter sterilized.
5. Complete medium (see section III.B)
6. 2× Maintenance medium: 2× MEM (Gibco BRL 61100) supplement with 10% horse serum (heat inactivated), 200 units/mL penicillin/streptomycin, 4 mM L-glutamine, 5 μg/mL fungizone, and 0.2% yeast extract.
7. 1% Agarose solution, autoclave sterilized. Store at room temperature and melt in a microwave oven prior to use.
8. Ad genomic plasmid DNA (see section II.C).
9. Shuttle plasmid DNA with the desired foreign sequence inserted (see section II.D).

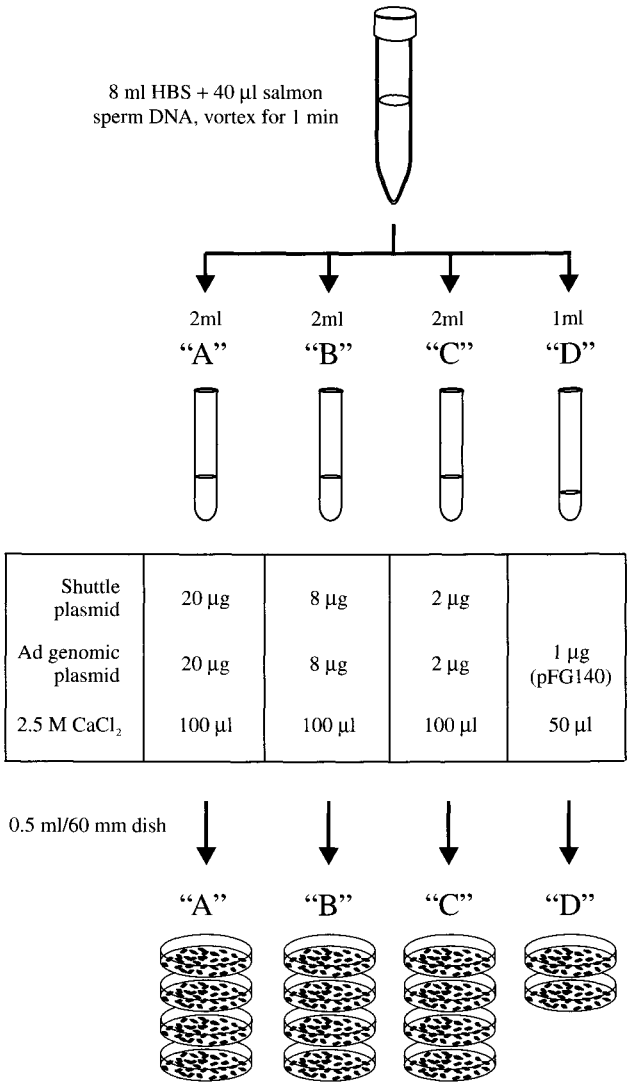

Figure 8 Standard protocol for Ad vector rescue by *in vivo* site-specific recombination.

10. Phosphate-buffered saline (PBS): 137 mM NaCl, 8.2 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, autoclave sterilized.
11. PBS^{++}: PBS supplemented with 0.68 mM sterile $MgCl_2$ and 0.5 mM sterile $CaCl_2$.
12. Glycerol, autoclave sterilized.

METHOD

1. Label four 60-mm dishes "A", four dishes "B", four dishes "C", and two dishes "D". Seed these dishes with 293 cells to reach ~80% confluency in 1 to 2 days for cotransfection.
2. In the late afternoon, 1 hour prior to cotransfection, replace the medium from the 60-mm dishes of 293 cells with 5 mL of freshly prepared complete medium without washing.
3. Meanwhile combine in a 15 mL conical tube 8 mL of HBS and 40 μL of salmon sperm DNA and vortex at maximum setting for 1 min.
4. Add 2 mL of the above solution to each of three polystyrene tube labeled "A", "B", and "C"and 1 mL to a fourth polystyrene tube labeled "D".
5. Add 2 μg of shuttle plasmid DNA and 2 μg of Ad genomic plasmid DNA to tube "A" (this will result in 0.5 μg of each plasmid per dish). Add 8 μg of shuttle plasmid DNA and 8 μg of Ad genomic plasmid DNA to tube "B" (2 μg of each plasmid per dish). Add 20 μg of shuttle plasmid DNA and 20 μg of Ad genomic plasmid DNA to tube "C" (5 μg of plasmid per dish). Add 1 μg of pFG140 DNA to tube "D". Gently mix each tube thoroughly.
6. To tubes "A", "B", and "C" add 100 μL of 2.5 M $CaCl_2$ dropwise with gentle mixing. To tube "C" add 50 μL of 2.5 M $CaCl_2$ dropwise with gentle mixing. Incubate the tubes at room temperature for 30 min. The solutions should become slightly cloudy.
7. Add 0.5 mL of the contents in tube "A"dropwise to the monolayer in each of the dishes labeled "A" without removing the medium. Repeat for tubes "B", "C", and "D". Distribute the precipitate evenly by rocking the dishes and return to the incubator.
8. The following morning, melt 1% agarose solution in a microwave oven and allow it to equilibrate to 45°C. Equilibrate 2× maintenance medium to 37°C. Prepare overlay solution by combining 75 mL of melted 1% agarose and 75 mL of 2× maintenance medium.
9. Remove the medium from each of the cotransfected dishes and add 10 mL of overlay solution prepared in step 8. Perform this step quickly to prevent the overlay solution from prematurely solidifying but gently to prevent disturbing the monolayer.
10. Allow the overlay to solidify at room temperature (10 to 15 min) and then return the dishes to the incubator. Plaques should begin to appear in 5 days and will continue to appear until about 12 to 14 days post-cotransfection.
11. Ten days post-cotransfection, pick well isolated plaques from the monolayer by punching out agar plugs with a sterile cotton plugged Pasteur pipet attached to a rubber bulb. It is recommended that

plaques be isolated at about 10 days post-cotransfection to ensure that those chosen are well isolated with no plaques overlapping.

12. Transfer the agar plugs into 0.5 mL PBS^{++} supplemented with glycerol to 10% in a suitable vial. Vortex briefly and store at −70°C.

D. Analysis of Recombinant Vectors and Preparation of Working Vector Stocks

Once plaques have been isolated, the viruses are expanded for extraction of vector DNA for analysis and to yield a working vector stock.

MATERIALS

1. 90% Confluent 60-mm dishes of 293 cells.
2. TE (see section II.A).
3. Complete medium (see section II.A).
4. Maintenance medium: MEM (Gibco BRL 61100) containing 5% horse serum (HS) (heat inactivated), 100 units/mL penicillin/streptomycin, 2 mM L-glutamine, and 2.5 μg/mL fungizone.
5. PBS^{++} (see section II.C).
6. Pronase–SDS solution (see section II.A).

METHOD

1. Seed 60-mm dishes of 293 cells (one per plaque) to reach ~90% confluency on the day of use.
2. Thaw virus plaque picks and vortex briefly. Remove medium from the 60-mm dishes of 293 cells and add 250 μL of the plaque pick. Adsorb for 1 h in the incubator rocking the dishes every 10 to 15 min.
3. Following adsorption, add 5 mL of maintenance medium and return dishes to incubator until complete cytopathic effect (CPE) is observed (≥90% cells rounded up and detached from dish, usually 4 to 5 days postinfection). It is important that the DNA be extracted following complete CPE so that vector DNA bands are clearly visible above the background of cellular DNA. If complete CPE is not reached by 5 days postinfection (most likely due to low multiplicity) then scrape the monolayer into the medium and transfer the cell suspension into a suitable vial and supplement with glycerol to 10%. Freeze (−70°C)–thaw the cell suspension and infect 60-mm dishes of 90% confluent 293 cells with 0.2 to 0.4 mL as described above. Complete CPE should be observed within 5 days and the vector DNA can be extracted.
4. Once complete CPE is reached, the dishes are processed as follows: Scrape the cells into the medium and transfer 1.5 mL of the cell suspension into an eppendorff tube for vector DNA extraction (see

step 5). Transfer the remainder of the cell suspension into a suitable vial, supplement with glycerol to 10% and store at −70°C. This lysate should contain a significant amount of virus (~10^8 pfu/mL) and can be used for plaque purification of the vector (section III.E) or can be used in preliminary experiments or for further vector expansion (section III.F).

5. To extract vector DNA, pellet cells by spinning at 3000 rpm in a microcentrifuge for 5 min.
6. Discard supernatant, resuspend the cell pellet in 0.2 mL pronase–SDS solution and incubate tubes at 37°C overnight.
7. Add 0.2 mL dH_2O and 1 mL 95% ethanol and mixing by inversion until the DNA precipitate is formed.
8. Pellet DNA by spinning in a microcentrifuge (maximum speed for 2 min) and wash pellet twice with 70% ethanol. Let the pellet dry and resuspend in an appropriate volume of TE (~35 μL). Dissolve DNA by heating at 65°C with occasional vortexing.
9. Digest 5 to 10 μL of the DNA with an appropriate restriction enzyme. Analyze the DNA structure by agarose gel electrophoresis to verify that the DNA structure of the recombinant virus is correct. If the infection of 293 cells has been complete, viral DNA bands should be readily visible superimposed on a smear of cellular DNA.

Once the DNA structure of the vector has been verified the virus can be plaque purified (section III.E).

E. Titration of Adenovirus

The procedure outlined below is used to plaque purify recombinant vectors as well as to determine the concentration of vector stocks. To accurately determine vector concentration, titrations should be performed in duplicate.

MATERIALS

1. 80 to 90% Confluent 60-mm dishes of 293 cells.
2. PBS^{++} (see section III.C).
3. 1% Agarose solution (see section III.C).
4. 2× Maintenance medium (see section III.C).
5. Glycerol, autoclave sterilized.

METHOD

1. Seed 60-mm dishes of 293 cells to reach ~80 to 90% confluency in 1 to 2 days for titration.
2. Prepare serial dilutions of the recombinant virus in PBS^{++} (10^{-2} to 10^{-6} for samples prepared in section III.D and 10^{-4} to 10^{-10} for samples prepared in sections III.F and III.G).

3. Remove the medium from the 60-mm dishes of 293 cells and infect with 0.2 mL of the diluted samples. Return dishes to the incubator and adsorb for 1 h, rocking the dishes every 10 to 15 min.
4. During the adsorption period, melt 1% agarose solution in a microwave oven and equilibrate to 45°C. Equilibrate 2× maintenance medium to 37°C.
5. Following 1 h adsorption, combine equal volumes of melted agarose solution with 2× maintenance medium, mix well, and gently overlay dishes with 10 mL. Perform this step quickly to prevent the overlay solution from solidifying prematurely but gently to prevent disturbing the monolayer.
6. Allow overlay to solidify for 10 to 15 min at room temperature and then return dishes to the incubator.
7. Plaques should start to appear about 4 days postinfection and should be counted 10 to 12 days postinfection. For isolation of recombinant virus by plaque purification well isolated plaques should be picked according to steps 11 and 12 of section III.C around 10 days postinfection. The plaque purified vectors are expanded according to section III.D and used as inoculum for the preparation of high-titer viral stocks (section III.F).
8. Determine the vector concentration in plaque forming units per ml (pfu/mL) as follows:

   titer = (number of plaques)(dilution factor)/(infection volume)

   Calculate the titer from dishes bearing approximately 20 to 80 plaques.

The number of plaques should vary in direct proportion to the dilution factor; otherwise, repeat the titration making sure that the samples are thoroughly mixed when setting up the serial dilutions.

F. Preparation of High-Titer Viral Stocks (Crude Lysate)

Since most of the virus remains associated with the infected cells until very late in infection (i.e., until the cells lyse), high-titer stocks can be easily prepared by concentrating infected 293 cells. The following protocol describes the production of high titer virus preparations using either monolayers of 293 cells or suspension cultures of 293N3S cells. 293N3S cells are preferable for the production of very large amounts of high-titer viral stocks due to the greater ease of handling suspension cultures. The following describes protocols for the preparation of crude lysates of high-titer vector stocks that are suitable for most experiments. Prior to the preparation of high-titer stocks, confirm that enough inoculum is available and if not, prepare an intermediate-scale virus stock by infecting two to three 150-mm dishes of 293 cells.

1. Preparation of High-Titer Viral Stocks (Crude Lysate) from Cells in Monolayer

MATERIALS

1. PBS^{++} (see section III.C).
2. Glycerol, autoclave sterilized.
3. 150-mm dishes of 80 to 90% confluent 293 cells.
4. Maintenance medium (see section III.D).

METHOD

1. Seed 150-mm dishes with 293 cells to be 80–90% confluent at time of infection. The number of dishes is dictated by the amount of vector desired.
2. Dilute vector sample prepared in section III.E, step 7 1:8 with PBS^{++}.
3. Remove medium from the 293 cells and add 1 mL of the diluted vector sample prepared in step 2 to each 150-mm dish of cells (moi of 1–10 pfu/cell).
4. Adsorb for 1 h in the incubator, rocking the dishes every 10 to 15 min. Following adsorption, add 25 mL maintaince medium and return dishes to the incubator. Examine daily for signs of CPE.
5. When CPE is nearly complete (most cells rounded but not yet detached) harvest by scraping the cells into the medium and centrifuging the cell suspension at 800*g* for 15 min.
6. Discard the supernatant and resuspend the cell pellet in 2 mL PBS^{++} supplemented with glycerol to 10% for each 150-mm dish infected. Freeze (−70°C) and thaw the crude virus stock prior to characterization of the vector (section III.H). Store aliquots at −70°C.

2. Preparation of High-Titer Viral Stocks (Crude Lysate) from Cells in Suspension

MATERIALS

1. 293N3S cells (Microbix Biosystems Inc.).
2. Joklik's modified MEM supplemented with 10% horse serum (heat inactivated) (see section III.B).
3. Spinner flasks (Bellco)
4. 1% Sodium citrate.
5. Carnoy's fixative: add 25 mL glacial acetic acid to 75 mL methanol.
6. Orcein solution: add 1 g orcein dye to 25 mL glacial acetic acid plus 25 mL dH_2O; filter through Whatman No. 1 paper.

METHOD

1. Grow 293N3S cells to a density of $2-4 \times 10^5$ cells/mL in 4 L complete Joklik's modified MEM supplemented with 10% HS. Centrifuge cell suspension at 750*g* for 20 min and save half of the conditioned medium. Resuspend the cell pellet in 0.1 vol fresh medium, and transfer to a sterile 500-mL bottle containing a sterile stir bar.
2. Add virus at an MOI of 1–20 pfu/cell and stir gently at 37°C. After 1 h, return the cells to the 4-L spinner flask and bring to the original volume using 50% conditioned medium and 50% fresh medium. Continue stirring at 37°C.
3. Monitor infection daily by inclusion body staining as follows:
   (a) Remove 5 mL from the infected spinner culture. Spin for 10 min at 750*g* and resuspend the cell pellet in 0.5 mL of 1% sodium citrate.
   (b) Incubate at room temperature for 10 min and then add 0.5 mL Carnoy's fixative and fix for 10 min at room temperature.
   (c) Add 2 mL Carnoy's fixative and spin 10 min at 750*g*. Discard supernatant and resuspend the pellet in a few drops of Carnoy's fixative. Add one drop of fixed cells to a slide and air dry for about 10 min Add one drop orcein solution and a coverslip and examine using a microscope. Inclusion bodies appear as densely staining nuclear structures resulting from accumulation of large amounts of virus and viral products at late times postinfection. Include a negative control in initial tests.
4. When inclusion bodies are visible in 80–90% of the cells (~3 days depending on the input MOI), harvest by centrifugation at 750*g* for 20 min in sterile 1-L bottles. Combine pellets in a small volume of medium, and spin again.
5. Discard supernatant and resuspend pellet in 20 ml PBS^{++} supplemented with 10% glycerol. Freeze (−70°C)–thaw and then aliquot and store at −70°C and characterize vector as described in section III.H.

G. Purification of Adenovirus by CsCl Banding

Many experimental studies can be performed using virus in the form of crude infected cell lysates prepared as described in sections III.E and III.F. However, for some experiments, particularly for animal work, it is desirable to use purified virus. The following protocol describes a method for purifying vectors obtained from 4-L of infected 293N3S cells or 30 × 150-mm dishes of 293 cells by CsCl banding.

MATERIALS

1. 10 and 100 mM Tris, pH 8.0, autoclave sterilized.
2. 5% Sodium deoxycholate, filter sterilized.
3. 2 M $MgCl_2$, autoclave sterilized.
4. DNAase I (100 mg bovine pancreatic deoxyribosenuclease I in 10 mL of 20 mM Tris, pH 7.4, 50 mM NaCl, 1 mM dithiothreitol, 0.1 mg/mL bovine serum albumin, 50% glycerol, aliquoted and stored at −20°C).
5. CsCl solutions:

Density (g/cc)	CsCl (g)	10 mM Tris, pH 8.0 (g)
1.5	90.8	109.2
1.35	70.4	129.6
1.25	54.0	146.0

   Dissolve CsCl into 10 mM Tris, pH 8.0, solution in the amounts indicated above to achieve the desired density solution and filter sterilize. Weigh 1.00 mL to confirm density.
6. Glycerol, autoclave sterilized.
7. Beckman SW41 and SW50 rotor and ultraclear tubes.
8. Slide-A-Lyzer dialysis cassettes (Pierce).

METHOD

1. Prepare crude cell lysate from infected cells as follows:
   (a) For 30 × 150-mm dishes as prepared in section III.E.1: when complete CPE is evident, scrape the cells into the medium, transfer the cell suspension to a centrifuge bottle, and spin for 10 min at 750*g*. Resuspend the cell pellet in 15 ml 0.1 M Tris–Cl, pH 8.0. Sample can be stored at −70°C.
   (b) For 4-L spinner cultures prepared in section III.E.2: when inclusion bodies are visible in 80–90% of the cells, harvest cells by centrifugation at 750*g* for 20 min in sterile bottles. Resuspend pellet in 15 mL 0.1 M Tris, pH 8.0. Samples can be store at −70°C.
2. Thaw sample and add 1.5 mL 5% Na deoxycholate for each 15 mL of cell lysate. Mix well and incubate at room temperature for 30 min. This results in a highly viscous suspension.
3. Add 150 μL 2 M $MgCl_2$ and 75 μL DNAase I solution to each 15 mL of cell lysate, mix well, and incubate at 37°C for 60 min, mixing every 10 min. The viscosity should be greatly reduced.
4. Spin at 3000*g* for 15 min at 5°C in the Beckman table-top centrifuge.
5. Meanwhile, prepare CsCl step gradients (one SW41 ultraclear tube for each 5 mL of sample): Add 0.5 mL of 1.5 g/cc solution to each tube. Gently overlay with 3.0 mL of 1.35 g/cc solution. Gently overlay this with 3.0 mL of 1.25 g/cc solution.

6. Apply 5 mL of supernatant from step 4 to the top of each gradient. If necessary, top off tubes with 0.1 M Tris, pH 8.
7. Spin at 35,000 rpm in an SW41 rotor at 10°C, for 1 h.
8. Collect virus band (should be at 1.25 d/1.35 d interface) with a needle and syringe by piercing the side of the tube. The volume collected is unimportant at this stage so try to recover as much of the virus band as possible. If more than one tube was used, pool virus bands into a single SW50.1 ultraclear tube.
9. Top off tubes with 1.35 g/cc CsCl solution if necessary and centrifuge in a SW50.1 rotor at 35,000 rpm, 4°C, for 16–20 h. (Alternatively, the pooled virus can be centrifuged in the SW41 rotor at 35,000 rpm, 10°C, 16–24 h.)
10. To collect the virus band, puncture the side of the tube just below the virus band with a needle and syringe. Collect the virus band in the smallest volume possible and transfer to a Slide-A-Lyzer dialysis cassette. Dialyze at 4°C against three changes of 500 mL 10 mM Tris, pH 8.0, for at least 24 h total.
11. After dialysis, transfer the virus to a suitable vial and add sterile glycerol to a final concentration of 10%. Store the purified virus in small aliquots at −70°C.

H. Characterization of Adenoviral Vector Preparations

Before the recombinant vector is used for experimentation the concentration should be determined, the DNA structure should be confirmed and expression of the transgene should be ascertained.

MATERIALS

1. All materials listed in section III.E.
2. TE (see section III.A).
3. 10 mM Tris, pH 8.0
4. 10% SDS
5. Pronase–SDS solution (see section III.A).
6. 3 M Sodium acetate, pH 5.2, autoclave sterilized.
7. 95 and 70% Ethanol.

METHOD

The concentration in pfu/ml is determined by titration on 293 cells as describe in section III.E. The concentration of virus particles, based on DNA content at OD_{260} can also be determine spectrophotometrically as follows:

1. Dilute (usually 20-fold) purified virus with TE supplemented with SDS to 0.1%. Set up blank the same except add virus storage buffer

(10 mM Tris, pH 8.0, supplemented with glycerol to 10%) instead of virus.
2. Incubate for 10 min at 56°C.
3. Vortex sample briefly.
4. Determine OD_{260}.
5. Calculate the number of particles/mL, based on the extinction coefficient of wildtype Ad as determined by Maizel *et al.* [41] as follows:

$$(OD_{260})(\text{dilution factor})(1.1 \times 10^{12}).$$

The DNA structure of the recombinant vector should be confirmed following large-scale preparation. Virion DNA can be extracted from CsCl banded virus for analysis as follows:

1. An appropriate volume (~25 μL depending on the concentration of the virus) of the purified virus is added to pronase–SDS solution to a final volume of 0.4 mL and incubated overnight at 37°C to lyse the virions and digest virion proteins.
2. Virion DNA is precipitated by adding 1/10 vol 3 M sodium acetate, pH 5.2, and 2.5 vol 95% ethanol and incubating at −20°C for 15 to 30 min.
3. Spin in microcentrifuge for 10 to 15 min at maximum speed.
4. Discard supernatant and wash DNA pellet twice with 70% ethanol.
5. Dry DNA pellet and resuspend in an appropriate volume of TE.

For crude preparations, viral DNA can be extracted following infection of 293 cells as described in section III.D. A sample of the vector DNA is digested with the appropriate diagnostic restriction enzyme(s) and the structure of the DNA is analyzed by agarose gel electrophoresis.

293 cells contain nts 1–4344 bp of Ad5 DNA [42] with consequent homology flanking the expression cassette of generation vectors. Therefore, the possibility exists that homologous recombination between the Ad vector and the Ad sequences present in 293 cells may result in the formation of $E1^+$ replication-competent Ad (RCA). The frequency with which Ad vectors recombine with Ad sequences in 293 cells is unknown but in general $E1^+$ RCA replicate faster than $E1^-$ vectors. Consequently the proportion of RCA increases with prolonged propagation of the vectors in 293 cells. RCA can act as a "helper" virus resulting in the mobilization of the replication-deficient E1-substituted vector in coinfected cells as well as cause tissue damage and pathogenicity. To minimize RCA contamination, vectors should not be serially propagated indefinitely. It is recommended that large-scale vector preparations be initiated from a stock prepared immediately after plaque purification (section III.E). If the original plaque purified stock is exhausted, plaque purification can be repeated. The presence and level of RCA contamination in vector stocks should be determined, especially if the vector is to be used for extensive

experimentation. A number of different approaches has been developed for the detection of RCA, including Southern blot hybridization [43], quantitative PCR [43] and biological assays [44].

I. Alternative Procedures to Expedite Vector Production

It is recommended that the steps outlined in Fig. 7 and detailed in the preceding sections be followed as they are well proven. However, since only the correct recombinant vector should be generated following cotransfection [29, 31, 39], several alternative procedures are acceptable to expedite vector production (Fig. 7).

1. Once the vector has been rescued following cotransfection (section III.C), it can be immediately titrated for plaque purification and the DNA structure can be checked afterward.
2. While plaque purification is strongly recommended (section III.E), especially if large quantities of the vector are to be generated for extensive experimentation, this step is not absolutely essential since all infectious virus generated should be the desired recombinant. Optionally, therefore, high-titer stocks can be generated directly from the plaques isolated following cotransfection.
3. As mentioned in section III.F, vector purification by CsCl banding, while recommended, may not be necessary for many experiments.
4. It is strongly recommended that the recombinant vector be isolated from individual plaques following cotransfection using the method described in section III.C. However, vector production can be expedited by omitting steps 8 through 12 in section III.C. In this case, following overnight cotransfection, remove the medium from the monolayers and add 5 mL of maintenance medium. If complete CPE is observed within 7 days post-cotransfection, then proceed from step 4 in section III.D. If complete CPE is not observed by 7 days post-cotransfection see section III.D, step 3.

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CHAPTER 5

Adenoviral Vector Construction II: Bacterial Systems

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I. Introduction

The use of adenovirus (Ad) as a vector for *in vitro* and *in vivo* gene delivery is expanding rapidly. Besides the use of Ad for gene therapy, it is a highly efficient tool to study *in vitro* and *in vivo* gene expression in cell types or tissues not easily transduced by other methods. Other purposes include the use of Ad for the production of high levels of recombinant, potentially therapeutic, proteins and for *in vivo* vaccination [1–6]. In fact, pioneering the applications of Ad as a gene expression vector were studies which demonstrated that high levels of expression of the SV40 large T antigen in Ad vectors could be achieved. This has become an important source for the biochemical analysis of SV40 T antigen [7, 8]. The ability of Ad vectors to efficiently transduce a variety of cell types and many different target organs *in vivo*, independent of active cell division, is considered an advantage over other vectors. Furthermore, high titers of virus and high levels of transgene expression can easily be obtained [3].

Extensive genetic and molecular analyses of adenovirus have resulted in a detailed knowledge of the viral life cycle and the function of the majority of viral proteins, further stimulating the use and modifications of Ad vectors [9, 10; Chapters 1 and 2, this volume]. The genome of the most commonly used human adenovirus (group C, serotype 5) consists of a linear 36-kb double-stranded DNA molecule. Transcription of the viral genome occurs on both

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strands and viral gene expression is coordinated through a precisely temporally regulated splicing program of almost all the transcripts. Early transcription units (E1, E2, E3, E4) are differentiated from late ones, depending on the expression pattern relative to the onset of viral DNA synthesis [9, 10]. The overlapping location of viral genes on the viral genome limits the molecular manipulations for vector constructions to the E1, E2, E3, and E4 regions. The earliest, first-generation Ad vectors have the E1 region deleted (E1°), rendering such vectors replication-deficient. In addition, in most AdE1° vectors the viral E3 region is also deleted, as the E3 functions are not required for the viral life cycle *in vitro* [9, 10]. In most cases a heterologous expression cassette with a transgene is inserted in place of the E1 region. Such AdE1° and AdE1°E3° vectors can be propagated to high yields in permissive E1-complementation cell lines, such as 293 cells [11] or PER.C6 cells [12; Chapter 6, this volume]. The latter prevent the occurrence of replication-competent adenovirus by recombination, allowing the production of safe, clinical-grade batches of Ad vectors. However, the high level of tissue toxicity and inflammation associated with first generation Ad vectors have stimulated further manipulation of the viral genome, resulting in vectors with simultaneous deletions of several regulatory regions, AdE1°E3°E2A° or AdE1°E3°E4° [13–25]. Importantly, AdE1°E3°E4-modified vectors, carrying the E4 ORF3 or E4ORF3 + ORF4 functions were able to allow persistent transgene expression *in vivo*, in selected animal models, in the absence of vector-induced toxicity and inflammation [14, 21, 26]. This renders these types of vectors, with a cloning capacity of approximately 11 kb, attractive for certain, such as liver-selective, gene therapy applications [27]. In this context, various studies have shown that high-capacity or gutless vectors, devoid of all viral genes [28, 29], also combine long-term transgene expression with reduced toxicity [30, 31]. The generation of gutless vectors will be described in Chapter 15.

This overview summarizes the recent development of novel technologies, which efficiently permit the rapid construction and generation of single or multiply deleted Ad in *Escherichia coli*. The construction of Ad in *E. coli* by various recombination techniques, emphasizing homologous recombination, will be summarized and compared to direct *in vitro* cloning technologies by ligation.

II. Generation of Ad: Traditional Approaches

Initially Ad E1° vectors were generated in eukaryotic cells, such as in 293 cells using two approaches: (i) the *in vitro* ligation method [32–35] and (ii) the homologous recombination method in 293 cells [36–39]. The *in vitro* ligation method uses whole viral DNA, cut at a unique site downstream from the viral E1 region, and ligated directly to a DNA fragment containing the viral left end

joined to a transgene; the ligation product is used to transfect 293 cells. This method is hampered by the large size of the Ad genome which limits the number of useful restriction sites available for *in vitro* ligation and contamination with wild-type virus. Efficient and improved *in vitro* ligation techniques for the construction of vectors in bacterial systems have been reported [40–42] and will be described below.

Alternatively, cotransfection into the complementation cells of the viral genome and plasmid molecules can generate the Ad by homologous recombination *in vivo*. These methods frequently generate a background of parental virus and repeated screening of many plaques is often required to isolate pure recombinant vectors. However, the development of counterselective methods against the parental wild type vector [43–46] has facilitated the screening for the recombinant virus. The homologous recombination method described by Bett *et al.* [36] uses two plasmids with overlapping sequences of homology that recombine *in vivo*. The first plasmid carries the entire Ad genome with a deletion of the DNA packaging signal and the E1A region. The second plasmid contains the left inverted terminal repeat (ITR), packaging signal, transgene and overlapping sequence with the first plasmid. Both plasmids are cotransfected into 293 cells and pure Ad is then isolated by plaque purification. The major limitation of this approach remains the low frequency of the recombination event and the potential instability of the large plasmid due to the presence of a head-to-head ITR junction [47]. However, due to a variety of novel and improved techniques, highly efficient methods are now available to generate Ad in mammalian systems, reviewed in Chapter 4.

Another method is based on the manipulation of the entire viral genome as an infectious yeast artificial chromosome (YAC) [48]. Targeted modifications of the viral genome are introduced by homologous recombination in yeast cells and infectious virions are generated after transfection of the adenovirus genome, excised from the YAC vector. Although clearly pioneering the subsequent studies of viral vector construction in bacterial systems, the YAC system requires the use of an additional host (yeast) and DNA yields are relatively low.

III. Generation of Ad: Bacterial Systems

Recently, several novel methods based on bacterial systems have been developed for the generation of Ad. Three basic methods have evolved to enable the manipulation of the full-length adenoviral genome as a stable plasmid and facilitate the efficient construction of precisely tailored and infectious Ad in *E. coli*. These methods are based on: (i) homologous recombination, (ii) direct ligation, and (iii) cosmid technology. All three methods offer major advantages over traditional approaches: (i) Manipulation of the viral genome at any point

is possible. (ii) Recombinant viral DNA is purified from individual bacterial clones and therefore generates homogenous virus preparations, obviating the need for tedious plaque screening and purification. (iii) Importantly, and in contrast to the traditional *in vivo* approaches, these methods entirely separate viral vector construction from virus production. The first step is performed in bacteria and the second step takes place in the mammalian complementation cell line. Therefore, each step can be carefully controlled and optimized. Trouble shooting is facilitated; for example, failure of producing a virus cannot be associated with the inability to generate the desired genome. The recombination and direct ligation methods are described below.

IV. Homologous Recombination in *E. coli*

The use of classical molecular biology techniques for the manipulation of the Ad genome is limited by its large size. Homologous recombination presents an alternative way to engineer DNA. In yeast, homologous recombination is particularly flexible and recombination between linear DNA fragments flanked by short-homology arms and endogenous recipients such as the yeast genome or YACs or a gapped plasmid [49, 50] is routinely used. The concept was also applied in *E. coli* by the cloning of short DNA fragments [51] and of PCR products [52] into gapped plasmids as targets. We mentioned above the work of Ketner *et al.* [48], who reported in 1994 the cloning and further manipulation of the Ad genome as an infectious YAC clone, taking advantage of the very efficient *Saccharomyces cerevisiae* recombination machinery. The availability of specific mutant bacterial strains allowed us and others to transfer this technology into *E. coli* and to bring the adenovirus genome manipulation back to the level of standard molecular biology.

Recombination is an essential process involved in the repair of DNA lesions, such as double-stranded breaks (DSBs) and the restart of replication forks that failed to progress to completion. The *E. coli* recombination machinery includes at least 25 different proteins among which the RecBCD enzyme and the RecA protein are major components of the initiation and pairing steps [53]. In wild-type cells, the RecBCD enzyme binds to the end of a dsDNA substrate and initiates unwinding. RecBCD degrades the 3′-terminated strand during unwinding until it reaches a chi site. The chi sequence is a recombination hotspot which modifies the enzymatic activities of RecBCD enzyme [54]. The nuclease activity of RecBCD is attenuated and RecA is loaded on the 3′ end of ssDNA, allowing the essential steps of pairing and strand exchange. The RecBCD nuclease activity is also responsible for the degradation of foreign DNA that does not contain any chi site. This last property explains why, in contrast to yeast, most bacteria do not recombine transformed DNA readily and are not widely used for plasmid manipulation by homologous recombination [55]. However, some bacterial strains where the RecBCD enzyme is

inactivated have been shown to be recombination-proficient. They harbor an additional suppressor (sbcA or sbcB) mutation that activates an alternative recombination pathway [56]. The genotype (*endA, sbcBC, recBC, galK, met, thi-1, bioT, hsdR, str*^{r}) of the BJ5183 bacterial strain [57] used in most of the studies discussed below is RecBC sbcBC and contains in addition an activated RecF pathway. This pathway has been shown to direct nonconservative recombination which is defined as a homologous recombination event generating one duplex molecule out of two duplex DNA molecules [58]. Subsequently, taking advantage of such recombination-proficient *E. coli* strains [59], several systems have been developed to manipulate the Ad genome by homologous recombination.

The different systems applying homologous recombination for Ad construction can be put into two groups depending whether the targeted vector is transfected as a linear DNA fragment or as a circle. In the first case, the recombinant plasmid is rescued by recombination between two linear DNA fragments (the donor fragment and the linearized vector). In this approach the only selection necessary is the recircularization of the plasmid. The first application of this technology for Ad construction was described by Chartier *et al.* [60], demonstrating (i) stable cloning of the entire viral genome into a bacterial plasmid and (ii) the use of such infectious bacterial plasmids to further introduce alterations into the viral genome. Subsequently, the basic protocol was extended and modified by others [61]. A second approach where the plasmid to be modified is transformed as a circle allows more flexibility but requires the development of complex selection systems. Such approaches have been reported and will be discussed [62, 63].

V. Homologous Recombination with Linear Ad Vector Genome Plasmids

The work by Chartier *et al.* [60] showed that stable maintenance of plasmids containing the entire Ad viral genome is achieved through the separation of the viral ITRs by the bacterial plasmid backbone, confirming the observations made earlier by Hanahan and Gluzman [64]. This was accomplished by the insertion of the left and right end of the Ad genome in their normal orientation into a colEI-derived bacterial plasmid ppolyII [65], using conventional cloning techniques. Such a plasmid linearized between the two Ad ends served as a vector to eventually incorporate the entire Ad viral genome. Cotransfection of the linearized vector DNA and linear Ad5 genomic DNA into the *E. coli* strain BJ5183 generated a stable circular plasmid containing the full-length Ad5 genome through homologous recombination within the end fragments of Ad5 (Fig. 1). Engineering unique restriction sites such as *Pac*I (noncutting within the Ad5 viral genome) immediately adjacent to the viral ITRs enabled

the precise release of a fully infectious viral genome (Fig. 1). In contrast, the closed circular plasmid was unable to generate any infectious virus, confirming that at least one viral ITR extremity has to be in a free configuration to efficiently initiate the replication machinery of Ad [33, 64].

Various single-step replacement strategies exploiting the *E. coli* homologous recombination machinery were subsequently designed in our laboratory to selectively modify various genetic regions in Ad5. The principle for these manipulations is simple: the viral region to be modified is first subcloned into a bacterial shuttle plasmid containing sequences of the Ad genome to be targeted. The desired alterations, such as deletions, point mutations, or insertions of transgene-containing expression cassettes, are performed in this shuttle plasmid using standard molecular biology techniques. Subsequently, a restriction fragment containing the modified DNA segment and leaving sufficient sequences of Ad homology on either side of the modification is prepared,

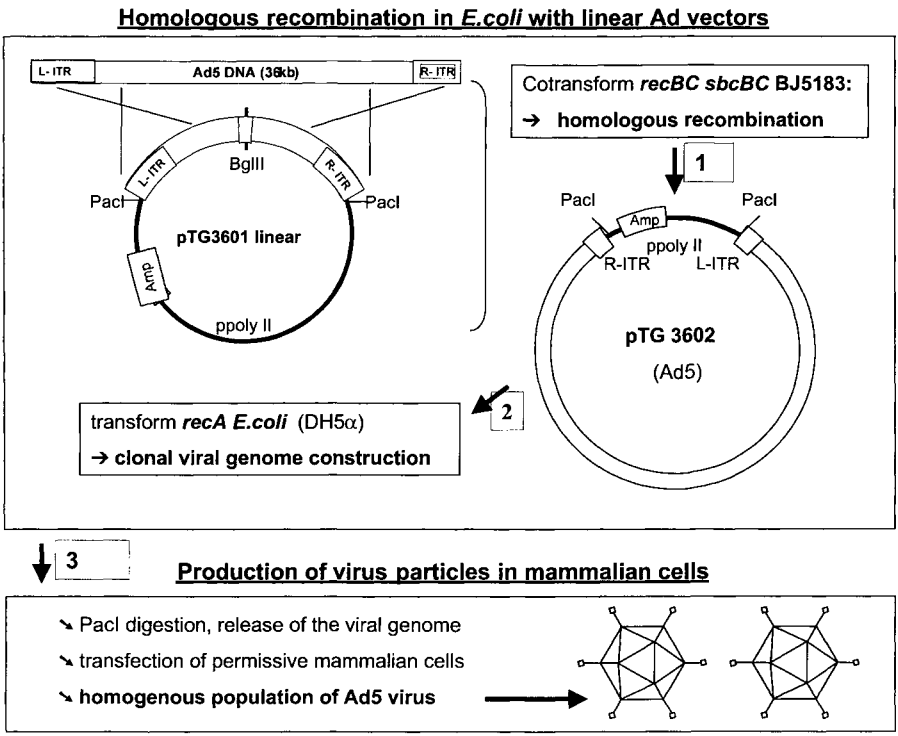

Figure 1 Cloning of infectious full-length Ad5 genome in *E. coli* by homologous recombination [60]. The vector plasmid pTG3601 contains 935 and 853 bp from the left and right ends of Ad5. Cotransfection of BglII - linearized pTG3601 with linear Ad5 DNA results in recombinants containing the full-length Ad5 genome.

purified, and cotransfected into BJ5183 along with the plasmid DNA containing the full length Ad genome to be modified. The Ad genome plasmid is linearized in the targeted region. After recombination occurred between the donor fragment and the linearized Ad plasmid, the expected recombinant is simply rescued by plating bacteria in the presence of the appropriate antibiotic. The circularization of the plasmid is the only selection pressure applied. A detailed example for the targeting of the E1 region is illustrated in Fig. 2.

Unique restriction sites available for the targeting of alterations into various regions of the Ad genome (Fig. 3) are *Cla*I (E1), *Sgf*I, *BamH*I (E2), *Srf*I, and *Spe*I (E3). In cases where the double strand break is located outside the targeted region, the percentage of rescue of the expected modification decreases with increasing distance of the linearization site with respect to the targeted region [60, 66]. In order to improve this and to very efficiently modify the E4

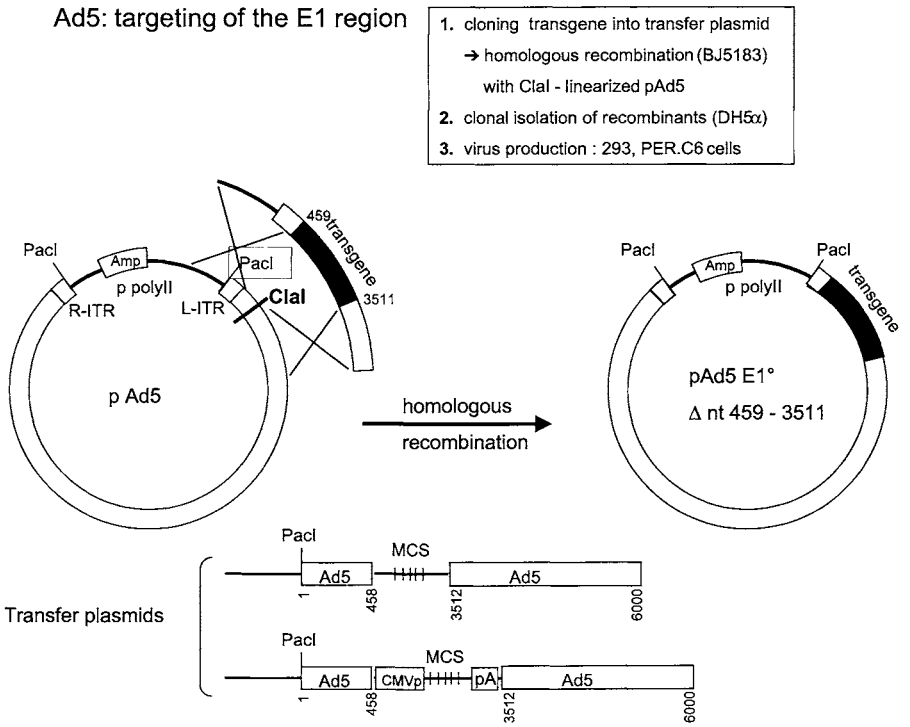

Figure 2 Targeting of the Ad5 E1 region by homologous recombination with linear vectors. Representative examples of transfer plasmids are schematically shown. The transfer plasmid is linearized or the expression cassette flanked with sequences of homology is excised. They are then cotransfected with the *Cla*I–linearized Ad5 genome vector plasmid. Upon homologous recombination in *E. coli* BJ5183 a recombinant viral genome plasmid is generated with the E1 region deleted and replaced by the foreign expression cassette.

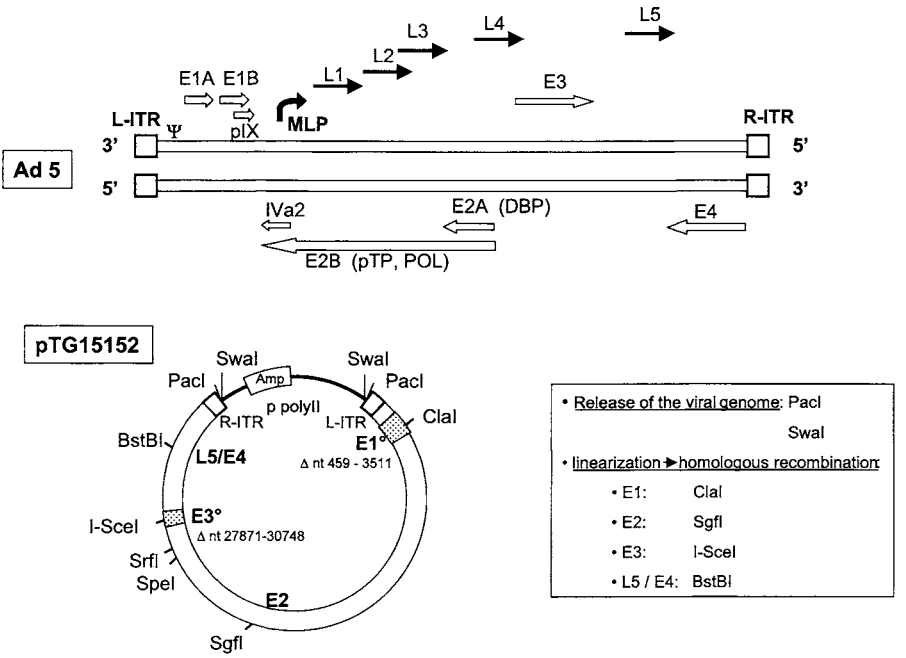

Figure 3 Schematic representation of the Ad5 genome and map of the basic Ad5 genome plasmid pTG15152 used as a standard vector in our laboratory for the construction of Ad vectors. The extent of the deletions in the E1 and the E3 regions is indicated.. With the exception of these deletions the entire sequence of Ad5 is contained in this plasmid. The viral genome can be released from the plasmid backbone by *Swa*I or *Pac*I digestions, located immediately adjacent to the left and right inverted terminal repeats (L-ITR, R-ITR). Unique restriction sites to target the E1, E2, E3 L5/E4 region are indicated. The *Cla*I site is resistant to Dam methylation. To facilitate efficient homologous recombination in the E3 region, the recognition site for the homing endonuclease I-*Sce*I [68] was introduced at the deletion point in E3. The *Bst*BI site, located exactly between the L5 and E4 transcription units enables the generation of recombinants in both regions.

region or introduce mutations into the fiber gene of Ad, we have introduced a *Bst*BI site just between the poly(A) sites of the fiber gene (L5) and the E4 region [21, 67]. In addition, a recognition site for the homing endonuclease I-*Sce*I [68] was introduced directly at the deletion point in E3 (Fig. 3) to improve the efficiency of targeting the E3 region. The single *Cla*I restriction site in the E1 region (G*ATCGATC*) is Dam methylation-sensitive. The *dam* gene of *Escherichia coli* encodes a DNA methyltransferase that methylates adenine in –GATC–sequences in double-stranded DNA [69]. Thus, the replacement of this Dam-methylation-sensitive *Cla*I site with a methylation-resistant *Cla*I site (T/A*ATCGATT*/A) represented another improvement of the procedure, alleviating the need for a cumbersome step of growing the Ad plasmids in

a *dam*$^-$ bacterial strain. Multiple transfer plasmids are now available in our laboratory to routinely target the viral E1, E2A, E3, L5, and E4 regions using the strategic sites indicated above. Thus, we have successfully introduced numerous alterations, deletions, as well as insertions of transgenes into the viral E1, E2A, E3, E4, and fiber regions [20, 21, 27, 60, 67; R. Rooke, unpublished observations]. The application of homologous recombination to modify the E4 region is illustrated in Fig. 4. Due to the availability of new endonucleases, such as intron-encoded homing endonucleases [70], the generation of further multiply altered Ad viral genomes can be envisioned, by insertion of such endonucleases at further strategic sites in the Ad vector genome.

Our experimental evidence suggests that a minimum of ~50 bp of complete homology on either side is required for significant recombination efficiency, consistent with Watt *et al.* [71]. There is an exponential increase in the frequency of recombination when the length of homologous DNA is increased to about 100 bp. Beyond this value, there is an apparent linear increase with longer DNA segments of homology [71;

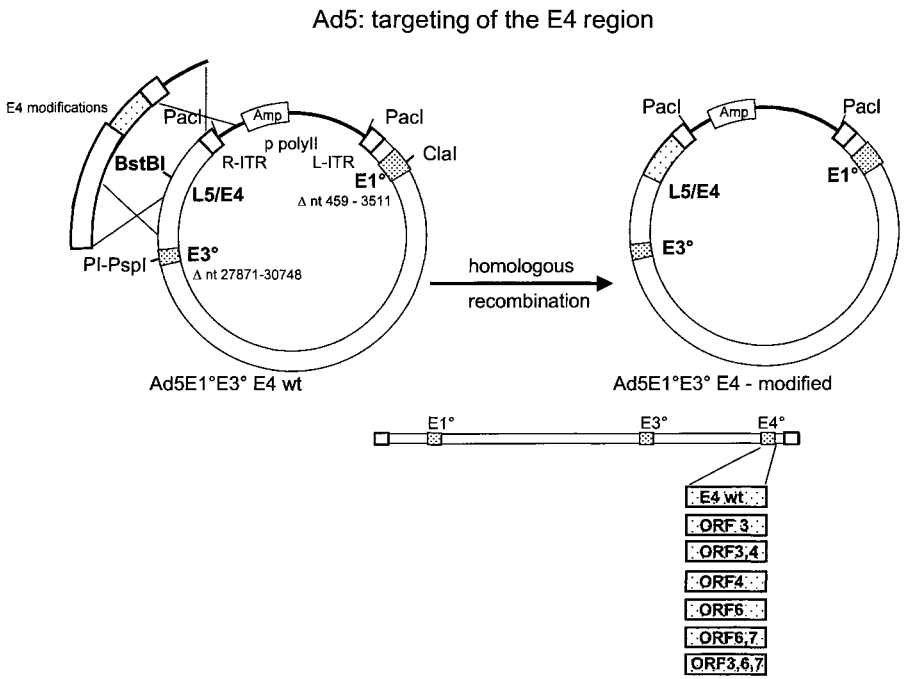

Figure 4 Targeting of the E4 region. E4 modifications are introduced in the appropriate transfer plasmid by standard cloning procedures. The vector genome plasmid (such as pTG15152) is linearized at the *Bst*BI site. Co-transfection into *E. coli* BJ5183 and homologous recombination introduces the E4 modifications indicated in place of E4.

M. Lusky and D. Dreyer, unpublished observations]. If the length of homologous DNA segments is too large (1 kb and beyond), double recombination events may result in the parental vector backbone, thus diminishing the yield of true recombinants (M. Lusky and D. Dreyer, unpublished observations).

The ratios of vector DNA (Ad5 plasmid, linearized) to transfer DNA and the absolute amounts also influence the efficiency of transformation as well as the frequency of homologous recombination in BJ1583. Our own experimental evidence suggests that a molar ratio of vector to transfer DNA of about 1:10 appears optimal. In addition, we routinely use about 3 to 10 ng of vector DNA to achieve optimal transformation and recombination efficiencies. This is consistent with the notion that the size of the Ad5 genome constitutes a limiting factor and increasing amounts of Ad vector DNA inhibit the transformation into *E. coli* BJ5183 [60].

One drawback of the method described above is that two transformation steps in *E. coli* are required prior to the use of high quality, infectious plasmid DNA for transfection into the appropriate mammalian complementation cells. This is due to the fact that the yield and quality of large plasmids, such as Ad plasmids, in the *E. coli* strain BJ5183 are low. The nonconservative recombination mechanism exploited for the manipulation of the Ad plasmids is responsible for this feature. During plasmid amplification in a RecBC sbcBC bacterial strain, nonconservative recombination occurs between a replicating circle and another circle. The end of the generated rolling circle undergoes further recombination with circles. This results in the accumulation of linear plasmid multimers and decreases the yield in circular plasmid DNA [58, 72]. Thus, for extensive characterization of a new recombinant clone, candidate plasmid DNAs derived upon homologous recombination in BJ5183 are transformed into the *recA*$^-$ *E. coli* strain DH5α [73] in our laboratory. In this strain large plasmids are stable and the yield and quality of plasmid DNA is high. Furthermore, due to the availability of state-of-the-art plasmid preparation kits, this second step becomes routine work, taking one extra day's worth of work.

Once the new recombinant viral genome is fully characterized, the viral DNA is transfected upon release with *Pac*I into the appropriate human complementation cells. Since production of recombinant virus is clonal there is no need for plaque purification. With simple E1-deleted or E1 replacement vectors complete CPE is usually obtained within 5 to 7 days after transfection into either 293 [11] or PER.C6 cells [12]. Since the screening and molecular analysis have already been carried out in the prokaryotic host, the eukaryotic cells merely serve to amplify the clonal recombinant. This leads to an enormous gain in time.

Taken together, the basic procedure developed by Chartier *et al.* [60] allows the rapid cloning and manipulation of full-length infectious Ad genomes in bacterial plasmids. The method combines the powerful genetic engineering techniques available in *E. coli* and the ability of this microorganism to recombine homologous sequences at high frequency. The advantages of this technology are multiple and evident: (i) all cloning and, more importantly, all recombination steps are carried out in *E. coli,* thus a high degree of control is possible at each step during the procedure, (ii) the frequency of bacterial colonies containing the plasmids with the modified Ad genome is very high (up to 90% efficiency), (iii) any genetic region of the viral genome can be specifically modified or deleted, including the introduction of point mutations, if appropriate restriction sites are available, (iv) plasmids containing full-length and modified Ad genomes can be introduced into appropriate bacterial strains for production of large amounts of infectious viral DNA, and (v) transfection of released recombinant Ad viral DNA into the appropriate complementation cell lines generates homogenous pure virus particles without the time-consuming need for plaque screening and purification. The method also has been successfully applied to the assembly and further modification of a variety of animal adenoviruses into infectious plasmids [74–78].

A direct modification of the technique developed by Chartier *et al.* [60] uses oligonucleotide site-directed cleavage of DNA and homologous recombination for the production of recombinant Ad vectors [61]. This procedure, schematically illustrated in Fig. 5, allows the introduction of modifications into the viral genome at virtually any predetermined sequence. Toward this goal, the authors describe the use of torsionally stressed supercoiled Ad plasmid DNA which will allow the stable strand displacement of a targeted sequence by hybridization with a complementary oligonucleotide. The hybridization induces the formation of a stable D-loop structure. The single-stranded DNA displaced in such D-loops is specifically cleaved by a single strand-specific nuclease, such as S1 nuclease (Fig. 5). This results in the linearization of the Ad plasmid at the targeted site and therefore provides a perfect template for homologous recombination. The recombination reaction is then performed as described above. The utility of this method was demonstrated by using an oligonucleotide complementary to the E3 transcription unit of Ad5 to incorporate the simian virus 40 (SV40) origin of replication into the E3 region by homologous recombination. The resulting cloning efficiency was about 60%, probably reflecting the relative inefficiency of the vector linearization. The clear advantage of this procedure is the complete independence of unique restriction sites in the vector backbone, thus enabling modification virtually anywhere in the vector genome.

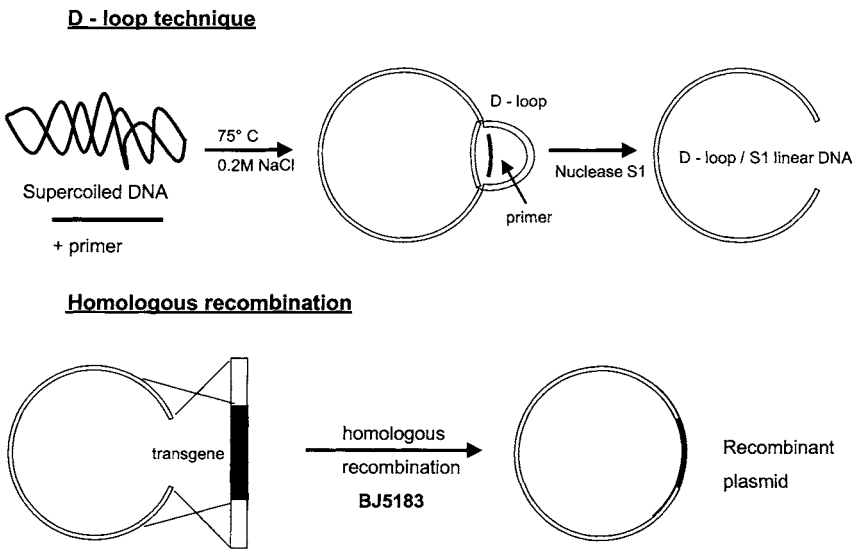

Figure 5 Schematic representation of the D-loop technique [61]. Linearization of the supercoiled target vector backbone is induced by oligonucleotide–mediated D-loop formation followed by S1 nuclease digestion at the target site. D-loop / S1-linearized DNA serves as a vector for subsequent homologous recombination events.

VI. Homologous Recombination with Circular Ad Vector Genome Plasmids

Another way to circumvent the need for single cutting sites is obviously to use a circular instead of a linear template for the homologous recombination. However, in that case the selection process will necessarily involve a step of counterselection against the parental Ad plasmid, rendering the selection process more complex than the simple recircularization to be selected for in the systems described above. Two different approaches where the viral backbone is used as a supercoiled circular plasmid have been described [62, 63]

The method by Crouzet *et al.* [62] for the clonal production of *E. coli*-derived Ad genomes (EDRAG) exploits the observation that incP-derived plasmids, but not colE1-derived plasmids, such as pBR322- or pUC-derivatives, replicate and are stably maintained in *E. coli polA* mutant hosts [79]. As a starting point, an incP plasmid [80, 81] with a tetracycline resistant marker, carrying the extremities of Ad5 was used as a vector to incorporate the entire Ad5 genome to generate a full-length Ad5 genomic infectious plasmid. Subsequently, any modification can be introduced in this incP-derived full-length viral genome plasmid by homologous recombination events between the incP-Ad5 replicon and a colE1 shuttle plasmid. The colE1 shuttle plasmid, carrying

a kanamycin resistance marker and a conditional suicide gene (the *sacB* gene of *Bacillus subtilis* [82]) is engineered by standard cloning techniques containing the specified modification flanked by appropriate homology sequences to the Ad genome. The colE1-derived shuttle plasmid is transformed into the *polA* host carrying the incP/Ad5 plasmid. Cointegrates are selected by growing the *polA* host in the presence of both antibiotics. Resolution, leading to loss of the colE1 replicon from the recombinant Ad plasmid, of this cointegrate is subsequently selected by growth of the cells in sucrose activating the *B. subtilis sacB* gene as suicide gene, leading to the concomitant loss of the *sacB* conditional suicide marker. Consecutive rounds of this two step recombination procedure allow the introduction of multiple independent modifications within the virus genome, with no requirement for an intermediate virus. The potential of this procedure was demonstrated by the recovery of various E1°E3°E4° vectors.

The second system [63] using circular viral genome plasmids as target for the homologous recombination is designed only to replace the viral E1 region with heterologous transgene expression cassettes (see Fig. 6). The vector backbones (pAdEasy) lack Ad nt 1–3533 and are further deleted of the E4 and/or E3 regions. The various shuttle vectors designed (pShuttle) contain a polylinker or a prepared expression cassette surrounded by adenoviral sequences ("arms"), allowing homologous recombination with the pAdEasy

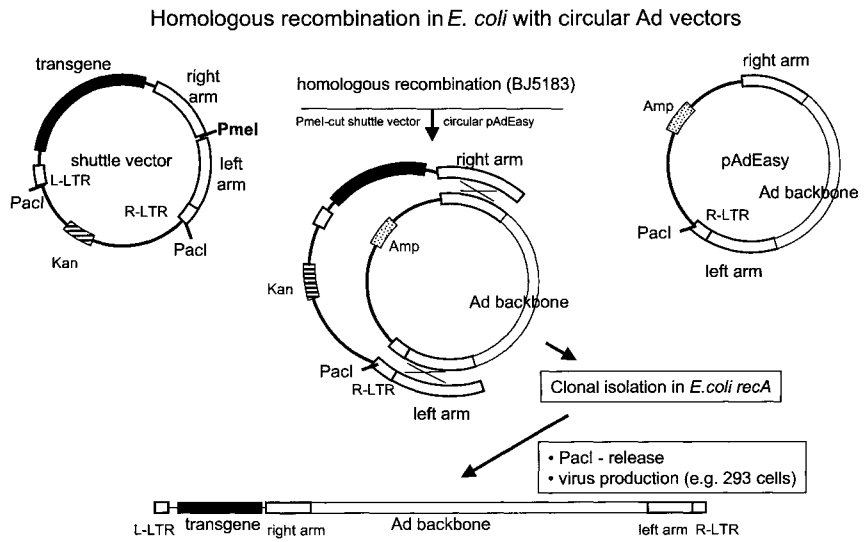

Figure 6 Homologous recombination in *E. coli* BJ5183 with circular target vectors [63]. The gene of interest is cloned into a shuttle vector. The resulting plasmid is linearized by PmeI and cotransfected into *E. coli* BJ5183 with the circular Ad backbone plasmid pAdEasy. Selection of recombinant Ad plasmids in the presence of Kanamycin allows the counterselection of the pAdEasy parental backbone.

system. The left arm contains Ad nt 34,931 to 35,935 (3′ end of Ad) joined to the plasmid backbone and joined to Ad nt 1–480 (5′ ITR, ori and packaging sequence). The right arm consists of Ad nt 3534–5790 joined to the expression cassette. *Pac*I sites are engineered immediately adjacent to the ITRs to facilitate the generation of infectious recombinant Ad genomes. Importantly, the shuttle plasmid backbone contains a kanamycin resistance gene for the selection of recombinants in *E. coli*. Upon linearization of the shuttle plasmid at a single restriction site between the "arms," the DNA is cotransfected with the supercoiled pAdEasy vector into *E. coli* BJ5183 (Fig. 6). Recombination occurs between the homologous arms, the pAdEasy plasmid backbone that harbors an ampicillir resistance gene is replaced by the pShuttle plasmid backbone that presents the kanonyan resistance gene and the expression cassette is introduced in the E1 deletion. Recombinant viral genomes are selected in the presence of kanamycin allowing efficient counterselection of the parental, circular pAdEasy vector. Contamination of the reaction with some uncut shuttle DNA can easily be distinguished from the recombinant DNA due to the size differences. Similar to the approach described by Chartier *et al.* [60], the new recombinant viral plasmid then needs to be transferred into a *recA*$^-$ strain, such as DH10B [73] for greater yields of DNA. Using this approach, a cloning efficiency of about 70% was reported. Upon release of the recombinant viral genome from the bacterial plasmid backbone, virus is produced in the appropriate human complementation cell lines as described above. In some of the vectors described by He *et al.* [63] the green fluorescent protein reporter gene is cointroduced with the expression cassette into the recombinant virus, facilitating the tracing at each step of the viral production. The system as outlined [63] is specifically designed to efficiently and easily introduce heterologous expression cassettes in place of the viral E1 region. Since this approach relies on the replacement of the plasmid backbone in between the two Ad ITRs for the selection of the recombinants, only viral regions located at the extremities of the genome (E1 region, E4 region) can easily be targeted. The targeting of any other region requires additional procedures to promote the loss of the prokaryotic selection marker from the viral genome as illustrated in the system described by Crouzet *et al.* [62].

VII. Ad Vector Construction by Transposon-Mediated Recombination

Recently a transposon-mediated recombination system has been developed which involves the generation of Ad by Tn7-mediated, site-specific transposition in *E. coli* [83]. The development of this system requires two plasmid components: (i) a low-copy-number, full-length, circular adenoviral plasmid (admid [83]) with a β-galactosidase marker replacing the E1 region

and containing the Tn7 attachment site (*lacZatt*Tn7) and (ii) an admid transfer vector with a mini-Tn7 containing an expression cassette with the gene of interest. Tn7-mediated transposition of the expression cassette into the *lacZatt*Tn7 site disrupts the lacZ coding region, resulting in a β-gal- phenotype of the newly generated admid. The authors describe a transposition frequency averaging 25%. After clonal isolation of the new recombinants, the Ad genome is released from the admid backbone and transduced into 293 cells for amplification and production. Described to carry out replacements in the E1 region, the admid system could also be adapted to alter other or additional viral regions.

VIII. Ad Vector Construction by *in Vitro* Ligation

The notion of separation of the molecular construction of the rAd vector from the virus production is also guiding the direct cloning procedures described below. Efficient and improved *in vitro* ligation techniques have recently been described [40–42]. Due to the paucity of unique restriction sites within the adenoviral genome, direct ligation of a transgene expression cassette into the viral vector backbone is facilitated by the use of intron-encoded endonucleases. The technique basically involves two cloning steps in *E. coli* followed by the transduction of the linear recombinant viral DNA into the permissive mammalian cell–complementation system (see Fig. 7).

Intron-encoded endonucleases are enzymes that are encoded within group I introns [70]. An unusually long homing sequence ranging from 15 to 39 bp renders these endonucleases rare cutting and ideal to use for cloning sites in adenoviral backbones. Two groups have described the use of intron-encoded endonucleases, such as I-*Ceu*I [84] and PI-*Sce*I [85] for the direct substitution of the viral E1 region by a reporter gene. Mizuguchi and Kay [40] have generated four basic viral backbones, pAdHM1–4 in a bacterial plasmid; all viral backbones are E1° with or without an E3 deletion and the viral DNA is flanked by *Pac*I and *Cla*I sites (pAdHM1, 2) or by *Pac*I sites at both ends (pAdHM3, 4). In each backbone three unique cleavage sites are introduced in to the E1-deleted region: I-*Ceu*I, *Swa*I, PI-*Sce*I. A basic shuttle plasmid (pHM3) contains a multiple cloning site flanked by I-*Ceu*I and PI-*Sce*I sites, respectively. The expression cassette of interest can be inserted in any site of the multiple cloning site. After cloning, the cassette-containing insert is excised from the shuttle plasmid using the intron-encoded endonucleases. The restricted fragment is then directly cloned into the vector backbone, which is linearized by I-*Ceu*I and PI-*Sce*I. This scheme permits a directed cloning by ligation of the expression cassette into the viral backbone and lowers the background of parental viral vector (null vector) due to different ends created

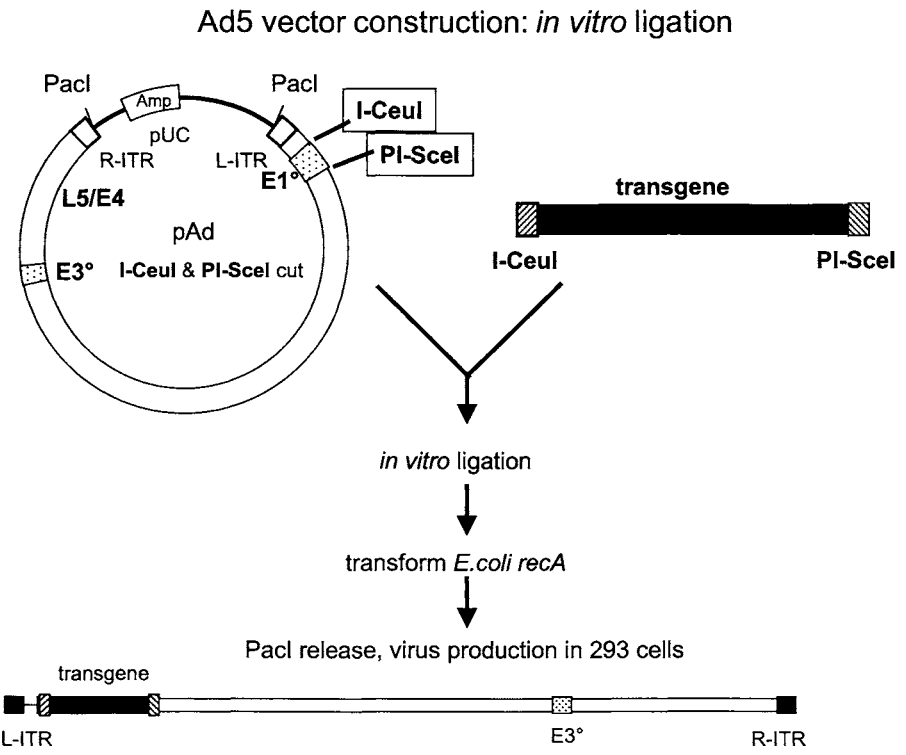

Figure 7 Construction of Ad by ligation *in vitro* [40–42]. A detailed description is found in the text.

by the two different intron-encoded endonucleases. Restriction of the ligation product with *Swa*I further diminishes the background of the parental null vector in the cases where the ligation products do not contain a *Swa*I site. The restricted ligation product is then directly transformed into *E. coli* DH5α. With this method the authors report a 90% cloning efficiency for recombinant viral genomes with the correct restriction pattern. The recombinant viral genome can subsequently be released from the plasmid backbone by *Pac*I or *Pac*I plus *Cla*I digestion followed by the generation of a homogenous Ad population upon transfection into 293 cells. The simplicity of the system is clearly emphasized by the careful choice of unique and strategic restriction sites. The authors have recently added new vector backbones including E1E3E4-deleted vectors as well as modified shuttle vectors to increase the variety of vector construction [41].

The $pAd_{vantage}$system described by Souza and Armentano [42] follows the same logic. Their basic viral E1° backbone ($pAd_{vantage}$) is based on Ad 2 (serotype 2) and contains an I-*Ceu*I site in the region of the E1 deletion, the ITRs of the viral genome are flanked by *Sna*BI sites (*Sna*BI does not

cleave within the Ad2 viral genome). A shuttle plasmid containing a polylinker flanked by I-*Ceu*I sites is used to introduce the gene expression cassette of interest. The ligation of the I-*Ceu*I restricted expression cassette into the I-*Ceu*I linearized vector is reported with an ~50% cloning efficiency.

The authors describing the *in vitro* ligation approach (Fig. 7) indicate that under optimal conditions the timing from start (cloning of the gene of interest into the shuttle vector) to finish (isolation of plaques, or passage 1 lysate) generally does not exceed 3 weeks. Taken together, the improved, direct *in vitro* ligation method is simple, straightforward, and appears very efficient, although restricted to the replacement of the E1 region [42] and to the E1 and E4 region [40, 41] in its current state. Although it will not allow more precise modification of the Ad genome, the system is clearly amenable to further developments and improvements. For example, additional introduction of noncutting endonucleases, such as intron-encoded or other nonconventional nucleases, into the viral E3 and E4 regions will enable the generation of Ad vectors containing multiple inserts.

IX. Conclusion

In summary, the concept of the separation of the steps of Ad genome construction in prokaryotic cells and virus amplification in eukaryotic cells has led to the exploitation of powerful methods, including homologous recombination, direct ligation and transposition technologies to clonally derive Ad constructs in *E. coli*. The advent of these technologies has enabled the construction and generation of numerous modified Ad vectors carrying many different transgenes. In addition, our understanding of the *in vivo* biology of these vectors has been advanced tremendously through the ease of introducing viral genome modifications and studying their effects. The methods in use are simple, highly efficient, and can generate Ad within a very short time period (Fig. 8). The choice of which method to use will depend on the vector region to be targeted and the type of modification to be introduced. Homologous recombination methods using linear vectors can target virtually any region in the Ad5 backbone and allow precise modification of the Ad genome sequence such as small deletions and point mutations. Homologous recombination methods with circular vectors and the *in vitro* ligation technique, although very efficient, are currently set up to target the ends of the viral genome (e.g., E1, E4 region) It is clear that these techniques will be further optimized, as research develops, and could be adapted for the generation of other viral vector systems.

Many applications of these methods can be envisioned, including the use of custom tailored Ad for the *in vivo* production of recombinant therapeutic proteins or the generation of custom-made libraries in Ad vectors to enable the high-throughput screening and applied functional analysis of many genes in the context of functional genomics projects.

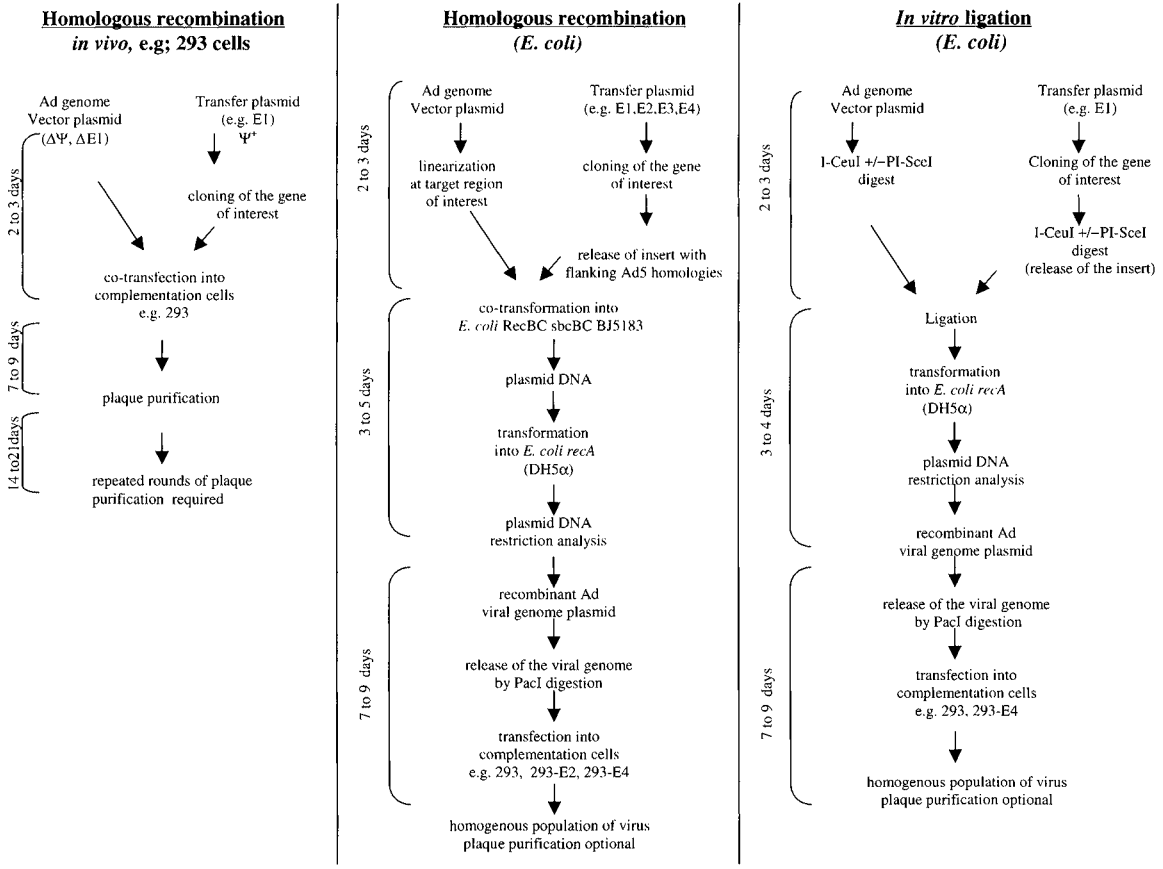

Figure 8 Overview of the basic techniques (homologous recombination and *in vitro* ligation) of Ad vector construction in *E. coli* compared to traditional methods of vector construction and virus generation in eukaryotic cells [36, 40, 42, 60].

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CHAPTER 6

Propagation of Adenoviral Vectors: Use of PER.C6 Cells

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I. Introduction

A. Scope of the Chapter

The goal of gene therapy is the introduction of genes into human somatic cells for therapeutic purposes. The success of gene therapy is therefore dependent on the efficiency by which a therapeutic gene can be transferred to the patient's target tissues. In many cases, viruses are exploited for gene transfer purposes and in particular gene transfer vectors derived from adenoviruses (adenoviral vectors) are often used to achieve this (for review see [1]).

The reason for this is that adenoviral vectors:

- efficiently transfer genes to many different cell types;
- can be propagated on well-defined production systems to high yields; and
- are very stable, which makes purification and long-term storage possible, thereby making pharmaceutical production feasible.

This contribution will focus on the production systems for clinical lots of adenoviral vectors. Particular attention will be paid to the generation and use of complementation cell lines that carry the E1 genes. Particular emphasis will be on the PER.C6 cell line, which was developed to prevent generation of replication-competent adenovirus (RCA) during propagation of E1-deleted adenoviral vectors. In addition, safety issues with respect to the use of the cell line for making clinical grade material will be addressed.

B. Adenoviruses

Human adenovirus was isolated for the first time in 1953 from cultured adenoidal tissue [2, 3]. Since then, 51 different serotypes have been isolated from various tissues and excretions of humans, of which serotypes 42–51 were obtained from immunocompromised individuals [4–6]. A serotype is defined on the basis of its immunological distinctiveness as judged by quantitative neutralization with animal antisera (horse, rabbit). If neutralization shows a certain degree of cross-reaction between two viruses, distinctiveness of serotype is assumed if (i) the hemagglutinins are unrelated, as shown by lack of cross-reaction on hemagglutination-inhibition, or (ii) substantial biophysical/biochemical differences in DNA exist [7].

Human adenoviruses are subdivided into six different groups (A–F), which are based mainly on differences in hemagglutination, restriction enzyme analysis, and DNA homology [8]. The adenoviruses were found to be associated with different disease patterns (see, e.g., [9, 10]). In addition to the human adenoviruses, some 40 different serotypes have been isolated from various animal species [11].

All adenoviruses possess a DNA molecule that is surrounded by a capsid consisting essentially of hexon, penton-base, and fiber proteins. The virion has an icosahedral symmetry and, depending on the serotype, a diameter of 60–90 nm.

The well characterized adenovirus serotypes 2 and 5 have a linear double-stranded DNA genome of approximately 36,000 base pairs (Fig. 1). Other adenoviruses have genome sizes ranging from 30 to 38 kb. The genome contains, at both its ends, identical inverted terminal repeats (ITRs) of approximately 90–140 base pairs with the exact length depending on the serotype. The viral origins of replication are within the ITRs exactly at the genome ends. Sequences required for encapsidation (Ψ) of the viral genome are located in a region of approximately 400 bp downstream of the left ITR.

The structure of the adenoviral genome is described on the basis of the adenovirus genes expressed following infection of human cells, which are called early (E) and late (L), according to whether transcription of these regions takes place prior to or after onset of DNA replication.

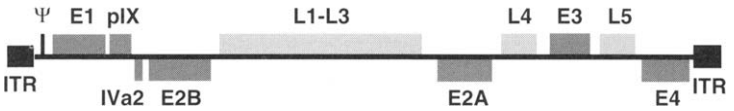


Figure 1 Map of the adenovirus genome. The 36-kb (for adenovirus type 5) double-stranded DNA molecule is usually divided into 100 map units (mu). The early (E) and late (L) regions are indicated on the map. The ITR sequences (inverted terminal repeats) are identical, inverted, terminal repeats of approximately 100 bp, depending on the serotype, which are required for replication. Ψ is a stretch of sequences involved in packaging of the viral DNA into particles. E1 comprises the E1A and E1B region, both encoding two proteins, which are described in detail in section I.C. E2A encodes the DNA binding protein, E2B the precursor terminal protein and DNA polymerase. E3 encodes a number of proteins that are predominantly involved in modulating the host's immune response against adenoviral infected cells. E4 proteins (six in total) are involved in modulation of gene expression and viral replication, mainly through interactions with the host cell. IVa2 (transcriptional activator of major late promoter) and pIX (essential for assembly of the virion) are intermediate proteins. L1–L5 encode the late proteins, which are mainly capsid proteins, including penton (L2), hexon (L3), hexon-assembly (L4), and fiber (L5) protein.

Infection of a target cell starts by interaction of the fiber with a receptor on the surface of the cell. Many, but not all [12], adenoviruses use the Coxsackie-adenovirus receptor (CAR) for this [13, 14], which is present on the cell surface. Integrins act as secondary receptors by binding to the viral penton-base protein. Subsequently, the virus is internalized by receptor-mediated endocytosis. The adenoviruses escape from the endocytic vesicles (or receptosomes) by virtue of a change in the configuration of the virion surface due to the low pH in these vesicles. As a consequence, the virus particles are released in the cytoplasm of the cell, where they are further degraded [15], with the DNA ending up in the nucleus, where a complex with histone proteins is formed, which may attach to the nuclear matrix for replication [16].

The adenovirus DNA is usually not integrated into the host cell chromosomal DNA but remains episomal (extrachromosomal) unless transformation or tumorigenesis has occurred.

C. Adenovirus Replication

As indicated before, a productive adenovirus infection is divided into two distinct phases: the early and the late phases. In the early phase, the so-called early genes (E1, E2, E3, and E4) of adenovirus are expressed to prepare the host cell for virus replication. During the late phase, actual viral DNA replication and production of viral structural proteins takes place, leading to the formation of new viral particles. Adenovirus replication requires both host-cell and viral proteins (see [8, 16] for reviews). The cellular proteins needed for replication are nuclear factors I, II, and III [16], which are involved in initiation of viral DNA replication and elongation, as well as in increasing the efficiency of replication.

Adenovirus DNA replication starts with expression of the "immediate–early" E1 genes. The E1 region comprises two different transcription units, E1A and E1B. The main functions of the E1A gene products are (i) to induce quiescent cells to enter the cell cycle and resume cellular DNA synthesis and (ii) to transcriptionally activate the E1B gene and the other early regions (E2, E3, E4). The E1A region encodes two major RNA products, 12S and 13S, which are generated by one transcription unit and which differ in size due to alternative splicing. The RNAs encode acidic proteins of 243 and 289 amino acids, respectively (for adenovirus 5). These are phosphorylated proteins, present in the nucleus of the cells. In addition, during lytic infection mRNAs of 9S, 10S, and 11S are produced, but these proteins were found to be not essential for adenoviral replication [17, 18]. The function of these proteins has not yet been resolved.

The E1B region codes for one 22S mRNA, which is translated into two proteins, with molecular weights (for adenovirus 5) of 21 and 55 kDa. E1B proteins assist E1A in redirecting the cellular functions to allow viral replication. The E1B 55-kDa protein forms a complex with the E4 open reading frame 6 (ORF6) 34-kDa protein, which is localized in the nucleus [19, 20]. Its main function is to inhibit the synthesis of host proteins and to facilitate the expression of viral genes. In addition, it also blocks the p53 tumor-suppressor protein, thereby inhibiting apoptosis [21]. The E1B 21-kDa protein is important for quenching the cytotoxic effects to the target cells induced by E1A proteins. It has anti-apoptotic functions similar to the human Bcl-2 protein, which is important for preventing premature death of the host cell before the virus life cycle has been completed [22]. Mutant viruses incapable of expressing the E1B 21-kDa gene-product exhibit a shortened infection cycle that is accompanied by excessive degradation of host cell chromosomal DNA (*deg*-phenotype) and an enhanced cytopathic effect (*cyt*-phenotype) [23].

The E2 region encodes three different proteins that function in viral DNA replication: an Ad-specific DNA polymerase, the precursor terminal protein (pTP), and the DNA-binding protein [16]. The DNA-binding protein, which is encoded by the E2A gene, binds to single-stranded DNA and is involved in unwinding duplex DNA. It might also be involved in the regulation of transcription. The precursor of the terminal protein (pTP) and the DNA polymerase, which are present as a heterodimer, are encoded by the E2B region. The pTP is attached to the adenoviral DNA and is cleaved by the viral protease late in infection. It has a function in protection of the DNA from nucleolytic breakdown and in attaching the adenoviral DNA to the nuclear matrix, which may localize the viral genome to areas of the nucleus in which high concentrations of replication and transcription factors are present. The polymerase is involved in the synthesis of new DNA strands.

None of the E3 products are required for virus replication. They do, however, play an important role in virus multiplication *in vivo*, since they protect virus-infected cells from being eradicated by the host's immune response (reviewed in [9]). Several differentially spliced mRNAs are synthesized from the E4 region during infection and six different polypeptides have been identified in infected cells [24]. These proteins are involved in modulation of gene expression and viral replication, mainly through interactions with the host cell.

The E4 ORF3 and E4 ORF6-encoded proteins are involved in post-transcriptional processes that increase viral late protein synthesis. They do so by facilitating the cytoplasmic accumulation of the mRNAs encoding these proteins and by expansion of the pool of late RNAs in the nucleus, most likely by influencing splicing. In addition, the E4 ORF6-encoded protein forms a complex with the E1B 55-kDa protein that selectively increases the rate of export of viral late mRNAs from the nucleus. The complex is located in so-called viral inclusion bodies, the region where viral DNA replication, viral late gene transcription, and RNA processing occur [25]. The E4 ORF6 protein, either alone or in a complex with the E1B 55-kDa protein, binds the cellular protein p53, thereby blocking its potential to activate the transcription of tumor-suppressing genes [26, 27].

E4 ORF1 sequences are related to dUTPase enzymes. It has been hypothesized that this gene has a role in stimulating quiescent cells [24].

The E4 ORF4 protein binds to protein phosphatase 2A, which results in hypophosphorylation of some proteins, including the adenovirus E1 proteins. This perhaps limits cytotoxic effects of E1A and may lead to a more productive infection. It is also in line with the observation that E4 ORF4 mutants are more effective than wild-type viruses in killing nonpermissive rodent cells [28]. E4 ORF4 also induces apoptosis in transformed cells like 293 cells [29].

The E4 ORF6/7 modulates the activity of the cellular transcription factor E2F, which may subsequently activate cellular genes which are important for the S phase [30]. The functions of E4 ORF1, ORF2, and ORF3/4 during lytic infection are less clear and are dispensable for growth of the virus in laboratory cell lines.

After onset of DNA replication, expression of the late genes L2–L5, which are all under the control of one promoter, is switched on. These genes encode the structural components of the virus particles, including L2 the penton, L3 the hexon, L4 the hexon assembly, and L5 the fiber protein. These proteins form the new virus particles into which the adenoviral DNA becomes entrapped. Depending on the serotype, 10,000–100,000 progeny adenovirus particles can be generated in a single cell. The adenoviral replication causes lysis of the cells.

II. Cells Expressing E1 of Adenovirus

A. Transformation of Cells by E1 of Adenovirus

In the previous section of this chapter, the function of adenoviral gene products in the replication of adenovirus was described. There is extensive influence of adenoviral proteins on a large number of cellular functions. In the absence of lytic viral replication, adenoviral genes may have a profound effect on cellular functions, the most striking being transformation by the adenoviral E1A and E1B proteins. Clearly, these proteins interfere with the regulatory mechanism of cellular proliferation.

Human adenoviruses have a narrow host range for productive infections, and can only be propagated in cells of human, chimpanzee [31], pig [32], and cotton rats [33]. In rodent cells, e.g., from rat (with the exception of the cotton rat), hamster, or mouse, they bring about an abortive infection, which occasionally leads to transformation [34]. In the transformed cells the adenoviral DNA is integrated into the genome and at least the genes of the viral E1 region are expressed (reviewed in [35]).

The viruses that were used for such studies were mainly adenovirus serotypes 2, 5, and 12. The various Ad serotypes differ in their ability to induce tumors upon inoculation into newborn hamsters; for example, Ad type 5 (Ad5) is nononcogenic [36], whereas Ad12 is highly oncogenic [34]. However, all Ad serotypes or their DNA can transform rodent cells [37, 38]. Ad5E1-transformed cells can form tumors only in immunodeficient mice and rats, whereas Ad12E1-transformed cells are oncogenic both in immunodeficient and in immunocompetent animals [39], which correlates with the ability of Ad12E1 to repress expression of MHC class I genes [40].

In culture, both rodent cells, e.g., from rat, mouse, or hamster, and human cells can be transformed by Ad DNA, although human cells, including fibroblasts and epithelial cells, are relatively refractory to transformation. Adenovirus DNA transformed human cell lines have been made from cultures of human embryonic kidney [41, 42], human embryonic retina [43–46], human embryonic lung [44], and recently, human amniocytes [47].

As described before, the E1 region consists of two transcriptional units, E1A and E1B. For complete morphological transformation, both regions are needed, but the E1A region by itself can immortalize rodent cells [48] and occasionally human cells [43], albeit with very low efficiency. Expression of E1A usually results in induction of programmed cell death (apoptosis), which can be prevented by coexpression of E1B [49]. The E1A associates with a number of cellular proteins, including the tumor suppressor gene product pRb, as well as p107, p130, cyclins A and E, cyclin-dependent kinase 2 (cdk2), and p300 (reviewed in [50–52]). Most of these proteins are involved in cell-cycle control, and, with the exception of p300, regulate the activity of the

transcription factor E2F [51]. The E1A proteins do not exert their activity in initiation of transcription by direct, sequence-specific binding to DNA, but rather do so by binding to cellular transcription factors.

The E1B 55-kDa [19] and 21-kDa [53] proteins cooperate independently with E1A in transformation, and are required to inhibit the apoptotic response initiated by E1A. The 55-kDa E1B protein inhibits apoptosis by blocking the function of the p53 tumor-suppressor protein, which mediates E1A-induced apoptosis [21]. The 21-kDa E1B protein inhibits apoptosis in a way similar to the cellular Bcl-2 protein [22].

B. E1-Expressing Cell Lines for Adenoviral Vector Production

Most adenoviral vectors currently used in gene therapy have a deletion in the E1 region, where novel genetic information can be introduced [54]. The E1 deletion renders the recombinant virus replication-defective, which is a prerequisite for most of the clinical applications. In order to be able to produce E1-deleted recombinant adenoviral vectors, complementing cell lines have to be used that express the E1 proteins of adenovirus. One of the main challenges here is to express sufficient levels of the E1 protein to achieve this. However, adenovirus E1 proteins, and in particular E1A proteins, are very toxic to cells. E1A has a profound effect on the transcription of many cellular genes, which leads to alteration of the morphology and growth of the cells and may lead to apoptosis.

A few examples have been reported in literature, where cells have been immortalized (but not transformed) with E1A only. This has been described both for rodent [48] and for human cells [43]. It is not known whether cells that express E1A only are able to complement adenoviral vectors that are deleted for both E1A and E1B. Attempts have also been made to express E1 proteins in established cell lines such as A549. Growth of established cells is not dependent on E1 expression and the toxicity of E1 proteins made it difficult to isolate clones that show stable expression of the E1 proteins, although a few papers report encouraging results [46, 55, 56]. To the best of our knowledge, there is limited use of such cells and therefore this chapter will deal mainly with the group of E1-expressing cells that use the transforming capacity of the adenoviral E1 genes.

Typical examples are the cell lines derived from human embryonic kidney (HEK) [41, 42], human embryonic retina (HER) [43–46], and human amniocytes [47]. The advantage of using E1 for immortalization is that such cells are dependent on E1 expression for growth, and therefore the levels of E1 expression are remarkably constant over time.

The vast majority of cell lines that were made by immortalization and transformation of primary cells, were made to study immortalization and transformation and were not made for propagation of E1-deleted adenoviral

vectors. The only documented cell lines based on the E1 immortalization principle, which were made specifically for use in gene therapy are the PER.C6 cell line [46] and the amniocyte-derived cell line [47].

These cell lines have been tailor-made for the manufacture of clinical lots of adenoviral vectors, with special attention to avoiding generation of RCA (see below). In addition, proper documentation and adequate safety testing are pivotal to ensure manufacture of safe batches of adenoviral vectors. As PER.C6 is the only cell line currently used for making clinical lots of adenoviral vectors, a description of the generation of PER.C6 is given below. Also, the performance of the cell line in production of recombinant adenovirus as well as results of safety and genetic testing are provided.

III. PER.C6 Prevents RCA during Vector Production

A. RCA

The majority of preparations of E1-deleted adenoviral vectors have been produced on 293 cells. This cell line was generated in Leiden in the group of Prof. Van der Eb, by transfection of E1 sequences of adenovirus type 5 into primary human embryonic kidney cells [41]. The aim of this experiment was to study the transforming potential of adenoviral E1 sequences, and the DNA used for it was sheared adenoviral DNA [41]. Precise mapping of the adenoviral sequences present in this cell line indicated that the cell line had integrated bases 1–4137 of the adenoviral DNA [57]. Adenoviral vectors carry a deletion in the E1 region that runs from approximately nt 400 to nt 3500 of the adenoviral genome. This means that there is a substantial sequence overlap between the E1 sequences present in the cell line and the adenoviral vector DNA (see Fig. 2). This sequence overlap may result in homologous recombination between the sequences. Due to a double crossover, the E1 region present in the cellular chromosome may end up into the E1-deleted adenoviral vector [58] (Fig. 2). The resulting virus is E1-positive and therefore capable of replicating independently in cells that do not contain E1 sequences in the chromosome. Several reports have described the occurrence of RCA in adenoviral vector batches produced on 293 cells [46, 55, 58–60].

RCA in clinical preps is unwanted, both from the manufacturing and the safety points of view.

Its appearance in batches is a chance process and is therefore unpredictable and difficult to control. This is a significant problem for GMP manufacturing, in particular if large-scale batches have to be prepared.

It is also unwanted from a safety point of view, as upon coinfection of a cell RCA causes the E1-deleted adenoviral vector to replicate in an

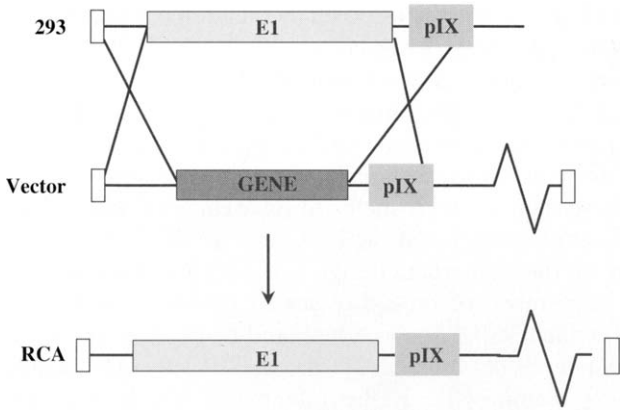


Figure 2 Mechanism of generation of RCA in 293 cells. Adenoviral vectors contain sequences that overlap with sequences present in the genome of 293 cells, indicated by the crossing lines. Due to the sequence homology, crossover events can occur, which lead to exchange of DNA. E1 sequences replace the transgene in the adenoviral vectors, resulting in E1-containing adenoviruses that are replication-competent.

uncontrollable way. It causes shedding of the vector [61]. In addition, RCA has been shown to cause inflammatory responses [59, 62]. Therefore, RCA generation during production of E1-deleted adenoviral vectors has to be circumvented.

B. PER.C6: Absence of Sequence Overlap Eliminates RCA Generation

The strategy to prevent RCA occurrence was to eliminate sequence overlap between the E1 sequences present in the cellular genome and the adenoviral vector [46]. A potential hurdle to do this is the way the E1B and pIX gene are regulated. Both E1B and pIX use the same poly(A) sequences [63]. Furthermore, the pIX gene is not expressed upon transfection in cultured cells [64], but can be expressed only if present in an adenoviral genome. Therefore, an RCA-free packaging system should consist of two components: (i) an adenoviral vector that is deleted for E1A and E1B, but contains the pIX expression cassette and (ii) a cell line that expresses E1A and E1B and is devoid of pIX sequences.

1. E1 Construct Used for Making PER.C6

To create the novel cell line, the aim was to use only a minimal number of human adenovirus-type-5-derived sequences, i.e., the E1 protein coding sequences only, to prevent sequence overlap with E1-deleted Ad. The E1 promoter and poly(A) sequences were therefore obtained from nonadenovirus

sources. The E1 promoter was replaced by the human phosphoglycerate kinase (PGK) promoter (see below), which is a known housekeeping promoter [65] and the poly(A) sequences were isolated from hepatitis B virus [66, 67]. The construct pIG.E1A.E1B contains, in addition to the E1A and E1B coding regions, sequences upstream of the E1A gene, including E1A enhancer elements and the cap sequence. Untranslated E1A sequences were also retained in the construct. These elements were included since earlier studies indicated that this results in efficient expression of the E1A gene [68].

A map of the construct, designated as pIG.E1A.E1B, is presented in Fig. 3. Despite removal of the splice site at position 3509 of the adenoviral genome [63], which is highly conserved, and truncation of the E1B transcript, high expression levels of both E1B 21 kDa and E1B 55 kDa were obtained [46]. In fact, the expression of the E1B proteins was even higher than in 293 and 911 cells, whereas equal expression levels of E1A were observed [46].

To prevent sequence overlap with E1 present in PER.C6 cells, adenoviral vectors were constructed that carry a deletion of the complete E1 region. These vectors were shown to propagate efficiently in PER.C6 cells (see below) and were found to express the pIX gene [46].

2. Generation of PER.C6

The primary cells selected for making a new E1-complementing cell line were human embryonic retinoblasts (HER). The choice for retinoblasts [43] was based on the observation that Ad12 could transform hamster retinal cells *in vitro* [69] and induce retinoblastomas following intraocular injection into newborn baboons [70]. It has been described that these cells can be immortalized relatively easily by E1 of human Ad5 [43, 44, 71] and Ad12 [72]. In addition, the 911 cells, which are derived from HER cells, are very efficient in production of recombinant adenoviral vectors and easy to use [71], thus providing a second argument for the use of primary HER cells as the source of primary cells to make a novel cell line.

Primary HER cells have a limited life span. Such cells can be cultured for only a few passages, after which the cells senesce. Transfection of HER cells with E1 constructs results in immortalization and transformation of the cells, reflected by focus formation in the cultures. This is easily recognized by both macroscopic and microscopic examination of the cultures. Such foci

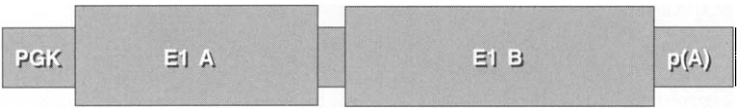


Figure 3 The E1 construct used to generate PER.C6. The pIG.E1A.E1B construct contains adenovirus type 5 sequences 459–3510. E1A expression is driven by the human PGK promoter. E1B transcription is terminated by hepatitis B virus-derived poly(A) sequences.

can be isolated and cultured further. Therefore, the pIG.E1A.E1B construct was transfected into primary HER cells, and PER.C6 cells were isolated as described in detail before [46].

After propagation of the cells to passage number 29, a research master cell bank was laid down, which was extensively characterized and tested for safety, including sterility testings (see below).

Immortalization of primary cells with E1 sequences of adenovirus guarantees (i) a stable expression of E1 proteins, as the cells need E1 expression for growth, and (ii) that no external selection marker is needed to distinguish E1 expressing from nonexpressing cells. Human adenovirus serotype 5 was taken as the donor for E1 sequences.

C. Frequency of RCA Occurrence

In order to test whether PER.C6 cells are able to propagate adenoviral vectors without concomitant generation of RCA, E1-deleted adenoviral vectors were propagated on 293 cells and on PER.C6 cells. The adenoviral vectors used did not have any sequence overlap with E1 sequences in PER.C6. The batches of vector were analyzed for the presence of RCA, using cell culture based assays, as described before [46, 58]. The results (summarized in Table I) clearly indicate that adenoviral vectors when propagated on 293 cells, get contaminated with RCA. On the other hand, the data provided in Table I clearly demonstrate that PER.C6 cells support RCA-free propagation of E1 deleted adenoviral vectors, even if large-scale batches (produced on 1–3E10

Table I
Frequency of RCA Occurrence in 293 Cells and in PER.C6 Cells

Helper cell	No. of productions	No. of cells per production	No. of RCA positive batches	
			2.5E9 IU	2.5E10 IU
293	22	1E8–3E9	13/22	ND
PER.C6	8	1E8–3E9	0/8	0/2
PER.C6	3	1E10–3E10	ND	0/3

Note. Batches of E1 deleted adenoviral vectors, propagated on either 293 and PER.C6 cells, were tested for the presence of RCA at a level of sensitivity of either 1 RCA in 2.5E9 infectious units (IU) or 1 RCA in 2.5E10 IU of E1 deleted adenoviral vector. The number of batches that were produced on either cell line, as well as the number of cells used for the production, are indicated as well.

PER.C6 cells) were tested for RCA in a very sensitive assay (1 RCA/2.5E10 infectious units).

In a separate experiment, an E1- and E3-deleted Ad5 vector was derived and propagated in PER.C6 cells. A master virus seed (MVS), prepared from passage 12, was used to generate 8 virus-production lots (passage 13). The unprocessed virus harvest (vector-infected suspension culture) of the MVS and the virus-production lots were tested for RCA. In brief, test articles were frozen and thawed and then assayed by inoculation onto the human-lung-carcinoma (A549, ATCC CCL 185) cell line for approximately 1–2 h at 37°C, after which the inoculum was removed and the culture was refed with medium. Cultures were passaged three times to amplify any putative RCA present, with incubation times ranging from 4 to 7 days for the early passages and 2 to 5 days for the final passage. The cultures were examined for cytopathic effects at each passage. The virus-production scale was approximately 20 L and a 60 mL volume (diluted to 600 mL to avoid toxicity and interference with detection of RCA) was tested for RCA for each lot. The testing volume was selected on the basis of a worst-case calculation to ensure the testing of at least three dose equivalents of virus. Earlier virus-production studies suggested that the freeze–thaw extract would contain at least 5×10^9 particles/mL (or 10^{11} particles/20 mL). Thus, at least 3×10^{11} Ad5 particles (three dose equivalents) would be tested. Assuming a random (Poisson) distribution of RCA, if there were an average of one RCA per 1×10^{11} particles (20 mL), one would predict the probability of not detecting it by testing only 1×10^{11} particles to be $= e^{-1}$ or 0.3679 (36.79% chance). By testing 3×10^{11} particles (60 mL), the (binomial) probability of not detecting 1 RCA/1 $\times 10^{11}$ particles is reduced to $= e^{-3}$ or 0.04979 (4.98% chance). Mathematically, this is equivalent to three independent tests of 20 mL each (60 mL total).

No RCA was detected in the MVS or in any of eight virus-production lots assayed. Using the ratio of particle/TCID50 determined for purified virus (15.6 particles/infectious units), the virus-production lots were estimated to have an average of 1.9×10^{10} particles/mL. It was estimated that the mean probability of not detecting at least one RCA in a dose of 10^{11} particles of virus-production lots was 0.000887%. Besides having directly tested the infected cell suspension of the MVS for RCA, the repeated inability to detect RCA in the various clinical batches bodes well for the RCA-free nature of the MVS. For the clinical production runs, 1 mL of MVS is used to inoculate each of 100 roller bottles (RBs). This means a total of 800 mL of MVS have been used for these "clinical lots." Following the same calculation scheme as above, if there were one RCA per 20 mL of the MVS, there would be $e^{-1\times5}$ or 0.00674 probability (0.674% chance) of not transmitting an RCA when preparing a single clinical batch. Moreover, cumulatively across the eight clinical production runs, there would be only $(e^{-1\times5})^8$ or a 4.25×10^{-18} probability (4.25×10^{-16}%) chance of not

transmitting RCA in the preparation of eight lots. In conclusion, the 60-mL freeze–thaw sample used for RCA testing provided adequate assurance for the detection of RCA in virus-production lots, at a level of one RCA for a 10^{11} dose. However, for testing of future Ad5 vector lots, we plan to use a clarified lysate. In this case, the probability estimated for detection of RCA will be based on more direct measurement of virus concentration.

In summary, eliminating overlap between E1 sequences in the cell and the E1-deleted adenoviral vector eliminates RCA.

IV. Production of Adenoviral Vectors

A. Vector Stability

When constructing E1-deleted adenoviral vectors, a number of choices must be made regarding the structure of the vector backbone and the composition of the transgene. One must determine if the size of the E1 deletion will be adequate to accommodate the size of the transgene or if additional deletions, such as in the E3 region, will be needed. One must also decide on the placement of the transgene within the genome (E1 vs E3) and the orientation of the transgene (E1 parallel vs E1 antiparallel). Finally one must decide on the composition of the transgene in terms of the transcriptional regulation elements that are utilized (promoter and polyadenylation signals).

All of these parameters make constructing adenoviral vectors that express the transgene to the desired level, are genetically stable and propagate well enough to allow high-level production, a somewhat empirical process. The net genome size of the vector, the deletions used, transgene orientation, the composition of the transgene and the transgene product itself can all affect the growth and productivity of the vector. The degree to which vector and transgene structure can effect genomic stability and productivity is illustrated by our experience with Ad5 vector 1 (Fig. 4). Vector 1 contains an E1 deletion into which the transgene was introduced in the E1 antiparallel orientation. The transgene is composed of our gene of interest flanked by the immediate-early gene promoter and intron A from the human cytomegalovirus, and the bovine growth hormone polyadenylation signal sequence. In addition to the deletion of the E1 region, the vector has an E3 deletion [73].

When the genetic stability of vector 1 was assessed after serial passage in PER.C6 it was found to be unstable. Restriction analysis of purified viral DNA recovered from passages 12 to 19 indicated that the virus population contained genetic variants (Fig. 5). Over this passage series, the proportion and number of variants appeared to increase. An analysis of the novel restriction fragments and close to 1000 individually recovered, circularized viral genomes, indicated that two genetic mechanisms could account for all of the observed

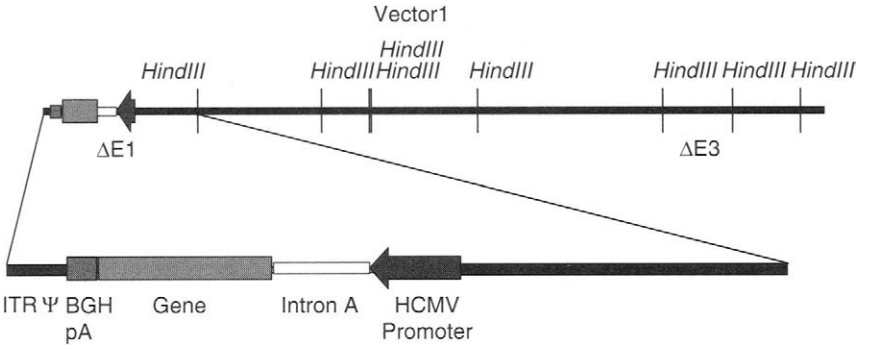


Figure 4 Genetic structure of Ad5 vector 1.

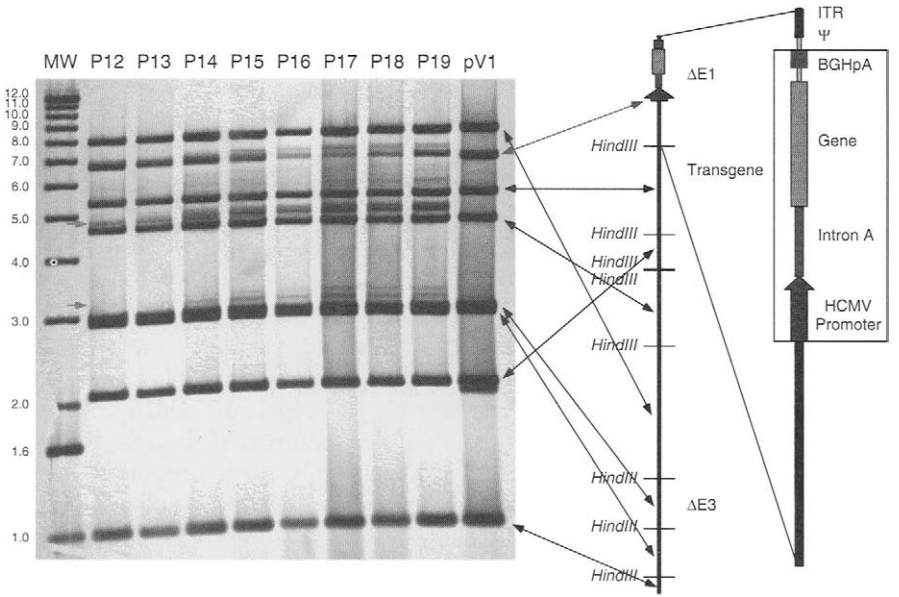


Figure 5 Genetic structure of serially passaged vector 1. Viral DNA was purified from passages 12 to 19 of vector 1 digested with *Hin*dIII and end-labeled with [P^{32}]-dATP. The end-labeled restriction fragments were then size-fractionated by gel electrophoresis and detected by autoradiography. pV1, the plasmid used to derive Vector 1 is shown for comparison. The position in the vector 1 genome to which the restriction fragments correspond is indicated on the right. The reduction and upward shift in the 6.6-kb transgene-containing restriction fragment (uppermost double arrow) is due to amplification of the 107-bp sequence in the packaging region. Novel bands seen at approximately 4.8 and 3.2 kb (arrowheads) are due to deletions in the transgene in association with amplification in the packaging region.

RFLPs: (i) deletions of the transgene expression cassette, particularly in the region of the hCMV promotor and intron A, and, in two instances, deletion of only adenovirus sequence; and (ii) amplification (two to four repeats) of a 107-bp sequence in the region containing the viral packaging elements. No rearrangements or insertions in the E3 region were detected.

The genetic analysis of vector 1 has led to the development of highly stable vectors that can be easily propagated in PER.C6 cells, suggesting that the genetic instability can be overcome by vector design and is not necessarily related to the use of PER.C6 cells.

B. The Production Process

To make E1-deleted adenoviral vectors for human gene therapy, a scaleable process suitable for commercial manufacturing under GMP conditions was developed. One of the key factors in the development of cell-culture-based production processes is the culture system. In particular, if scaling of the process is needed, culture of the cells in a bioreactor is highly desired. For robust and scaleable systems, suspension growth of the required cell line is extremely advantageous. PER.C6 cells can be cultured both as adherent cells and in suspension culture. For suspension growth, specific well-defined serum-free media have been developed (e.g., ExCell 525; JRH Biosciences). These media do not contain any protein that is derived from human or animal tissues or specimens. This results not only in many fewer contaminants to be removed during downstream processing but also a favorable safety profile with respect to pathogens which might be introduced by animal/human-derived components.

The serum-free culture medium (SF-medium) supports the growth of PER.C6 cells to densities of $1.5–2.5 \times 1^6$ cells/mL in routine T-flask and roller bottle cultures. In perfused bioreactor systems, cell densities up to 1^7 cell/mL are easily obtained.

An overview of the process of production of E1-deleted recombinant adenoviruses is presented in Fig. 6 and is summarized below.

After thawing a vial of PER.C6, expansion in a T-flask containing SF-medium is done, followed by transfer of the suspension culture to roller bottles. Then these roller bottles are cultured until sufficient cells are generated to inoculate a bioreactor. In the standard batch-wise production process (e.g., in 2- or 20-L bioreactor) half of the bioreactor working volume is inoculated at 0.5×10^6 cells/mL. Then PER.C6 is grown in 2 days to 2×10^6 cells/mL and diluted once to 1×10^6 cells/mL by adding the same volume of fresh medium. Then the seed virus is added and temperature is lowered from 37° to 35°C, followed by harvest after 3 days by pelleting. The latter is necessary if the purification process consists of ultracentrifugation with CsCl density gradients. After these 3 days, the virus particles become suspended utilizing

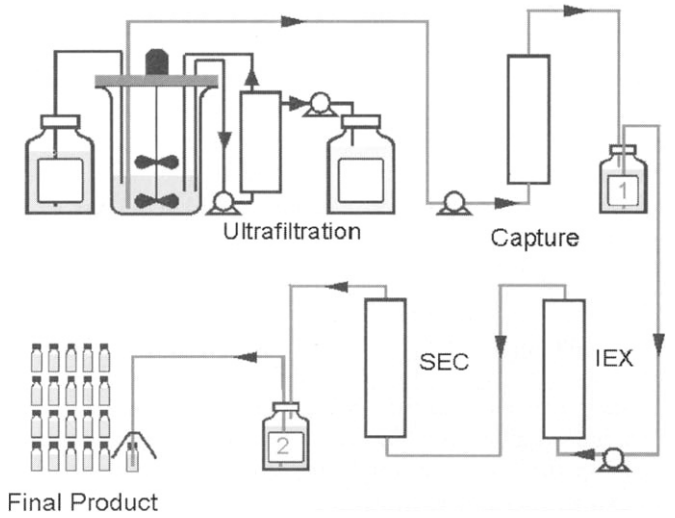


Figure 6 Overview of the process of production of E1-deleted recombinant adenovirus. The process is described in section 4B of this chapter.

cell lysis. The batch process is very robust but not economical since only low cell densities can be obtained due to the rapid consumption of nutrients from the medium. When high cell densities are required a perfusion system can be used. Nutrients are replaced and metabolites removed by perfusion of fresh medium. A suitable perfusion system can be obtained with hollow fiber modules. These modules are operated externally on the bioreactor and can therefore easily be replaced when malfunction occurs. Hollow-fiber technology also has the opportunity for virus retention, easy scale-up, and its potential application as a first step in the virus isolation. To take full advantage of high-density cultures the virus replication should last longer than 3 days to enable the utilization of all cells present because a repeated infection can occur with newly released particles from lysed cells. A typical example of a 20-L bioreactor run is presented in Fig. 7. Because a large part of the total produced virus will be in suspension, the volume of such a culture is too large to enable purification by ultracentrifugation. Hollow-fiber ultrafiltration and chromatography are methods of choice for virus isolation and purification. With these systems directly connected to the bioreactor, thereby ensuring a closed system, all virus can be isolated from the culture medium. After capture of the virus, the bulk product can be further purified utilizing ion exchange chromatography and/or size exclusion chromatography systems. The obtained product is of high purity and infectivity. Final formulation can be done by ultrafiltration, bringing the product to the final concentration in the required buffer.

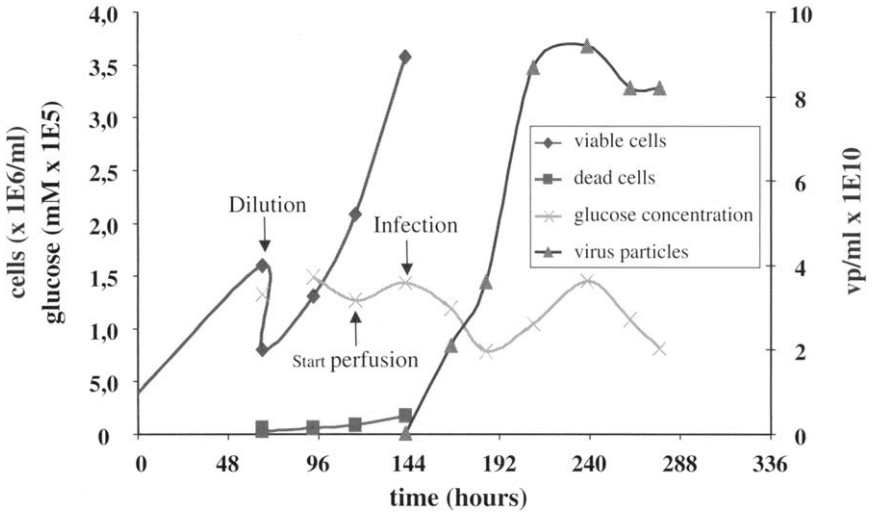


Figure 7 Example of production of E1-deleted adenoviral vectors in PER.C6 in a 20-L bioreactor. PER.C6 cells are seeded at a density of 0.5E6 cells/mL, in ExCell525 culture medium. Perfusion is started 48 h later, at a rate of 1 bioreactor volume/24 h. The glucose concentration remains constant during perfusion. Under these conditions, cell densities of 1×10^7 cells/ml are obtained.

C. Yields of Adenoviral Vectors

The yields of virus obtained after propagation in PER.C6 cells in 20-L suspension cultures ranges from 0.6×10^{11} to 1.1×10^{11} vp/mL culture medium with an average yield of 0.8×10^{11} vp/mL ($n = 5$). The cell density during infection was approximately 3×10^6 cells/mL. The calculated virus yield per cell is therefore 0.2×10^5–0.4×10^5 vp/cell. As the cultures are inoculated at a multiplicity of infection of 40 vp/cell, an amplification factor of 500 was achieved. The loss during isolation and purification can be held to 70–80%. This figure was consistently obtained in multiple runs for three different adenoviral vectors.

Similar yields of E1-deleted adenoviral vectors obtained on PER.C6 have been obtained by others [74].

D. Scale of Adenoviral Vector Production

The estimated scale of the required bioreactor and cell-line stability is calculated as follows. The cell density used for virus production in perfusion mode is $3–6 \times 10^6$ cell/mL. Therefore, assuming at least 20,000 virus particles per cell yield, the overall expected yield in the crude bioreactor harvest is 2×10^4 vp/cell $\times 5 \times 10^6$ cell/mL $= 1 \times 10^{11}$ vp/mL. Further, after optimization,

maximum expected loss of virus particles after downstream processing (DSP) by column chromatography is 75%. Therefore, from a 20-L perfusion bioreactor 1×10^{11} vp/mL $\times$ 0.25 (recovery) $\times$ 10^4 mL $= 5 \times 10^{14}$ vp can be obtained. This gives 5×10^{14} vp/1×10^{10} vp/*dose* = 50,000 doses (assuming 1×10^{10} vp/dose). When during product development 40% of the batch is retained for QC and archiving purposes 3000 patients can receive 50,000 $\times$ 0.6/3000 = 10 doses each. Therefore, using the currently developed technology, this 20-L bioreactor is sufficient for the generation of material for the first clinical studies. However, to be able to do process development on a larger scale, needed for full commercial production, a larger vessel is required. Full production scale is expected to be about five times larger, and therefore a 100-L bioreactor is expected to be the maximum volume required for application with single doses up to 10^{10} vp. To propagate the cells from a working cell bank ampoule, containing 5^6 cells, to a 5E6 cell/mL culture in a 100-L bioreactor would take 17 cell doublings. So a reliable production process would require a cell line which is at least stable over 20 cell doublings. PER.C6 was shown to be stable with respect to E1 expression for at least 98 cell doublings.

V. Safety Considerations of PER.C6

A. QC Testing of PER.C6 Cells for Use in the Manufacture of Biologicals and Vaccines

The safety of vaccines and biologicals manufactured in continuous cell lines of animal or human origin is of *paramount importance* and *must be ensured* by the manufacturer through a program of quality control (QC) testing applied to the product before release for human administration. This QC testing is intended to (i) ensure the *identity* of the product, (ii) ensure the *safety and sterility* of the product by demonstrating the absence of adventitious microbial agents, and (iii) ensure the *safety and sterility* of the product by demonstrating the absence of adventitious viral agents. The program for QC testing applied to a biological product, formalized as a *release protocol*, is developed as a responsibility of a Department of BioAnalytical Development. The release protocol is developed through an evaluation and integration of (i) relevant compendial literature and precedents, (ii) the origin of the cell line used for production and its development as a master cell bank, (iii) the sourcing and quality control testing of raw materials of animal origin used in manufacture, and (iv) the method of good manufacturing practice (cGMP) manufacture of the bulk and intermediate and final product considering, among other things, the quality of environment in which bioprocessing is conducted, the method of manufacture, in particular the isolation of the culture system from operators, and the consistency of preparation.

The release protocol prescribes the QC testing to be applied not only to final product but, importantly, master cell banks, master virus seeds, and other bioprocess inputs, raw materials of animal origin, and intermediate bulk products developed during downstream processing, purification and formulation. The release protocol specifies testing methods and volumes to be tested relying upon bacterial broth and agar cultures, embryonated eggs, small animals, and *in vitro* cell culture in a variety of primary and continuous cell lines of mammalian or human origin. These methods are well known to be sensitive to the detection of a variety of bacterial and viral agents and applied in concert provide a comprehensive and sensitive analytical approach upon which to ensure product safety. More recently, with the development of exquisitely sensitive polymerase chain reaction (PCR) methods for the detection of agents which are refractory to animal or cell culture, these classical propagation methods are commonly supplemented with agent-specific testing, using PCR and polymerase-enhanced reverse transcriptase (PERT) assays. The general methods of testing to ensure product safety are presented in illustrated form in Fig. 8.

1. QC Testing for the Release of PER.C6 Master Cell Bank

The development of PER.C6 research master cell bank (rMCB) A068-016 to support manufacture of biologics has been previously described. The release protocol to ensure the (i) identity, (ii) sterility, and (iii) viral safety of the rMCB is presented in Table II. The QC testing was conducted by contract at Inveresk Research (Tranent, Scotland) and at MicroSafe (Leiden, The Netherlands).

Table II
Release Protocol for Crucell rMCB A068-016

Test	Method
Identity	Isoenzyme analysis
Sterility	Broth and agar for cultivation of bacteria, fungi, mycoplasma
	In vitro indicator cells for detection of mycoplasma using Hoechst stain
Viral safety	
In vivo eggs	Eggs (allantoic and yolk sac)
In vitro cell culture	MRC-5, HeLa, Vero, bovine cells
Agent-specific testing using PCR	HBV, HCV, EBV, HHV6, HIV-1, HIV-2, HTLV-1, HTLV-2, AAV, B19, SV40
Agent-specific testing for retroviruses	PERT, S^+L^-, XC testing

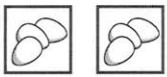
Method	*Criteria for Evaluation*
 ***Sterility*** *Inoculation of Broth and Agar Culture and Cell Cultures with Observation of 14-21 days*	***Turbidity, Colony Formation Cytoplasmic Fluoresence***
 ***In Vivo Testing in Eggs*** *Injection of Eggs by Amniotic, Allantoic or Yolk Sac Routes with Observation for 7-14 days*	***Viability Gross morphology Hemagluttination***
 ***In Vivo Testing in Animals*** *Injection of Adult or Suckling mice, Guinea pigs or Rabbits by IM, IP, or SC Routes with Observation for 7-60 days*	***Viability Fitness Evidence of Disease***
 ***In Vitro Testing in Cell Culture*** *Inoculation of Primary or Continuous Cell Lines of Human, Primate or Animal Origin with Observation for 14-28 days*	***Evidence of Cytopathology Hemadsorption Hemagluttination***
 ***Testing for Specific Virus Agents*** *Use of Sequence Specific Primers for PCR Amplification or PERT, or TEM*	***Evidence of Gene Specific Product*** ***Evidence of Enzymatic Activity of RT***

Figure 8 Testing methods for the demonstration of product safety.

2. QC Testing for the Release of a PER.C6 Working Cell Bank

The release protocol of research working cell bank (rWCB) A068-043W, according to the panel of testing, is presented in Table III. The QC testing was conducted by contract at Inveresk Research and at MicroSafe. This testing included tests for (i) identity, (ii) sterility, and (iii) viral safety in cells of human and simian origin.

3. Development of a Master Cell Bank at the Merck Research Laboratories

Cryopreserved vials of the rWCB were obtained from Crucell by the Merck Research Laboratories and expanded under conditions of cGMP manufacture to create a master cell bank (MCB) for future manufacturing use. This MCB has been released for use in the propagation of recombinant adenovirus according to a release protocol presented in Table IV. The preponderance of this QC testing was conducted by Q-One BioTech (Glasgow, Scotland).

This release protocol for the rWCB provides persuasive demonstration of the (i) identity, (ii) sterility, and (iii) viral safety of the PER.C6 MCB. This release protocol specifies animal testing in small animals to supplement the egg safety testing applied to the rWCB, expands the variety of primary and continuous cell lines used for viral safety using *in vitro* cell culture, and greatly broadens the variety of agent-specific testing using PCR-based testing and biochemical testing for retroviruses. The human cell line 293 was included in the panel of tissue culture cell lines in an attempt to detect the presence of any defective adventitious virus that requires the presence of E1 in the host cell. The direct assay for reverse transcriptase, as well as the detection of RT in cocultivation supernatant fluids, was done with the highly sensitive PCR-based reverse transcriptase (PBRT) assay. The supplemental PCR tests were included

Table III
Release Protocol for Crucell rWCB A068-043W

Test	Method
Identity	Isozyme
Sterility	Broth and agar for cultivation of bacteria, fungi, mycoplasma
	In vitro cell culture testing for mycoplasma
Viral Safety	
In vitro cell culture	Vero, MRC-5, PER.C6
Agent-specific testing using PCR	Adeno-associated virus

Table IV
Protocol for Release for the a PER.C6 Master Cell Bank

Test	Method
Identity	Isozyme analysis DNA Fingerprinting PCR-Based Test for E1
Sterility	Broth and agar for cultivation of bacteria, fungi, mycoplasma
	In vitro cell culture testing for mycoplasma
Viral safety	
In vivo eggs	Eggs (allantoic and yolk sac)
In vivo animals	Guinea pig, adult and suckling mouse
In vitro cell culture	VERO, MRC-5, 293, Rabbit Kidney 13, Vero, bovine turbinate, porcine kidney
Agent-specific testing	Transmission electron microscopy PERT for RT
	Raji, RD, H9 cell-cocultivation for retroviruses
	PCR for HBV, HCV CMV, EBV, HHV6, HHV7, HHV8, HIV-1, HIV-2, HTLV-1 & HTLV-2, SiFV, SFV, AAV, B19, bovine polyoma, SV40

with due consideration for the human origin of the cell line and the use of bovine serum for the derivation of the cell line. The tumorigenic potential of the cell line was tested beyond the anticipated manufacturing cell-passage level.

Satisfactory results were obtained from all QC testing. The results of the testings are presented in Table V.

B. Tumorigenicity

1. Tumorigenicity Studies of PER.C6 Cells

Three tumorigenicity studies were carried out on the PER.C6 cell line. The results of these studies are summarized in Table VI. In the first study, nude (nu/nu) mice were injected subcutaneously with 10^7 PER.C6 cells. Positive control animals were injected subcutaneously with 10^7 KB cells. KB is a known tumor-producing cell line derived from an epidermoid carcinoma (American Type Culture Collection; CCL-121). The study was conducted over 28 days, at which point all animals were necropsied and examined grossly and histologically. All of the positive control animals had growing nodules, and 8 of 10 male mice and 7 of 10 female mice receiving PER.C6 cells had growing nodules, thus producing a positive test (Table VIA).

At the time of the first study, 21 or 28 days was the duration that was usually used. Subsequently the Center for Biologics Evaluation and

Table V
Summary of Testing of PER.C6 Research Master Cell Bank (Passage No. 29)

Test	Specification	Result
Sterility (EP)	Negative	Negative
Mycoplasma (broth, agar and DNA staining)	Negative	Negative
In vitro virology for adventitious viruses (28 days, with cytopathic effect and haemadsorption) on Vero, MRC-5, HeLa and PER.C6 cells (PTC)	Negative	Negative
Specific viruses		
Human immunodeficiency virus types 1 and 2	Negative	Negative
Human T-lymphotropic virus types 1 and 2	Negative	Negative
Human hepatitis B + C	Negative	Negative
Human cytomegalovirus	Negative	Negative
Human parvovirus B 19	Negative	Negative
Human herpes virus 6	Negative	Negative
Simian virus 40	Negative	Negative
Adeno-associated virus	Negative	Negative
Epstein–Barr virus	Negative	Negative
Bovine viruses (BVD, IBR and PI3)	Negative	Negative
In vivo virology in suckling mice (i.c. and i.p.), and embryonated eggs, allantoic and yolk sac injections (PTC)	Negative	Negative
Isoenzyme test for human origin	Confirmed	Confirmed
In vivo virology (adult mice, guinea pigs and suckling mice) and transmission electron microscopy (TEM)	Absence of adventitious microbial contamination	Free from infectious adventitious microbial contamination
Reverse transcriptase assay	Negative	Negative
S^+L^- focus forming assay and XC plaque assay	Negative	Negative
Tumorigenicity in nude mice	Report result	Tumorigenic
Restriction analysis	No evidence of mutation or rearrangements	No evidence of mutation or rearrangements
Sequencing	Report sequence	Sequence reported

(*continued*)

Table V
(continued)

Test	Specification	Result
DNA profiling rMCB (passage 29) and late passage cells (passage 98)	Late passage banding pattern resembles rMCB	Late passage banding pattern resembles rMCB
Karyotyping/chromosomal analysis	Report chromosome numbers	Modal No. 86. Range 68–106
Fluorescent product enhanced reverse transcriptase (PERT) assay	Negative	Negative
S^+L^- focus forming assay and XC plaque assay	Negative	Negative
Multicolor fluorescent *in situ* hybridization (M-FISH)	Report integration site	Chromosome 14
Copy no. determination (fiber FISH analysis)	Report results	13.6 ± 6.1
Prions Determination of prions Sequence analysis	No evidence for infectious PrP^{Sc}	Confirmed

Research (CBER) of the Food and Drug Administration had suggested the observation period be extended to 84 days. This was to give more time for slow growing tumors to appear and for nontumorigenic nodules to regress or disappear. Therefore, the tumorigenicity study on the PER.C6 cells was repeated.

The second study was performed in nude (nu/nu) mice over an 84-day period. Thirty nude mice were injected subcutaneously with 10^7 PER.C6 cells in 0.2 mL of serum-free medium. As a positive control, 10 mice were injected subcutaneously with 10^6 HeLa cells in 0.2 mL of serum-free medium. As a negative control, 30 mice were injected with 0.2 mL of medium. The mice were palpated at the injection site every 3 to 7 days and any nodules found were measured in two dimensions. The PER.C6 cell test arm and the negative control arm had 10 mice necropsied 21, 42, and 84 days postinjections. The positive control arm was necropsied at 42 days postinjection. Gross and histological examinations were performed on all injection sites and nodules if they appeared. During the initial days after injection, palpable nodules were present at the subcutaneous injection sites in all animals inoculated with PER.C6 cells. Between postinjection days 5 and 14, the detectable masses disappeared from the injection sites. However, in several of these mice, the masses subsequently reappeared by around day 21 and continued to enlarge until the animals were necropsied. Of the mice injected with PER.C6 cells, 5 of 10 sacrificed on day 21, 5 of 10 sacrificed on day 42, and 1 of 10

Table VI
Tumorigenicity of PER.C6 Cells

A. Day 28 tumorigenicity of PER.C6 and KB cells in nude mice

Cell type	No. of cells	Male	Female
KB	1×10^7	10/10	10/10
PER.C6	1×10^7	9/10	7/10

B. 84-Day tumorigenicity study of PER.C6 and HeLa cells

Cell type	No. of cells	Day 21	Day 42	Day 84
HeLa	1×10^6	NA	10/10	NA
PER.C6	1×10^7	5/10	5/10	1/10
Medium control	—	1/10[a]	0/10	0/10

C. Titration tumorigenicity study of PER.C6 cells in nude mice

Cell type	No. of cells	Day 21	Day 42	Day 84
PER.C6	1×10^3	0/10	0/10	0/10
PER.C6	1×10^5	0/10	0/10	0/10
PER.C6	1×10^7	5/10	9/10	7/10[b]
Medium	—	0/10	0/10	0/10

Note. Details of the experiment are presented in Section 5B.
[a] Benign lung adenoma.
[b] Seven animals sacrificed, with tumors on day 56 and leaving 0/3 at day 84.

sacrificed on day 84 (actually sacrificed on day 49 due to tumor size) had gross or microscopic evidence of a tumor (Table VI B). Histologically, these recurrent nodules were composed of sheets of large pleomorphic cells with numerous, sometimes abnormal, mitotic figures. These masses compressed the surrounding tissues but were not invasive. No tumors were observed outside the injection sites. The interpretation of the test is that PER.C6 cells are positive for tumorigenicity.

In view of the positive tumorigenicity results obtained following injection of 10^7 PER.C6 cells, a titration study was performed in which nude mice were injected with PER.C6 cells at doses of 10^7, 10^5, or 10^3 cells per animal. Mice were necropsied 21, 42, or 84 days postinjection. No animals receiving 10^3 PER.C6 cells had palpable masses at the injection site from the first palpation day until necropsy. None of these animals had gross or microscopic evidence of nodules or tumor cell collections at any necropsy time point. Two of the 30 mice receiving 10^5 PER.C6 cells had palpable nodules on postinjection day 3.

These masses disappeared by day 7 and did not recur. Gross and histological examination of the injection sites were negative at all necropsy time points. In the mice that received 10^7 PER.C6 cells, 29 of 30 animals had palpable nodules on Day 3—some of which disappeared or became smaller but most of these recurred and grew progressively until necropsy. At necropsy, 5 of 10 mice on day 21 had tumors, 9 of 10 mice sacrificed on day 42 had tumors, and 7 of 10 in the group scheduled for day 84 had tumors but were sacrificed on day 56 because of tumor size (Table VI C). The histological and gross features of the PER.C6 cell tumors were similar to those described for the previous study (above). No metastatic nodules were found. Thus, the tumorigenicity studies of PER.C6 cells were positive at 10^7 cells per animal and negative at 10^5 and 10^3 cells per animal. This would indicate that not all of the PER.C6 cells are tumorigenic and/or a critical mass of tumorigenic cells are necessary for tumor formation.

2. Tumorigenicity Studies of Residual DNA from PER.C6 Cells

In view of the positive tumorigenicity studies with 10^7 PER.C6 cells, the oncogenic potential of residual DNA from these cells was tested in both nude mice and newborn hamsters. For these studies, DNA was isolated from passage 61 PER.C6 cells using standard procedures. The DNA preparation was shown to be of high molecular weight (average size ~100 kb) and devoid of significant protein or RNA impurities. In the nude mouse study, 20 female nude (nu/nu) mice were injected subcutaneously with 225 μg of PER.C6 DNA (in a volume of 0.25 mL). For negative controls, two groups of 20 female mice each were injected subcutaneously with 0.25 mL of vehicle. Approximately 5 months after injection, the mice were necropsied and examined histologically for tumor growth. None of the mice in this study exhibited gross or microscopic evidence of tumors at the injection site. One treated mouse had a lymphoma at a distant site. However, nude mice—particularly females—are known to have a high incidence of spontaneous lymphoma [75–78], and the occurrence of a single lymphoma in 20 treated mice is consistent with the spontaneous incidence. Although the lymphoma was almost certainly a spontaneous event, a polymerase chain reaction (PCR) study was performed on the lymphoma DNA to determine if there was any evidence for the presence of the adenovirus E1 region—the transforming agent of PER.C6 cells. The study was negative, with a sensitivity of approximately one copy of E1 per 750 tumor cells. Previously, E1 expression has been shown to be necessary to maintain the transformed state of 293 cells, which, like PER.C6 cells, were transformed by E1 [79]. The results of the PCR analysis support the conclusion that the lymphoma was a spontaneous event, not induced by PER.C6 DNA.

A second tumorigenicity study using DNA from PER.C6 cells was carried out in newborn hamsters. Between 18 and 36 h after birth, female and male

hamsters (28 total) were injected subcutaneously with approximately 100 μg of PER.C6 DNA (in a volume of 110 μL). Two groups of control hamsters (50, mixed sex, per group) were injected with 100 μL of vehicle. Several pups in each group were lost due to maternal cannibalism, reducing the group sizes to 20 (11 female, 9 male) in the PER.C6 DNA group, 40 (19 female, 21 male) in control group 1, and 45 (27 female, 18 male) in control group 2. After weaning, the hamsters were palpated on a weekly basis. The hamsters were necropsied approximately 5 months after injection and examined grossly and histologically for tumor growth. One female hamster in control group 2 died approximately 21 weeks after injection of a malignant ovarian teratoma. No evidence of tumors was found in the 20 hamsters that were injected with PER.C6 DNA.

3. Concerns about Using a Tumorigenic Cell Substrate

The basis for concern about using a tumorigenic cell substrate to produce a vaccine includes three theoretical possibilities. First, DNA from the cells carrying a putative activated oncogene or cancer-causing mutation could be integrated into the recipient's genome and produce a tumor. Second, a transforming protein in the cells could be transmitted and result in a tumor. Third, an adventitious tumor virus may be present and could be transmitted to the recipient and produce a tumor.

Concerning residual DNA from a tumorigenic cell substrate, there have now been several reports demonstrating that DNA extracted from tumorigenic cell lines or tumors growing *in vivo*—and even purified activated oncogenes—do not produce tumors when injected into animals at levels up to 1000 μg of DNA [80–87]. The negative results obtained with PER.C6 DNA in nude mice and newborn hamsters are consistent with these findings. In the case of the PER.C6 studies, the amount of DNA injected (~100 or 225 μg) represents a $>10^6$-fold excess compared to the amount of residual DNA present in a dose of vaccine produced on this cell substrate. Others have calculated that 100 pg of residual DNA from tumorigenic cells would be equal to less than a billionth of a tumor-producing dose [80–87].

The second concern, transforming proteins or growth factors, has been considered by a WHO study group to be significant only if they are continually produced by cells or have continued administration [80, 81]. The study group did not consider the presence of contaminating known growth factors, in the concentrations that they would be found, to constitute a serious risk in biological products prepared from continuous cell lines.

The third category of concern, viruses or other adventitial agents, does present a potential risk. This risk is greatest when primary cells are used because of the frequent need for newly acquired cells that require repeats of the extensive testing for adventitial agents. Human diploid cell lines and continuous tumorigenic cell lines are thoroughly and routinely tested for a

wide variety of known and unknown adventitial agents in a series of *in vitro* and *in vivo* assays, thus providing adequate assurance that adventitial agents will not be transmitted.

C. Prion-Related Issues

It is now generally accepted that an abnormal form of the cell surface glycoprotein PrP, or prion protein, is the main infectious agent in transmissible spongiform encephalopathies like scrapie, bovine spongiform encephalopathy (BSE), and Creutzfeldt–Jakob disease (CJD) ([88] and reviewed in [89]). The abnormal form of PrP, called PrP^{sc} or PrP-res, is characterized by a remarkable resistance to denaturing agents and to degradation by Proteinase K (Prot K). Diagnostic tests take advantage of this unusual stability that allows a distinction between PrP^{c} and PrP^{sc} using antibodies that recognize both forms of PrP (e.g., [90]).

Human prion diseases occur in sporadic, acquired or inherited forms with different clinical and pathological phenotypes (reviewed in [91]). In 1996 a new variant of CJD (vCJD) was reported in the United Kingdom in relatively young patients with clinical features different from the known CJD forms [92]. It was also found by strain typing that the prion protein of these patients was indistinguishable from the one that causes BSE, thus raising the question whether vCJD could be acquired by consumption of meat from cattle suffering from BSE [93, 94]. The possibility of transmission of PrP^{sc} from bovine to human raises safety issues for cultured cell lines used for the production of human drugs.

Therefore, PER.C6 cells were carefully examined for the PrP phenotype (see below) as well as genotype. It has been found that specific mutations in the PrP gene are associated with hereditary forms of human prion disease (reviewed in [89] and [91]). Furthermore, a common methionine/valine polymorphism at codon 129 of the PrP gene appears to be associated with phenotypic variability and susceptibility to sporadic and iatrogenic CJD. The vast majority of patients suffering from sCJD and also from vCJD were found to be homozygous for 129 M, whereas patients heterozygous at codon 129 were strikingly underrepresented [95–97]. To examine whether the PER.C6 PrP gene contains any of the known mutations associated with susceptibility to prion diseases, the PER.C6 PrP gene was sequenced. For these sequencing studies, genomic DNA from PER.C6 cells was isolated, and used to amplify the PrP gene sequences by PCR. The resulting PCR product was cloned into a vector, and the PrP gene in each of 13 PrP-containing clones was sequenced by BaseClear (Leiden, The Netherlands). Five of these clones contained sequences coding for the 129 Methionine PrP^{c} protein, while the other eight contained the 129 Valine PrP^{c} sequence, demonstrating the heterozygosity at this position. To confirm this observation, the resulting PCR product was also sequenced. As expected, a double peak (g/a) was observed in the 129 codon at a position

defining it as a valine (if the nucleotide is a guanine) or as methionine (if the nucleotide is an adenine). The PER.C6 PrP gene sequence was then compared to the wild-type sequence published in GenBank (Accession No. M12899) and was found to be identical to the wild type gene; thus, ruling out the possibility that these cells possessed a hereditary mutation that would be predisposing for prion diseases. The sequence also revealed that PER.C6 cells are heterozygous for methionine/valine at codon 129.

PrP^c is constitutively expressed in adult brain [90, 98, 99] and at lower levels in other tissues like liver and spleen [100]. PrP expression has also been found in a variety of rodent and human cell lines. Our studies on PER.C6 and 293 cells have shown that these cells also express the cellular form of PrP. A validated Western blot analysis of Prot K-treated protein extracts of PER.C6 cells and their parental HER cells has failed to detect any Prot K-resistant forms of PrP at passages 33 and 36 of PER.C6 cells and passage 6 of their parental HER cells.

In addition to the sequencing of the prion gene and testing for the presence of abnormal prion protein in the PER.C6 cells at an early and late passage level of the culture, serum and trypsin batches that were used were traced to see if any were derived in the United Kingdom.

Finally, it has been possible to adopt the PER.C6 cells to serum-free suspension so that bovine sera can be completely avoided in the future if desired.

The above-mentioned characteristics of PER.C6 make it a safe manufacturing cell line in this respect.

D. Genetic Characterization of PER.C6 Cells

1. Sequence Analysis of E1

The integrity of the E1A and E1B coding regions present in PER.C6 was tested by sequence analysis. This was done by bidirectional sequencing of PCR fragments generated from these regions, and the sequence of these fragments was compared to the original pIG.E1A.E1B sequence, the construct that was initially used in transfection.

No mutations, deletions, or insertions were detected between the sequence of the PCR fragments and pIG.E1A.E1B, indicating that no genetic alterations were introduced in the E1A and E1B regions during transfection and subsequent culture of the cells.

2. Site of Integration of E1

The chromosomal integration site of the plasmid pIG.E1A.E1B in PER.C6 was determined by using the multicolor fluorescent *in situ* hybridization (MFISH) technique in combination with the principle of combined binary ratio labeling (COBRA) [101]. This technique combines 24-color COBRA-MFISH

using chromosome-specific painting probes for all human chromosomes with plasmid probe (pIG.E1A.E1B) visualization (25th color).

The pIG.E1A.E1B integration site was determined using PER.C6 cells that are derived from the research master cell bank (passage number 29). Cells were analyzed at passage numbers 31, 41, 55, and 99. Two hundred and fifty metaphases and interphases were studied.

pIG.E1A.E1B integration was detected only on chromosome 14 (Fig. 9, see color insert) and in both sister chromatids of the chromosome in all PER.C6 passage numbers screened. Of the 47 metaphases and 203 interphases, 75–80% consisted of integration of pIG.E1A.E1B in one chromosome 14, whereas 20–25% consisted of integration in two chromosomes 14 [102].

3. Copy Number of the E1 Construct

The number of copies of pIG.E1A.E1B present in the PER.C6 chromosome was studied by Southern blot analysis, dot bot analysis and fiber FISH analysis [102]. Southern hybridization revealed the presence of several integrated copies of pIG.E1A.E1B in the genome of PER.C6 [46].

In addition, dot blot analysis showed a pIG.E1A.E1B plasmid copy number of 19 ± 3 (research master cell bank) and 24 ± 16 (extended cell bank, passage number 99) per genome.

From the results it was concluded that PER.C6 consists of five to six copies of pIG.E1A.E1B per haploid genome.

Fiber FISH enables physical length measurements of *in situ*-hybridized DNA probes on linearized DNA fibers with a resolution equal to the theoretical length of a linearized DNA molecule according the model of Watson and Crick (1 kb is 0.34 μm). Therefore, fiber FISH was conducted to measure the length of the integrated construct in the PER.C6 cell line at passage numbers (pns) 31, 41, and 99. Twenty fibers were measured. It was determined that pIG.E1A.E1B was integrated in tandem copies in chromosome 14 of PER.C6. The copy number of these in-tandem integrations was determined to be as follows: pn31, 13.6 ± 6.1; pn41, 18 ± 4.5; and pn99, 20.1 ± 7.9.

4. Chromosome Analysis

PER.C6 cells from cellular passages 44 and 66 were harvested for chromosome analysis to determine the modal chromosome number and the karyotype in a sample of metaphase plates. Cells were harvested, and slides were prepared and stained using a standard giemsa banding (GTG) technique. At each passage level, the chromosomes in 50 metaphase plates were counted. Also, full karyotypes were prepared from each passage level.

At passage level 44, the chromosome number ranged from 43 to 160. The mean number of chromosomes was 72 and the modal number was

61. All metaphase plates examined had structural chromosomal changes and rearrangements. A marker chromosome 19 with additional material in the long arm ($19q^+$) was the most common alteration and was found in 14 of the 20 metaphase plates that were karyotyped.

At passage 66, the chromosome number ranged from 42 to 112. The mean number of chromosomes was 63 and the modal number was 64. All metaphase plates karyotyped again were found to have structural changes. The $19q^+$ was again the most common change, observed in 15 of 20 karyotypes. There was also a marker chromosome 11 with extra material in the short arm ($11p^+$) in 14 of the 20 karyotypes and a marker chromosome 9 with additional material in the short arm ($9p+$) in 8 of the 20 karyotypes.

Several of the markers differed at the two passage levels, but the most common marker, $19q^+$, was the same. The continuing changes seen as passage level increases is typical of heteroploid continuous cell lines.

5. DNA Fingerprinting

PER.C6 cells were also analyzed on two occasions by DNA fingerprinting. DNA profile analysis of PER.C6 indicated no changes in the banding pattern obtained between the research master cell bank (pn 29) and an extended cell bank that was laid down at passage number 99. On a second occasion, a consistent DNA fingerprint was obtained between pn 45 and pn 67. There was no evidence of cross contamination with other cell lines.

VI. Conclusions

At the present time, the PER.C6 cell line is the best substrate for the production of adenoviral vectors for gene therapy or vaccines. This conclusion is based on the ability to obtain good yields and safety considerations.

The major safety considerations are the possibility of:

i. the production of replication-competent adeno virus (RCA);
ii. a tumorigenic risk from the transformed cell line;
iii. the presence of abnormal prions;
iv. contamination by adventitial agents.

As described in this chapter, the lack of any overlap between the genome of the adenoviral vectors that carry the E1 deletion and the adenoviral E1 sequences carried in the PER.C6 cells makes homologous recombination impossible, thereby preventing the formation of RCA.

It is well known that many transformed cell lines can produce tumors when injected into immunodeficent animals. As described, PER.C6 cells produce tumors in nude mice when 10^7 cells are injected. They do not produce

tumors, however, when 10^5 or 10^3 cells are injected. Since it is not anticipated that there will be any PER.C6 cells in a final product, this leaves the question of possible tumorigenicity of residual PER.C6 cellular DNA. Studies in nude mice and newborn hamsters in which DNA from PER.C6 cells was injected were negative for tumor production.

The possibility of the presence of abnormal prions that could produce a neurodegenerative disease was also considered. This could occur if the PER.C6 cells had a mutation in a prion gene or if the cells were contaminated with abnormal prions such as in bovine spongioform encephalopathy. As far as possible, all serum and trypsin batches used from the time of origin of the culture were traced and no contact of serum from British sources was identified. The PER.C6 cell line was also adapted to serum-free suspension cultures.

The prion protein gene of PER.C6 cells was sequenced and no mutations were found and the cell line was shown to be heterozygous for the 129 M/V polymorphism. The cell line was also analyzed for the presence of abnormal prions at an early and late passage and an early passage of the HER parental line and none were found. In total, these studies indicate that the risk of a prion disease from the use of PER.C6 cells is vanishingly small.

Finally, extensive studies for known and unknown adventitial agents have been documented and are negative.

While there can be no absolute elimination of risk, this body of studies indicates a minimal, if any, risk from the use of this cell substrate for the production of adenoviral vectors. As new studies are developed they will also be applied to ensure that no hazards are present. It has often been pointed out that a continuous cell line such as PER.C6 permits extensive analysis for adventitial agents and other safety concerns and thus is less hazardous than short-lived primary cell cultures for which testing must be repeated for each newly established culture.

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CHAPTER 7

Purification of Adenovirus

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I. Introduction

Since the late 1950s, adenoviruses have been purified using classical methods of density gradient ultracentrifugation. These methods were efficient and could supply the quantities of highly purified viral particles necessary for research. The need for larger quantities has arisen with the advent of the use of adenoviral vectors for gene therapy trials. In this chapter, we discuss the techniques for extracting adenoviral particles from a complex milieu. Selecting the best technique for purification requires an understanding of the physical nature of the particles as well as the nature of contaminants. Knowledge of these properties is essential for developing a purification process that is sufficient to supply the commercial market for a therapeutic adenovirus.

A. The Physical Characteristics of the Adenovirus Particle in Solution

There are numerous adenoviruses that possess specific tropism for many species of animals including human, bovine, ovine, equine, canine, porcine, murine, and simian adenovirus subgenera. Although many of these adenoviruses are capable of delivering a transgene to human tissues, the development of clinical adenoviral agents most often employs human adenoviral vectors derived from human adenovirus serotypes 2 or 5. Consequently, for the purposes of this discussion, the information given here refers to human adenovirus types 2 and 5.

1. Particle Size

Size and shape are key factors involved in purifying any macromolecule. These factors are equally applicable to adenoviruses, which are much larger than most biomolecules commonly purified. Adenoviruses are icosahedral in shape with fiber-like extensions from each of the 12 vertices. The adenovirus is composed of DNA, protein, and carbohydrate. The viral DNA is packaged in a highly organized protein coat termed the "capsid." Negative staining electron microscopy of the adenovirus capsid was used to estimate a diameter of 73 nm along a fivefold symmetry axis with a vertex-to-vertex diameter of about 83 nm [1]. Freeze–fracture studies demonstrate a slightly larger capsid diameter. Fibers extending from each of 12 vertices increase vertex-to-vertex diameter by about 40 nm for human adenovirus serotype 5. Oliver *et al.* [2] employed photon correlation spectroscopy to characterize the adenovirus type 5 particles in solution, reporting a molecular weight of 167×10^6 Da and a corresponding particle diameter of 98 nm.

2. Diffusion of Adenovirus Particles

The adenovirus particle diffuses very slowly in solution. The diffusion coefficient for Ad-5 is 4.46×10^{-12} m^2/s in serum containing media at 37°C [2, 3]. Figure 1 compares the diffusion constants of some well-known

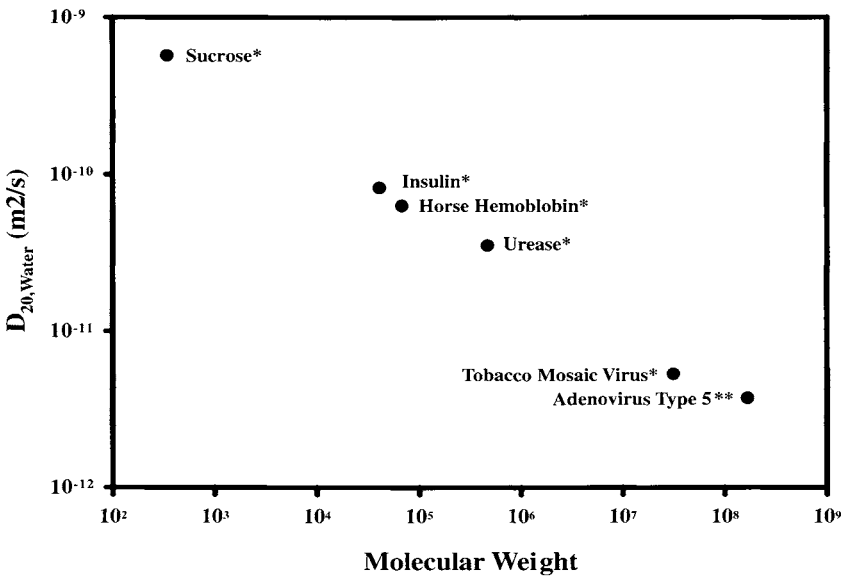

Figure 1 The diffusion coefficients of macromolecules are related to their sizes. Adenoviral particles diffuse very slowly in solution. This slow diffusion rate affects mixing, separation, and analytical methods.

macromolecules to adenovirus. The large size and corresponding slow diffusion of adenovirus particles in solution require consideration in mixing because, given the density of the particle and its slow rate of diffusion, Brownian motion cannot be relied upon to disperse the particle. If left alone the particles in solution would take weeks to reach equilibrium. However, gravity will intervene and cause the particles to sediment to the bottom of the container. Consequently, adenovirus solutions require greater agitation than protein solutions to disperse the particles evenly.

The slow diffusion rate also complicates analytical methods. For example, the interaction between the virus and a cell takes much longer than protein–cell interactions because of the slow diffusion rate. Typical biological methods for quantifying particles depend on Brownian motion for bringing about virus–cell interaction. Without accounting for this slow diffusion, the titer of the material tested may be underestimated. This will be discussed in more detail below (see section III.B.2 of this chapter).

3. Capsid Surface

The surface of the adenovirus capsid is of particular interest when selecting a separation technique since there are many binding methods available. The adenovirus capsid consists of 252 capsomeres. Two hundred and forty of these capsomeres are hexons and 12 are pentons. Hexons are trimers of protein II and are the main structural component of the capsid. Pentons, constructed from penton base and fiber proteins, are prominent features as these structures protrude and add to the hydrodynamic radius of the virus particle. In addition, protein IIIa and protein IX may also contribute to surface characteristics. Protein IIIa is essential and two of these proteins are found at the junction joining adjacent facets together like stitching. Protein IX is not essential for capsid assembly but enhances the stability of the virus at higher temperatures. Four protein IX trimers stabilize a group of nine hexons (ninemers) that have assembled into a facet [4].

The hexon, which comprises about 50% of the total virion protein, dominates the charge characteristics of the particle. Hexon capsomeres possess an isoelectric point (pI) near pH 6 [1]. At physiological pH, the capsomere would be expected to bear a negative charge. The complete adenovirus structure would also be expected to show a net anionic surface charge under physiological conditions. It is generally advisable to avoid exposing the proteins to solutions at or near the pI because many proteins change conformation, degrade, or precipitate when titrated through it.

Ninemers, hexons, and complete virions precipitate from solution when titrating the solution from pH 7 to pH 5 [5, 6]. If the particle is titrated through the pI rapidly and further lowered, losses occur but active particles can be recovered from solutions such as acetic acid. The virus is also stable up to around pH 8; the exact threshold being dependent upon the composition of

the solution. Exposing particles to a pH greater than 8 generally leads to a loss of activity and particle disruption.

4. Hydration of the Adenovirus Particle

The degree of hydration of the particle is an important consideration for both purification and stability of the adenovirus. Sedimentation by ultracentrifugation using Schlieren optics [2] suggested that the adenovirus particle contains a "hard core" (water excluded) with a diameter of 76 nm, similar to that obtained using negatively stained electron microscopy. The difference between the hard core diameter of 76 nm and the light scattering diameter of 98 nm is significant because it suggests that the particle contains water. The amount of water represented by the difference suggests that the virus particle contains 2.3 g of water for every gram of virus [2]. These observations are consistent with measurements of other viruses [7, 8].

While proteins are stabilized by the incorporation of a few water molecules ("waters of hydration"), the amount of water suggested by these studies is more than 21 million water molecules per virion. This amount far exceeds water typically bound to proteins. This degree of hydration corresponds to a theoretical density calculation of about 1.4 g/mL, which is very close to the observed density of 1.34 g/mL. The additional water should not be surprising since the virus exists in an aqueous environment. With these data in mind, however, the particle would be expected to show unique properties.

The adenovirus particle is generally considered rigid. However, the degree of hydration suggests that a certain amount of flexibility should be considered. Since the particle is not encapsulated in a membrane small ions may have ready access to the core (see section IV). Changes in ionic strength may induce conformational changes in the capsid proteins; some of these may be beneficial for separation and some may be catastrophic. The particle may also be sensitive to rapid changes in salt concentrations. One might also predict that hydrophobic solvents should be avoided.

B. Features of the Milieu

Effective virus purification capitalizes on the differences between the physical properties of the adenovirus relative to the components of the mixture from which it is being isolated. The exact composition of the milieu varies with the cell culture process, and, to a lesser extent, every batch. In general, the large-scale purification of adenovirus requires the isolation of the virus from infected cell lysate taken from a bioreactor. This mixture consists of a formulated medium usually containing bovine serum, and less frequently antifoaming agents, or anti-clumping agents (pluronics). Significant amounts of additives, however, present difficult challenges for any recovery procedure. Efficient large-scale production requires high cell densities which in turn require

high gas exchange rates. This can cause severe foaming and necessitate the addition of agents to control it. Other additives, such as anti-clumping agents and lipids, adapt the media for large-scale cell culture. Cell lysis, necessary to release the adenovirus from the host cell, results in the additional release of DNA, protein, lipids, carbohydrates, and other cellular components. Culture conditions, media components, cell derived contaminants, and additives may have a significant impact on downstream processing.

1. Culture Conditions

Adenoviruses are produced by infection of cell lines in culture with a viral seed stock. The particular cell line used requires a highly developed cell culture method to achieve maximum yield. Flat stock culture, although useful for small-scale work, is generally not sufficient for larger scale applications. Many of the cell lines used in flat stock culture have resisted attempts to adapt them to the suspension and serum free conditions preferred for large-scale processes. A compromise is struck by the culture of attachment-dependent cells using microcarriers in a bioreactor. This microcarrier-based process introduces yet another component that must be separated from the adenovirus. Similarly, if serum is utilized, it will be necessary to consider the effective removal of its components.

2. Construct Induced Contaminants and Considerations

The viral construct may also contribute to the milieu as the viral DNA backbone may lead to the contribution of many more contaminants. For downstream purification, higher titers favor better recovery and cleaner preparations because recovery and purification is an enrichment process. Even with maximum productivity, however, adenoviral particles represent a small fraction of molecular entities produced by the end of the culture process. Therefore, factors affecting the end titer can also affect the process.

The majority of adenoviral vectors for gene therapy are serotype 5 and have been rendered deficient for replication in most cells. With the exception of replication-competent adenoviruses, most vectors have been crippled to eliminate their replication in normal human cells. In general, when compared to wild-type virus, deletions or mutations in the early genes tend to attenuate viral replication in all cell lines. Attenuation for replication is typically achieved by large deletions in the immediate-early region E1. These vectors require specialized packaging cell lines for efficient production. Cell lines such as HEK 293 [9] or PER.C6 [10] have been transformed with adenoviral DNA and provide sufficient E1 function *in trans* to enable replication.

In addition to E1 deletions, many vectors possess deletions for much of the E3 region. The deleted E3 genes are considered nonessential for viral replication and these deletions allow for larger transgene packaging capacity. Other deletions have been made to reduce the frequency of recombination

during culture. So-called "second generation" viruses may have additional early gene deletions (e.g., in E4) as well as a deletion of protein IX encoding sequences [11]. Elimination of certain essential genes from the virus requires that the cell line be able to complement these protein functions *in trans* to package the virus [12].

A significant unwanted by-product of adenoviral replication is DNA. Wild-type human adenoviruses are able to replicate in a variety of both quiescent and proliferating human cells due to the function of adenoviral immediate-early genes. E1a proteins can be observed within 1 h after infection, as cellular transcription factors are sufficient to transcribe the E1a genes. E1 expression initiates the adenoviral life cycle by altering the cell cycle machinery to induce cellular DNA replication even in quiescent cells. Viral and cellular proteins activate subsequent viral transcription. New copies of viral DNA are synthesized and viral production proceeds in a replication cascade. By the end of viral DNA replication, a large amount of DNA is present in the infected cell.

The purpose of a gene therapy vector is to convey a therapeutic effect by the delivery and expression of therapeutic genes. Many of these transgenes have a significant effect on the cells and adenoviral life cycle. Some genes, such as the retinoblastoma protein and the cyclin-dependent kinase inhibitor p21 directly affect the levels of activated E2F. E2F is a cellular transcription factor that is necessary for the transactivation of the adenoviral E2 promoter and thus the expression of viral DNA replication proteins. Other transgene products, such as proapoptotic proteins, can overcome the adenoviral block to apoptosis leading to early cell death and can interfere with adenoviral particle assembly. Secreted pleiotropic transgene products, such as growth factors, can trigger undesired effects in the packaging cell and severely attenuate production. Before adenoviral DNA can be coated with viral core proteins, the DNA is available for transcription. In this way, some adenoviral genes, especially those encoding capsid components, are not expressed until DNA replication occurs [13]. During this phase, the expression of transgene product is enhanced by the replication cycle itself by expanding the copy number of the transgene with each copy becoming available for transcription. Strong exogenous promoters may also sequester transcription factors and the cellular protein synthesis machinery can become clogged with transgene expression leading to attenuated adenoviral protein production. If the sequence of events leading to particle maturation is disturbed, even by an imbalance in protein production, large quantities of viral proteins, incomplete particle assemblies, transgene products, abnormal cellular structures, and in some cases extreme amounts of extracellular proteins can be added to the milieu. These complications can significantly impede purification and add to the analytical requirements.

C. Summary of Characteristics

The attributes of the adenoviral particle, the culture process, components of the media and properties of the vector itself have significant impacts on the design of a purification process. Table I outlines the salient aspects of the particle and the lysate.

II. Recovery and Purification of Adenoviral Particles

Purification must take place in the context of a complicated lysate. Table II summarizes the key features of the adenovirus particle and suggests recovery techniques that may be employed. Together, the features of the particle and the milieu point to a sequence of process steps that yield purified adenovirus (Table I). Since the particles are produced in cells, the first recovery step is harvesting of the infected cells. The next step requires lysis of the cell to release the virus. The cell lysate contains cell debris so a clarification step is necessary to protect downstream steps. A substantial amount of DNA is present and must be eliminated early in the process. The clarified lysate is too crude for high-resolution purification so an initial purification is needed. Once the preparation has been simplified by the initial purification, a fine separation step removes the remaining contamination. The purification may utilize salts or buffers that are undesirable for use in the clinic. These components may need to be exchanged for a final formulation. The following sections provide techniques to accomplish this sequence.

A. Harvest Methods

Cells grown and infected in large-scale flat stock culture eventually detach from the surface. Alternatively, trypsin may be used to detach cells before the onset of cytopathic effect (CPE). Cells free in the medium may be collected by centrifugation or filtration. Cells grown in suspension are also harvested in the same manner. Cells grown on microcarriers may be harvested by allowing the cells to settle so the spent medium can be decanted. Infected cells may be removed from the microcarriers by trypsinization or processed while still on the carrier. Infected cells remain suspended and therefore can be decanted with the spent medium.

In order to maximize the harvest yield, one should consider the point at which harvest occurs. The life cycle of the adenovirus was thought to terminate at the time cytopathic effect (CPE) is observed. Analysis using AEHPLC (section III.F of this chapter), however, demonstrated that the peak

Table I
Properties of Adenoviral Particles

Property	Data	Consideration	Issues
Diffusion	4.46×10^{-12} m^2/s	Filtration, centrifugation, chromatography, freezing, and thawing	• Slow diffusion rate may provide a means for separation on filters and chromatography resins. • Centrifugation not counter acted by diffusion. Mixing will be problematic especially during freeze–thaw operations. • Slow diffusion may decrease concentration dependent aggregation. • Assays are hindered.
Water content	High compared to proteins	Fragility, salt concentration, small ions, solvents, freezing, and thawing	• Particle may be swelled with water. • High salt and solvents may result in degradation. • Sensitivity to shearing forces.
Viral genome alterations	Viral, cellular, and transgene expression	Early gene alterations and deletions overexpression of transgene products	• Cell lysate may contain many incorrectly assembled particles, adenoviral protein structures, and unusual cellular structures that are similar in size to adenoviral particles. • Transgene product may be a contaminant which could confound potency assays. • High titers result in less difficult purification
Lysate	Complex	Contaminants	• Particle assay for crude materials. • DNA, lipids, BSA, antifoaming agents, anti-clumping agents, and other contaminants may bind to the particle and co-purify or foul filters and resins.

of particle production occurs before cells begin to detach. Figure 2 shows the particle count taken at various time points during a bioreactor process. In most bioreactor runs the particle concentration drops slightly from the peak, but in some cases the particle concentration falls to as low as 10% of the peak value

Table II
Physical Properties of Adenovirus Particles That Can Be Exploited for Purification

Property	Data	Method	Issues
Density	1.34 g/mL	Density gradient ultra-centrifugation	• Classical method—small scale.
Size	~100 nm	Filtration	• Some cell debris similar size, lipids, and cell culture additives may interfere.
		Size exclusion chromatography	• Particle larger than resin pore sizes. • Could be used for buffer exchange.
Surface	Protein: ionic, hydrophobic, specific surface chemistry	Ion exchange, hydrophobic interaction, affinity chromatography ligand binding filtration	• Scaleable methods employed for protein purification. • Well established literature to predict behavior of cell culture components. Particle size larger than pore sizes. • Aggregation may occur.
		Reversed-phase chromatography, solvent extraction	• Solvents may be problematic.

in just a few hours. This phenomenon underscores the need to monitor the process carefully to obtain maximum yield.

B. Lysis Methods

Following recovery of the infected cells, the adenovirus must be released from the cell. This is accomplished by lysing the cell. There are many methods available for both large- and small-scale cell lysis. The most useful of these methods are discussed below.

1. Freeze–Thaw

Cells burdened with a full viral load are fragile and easily disrupted. Freezing and thawing the infected cells achieves the release of virus. This method is attractive at small scale because it does not require specialized equipment. It is less attractive at large scale because the freezing and thawing of a large sample is difficult to control. Consequently other methods are preferred for large scale.

The freeze–thaw lysis method requires that one observe how the solution freezes and how it thaws. During the freezing process, solutes, such as salts, proteins, and free viral particles, depress the freezing point of the solution.

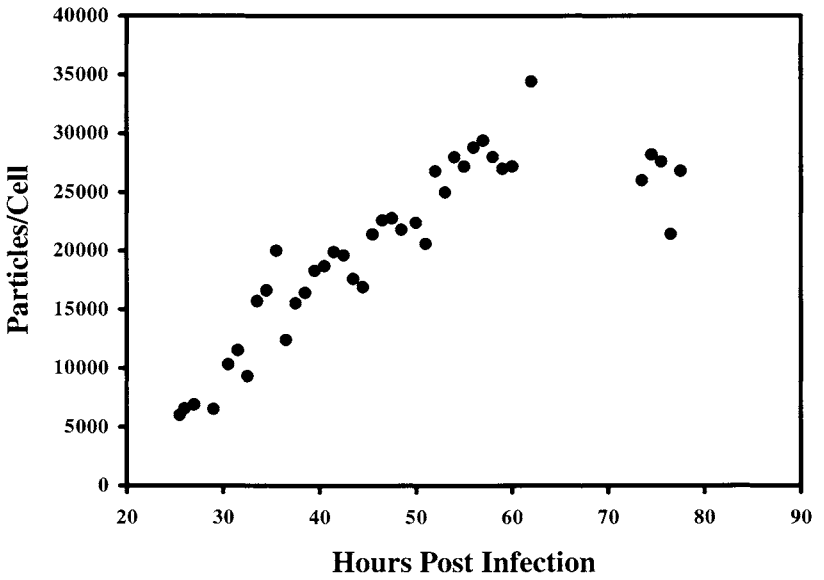

Figure 2 Adenoviral particle production can be followed using anion-exchange high-performance liquid chromatography (HPLC) as discussed in section III.F. The per cell productivity was monitored during a 5 L bioreactor run with a replication deficient type 5 adenovirus grown in HEK 293 cells. The completion of particle production occurs before obvious signs of cytopathic effects are manifested.

Small ice crystals of pure water begin to form. The solutes excluded from the ice tend to concentrate in spaces between the ice crystals. These areas of concentrated solutes experience freezing point depression. If the freezing process is too slow, the virus may be found in highly concentrated bands. Once the thawing process begins, low-molecular-weight solutes are free to diffuse away rapidly, but adenovirus particles remain roughly in the same place, owing to their large size and slow diffusion constant. Given that nearly all proteins precipitate from solution at a critical concentration, one would expect that the virus particle would also be similarly limited. At temperatures above 0°C, frequent collisions among particles lead to an aggregation cascade. While freeze–thaw releases more than 90% of the virus in three cycles under favorable conditions, improper control may lead to greater than 50% loss.

Consideration for damage and loss of the particles represents the greatest concern. Damage may not be obvious at first when a structure as large as a virus is involved; rather, damage suffered during early recovery steps may manifest itself as reduced stability of the purified virus. Particles are packed into the cell in a tight array; once released the viral particle can aggregate. Collisions leading to aggregation in the cell may be limited by mediating proteins, which impede movement and hold the particle in the soluble array.

The control of pH and salt concentration is also critical for freeze–thaw. Some buffers such as phosphate do not maintain buffering capacity when the solution freezes. This may be because sodium phosphates precipitate at low temperature.

2. Homogenization

A useful method for cell disruption used for recovery of recombinant proteins involves passage of bacterial or yeast cells through a small orifice under pressures up to 20,000 psi. Upon passage through the orifice, the cells expand and rupture as they experience a sudden drop in pressure. The French press has been used for this purpose at laboratory scale for many years [14]. Large-scale processing using this principle may be accomplished using a Gaulin homogenizer (APV Gaulin, Wilmington, MA). Lysis of infected mammalian cells can also be achieved using a similar device. However, at the pressures under which this method is normally used to rupture bacterial cells viral loss is observed. To protect the virus the pressures must be minimized. Figure 3 illustrates this affect by comparing the recovery of adenovirus particles after lysis at different fluidization pressures in a microfluidizer.

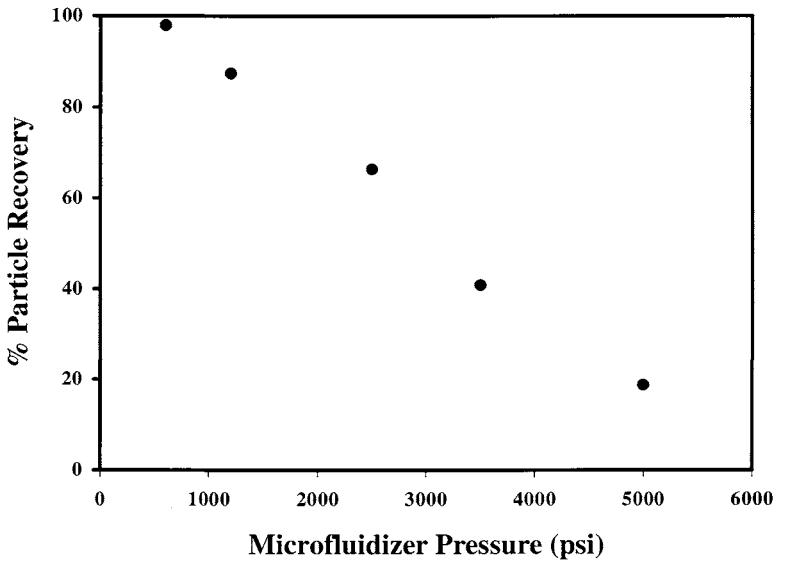

Figure 3 Methods to lyse infected cells vary from repeated cycles of freezing and thawing to microfluidization. The cell can be lysed by a sudden pressure drop generated by several different means. This experiment used anion-exchange HPLC to measure the recovery of particles over a range of pressures in a microfluidizer. Samples were also submitted for titer and showed the same trend. Particle recovery declines at differential pressures above 600 psi. Similar results were obtained using a nitrogen bomb apparatus (data not shown).

3. Sonication

Sonication is widely used for breaking small quantities of cells for research [14] because it is rapid and convenient for small samples. This method results in excellent release of virus from infected cells. Disadvantages of this method include the generation of heat, production of free radicals, and attendant chemical damage, as well as the lack of equipment suitable for large-scale applications.

4. Simultaneous Harvest and Lysis Using a Continuous Flow Centrifuge

Bacterial and yeast cells can be readily harvested at large scale by the use of continuous flow centrifuges. The use of these devices to harvest intact mammalian cells is less certain. These centrifuges are configured to concentrate and harvest cells by different means. To aid in the separation these centrifuges often are fitted with a series of conical discs inside the rotor. All systems expose the cells and liquid to a centrifugal field allowing the cells to be concentrated in the interior of a hollow rotor. Supernatant, which has a lower buoyant density, flows out of the rotor. The "pellet," which remains liquid, either collects in the rotor, exits through restrictions on the outer edge of the rotor, or is ejected in a discharge cycle as the rotor opens. This third method is to briefly open the rotor on the outer edge while it is spinning. If the rotor is opened for just a fraction of a second, the pellet is discharged and little supernatant is lost. Generally, these centrifuges develop between 10,000 and 20,000 *g*. Fragile cells, such as mammalian cells, are particularly at risk of lysis in both the discharge and nozzle-type disc stack machines because of the shear forces and rapid pressure changes. For the same reason these systems are ideal for large-scale concentration and lysis of infected cells [15]. Shear from the rotating discs provide some cellular disruption and the rapid pressure drop from discharge finishes the job. The resulting lysate may be further clarified.

5. Lysis during Filtration

Another method used for lysis is cross-flow filtration. Cross-flow filtration, also known as tangential-flow filtration, separates particles in solution by passing the solution along the surface of a membrane. Liquid passes through the membrane because of the pressure differential across the membrane. Particles and solutes are retained if they are larger than the "cutoff size" of the membrane. Cross-flow systems are configured to allow for recirculation of material along the membrane surface. In this way, larger components are retained (retentate) and smaller components are collected in the passed liquid (permeate). Membranes may be configured in flat plate, spiral wound, or hollow fiber systems. Cutoff sizes vary from particles visible to the eye down to molecules as small as 300 molecular weight. A typical clarification operation can be achieved by using either a 0.45- or a 0.2-μm nominal cutoff

size. Infected cells in this system are exposed to both shear and rapid pressure drops. The advantage of these systems is that the viral particles, which are slightly less than 0.1 μm in diameter, are not allowed to traverse the filter until they have been released from the cell. Cellular debris is retained and therefore separated from the virus.

C. Clarification

The lysis procedure releases the virus from the packed array inside the cell into the medium. All procedures produce cell debris, which must be removed before purification. A common procedure for lysis includes concentration of the intact cells followed by lysis. This method works well at the laboratory scale and may be necessary for production with viral vectors that exhibit poor per cell productivity. With optimized culture methods overconcentration becomes a concern. Highly concentrated lysates exhibit significant losses during lysis and clarification. The loss seems to be associated with aggregation of the virus particle.

1. Centrifugation

Centrifugation at low relative centrifugal force (RCF, ~1000 $g \cdot$ min.) is sufficient for cell debris removal. Centrifugation in a swinging bucket centrifuge is a common method and can be efficient for volumes below 5 L. The disadvantages include performing this operation using an aseptic technique and the possible generation of an aerosol of virus particles. Centrifugation at higher RCF can lead to the loss of virus. Figure 4 shows relative yield loss over time of particles after centrifugation for 5 min at increasing RCF.

2. Filtration

Filtration is another clarification method. Either cross-flow filtration or dead end single-pass filtration can be used to remove debris [16]. A study employing a variety of membranes with varying compositions is necessary for optimizing yield. Some membrane materials bind proteins and may also bind adenovirus. Membranes composed of polyethersulfone possess low protein binding characteristics. Adenovirus will pass through these membranes with excellent yields if the concentration of the adenoviral particles is kept below 5×10^{11} particles/mL.

Most filters are rated by their performance in passing dyes or particles of standard sizes. However, manufacturers generate membrane pores in different ways. Some membranes possess pore sizes similar to the cutoff size; while most possess pores much larger than the cutoff. These types of membranes rely on the torturous path the solute must follow to get through the membrane. Proteins and other contaminants can interfere with filtration by forming a barrier that effectively reduces the pore size. Adenovirus can be filtered through some

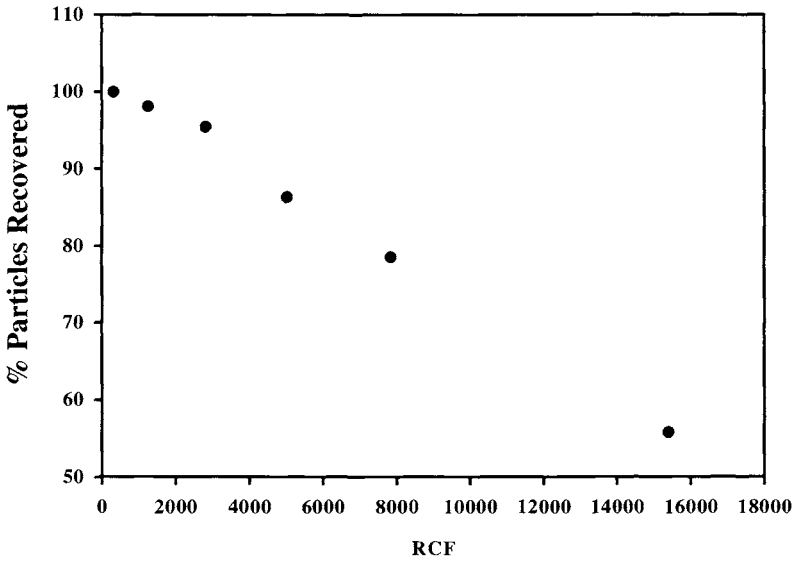

Figure 4 The density of adenoviral particles is significantly higher than most lysate components. Samples of infected cell lystate were spun in an Eppendorf Microfuge model 5415c at 4°C for 5 min at different speeds. Recovery of particles was measured using anion-exchange HPLC. Yields were plotted against relative centrifugal force (RCF). Particle loss occurred when the sample was subject to RCF greater than 3000 g min.

0.22-μm membranes with better than 90% yield; but the membrane must be selected by experimentation. Fortunately, adenoviral preparations may be sterilized by 0.22-μm filtration with filters available from many manufacturers such as Millipore, Gelman, or Sartorius.

3. Expanded Bed Chromatography

Expanded bed chromatography removes cell debris using an upward-flowing chromatography column partially filled with large or dense beads [17–19]. "Expanded bed" differs from "fluidized bed" in that the suspended bed is stabilized by a gradient of bead densities so that mixing is minimized. Resin of this type is available from Pharmacia (Piscataway, NJ). In this mode of operation, the column is fed from the bottom at a flow rate sufficient to suspend the beads throughout the column but not cause the resin to pack at the top. Supernatant exits through the top frit. After absorption of the entire volume to be clarified, the direction of flow reverses and the column is packed, washed, and eluted. The main advantage of this technique is that it accomplishes debris removal and column chromatography in one unit operation. Drawbacks include the need for a special column and a special resin, limiting flexibility. The flow rate for loading the column is restricted to

a rather narrow range by the requirements to keep the bed suspended but not packed at the top of the column.

4. Digestion of DNA

Lysis releases a large amount of DNA in both large and small fragments. Some of the DNA associates with DNA binding proteins and are found in large structures. DNA (and RNA) digestion is necessary because this contaminant promotes aggregation and complicates downstream processing. The adenovirus particle possesses sufficient surface area that significant amounts of DNA bind to the capsid in spite of the anionic surface charge of the particle. Much of this DNA is viral and if not separated can be taken up by the target cell causing abnormal replication. In some cases, E1 can be cotransfected with an E1-deficient particle and can give rise to the generation of replication-competent adenovirus. Consequently, it is important to eliminate as much of the exogenous DNA as is practical.

Fortunately, nucleases such as Benzonase (Merck KGaA, Darmstadt, Germany) are available in highly pure forms able to digest the majority of the complicating nucleic acids. The enzymes function best at 37°C near pH 7 in the presence of magnesium ion. The salt concentration is also critical as high salt inhibits the enzymes. Aggregation of the adenovirus, which depends upon collisions, occurs more rapidly at warmer temperatures, while the enzymes function poorly below 15°C. The lysate must be cleared of debris since the quantity of host cell and free viral DNA competes for enzyme and results in incomplete digestion. A compromise is to perform a modest clarification (see above), adjust the salt concentration by dilution, buffer the solution to maintain physiological pH, add magnesium ion, and then perform the digestion at room temperature. The final concentration of nuclease can be increased to accelerate the process.

D. Purification

Once the clarified lysate is free of exogenous DNA, substantial purification can proceed by a variety of techniques. At small scale both chromatographic or density gradient centrifugation methods are effective. Large scale, however, favors chromatography.

1. Ultracentrifugation

Meselson *et al.* [20] presented a new method of determining the molecular weight and partial specific volume of macromolecules by density gradient centrifugation. This technique has been particularly useful for macromolecules such as DNA and viruses. Salts, such as Cs_2SO_4 and CsCl, form density gradients when subjected to a strong centrifugal field. Macromolecules separate from contaminates on the basis of their respective buoyant densities and collect in bands at their own density if the sample is centrifuged to equilibrium.

Adenoviruses can be purified using this technique since the buoyant density of the particles is approximately 1.34 g/mL. A typical purification scheme is a three-step process where the infected cell is lysed and the DNA is digested. The sample is then applied to a step gradient of CsCl in a tube where the density of the bottom layer of CsCl is around 1.4 g/mL and that of the top layer is around 1.25 g/mL (both layers are buffered with Tris to approximately pH 8). After spinning at approximately 150,000 *g* for 1–2 h, the virus separates from cellular debris and collects in a band between the CsCl layers. The band is collected by puncturing the tube and drawing the material out with a syringe. This collected band is then mixed with CsCl at 1.35 g/mL, placed in a centrifuge tube and subjected to 200,000 *g* overnight. The intact virus separates from DNA, proteins, and defective particles and is collected as before. CsCl is then removed by dialysis.

This method is easy to perform and yields high-purity virus preparations. Unfortunately, the process time required is long, CsCl must be removed from the final product, and specialized equipment is required. The main disadvantage is that this process cannot be performed at the large scales demanded for pivotal clinical trial or market use.

2. Purification by Chromatography

Column chromatography is by far the most versatile and powerful method for purification of viruses. The methods described above serve to prepare the viral preparation for chromatography by freeing it from cells, cell debris, and interfering substances. The clarified lysate must be in a buffer suitable for application to a chromatography column. Modes of chromatography applicable to viruses include ion exchange, reversed phase, hydrophobic interaction, size exclusion (gel filtration), immobilized metal chelate, affinity, etc. For each mode, one could choose from many commercially available resins and mobile phases. The selection of an optimal sequence of chromatography steps has been made easier by commercial instruments that are able to perform a systematic search of columns and gradient conditions. Books and review articles describe these modes of chromatography and offer strategies for selecting the best combination [21–27]. Specific purification methods for adenoviruses are also found in the literature [28].

Fundamental differences distinguish analytical and preparative chromatography [29]. Analytical runs are performed by injecting a small amount of sample onto a column with high resolving power. Such columns typically have very small particle size beads (3–10 μm), high theoretical plate numbers, and rapid run times under high pressure. The result of the run is judged by the appearance of the chromatogram; that is, the peaks should be symmetrical, narrow, and well resolved. Preparative runs, in contrast, are carried out by applying a large sample load (usually near the maximum for the column) using columns of 1 to 500-L bed volume packed with larger beads (20–90 μm

or larger). The cost of the packing becomes more significant for such large columns. The result of the preparative run is measured by the ability to recover pure fractions from the column with high yield. This means that an analytical technique is needed to judge purity and yield of the fractions. Anion exchange HPLC and reversed-phase HPLC [30–32] serve as the two most powerful analytical techniques (see section III of this chapter). Without such information, the outcome of a preparative separation is not known because the chromatograms for many preparative separations are complex and difficult to interpret.

Method scouting is conveniently done on small columns in high-pressure systems (HPLC, FPLC) in order to speed development and conserve material. Media with smaller bead sizes may be used provided larger bead sizes are also available. Preliminary screening should identify two modes of chromatography able to resolve the virus of interest from the contaminants. The first column step usually employs a resin with high binding capacity and/or high selectivity for the product; otherwise, a very large column may be needed. Anion exchange chromatography is often selected as the first step. Adenoviruses do not adsorb to cation exchange resins at physiological pH; but this type of resin may be used as a first step. While the adenovirus passes through cation exchange columns, some protein contaminants will be removed from the viral solution. For adenovirus, this method is not needed. Resins for each step are then selected based on resolution, recovery, speed and cost, and possibly other factors such as freedom from extractable materials and availability of documentation required for the production of clinical material for human trials under current Good Manufacturing Practices (cGMP, Part 21CFR). The mobile phase is selected and the gradient optimized. Sample volume and concentration influence resolution in column chromatography so both of these parameters must be optimized as well. All of this work can be done on relatively small columns. The product produced at a small-scale should meet all of the purity requirements desired for the final product.

Scale-up of chromatography steps is performed by maintaining the media bed height and linear flow rate of the mobile phase while increasing the cross-sectional area (hence column volume) of the column. Fine-tuning the process is usually done at the production scale; only minor adjustments should be needed.

Column packing instructions depend on the particle size and nature of the resin [25]. The resin manufacturer's instructions should be followed and then checked by measuring theoretical plates, n, for the column with acetone (UV detection) or sodium acetate or sodium chloride (conductivity detection). The theoretical plate number can be measured from the chromatogram by the formula [29]

$$n = 5.55(t_r/_{w1/2}).$$

where t is the retention time and $w_{1/2}$ is the width of the peak at half of its maximum height. Sometimes the width is measured at the baseline. In this case, the constant in the equation changes from 5.54 to 16. The plate number increases with column length. Often it is useful to correct the plate number of column length, yielding a parameter known as "height equivalent theoretical plate" (HETP), given by

$$\text{HETP} = \frac{L\,(\text{cm})}{N}.$$

These parameters offer a simple way to monitor column performance over time. Care should be taken to avoid introducing air into the column because air pockets degrade performance and may necessitate repacking of the column.

Columns should be cleaned after use and stored in a suitable bacteriostatic environment [25] following the manufacturer's directions. Most process resins can be cleaned and sanitized with sodium hydroxide solutions in the range of 0.1–1 N (exception: silica-based materials dissolve at alkaline pH).

a. Ion-Exchange Chromatography of Adenovirus Ion-exchange chromatography offers a powerful method for adenoviral fractionation because of its high capacity and resolution. Ion-exchange chromatography exploits the charge that proteins carry on their surface. The net charge of these groups varies with pH and the amino acids exposed in the protein surface [27, 33]. Adenoviral capsids are highly anionic in nature making anion exchange ideal for purifying them. Anion exchange resins carry positively charged groups such as diethylaminoethyl (DEAE) or quaternary amino ethyl (QAE), which bind anionic proteins in a manner that depends on pH. Elution may also be accomplished by changing pH to eliminate the ionic interaction with the protein. For proteins, it is helpful to know both the isoelectric point of interest and how the protein charge varies with pH. These properties can be measured by isoelectric focusing and electrophoretic titration [25]. Good binding and elution characteristics are often obtained about 1–1.5 pH units above the isoelectric point for anion exchange or an equal increment below the isoelectric point for cation exchange.

The predominate capsid protein is the hexon which possesses an isoelectric point near pH 6. As mentioned above, the particle is only stable in a narrow pH range near pH 7. Ion exchange resins also bind protons (cation exchange) or hydroxyl ions (anion exchange). Increasing salt concentration may lead to large changes in pH because salt competes with protons or hydroxyl ions for binding sites on the resin. One should be alert to the possibility of pH changes, possibly as much as one pH unit during chromatography. Maintaining the pH with the correct buffer at sufficient concentration is important for stabilizing the pH during elution.

Several ion exchange resins should be tested for binding capacity and resolution at a constant flow rate because resins with the same functional

group may differ considerably in these properties owing to differences in their backbone, density of substitution, or other factors. After selection of the resin and mobile phase the other critical parameters can be optimized: sample load and volume, flow rates for absorption and elution, and elution gradient.

Hexon is a noncovalent trimer that is anionic at pH 7. The capsid is composed of 240 of these capsomeres and gives the particle a large number of negative charges on the surface at neutral pH. Proteins bind to ion exchange resins in low salt (5–50 mM NaCl) and elute with high salt (0.1–1 M). Concerted binding of capsomeres in the capsid to the resin allows the particle to adsorb at higher salt concentrations than those used to elute endotoxins and most proteins. This allows easy separation of viral particles from proteins. Most chromatographic resins are optimized for different classes of ligands by making the resin particle with various pore sizes. Proteins and other ligands have access to a substantial amount of resin surface area inside the pores. Adenoviral particles do not have access and are limited to the outer surface of the resin. Large fragments of DNA, however, are also highly anionic but with a higher charge density. Consequently, DNA elutes at higher salt concentrations than adenovirus. These properties result in an order of binding and elution for the constituents of a clarified lysate. At buffered salt concentrations as high as 350 mM NaCl, the particle binds to the column while nearly no free proteins bind under these conditions. Large DNA–protein complexes such as incorrectly assembled particles and some cellular structures bind under these conditions. DNA binds tightly. Using a linear salt gradient the order of elution will generally be proteins first, followed by complex contaminants, viral particles, other cellular-derived structures, and, last, DNA that has escaped digestion. The elution of these components results in excellent separation between peaks (Fig. 5). Purification yields are as great as 99% but can be lower if the peak must be trimmed to improve purity.

b. Immobilized Metal Affinity Chromatography of Adenovirus In 1975, Porath showed that metal ions could be linked to a column in a 1:1 complex with a chelating ligand, iminodiacetic acid, bound to the column [34, 35]. These columns had unique properties for fractionation and provide orthogonal methods for purification. This technique is referred to as "immobilized metal affinity chromatography" or IMAC. Beaded agarose is the most common support and iminodiacetic acid remains the most popular chelating ligand. Such columns can be charged with a variety of divalent metal ions, Zn^{2+} and Cu^{2+} being preferred for protein chromatography [36]. Adenovirus particles bind readily to Zn^{2+} charged resin, whereas Cu^{2+} is not as efficient. Excess metal ion is removed by washing before applying protein. Bound metal ion forms a coordination complex, leaving some coordination sites free to interact with proteins. Protein binding typically occurs through histidine residues [23, 36], which occupy the free coordination sites. However, coordination with epsilon

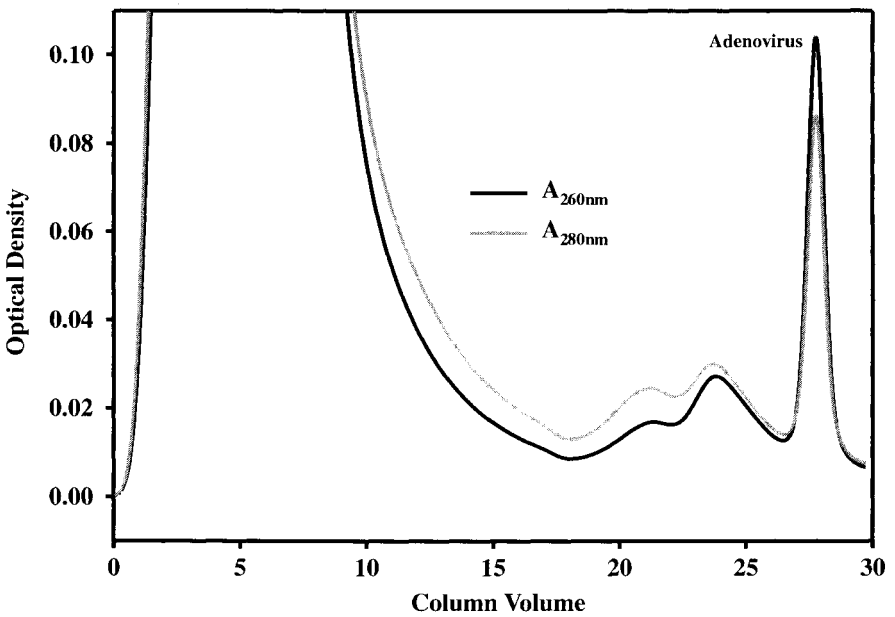

Figure 5 Anion exchange chromatography is the most robust method to recover and purify adenoviral particles from crude stocks. Monitoring the optical density at 280 and 260 nm allows the chromatographer to easily recognize the fractions containing adenovirus by taking a ratio of A_{260} to A_{280}. The ratio for pure virus is around 1.25. The chromatogram above is plotted as the optical density vs the number of column volumes of materials that have been pumped through the column. In this example, DEAE Fractogel 650 M (EM Sciences) was used. The column was buffered in 50 mM Hepes, pH 7.5, at room temperature throughout the process. Infected cell lysate was loaded onto the column with an adjusted salt concentration of approximately 350 mM NaCl. The load produced significant absorbance as the majority of contaminants passed through the column. More contaminants were eluted during a post loading wash with equilibration buffer. Elution was with a linear salt gradient from 350 to 600 mM NaCl. The adenovirus peak is well resolved at the end of the chromatogram. The approximate salt concentration of the collected peak was about 450 mM NaCl. Column cleaning was achieved with 1 M NaCl and 0.5 N NaOH (data not shown). The column height for this chromatography was about 5 cm. The chromatography looks the same, however at 10 cm bed height. Scale up produces the same chromatogram if the column diameter is increased using the same bed height and the flow rate is adjusted accordingly.

amino groups is also probable. Elution can be achieved either by changing pH or by adding competitors such as imidazole or glycine, for the binding sites. Ethylenediaminetetraacetic acid (EDTA) can be used to elute the column, as EDTA strips the metal from the column and the protein.

IMAC works well as a polishing step for purification since it removes residual host cell contaminants. Since IMAC can be operated in high salt conditions, fractions from an initial recovery column, such as anion exchange eluate, can be loaded directly on the equilibrated and charged column. The

buffer selected must not strongly chelate metals, of course. The yields typically fall in the range of 60–80% and the purity is greater than with CsCl ultracentrifugation methods. Figure 6 shows a chromatogram of adenovirus type 5 purified with zinc charged IMAC and eluted with a step gradient of glycine.

Modifications to the fiber can also affect the chromatography. Interestingly, "fiberless" adenovirus, vectors that have been altered to express truncated fiber protein, bind normally to anion exchange resin, but do not bind at all to IMAC charged with zinc. Presumably the residues serving as zinc binding sites have been removed from the surface of the fiber by mutation. Alternatively, the adenovirus fiber can be engineered to provide histidine repeats that will bind to Ni^{2+} very effectively [37].

c. Reversed-Phase Chromatography Reversed-phase HPLC can be used for analysis of adenovirus particles or as a polishing step after initial purification

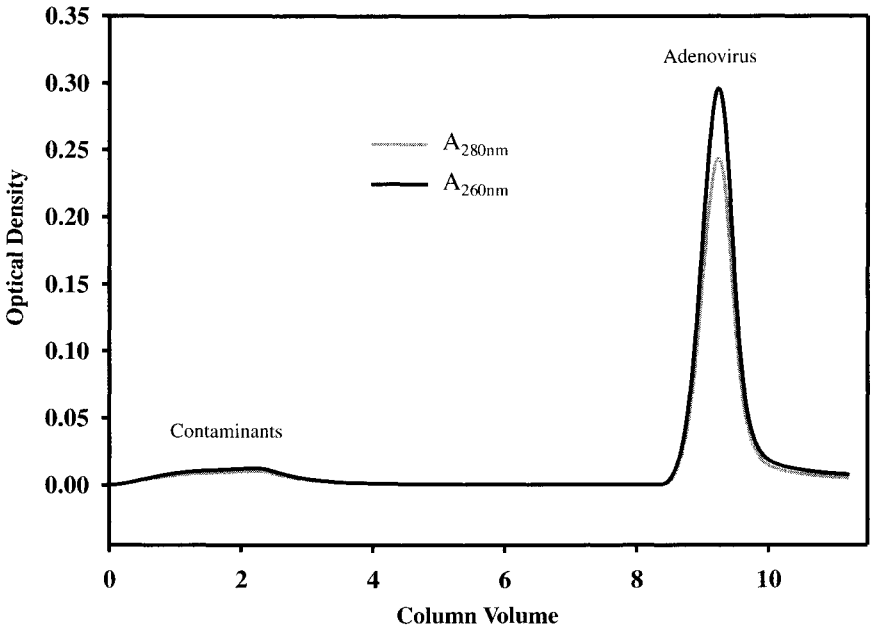

Figure 6 High-resolution techniques, such as zinc metal affinity chromatography are needed to complete the purification of adenoviral particles. TosoHaas AF chelate 650 M immobilized metal affinity resin was charged with divalent zinc. The column was equilibrated at room temperature with 450 mM NaCl in 50 mM Hepes at pH 7.5. DEAE adenovirus fractions (Fig. 5) were loaded onto the column followed by a wash with equilibration buffer. Remaining contaminants eluted from the column during the load and wash. The adenovirus was eluted from the column with a 500 mM glycine step gradient. After elution, the column was stripped with EDTA followed by 1 M NaCl then 0.5 N NaOH (data not shown).

by anion exchange chromatography. The recoveries for the preparative method run in the range of 20%, which is poor compared to other methods. The lower recovery may be related to the presence of organic solvents in the mobile phase as high-molecular-weight proteins tend to denature or precipitate. Recoveries may be improved through careful selection of column, solvent, ion-pairing agent, and pH.

d. Hydrophobic Interaction Chromatography of Adenovirus The discovery of hydrophobic interaction chromatography (HIC) resulted from an attempt to make affinity columns [38]. This fortunate accident uncovered a unique mode of protein chromatography. On the surface, HIC resembles reversed-phase chromatography [27, 39, 40] in that the protein binds to the column through hydrophobic interactions in an aqueous solvent. Both resin types consist of a stationary phase with a hydrophobic surface. Thereafter, the two techniques diverge [23]. HIC resins are typically constructed from polysaccharide or polymeric material. Reversed-phase resins are typically bonded silicas. HIC resins have a lower density of substitution and they tend to be less hydrophobic than reversed-phase media.

Typically, the conformational changes are driven by high salts (such as ammonium sulfate). The salt presents an ionic environment that is favorable to hydrophilic surfaces. Hydrophobic surfaces are driven together so that exposure to the environment is reduced. In this way, the proteins are partially "salted out" and adsorb to the resin. The use of ammonium sulfate is relatively gentle because most proteins are stabilized in the presence of high concentrations of ammonium sulfate. High salt, however, may destabilize the particle (see section IV, below), possibly because of its high water content or because the capsid proteins are twisted into destabilized conformations. The loading material may be adjusted to high salt prior to application to the column. Alternatively, small amounts may be applied to the column repeatedly, washing with equilibration buffer, or the material could also be diluted in equilibration buffer inline with the load. The advantage of the two later methods is that protein precipitation occurs slowly from the time of salt addition. Limiting the time of exposure to high salt may improve the chromatography and mitigate yield loss. Elution is achieved by reducing the salt concentration with a reverse gradient.

With the exception of the direction of the gradient, HIC columns are optimized and operated along the same lines as ion exchange columns. Residence time on the column should be minimized because of the possibility of denaturation [23]. Yields of virus from this type of chromatography typically range between 20 and 60%.

e. Size-Exclusion Chromatography (SEC) of Adenovirus Size exclusion, also called gel filtration or gel permeation, is the only mode of

chromatography that is not intended to involve binding of proteins to the resin [41, 42]. The pore structure of the resin provides a molecular sieve, where smaller molecules can access the entire volume of the pores and large molecules are excluded from the pores. If a mixture of proteins differing in size is applied to a size exclusion column, the largest proteins will emerge first and smallest last. Molecules above a certain size do not penetrate the pores at all. Normally, these are the first to elute from the column; the volume at which they elute is termed the "excluded volume." Adenovirus particles, perhaps owing to the slow rate of diffusion may not elute in the excluded volume of the column. Instead, the particles may elute as much as a column volume beyond the excluded volume. Late elution from SEC often results from charge interactions between the sample and the column. This effect may be reversed by raising salt concentration. Similarly, molecules below a certain size all elute at the "included volume."

All other species elute between the excluded volume and the included volume. This property limits the resolving power of size-exclusion chromatography because the number of peaks that can fit into the volume allowed is small. A further limitation of size exclusion chromatography is that resolution deteriorates if sample volume exceeds 4–5% of the bed volume of the column. Another limitation is that resolution deteriorates with increasing sample viscosity. The maximum protein concentration allowing good resolution is usually in the range of 5–10 mg/mL protein. Taken together, these two factors mean that a size exclusion column has 1–5% as much protein capacity as an ion-exchange column of the same size! Hence, size exclusion has limited utility for purifying adenoviral particles or proteins and is usually reserved as a last step.

Sample load volume, maximum protein concentration and flow rate should be determined by experiment. Careful column packing technique is critical for good results. Plate number should be measured for a new column and at regular intervals during use. Despite these limitations, size-exclusion chromatography has an important place in the arsenal. It is gentle and rapid so yields are nearly quantitative. Additionally, it provides an opportunity to exchange the buffer to the desired formulation because SEC is compatible with a wide range of aqueous buffers.

E. Buffer Exchange

Every process confronts the problems of removing low-MW species and/or concentrating the desired fractions. Dialysis [43–45] or gel filtration (section II.D.2.e, above) may be used to remove small molecules or exchange buffers when the sample volume is in the range of 1 L or less. Several types of ultrafiltration devices are available for concentration of proteins on a laboratory scale, including pressurized stirred cells and filters driven by centrifugal force or other means.

Concentration of protein solutions at a process scale is usually done by ultrafiltration using a tangential flow filter [16, 27, 46]. Buffer exchange and removal of low-MW species are usually done by diafiltration; both concentration and diafiltration may be done on the same device and combined as a unit operation. Diafiltration is more efficient than dialysis in that less buffer and less time is needed to achieve a given level of solute removal. The concentration of solute remaining in the retentate after 5 vol of continuous diafiltration is given by [47]

$$C_R = C_0 e^{-V(1-\sigma)},$$

where σ represents the rejection of the solute by the membrane. For solutes freely permeable to the membrane, $\sigma = 0$. Under these conditions, diafiltration with 3 vol of buffer reduces the concentration of solute by 95%. In contrast, 20 vol of buffer would be required for the same result by standard dialysis. In either case, buffer exchange of viral particles can be achieved. Care must be taken to avoid foaming or excessive shear. Special attention should be devoted to pH control. The process should be monitored to avoid overconcentration and possible loss of product through precipitation.

III. Analytical Methods for Process Development and Process Tracking

Analytical methods are as important for purification as the process steps themselves. Analytical methods are essential for following the process and assessing the purity of adenovirus particles throughout the process. The methods must be rapid, reliable, and informative about the quantity and quality of adenovirus particles. They should be sensitive enough to detect subtle changes. As with the process techniques, characteristics of the virus are useful for selecting analytical techniques. In this section we have highlighted several assays not only because their usefulness for a process, but because an understanding of what these assays mean is critical in producing vector with the quality required for use in humans.

A. Plaque-Forming Titer Assays

Plaque forming assays have been in use as biological assays since early in the 20th century. A common method for many viruses, this type of assay has been employed with adenovirus since they were discovered. The plaque assay is performed using many variations but generally consists of diluting the virus preparation to a point that a thin layer placed over sensitive cells will result in a countable number of infection events. This is usually accomplished in a petri dish or in six-well plates. Once cells have been exposed, the viral

solution is removed and a layer of warm agar applied on top of the cells. After 1 to 2 weeks of incubation, the cells are stained with a dye, such as neutral red, and plaques of lysed cells become visible. It is assumed that because of the extreme dilution, each plaque is the result of a single viral particle infecting a cell. The plaque arises following the replication of that viral particle and subsequent infection of adjacent cells by virus progeny. The number of plaques in a well divided by the inoculum volume and corrected for dilution yields a titer. This method is simple but relatively insensitive. If the cells are robust, the inoculum layer may be very thin and the exposure reasonably long. The slow diffusion rate of the particle and the formation of a meniscus in the well limit the method to sampling about 10% of the virions in a sample.

B. Adenovirus 96-Well Titer Plate Assay

A convenient method for estimating adenoviral titers uses cells (of an appropriate cell line) in the wells of a 96-well plates. The cells are plated such that they reach 50% confluence after 1 day of growth. The sample is then diluted so that the final particle concentration falls between 5 and 1000 particles/mL. Several different dilutions are prepared. It is best to perform an initial dilution in the original sample tube using the whole sample because the freeze–thaw process concentrates the virus into bands that will not disperse without substantial mixing. The largest error in dilution usually occurs with this first dilution. The initial dilution should be limited so that the concentration after the initial dilution can be verified using a particle assay. Thereafter, dilution of the sample is not problematic. To infect the cells the entire medium is first removed from the seeded wells. Each different dilution of virus is pipetted into at least 10 wells per dilution. When using a diffusion-adjusted calculation (see section III.2, below) the wells should be filled to the top. It is common in practice, however, to inoculate 100–200 μL of medium with 10–50 μL microliters of virus solution. For diffusion-corrected calculation the infection time is limited to 1 h or less (15 min is convenient) and then the virus solution is replaced with medium. For methods using Spearman-Karber titer calculations, such as the Lynn Titerpint analysis [48], analysts typically leave the virus solution on the plate for the duration of the assay. Incubation of cells takes place at 37°C, 7–10% CO_2, 90–100% humidity for varying times depending on the method of detection. One method is to fix the cells after 3 to 5 days with methanol and acetone followed by staining with a FITC-conjugated anti-adenovirus antibody. This method of detection requires microscopic examination of each well under ultraviolet light. A well is counted if one or more fluorescent cells as positive. If cytopathic effect is used for detection, 1 to 2 weeks of incubation will be required. The dilutions that produce fewer than 100% positive wells are used in the titer calculations.

1. Spearman–Karber Analysis

Spearman–Karber analysis, based on Finney [49], essentially converts data such that graphing the data as log dilution vurses positive wells approaches a straight line. Spearman–Karber performs an interpolation to a midpoint. Thus, Spearman–Karber gives a log dilution where 50% of the wells would have been positive. Titer is expressed as a negative log of the dilution. The Lynn program transforms the number by taking the reciprocal of the dilution (10 raised to the power of the Spearman–Karber number) and divides by the inoculum amount ostensibly to get the inoculum concentration. However, this value is expressed as ED_{50} (or $TCID_{50}$) per milliliter and is often substituted for a virus concentration. This assumes that everything that was put into the well is measured by the assay and the fact that positive wells follow a Poisson distribution is ignored. A more appropriate analysis accepts the Poisson distribution in that even if the average number of virions per well is one, not every well would get a virion; but the Poisson distribution is applicable for this kind of assay. The Poisson distribution is given by

$$p = \frac{S^r e^{-s}}{r!},$$

where p is probability or fraction of positive wells, S is the event density or average virion per well, and r is the number of virion in a particular well. For the number of wells that get zero particles, $r = 0$, $S^r = 1$, and $r! = 1$ so the fraction of positive wells is given by $1 - e^{-S}$. The Spearman–Karber analysis gives us a convenient way to take all the data into account, calculate a standard error, and then apply the Poisson distribution to get a concentration. Since the value obtained gives the dilution where the fraction of positive wells is 0.5, $p = 0.5$. Solving for S yields $S = 0.69$ virion per well. This is always the case when 50% of the wells are positive. If, for example, the inoculum were 50 μL, then the concentration of the inoculum would be 13.8 particles per milliliters. Using the dilution obtained by the Spearman–Karber number the original concentration can be calculated. As in the plaque assay, the key assumption is that no virion in a well escapes detection. In the time frame of these assays, this is clearly not possible. A more precise analysis must take into account Brownian motion (diffusion).

2. Diffusion-Normalized Calculation

In the older animal virus literature the methodology used for measuring infectious titer was simple: a thin-layer of a viral preparation was placed over the target cells for as long as practical. It was intuitively understood that the infection process was diffusion limited. This methodology would help minimize underrepresenting the titer. Done properly, these assays may underestimate by 10 to 100-fold; but the values obtained in a given experiment were useful in a relative sense. However, many of the cells used for replication-deficient

adenoviruses cannot be maintained with very low media levels. Unfortunately, the adenoviral particle, or any particle of similar size, diffuses very slowly in solution. Adding more media mitigated the sensitivity of the cells, but resulted in substantially greater underestimations of titer. This is because the probability of infecting a given cell is dependent on the concentration of the virus and the time of exposure.

The discrepancy between adenoviral titers and the more precise particle assays (see below) has furthered the concept of particle to infectious unit ratio, or PIU. In the case of adenoviral vectors, this concept is based on the supposition that, in a population of intact and otherwise complete particles, most are not infective. However, a search of the literature does not support this supposition. To the contrary, work by Nyberg-Hoffman *et al.* [3] used a model derived from Fick's Laws of Diffusion to demonstrate that most, if not all, adenovirus particles are indeed infective. That work, and others [50], showed the importance of diffusion mechanics for virus binding and demonstrated the dependence of titer determination on often ignored experimental conditions.

The defective particle misconception neglects the important factor that most of the added particles in a well never contact the target cell during the critical period of the assay. Changes in assay conditions such as particle concentration or exposure time can have a dramatic effect the results. A significant outcome of the misconception has been the requirement by regulatory agencies to demand that there be at least one infective particle per hundred total particles (FDA Guidelines). Substantial resources have been spent in attempts to purify away the "defective particles." Unfortunately, in many cases these particles are merely experimental artifacts and thus cannot be removed by purification. Other ramifications of this misconception have been discussed in the literature [50, 51].

Diffusion can be accounted for by the use of diffusion-normalized analysis [3]. This analysis takes into account the diffusion of the particle under the conditions of the assay by deriving normalization equations from Fick's Laws of Diffusion. For a titer plate assay the equation is given by

$$V = -\frac{\ln\left(1 - \frac{p_w}{n}\right)}{A_w \cdot C_w \cdot I \cdot \sqrt{t}} \times \text{dilution factor},$$

where p_w is the number of positive wells per dilution, n is the total number of wells per dilution, A_w is the area of the bottom of the well in cm^2, C_w is the confluence of the well at the time of infection, I is a constant incorporating the diffusion coefficient and is equal to 2.38×10^{-4} cm/particles $s^{1/2}$, and t is the exposure time in seconds. From the equation, one can see that p_w must be less than n and greater than zero. Optimally, the number of positive wells should be between 20 and 80% of the total wells in a dilution. This method yields titers that are up to 50% of the particle concentration.

C. Flow Cytometry

Fluorescence activated cell sorting (FACS) offers another sensitive method to assess infective titer [52]. Sufficient quantities of permissive cells for analysis can be grown and infected in six-well plates. Infected cells are harvested, fixed, and stained with a FITC-conjugated anti-adenovirus antibody. Infected cells are brightly stained while uninfected cells are not. FACS analysis determines the fraction of cells infected at the time of harvest. The fraction of infected cells at the time of infection is diluted at the time of harvest because uninfected cells continue to divide. By performing cell counts at infection and at harvest times, one can then calculate the proportion of cells infected at infection time by using the fact that the number of infected cells does not increase during the incubation period. Multiplying the proportion of infected cells by the number of cells at infection time yields the number of cells originally infected. Since the total number of cells at infection time is also known, the proportion of infected cells at infection time is also known. This value can be used to calculate a titer.

Cells are infected with a virus concentration high enough to get 5–10% of cells to stain positive. Exposure time ranges from 30 s to 60 min. Incubation is up to 50 h to avoid secondary infections. The titer is given by the diffusion-adjusted equation:

$$V = -\frac{\ln(1 - F)}{I\varphi\sqrt{t}} \times \text{dilution factor.}$$

The average cell area (two-dimensional footprint), symbolized by φ, is a variable that must be determined for each cell line. Subclones of HEK 293 cells, for example can display morphology differences from the parent stock. Cell area can be determined using image analysis to analyze micrographs of cells. HEK 293 cells obtained from ATCC and at low passage number possess a cell area of approximately 6.3×10^{-6} cm^2. Other subclones of 293 cells can have larger or smaller areas. F is the final adjusted fraction of positive cells detected. I is 2.38×10^{-4} cm/particles s$^{1/2}$ as above. This method yields titers that are 50 to 80% of the particle concentration. This is probably the best value that can be obtained considering that no step in the infection process is 100% efficient.

D. Particle Concentration Determination by Ultraviolet Absorbance

The most common method for measuring particle concentration is to disrupt the particles using SDS followed by absorbance measurement at 260 nm. Maizel [53] determined that the absorptivity of adenovirus was 1.1×10^{12} particles/mL/absorbance unit at 260 nm. This method is convenient and rapid but not without limitations. The sample must be pure and free of particulates and aggregates in order to obtain an accurate reading. The buffer formulation of the sample can affect the reading as salt concentration partially determines

the concentration at which SDS may form micelles. This method cannot distinguish disrupted particles from intact particles. Contaminating DNA may increase the absorbance and lead to an overestimation of particle concentration. Absorbance readings at several wavelengths provide a check on the validity of the assay. Absorbance in the longer ultraviolet and visible regions indicates light scattering. The ratio of 260 nm (DNA) to 280 nm (protein) should fall between 1.2 and 1.3. A ratio outside this range indicates contamination.

E. Analytical Reverse-Phase HPLC

Reversed-phase high-performance liquid chromatography (RPHPLC) first achieved prominence as an analytical technique because of its wide applicability and ability to resolve a large number of components in a single chromatographic run [54]. This excellent technique also works well as a preparative method for some proteins, mainly those of lower molecular weights (<30,000) [25, 55]. RPPHPLC is the dominant method for the purification of peptides and protein of MW <10,000 [56]. For example, human insulin is produced at a level of tons per year using RPHPLC. Application to larger proteins is limited by the denaturing tendency of organic solvents. For this reason RPHPLC is considered a denaturing technique. The binding of proteins to reversed-phase columns results from a hydrophobic interaction between exposed regions of the protein and the hydrophobic surface of the stationary phase (the resin). Denaturation by the mobile phase exposes hydrophobic regions buried within the protein. Elution is achieved by applying a gradient of increasing concentration of organic solvent, usually acetonitrile. Proteins tend to elute as broad, asymmetrical peaks unless an ion pairing agent, such as trifluoroacetic acid (TFA) is included. TFA is thought to bind to the positive charges on the protein, masking negative charges on the resin matrix and providing additional hydrophobic surface to interact with the column [54].

RPHPLC is a powerful analytical tool to use for adenoviral samples because it resolves the proteins contained in the virus particle. This method, described in Lehmberg *et al.* [31], has provided detailed insights into the nature of adenovirus preparations. The method consists of injecting the sample onto a C4 reversed-phase column that has been equilibrated in 20% acetonitrile at a constant TFA concentration of 0.1%. The column temperature, a critical parameter for RPHPLC, is kept at 40°C. These conditions dissociate the particle into proteins that in turn bind to the column. The column is then eluted with an acetonitrile gradient beginning at 20% and ending at 60%. The TFA concentration is maintained at 0.1%. The absorbance is monitored at 214 nm and can be monitored at 260 and 280 nm. The resultant chromatogram gives a characteristic fingerprint of the adenovirus proteins. Mass spectrometry and N-terminal sequencing have identified 14 major peaks. These proteins can be recovered quantitatively enabling this assay to be used as a quantitative

method for determining particle concentration. The relative peak areas of the proteins can be compared to a known standard to assess particle quality.

F. Analytical Anion-Exchange HPLC

The anionic nature of the adenoviral particle lends itself to analysis by anion-exchange HPLC [30, 32]. This method is nondestructive and yields a wealth of information. Before this method, monitoring the production and purification of adenovirus particles was limited to infectious titer assays. For the purposes of process development and monitoring, infectious titer assays were too slow, resulting in low throughput. They were not sensitive enough to distinguish small differences among samples. Protein analysis methods, such as SDS–PAGE, could not quantitate virus in crude samples because most of the viral protein is not incorporated into whole virus [1]. Absorbance techniques to assess viral particles based upon the UV absorption of DNA were not applicable to crude samples. This is because free viral and host cell DNA is present in the sample. These limitations are overcome by AEHPLC.

A 1-mL Resource Q anion exchange column (Pharmacia Biotech, Piscataway, NJ) is convenient for HPLC analysis of samples. Lysates are prepared by treatment with nuclease. Semipure or pure virus can be injected directly because they do not require nuclease treatment. As long as the column is not reequilibrated during injection, the assay is independent of the injection volume. After sample loading, the column is washed with equilibration buffer (Hepes or Tris, at approximately pH 7.5) followed by linear salt gradient elution. The chromatography can be monitored on a standard ultraviolet detector. Significantly, more information can be gleaned by the use of a photo diode array (PDA) detector scanning from 210 to 300 nm. The retention time of the adenovirus peak varies with the serotype of the virus.

The AEHPLC chromatogram reveals information about purity, particle integrity, and particle quantity. Figure 7 shows the chromatograms of purified adenovirus type 5 and infected cell lysate. Other serotypes give slightly different peak elution times. Provided that the HPLC system remains below the pressure limits of the resin, the chromatography can be performed in less than 6 min. Monitoring the process by anion exchange HPLC enables the production staff to rapidly obtain a picture of the progress of the purification.

IV. Formulation and Stability

Process development must determine product stability over a wide range of conditions. The time course of the purification must be scrutinized for excessive run times and delays because rapid processing favors high yields. A well-designed process includes holding points selected such that the viral

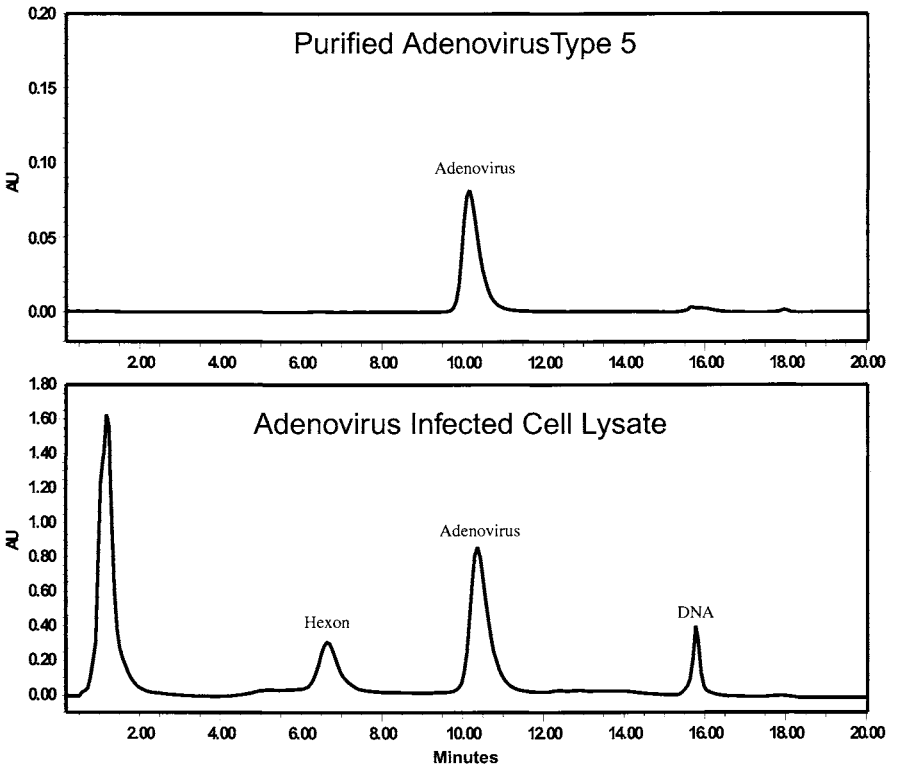

Figure 7 The most useful method for analyzing both crude and pure samples of adenovirus is anion-exchange HPLC. Pure adenovirus elutes in a nearly symmetrical peak. The adenoviral peak from lysate elutes with baseline separation from contaminants such as hexon and undigested DNA. The method is detailed in Shabram *et al.* [30].

preparation can be safely stored in the event of a planned or unplanned delay. Stability studies are critical to identify those steps at which the virus is at risk. Additionally, the lessons gleaned from these studies point to a stabilizing formulation needed for clinical trials and beyond.

Aggregation of adenovirus particles is well known to those in the field. The adenovirus demonstrates low stability at 4°C. This phenomenon has yet to be explained, although there are examples of cold-sensitive enzymes in the literature [57, 58]. The instability seems to be related to the tendency of adenovirus particles to aggregate. Aggregation requires that particles bind to each other after a collision. As the collisions proceed the aggregating particle grows. A systematic analysis of aggregation was published in 1917 by Smoluchowski [59]. Using Smoluchowski's coagulation model the aggregation frequency of adenoviral particle can be roughly estimated at one per every 50

collisions! This may seem surprisingly frequent until one realizes that collisions between particles are relatively rare due to the slow diffusivity of the particle. It also explains the observation that aggregation is dependent on particle concentration.

Aggregation may also be the end result of damage that occurs early in the production process but does not manifest itself until the sample is concentrated. Aggregation can be mitigated by changing the conditions of the formulation such that aggregation events are not favored. Collision frequency can be reduced by increasing the viscosity of the solution. Figure 8 shows the effect of concentration on the stability of virus in phosphate-buffered saline with 2% sucrose at 4°C. Aggregation was measured as disappearance on AEHPLC. Aggregation accelerated at concentrations greater than 5×10^{11} particles per milliliter. The addition of glycerol increased the virus stability (Fig. 9). This is likely due to the ability of glycerol to cause preferential hydration of protein surfaces [60, 61], leading to a tighter association of the capsid subunit structure. Additionally, an increase in viscosity reduces the number of collisions between particles.

Salt concentration plays a significant role in the stability of the particle in that stability is reduced in salt solutions above 300 mM. Stability at these salt levels was increased in the presence of glycerol. The putative damage may be due to an anion-specific effect and probably follows the Hoffmeister series. Studies with potassium chloride, sodium chloride, and cesium chloride showed that while chloride concentrations above 1.5 M were not destabilizing, concentrations between approximately 0.4 and 1.0 M could be harmful

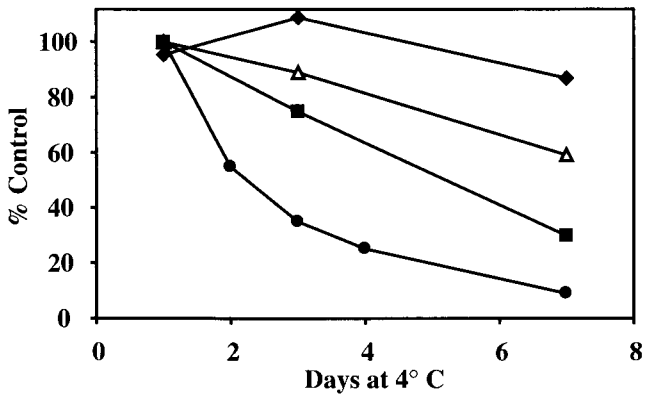

Figure 8 Adenovirus stability is affected by concentration. Recombinant adenovirus was diluted to 4×10^{11} (♦), 5×10^{11} (△), 6×10^{11} (■), or 8×10^{11} (●) particles/mL and was stored at 4°C. On the indicated days aliquots were assayed for virus concentration with the anion exchange HPLC assay. The buffer was 20 mM sodium phosphate, pH 8, 100 mM NaCl, 2 mM $MgCl_2$, and 2% sucrose.

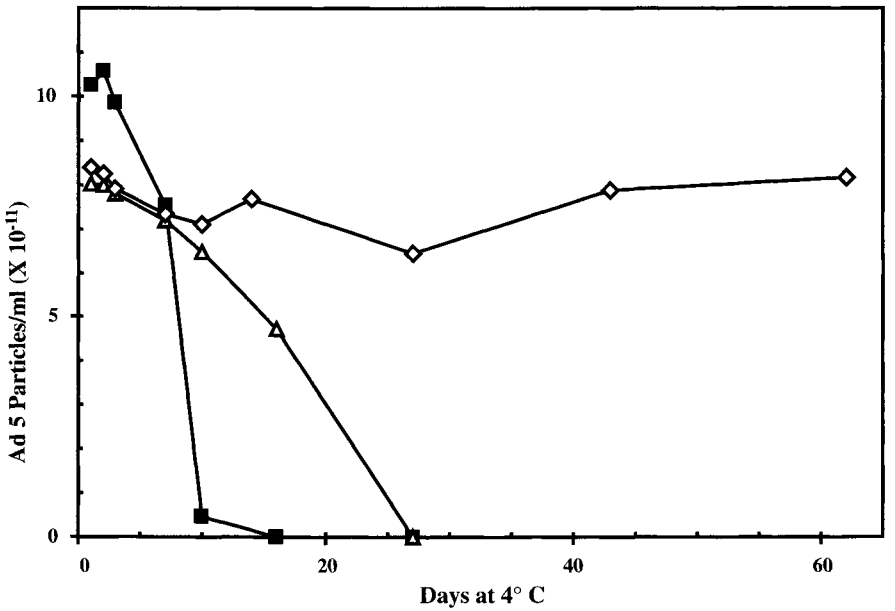

Figure 9 Glycerol stabilizes the adenovirus in solution. Recombinant adenovirus at 10^{12} particles/mL was stored at 4°C in aliquots: undiluted (■), diluted 20% with water (△), or diluted with 50% glycerol to a final concentration of 10% (v/v) glycerol (◇). On the indicated days aliquots were assayed for virus concentration with the anion exchange HPLC assay. The buffer was 20 mM sodium phosphate, pH 8, 100 mM NaCl, 2 mM $MgCl_2$, and 2% sucrose.

regardless of the cation. In contrast high concentrations of sodium sulfate, sodium phosphate or potassium phosphate were not destabilizing.

A major consideration is the exposure to pH outside the physiological range. As mentioned earlier, phosphate buffer at pH 7.2 lacks buffering capacity during freeze–thaw. Hence, the rate of freezing and thawing are critical. In general, fast freezing and fast thawing improves the stability of the material. Buffers such as Hepes or Tris maintain buffering capacity during freeze–thaw and therefore are preferable for stabilizing pH. One might suspect that the lower stability at 4°C may be related to pH stability and therefore the buffering capacity of the formulation. While phosphate may be problematic for freeze–thaw it is interesting that substituting Tris for phosphate does not affect the stability at 4°C.

Freezing and thawing was mentioned earlier as a potential risk for the viral preparation. Cryoprotection agents are often used to mitigate the risk by disturbing ice crystal formation and providing for an amorphous frozen solid. Typically carbohydrates are use to accomplish this. Sucrose and mannitol are often found in formulations where the freezing process is

critical. Typically, mannitol provides slightly better protection with proteins than sucrose and is preferred for its superior cake formation in a lyophilized product. With adenovirus, however, the opposite is true. Sucrose provides moderate protection but mannitol has a clear negative affect.

V. Conclusions

The use of adenovirus vectors for gene therapy has placed increased demands upon the technology for production, purification, and characterization of virus particles. Some of the classic technology has been reexamined and improved. A new class of methods based upon column chromatography has added a powerful set of tools to this array. In large part, the chromatographic methods are based upon modes of chromatography and resins originally developed for protein purification. With proper consideration for the size and other characteristics of adenovirus particles column chromatography may be applied with considerable success. The past decade has witnessed rapid advances in this area. Column chromatography is now a preferred method for adenovirus purification because of its versatility and ability to purify large amounts of virus to a high state of purity while retaining biological activity.

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CHAPTER 8

Targeted Adenoviral Vectors I: Transductional Targeting

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I. Introduction

The extensive use of human adenoviruses (Ads) for gene therapy is largely due to the fact that the biology of these viruses has been extensively studied for decades. Therefore, at the time when gene therapy emerged, these viral agents represented a rational choice as a candidate system for delivery of therapeutic genes to diseased tissues. Compared to other gene transfer vectors, both viral and nonviral, adenoviruses possess a number of properties which make them a preferred means of cell transduction. In addition to the well understood biology of Ad and extensively developed methods for the generation, propagation, and purification of these vectors, the *in vivo* stability of Ad, the capacity to accommodate significant amounts of heterologous DNA, and the ability to efficiently infect a wide variety of different cell types at various points of the cell cycle, have significantly facilitated progress in Ad-based gene therapy in the early stages of its development. A critical overview of the results achieved by gene therapy during the past decade clearly shows that although it has not yet lived up to most of the expectations raised at the time it was conceived and even has resulted in the death of a human being, it has, in fact, shown quite impressive results in some applications. Importantly, being a new field of experimental biology and a promising new direction in modern medical science, gene therapy has attracted a substantial additional workforce, talent

and funds to not only use the knowledge previously generated by classical virologists, but also to foster further efforts in studying the biology of Ad, thereby creating a positive feedback to basic science.

Despite recent successes in employing Ad vectors for developing new treatment modalities, progress in this direction has clearly been hampered by a number of deficiencies of therapeutic Ad vectors. The list of these limitations includes at least three major points. First of all, Ad vectors are not as safe as desired. Even though Ads, which are considered mild pathogens in the first place, are used for gene delivery in the form of highly attenuated derivatives of the wild-type viruses, when used at high doses they can potentially lead to quite deleterious side effects, including lethal outcomes. This is due in part to the fact that even replication-deficient versions of these vectors are capable of low-level expression of viral genes in the infected cells, thereby causing vector-associated cell toxicity and death. In addition, since Ad virions contain hundreds of copies of viral proteins, they are highly immunogenic and as such can cause acute immune responses in a patient, which result in rapid clearance of both the injected agent and the virus-infected cells. Finally, the promiscuous tropism of Ad vectors may result in the widespread dissemination of the vector upon delivery to the patient and may potentially lead to random infection of normal tissues, thereby further complicating the safety and toxicity issues.

The purpose of this chapter is to demonstrate that by addressing the deficiency of Ad vectors related to their natural tropism, one can potentially improve the specificity, immunogenicity, and safety of these agents and thus further increase their overall utility for gene therapy.

II. The Pathway of Adenoviral Infection

Strategies to retarget Ad vectors should be based on an understanding of the biology of Ad infection and the roles played by the viral capsid proteins involved in determining tropism. These topics are reviewed elsewhere in this volume and will therefore be described only briefly here.

Adenoviruses are nonenveloped viruses with double-stranded DNA genomes packaged into icosahedral capsids. The major protein component of the Ad particle is the hexon protein, which forms the planes of the capsid and appears to play a predominantly structural role in the virion. At each of the 12 vertices of the capsid is a penton complex consisting of a pentameric penton base associated with a trimeric fiber protein which projects from the viral surface.

The entry of Ad into susceptible cells requires two distinct, sequential steps—binding and internalization—each mediated by the interaction of a specific component of the Ad penton complex with a cellular receptor. The initial high-affinity binding of Ad to the primary cellular receptor occurs via

the carboxy (C)-terminal knob domain of the fiber capsid protein [1, 2]. The knob domain also initiates and maintains the trimeric configuration of the fiber molecule [3, 4], which is critical for the ability of the fiber to associate with the penton base, since fiber monomers cannot be incorporated into mature viral particles [5]. The central shaft domain of the fiber serves to extend the knob away from the surface of the virion, thereby facilitating its interaction with the primary receptor. The amino (N)-terminal tail domain is associated with the penton base and also contains a nuclear localization signal, which directs the newly synthesized fiber polypeptides to the nucleus of the infected cell, where the assembly of the viral particle occurs [6]. Thus, the three domains of the fiber protein fulfill well-defined and distinct functions.

The cellular fiber receptor for the two human Ad serotypes which are most commonly used as gene delivery vectors, Ad2 and Ad5 from subgroup C, has been identified as the coxsackievirus and adenovirus receptor, CAR [7, 8]. CAR also serves as the primary receptor for Ad serotypes from subgroups A, D, E, and F, but not subgroup B [9]. CAR appears to function purely as a high-affinity docking site for Ad on the cell surface: the cytoplasmic and transmembrane domains of the molecule are not essential for Ad infection [10, 11]. CAR has two extracellular immunoglobulin-like domains, of which the N-terminal domain, D1, which is distal to the cell surface, is responsible for binding the Ad knob [12].

Following attachment, the next step in Ad infection is internalization by receptor-mediated endocytosis potentiated by the interaction of Arg-Gly-Asp (RGD) peptide sequences at the apex of protruding loops of the Ad penton base with secondary host cell receptors, integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ [13, 14]. After internalization, the virus is localized within the cellular vesicle system, initially in clathrin-coated vesicles and then in endosomes. Acidification of the endosomes allows the virions to escape and enter the cytosol. The virions are then translocated along microtubules to nuclear pore complexes where the capsid is disassembled and the DNA genome is imported into the nucleus [15].

III. Strategies and Considerations

The capacity of an Ad vector to infect a given cell is therefore dictated by the CAR- and integrin-expression levels of the cell. It has been shown that cells expressing both receptors below a certain threshold level are refractory to Ad infection [16]. Recent studies have also demonstrated that a number of cell types such as endothelial, smooth muscle cells, differentiated airway epithelium cells, lymphocytes, fibroblasts, hematopoietic cells, and some others demonstrate either complete or partial resistance to Ad infection [10, 17–23]. Importantly, the employment of Ad vectors for cancer gene therapy has revealed that many types of tumor cells express CAR at marginal or even

undetectable levels and are thus Ad-refractory [24–26]. An interesting finding in this regard was recently published by Okegawa *et al.*, who demonstrated a striking inverse correlation between the level of CAR expression by prostate cancer cell lines and their tumorigenicity, thereby suggesting that in general the most aggressive tumors may be CAR-deficient and therefore refractory to therapeutic intervention utilizing unmodified Ad2- or Ad5-derived vectors [27]. The authors have also observed the same phenomenon on human breast cancer cells. If the results of this work are further corroborated by data from other laboratories, CAR deficiency in tumors may become a major obstacle in employing Ad vectors for cancer gene therapy, therefore necessitating the derivation of Ad vectors capable of infecting these tumors in a CAR-independent fashion. Another reason to develop tropism-modified Ad vectors is the fact that many normal human tissues express high levels of CAR [7] and may thus become random targets for therapeutic Ad agents. As the products of some therapeutic genes may be toxic or otherwise deleterious to normal cells, such uncontrolled transduction may result in destructive side-effects which can compromise the efficiency of the therapy.

An overview of the native cell-entry pathway utilized by Ad suggests that it may be modified by altering the mechanism of the virus–cell interaction. Theoretically, this goal may be achieved by modifying the structure of the receptor-binding components of the Ad virion, the fiber and the penton base, in a way which promotes interactions of the modified capsids with cell surface-localized molecules distinct from the native Ad receptors. The accomplishment of this goal would result in Ad vectors possessing expanded tropism, which would be able to achieve cell entry by either of two routes, the natural or newly created pathway. Obviously, although such infectivity-enhanced vectors would be of utility in those clinical applications where tropism to CAR is not a confounding issue, they would still be a suboptimal means of cell-specific gene delivery in most therapeutic strategies requiring stringent control over vector dissemination in patients. Therefore, in order to achieve the maximum targeting gain, the development of truly targeted Ad vectors will necessitate the ablation of the native CAR tropism of the vector.

These two goals may be realized by a variety of strategies, which differ from each other in the means utilized for engineering the novel viral tropism and the ablation of CAR tropism. In essence, there are two conceptually different approaches, which may be referred to as conjugate-based targeting and genetic targeting. These strategies are similar in that they are both based on establishing a physical link between the Ad virion and a targeting molecule, or ligand, such that binding of the ligand to a target receptor attaches the virion to the cell expressing that receptor. The basic difference between these strategies is that whereas the conjugate-based approach employs methods of complexing the Ad vector with the targeting moieties which do not usually require any modifications of the Ad virion, and results in a multicomponent vector, in

genetic targeting no extraneous complexes or conjugates are involved as the targeting is achieved by genetic modification of the Ad virion itself, thereby resulting in a single-component vector.

A variety of different types of molecules may be employed as targeting ligands in these two approaches. Perhaps with the exception of small inorganic molecules which possess specificity to selected cell surface receptors, any natural receptor-binding ligand can be linked to an Ad capsid. This covers a wide spectrum of ligands ranging from relatively simple organic substances such as folate to complex chemical conjugates or genetic fusions of antibodies (see "Conjugate-Based Targeting" below). However, this spectrum of naturally available targeting moieties, although quite broad, cannot meet the needs of Ad targeting in the most general sense, as it is not applicable to cell surface molecules which do not perform any receptor functions and thus do not have any natural ligands. To direct Ad vectors to this type of molecule, relevant targeting ligands should be engineered *de novo*. This task may be achieved by developing mono- or polyclonal antibodies against the target molecule and using these antibodies for Ad targeting. However, this approach can be used only in a conjugate-based strategy, since the incorporation of an entire antibody molecule into the Ad capsid is not yet possible (see discussion below). Alternatively, a more versatile approach which is compatible with both targeting strategies may be employed for the identification of ligands. Specifically, phage libraries which are designed to display an enormous diversity of random peptides or single-chain antibodies may be utilized for the identification of the ligands of interest in a so-called "biopanning" procedure. Such biopanning usually involves several rounds of interactions between the phages constituting the library with the target, which may be represented either by purified target molecules or cells expressing these molecules or, in the extreme, the entire organism [28–34]. Each round of selection leads to the isolation of an enriched subpopulation of phage particles demonstrating some degree of binding to the target, which is then used in a subsequent round of selection. After being repeated several times, this sequential procedure normally results in the identification of ligands possessing specificity to selected targets, which may be used for the Ad rerouting strategies.

IV. Conjugate-Based Targeting

A. Bispecific Chemical Conjugates

In order to restrict gene delivery exclusively to the target cells, it is necessary to prevent the interaction between the knob domain of the Ad fiber and its cellular receptor, CAR, which plays the major role in the determination of Ad tropism. Douglas *et al.* were the first to show that it is possible

to redirect Ad infection by employing the Fab fragment of a neutralizing anti-knob monoclonal antibody (mAb) chemically conjugated to a receptor-specific ligand, in this case folate [35]. When complexed with Ad vector particles, the bispecific conjugates simultaneously ablate endogenous viral tropism and introduce novel tropism, thereby resulting in a truly targeted Ad vector (Fig. 1). In this approach, the Fab fragment is employed in preference to the intact anti-knob mAb, in order to prevent the two antigen-binding arms of the parent antibody cross-linking different viruses to form large complexes which might prove refractory to cellular uptake.

Since native Ad entry is a two-step process in which the primary cellular receptor serves merely as a docking site for Ad on the cell surface, by analogy it is possible to retarget an Ad vector simply by redirecting binding to an alternative cellular receptor, with subsequent internalization mediated by the interaction between the penton base and cellular integrins. Hence, it has been possible to retarget Ad vectors by conjugating the anti-knob Fab fragment to a wide variety of ligands. In many cases, such bispecific conjugates have been demonstrated to enhance Ad-mediated gene transfer to

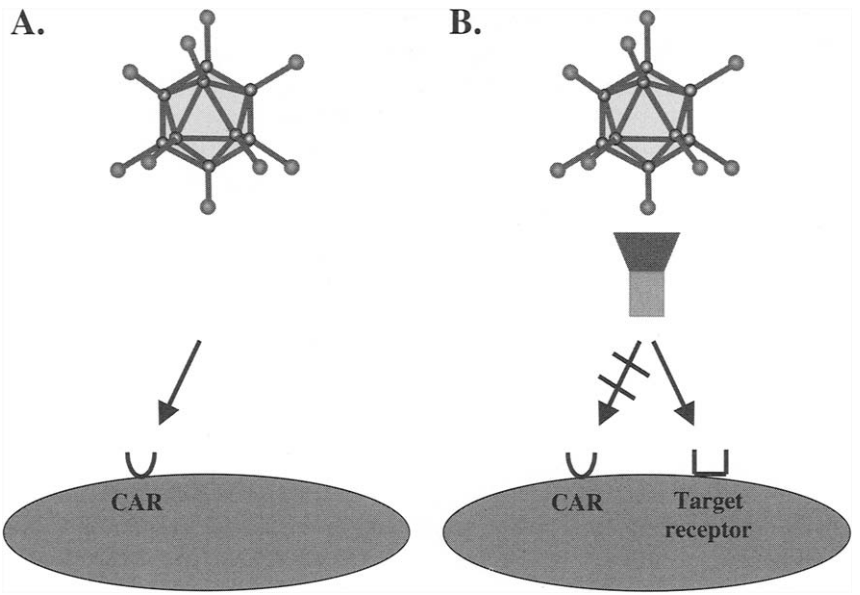

Figure 1 Strategy for targeting of adenoviral vectors using Fab fragment of a neutralizing anti-knob monoclonal antibody (mAb) chemically conjugated to a receptor-specific ligand. (A) Ad attachment to cells is accomplished by the high-affinity binding of the knob domain of the fiber to the primary cellular receptor, CAR. (B) When complexed with the Fab fragment of a neutralizing anti-knob antibody conjugated to a receptor-specific ligand, the Ad vector is unable to bind CAR and is directed to a novel target receptor on the cell surface.

target cells which are refractory to native Ad infection due a low level of CAR. For example, a bispecific conjugate in which the anti-knob Fab fragment is chemically conjugated to basic fibroblast growth factor, FGF2, has been shown to mediate enhanced Ad infection of Kaposi's sarcoma cell lines [36] and vascular endothelial and smooth muscle cells [37], which are only poorly infected by the unmodified vector. In these instances, retargeting of the Ad vector with FGF2 permits a given level of gene transfer to be achieved with a lower dose of virus.

The Fab–FGF2 conjugate has also been employed to demonstrate that enhancement of Ad infection can be exploited for therapeutic advantage. To this end, it is well recognized that Ad vectors produce a dose-dependent inflammatory response in rodents and primates. Vector-associated toxicity has also been observed in human clinical trials and threatens to prevent Ad from realizing its full potential as a vector for human gene therapy. This suggests that it would be advantageous to reduce the number of Ad particles required for a given level of gene transfer *in vivo*. Preliminary *in vitro* and *in vivo* studies demonstrated that Ad-mediated gene transfer to the human ovarian cancer cell line SKOV3.ip1 could be significantly enhanced by using basic fibroblast growth factor (FGF2) as the targeting ligand, permitting the transduction of a given number of target cells to be achieved by a lower dose of virus [38–40]. Rancourt *et al.* subsequently demonstrated that intraperitoneal administration of an FGF2-redirected Ad vector carrying the gene for herpes simplex virus thymidine kinase, AdTK, resulted in a significant prolongation of survival in a murine model of human ovarian cancer compared to the same number of particles of the unmodified vector [39]. In addition, the enhanced Ad infection permitted an equivalent therapeutic effect using a 10-fold lower dose of the vector. Similar results have been reported by other investigators [41, 42]. Moreover, intravenous administration of the FGF2-targeted AdTK vector led to markedly decreased hepatic toxicity and liver transgene expression compared with the untargeted vector [41, 42]. Thus, these findings suggest that strategies to enhance the efficiency of infection of recombinant Ad vectors may be of general clinical utility, by permitting therapeutically significant levels of gene transfer while minimizing the toxicity associated with high numbers of virus particles. These benefits are not limited to the field of cancer gene therapy: FGF2-mediated augmentation of gene transfer by an Ad vector encoding platelet-derived growth factor-B has been shown to enhance infection of target cells involved in tissue repair, resulting in an improved therapeutic outcome and potentially overcoming the safety and efficacy limitations of unmodified Ad vectors [43].

Bispecific moieties consisting of the anti-knob Fab fragment chemically conjugated to a mAb directed against the epidermal growth factor receptor (EGFR) have been employed to retarget Ad vectors to primary and established glioma cells and squamous cell carcinoma of the head and neck (SCCHN)

cells [25, 44]. In addition to EGFR-specific infection, the retargeted vectors increased gene transfer to CAR-deficient cancer cells by up to 66-fold relative to the unmodified vector. Furthermore, retargeting enhanced the selectivity of Ad infection for tumor tissue relative to normal tissue from the same patient [44]. The value of retargeting Ad vectors to achieve efficient and specific gene transfer to cancer cells has further been demonstrated by means of a bispecific conjugate targeted to the pan-carcinoma antigen EpCAM [45]. The conjugation of the anti-knob Fab fragment to mAb CC49 permits enhanced Ad infection of primary ovarian carcinoma cells, which express the cognate TAG-72 receptor, while decreasing gene transfer to normal peritoneal mesothelial cells, relative to untargeted Ad [46]. In this case, the selectivity of the targeted vector for cancer versus normal cells was enhanced up to more than 200-fold relative to the unmodified vector.

In the field of cancer immunotherapy, the relative resistance of CAR-deficient dendritic cells (DCs) to Ad infection has limited the application of gene-based vaccination. Using a bispecific antibody consisting of the anti-knob Fab fragment conjugated to an anti-CD40 mAb, Tillman *et al.* observed highly augmented Ad-mediated gene transfer to monocyte-derived dendritic cells [47]. Importantly, this efficient gene transfer was accompanied by the maturation of the DCs, resulting in an enhancement in the efficacy of DC-based vaccination against human papilloma virus 16-induced tumor cells in a murine model [48].

The anti-knob Fab fragment has also been chemically conjugated to the H_c fragment of tetanus toxin, permitting Ad vectors to be retargeted to neurons following intramuscular injection into mouse tongues [49]. This result further demonstrates the versatility of this targeting strategy, the aim of which is to redirect Ad binding to the surface of the target cells. The universality of this approach is further exemplified by the chemical conjugation of an anti-knob Fab fragment to a peptide with specificity for the lung endothelium, which was identified by *in vivo* phage display [50]. In an *in vitro* study, the redirected Ad vector exhibited specificity for cells expressing the target receptor for this peptide.

Reynolds *et al.* have recently reported that a bispecific antibody consisting of the anti-knob Fab fragment chemically conjugated to a mAb directed against angiotensin-converting enzyme (ACE), which is preferentially expressed on pulmonary capillary endothelium, was able to mediate targeted Ad infection of pulmonary endothelial cells following tail vein injection into rats [51]. This shows that an Ad vector complexed with a bispecific conjugate maintains its targeting fidelity upon systemic vascular administration, a result which clearly has important and encouraging implications.

Ad vectors have also been retargeted by bispecific antibodies in which the anti-Ad mAb is directed against the penton base. In this case, the full-length mAbs are employed, the combined length of which allows the retargeting moiety to extend beyond the fiber knob. Wickham *et al.* incorporated the

FLAG peptide epitope into the penton base and then generated bispecific antibodies comprising an anti-FLAG mAb conjugated to a mAb specific for the target receptor. The retargeting of Ad binding to αv integrins augmented gene transfer to CAR-deficient endothelial and smooth muscle cells by 7- to 9-fold [52], while retargeting of Ad to E-selectin increased gene transfer to endothelial cells by 20-fold [53]. An anti-FLAG × anti-CD3 bispecific antibody resulted in a 100- to 500-fold increase in gene transfer to T cells, another cell type poorly infected by unmodified Ad vectors [54].

Yoon *et al.* have described a strategy to target Ad to human hepatocellular carcinoma cells by means of a bispecific antibody comprising the Fab fragment of an anti-hexon antibody chemically conjugated to a mAb that binds to an antigen that is highly expressed on the target cells [55]. However, the Ad vector in this case was not truly targeted since it retained the ability to bind the native receptor, CAR.

The relatively large number of published studies in which the tropism of Ad vectors has been modified by means of bispecific moieties consisting of an anti-knob Fab fragment chemically conjugated to a ligand largely reflects the historical primacy of this strategy. The chief advantage of this approach is that a variety of ligands, including vitamins, growth factors, antibodies, and peptides, can be chemically conjugated to the anti-knob Fab fragment and used to redirect Ad binding. However, the chemical conjugation results in a heterogeneous population of molecules, which presents a problem in obtaining regulatory approval for a clinical trial employing these conjugates. Moreover, the yield of appropriately conjugated bispecific molecules can be low.

B. Bispecific Recombinant Fusion Proteins

In recognition of the disadvantages associated with chemical conjugation strategies, a number of groups have generated bispecific targeting moieties in the form of recombinant fusion proteins. This permits the expression and purification of a homogenous population of retargeting molecules. The principle on which the design of these bispecific proteins is based is the same as that underlying the construction of chemically cross-linked targeting agents: one site of the protein is directed against an Ad capsid protein, while a second site is specific for a cell surface molecule. Again, the derivation of a truly targeted vector requires that the bispecific molecule block the binding of Ad to its native primary receptor, CAR.

One class of bispecific fusion proteins used to retarget Ad vectors consists of a neutralizing anti-Ad knob single-chain antibody (scFv) genetically fused to a cell receptor-specific ligand or scFv. Watkins *et al.* were the first to describe this type of fusion protein, for which they coined the term "adenobody"[56]. They isolated a neutralizing anti-Ad5 knob scFv, designated s11, from a phage library and then fused epidermal growth factor (EGF) to its C-terminal. The resultant fusion protein was expressed in bacteria and purified from the

periplasmic fraction. As expected, this adenobody bound both the Ad5 knob and the EGFR on target cells, and was therefore able to redirect Ad infection via this target receptor [56]. Nicklin *et al.* fused this anti-Ad5 knob scFv with a heptapeptide identified by biopanning a phage display library on human umbilical vein endothelial cells (HUVECs) [57]. The fusion protein retargeted Ad infection of HUVECs, resulting in a 15-fold increase in the efficiency of transduction of these CAR-deficient cells, relative to the unmodified vector. Haisma *et al.* constructed a bispecific scFv by fusing the anti-Ad5 knob scFv s11 to an scFv directed against EGFR [58]. Two versions of the bispecific scFv were constructed, with the anti-EGFR scFv at either the N- or C-terminal of s11. In this case, the bispecific scFvs were expressed in mammalian COS-7 cells and purified from the conditioned medium. Both forms of the scFv were able to retarget Ad infection via EGFR. One disadvantage of the adenobody approach is that the incorporation of different targeting moieties can have a big impact on the solubility properties on the resultant fusion protein. Hence, it can be difficult to predict whether the fusion of a given targeting scFv or ligand to the anti-Ad5 knob scFv will yield a soluble molecule which can be purified and will be functional.

A second class of bispecific fusion proteins permitting the derivation of a truly targeted Ad vector consists of the extracellular domain of CAR genetically fused to a receptor-targeting moiety. By definition, once complexed with a CAR–ligand fusion protein, an Ad vector will not be able to bind to its native primary receptor on the cell surface. Dmitriev *et al.* genetically fused EGF at the C-terminal of the extracellular domain of CAR [59]. The soluble CAR–EGF fusion protein was expressed in insect cells using a baculovirus expression system. The bispecific fusion protein mediated EGFR-specific, CAR-independent Ad infection of target cells [59]. In a similar approach, Ebbinghaus *et al.* fused the extracellular domain of CAR to the Fc region of human immunoglobulin 1 [60]. When complexed with an Ad vector, this fusion protein mediated up to a 250-fold increase of transgene expression in CAR-negative, Fcγ receptor I-positive human monocyte cell lines.

The third class of bispecific fusion protein employed to retarget Ad infection is rather different from those described above. Li *et al.* chose to exploit the common signaling pathways triggered by ligation of αv integrins and growth factor receptors [61]. Consequently, they fused a mAb specific for the integrin-binding site on the Ad2/5 penton base to recombinant growth factors and cytokines (TNF-α, IGF-1, and EGF) which trigger the activation of phosphatidylinositol-3-OH kinase (PI3K), a signaling molecule involved in Ad internalization. The bifunctional antibodies were expressed in insect cells as secreted proteins. Ad vectors complexed with these bispecific molecules increased gene delivery 10- to 50-fold to human melanoma cells lacking αv integrins [61]. Thus, whereas other strategies to retarget Ad vectors are based on redirecting Ad binding, with internalization mediated by αv integrins, the

use of bispecific molecules to trigger alternative cell signaling pathways enabled the native secondary Ad receptors to be bypassed. However, it would appear that the range of receptors which could be targeted in this manner is somewhat limited and lacking in cell specificity.

C. Bispecific Peptides

Hong *et al.* have retargeted Ad infection by means of a bispecific 35-mer oligopeptide comprising two distinct peptide domains [62]. One domain was a 20-mer peptide isolated from a phage library on the basis of its ability to recognize the receptor-binding region of the Ad5 knob, while the second domain corresponded to the gastrin-releasing peptide (GRP). The authors demonstrated that the relative orientation of the two domains of the bispecific peptide was crucial: only the peptide with the GRP domain at the N-terminal was capable of mediating Ad infection in a GRP receptor-dependent manner [62]. In contrast to other targeting strategies in which the bispecific molecules were complexed with the Ad vector prior to infection, in this case the bifunctional peptide was bound to the target cellular receptors prior to the addition of Ad. It is not clear whether this approach was mandated by the small size of the oligopeptide.

D. Polymer-Mediated Coupling of Ligands to Ad Capsid Proteins

An alternative strategy to link receptor-specific targeting peptides to the Ad capsid involves the use of polyethylene glycol (PEG). Romanczuk *et al.* used bifunctional PEG to couple Ad to a peptide identified by biopanning a phage display library against differentiated, ciliated airway epithelial cells [63]. Similarly, Drapkin *et al.* used PEG to couple Ad to a seven-residue peptide derived from urokinase plasminogen activator [64]. In both cases, infection of the CAR-deficient target cells by the modified vector was significantly enhanced over the unmodified vector. However, the modified vectors were still able to infect HeLa cells in a CAR-dependent manner: hence, the native tropism of the modified vectors had not been abolished. Nevertheless, now that the residues of the Ad5 knob responsible for binding CAR have been identified, a truly targeted vector could readily be generated by coupling peptides to the capsid of a virion whose knob domain had been mutagenized to prevent binding to CAR. A significant advantage of the use of PEG to couple ligands to the Ad capsid is that PEGylation partially protects the virus from neutralizing antibodies both *in vitro* and *in vivo* [65]. In a similar approach, Fisher *et al.* have shown that incorporation of targeting ligands such as basic FGF and vascular endothelial growth factor on to Ad vectors coated with a multivalent hydrophilic polymer leads to ligand-mediated, CAR-independent gene transfer to target cells [66]. Importantly, the polymer-coated, retargeted vector was resistant to neutralizing antibodies.

E. Biotinylated Ad/Avidin Bridge/Biotinylated Ligand

Whereas the targeting complexes described above have comprised two components, three-component complexes have also been described. In this case, an avidin or streptavidin molecule serves as a bridge to link a biotinylated Ad vector to a biotinylated ligand. Smith *et al.* used this strategy to enhance Ad infection of CAR-negative primitive hematopoietic cells which express the c-Kit receptor on the cell surface [67]. Biotinylated Ad vectors were linked via an avidin bridge to the biotinylated cognate ligand for the c-Kit receptor, stem cell factor. Ad-mediated gene transfer was targeted specifically to c-Kit-positive hematopoietic cell lines, resulting in up to a 2440-fold increase in gene expression. In a second example of the versatility of this strategy, Smith *et al.* infected CAR-negative primary T cells with Ad vectors targeted with biotinylated antibodies to CD44 (resting and activated T cells) or with biotinylated IL-2 (activated T cells only) [67]. Kreda *et al.* have employed a streptavidin bridge to link a biotinylated Ad vector to a small molecule agonist of the G-protein-coupled $P2Y_2$ receptor [68]. The tropism-modified vector was able to infect Ad-resistant, well-differentiated airway epithelia cells in a $P2Y_2$ receptor-specific manner.

A key attribute of this targeting strategy is that it is fairly straightforward to biotinylate a range of molecules. One disadvantage is the potential problem of scaling up the procedure, but the major limitation is the fact that three components are involved, each of which would have to meet the standards laid down by the regulatory authorities before the targeted vectors could be employed in a clinical context.

Overall, conjugate-based targeting of Ad vectors possesses the advantage that major structural alterations of the Ad capsid are not involved. Consequently, a preexisting Ad vector can be complexed with a variety of bispecific targeting molecules in order to redirect infection to a number of cell or receptor types. However, the major problem is that this targeting approach employs at least two components, vector and targeting moiety, which must be produced independently and then complexed together. This can lead to significant variation between batches of tropism-modified vector. In contrast, a single-component targeted vector can be derived by genetic modification of the Ad capsid.

V. Genetic Targeting

As mentioned above, this Ad targeting strategy involves genetic ablation of the virus' tropism to CAR and simultaneous engineering of an alternative receptor-binding specificity to the vector by genetic engrafting of targeting ligands into protein components of the Ad capsid. Whereas the means for accomplishing the first of these tasks was a real challenge until just a

couple of years ago, nowadays it may be achieved rather trivially. This has become possible largely due to systematic efforts undertaken at the Brookhaven National Laboratory (BNL) and GenVec, Inc., to identify those amino acids in the Ad fiber knob domain which mediate the interaction with CAR. Specifically, the BNL team used recombinant forms of the Ad12 fiber knob and the D1 domain of CAR protein to obtain crystal structures of knob–D1 complexes [69]. The resultant three-dimensional model of the complex has allowed the identification of CAR-binding residues within the knob. The importance of those amino acids in CAR recognition by Ad was then confirmed by the generation of Ad virions incorporating mutated fiber proteins. The same goal has been reached by Roelvink *et al.* in a large-scale project on Ad5 knob mutagenesis which was rationalized by the identification of amino acid residues conserved in the fiber knobs of CAR-binding human Ad serotypes [70]. Although these groups utilized totally different approaches, the results of the two studies corroborate each other quite nicely. It has been shown that the CAR-binding site within the Ad5 knob domain involves the AB- and DE- loop, as well as β-strands B, E, and F (Fig. 2, see color insert). Mutations of the key amino acids identified in these studies quite dramatically decrease the capacity of recombinant knob proteins or complete Ad virions bearing such mutations to bind CAR, thereby providing a simple means to ablate native Ad tropism to CAR.

While this knob mutagenesis represented a universal approach to CAR ablation, engineering of novel Ad tropism may be achieved in a variety of distinct strategies. These targeting maneuvers are distinguished by genetic modifications of different components of the Ad capsid. It has been demonstrated that genetic modifications of the three major proteins constituting an Ad virion, the hexon, the fiber and the penton base, may be employed to redirect the virus.

A. Ad-Targeting Strategies Involving Genetic Manipulations of the Fiber Protein

Logically, as the fiber normally plays the role of primary attachment of the virion to the cell, the majority of Ad targeting efforts have been based on fiber modification. To date, at least three distinct strategies of Ad targeting involving fiber modification have been used: fiber or knob shuffling, fiber modifications via the incorporation of targeting ligands, and fiber- or knob-replacement strategies.

1. Fiber- and Knob-Shuffling Approaches

This is the most obvious and straightforward strategy, which is based on the overall structural similarity of the fibers of different Ad serotypes and is further rationalized by the fact that representatives of these serotypes

use different receptors to infect permissive cells. In this regard, the high degree of homology between fiber tail domains suggests that the penton base protein of a given Ad serotype may associate quite efficiently with fibers derived from different serotypes. Furthermore, the junctions between the tail and shaft, and shaft and knob, domains within the fiber protein represent convenient fusion points in those instances when a mosaic fiber protein incorporating individual domains derived from different Ad serotype fibers is designed. These considerations have resulted in a number of studies employing Ad vectors whose fibers have been either completely replaced with heterologous fiber proteins or modified to contain knobs originating from other Ad serotype fibers.

The feasibility of Ad retargeting via fiber shuffling was demonstrated in pioneering work by Gall *et al.* who showed that by replacing the fiber gene in Ad5 genome with the gene from Ad7, a chimeric Ad vector incorporating Ad7 fibers may be generated [71]. By using two types of competition assays, the authors demonstrated that this fiber substitution resulted in the alteration of the tropism of the Ad vector. It was thus shown that exchange of the fiber is a strategy that may be used to manipulate native Ad tropism.

This approach was later utilized in collaborative work conducted by researchers at the University of Iowa and Genzyme Corp. to develop better Ad vectors for human gene therapy. To this end, by screening a number of Ad serotypes for an enhanced ability to infect either the well-differentiated ciliated human airway epithelia (CHAE) or fetal rat central nervous system (CNS) cells, the authors identified Ad17 as one of the best agents for transduction of both types of cell targets [72, 73]. Based on these data, an Ad2 vector was then genetically modified to contain the Ad17 fiber in place of the native Ad2 fiber. The resultant virus possessed the receptor specificity and infection capacity of Ad17. Specifically, the levels of reporter gene expression directed by this modified vector in CHAE cells were 15- to 95-fold higher than those achieved by the parental Ad2 vector [73]. Additionally, this tropism-modified vector was sevenfold more efficient than its unmodified Ad2 counterpart in transducing CNS cells *in vitro* [72]. These findings provide a rationale for the utilization of similar Ad vectors incorporating Ad17 fiber proteins for gene therapy approaches to the treatment of cystic fibrosis and CNS disorders.

The poor transducibility of human hematopoietic stem cells (HSCs) by commonly used Ad vectors was addressed by the utilization of the fiber swapping approach in work by Shayakhmetov *et al.* [22]. An analysis of the binding and internalization of six different Ad serotypes (3, 4, 5, 9, 35, and 41) performed on $CD34^+$ cells identified Ad35 as the best overall vehicle for transduction of HSCs. The subsequent generation of an Ad5 vector incorporating the Ad35 fiber protein and evaluation of its transduction capacity on $CD34^+$ cells showed that this vector was several-fold more efficient than a control vector incorporating Ad5 fibers.

Another example of the successful use of the fiber shuffling strategy is the result of extensive studies originated at Crucell Holland (Leiden, The Netherlands). A panel of Ad5-based vectors incorporating fibers from other serotypes was employed in order to identify those Ad fibers which would be the most efficient in mediating infection of human DCs and cardiovascular or synovial tissues [22, 74–76]. An initial screening resulted in the Ad35 fiber being the most efficient in directing Ad binding to DCs, while synovial and cardiovascular tissues were most sensitive to a chimeric vector containing Ad16 fibers. A chimeric Ad5/35 vector proved to be 100-fold more potent than an Ad5 control for gene transfer and expression in human DCs [76]. However, no maturation of the DCs was observed, in marked contrast to the studies in which Ad was targeted to CD40 [47]. In a parallel study, an Ad5 vector carrying the fiber of Ad16, showed on average, an 8- and 64-fold increase in the reporter gene expression in endothelial and smooth muscle cells, compared to the parental Ad5 vector [75]. Therefore, these findings suggest that Ad5/16 may serve as a prototype for the generation of derivative vectors carrying relevant therapeutic transgenes, which may be quite efficient as a means to treat cardiovascular disorders. According to a study by Goosens *et al.*, the same vector platform shows promise as an efficient system for gene delivery to synoviocytes which do not express CAR and thus are refractory to infection by Ad5-based vectors [74]. Specifically, the authors demonstrated that an Ad5/16 vector was more potent in transducing cultured synoviocytes compared to Ad5. An observed 150-fold increase in transgene expression was caused by both the transduction of a higher percentage of synoviocytes and higher level of gene expression per transduced cell.

An approach similar to fiber shuffling was realized in studies which employed genetic replacement of the Ad5 fiber knob domain with the Ad3 knob as a way to achieve the goal of Ad5 rerouting via an alternative Ad receptor [77, 78]. In these reports, the retargeting of the vector to the, as yet unidentified, Ad3 fiber receptor suggested the utility of this knob-swapping approach in those instances where preferential expression of the Ad3 fiber receptor favors infection of Ad5-refractory cells. For instance, the vector-containing chimeric fibers outperformed the control vector incorporating Ad5 fibers in gene delivery to human fibroblasts and head and neck cancer cells [78]. Similarly, in their efforts to develop a gene delivery system for treatment of lymphoproliferative disorders, Von Seggern *et al.* also derived an Ad vector containing Ad5/Ad3 fiber chimeras and demonstrated its superior gene transfer properties on Epstein–Barr virus-transformed B lymphocytes [79].

Despite their attractive simplicity, the fiber- and knob-shuffling approaches are limited in their utility for gene therapy and may hardly be viewed as universal strategies with the potential to direct Ad vectors to any given type of cell target. This is due to the fact that the repertoire of target receptors is dictated by the diversity of targeting ligands available. However

significant the transduction efficiency gains achieved in the published studies, they cannot be further improved after all available Ad serotypes have been tested in the cell system of interest and the best overall candidate fiber has been identified.

Whereas in the context of the fiber- or knob-shuffling approaches an investigator is strictly limited in the capacity to improve upon the vector specificity, this problem may be overcome by expanding the repertoire of targeting ligands beyond the limits of the natural diversity of Ad fibers. This concept is realized in strategies of Ad targeting based on the incorporation of targeting ligands into the Ad fiber protein or fiber-replacing molecules.

2. Ad Fiber Modifications

The most significant advantage of this approach compared to the fiber- or knob-shuffling strategies is that it potentially permits the utilization of a wide range of ligands for Ad targeting. As mentioned above, these may be the natural ligands for target receptors, or alternatively they may be identified via the use of phage display technology. From a theoretical standpoint, if these two methodologies are utilized in a rational manner, a virtually unlimited number of targeted Ad vectors may be derived. There are, however, practical considerations which may limit the usefulness of this approach. Evidently, the genetic fusion of a targeting ligand with the fiber protein should be accomplished in a way which would retain a functional configuration of both fusion partners. Therefore, the specific site in the fiber protein chosen for ligand insertion should not play any vital function which could potentially be abrogated by the ligand insertions. Furthermore, the configuration of such a site should favor the presentation of a ligand on the surface of the fiber molecule, thereby facilitating its interaction with the cognate receptor. In addition, the architecture of an Ad virion in general and the receptor-binding function of the knob in particular imply that of the three structural domains of the fiber protein, the knob is the most logical locale for presentation of targeting ligands. Thus, the configuration of the knob may apply additional limitations on the design of functional fiber–ligand fusions.

Although these consideration are quite obvious, they were of little use at the time when the first attempts to modify the fiber were undertaken, as the work was initiated well before the three-dimensional model of the fiber knob domain became available. Modification of the Ad5 fiber protein was first endeavored by Michael *et al.*, who demonstrated that a short peptide ligand, the gastrin-releasing peptide (GRP), genetically fused to the C-terminal of a recombinant fiber protein does not interfere with fiber trimerization and is available for binding with a GRP-specific antibody [80]. In the absence of any structural information about the knob domain, the choice of the C-terminal was rationalized by previous findings that the N-terminal of the protein is embedded in the capsid, while the C-terminal is exposed outside

the virion. Wickham *et al.* subsequently derived an Ad vector incorporating a fiber extended at the C-terminal with a stretch of lysine residues in order to target this vector to heparin-containing cellular receptors [17]. This tropism expansion maneuver resulted in a vector which was able to efficiently transduce a number of Ad5-refractory cells, thereby establishing the feasibility of this genetic approach to Ad targeting. Specifically, the transduction capacity of this new vector on endothelial cells, glioblastomas, smooth muscle cells, and fibroblasts was 9- to 311-fold higher than that of unmodified control vector bearing wild-type Ad5 fibers. The same group of investigators subsequently redesigned the oligo-lysine modified vector by making AdZ.F(pK7), a vector containing seven C-terminal lysine residues, and also constructed a similar vector, designated AdZ.F(RGD), incorporating an RGD-containing peptide at the C-terminal of the fiber [18]. The latter vector was designed to target αv-integrins expressed by a number of cell targets whose infection with unmodified Ad vectors is inefficient. Evaluation of these two vectors on a panel of cells which are poorly infected with Ad5 showed that they both were equally efficient in transducing endothelial cells, while AdZ.F(pK7) clearly outperformed AdZ.F(RGD) on smooth muscle cells and macrophages [18]. The highly augmented transduction efficiency of AdZ.F(pK7) on smooth muscle cells in tissue culture encouraged the authors to employ this vector to transduce pig iliac arteries injured by a balloon catheter. As expected from the data obtained in the *in vitro* studies, AdZ.F(pK7) proved to be more efficacious than its unmodified counterpart in delivering a transgene to the target tissue.

This proof of concept work led to a number of spin-off studies utilizing AdZ.F(pK7) and AdZ.F(RGD) for gene delivery to a variety of CAR-deficient tissues. For instance, AdZ.F(RGD) showed superior *in vivo* gene transfer to the cortical vasculature in rats [81], whereas AdZ.F(pK7) proved to be useful for transduction of muscle cells at all stages of differentiation as well as mature skeletal muscle [82], myeloma cells [83], myeloid leukemic cells [84], and malignant glioma cells [85]. However, the magnitude of enhancement achieved by AdZ.F(pK7) *in vivo* was somewhat less than might have been anticipated from the *in vitro* augmentation.

A series of Ad vectors similar to AdZ.F(pK7) designed by Hamada's group was used in extensive work aimed to develop gene therapy for gliomas. All the vectors used in these studies were designed on an Ad5 platform and incorporated a fiber protein with a stretch of 20 lysine residues fused via a peptide linker to the C-terminal of the fiber. The employment of this prototype vector for delivery of genes encoding cytokines IL-2 and IL-12 [86], the p53 tumor suppressor [86], prodrug-converting herpes simplex virus thymidine kinase [87], or a conditionally replicative Ad genome [88] has led to significant improvements of transgene expression and Ad-mediated killing of glioma cells.

Despite the fact that the utility of C-terminal modifications of the fiber for Ad targeting has been clearly demonstrated, it has also been reported that some

of the attempted modifications employing rather long targeting peptides did not result in viable Ad virions [18]. Moreover, fiber modification studies performed by Hong and Engler showed that the addition of a 27-amino-acid residue peptide sequence to the C-terminal of the fiber resulted in a protein incapable of assembly into trimers [4]. This led to the hypothesis that ligands exceeding a threshold of about 30 amino acids in length cannot be successfully incorporated into this locale in the fiber protein without detrimental consequences for the structure of the fiber.

Evidently, these findings would limit the applicability of the fiber modification approach should the C-terminal of the fiber be the only locale within the molecule suitable for the presentation of ligands. Fortunately, the three-dimensional model of the Ad5 fiber knob proposed by Xia *et al.* [89] provided the rationale for the evaluation of other sites within this domain for the purpose of ligand incorporation. According to this model, the loops which connect the β-sheets R and V within each fiber knob monomer may function well as ligand-presenting structures. These loops are localized on the surface of the knob and are thus readily accessible for interactions with potential receptors (Fig. 2). The flexibility of the loops suggests that ligands incorporated within the loops would be able to assume the proper configuration required for the interaction with their cognate receptors. The loops are not involved in intramolecular interactions; therefore, modifications of their structure should not affect the stability of the fiber. Additionally, alignment of the primary sequences of the knob domains of various Ad serotypes reveals that the length and amino acid composition of the loops varies quite significantly, implying that the incorporation of targeting ligands into the loops would be well tolerated by the knob structure. These considerations encouraged Krasnykh *et al.* to conduct a proof of concept study aimed to show the feasibility of Ad targeting via genetic modification of the loops within the fiber knob [90]. This was achieved by genetic incorporation of a ligand-mimicking octapeptide FLAG tag into the HI loop of the knob domain. First, it was shown that a fiber protein incorporating the FLAG tag expressed in baculovirus-infected insect cells retains its native trimeric configuration and binds to an affinity matrix containing an anti-FLAG antibody. It was then shown that incorporation of the peptide did not affect the ability of the modified fiber to bind to CAR-positive cells. Hence, the two key features of the fiber, trimerization and receptor binding, were both preserved in the fiber-FLAG protein, thereby rationalizing the generation of an Ad vector containing such fibers. A virus containing chimeric fiber-FLAG proteins was shown to be viable and was able to infect cells via CAR-mediated pathway. Importantly, the FLAG peptide engrafted in the virion retained its ability to bind an anti-FLAG mAb, suggesting that a peptide of a similar size possessing targeting properties should function well in the context of the HI loop of the fiber.

This concept was proved in a subsequent study by the same team of investigators [91], who derived an Ad vector, Ad5lucRGD, incorporating

within the HI loop of the fiber knob the targeting peptide CDCRGDCFC, known as RGD-4C. This ligand was chosen for Ad targeting based on its small size and well-documented ability to bind quite efficiently with a number of cellular integrins, which were used as target receptors in these studies. Therefore, the rationale behind this work was to target the virus directly to integrins, which normally function as the secondary Ad receptors. It was expected that the resultant virus would thus possess the capacity to enter the cell via a CAR-independent, integrin-mediated pathway. The employment of the resultant virus for gene transfer to a panel of cell targets expressing various levels of CAR and integrins proved that the HI loop-localized RGD-4C peptide was indeed able to direct the vector to integrins, thereby expanding its tropism. Importantly, it was demonstrated that as a result of this tropism expansion, the virus gained a significant advantage over its unmodified counterpart in transducing cell targets which are low in CAR expression. This infectivity enhancement was most dramatic when both viruses were applied to primary tumor cells isolated from patients with ovarian cancer. In this experiment, the reporter gene expression detected in Ad5lucRGD-transduced cells was two orders of magnitude higher than that in the cells infected with the control vector.

The superior transduction efficiency of Ad5lucRGD was subsequently exploited in more extensive studies in established cell lines and primary samples of ovarian tumors [92]. Furthermore, it was later demonstrated that this vector offers another advantage in the treatment of ovarian cancer by circumventing the inhibition of Ad infection of tumor cells by neutralizing anti-fiber antibodies present in ascitic fluids in the patient's peritoneum [93].

The successful utilization of Ad5lucRGD to augment gene transfer to squamous cell carcinoma of head and neck cell lines [94], myelomonocytic leukemia cells [95], rhabdomyosarcoma cells [96], and glioma cells [97] by up to three orders of magnitude suggests that this vector may serve as a prototype for the derivation of agents suitable for gene therapy of various types of cancer, where CAR deficiency of target tumors undermines the efficacy of unmodified Ad5 vectors.

A recent report by Asada-Mikami *et al.* showed another use for Ad vectors based on the Ad5lucRGD platform by demonstrating the improved capacity of this vector to transduce dendritic cells [98].

A further demonstration of the utility of HI loop modifications for Ad targeting, as well as the compatibility of this approach with the ligand definition strategy based on phage library biopanning, was provided by Xia *et al.* [99]. In order to design an Ad vector suitable for transduction of brain microcapillary endothelium (BME) via the transferrin receptor (TfR)-mediated pathway, these investigators screened a phage library displaying linear, nonconstrained nonapeptides on a recombinant form of the extracellular domain of human TfR and isolated a total of 42 phage clones demonstrating significant binding to

this target. Ten of these peptides were then incorporated into the HI loop of the fiber as potential targeting ligands. Notably, the authors succeeded in rescuing only 7 of the 10 viral vectors; moreover, 2 of the rescued vectors were only amplified quite poorly on 293 cells. The rescued vectors were then employed for gene transfer to CAR-negative cells expressing TfR. These experiments showed that by using TfR as a primary binding receptor, the peptide-modified Ad vectors were 3- to 34-fold more efficient than unmodified vectors in transducing the target TfR-positive cells. The successful use of these vectors for gene delivery to human BME cells proved the suitability of these agents for the purposes of gene therapy of inherited metabolic disorders causing malfunction of the central nervous system.

Since the work with Ad vectors incorporating fibers with C-terminal modifications revealed the limitations relating to the size of the ligands which could be incorporated at this locale, it would be logical to address this ligand size issue in the context of HI loop modifications of the fiber. In this regard, a pilot study using incremental increases in the size of this loop via the incorporation of heterologous protein sequences with the subsequent characterization of the yields and infectivities of the resultant vectors would be of high relevance. This task has been achieved in a recent study by Belousova and Krasnykh [100], who generated a panel of Ad vectors incorporating within the HI loop a series of fragments of the RGD-containing loop of the Ad5 penton base protein. The results of this study showed that heterologous protein sequences up to at least 83 amino acids long may be incorporated into the HI loop of the fiber protein without any significant negative consequences on the viability, yield, and infectivity of the resultant vector. However, these studies also revealed that the stepwise increments in the insert size have some adverse effects on these properties of the vector. These findings led to the conclusion that although the capacity of the HI loop to accommodate targeting ligands of moderate size is superior to that of the C-terminal of the fiber, ligands whose size exceeds a certain limit will not fit into this loop. Additionally, not only the size but also the configuration of a targeting ligand may become an issue when the HI loop is used for ligand presentation. For instance, the functional configuration of some ligands may conflict with the framework of the HI loop. This conflict may arise when the C-terminal of a ligand needs to be directly involved in receptor binding: if this is the case, then the covalent bond between the ligand and the loop would prevent the efficient interaction of the modified Ad vector with the target cell.

These considerations, together with the problems with the rescue and propagation of the Ad vectors observed in the work by Xia *et al.* [99], suggest that in order to increase the likelihood of the successful generation of fiber-modified Ad vectors, it will be necessary to develop an approach which would allow for the easy and fast high-throughput evaluation of newly identified targeting ligands in the context of the Ad fiber knob. The development of such

a method would streamline the generation of targeted Ad vectors by narrowing the range of candidate ligands to those which have maximum probability of functioning upon engrafting into the Ad capsid.

Perhaps the most rational way to solve this problem would involve the generation of ligand display libraries using Ad as the vector. In such libraries, a variety of ligands would be randomly incorporated into specific locales of the Ad fiber protein and, upon rescue, the resultant diverse repertoire of modified Ad vectors would be screened for specificity for the receptors or cells of interest. However, at the present stage of development of the technology for the generation and characterization of recombinant Ad vectors, the derivation and screening of such a library presents a challenge of enormous complexity and is hardly achievable. Nevertheless, recent advances in the field have shown that there may exist some alternative approaches potentially useful in the rationalization and facilitation of the selection of ligands for Ad targeting via fiber modification.

A first step in this direction was taken by Jakubczak *et al.* who described a method which allows the fast and easy generation of fiber-modified Ad vectors without the need to construct recombinant viral genomes and rescue recombinant virions [101]. This approach capitalizes on two key advances previously made in the Scripps Research Institute by Von Seggern *et al.* [79, 102]. First, the strategy takes advantage of a plasmid vector designed to express high levels of Ad fiber protein in eukaryotic cells [79]. This plasmid is used to direct the expression of the candidate modified fiber protein. Second, a helper Ad vector containing a fiber gene-deleted genome packaged into the wild type Ad5 capsid by propagation in the fiber-complementing cell line (for details see below) is used to produce fiberless Ad capsids into which the plasmid-encoded modified fiber proteins will be incorporated. The method works in the following manner: (i) a candidate fiber gene is cloned into the expression plasmid; (ii) the plasmid is then used for transfection of 293 cells, resulting in the expression of the fiber protein of interest; (iii) the transfected cells are infected with wild-type Ad virions encapsidating fiber gene–deleted genomes. If the fiber protein expressed by the plasmid retains the configuration necessary for its efficient incorporation into an Ad capsid, it transcomplements the deletion in the genome of the helper virus and is incorporated into Ad virions. The resultant virions may then be subjected to screening on a target of interest. Although the throughput of the system cannot match that of the phage display library, this strategy provides an excellent means for the generation of genetically modified fibers and their preliminary characterization in the context of complete Ad particles.

Another promising approach addressing the same issue of the ligand–fiber compatibility was developed in a recent study by Pereboev *et al.* [103]. The rationale for this work is to expand the utility of a traditional phage display system for the identification of targeting ligands by tailoring its format such that

it closely mimics the ligand presentation by the Ad fiber protein. In contrast to the classical approach, where randomized ligands are genetically incorporated into one of the phage coat proteins, in this novel strategy a diversity of targeting ligands is created within the Ad5 fiber knob domain attached to the surface of the phage particle by Jun and Fos leucine zippers. Therefore, the subsequent screening of the library leads to the identification of ligands which demonstrate the ability to bind to a target receptor while engrafted directly in the fiber knob. This approach thereby counterselects against those ligands which could be identified in a biopanning experiment employing a traditional phage library, but would then fail to recognize the target upon incorporation into the Ad fiber. Further development of this proof of concept study should result in the derivation of phage libraries which would meet the most stringent criteria of selection for Ad targeting ligands.

3. Fiber- and Knob-Replacement Strategies

While the new approaches developed to facilitate the identification of fiber-compatible targeting ligands may be very efficient and useful, they cannot solve the problem of the structural incompatibility of the ligand and the fiber. Furthermore, extensive use of these methods may soon show that a significant proportion of ligands is not suitable for fiber modification because of serious structural conflicts between the fiber and the ligand. The high rate of failures in documented attempts to incorporate a targeting ligand into the rather complex framework of the fiber knob domain makes these expectations quite real. Therefore, it is rational to hypothesize that the tropism of the Ad vector might be manipulated more easily and much more efficiently, if the receptor binding function in the resultant fiber molecule were structurally disengaged from the trimerization function. This goal may be achieved by generating a fiber-derived molecule in which the functions of receptor recognition and trimerization would be delegated to distinct domains within the protein. Therefore, the trimerization of such a fiber would be secured by a protein moiety introduced into the design of the "platform" fiber and would not be affected by subsequent modifications of the other domain of the chimera, which would define the vector tropism. The practical realization of this strategy would dramatically expand the range of targeting ligands compatible with the fiber modification strategy, thereby diversifying the repertoire of target receptors and target cells. Obviously, the knob domain, whose complex structure seems to restrict the range of targeting ligands and targeting approaches, should be deleted from the resultant protein. In a more general sense, only the fiber tail domain which anchors the fiber in the capsid and is thus indispensable, should be retained in the modified protein, implying that the shaft of the fiber may be replaced too. In order to maintain the trimeric structure of a knob-deleted protein, the loss of the trimerization function normally provided by the knob should be compensated by the incorporation of a heterologous protein moiety

into this protein. This may be achieved by designing a knob-deleted fiber containing a protein or peptide motif known to form stable homotrimers upon self-association. Two recent studies illustrate this general concept.

The work by Krasnykh *et al.* [104] involved the generation of an Ad vector whose capsid lacked wild-type fibers but instead incorporated chimeric molecules designed to fulfill the functions normally performed by the Ad fiber. These chimeras comprised the N-terminal portion of the Ad5 fiber protein, including the tail domain and two pseudorepeats of the shaft domain, genetically fused to the bacteriophage T4 fibritin protein deleted at the N-terminal. The entire knob domain and most of the shaft domain of the fiber were deleted in this protein. Truncated fibritin was incorporated into this chimeric protein in order to provide trimerization of the resultant molecule. Previous studies on the structure of fibritin showed that, owing to the presence of the C-terminal "foldon" domain, fibritin is capable of forming homotrimers which are extremely stable under a variety of different conditions. Most importantly, it had also been demonstrated that the trimeric structure of fibritin is not compromised by either extensive N-terminal deletions or extensions of its C-terminal, thereby making fibritin an ideal "stuffer" between the fiber tail and the targeting ligand positioned at the C-terminal of the resultant chimera. A sequence of six histidine residues connected to the fibritin protein via a short peptide linker was used to demonstrate the feasibility of targeting of fibritin-containing Ad vectors to alternative cell-surface receptors by directing the modified vector to an artificial receptor, whose extracellular domain consists of an anti-His scFv [105]. First, it was shown that the fiber–fibritin 6H chimera expressed in bacteria is trimeric and binds to affinity matrix via the 6His ligand present in the protein. The subsequent incorporation of this chimera into the Ad5 capsid resulted in a stable virion capable of infecting target cells expressing the complementary receptor in a CAR-independent manner. Although fiber replacement did not affect the stability of the virus or its yield, the efficiency of infection by the new virus was lower than that by the control Ad vector containing wild-type Ad5 fibers. This decrease in infectivity may be due to the previously reported low affinity of interaction between the targeting ligand, 6His, and the ligand-binding scFv component of the artificial receptor used in the study. Fortunately, the ligand-accommodating capacity of the described fiber–fibritin chimera extends well beyond the 6His sequence, thereby allowing utilization of a wide variety of targeting ligands in the context of this prototype molecule. To this end, it has been previously demonstrated that C-terminal insertions up to at least 163 amino acids long are well tolerated by the fibritin structure [106].

A similar study was reported by Van Beusechem *et al.* [107], who attempted to replace the fiber knob domain with trimerizing α-helical coiled-coil domain of the Moloney murine leukemia virus p15 envelope protein. Since p15 is known to have low thermostability, the resultant fiber chimeras were

rather unstable with only 5–10% of the protein being assembled into trimers. Expression of these proteins by the E1-deleted Ad vectors, which also expressed the wild-type fiber, demonstrated their nuclear localization and some degree of incorporation into complete Ad virions.

Importantly, the knob- or fiber-replacement strategies allow for the simultaneous fulfillment of the two tasks required for the generation of truly targeted Ad vectors: ablation of native Ad tropism to CAR and introduction of novel tropism. Equally importantly, these approaches may be applied for the generation of truly targeted vectors derived from any Ad serotype without the prior identification and ablation of the receptor-binding site within a given fiber.

Although the studies described herein have demonstrated only the feasibility of the fiber- and knob-replacement approaches for Ad targeting, the further development of these novel technologies may eventually result in significant improvements to the utility of the present generation of Ad vectors.

4. Strategies to Rescue and Propagate Truly Targeted Ad Vectors

Regardless of the approach chosen for the derivation of truly targeted Ad vectors, the resultant virus should somehow be rescued and propagated. This constitutes a serious technical problem, as such a vector is, by definition, not able to infect cells via the native cell entry pathway and thus cannot be amplified in any of the cell lines normally used for this purpose. It would theoretically be possible to solve this problem on a case-by-case basis by first deriving an E1-complementing cell line expressing the target receptor and then using this cell line for the rescue of the targeted Ad vector of interest. However, the practical execution of this strategy would be extremely laborious, cumbersome and thus highly inefficient. In addition, this approach would not work in those instances when an Ad vector targeted to unknown cell surface molecule was being derived. Therefore, a universal solution to the problem would be highly desirable.

At least two distinct approaches have been developed to address this problem. The first strategy involves the generation of a packaging cell line which expresses the wild-type Ad fiber and may be used in the rescue and initial propagation of truly targeted Ad vectors (Fig. 3). Ideally, this cell line should be designed to express an Ad fiber whose receptor is naturally produced by this cell line. The utilization of such a cell line will result in the production of mosaic Ad virions randomly incorporating both wild-type and modified fibers. The presence of the wild-type fiber in the resultant virions will allow the efficient infection of any cell line expressing native Ad fiber receptor, including the packaging line. After a sufficient amount of mosaic virus has been generated, this vector may then be converted into the truly targeted configuration by a final amplification step on a cell line which does not express any Ad fiber, for

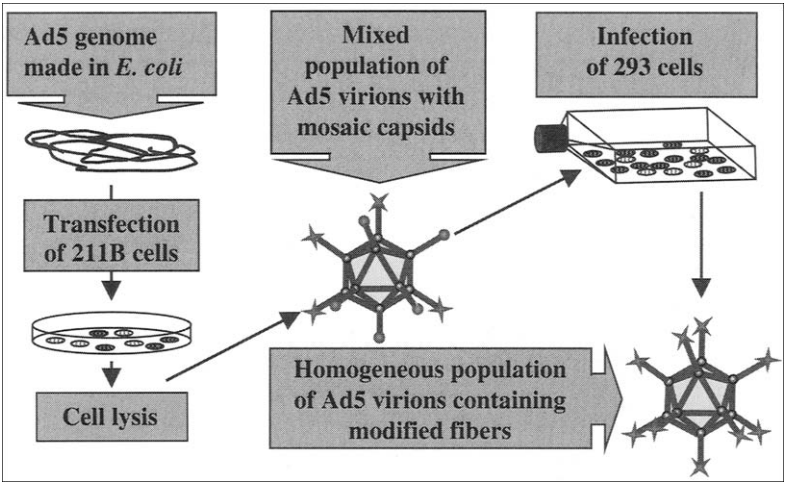

Figure 3 Strategy employing a packaging cell line which expresses the wild-type Ad fiber to enable the rescue and initial propagation of truly targeted Ad vectors. In this approach, 211B cells, a derivative of 293 cells which constitutively express the Ad5 fiber protein, are transfected with an Ad5 genome containing a modified fiber gene. This results in the production of mosaic Ad virions randomly incorporating both wild-type and modified fibers. The presence of the wild-type fiber in the resultant virions allows the efficient infection of any cell line expressing native Ad fiber receptor, including the packaging line. After a sufficient amount of mosaic virus has been generated, this vector may then be converted into the truly targeted configuration by a final amplification step on a cell line which does not express any Ad fiber, for example 293 cells.

example 293 [108], 911 [109], or PerC6 [110]. The feasibility and utility of this approach was first demonstrated by two groups of investigators [102, 111] who designed derivatives of the E1-complementing 293 cell line, designated 211 and 293-Fib, respectively, which constitutively express the wild-type Ad5 fiber protein to enable the propagation of fiber-deleted Ad vectors. Both cell lines allowed the efficient rescue and amplification of Ad virions containing fiber gene-deleted genomes. Therefore, this strategy is quite efficient and may be used for the generation of Ad targeting vectors derived from virtually any Ad serotype. The only drawback of this approach, although purely hypothetical at this point, is that homology between the modified fiber gene contained in the genome of the targeted Ad vector and the wild-type fiber gene incorporated into the genome of the packaging cell may result in recombination and restoration of the wild-type fiber gene in the viral genome, thereby negating the whole targeting effort.

In an alternative strategy, the targeted Ad vector is designed to have two different tropisms. One of the receptor specificities engineered into the virion provides virus binding to an artificial receptor, which is expressed by the

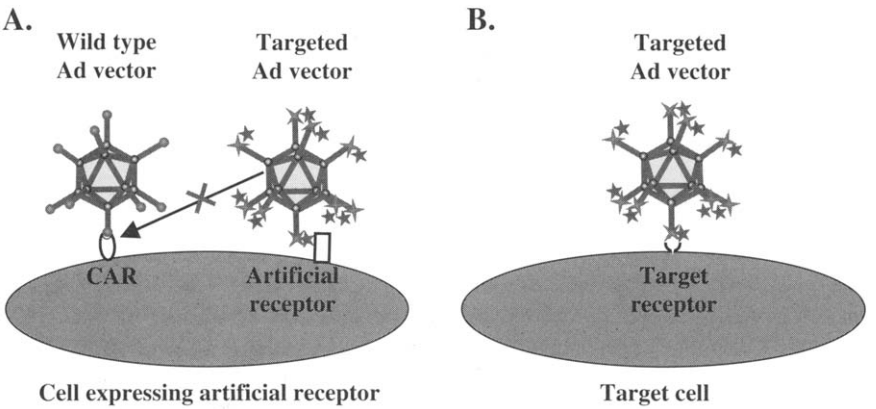

Figure 4 Strategy for utilization of an artificial receptor to provide a CAR-independent pathway of cellular entry to enable propagation of truly targeted Ad vector lacking native tropism. (A) In contrast to the wild-type vector, targeted Ad cannot utilize CAR for cell attachment and, therefore, achieves cell entry during rescue and amplification in cell culture via binding to artificial receptor. This attachment is mediated by a propagation ligand (five-pointed star) incorporated into the Ad capsid. (B) For gene delivery to target cells, the vector employs a targeting ligand (four-pointed star), which recognizes cognate receptor molecule distinct from the artificial receptor. Reproduced in modified form with permission from [141].

correspondingly modified packaging cell line (Fig. 4). This feature of the vector is only used during vector amplification in the laboratory. The other receptor specificity defines the vector tropism in the context of targeted gene delivery for gene therapy purposes. Therefore, the resultant vector contains two targeting ligands of which one (the "propagation ligand") is needed during vector rescue and amplification, whereas the second (the "targeting ligand") directs the vector to a receptor naturally expressed by the target tissue. Importantly, the receptor chosen to facilitate Ad propagation should be entirely artificial and should not have any natural analogs. Otherwise, the propagation ligand may compromise the truly targeted status of the vector by randomly binding *in vivo* to receptors analogous to the artificial receptor expressed by the packaging cells.

This approach has been realized by Douglas *et al.* [105], who generated a number of cell lines expressing an artificial receptor (AR), which binds proteins containing C-terminal 6His tags. This receptor was designed by genetically fusing an anti-6His single chain antibody, scFv, with the transmembrane domain of the platelet-derived growth factor receptor. The functional utility of this receptor was demonstrated by constructing an Ad vector containing 6His tag at the C-terminal of the fiber and using this vector for gene delivery to AR-expressing cells as well as to the parental cells lacking the AR. These studies showed that the 6His-modified Ad vector can efficiently infect AR-expressing cells in a CAR-independent fashion.

A similar system employing a hemagglutinin (HA) epitope incorporated into the Ad capsid and a complementary receptor embodying an anti-HA scFv was generated by Einfeld *et al.* and used for the rescue and amplification of Ad vectors lacking tropism to CAR [112].

The disadvantage of the approach using artificial receptor-expressing cell lines is that it requires additional modification of the Ad vector to carry a propagation ligand. However, this is just a minor limitation of an otherwise very efficient strategy, as the propagation tag may first be incorporated into the capsid of the prototype Ad vector, which can subsequently be used for the generation of various targeted derivatives.

B. Ad-Targeting Strategies Involving Genetic Manipulations of the Hexon and Penton Base Proteins

The genetic targeting of Ad virions via modifications of the hexon or penton base proteins has not been studied as extensively as fiber-based targeting. This is primarily due to the fact that these proteins do not play a significant role in the attachment of Ad to the primary cellular receptors. Therefore their utility for Ad targeting is not immediately apparent. However, a number of considerations imply that attempts to modify Ad vector tropism by alterations to these proteins may be successful. Although the accessibility of both proteins for binding to a putative target receptor may be an issue because of structural interference with the fiber protein, it may be significantly improved by shortening the shaft of the fiber by genetic means, thereby reducing the length of the entire fiber protein. The direct binding with cellular integrins of the penton base protein of human Ad9 [113], whose fibers are significantly shorter than those in Ad5 or Ad2 virions, suggests that shortening of the fiber may be a general strategy to facilitate interactions between the penton base and a cellular receptor. The fact that Ad2 binds to β2 integrins via its penton base protein [114] indicates that under certain circumstances the shortening of the fiber is not even required for such direct binding to occur. This is further supported by the finding by Einfeld *et al.* that a peptide ligand incorporated into the RGD-containing loop within the penton base of Ad5 vector binds quite efficiently to an artificial receptor expressed by the target cell [112]. It has also been previously reported that genetic modifications of recombinant penton base proteins result in alterations of their binding specificity [115].

Although the hexon protein does not play a documented role in the cell entry pathway used by Ad, its abundance in the Ad capsid makes the hexon a very attractive candidate as a ligand-presenting molecule. In addition, a comparison of the amino acid sequences of several known hexons reveals the presence of a number of hypervariable regions (HVRs) in these otherwise highly conserved proteins [116]. There are significant differences in the length and amino acid composition of these regions, strongly suggesting that they

may be used as sites for genetic alterations of the protein. A recent study by Rux and Burnett further rationalized the use of these HVRs as potential sites for the incorporation of targeting ligands by showing their localization on the surface of the Ad virion [117]. A practical demonstration of the feasibility of Ad targeting via hexon modification was performed by Vigne *et al.*, who replaced HVR5 in the Ad5 hexon protein with an RGD-containing peptide flanked with flexible linkers and demonstrated the ability of an Ad vector incorporating the modified hexon to achieve fiber-independent transduction of vascular smooth muscle cells [118].

In the aggregate, it appears that genetic modifications of the penton base and hexon proteins may eventually develop into an alternative strategy of Ad targeting, which may be used instead of, or in addition to, the fiber-based targeting approaches.

VI. Transductionally Targeted Ad Vectors for Clinical Gene Therapy Applications

As discussed above, the poor efficiency of Ad-mediated gene transfer in several human gene therapy trials has been correlated with a low level of expression of CAR by the target cells. Strategies to accomplish efficient cell-specific gene transfer by Ad vectors *in vivo* merely by exploiting physical methods to confine vector administration to isolated body compartments have proven inadequate. For example, locally administered Ad vectors carrying the herpes simplex virus thymidine kinase (HSV-TK) gene have been shown to disseminate, probably as a result of leakage into the bloodstream, resulting in a high level of liver-associated toxicity [119]. Substantial hepatic toxicity related to the absence of tumor cell-specific targeting has also been demonstrated in Ad-mediated transfer of the HSV-TK gene in an ascites model of human breast cancer [120]. Thus, targeted Ad vectors capable of efficient and cell-specific CAR-independent gene transfer are required for clinical gene therapy applications.

The benefits accrued in preclinical studies using tropism-modified Ad vectors provide a strong rationale for the immediate employment of these vectors in clinical trials. As discussed above, Ad vectors modified to contain the integrin-targeting RGD motif within the HI loop of the fiber knob have permitted levels of gene transfer to CAR-deficient primary cells to be enhanced more than two orders of magnitude over unmodified vectors. Based on these observations, the University of Alabama at Birmingham is currently employing this vector backbone in Phase I clinical trials for ovarian cancer and recurrent cancer of the oral cavity and oropharynx. These trials are the first to employ tropism-modified viral vectors in human patients. It is hypothesized that the tropism-modified vectors will allow augmented transfer of the herpes simplex virus thymidine kinase and cytosine deaminase genes, respectively,

at lower vector doses, thereby leading to increased efficacy and reduced toxicity. These two diseases represent ideal opportunities to perform the initial studies of tropism-modified Ad vectors in the clinical context. In this regard, ovarian cancer is generally confined to the peritoneal cavity, permitting vector administration by injection into that body compartment. Cancer of the oral cavity and oropharynx is a locoregional disease accessible to direct intratumoral injection of Ad vectors. Thus, the anatomical isolation of the disease targets facilitates vector administration.

However, it is apparent that additional requirements will be imposed upon targeted Ad vectors designed for clinical use in disease settings for which systemic vector administration is mandated. It has been reported that intravenous administration of untargeted Ad5 vectors delivers more than 90% of the input virus to the liver, thereby reducing the titer of virus particles available for transduction of the target disease cells [121–123]. Importantly, several studies have shown that the intravenous administration of Ad vectors leads to liver toxicity [124, 125]. Thus, one of the barriers to intravenous delivery of Ad vectors *in vivo* is the high degree of sequestration by the liver.

Zinn *et al.* have demonstrated that the liver uptake of intravenously administered technetium (Tc)-99m-labeled recombinant Ad5 knob in mice is significantly reduced upon coinjection of unlabeled Ad5 knob, but is not affected by Ad3 knob, which recognizes a different primary receptor [126]. This indicates that the liver possesses specific receptors for the Ad5 knob, an observation supported by the subsequent reports of high levels of CAR mRNA in the liver [7, 8]. These findings seemed to suggest that successful strategies to reduce liver sequestration and achieve cell-specific targeting following intravenous injection of Ad vectors will necessitate modifications to the knob domain to prevent recognition of CAR. In support of this, Printz *et al.* [42] and Reynolds *et al.* [51] have observed significantly reduced transgene expression in the livers of mice injected with Ad5 vectors which are retargeted by bispecific conjugates which prevent binding to CAR. However, the effect of the conjugates in reducing hepatocytes transgene expression may not be due to CAR blockade alone. It is possible that the size of the antibody-complexed vector contributes to the reduction in hepatocyte transduction by effectively enlarging the vector particle such that it less readily transverses the small fenestrations of the mouse liver sinusoidal epithelium.

The reasons for hypothesizing a mechanism other than (or in addition to) CAR blockade stem from the emerging results of studies using Ad vectors whose fibers have been genetically modified so that they no longer recognize CAR. Somewhat surprisingly, Leissner *et al.* observed that hepatic transgene expression mediated by CAR-ablated vectors following intravenous administration into the tail vein of mice was not significantly reduced compared to unmodified vectors [127]. While the CAR-ablated vectors used in this study were suboptimal in that they did not contain a targeting ligand with specificity

for an alternate receptor (and thus may eventually have accumulated in the liver "by default"), these results have called into question the notion that liver transduction by Ad vectors is purely due to the high level of CAR on hepatocytes. We have in fact found that complexing Ad with the Fab fragment of a neutralizing anti-knob mAb was not sufficient to reduce liver transgene expression, whereas when conjugated to a ligand, the same Fab fragment achieved the desired reduction. Whether this is due to particle size or the need for an effective alternate ligand is as yet unclear. Hence, modification of Ad vectors to avoid hepatocyte transduction does not appear to be as straightforward as first thought. Additional mutations such as ablating the RGD motif in the penton base to avoid interaction with cellular integrins have been proposed and are currently under evaluation, as are studies using vectors genetically modified to increase particle size by extending shaft length with a view to diminishing viral penetration of the hepatic fenestrations. The combination of "liver untargeting" approaches with a genetically incorporated, truly specific ligand are eagerly awaited. It is clear that the development of rational strategies to facilitate the clinical application of systemically administered Ad vectors will be dependent on a better understanding of the biological basis of hepatic vector localization.

The problem of liver sequestration of Ad vectors is not an issue which relates only to hepatocytes. In this regard, Reynolds *et al.* showed that while transductional targeting of Ad led to a reduction in hepatic transgene expression, the biodistribution of viral DNA 1.5 h after intravenous administration was not significantly altered [51]. The authors hypothesized that this could reflect nonspecific phagocytic uptake by Kupffer cells. It has previously been shown that 90% of Ad DNA is eliminated by the liver within 24 h in an early innate immune response and does not lead to transgene expression [128]. Inhibition of Kupffer cells reduces the elimination of Ad DNA from the liver and leads to a three- to fourfold increase in hepatic transgene expression [129, 130]. This suggests that further improvements in the use of targeted Ad vectors for systemic gene delivery might necessitate strategies to mitigate against nonspecific sequestration of the vector by the reticuloendothelial system (RES). In accordance with this, Tao *et al.* have generated data in mice suggesting that low doses of Ad ($1–3 \times 10^{10}$ viral particles) are efficiently taken up by the RES/Kupffer cells, whereas high doses (1×10^{11} viral particles) saturate these cells [131].

While the hepatic sinusoids and their fenestrations constitute a highly favorable anatomic environment for Ad entry and are thus a problem in the context of liver sequestration of the vector, anatomical factors in other tissues may actually impede Ad transduction. In support of this idea, Fechner *et al.* have reported that expression of CAR and αv integrins does not correlate with Ad vector-mediated gene delivery *in vivo* [132], suggesting that anatomical barriers, in particular the endothelium and the subendothelial matrix,

need to be overcome in order to achieve organ-specific gene delivery. Thus, efficient gene transfer by targeted Ad vectors might require the implementation of additional methods to permeabilize anatomical barriers. In this regard, Maillard *et al.* have shown that pretreatment of the rabbit iliac artery with elastase could enhance Ad-mediated gene transfer to arterial smooth muscle cells after balloon abrasion [133]. In a similar approach, Kuriyama *et al.* have reported that the administration of proteases to degrade the fibrous proteins of the extracellular matrix prior to intratumoral injection of Ad vectors leads to increased Ad infection [134]. An *in vitro* study by Nevo *et al.* demonstrated that the endothelial cell monolayer presents a physical barrier to Ad infection of myocytes, which could be partially overcome by increasing endothelial permeability with α-thrombin [135]. Protease digestion might also prove a rational strategy to increase the permeability of the basal lamina, which has been shown by Huard's group to present a physical barrier to the transduction of mature skeletal muscle by both untargeted and tropism-expanded Ad vectors [136, 137]. In a quite different approach, Cho *et al.* demonstrated that the efficiency of transduction of mature skeletal muscle could be enhanced by administering Ad vectors in a large solvent volume, thereby increasing the hydrostatic pressure and favoring vector egress out of the intravascular compartment [138]. In contrast to the anatomical situations described above, the "leaky" vasculature associated with solid tumors [139] is hypothetically favorable for the vascular egress of Ad tumor-targeted Ad vectors.

Thus, it is apparent that the success of systemic administration of targeted Ad vectors will depend on a greater understanding of the receptor-independent biological factors such as vascular pharmacodynamics and anatomical barriers limiting their utility, which should, in turn, facilitate the development of rational strategies whereby these obstacles might be overcome.

VII. Conclusion

Key to the realization of the full potential of gene therapy is the development of gene delivery vectors possessing the requisite level of efficiency and specificity. Despite a number of important biological features that make human adenoviruses a promising vector system for gene therapy, the CAR-dependence of Ad infection has been recognized as one of the major disadvantages of this vector system. In order to overcome this limitation, the concept of targeted Ad vectors capable of delivering therapeutic genes to specific subsets of cells affected by a disease was proposed in the early 1990s. A number of successful studies in recent years have shown the feasibility of the concept and provided the rationale for further improvements to the currently available vectors. Moreover, the degree of specificity and efficiency of Ad-mediated

gene transfer from currently available vectors could be refined by combining existing strategies. For example, Ad-mediated gene transfer to a heterogeneous population of cells, such as found in a tumor, could be increased by simultaneous targeting with two or more vectors targeted against distinct cell surface receptors, as described by Grill *et al.* [97]. Furthermore, a more exquisite level of specificity could be imposed on a transductionally targeted Ad vector by placing the expression of the transgene under the control of a tumor- or tissue-selective promoter, as described elsewhere in this volume. Additionally, while this chapter has focused on the transductional targeting of replication-defective Ad vectors, it is also recognized that strategies to redirect replicating adenoviruses to achieve CAR-independent infection will be necessary to realize the full potential of replicating adenoviruses in the clinical setting [140].

The studies described in this chapter have taken the field of Ad targeting from its conceptual stage to the state of a rapidly evolving branch of experimental virology. It is now becoming apparent that anatomical barriers will need to be overcome to enhance the utility of tropism-modified Ad vectors in the context of systemic delivery. Future work should lead to the derivation of truly targeted Ad vectors suitable for a variety of clinical applications requiring highly efficient, cell-specific delivery of therapeutic genes *in vivo*.

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CHAPTER 9

Targeted Adenoviral Vectors II: Transcriptional Targeting

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I. Introduction: Rationale of Transcriptional Targeting

Gene therapy is an innovative approach aimed at introducing genetic material into an organism for therapeutic intent. Still in its infancy, this novel concept has witnessed fundamental preclinical success with numerous ongoing clinical trials to confirm these findings. Critical to the success of gene therapy trials are issues relating to specific delivery of physiologically active biomolecules at therapeutically significant concentrations. Initially this was achieved by using direct intralesional injections of vectors to localize the delivery to the target tissue and universal promoters to maximize expression at that site. Over the past several years, we and several other investigators have investigated the potential of tumor-specific promoters to transcriptionally regulate gene expression in the laboratory and in clinical trials. The safety demonstrated by these trials using tumor/tissue-specific promoters has led to the recent approval of a trial administering a conditionally replicative adenovirus systemically for the treatment of metastatic prostate cancer.

In order for gene therapy to be widely applicable, there is an urgent need to develop a new generation of viral vectors capable of achieving these goals of targeted delivery and controlled gene expression at the target site. The aim of this chapter is to discuss various potential strategies that have been utilized to achieve tissue/tumor-specific expression using adenoviral vectors. A better understanding of tissue specific gene expression necessitates a basic review of the eukaryotic transcription process at the molecular level. Consequently, we

ADENOVIRAL VECTORS FOR GENE THERAPY

begin by examining the molecular architecture of DNA and its relationship with the transcriptional mechanism.

II. Regulation of Transcription in Eukaryotes

To fully understand the complexity underlining transcriptional targeting a brief review of the mammalian transcriptional process follows:

A. Molecular Organization of DNA

During interphase the genetic material in association with proteins is dispersed throughout the nucleus in the form of chromatin. At the onset of mitosis, chromatin condensation takes place and during prophase it undergoes further compression into recognizable chromosomes. The associated proteins are basic, positively charged (lysine- and arginine-containing) histones and less positively charged nonhistones including high-mobility group (HMG) proteins. Histones play a key role in chromatin structural organization and are subject to various posttranslational modifications like acetylation, phosphorylation, and ubiquitination. Histones constitute nearly half of all the chromatin protein by weight and can be divided into six types: H1, H2A, H2B, H3, H4, and H5. DNA is incorporated into a 100 Å nucleosomal fiber comprising of two molecules each of H2A, H2B, H3, and H4 which form the core histone octamer along with one linker histone H1 or H5. The nucleosome core particle consists of 146 base pairs of DNA while the core histone octamer interacts with about 200 base pairs of DNA. While the histones function by interacting with DNA to form nucleosome, the nonhistone proteins are responsible for performing diverse functions including tissue-specific transcription. The 100-Å nucleosomal fiber is arranged into a higher order structure termed a 300-Å supercoiled filament or solenoid. Evidence indicates that certain nonhistone proteins including topoisomerase II bind to chromatin every 60–100 kilobases and tether the supercoiled, 300-Å filament into structural loops. Further interaction with other nonhistone proteins leads to gathering of loops into rosettes, which in association with additional nonhistones undergo condensation forming a scaffold. This is known as the radical loop–scaffold model of compaction. Special, irregularly spaced repetitive base sequences associate with nonhistone proteins to define chromatin loops. These stretches of DNA are known as scaffold-associated regions (SARs). In order to be competent for transcription, the 300-Å chromatin filament must undergo decondensation.

B. The Central Dogma

According to the central dogma, the genetic information flows from (1) DNA to DNA during genomic replication and (2) DNA to protein during

gene expression. Gene expression can be simply defined as a phenomenon by which the genetic information stored in DNA is transferred to a protein. It involves two distinct processes. The process by which cells convert genetic information from DNA to RNA is called *transcription* and the decoding of RNA information to generate a specific sequence of amino acids is called *translation*. In addition, the flow of genetic information from RNA to DNA has been demonstrated in the case of retroviruses. Thus, the flow of genetic information from DNA to RNA is sometimes reversible. However, this flow is unidirectional from RNA to protein and irreversible since, normally, the genetic information within the messenger RNA (mRNA) intermediate is not altered. However, a few exceptions in the form of RNA editing seem to challenge the present concept. RNA editing has been shown to alter the information content of the gene transcripts by changing the structures of individual bases and by inserting or deleting uridine monophosphate residues.

Gene expression in eukaryotes is a spatially and temporally regulated process. Gene expression is regulated at multiple levels including transcription, posttranscriptional processing, nucleocytoplasmic transport, mRNA stability, translation, posttranslational modification, and intracellular trafficking of the protein.

C. Transcription (Fig. 1)

In eukaryotes, transcription occurs in the nucleus with the help of RNA polymerase to generate a single-stranded RNA molecule that is complementary in base sequence to the DNA template strand. There are three different types of RNA polymerases for the transcription of different types of genes. *RNA polymerase I* functions to transcribe ribosomal RNA (rRNA) genes to generate a large rRNA primary transcript which undergoes processing within the nucleolus to generate a 28S rRNA, a 5.8S rRNA, and an 18S rRNA. *RNA polymerase II* transcribes all of the protein coding genes into primary transcripts called pre-mRNAs that upon posttranscriptional processing generate mRNAs. While *RNA polymerase III* is known to transcribe transfer RNAs (tRNAs), 5S rRNA, and small nuclear RNAs (snRNAs).

There are two main types of *cis*-acting elements in all polII-transcribed genes: promoters and enhancers. The promoter, which is in close proximity to the protein coding region, consists of nucleotide sequences spanning approximately −40 and +50 nucleotides relative to a transcription initiation site. A typical core promoter consists of four distinct elements: (1) A unique sequence called Goldberg–Hogness or the TATA box which has a consensus sequence TATAAAA and is located about −25 to −30 nucleotides upstream of the transcription initiation site. The TATA box alone is sufficient for independently directing a low-level polII-mediated transcription. (2) An initiator element that is functionally analogous to the TATA box and directly

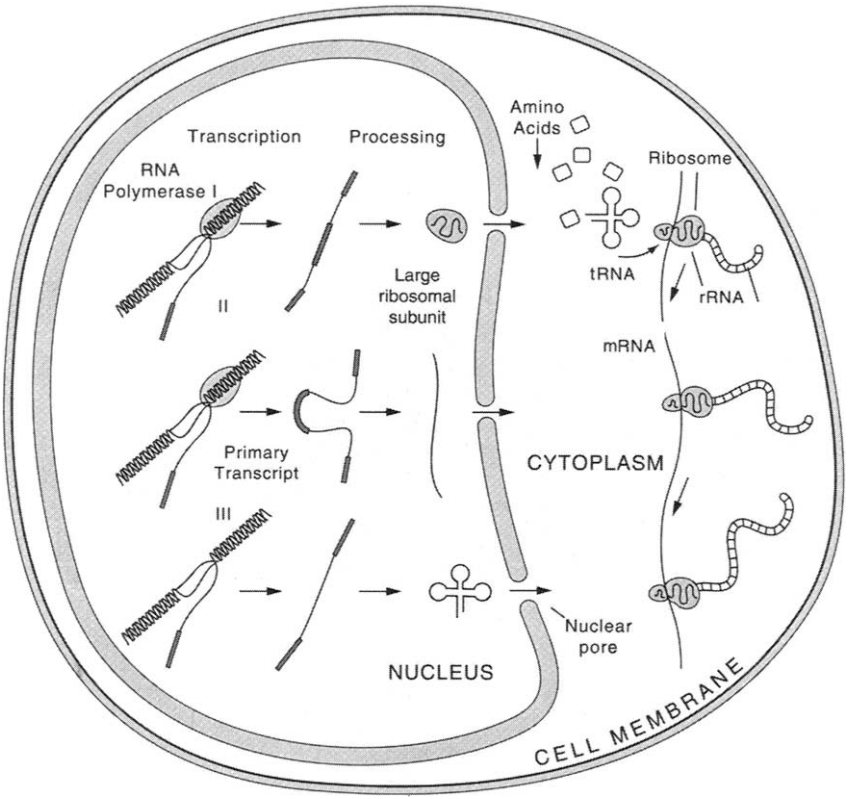

Figure 1 The transcriptional process of the RNA polymerase I, II, and III transcribing unwound DNA to rRNA, mRNA, and tRNA, respectively.

overlaps the transcription start site and has the loose consensus sequence PyPyA+1NT/APyPy. (3) The downstream core promoter element which is located approximately at position +30 downstream of the initiation site and acts in conjunction with the initiator element to direct transcription initiation. (4) The TFIIB recognition element, which has the consensus sequence $^{G}/_{C}{}^{G}/_{C}{}^{G}/_{A}$CGCC and is located from −32 to −38 upstream of the TATA box. Another cis-acting element called the CAAT box has a consensus sequence GGCCAATCT and is located near position −70 to −80 relative to transcription initiation site. Mutagenesis studies suggest a critical role of the CAAT box in modulating the promoter's ability to facilitate transcription.

In addition, polII promoters often contain two conserved sequences, the SP1 or GC box (GGGCGG) at about position −110 and the octamer box (ATTTGCAT); however, their positions are variable and they may occur either singly or in multiple copies. These consensus sequences are known to influence

the efficiency of the promoter in initiating transcription. In addition, the regulatory regions termed enhancers are located farther upstream, downstream, or within the gene. The activity of the enhancers is independent of the location, orientation, and gene type. Although they may not be involved directly in template binding, they are capable of modulating highly efficient transcription initiation.

The promoter regions are normally sequestered within the nucleosome and thus are rarely able to bind to basal transcription factors and RNA polymerase, thereby leading to transcriptional repression or silencing. In order for transcription initiation to occur, the sequestered promoter must be exposed so that it can readily bind basal factors and this is achieved by chromatin remodeling. The DNA in highly compacted chromatin is relatively resistant to nuclease DNaseI digestion. Thus, sensitivity of the DNA to DNaseI reveals the degree of chromatin condensation and is directly proportional to the transcriptional activity of a particular gene. Chromatin remodeling by acetylation and deacetylation of the histone proteins represents a major regulatory mechanism during gene activation and repression, respectively. The acetylation of histones by histone acetylase causes neutralization of the lysine basic charge, which in turn causes relaxation of contacts between the histones and the DNA. Thus, acetylated histones are preferentially found in active or potentially active genes where the chromatin is less tightly packed. Further, treatment of cultured cells with compounds like sodium butyrate, which enhances histone acetylation, leads to activation of previously silenced cellular genes.

The normal chromatin in the nucleosomal conformation can be converted into highly condensed heterochromatin which is transcriptionally inactive by the addition of methyl groups to a series of cytosine residues in the CpG dinucleotides found in tissue-specific genes. Thus, methylation and demethylation may play a crucial role in tissue-specific gene regulation. Locus control regions (LCRs) are specialized regulatory sequences located several kilobases upstream of the gene and capable of modulating transcription of gene clusters by influencing the chromatin structure. An assembled LCR–transcription factor complex is called an enhanceosome and if any of the components of this complex are missing, transcriptional activation of the gene cluster cannot occur. Insulators or boundary elements are regulatory sequences located in the vicinity of junctions between condensed and decondensed chromatin, which represent transcriptionally active and inactive loci, respectively. Insulators do not enhance transcription and are responsible for position-independent effects, but can prevent transcription when placed between an enhancer and a promoter.

D. Mechanism of Transcription

Eukaryotic transcription by RNA polII involves five stages: (a) formation of the preinitiation complex, (b) initiation, (c) promoter clearance, (d) elongation, and (e) termination. RNA polII cannot interact directly with the promoter to initiate transcription but requires recruitment to the promoter by interacting

with transcription factors. Transcription initiation is precisely controlled by the binding of a variety of *trans*-acting proteins termed transcription factors to the promoter and the enhancer. Transcription factors that assist the binding of RNA polymerase II to the promoter and initiate low levels of transcription are called basal factors, while other transcription factors are termed activators and repressors by binding to the enhancers. The transcription factors that bind to the TATA box are known as the TATA-binding protein (TBP) and are essential to the initiation of transcription from all pol II genes. A number of other basal factors that associate with TBP are called TBP-associated factors ($TAF_{II}s$) and help in the assembly and binding of the complex to the promoter, which in turn leads to transcription initiation.

The first event in the formation of preinitiation complex involves recognition of the TATA box by a multisubunit TFIID complex. A complex consisting of TBP and $TAF_{II}s$ called TFIID specifically binds to the TATA box to induce conformational changes that favor the binding of other transcription factors like TFIIA and TFIIB, both of which can interact directly with TFIID. TFIIB serves two critical roles in transcription initiation: (a) It acts as a bridge and recruits TFIIF/RNA polII to the promoter; and (b) it aids in the selection of the transcriptional start site. TFIIB interacts asymmetrically with TFIID–DNA and contacts the phosphodiester backbone of DNA both upstream and downstream of TATA box. The position of the amino terminus of TFIIB in the DNA–TFIID–TFIIB complex is located near the transcription start site, which might explain the role of TFIIB in stabilizing the melting of the promoter prior to RNA synthesis.

Following the assembly of the DNA–TFIID–TFIIA–TFIIB complex, RNApolII is recruited to the promoter by TFIIF. TFIIF has two subunits: (1) the larger subunit, RAP74, which has an ATP-dependent DNA helicase activity which may catalyze the local unwinding of the DNA to initiate transcription, and (2) the smaller subunit, RAP38, by which it binds tightly to the RNA polII. This is followed by binding of TFIIE to the DNA downstream from the transcriptional start point. Two other factors, TFIIH and TFIIJ are recruited to the initiation complex but their locations in the complex are unknown. The interaction of the preinitiation complex with the core promoter alone is not sufficient to initiate transcription. A sequence of events beginning with the phosphorylation of the carboxy-terminal domain of RNApolII by TFIIF followed by ATP hydrolysis set the stage for DNA melting, initiation of synthesis and promoter clearance. Most of the TFII factors dissociate before RNApolII leaves the promoter. The carboxy-terminal domain coordinates processing of RNA with transcription.

The general process of transcription initiation is similar to that catalyzed by bacterial RNA polymerase. Binding of the RNApolII generates a closed complex, which is converted at a later stage to an open complex in which the DNA strands have been separated. TFIIE and TFIIH are involved in an

extension of the unwound region of the DNA to allow the polymerase to begin transcription elongation. Several elongation factors including TFIIF, SII, SIII, ELL, and P-TEFb function to suppress or prevent premature pausing of RNApolII as it traverses the DNA template. Early in the elongation process when the growing RNA chains are about 30 nucleotides long, the 5′ ends of the pre-mRNAs are modified by the addition of 7-methyl guanosine caps. The 7-methyl guanosine cap contains an unusual 5′–5′ triphosphate linkage and two methyl groups are added posttranscriptionally. The 7-methyl guanosine caps are recognized by protein factors involved in the initiation of translation and also help by protecting the growing RNA chains from degradation by nucleases.

The 3′ ends of the RNA transcripts are produced by endonucleolytic cleavage of the primary transcripts rather than by the termination of transcription. The transcription termination occurs at multiple sites located 1000 to 2000 nucleotides downstream from the site that will eventually become the 3′ end of the mature transcript. The endonucleolytic cleavage occurs 11 to 30 nucleotides downstream from the conserved consensus sequence AAUAAA, which is located near the end of the transcription unit. Following endonucleolytic cleavage, the enzyme poly(A) polymerase adds about a 200-nucleotide-long poly(A) tail to the 3′ ends of the transcript in a process termed polyadenylation.

E. Structural Motifs (Fig. 2)

The transcription factors are modular in nature and contain characteristic structural motifs. The DNA binding domain as the name implies, binds to the DNA sequences present in the promoters and enhancers while the *trans*-activation domain is responsible for the activation of transcription via protein–protein interactions. The DNA binding domains have characteristic three-dimensional motifs, which result from associations between amino acids present within the polypeptide chains. Thus far, at least five types of DNA binding motifs have been extensively characterized. These include (1) helix–turn–helix (HTH) motif, (2) leucine zipper motif, (3) helix–loop–helix (HLH) motif, (4) zinc-finger motif, and (5) steroid hormone-binding motif.

1. Helix–turn–helix motif (HTH) was first discovered as the DNA-binding domain of phage repressor proteins. It is characterized by a geometric conformation that consists of two α-helical regions separated by a turn of several amino acids, which enable it to bind to DNA. Unlike other DNA binding motifs, HTH cannot function alone, but as part of a larger DNA-binding domain it fits well into the major groove of the DNA. The HTH motif has been identified in a 180-bp sequence called the homeobox, which specifies a 60-amino-acid homeodomain sequence in a large number of eukaryotic transcription factors involved in developmentally regulated genes.

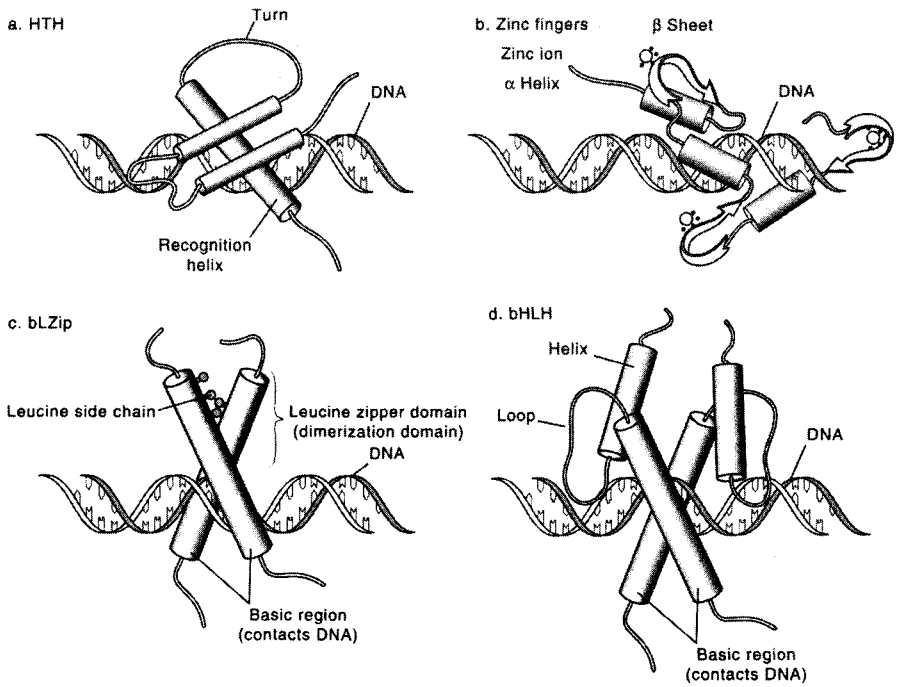

Figure 2 The structural motifs exhibited by transcriptional factors.

2. Leucine zipper motif consists of a stretch of amino acids with a leucine residue in every seventh position. The leucine-rich regions form an α-helix with a leucine residue protruding at every other turn and when two such molecules dimerize, the leucine residues zip together. The dimer contains two alpha-helical regions adjacent to the zipper, which bind to phosphate residues and specific bases in DNA, giving it a scissors-like appearance. The transcription factor AP1 has two major components: Jun and Fos polypeptides encoded by c-*jun* and c-*fos* genes, respectively. Both Jun and Fos contain leucine zippers in their dimerization domains. A Jun leucine zipper can interact with another Jun leucine zipper to form a homodimer or with a Fos leucine zipper to form a heterodimer; however, a Fos leucine zipper is unable to interact with another Fos leucine zipper to form a homodimer. Neither Jun nor Fos alone can bind to DNA and thus in their monomeric forms, they are unable to act as transcription factors. However, Jun–Jun homodimers or Jun–Fos heterodimers are both transcription factors and bind to DNA with the same target specificity but with different affinities. The ability to form homo- or heterodimers greatly increases the repertoire of potential transcription factors a cell can assemble from a limited number of gene products.

3. Helix–loop–helix motif consists of a stretch 40–50 amino acids containing two amphipathic α-helices separated by a 12- to 28-amino-acid long nonhelical loop. The proteins bearing HLH form both homodimers and heterodimers by means of interactions between the hydrophobic residues on the corresponding faces of the two helices. The HLH proteins that contain a stretch of highly basic amino acids adjacent to the HLH motif are termed bHLH proteins. These bHLH proteins are of two types: Class A consists of proteins that are ubiquitously expressed (e.g., mammalian E12/E47), while, Class B consists of proteins that are expressed in a tissue-specific manner (e.g., mammalian MyoD, myogenin, and Myf-5).

4. Zinc-finger motif was first recognized in the *Xenopus* RNA polIII transcription factor TFIIIA. There are several types of zinc-finger proteins, however, the classic zinc-finger consists of about 23 amino acids with a loop of 12 to 14 amino acids between the Cys and His residues and a 7–8-amino-acid linker between the loops. The consensus sequence of a typical zinc finger is Cys-X_{2-4}-Cys-X_3-Phe-X_3-Leu-X_2-His-X_3-His. The interspersed cysteine and histidine residues covalently bind a single zinc ion to form a tetrahedral structure thereby folding the amino acids into loops. The crystal structure analysis of DNA bound by zinc fingers suggests that the C-terminal part of each finger forms α-helices that bind DNA while the N-terminal part forms a β-sheet. Three α-helices fit into one turn of the major groove and each α-helix makes two sequence-specific contacts with DNA. A zinc finger transcription factor may contain anywhere from 2 to 13 zinc fingers. Thus zinc fingers bind to DNA and also control the specificity of dimerization. Therefore, a zinc finger motif offers a novel strategy to design an artificial sequence-specific DNA-binding protein aimed at regulating specific gene expression.

Recent studies indicate that it is possible to engineer zinc finger protein-based gene switches for precise and specific regulation of gene expression. Beerli *et al* [1] have utilized zinc-finger domains to design a polydactyl protein specifically recognizing 9- or 18-bp sequences in the 5′ untranslated region of the erbB-2/HER-2 promoter. They have evaluated the efficacy of gene regulation by converting the polydactyl finger into a transcriptional repressor by fusion with Kruppel-associated box (KRAB), ERF repressor domain (ERD), or mSIN3 interaction domain (SID) repressor domains. Transcriptional activators were generated by fusion with the HSV VP16 activation domain or with a tetrameric repeat of VP16's minimal activation domain, termed VP64. Their results indicate that both gene repression and activation can be achieved by targeting designed proteins to a single site within the transcribed region of a gene. Kang and Kim [2] examined the ability of designer zinc-finger transcription factors to regulate transcription *in vitro* using an ecdysone-inducible system. They constructed a 268/NRE chimeric peptide by linking the three-finger peptide from Zif268, which recognizes the site 5′-GCGTGGGCG-3′, and the three-finger NRE peptide (a variant of the Zif268 peptide), which binds specifically to part

of a nuclear hormone response element 5′-AAGGGTTCA-3′. By incorporating a 19-bp binding site for the 268/NRE near the transcriptional start site in the luciferase reporter vectors >99% repression of activated transcription was observed *in vivo*. Earlier studies have shown that 268/NRE peptide binds to the 19-bp recognition sequence about 6000-fold more tightly than the Zif268 peptide [3]. Imanishi *et al.* [4] utilized zinc fingers to create six-zinc-finger proteins Sp1ZF6$(Gly)_n$ by connecting two DNA-binding domains of transcription factor Sp1 with flexible polyglycine peptide linkers. These peptides were capable of inducing specific DNA bending by binding to two GC boxes and may provide an optimized approach to control gene expression by changing the DNA bending direction.

Corbi *et al.* [5] engineered a novel gene, "Jazz," that encodes for a three-zinc-finger peptide capable of binding the 9-bp DNA sequence 5′-GCTGCTGCG-3′ present in the promoter region of the human and murine utrophin genes. Chimeric transcription factors Gal4-Jazz and Sp1-Jazz were able to drive the expression of luciferase from the human utrophin promoter. Moore *et al.* [6] addressed the issue of zinc-finger DNA-binding specificity by altering the way in which zinc-finger arrays are constructed. Their results suggest that by linking three two-finger domains rather than two three-finger units, far greater target specificity and binding with picomolar affinity can be achieved through increased discrimination against mutated or closely related sequences. Taken together, the overall results suggest the potential utility of zinc-finger-based designer transcription factors in achieving regulation of gene-specific expression in diverse applications including gene therapy, functional genomics, and transgenic organisms.

F. Regulation of Adenoviral DNA Transcription Process

The adenovirus is a double-stranded DNA virus that has evolved to infect a host cell, transport its DNA into the nucleus of the host, replicate its DNA, use the host transcriptional apparatus to produce necessary structural proteins for replication, assemble itself, and destroy the host to release the newly formed infectious particles to perpetuate the process further. This process has been described in detail in Chapter 3.

III. Approaches of Transcriptional Regulation

A. Prior Rationale: Universal Promoters

Several universal promoters have been utilized to attempt to maximize gene expression. The LTR, CMV, and RSV promoters were isolated from Maloney retrovirus, cytomegalovirus, and Rous sarcoma virus, respectively.

These promoter elements were used because of the universal transcriptional activation over a broad host range. This universal transcription allowed for excellent but nondiscriminatory gene transcription and subsequent transgene expression. Because of the high levels of gene expression within several DNA constructs (i.e., viruses, cosmids, plasmids, etc.), these promoters are still used daily throughout the scientific community to test hypotheses which require uniform and high-level gene transcription. These were the promoters utilized in the first wave of gene therapy clinical trials, which focused on maximal gene expression and used local injection techniques to control the region of gene expression achieved. The LTR promoter was used to control herpes simplex virus thymidine kinase (HSV-TK) expression in a retroviral vector by placing retroviral producer cells into residual brain tumors to confer TK expression to the brain tumor, which could lead to conversion of a prodrug and subsequent tumor cell death. The CMV promoter was used in a replication-deficient adenovirus to deliver p53 gene expression after intralesional delivery to patients with both lung and head and neck tumors and is still under clinical investigation. The RSV promoter was employed to express HSV-TK after intralesional delivery in patients with several different tumor types.

B. Current Rationale of Tissue-Specific Promoters

A major challenge facing gene therapy is to generate vectors capable of achieving tissue- or tumor-specific expression. Initial gene therapy strategies utilized universal promoters that demonstrated gene transfer, but were associated with toxicity associated with nonspecific gene transduction (section III.A, above). Tissue-specific promoters offer a novel approach to developing transcriptionally targeted viral vectors with enhanced potential for human gene therapy applications as described below. Several important characteristics are required to develop a tissue/tumor-specific strategy for a particular disease. Fortunately, the recent explosion in our understanding of molecular events that are present in a variety of disease processes has simplified the identification of suitable promoters. Additionally the completion of the genome project and the utilization of microarray technology have enhanced the development of tissue- or tumor-specific promoters by allowing for the identification of novel but specific molecules associated with a particular disease (e.g., cancer). The advancements in molecular cloning techniques (e.g., PCR) has allowed the investigator to extract regulatory sequences from genomic DNA and evaluate each component through site directed mutagenesis analysis in plasmid expression vectors. Additionally, the development of luciferase and green fluorescent protein as well as other quantifiable transgenes has enabled the investigator to test the tissue- or tumor-specific nature of a particular promoter.

To illustrate the concept and utility of a tissue/tumor-specific promoter five such promoters have been selected from Table I. The basic rationale for

Table I
Gene Therapy Applications Of Tissue-Specific Promoters for Transcriptional Targeting

Promoter	Tissue-Specificity	Transgene	Vector	References
AFP	HCC	HSV-TK	Adenoviral	[27, 126, 134]
		CD, IL-2	Adenoviral	[28, 30]
		E1A	Adenoviral	[133]
Albumin	Liver	factor VIII	Adenoviral	[135, 136]
α-Actin	Muscle	GHRH	Nonviral	[137]
α-Lactalbumin	Breast cancer	CD	Adenoviral	[138]
β-Lactoglobulin	Breast cancer	HSV-TK	Adenoviral	[139]
β-Globin	Erythroid cells	β-globin	Retroviral	[140]
c-erbB2	Breast and pancreatic cancer	HSV-TK	Adenoviral	[141, 142]
CEA	Breast, pancreatic, lung, and colorectal carcinoma	HSV-TK, Cre H-ras mutant	Adenoviral	[15, 19, 21]
Egr-1	Radiation induced	TNF-α, LacZ	Adenoviral	[143, 144]
E-Selectin	Tumor endothelium	TNF-α	Retroviral	[145]
Flt-1	Vascular endothelial growth factor receptor type-1	Luciferase	Adenoviral	[146]
GFAP	Glial cells	FasL	Adenoviral	[147, 148]
		TH	Retroviral	
Grp78 (BIP)	Anoxic/acidic tumor tissue	HSV-TK	Adenoviral	[149, 150]
		HSV-TK	Retroviral	
hAAT	Hepatocytes	FactorIX	Nonviral	[151]
HGH and HGPH-α	Pituitary	HSV-TK,	Adenoviral	[152, 153]
HIF-1α/HRE	Hypoxia inducible	Erythropoietin	Nonviral	[154]
hK2	Prostate	EGFP, E1A, E1B	Adenoviral	[132, 155, 156]
HSP	Heat induced	p53, TNF-α	Nonviral	[157]
Hybrid ERE-HRE	Breast Cancer	Harakiri	Adenoviral	[158]
L-Plastin	Epithelial tumors	LacZ	Adenoviral	[159]
MBP	Oligodendrocytes	Caspase 8	Adenoviral	[160]
		GFP	AAV	[161]
MCK	Undifferentiated muscle	LacZ	Adenoviral	[162]
MMTV-LTR	Prostated cancer	antisense c-myc	Retroviral	[163]
MN/CA9	Renal cell carcinoma	E1A	Adenoviral	[164, 165]
MUC1 (DF3)	Breast cancer	E1A	Adenoviral	[166]
		HSV-TK	Retroviral	[167]
Nestin	Glioma, glioblastoma	Cre, LacZ	Adenoviral	[168]

Table I
(continued)

Promoter	Tissue-Specificity	Transgene	Vector	References
NSE	Neurons	FasL,	Adenoviral	[169]
		BDNF	AAV	[170]
Osteocalcin	Osteosarcoma	HSV-TK	Adenoviral	[77, 79, 171–173]
	Prostate	HSV-TK	Adenoviral	[174–176]
		E1A	Adenoviral	[177]
PEPCK	Hepatocytes	Insulin	Adenoviral	[178]
PSA	Prostate	Nitroreductase	Adenoviral	[46, 48, 49]
		HSV-TK, PNP	Adenoviral	
Preproenkephalin	CNS	LacZ	HSV	[179]
Probasin	Prostate	E1A	Adenoviral	[156, 180]
		Caspase 9		
Prolactin	Pituitary lactotrophic cells	HSV-TK	Adenoviral	[181]
SLPI	Ovarian, cervical carcinoma	HSV-TK	Nonviral	[182]
SM22α	Smooth muscle cells	LacZ	Adenoviral	[183]
Surfactant protein C	Respiratory epithelium	HSV-TK	Adenoviral	[184]
Tyrosinase	Melanocytes	Luc, PNP	Nonviral	[185, 186]
		GALV-FMG	Retroviral	
Tyrosine hydroxylase	Sympathetic nervous system	LacZ	HSV	[122]

selection, *in vitro* and *in vivo* laboratory investigation and the clinical testing associated with each, will be briefly reviewed below.

1. Carcinoembryonic Antigen (CEA) Promoter

a. Rationale Carcinoembryonic antigen is a 180-kDa cell surface glycoprotein overexpressed in 90% of gastrointestinal malignancies, including colon, gastric, rectal, and pancreatic tumors, 70% of lung cancers, and about 50% of breast cancers [7]. Thompson *et al.* [8] initially reported on the molecular cloning of the CEA gene from a human genomic library. Subsequently, Schrewe *et al.* [9] also isolated and characterized a cosmid clone containing the entire coding region of the CEA gene including its promoter. The CEA promoter region encompasses 400 bp upstream of the translational start site and is known to confer tissue-specific CEA expression. Hauck and Stanners [10] demonstrated that the CEA promoter region located between −403 and −124 bp upstream of the translational initiation site is capable of directing high levels of gene expression in CEA-expressing human colon cancer CRC cells. Chen *et al.* [11] showed the CEA promoter region to lie between −123 and −28 bp upstream from the transcriptional start site and have demonstrated

the presence of SP1 and upstream stimulatory factor binding sites. According to Richards *et al.* [12] the CEA promoter is located between −90 and +69 bp upstream from the transcriptional start site and the essential sequences of the CEA promoter reside between −90 and −17 bp upstream of the transcriptional start site of the CEA gene. Cao *et al.* [13] compared the CEA core promoter regions between −135 and +69 bp isolated from human colorectal carcinoma and normal adjacent mucosa and found that both the sequences were identical and without any mutations. Taking advantage of this fact, various studies have suggested the potential utility of the CEA promoter for restricted expression of heterologous genes (14, 10, 12).

b. In Vitro and in Vivo Experiments with CEA Promoter Takeuichi *et al.* [15] demonstrated that an adenoviral vector encoding a CEA promoter-driven N116Y dominant-negative H-Ras mutant was capable of suppressing liver metastasis by the human pancreatic cancer cell line PCI-43 in a nude mice model. Lan *et al.* [16, 17] demonstrated successful adenoviral-mediated transduction of *E. coli* cytosine deaminase (CD) *in vitro* as well as in an immunodeficient *in vivo* model of MKN45 gastric carcinoma. As compared to an adenoviral vector in which CD expression is driven by the constitutive CAG promoter, the expression of CD under the control of CEA promoter was confined to tumor xenografts. However, the reduction in tumor burden by AdCEA-CD/5-fluoro-cytosine (5FC), although significant, was not as great as that induced by AdCAG-CD/5FC. In fact, the CEA promoter was shown to be 200 times less active than the CAG promoter.

Similar results have been described in mice bearing xenografts that were transfected with CEA-CD constructs and subsequently treated with 5-FC (18, and 12). Tanaka and colleagues [19] have used the CEA promoter sequence located between −424 and −2 bp upstream of translational start site to generate an adenoviral vector expressing HSV-TK and examined its efficacy in killing CEA-producing cancer cells *in vitro* and *in vivo*. By employing intratumoral Ad-CEA-TK injection and gancyclovir (GCV) administration, the growth of the tumors was inhibited by 20% as compared to untreated tumors. Brand *et al.* [20] have used the CEA promoter (−296 to +102 bp with respect to transcriptional start site) to drive the expression of HSV-TK in an adenoviral vector. Their results indicate that the CEA promoter was active in several human and rat tumor-derived cell lines but not in rat primary hepatocytes and in mouse liver, while the CMV promoter was highly active in all cell types. Although the CEA promoter-driven TK expression was less, it was sufficient to kill 100% of cancer cells, indicating a significant bystander effect. Treatment of subcutaneous tumors in SCID mice with Ad-CEA-TK was able to significantly reduce tumor growth and the tail vein injection of a high dose of this virus caused no side-effects in the liver.

Kijima *et al.* [21] have utilized a novel Cre-lox-based strategy to achieve enhanced antitumor effect against CEA-producing human lung and colon

cancer cell lines. Their strategy involved generation of two recombinant adenoviral vectors: one expressing the Cre recombinase gene under the control of the CEA promoter while the second adenoviral vector is designed to express HSV-TK gene from the CAG promoter only after Cre excises the neomycin resistance gene (inserted between the CAG promoter and HSV-TK) in a loxP site-specific manner. (Cre recombinase derived from bacteriophage P1 mediates site-specific excisional deletion of a DNA sequence that is flanked by a pair of loxP sites composed of 34 nucleotides.) This novel approach requires simultaneous infection of a cell by the two adenoviral vectors. Using this approach, a CEA-producing human cancer cell line was rendered 8.4-fold more sensitive to GCV than infection by Ad-CEA-TK alone. Intratumoral injection of Ad-CEA-Cre along with Ad-lox-TK followed by GCV treatment almost completely eradicated CEA-producing tumors in an athymic subcutaneous tumor model, whereas intratumoral injection of Ad-CEA-TK with GCV treatment showed reduced tumor growth.

2. α-Fetoprotein (AFP) Promoter

a. Rationale The human AFP gene is developmentally regulated and is expressed at high levels in the fetal liver but its transcription declines rapidly after birth and is barely detectable in adult life [22, 23]. However, overexpression of the AFP gene is a characteristic feature of human hepatocellular carcinoma. The human AFP gene is about 20 kb long and contains 15 exons and 14 introns [24]. The cap site is located 44 nucleotides upstream of the translation initiation site and the TATA box is located 27 nucleotides upstream from the cap site and is flanked by sequences with dyad symmetry. Other sequences in the 5′ untranslated region include a CCAAC pentamer, a 14-bp enhancer-like sequence, a 9-bp sequence homologous to the glucocorticoid responsive element, a 90-bp direct repeat, and several alternating purine/pyrimidine sequences.

The AFP promoter is 200 bp upstream of the transcriptional start site. It is regulated by hepatocyte nuclear factor 1 (HNF1), nuclear factor 1 (NF1), and CCAAT/enhancer binding protein (C/EBP). The human AFP enhancer is located between −4.9 and −3.0 kb upstream of the transcriptional start site and consists of at least two functional domains designated A and B which have binding sites for at least four transcription factors, including HNF1, HNF3, HNF4, and C/EBP. The domain B is located at −3.7 to −3.3 kb upstream of the transcriptional start site and is solely responsible for typical enhancer effects, but maximum enhancer activity is observed together with domain A located at −5.1 to −3.7 kb. A hepatoma-specific nuclear factor termed AFP1 is known to bind to an AT-rich sequence, TGATTAATAATTACA, in the B domain of the human AFP enhancer. The AFP enhancer plays a critical role in enhancing AFP gene expression in the fetal liver as well as in hepatocellular carcinoma. The AFP silencer, which is a negative *cis*-acting element with a consensus sequence, 5′-CTTCATAACCTAATACTT- 3′, has been identified [25]. Two

transcriptional silencer elements have been identified: the proximal silencer which contains a single copy of the consensus sequence at −0.31 kb and the distal silencer at −1.75 kb which carries four copies of the consensus sequence. Of the two silencers, the distal silencer, exhibits a higher suppressive activity than the proximal silencer. The silencer activity is manifested only when the silencer is located downstream of the enhancer and upstream of the promoter. An inverse correlation exists between the silencer activity and the AFP expression levels in hepatocellular carcinoma cell lines, thereby suggesting the role of the silencer in downregulating the level of AFP expression.

b. In Vitro and in Vivo Experiments with the AFP Promoter Because of its tissue-specific nature, the AFP promoter has been used in adenoviral vectors for transcriptional targeting of suicide genes in AFP-producing hepatocellular carcinoma (HCC) cells *in vitro* as well as *in vivo*. Kaneko *et al.* [26] developed adenoviral vectors using either the 4.9-kb AFP promoter (Av1AFPTK1) or RSV promoter (Av1TK1) to express HSV-TK gene. *In vitro* and *in vivo* cell-specific killing was observed in AFP-producing HuH7 hepatocellular carcinoma cells transduced with Av1AFPTK1 and treated with GCV. In contrast to HuH7 tumors, AFP-nonproducing hepatocellular carcinoma SK-Hep-1 cells did not show complete regression when treated with Av1AFPTK1. Av1TK1 was able to cause complete regression in SK-Hep-1 tumors. Using a similar approach, Kanai *et al.* [27] developed adenoviral vectors by incorporating AFP enhancer domains A and B (−4.0 to −3.3 kb) and a 0.17-kb AFP promoter to drive the expression of HSV-TK. These vectors conferred cell-specific killing in AFP-producing HuH-7 and HepG2 cell lines but not in non-AFP-producing HLE and HLF cell lines. Kanai *et al.* [28] have also reported on the development of adenoviral vectors in which the expression of *E. coli* CD is driven by the AFP promoter. These vectors were capable of causing regression of HCC xenografts following treatment with 5FC. Arbuthnot *et al.* [29] analyzed *in vitro* and *in vivo* cell-specific expression of the nuclear β-galactosidase using adenoviral vectors containing transcriptional elements derived from either rat AFP or the human insulin-like growth factor II genes. Their results indicate hepatoma cell-specific expression using AFP promoter; however, primary hepatoma cells were poorly infected by these adenoviral vectors. Bui *et al.* [30] compared adenoviral vector-mediated expression of IL-2 under the transcriptional control of murine AFP promoter and CMV promoters for the treatment of established human hepatocellular xenografts in CB-17/SCID mice. Intratumoral injection of these adenoviral vectors resulted in growth retardation and regression in a majority of animals but with a wider therapeutic index and less systemic toxicity using the AFP vector. Using the AFP promoter and cre-lox based approach Sato *et al.* [31] were able to achieve strictly tissue-specific expression of LacZ in AFP-producing cells *in vitro* as well as *in vivo* in nude mice bearing AFP-producing tumor xenografts.

3. Prostate-Specific Antigen (PSA) Promoter

a. Rationale The gene for prostate-specific antigen, a member of the glandular kallikrein family, was independently characterized by Riegman *et al.* [32, 33] and Lundwall [34] from a human genomic library. The gene contains five exons and is located on the long arm of chromosome 19, in the region *q*13.3–*q*ter [33]. The gene is 7130 bp long and includes 633 bp of 5′ and 639 bp of 3′ flanking sequence. The promoter region contains a variant TATA box (TTTATA) at position −28 to −23, a GC box at −53 to −48, a CACCC box at −129 to −125. An imperfect palindromic sequence (AGAACAGCAAGTGCT) closely related to the reverse complement of the consensus sequence for steroid hormone receptor binding (TGTACANNNTGTC/TCT), is found at position −170 to −156. In addition, GGGAGGG and CAGCCTC repeats are located in the region −123 to −72. Expression of PSA is primarily detected in human prostate [35–37]. Further, PSA expression has been shown to be androgen-responsive [38]. This is achieved by several transcription factors that are involved in regulating prostate-specific antigen gene.

Two functionally active androgen receptor-binding sites or androgen response elements (AREs) have been identified at positions −170 (ARE-I) and −394 (ARE-II) [38–41]. Cleutjens *et al.* have identified a complex, androgen-regulated 440-bp enhancer (−4366 to −3874) which contains a high-affinity AR-binding site, ARE-III (5′-GGAGGAACATATTGTATCGAT-3′), at position −4200. In subsequent studies, a 6-kb PSA promoter fragment has been shown to confer prostate-specific and androgen-regulated expression of β-galactosidase in transgenic mice [42]. Pang *et al.* [43] identified an 822-bp PSA gene regulatory sequence, PSAR which when combined with the PSA promoter (PCPSA-P) exhibited an enhanced luciferase activity in LNCaP cells. Upon stimulation with 10 to 100 nM dihydrotestosterone, a more than 1000-fold increase in expression was observed as compared to androgen-negative controls. Their studies further suggest that this 822-bp sequence alone could serve as a promoter, thereby indicating that the complete PSA promoter contains two functional domains: a proximal promoter and a distal promoter, which can also function as an enhancer.

Yeung *et al.* [44] have identified two *cis*-acting elements within the 5.8-kb PSA promoter that are essential for the androgen-independent activity of the PSA promoter in prostate cancer cells. Their studies provide evidence that androgen-independent activation of the PSA promoter in the androgen-independent prostate cancer cell line C4-2 involves two distinct regions, a 440-bp AREc and a 150-bp pN/H, which are responsible for upregulation of the PSA promoter activity by employing two different pathways. AREc confers high basal PSA promoter activity in C4-2 cells, while pN/H is a strong AR-independent positive-regulatory element of the PSA promoter in both LNCaP and C4-2 cells. Further, a 17-bp RI fragment within the pN/H region was identified as the key *cis* element, which interacts with a 45-kDa

prostate cancer cell-specific transcription factor to mediate androgen- and AR-independent transcriptional activation of the PSA promoter. By juxtaposing AREc and pN/H, a chimeric PSA promoter has been created that exhibits 2- to 3-fold higher activity than wild-type PSA promoter in both LNCaP and C4-2 cells. Oettgen *et al.* [45] have identified a novel prostate epithelial-specific Ets transcription factor, PDEF, that is involved in PSA gene regulation and acts as a coregulator of AR. PDEF acts as an androgen-independent transcriptional activator of the PSA promoter. It also directly interacts with the DNA-binding domain of AR and enhances androgen-mediated activation of the PSA promoter. Thus, strong tissue-specificity of the PSA promoter makes it an ideal candidate for prostate cancer gene therapy. Latham [46] compared tissue-specific expression of luciferase reporter vectors by employing PSA, human glandular kallikrein (hKLK2), and CMV promoters in PSA-positive LNCaP and PSA-negative CoLo320, DG75, A2780, and Jurkat cells. Their studies revealed that minimal 628-bp PSA and hKLK2 promoters showed only low-level androgen-independent expression in both PSA-positive and PSA-negative cell lines. Tandem duplication of the PSA promoter slightly increased expression in LNCaP cells. Addition of the CMV enhancer upstream of the PSA or hKLK2 promoter led to substantially enhanced and nonspecific luciferase expression in all the cell lines. By placing a 1455-bp PSA enhancer sequence upstream of either the PSA or the hKLK2 promoter, a 20-fold increase in tissue-specific luciferase expression was observed. Tandem duplication of the PSA enhancer increased the expression 50-fold higher than either promoter while retaining tissue specificity. The expression from all the enhancer constructs was 100-fold above the basal levels upon induction with androgen dihydrotestosterone.

b. In Vitro and in Vivo Experiments with the PSA Promoter These enhancer sequences were incorporated in adenoviral vectors to express enhanced green fluorescent protein (EGFP) and nitroreductase. The results indicate low-level expression of EGFP by PSA enhancer promoter in LNCaP cells and no expression in non-PSA-producing EJ cells when compared with CMV promoter-driven EGFP. However, the PSA enhancer promoter was able to direct expression of comparable levels of nitroreductase in a tissue-specific manner in LNCaP cells alone. These transduced LNCaP cells upon treatment with CB1954 exhibited cytotoxicity. A replication-competent adenoviral vector CN706 in which the E1A gene is under the transcriptional control of the PSA enhancer/promoter has been shown to exhibit selective toxicity toward PSA-expressing prostate cancer cells [47]. Martinello-Wilks *et al.* [48] examined the efficacy of adenoviral vectors with a 630-bp PSA promoter-driven HSV-TK and *E. coli* purine nucleoside phosphorylase (PNP) genes for their ability to kill androgen-insensitive prostate cancer cell line PC-3 tumor xenografts in a nude mouse model. Both HSV-TK and *E. coli* PNP-expressing adenoviral vectors were able to achieve significant tumor regression *in vivo* following

GCV or 6MPDR treatment. Gotoh *et al.* [49] developed transcriptionally targeted recombinant adenoviral vectors by incorporating either 5837-bp long or 642-bp short PSA promoter elements to drive the expression of HSV-TK. The long PSA promoter was shown to have superior activity over the short promoter and was more active in C4-2 cells than in LNCaP cells. *In vitro* expression of TK conferred marked killing of C4-2 cells upon acyclovir treatment. Administration of this virus in an *in vivo* subcutaneous C4-2 tumor model, followed by acyclovir treatment, revealed significant inhibition of tumor burden. Lee *et al.* [50] demonstrated tissue-specific growth suppression of PSA-positive and -negative cell lines by transfecting PSA promoter enhancer-driven p53 tumor-suppressor genes. Recently, human prostate cancer- and tissue-specific genes P503, P540S, and P510S have been identified using a combination of cDNA library subtraction and high-throughput microarray screening by Xu *et al.* [51]. It would be interesting to characterize the promoter region of these genes and use it in developing transcriptionally targeted adenoviral vectors.

4. Osteocalcin (OC) Promoter (Fig. 3)

a. Rationale Osteocalcin (bone γ-carboxyglutamic acid (Gla)-containing protein (BGP)) is a 50-amino-acid, 5.8-kDa, major noncollagenous protein found in adult bone and has been shown to be transcriptionally regulated by 1,25-dihydroxyvitamin D_3 [52, 53]. The human, rat, and murine osteocalcin genes have been cloned and each consists of four exons and three introns [54–57]. Montecino [58] reported that the key promoter elements are located in two DNase I-hypersensitive sites. The proximal hypersensitive site (−170 to −70) includes sequence motifs that specifically interact with basal transcription factors such as Msx [59–61], HLH protein Id-1 [62], AP-1 [63], a bone-specific nuclear-matrix-associated protein, NMP-2 [64],

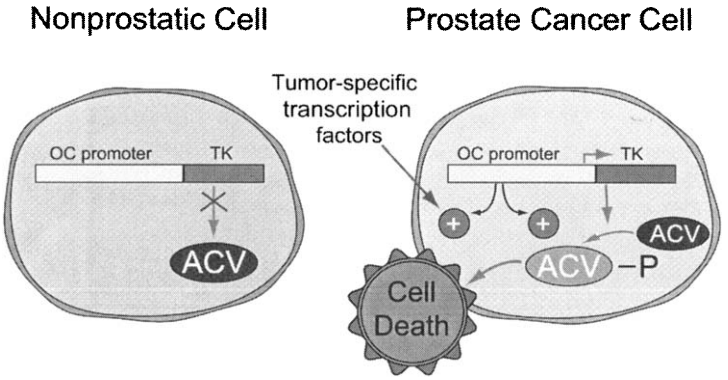

Figure 3 The specific ability of a tissue specific promoter such as the osteocalcin promoter to produce HSV-TK in a prostate cancer allowing cell death on prodrug (ACV) administration while sparing nonprostate cell by not allowing osteocalcin promoter activation.

and a member of the AML family of transcription factors [65, 66, 71]. The distal hypersensitive domain (−600 to −400) contains the vitamin D-responsive element (VDRE, −465 to −437), which interacts with the VDR-RXRα complex in a ligand-dependent manner [67–69]. Montecino *et al.* [70] have demonstrated that the promoter segment −343 to −108 is critical for inducing both proximal nuclease hypersensitivity and basal transcriptional activity and the DNase I hypersensitivity at −600 is not essential for vitamin D-dependent transcriptional upregulation. Two additional NMP-2 sites (site A, −604 to −599; site B, −440 to −435) have been identified in the sequences flanking the distal DNase I-hypersensitive domain that might support specific interactions between the nuclear matrix and the OC gene promoter [64, 71]. Analysis of the 5′ flanking sequence of rat osteocalcin gene reveals a modular organization of the promoter consisting of the TATAAAA sequence between −31 and −25 and the CCAAT sequence between −92 and −88 [72]. Lian *et al.* [55] identified a 24-nucleotide regulatory sequence, 5′-ATGACCCCCAATTAGTCCTGGCAG-3′, in the proximal promoter region with a CAAT motif as a central element, and have designated this sequence as an osteocalcin (OC) box since only two nucleotide substitutions are found in the rat and human osteocalcin genes in this region. Hoffman *et al.* [59] reported that the OC box is located at nucleotide positions between −99 and −76 and TATA box containing a consensus glucocorticoid-responsive element (GRE) between −44 and −31. The stimulation of osteocalcin gene expression by 1,25-dihydroxyvitamin D_3 is associated with sequence-specific binding of nuclear factors to a 26-bp sequence, 5′-CTGGGTGAATGAGGACATTACTGACC-3′, located between −462 and −437. This sequence contains a region of hyphenated dyad symmetry and shares homology with consensus steroid-responsive elements.The promoter region has been shown to contain two sites of an E-box motif (a consensus binding site for HLH proteins) termed OCE1 (CACATG at −102) and OCE2 (CAGCTG at −149) [62]. Mutagenesis studies have indicated that osteoblastic-specific gene transcription is regulated via the interaction between certain E-box binding transcription factors in osteoblasts and the OCE1 sequence in the promoter region of the osteocalcin gene. Banerjee *et al.* [63] demonstrated that an AML-1 binding sequence within the proximal promoter (nt −138 to −130) contributes to 75% of the level of osteocalcin gene expression. The promoter region is not GC-rich and does not contain a consensus sequence for the SP1 binding site [73]. Theofan *et al.* [74] performed a detailed analysis of the BGP promoter region. Three regulatory elements that share partial homology with the consensus sequence for the GRE have been identified at nucleotide positions −356, −178, and −68, respectively. In addition, two sequences related to the consensus sequence for the metal ion-responsive element (MRE) have been identified at positions −190 and −143. An octanucleotide sequence, TGCAGTCA, is located directly adjacent 3′ to the second MRE. Two other sequences that share homology

with the cAMP-responsive element are found at −437 (TGAGGACA) and −392 (TCACGGCA). The BGP promoter region also contains several pairs of inverted repeat sequences that form regions of dyad symmetry. Three particularly long regions of imperfect dyad symmetry are located between −523 and −504, −234 and −214, and −51 and −28. An octanucleotide palindromic sequence from −134 to −127 partially overlaps both a putative MRE and a cAMP-responsive element. A short sequence, GCAG, or its complement, CTGC, is repeated 17 times. A region of alternating purines and pyrimidines at location −90 to −81 from the CAT box has the potential to form a Z-DNA structure which may be important in gene regulation. A 7-bp osteocalcin silencer element, 5′-TGGCCCT-3′, has been located between +29 and +35 position in the first exon of the human osteocalcin gene, while two silencer elements, 5′-CCTCCT-3′ (nt +106 to +111 and +135 to +140) and 5′-TTTCTTT-3′ (nt +118 to +124), have been located in the first intron of the rat osteocalcin gene [75, 76].

b. In Vitro and in Vivo Experiments with the OC Promoter Ko *et al.* [77] developed an osteocalcin-promoter-driven TK-expressing recombinant adenoviral vector to achieve tissue-specific killing of osteosarcoma cells in experimental animal model. Administration of this vector followed by acyclovir treatment led to a significant growth inhibition of osteosarcoma in an experimental animal model. Cheon *et al.* [78] used a chemogene therapy approach by combining OC-promoter-driven TK expression and acyclovir with a methotrexate treatment regimen in nude mice bearing either subcutaneous human osteosarcoma (MG-63) or rat osteosarcoma (ROS) xenografts. Their results indicate that osteosarcoma tumor growth was more efficiently inhibited due to synergistic effects of combined methotrexate and acyclovir treatment. Shirakawa *et al.* [79] further demonstrated the potential utility of an adenoviral osteocalcin promoter-mediated suicide gene therapy for osteosarcoma pulmonary metastasis in nude mice. Hou *et al.* [80] demonstrated osteoblast-specific gene expression in adherent bone marrow cells using a 1.7-kb rat OC-CAT gene. Recipient mice were shown to be positive for osteoblast-specific expression following bone marrow transplantation.

Using a replication-defective adenovirus, Ad-OC-TK, we have completed a phase I clinical trial that demonstrated the expected safety profile and gene transfer that we expected. Eleven men with recurrent or metastatic prostate cancer were enrolled in a phase I intralesional dose-escalating trial, combining two Ad-OC-TK injections with 3 weeks of valacyclovir administration. In summary, this was well tolerated at all doses reaching a maximum of 5×10^{10} pfu (or 1×10^{12} vp) in patients in the high-dose group. Viral distribution studies revealed that after intralesional administration the patients demonstrated a measurable viremia for 2–3 days. Despite the presence of viral particles at these time points, no patient demonstrated hepatotoxity with valacyclovir administration. This is in direct contrast to intralesional delivery of Ad-RSV-TK

to the prostatic recurrence, in which patients experience hepatotoxicity upon prodrug administration. Finally, comparison of biopsy specimens prior to the first (day 0) and second (day 7) injection and at the end of the study (day 30) revealed successful gene transfer at day 7 by immunohistochemical staining for HSV-TK and some evidence of tumor destruction by day 30. These expected and encouraging results have led us to propose a phase I trial to test the transcriptional ability of the osteocalcin promoter to regulate adenoviral replication in a similar format.

5. MN/CA9 Promoter

a. Rationale The human MN/CA9 gene has been isolated, sequenced, and characterized by Opavsky [81]. This gene is a member of the carbonic anhydrase (CA) family, which codes for a diverse group of catalysts of the reversible conversion of carbon dioxide to carbonic acid. MN/CA9 expression has been detected in several types of carcinomas including renal, ovarian, and cervical, as well as in normal gastric mucosa [82–85]. The complete genomic sequence of the MN/CA9 gene including the 5′-flanking region encompasses 10.9 kb with a coding sequence comprising of 11 exons. The MN/CA9 protein contains 459 amino acids with a molecular weight ranging from 54 to 58 kDa. MN displays CA activity and binds zinc [86]. The nucleotide sequence close to the 5′ end shows 91.4% sequence homology to the U3 region of the long terminal repeats (LTRs) of the human HERV-K endogenous retroviruses [87]. This LTR-like sequence is 222 bp long with an A-rich tail at its 3′ end. Analysis of the MN/CA9 promoter region between −507 and +1 upstream of the transcription initiation site indicates that despite the presence of 60% GC residues, the additional features of TATA-less promoters are absent, but the presence of consensus sequences for AP1, AP2, and p53 transcription factor binding sites has been demonstrated [88–90]. Functional characterization of the 3.5-kb MN 5′ upstream region by deletion analysis led to the identification of −173 to +31 fragment as the MN promoter. The promoter region lacks the CpG-rich islands that are typical for TATA-less promoters but contains two nonoverlapping consensus initiator sequences required for promoter activity.

b. In Vitro and in Vivo Experiences with MN Initial *in vitro* studies with this promoter driving luciferase expression demonstrated tumor specificity for both renal cell carcinoma and cervical carcinoma. Based on the expression assays, we have constructed an oncolytic adenovirus with the MN promoter which has demonstrated 40- to 100-fold increased killing in human renal cell carcinomas compared to control cell lines not expressing this promoter activity. We are currently evaluating this oncolytic vector in animal models of human renal cell carcinoma.

C. Inducible Transcription

The ability to precisely regulate spatial and temporal expression of a particular gene is likely to have a significant impact in the field of human gene therapy. In order to be effective, such an approach must necessarily fulfill several criteria, including: (1) biological safety, (2) ease of administration, (3) low basal expression, (4) high and gene-specific inducibility, (5) reversibility, and (6) (preferably) of human origin to minimize immunogenicity. A wide variety of inducible systems for regulating gene expression have been developed. These include the use of metal response promoter [91], heat-shock promoter [92], the glucocorticoid-inducible promoter [93], IPTG-inducible lac repressor/operator system [94, 95], tetracycline-inducible system [96], RU486-inducible system [97], ecdysone-inducible system [100], FK506/rapamycin-inducible system [101], hypoxia-inducible factor 1 system [102], radiation-inducible system [103], and the tamoxifen-inducible system [104]. It is beyond the scope of this chapter to provide in-depth information on all of the above-mentioned inducible systems. Consequently, we will focus on those inducible systems that might have the greatest potential for human gene therapy applications.

1. Tetracycline-Inducible System

The tet-inducible system originally developed by Bujard and coworkers [94, 105] is widely used to regulate gene expression. The tet-inducible system is based on the tetracycline resistance operon of *E. coli*. The system utilizes the specificity of the tet repressor (tetR) for the tet operator sequence (tetO), the sensitivity of tetR to tetracycline, and the potent transactivator function of herpes simplex virus protein VP16. The system is based upon the concept of negatively regulating the transcription of the bacterial resistance gene by tetR protein binding to tetO DNA sequences. Addition of tetracycline or doxycycline causes derepression by binding to the tetR protein, thereby allowing transcription to proceed. This has been achieved by employing a tet transactivator (tTA) which is a chimeric tetracycline-repressed transactivator generated by fusing the carboxy terminal of tetR protein to the carboxy terminal 127 amino acids of VP16. The tTA, when bound to tetracycline, is prevented from binding to seven copies of tetO sequences, which are juxtaposed upstream of a minimal human cytomegalovirus promoter, thereby selectively turning off the transcription of the gene in question. Removal of tetracycline results in binding of tTA to the tetO sequences in the tet-inducible promoter, following which the VP16 moiety of tTA transactivates the target gene by promoting assembly of a transcriptional initiation complex, thereby selectively turning on the gene expression. A recent modification of this system allows for selective induction of gene expression in the presence of tetracycline. In this strategy, a mutated tetR, called reverse tTA (rtTA), has been generated by incorporating 4 amino acid changes into tTA, thereby facilitating rtTA

binding to the tetO sequence in the presence of tetracycline. Another variation involves fusion of tTA with the KRAB repressor domain of the human zinc-finger protein Kox1. Upon binding to tetO sequences, this protein is capable of blocking transcription as far as 3 kb downstream [106]. A further variation has revealed that by placing two minimal promoters in opposite orientations on either side of the tetO sequences, it is possible to simultaneously regulate the expression of two genes from a single plasmid [107]. Massie *et al.* [108] used the tet-inducible system to generate a recombinant adenoviral vector encoding a deletion in the R1 subunit of the herpes simplex virus type 2 ribonucleotide reductase. Topical and tetracycline-inducible gene expression in transgenic mice carrying a gene under the tet-inducible promoter has been achieved by adenovirus mediated gene transfer and expression of tTA [109]. Rubinchik *et al.* [110] developed a tet-inducible, double recombinant adenoviral vector expressing a fusion of murine FasL and green fluorescent protein. In this virus, the tet-responsive element and the transactivator element are built into opposite ends of the same vector to avoid enhancer interference. The *in vitro* expression of FasL-GFP in various cell lines could be conveniently regulated by tetracycline or doxycycline in a dose-dependent manner.

2. FK506/Rapamycin-Inducible System

The latest in the armamentarium of inducible gene expression systems are the chemical dimerizers that rely upon drug-dependent recruitment of a transactivation domain to a basal promoter to drive the expression of the therapeutic gene. The strategy is based upon generating a genetic fusion composed of a heterologous DNA-binding domain and an activation domain with the drug binding domain, thereby enabling a bivalent drug to crosslink the two proteins and reconstitute an active transcription factor. This is achieved by using small cell-permeable immunosuppressive molecules, FK506, rapamycin, and cyclosporine, to bind members of the immunophilin family. The FK506 molecule binds tightly to the cellular protein, FKBP12, while FK1012, a synthetic dimer of FK506, causes dimerization of several chimeric proteins containing FKBP12 [111]. Another synthetic compound, FKCsA, created by fusion between FK506 and cyclosporine A, binds with high affinity to FKBP12 and cyclophilin and has been used for inducible transcription of exogenous genes [112]. However, the most promising results have been obtained using the heterodimerizer rapamycin, which binds simultaneously to the human proteins FKBP and FRAP [113, 114]. In this system, transcriptional activation is achieved through rapamycin induced reconstitution of a transcription factor complex formed by coupling of (a) a unique DNA-binding domain, ZFHD, genetically fused to FKBP and (b) the activation domain of the p65 subunit of nuclear factor kappa B (NFκB), fused with the rapamycin-binding domain of FRAP. This novel approach has been successfully utilized for stable *in vivo* delivery of secreted alkaline phosphate, murine erythropoietin

and human growth hormone using eukaryotic expression vectors, adenoviral, retroviral, and adeno-associated viral vectors [115–117]. One of the limitations of this approach is the growth inhibitory and immunosuppressive activity of rapamycin which is due to the inhibition of endogenous FRAP activity [114]. This limitation can be overcome by nonimmunosuppressive analogs (rapalogs) of rapamycin by incorporating mutations in the FRAP domain that accommodate modified drugs [118, 119]. Considerable progress has also been made in designing novel synthetic dimerizers of the ligand for human FKBP12 and mutated FKBP [119–121]. These studies are suggestive of the potential utility of this novel approach for human gene therapy applications.

3. RU 486

Wang *et al.* [97] developed a novel regulated transcriptional activator consisting of a truncated ligand-binding domain of the human progesterone receptor, the DNA-binding domain of yeast transcriptional activator GAL4, and a C-terminal fragment of the herpes simplex virus VP16 transcriptional regulator protein. This novel transcriptional activator binds with high affinity to the synthetic progesterone antagonist RU 486 but binds very poorly to progesterone. In conjunction with the target gene containing four copies of the consensus GAL4 binding site, the gene expression was activated only in the presence of RU 486 [97, 98]. Wang *et al.* [99] also developed an inducible repressor system by substituting the KRAB transcriptional repressor domain for the VP16 transactivation domain. In addition to RU 486, this system can be activated by other synthetic progesterone antagonists at low concentration. The efficacy of this system has been demonstrated using an *ex vivo* transplantation approach in which cells containing stably integrated chimeric regulator GLVP and a target gene (tyrosine hydroxylase) were grafted in rats. One of the caveats of this system is the low but distinctive basal activity of the GAL4-responsive promoter in the absence of RU 486. Consequently, this system has been refined by designing a synthetic transcription factor which contains a 35-amino-acid truncation of the progesterone receptor rather than the 42-amino-acid truncation [123]. This system exhibits two- to threefold lower basal activities as compared to the earlier version.

IV. Enhanced Control of Transgene Expression

A. Safety Improvements

Prior to initiating our clinical trial with Ad-OC-TK, we performed a distribution study that measured TK activity in a variety of organs harvested 3 days after intravenous (iv) injection of Ad-CMV-TK (2×10^9 pfu) or

Ad-OC-TK (2×10^9 pfu) with three mice per group. TK enzymatic activity was detected only in the AdCMV-TK group (liver and spleen only), but not the Ad-OC-TK group. Next we performed a comparative study in which 10 C57/BL mice received one iv injection of 2×10^9 pfu of Ad-OC-TK or Ad-CMV-TK and intraperitoneal (ip) GCV. Significant mortality with severe hepatic histopathology was observed in the Ad-CMV-TK/GCV group (90% mortality), while the Ad-OC-TK/GCV administration did not affect survival of the treated animals (100% survival). These data and the above tissue distribution studies support the hypothesis that, in syngeneic hosts, the OC promoter is tissue-specific for tumors, since Ad-OC-TK inhibits tumor growth as effectively as do RSV-TK and CMV-TK, but without the generalized toxicity observed with these universal promoters. These findings paralleled the formal GLP toxicology study in mice and our toxicology profile in our clinical trial. Others have demonstrated the lethal effects of both universal promoter HSV-TK viruses in mice and rats and hepatotoxicity in humans after intraprostatic injections.

B. Potency Concerns

The initial concern with a tumor-specific promoter is that the magnitude of the transgene expression would decrease because of the specificity of the promoter. To address this issue, we compared the *in vivo* growth inhibition associated with intralesional administration of Ad-OC-TK with that of Ad-CMV-TK using a rat osteosarcoma (ROS 17/2.8) subcutaneous model. Ten athymic nude mice were injected with 1×10^6 ROS cells per site in four subcutaneous locations. After establishment of tumor growth at greater than 5 mm diameter, Ad-CMV-TK or Ad-OC-TK were injected intralesionally into five animals (or 20 tumors) each. After viral injection, the animals received ip GCV (three mice, 12 tumors) or phosphate-buffered saline (PBS; two mice, 8 tumors) for a 2-week period. The animals received one additional adenoviral injection 7 days after the first. The tumors were measured weekly and the animals were sacrificed after the second week of GCV or PBS administration. Both Ad-OC-TK and Ad-CMV-TK forms of therapy demonstrated a greater growth-inhibitory effect than was observed with PBS administration. The growth inhibition was superior with the Ad-OC-TK adenovirus. Therefore, the OC promoter has high intrinsic activity rivaling that of the strong universal CMV promoter, at least in ROS cells.

V. Future Directions

A. Enhancement of Weak But Specific Promoters

A wide variety of highly tissue-specific promoters have been evaluated for achieving transcriptional targeting, however, their applicability has been

hampered due to weak transcriptional activity. Enhancement of weak tissue specific promoters can be achieved by employing several different strategies. One of the simplest approach involves (a) deletion of those sequences from the promoter that do not contribute to tissue specificity or transcriptional activity and (b) incorporation of multiple copies of the enhancer and positive regulatory elements. This approach has been successfully used in the case of PSA promoter [43], tyrosinase promoter [124, 125], and CEA promoter [12].

Another approach involves generation of activating point mutations within the promoter region as has been in the case of AFP promoter [126] and the MDR 1 promoter [127]. Yet another strategy involves selective combination of multiple positive regulatory and tissue-specific elements to achieve enhancement of weak promoters. This strategy has shown promising results in augmenting melanoma-specific gene expression when the tyrosinase promoter, either alone or in combination with single or dual, tandem melanocyte-specific enhancer, was used to drive the expression of luciferase and the *E. coli* purine nucleoside phosphorylase gene.

Transient expression studies indicated 5- to 500-fold increase in luciferase activity following incorporation of either single or tandem enhancer elements. In another example, when 5–20 muscle-specific transcriptional elements were randomly assembled and linked to the minimal chicken α-actin promoter, sixfold higher activity was observed as compared to the CMV promoter [128]. In case of adenoviral vectors it might be possible to selectively increase specific expression from exogenous promoters by coexpression of modified VAI genes. Using this approach, Eloit *et al.* [129] were able to achieve 12.5- to 502-fold increased reporter gene expression. The fact that activity of certain E2F-responsive promoters in tumor cells exceeds that achieved in mitotically active normal cells has been exploited for tumor-selective transgene expression using an adenoviral vector in a malignant glioma model [130]. A novel approach involves development of dual-specificity promoters that are both cell-type-specific and cell-cycle-regulated. In this approach the transgene is under the transcriptional control of an artificial heterodimeric transcription factor whose DNA binding domain is expressed from a tissue-specific promoter, whereas the transactivating subunit is transcribed from a cell-cycle-regulated promoter. The feasibility of this approach has been successfully tested in a transient transfection system [131].

Transcriptional targeting of viral replication for selective killing of tumor cells can be achieved by deletion of adenoviral E1B/55-kDa protein which is essential for viral replication but is dispensable in p53-deficient tumor cells. An alternate approach involves generation of a replication-competent adenoviral vector in which E1A or E1A and E1B genes are under the transcriptional control of tumor-specific promoters like PSA, kallikrein-2, or AFP [47, 132, 133].

B. Improving Specificity with Multiple Promoter Segments

Several investigators have placed combinations of promoter sequences in tandem to derive more specific transgene expression. The authors of the following chapter in this book have both laboratory and clinical experience with this approach and this topic is well covered in their chapter.

C. Tumor-Specific Oncolysis

Several different approaches have been designed to achieved cancer-cell-specific adenoviral replication and subsequent tumor lysis. Based on our previous work in the laboratory and the clinic, we have designed an adenoviral vector that would only replicate in cells, which could activate the osteocalcin promoter. We have recently received approval for OBA #426 using the osteocalcin promoter to transcriptionally regulate adenoviral replication for the treatment of men with metastatic and recurrent prostate cancer. This approach is thoroughly reviewed elsewhere in this volume (see Chapter 10).

D. Combined Targeting Approaches

The preceding chapter describes elegant methods to achieve transductional targeting. These approaches will allow for the concentration of adenovirus at metastatic tumor deposits after a systemic administration. In collaboration with these investigators we have begun to combine both transductional and transcriptional targeting to allow for both tumor-specific concentration and tumor-specific oncolysis. This approach combines many of the individual strides achieved in adenoviral gene therapy in the past decade and holds great promise for the future of adenoviral cancer gene therapy.

VI. Summary

In summary, we believe that the success of gene therapy and its general applicability to medicine will be partially linked to the development of effective transcriptional targeting strategies. The main purpose of this chapter was to illustrate to the reader the benefits of transcriptional targeting and how this approach can be used to generate tumor- or tissue-specific gene expression. The main example of the osteocalcin promoter was used because of our laboratory's significant investigation of this promoter.

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CHAPTER 10

Development of Attenuated Replication Competent Adenoviruses (ARCAs) for the Treatment of Prostate Cancer

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I. Introduction

The specificity, or therapeutic index, of anticancer chemotherapy agents has long been problematic. The majority of cancer chemotherapy agents, such as alkylating agents, antimetabolites, antibiotics, plant alkyloids, and other cytotoxic agents, nonspecifically injure or kill dividing cells [1]. These agents are noted for their poor specificity and low therapeutic ratio of toxicity toward target cancer cells compared to normal cells (e.g., therapeutic ratios of 2:1 to 6:1). In some instances, hormonal anticancer agents offer improved specificity [2]. The few biologic response modifiers [3], particularly humanized monoclonal antibodies, also offer greater anti-cancer specificity. However, cytotoxic agents remain the mainstay of cancer chemotherapy. The unwanted toxicity problems, most notably the myeloid stem cell suppression characteristic of cytotoxic drugs, are so great that drugs designed to recover patients from the side-effects of cytotoxic anticancer agents such as G-CSF, GM-CSF, and erythropoetin [4] represent as significant a commercial market as the cytotoxic chemotherapy agents themselves.

Intense efforts to increase specific cancer cell cytotoxicity of anticancer agents have frustrated researchers for decades. One such effort is gene therapy [5, 6]. In experimental models of gene therapy using replication defective adenoviruses (Ads), the use of prodrug converting enzymes such as herpes simplex virus thymidine kinase [7–11] and cytosine deaminase [10] under the control of transcriptional response elements (TREs), has shown anticancer activity in animal models with significantly increased specificity. However, to

destroy a solid tumor in a human, replication-defective adenoviruses must deliver a therapeutic gene and initiate a significant bystander effect all before the host immune response to the adenovirus coat proteins limits further treatment. Unfortunately, in humans even when gene transfer was successful, gene expression from replication-defective vectors has been inadequate or too short-lived. Thus, clinically the limiting issue has been a lack of efficacy.

To address some of these shortcomings we have tried to design therapeutics with sufficient specificity and efficacy so that the short-term expression of adenovirus will be successful in killing enough target cancer cells to be medically useful. Physically, replicating adenoviruses can infect a broad range of human cells and produce infectious progeny that could attack adjacent tumor cells, leading to destruction of a solid tumor within a short period of time from a single virus treatment. In human patients, tumor-specific replicating adenoviruses would be expected to induce a strong cytotoxic T-cell response confined to the target tumor cells precipitating a cancer vaccine-like response that could help eliminate tumor cells.

Replicating viruses have been proposed to treat cancer for nearly a century [12]. The first sustained attempts to treat tumors in animal models occurred in the late 1940s and early 1950s when infections were induced with viruses such as avian pest, Russian Far East encephalitis, St. Louis encephalitis, Coxsackie, foot and mouth, herpes simplex, influenzae, West Nile, dengue, Newcastle disease, vaccinia, and rabies [13]. A significant early attempt was made to explore the cell-killing properties of replicating cytolytic adenoviruses for the treatment of cancer in humans. The first isolates of adenovirus were shown to grow "luxuriantly" on HeLa cells, cells originally derived from cervical cancer. It was proposed that perhaps adenoviruses would preferentially replicate in and destroy cervical cancers. Smith *et al.* [14] tested 10 different wild-type adenovirus serotypes, including adenovirus type 5 (Ad5), as a treatment for locally advanced cervical carcinoma. Virus was administered via intratumoral injection or intraarterial injection. The virus stocks used were unpurified lysates of tissue culture cells; the number of infectious viral particles (plaque-forming units) and the total number of virus particles in the injected dose were not determined. Although long-term clinical benefit was not achieved, tumor necrosis and cavity formation was observed in 65% of treated patients via intratumoral injection, and these effects were limited to the carcinoma tissue. Side effects, detected primarily in patients receiving immunosuppression with cortisone, included febrile illness and malaise; in all cases, the symptoms resolved in 7–9 days. Infectious virus was not recovered from any biopsy specimens or vaginal smears, but the titers of neutralizing antibodies were uniformly elevated by 5–7 days postinjection [14]. This study is significant, for it illustrates the promise and limitations of oncolytic adenoviruses while describing the

limited toxicity to be expected of replicating adenoviruses that contain the E3 region and do not contain transgenes encoding foreign proteins at these intermediate dose levels. However, adenoviruses were subsequently shown to replicate in many cell types and lacked the hoped-for specificity for cervical cancer cells.

A resurgence of interest in replicating adenoviruses has occurred in the past decade due to the ability to genetically manipulate viruses. In 1996, Bishoff *et al.* introduced the use of Ad5 deleted in the E1B-55kD protein so that the virus (ONYX-015 = dl1520) [15] preferentially replicates in $p53^-$ cells as compared to $p53^+$ cells by a factor of 100-fold. However, the mechanism of antitumor specificity of the ONYX-015 virus has come under criticism [16–20]. In 1997, Rodriquez *et al.* introduced transcriptional targeting of adenovirus using the enhancer/promoter of the human prostate-specific antigen gene to drive the Ad5 E1A gene [21].

We have focused on the use of transcriptional response elements to control the expression of virus genes required for virus replication [21–23]. To test this idea we initially chose prostate cancer and the regulatory enhancer and promoter elements (PSE) of prostate-specific antigen (PSA). PSA is the most widely used serum marker for the diagnosis and management of any form of cancer. It is produced in prostate cancer cells and normal prostate ductal epithelia (which represents less than 5% of the cells of the prostate); it is also produced in much smaller amounts in the periurethral glands (less than 0.01% compared to prostate epithelia) and very rarely in tumors of the skin and salivary gland, but frequently in tumors of the female breast [24]. Since the prostate is an accessory organ, removal or ablation of the entire gland has no serious health repercussions [2, 25–28], although the side-effects of incontinence and impotence are legendary. A virus destroying all PSA-producing cells would not be expected to attack cells leading to incontinence and impotence. Thus, the regulatory regions of the prostate-specific antigen gene are a reasonable choice for such an approach.

We reasoned that placing adenovirus genes under the control of the PSE would create host range mutants or a virus in which replication would be restricted primarily to PSA-producing (PSA^+) ductal epithelial cells within the prostate, and PSA^+ prostate cancer (PCA) cells. We refer to our genetically engineered viruses using transcriptional response elements as attenuated replication-competent adenoviruses (ARCAs). We describe CV706 (PSE driving the Ad5 E1A genes and deleted in the Ad5 E3 region), which is currently in clinical trials for localized prostate cancer. Since taking CV706 to clinic, we have focused on improving the specificity and efficacy of the ARCA platform. Below we describe additional prostate-specific viruses on the developmental pathway from CV706, leading to CV787 (probasin driving the E1A gene, PSE driving the E1B gene, and reintroduction of the Ad5 E3 region) and their toxicity and explore the possibility of achieving a better antitumor efficacy

by combining ARCA with conventional therapies including radiotherapy and chemotherapy. CV787 is also currently in the clinic.

II. ARCAs for Prostate Cancer: CV706 and CV787

A. Adenovirus: Gene Expression and Regulation

Members of the human Adenoviridae family were first cultured from the tonsils and adenoids of children in 1953 [29]. They represent 51 different serotypes which are distinguishable by antibody reactivity to epitopes on the virion surface. Each serotype is assigned to one of five subgroups (A–E). Adenovirus type 5 (Ad5), a member of Subgroup C, is associated with a self-limiting, febrile respiratory illness and ocular disease in humans; infectious virus can be recovered from the throat, sputum, urine, and rectum. Ad5 is also associated with renal impairment, hepatic necrosis, and gastric erosions in immunosuppressed individuals [30, 31]. Ad5 and the other Subgroup C viruses have little or no oncogenic potential in mammals [32].

The adenovirus type 5 genome is a double-stranded DNA molecule of 35,935 base pairs [33] containing short inverted terminal repeats [34]. Expression of the genome is a regulated cascade which is arbitrarily divided into early (E) and late (L) phases, with viral DNA replication required for maximal L gene expression. Related RNA transcripts are grouped according to the region of the genome from which they are transcribed as well as by the timing (E or L) of their expression. Viral gene expression is regulated at the levels of transcription, posttranscriptional modification (splicing), translation, and posttranslational modification. Products of the E1 region are essential for efficient expression of the other regions of the adenovirus genome. The E1A transcription unit is the first Ad sequence to be expressed during viral infection and its products play a crucial role in a number of important biological functions in adenovirus-infected cells. The E2 region encodes several proteins that are required for viral DNA replication. These include a DNA-binding protein [35], the viral DNA-dependent DNA polymerase, and the DNA terminal protein that are required for DNA replication [36–38]. The E3 region is not essential for replication in tissue culture and this region is deleted from most first-generation therapeutic adenoviruses [39, 40]. Proteins encoded by the E3 region modulate host immune responses to infection by inhibiting transport of the MHC class I protein to the cell surface, thereby impairing the cytotoxic T lymphocyte (CTL) response [41–43], and by blocking TNFΔ-induced cytolysis of infected cells [44–46]. Significantly, all natural isolates of adenovirus contain the E3 region. Seven transcripts of the E4 region have been identified. Some of the encoded proteins interact with and/or modulate the activity of E1 region proteins.

The onset of viral DNA replication signals the switch from E to L gene expression. Although the precise mechanisms are not fully understood, this transition requires both *cis*- and *trans*-acting factors [47–49]. Late genes primarily encode the structural components of the virion and the nonstructural scaffolding proteins that are essential for the assembly of infectious virus. It is estimated that up to 10,000 adenovirus virions accumulate per cell and most remain cell-associated [50]. The entire adenovirus replication cycle is complete in approximately 32–36 h [51]. Host range mutants of adenovirus have played a significant role in elucidation of virus functions. ARCAs using transcriptional response elements create host range mutants where replication is restricted to a particular cell type. The cytotoxicity associated with virus replication (lysis) and the vaccine nature of expressing highly visible foreign capsid antigens should be limited to a certain type of cell.

B. Tissue Specificity of ARCA

We hypothesized that tropism of a virus could be redirected if expression of an essential viral gene could be controlled. Viruses generated from this approach would have the same capsid as its parental virus and they should be able to penetrate all cell types that express the CAR receptor. Presumably, in all cells containing the CAR receptor, these viruses would follow the normal cell entry process: they would penetrate the endosome, fuse with the endosome membrane, reach the cytoplasm, find transport to the nucleus, and uncoat the viral DNA. In a normal adenovirus replication cycle the E1A gene is the only gene expressed during the first 2.5 h of infection [52–55]. In turn, the E1A proteins as transcription factors upregulate expression of the impending cascade of viral genes. However, we have genetically engineered prostate tissue-specific promoters and enhancers so as to drive the E1A genes. Viral replication should preferentially take place in cells that express the necessary transcription factors, thus enabling activation of the tissue or tumor-specific transcription regulatory elements. Thus, the E1A proteins should be preferentially expressed and the virus preferentially replicate in prostate cells.

There are several criteria important in regard to the transcriptional response element (TRE) necessary for the successful engineering of a therapeutic adenovirus: (1) the tissue-specific regulatory specificity must be tightly regulated, and transcription should be limited to tumor cells, or accessory cells with as few other sites of expression as medically tolerable, (2) the TRE must regulate the initiation of transcription of the adjacent gene, (3) the promoter must be strong enough to drive sufficient expression of essential viral genes, and (4) the TRE must be small enough to fit within the packaging limits of adenovirus. We chose prostate cancer and the TREs of PSA as our initial target.

Expression of the PSA gene is modulated by the prostate-specific enhancer (PSE) element that is located several thousand nucleotides upstream of the PSA

promoter [56]. When fused to a fragment (position −230 to +7, relative to the start of transcription) containing the PSA promoter, the PSE (position −5322 to −3875, relative to the start of transcription) confers tissue-specific expression on the reporter gene chloramphenicol acetyl-transferase (CAT) [56]. Sequence analysis of the PSE reveals the presence of regions with homology to steroid-response elements (SREs) and to binding sites for several cellular transcription factors including c-Fos and AP-1 [23, 56, 57]. A functional androgen-response element (ARE) within the PSE increases expression up to 100-fold in the presence of testosterone or the nonmetabolized testosterone analog R1881.

1. ARCAs Containing One Prostate-Specific Transcriptional Response Element

To test the feasibility of the ARCA technology, we engineered the PSE fragment into the adenovirus genome and generated a first generation virus, CV706. CV706 contains the PSE fragment (PSA promoter and enhancer) inserted immediately upstream of the E1A region and transcription of the E1A region is regulated by the PSE (Table I). Virus characterization showed that CV706 was able to efficiently replicate in PSA^+ prostate carcinoma cell lines but not in the other PSA^- human cell lines HBL-100, MCF-7, PANC-1, OVCAR-3. CV706 also does not replicate efficiently in DU-145, a prostate cancer cell line which does not express PSA and does not contain the androgen receptor [21]. Further study indicated that the transcription of the E1A mRNA was regulated by the PSE. E1A mRNA was detectable in PSA^+ LNCaP cells,

Table I
ARCAs for Prostate Cancer

Virus	E1A driven by	E1B driven by	E3 region	E4 driven by	Targeting cell
CV702	wt	wt	Deleted	wt	N/A
CV706	PSE	wt	Deleted	wt	Prostate cancer
CV711	wt	PSA	Deleted	wt	Prostate cancer
CV716	PSE	PSE	Deleted	wt	Prostate cancer
CV730	No E1A	wt	Deleted	wt	N/A
CV737	PB	wt	Deleted	wt	Prostate cancer
CV738	wt	PB	Deleted	wt	Prostate cancer
CV739	PB	PSE	Deleted	wt	Prostate cancer
CV740	PB	PB	Deleted	wt	Prostate cancer
CV757	wt	wt	Deleted	PSE	Prostate cancer
CV763	HK2	wt	Deleted	wt	Prostate cancer
CV764	PSE	HK2	Deleted	wt	Prostate cancer
CV787	PB	PSE	Full-length	wt	Prostate cancer
CV802	wt	wt	Full-length	wt	N/A

but was not detectable in PSA^- cells. E1A protein was also reduced by 99% in PSA^- cells, compared to that in the PSA^+LNCaP cells [21]. This indicates that the inserted PSE has successfully controlled expression of the E1A gene and the host range of this adenovirus mutant has been confined to a particular cell type.

We also showed that the tropism of adenovirus could also be changed when the E1B gene or the E4 gene was placed under the control of the PSE TRE. CV711, whose E1B gene is placed under the control of PSE, and CV757, whose E4 genes are driven by PSE, both replicate similarly to wild-type adenovirus in PSA^+ cells but are highly attenuated in PSA^- cells. Cell specificity of CV711 viruses is similar to CV706 and replicates similarly to wild-type virus in PSA^+cells. In contrast, CV757, shows significantly greater specificity for PSA^+cells. While CV757 grows similarly to wild-type in PSA^+ cells, it suffers a very large reduction in the ability to replicate in PSA^- cells (data not shown). Thus, adenovirus mutants can be generated to target PSA^+ cells when any one of the E1A, E1B, of E4 genes are driven by the PSE.

These observations have been confirmed with other prostate-specific TREs including the TREs for probasin and hK2. The rat probasin (PB) gene is developmentally regulated in the prostate by androgens. Induction of the rat probasin gene by androgens was shown to involve the participation of two different *cis*-acting DNA elements that bind the androgen receptor. An expression cassette carrying 426 bp of the PB gene promoter and 28 bp of the 5′-untranslated region was found to be sufficient to target expression of a bacterial CAT reporter gene specifically to the prostate epithelium [58]. It was also shown that the same 5′-flanking region of PB gene promoter fragment fused to the SV40 TAg gene could lead to the development of progressive forms of prostate disease that histologically resemble human prostate cancer in transgenic animals [58]. The promoter of the rat probasin gene was engineered into adenovirus to drive the expression of either the E1A gene or the E1B gene to generate CV737 and CV738, respectively. Both CV737 and CV738 showed significant specificity to PSA^+prostate carcinoma cells.

We also recently cloned the TRE of the human glandular kallikrein (hK2) gene. The hK2 gene is located 12 kb downstream from the PSA gene in a head-to-tail fashion, whereas the hK1 gene is located 30 kb upstream of the PSA gene in head-to-head fashion [59]. The PSA and hK2 gene share DNA (80%) and amino acid (78%) sequence homologies that suggests they evolved by gene duplication from the same ancestral gene [60, 61]. Interestingly, the hK2 protein was recently shown to be expressed in every prostate cancer, and the expression of hK2 protein incrementally increased from benign epithelium, to high-grade prostatic intraepithelial neoplasia, to adenocarcinoma. We recently described CV763 containing the hK2 promoter and enhancer driving the Ad5 E1 gene. CV763 behaved identically to CV706 [23].

Thus, the replication of adenovirus can be restricted to prostate cancer cells when one of the essential adenovirus genes E1A, E1B, or E4 is placed under the control of any one of three different prostate-specific TREs.

2. ARCAs Containing Two Prostate-Specific Transcriptional Response Elements

Since both the E1A and E1B genes are essential for adenovirus replication, we reasoned that it was possible to create a virus with significantly higher specificity if both the E1A and E1B genes were under independent control of two TREs. To test this hypothesis, we generated an adenovirus mutant CV716, in which both the E1A gene and the E1B gene were under the control of PSE. *In vitro* study showed that CV716 replicated well in the PSA-producing prostate cancer cells. However, replication of CV716 was highly attenuated in nonprostate human cell lines. Compared to CV706, the efficiency of CV716 replication in nonprostate cancer cells has been further reduced by another 100-fold, giving specificity for PSA^+ cells compared to PSA^- cells of nearly 10,000:1 (data not shown). The high degree of specificity for PSA^+ cells of CV716 as compared to PSA^-cells was found to be universally true [22, 23]. CV740, containing duplicate copies of the rat probasin promoter, also showed this high level of specificity. Unfortunately, CV716 and CV740 are genetically unstable, resulting in self-inactivation of the virus. The E1A gene and one copy of the tissue-specific TRE inserts are deleted during replication. Southern blot analysis of stocks of CV716 indicated a new band when annealed with an E1B-labeled probe. DNA sequence analysis of the cloned deletion mutant indicated that self-inactivation is due to homologous recombination between two identical inserted TREs.

In order to make a stable tissue-specific adenovirus we employed two different TREs to drive expression of early essential viral genes. In CV739 the E1A gene and the E1B genes are under the control of the TRE of the rat probasin gene and PSA gene, respectively. CV739 replicates well in PSA^+ prostate cancer cells, but poorly in nonprostate human cancer cell lines. The cell specificity of CV739 was similar to that of CV716, again showing the roughly 10,000:1 selectivity for PSA^+ cells as compared to PSA^- cells. However, CV739 is stable. No replication-defective mutants with deleted genomes were found after extensive passages. The same is true for other CV739-like viruses including CV764. CV764 is a stable ARCA variant containing the PSE driving the E1A genes and the hK2 promoter and enhancer driving the E1B genes. The sequences of the PSE and hK2 promoter and enhancer are 80% identical, yet the virus is genetically stable. Again, CV764 has the high therapeutic index of the other viruses containing two prostate-specific TREs with a cell specificity of 10,000:1 for PSA^+ cells compared to PSA^-cells [22, 23].

Taken together, these adenovirus variants show that tropism of adenovirus can be redirected by placing essential viral genes under the control of

tissue-specific regulators. The cell selectivity of a stable oncolytic virus can be over 10,000:1 when the expression of more than one viral gene is driven by two different tissue-specific TREs. The phenomenal success of creating adenovirus host range mutants with specificity for target cells compared to nontarget cells of over 10,000:1 is one of the major achievements of the ARCA technology.

C. Antitumoral Efficacy of ARCA

In vivo studies evaluating intratumoral and intravenous administration of prostate-specific adenoviruses were conducted in the *nu/nu* mouse containing human tumor xenografts. Tumors were produced by subcutaneous injection of PSA^+-producing prostate cancer LNCaP cells into each flank of each mouse, and after establishment of palpable tumors (mean tumor volume 300 mm^3), the tumors were directly injected with purified virus or vehicle (PBS and 10% glycerol). Tumor growth was then followed for 6 weeks, at which time the mean tumor volume in each group was determined. A significant antitumoral activity was observed in the *in vivo* study for CV706. Tumor volume dropped by more than 80% in the animal group that was treated with CV706 by a single intratumoral injection. These residual tumor masses were shown by histology to be scar tissue devoid of PSA^+ cells. After 6 weeks, 5 of 10 mice were visually free of tumor [21].

In contrast, DU145 is a prostate cancer cell line that is PSA^- and does not produce the androgen receptor. Tumors of DU145 cells were induced in nude mice and challenged with buffer, wild-type Ad5 but E3 virus CV702 and CV706. The results showed that CV702 inhibited growth of DU145 tumors, whereas CV706 has no effect on tumor growth. Thus, the prostate-specific CV706 virus not only shows efficacy but also selectivity for PSA^+cells *in vivo* [21].

The E3 region has long been considered unnecessary for replication of adenovirus *in vitro*. It has been universally deleted from Ad5 gene therapy constructs until recent efforts to prolong transgene expression from replication-defective Ad5 gene therapy constructs [39, 40, 62–64]. To test the possibility of increasing virus cytotoxicity, we created CV787 from its parent virus CV739 by engineering the full-length E3 region back into the viral genome. Thus CV787 contains the rat probasin promoter driving the E1A gene the PSE driving the E1B gene. Otherwise, CV787 is identical to the recombinant wild-type adenovirus CV802. CV787 retained the high specificity of characteristics of two TRE-containing viruses driving the E1A and E1B genes. Cell viability assay and virus yield assay demonstrated that addition of E3 aids virus replication and increases virus cytotoxicity. Thus CV787 has a stronger cytotoxicity than CV739 [22].

The increased cytotoxicity due to the Ad5 E3 region was also confirmed *in vivo* in the LNCaP xenograft animal model. A single intratumoral injection

of CV739 and CV787 yielded identical reduction of LNCaP xenografts. However, CV739 required 100-fold more virus to achieve the same effect as CV787 [22]. A single intratumoral CV787 at a dose of 1×10^8 particles/mm^3 was curative for animals 6 weeks after treatment ($n = 8$). A single intravenous injection of CV739 at a dose of 5×10^{10} particles could stop tumor growth, whereas CV787 at this dose level caused a fourfold reduction in tumor volume [22]. Six weeks following a single intravenous injection of 1×10^{11} particles, the sizes of tumors were reduced to less than 5% of their original size, and 8 of 14 mice were visually free of tumors. The residual tumors measurably present were immunohistologically devoid of PSA [22]. The serum PSA levels in mice injected intravenously with CV787 decreased to 5% of their starting values within 4 weeks. Intravenous administration of CV787 designed to treat LNCaP xenografts showed that 1×10^{11} particles could eliminate 300 mm^3 preexistent LNCaP xenografts, whereas 1×10^{11} particles of CV706 administered intravenously only stabilizes tumors. A dose–response curve of 1×10^9 and 1×10^{10} CV787 particles administered as a single intravenous dose can stabilize and regress tumors, respectively, but not eliminate tumors. These data indicate that CV787 has a significantly improved antitumor activity and a single dose of intratumoral or intravenous administration can eliminate pre-existent tumors in animal models.

D. Mechanism for Cell-Killing of ARCA

Infection with adenovirus causes profound changes in host-cell macromolecular synthesis that ultimately lead to cell death. Virion fiber protein inhibits macromolecular synthesis when applied directly to cells bearing the adenovirus receptor [65]; soluble penton protein causes cytopathic effects (CPEs) in susceptible cells that are similar to those caused by infectious virus [66]. Cell-specific DNA synthesis, export of cellular mRNAs from the nucleus to the cytoplasm, and cell-specific translation are all inhibited after infection, but the precise mechanisms are not completely understood.

The 243E1A protein induces the full range of classical apoptotic events by increasing the level of the host cellular tumor suppressor protein p53. The 289E1A protein induces apoptosis by a p53-independent mechanism that requires a product of the E4 region [67, 68]. The E1A-induced activation of the apoptosis pathway(s) must be modulated by E1B proteins to ensure efficient virus replication prior to cell death [69]. Activation of the interferon-inducible RNase L pathway by the adenovirus-associated type I (VAI) RNA [70] may also contribute to the stimulation of apoptotic pathways in adenovirus-infected cells [71]. The E3 11.6-kDa adenovirus death protein also has a role in cell-killing and promotes the release of progeny virions from the cell [72, 73].

We have investigated how our oncolytic viruses kill tumors in the *nu/nu* mouse model. Immunohistochemical analyses were performed to assay for the

de novo synthesis of CV787-encoded proteins in tumor xenografts and to examine the effects of treatment with CV787 on tumor morphology *in vivo*. The mice bearing human LNCaP tumors were injected intravenously on day 0 with 1×10^{11} particles of CV787 per animal. Tumors were excised from two animals on days 1, 3, 7, 14, 21, and 28. The tumors were cut into six pieces and each piece fixed, embedded in paraffin, and sectioned. Sections were stained for the presence of adenovirus protein by a double-antibody protocol with rabbit anti-Ad antibodies and Fast Red stain followed by a hematoxylin counterstain.

On day 1, intracellular staining for adenovirus protein was detected in less than 1% of the tumor cells examined in 12 sections from two tumors. Occasional small clusters of stained cells, as well as dispersed single stained cells, were visible. By day 3, large clusters of cells expressing adenovirus proteins were detected in one of the two excised tumors. In some instances, areas of tumor necrosis were adjacent to clusters of adenovirus protein positive cells. On day 7, intracellular staining for adenovirus proteins was detected in greater than 10% of the tumor cells examined in 12 sections from both excised tumors. Virus-infected cells within the tumor sections were prominent on Day 21 and increased to more than 90% of the microscopic field of the section by Day 28. These results demonstrated that CV787 replicated in and expressed virus-encoded gene products in the LNCaP xenografts. The increased distribution of virus protein-positive cells indicated that infectious progeny CV787 spread to adjacent cells within the tumor which was associated with progressive necrosis *in vivo* [22].

Adenovirus-induced apoptosis causes cell death *in vitro*, specifically at the late stage of infection [67], and this process may contribute to the therapeutic effect of oncolytic virus *in vivo*. LNCaP xenografts in athymic mice were treated on day 0 with vehicle alone or a total dose of 3.2×10^7 particles of CV706 per mm^3 of tumor. Tumor biopsy specimens were taken on day 14, and 5-μm sections were prepared and examined for apoptosis. Extensive areas containing apoptotic nuclei were detected in sections of tumors treated with CV706. More than 25% of nuclei were apoptotic in some sections from CV706-treated tumors. In contrast, less than 2% of nuclei were apoptotic in sections from tumors treated with vehicle alone.

Additionally, a visual change of tumor color has been documented when the animals receive tumor specific adenovirus variants. For example, the color of the established human LNCaP xenografts is black. However, the tumors will become white within 1 week after receiving a dose of CV706 or CV787 through either intratumoral or intravenous administration. Histological H&E staining analysis found that the virus-treated tumors had significantly fewer numbers of blood vessels when compared to the tumors treated with vehicle. It is unclear at this time as to the precise mechanism by which this reduction in blood vessel number is achieved. This can achieved either though direct

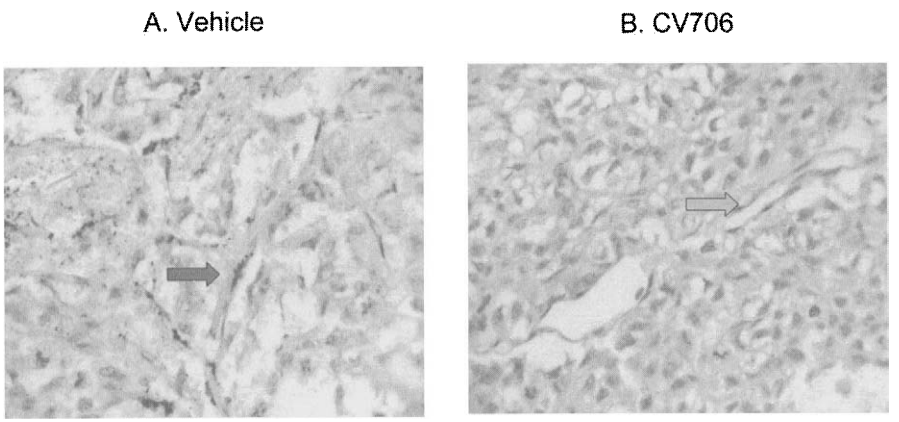


Figure 1 CD31 staining for newly developed blood vessel. Tumors were harvested 14 days after receiving (A) vehicle or (B) 1×10^7 particles/mm^3 tumor of CV706 and stained with anti-CD31 monoclonal antibody by immunohistochemical analysis.

damage of endothelial cells or indirectly through the destruction of tumor vasculature by extensive necrosis. CD31 is expressed constituitively on the surface of adult and embryonic endothelial cells and has been used as a marker to detect angiogenesis [74]. Immunohistochemical staining was performed to examine the effect of virus treatment on tumor angiogenesis by using monoclonal antibody against CD31. Tumors treated with CV706 showed a significantly lower level of CD31-positive cells in the vessel when compared to vehicle treated tumors (Fig. 1). This observation suggests that CV706 may be inhibiting tumor angiogenesis to a significant extent. The change of tumor color from black to white has been a reliable early indication for antitumor efficacy of a virus.

III. Synergy of ARCA and Conventional Therapy

Although conventional cancer therapies (surgery, chemotherapy, and radiation) are effective at curing early-stage disease, few human cancers are curable with a single modality. The utilization of a replication-competent cytolytic adenovirus as a therapeutic modality shows much promise. In our laboratory and clinical trials, CV706 and CV787 have been shown to be effective against human prostate tumors [21–23]. However, single-dose total eradication of human LNCaP xenografts in our animal model required as much as one-third of the lethal dose of virus alone [22, 75]. To set the bar for efficacy high, namely single-dose efficacy, is deliberate. While multiple doses

always allowed total eradication of tumors with lower doses, in the clinic the patient immune response will tend to limit the number of doses that can be effectively delivered despite the SIAPA technology described below. Certainly, strategies to improve the efficacy of these viruses are desirable. However, in preclinical experimental animal models, it can be readily appreciated that genetic engineering allows creation of many different viruses with an associated rationale for why the new mutant may be an improvement with greater efficacy over its predecessor. Experimentally it is impossible to compare different viruses for their efficacy in animal models unless a standardized treatment regimen is used. Thus, we use only single-dose treatment regimens in our animal studies. Small changes in efficacy do not warrant replacing current clinical candidates; large changes in efficacy are most interesting. The largest changes in efficacy we have seen came with the addition of the E3 region and the synergy afforded by radiation and chemotherapy.

We studied neoadjuvant therapy consisting of combining ARCA with conventional therapy for additional reasons as well: (1) combinations of agents with different toxicological profiles can result in increased efficacy without increasing overall toxicity to the host; (2) overlapping resistance between ARCA and conventional therapeutics has not been described previously and a combination of agents may thwart the development of drug resistance; and (3) tumor cell populations have different drug sensitivity profiles, which allow the physician to take advantage of possible synergies between drugs, resulting in increased anticancer efficacy in patients.

A. Synergy of CV706 and Irradiation

Radiation therapy, either administered through external beam or seed implantation (brachytherapy), is one of the most widely used therapeutic modalities for treatment of clinically localized prostate cancer [76, 77]. However, nearly 30% of patients treated with potentially curative radiation doses suffer relapse as defined as three serial rises in serum PSA levels (biochemical failure). Specifically, the eradication of locally advanced or high-risk prostate cancer with radiation has proven more difficult than believed previously. The gains with high radiation dose have been modest and fraught with significant side-effects [78]. It is apparent that there is a need for novel methods of radiosensitization. We have examined the effects of a combination treatment involving CV706 and irradiation for localized prostate cancer. We have demonstrated a very high synergy when irradiation treatment is complemented with CV706.

Initial *in vitro* experiments demonstrated a synergistic cytotoxicity to LNCaP prostate cancer cells when CV706 was combined with radiation. LNCaP cells treated with CV706 and radiation had significantly decreased viability compared to cells treated with either agent alone. LNCaP cells

treated with radiation exhibited a significantly greater burst size of CV706 (m.o.i. = 0.1) with more than 100 times more virus produced per cell. In addition, the combination treatment of CV706 with radiation did not alter the specificity of replication-mediated cytotoxicity.

A similar synergistic anti-tumor efficacy of combination therapy with CV706 and radiation was also observed *in vivo* in prostate LNCaP xenografts. Previous *in vivo* studies demonstrated that prostate tumors were completely eliminated within 6 weeks by a single intratumoral administration of CV706 at a dose 5×10^8 particles per mm^3. Combination treatment of CV706 at a dose of 1×10^7 particles per mm^3 with radiation 10 Gy eliminated tumors within 6 weeks. Under similar conditions CV706 or radiation alone could only slow down or inhibit further tumor growth (Fig. 2). Thus, a 50-fold lower dose of virus (1×10^7 particles) can be used in the combination treatment modality to achieve the same curative effect. Statistical analysis of the *in vivo* studies indicated that CV706 and the radiation combination group showed a significant anti-tumor synergy with a 1.5- to 6.7-fold higher inhibition of tumor

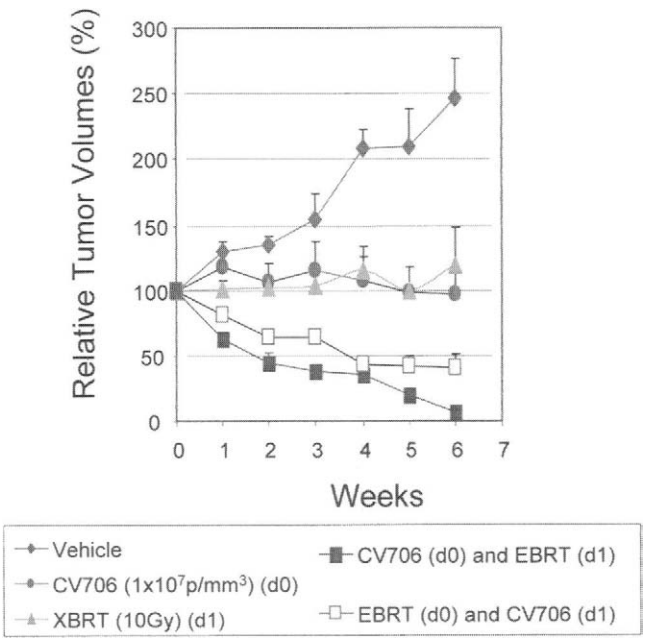


Figure 2 *In vivo* efficacy of intratumorally administered CV706 and radiation against nude mouse LNCaP xenografts. Tumor volume of LNCaP xenografts treated with either vehicle (diamond), CV706 alone (1×10^7 particles per mm^3 of tumor, circle), radiation alone (10 Gy, triangle), CV706 (1×10^7 particles per mm^3 of tumor) at day 0 plus radiation (10 Gy) at day 1 (close square), and radiation at day 0 plus CV706 at day 1 (open square).

growth over the additive effect during the entire treatment period. Subsequent studies have shown that a synergistic antitumor efficacy could be achieved when 1×10^7 particle CV706 was combined with only 5 Gy radiation (unpublished data, Chen *et al.*). Further *in vivo* studies are in progress to determine the effective minimum dose of CV706 in combination with radiation required for complete regression of tumors.

A series of experiments was then designed to examine the effects of the sequencing of the agents, the timing of radiation following virus administration, and radiation fractionation. Efficacy was highly dependent on the sequencing of the agents; treatment with CV706 24 h prior to radiation was significantly superior to radiation followed by CV706. The antitumor activity was decreased when the tumors were treated with radiation 7 days after CV706 administration. However, there was no significant difference in antitumor efficacy when the tumors were treated with CV706 followed by a single dose of radiation or four sequential daily fractional doses of radiation as long as the total does of radiation was the same.

A preliminary assessment of synergistic activity in the CV706 and radiation combination treatment reveals several mechanistic possibilities. First, radiation at the synergistic dose significantly increases virus replication. One-step growth curve study shows that although synergistic doses of irradiation did not alter virus replication kinetics, the irradiation significantly increases the burst size of CV706 in LNCaP cells. For example, burst size of CV706 in LNCaP cells treated with CV706 for 24 h followed by irradiation is 500-fold higher than that in cells treated with CV706 alone (0.01 m.o.i.). Irradiation kills mammalian cells in the reproductive (also known as clonogenic) death pathway, and breaks down DNA strands. Most radiation induced DNA double-stranded breaks are rapidly repaired by constituitively expressed DNA repair mechanisms [79]. DNA repair machinery becomes more active in irradiated cells, thus allowing for greater replication/multiplication of the episomal adenoviral DNA. Because of its small target size, the adenoviral genome (36 kb) is far less likely to sustain radiation-induced damage as it is 10^5-fold smaller than that of human cells (3×10^6 kb). Therefore, the more active cellular DNA synthesis machinery in the irradiated cells may facilitate viral DNA synthesis and virus replication. Second, CV706 may be augmenting the anti-tumor activity of radiation. The adenovirus E1A gene is the only viral gene expressed during the first 2.5 h of infection and encodes a multifunctional transcriptional factor also known to induce apoptosis [32, 80]. It is believed that the adenovirus E1A gene is a potent inducer of radiosensitivity through p53-dependent and -independent mechanisms. Malignant tumors, when expressing adenovirus E1A, are very sensitive to treatment with DNA-damaging agents *in vivo*, including irradiation [81, 82]. In the tumors treated with CV706 and radiation, the histological features included intravascular thrombosis and massive necrosis positioned more centrally within tumors. This supports a

growing concept for the involvement of an effect on the destruction of the vasculature leading to tumor reduction and elimination. This seems to be in agreement with a recent finding that inhibition of angiogenesis led to increased tumor radiosensitivity [83]. Indeed, CV706 in combination with radiation exerted a significant reduction of CD31 positive blood vessels, indicating an anti-angiogenesis effect of the combination treatment [84, 85].

As human tumors are composed of a mixture of cells having different genetic makeup, this inherent intratumoral heterogeneity may need treatment with multiple therapeutic modalities. As demonstrated here, radiation can be successfully combined with cytolytic adenoviral therapy. The advantages are as follows: the combination of CV706 with irradiation still limits the damaging effects of CV706 to the irradiated cells; the combination of CV706 with irradiation leads to significantly increased necrosis, marked decrease of blood vessel number, and inhibition of angiogenesis; The combination of CV706 with irradiation allows at least a 50-fold reduction in the amount of virus to achieve the same curative effect; animals receiving combination treatment appear healthier, characterized by a significant weight gain compared to the groups treated with either agent alone. Thus, combining cytolytic adenoviral therapy with radiation may augment the efficacy of standard cancer modalities.

B. Synergy of CV787 and Chemotherapy

Once prostate cancer enters a metastatic stage, the current treatment is androgen ablation therapy, which in 70% of men provides relief from otherwise uncontrollable bone pain and increases life expectancy by 6–18 months [86, 87]. For many years, chemotherapy was felt not to play any role in the treatment of advanced prostate cancer. However, this negative impression is apparently changing, as significant activity is being seen with new drugs and drug combinations [87]. Interim analysis of an ongoing Phase III trial of docetaxel in end-stage hormone refractory prostate cancer patients shows an improvement in life span to 30 months (Dan Petrylak, pers. commun.).

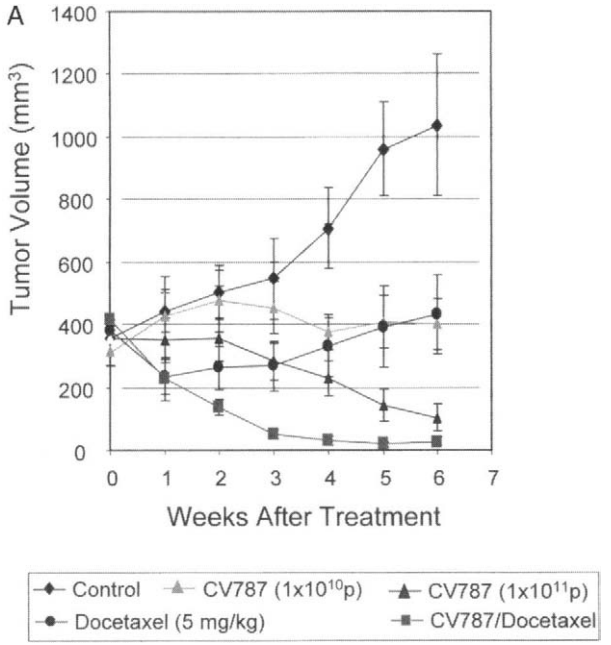
A synergistic antiproliferative effect in LNCaP prostate cancer cells was observed when CV787 was combined with various chemotherapeutic agents including cisplatin (Platinol), docetaxel (Taxotere), doxorubicin (Adriamycin), estramustine (Emcyt capsules), etoposide (VePesid), gemcitabine HCl (Gemzar), mitoxantrone (Novantrone), and paclitaxel (Taxol). In this chapter, we will discuss the synergistic antitumor efficacy of CV787 only when combined with the taxanes.

In vitro experiments demonstrated that LNCaP cells cultured with paclitaxel or docetaxel for 24 h before or after infection with CV787 had significantly lower rates of proliferation than cells treated with either agent alone [88]. Also, LNCaP cells exhibited a greater burst size of CV787, whereas no significant effect on viral growth kinetics was seen. No significant difference

in the effectiveness of the combined therapy of taxane and CV787 infection was observed by varying the time of taxane administration *in vitro*, whereas varying the administration schedule of paclitaxel with Ad-p53 gene therapy can modulate the synergistic activity between these two agents in ovarian cancer [89]. Furthermore, *in vitro* combination treatment of CV787 with taxane did not alter the specificity of replication-mediated cytotoxicity. CV787 has been shown previously to replicate preferentially in PSA-producing human prostate cancer cells 10,000 times more efficiently than in non-PSA-producing cells [22]. In the presence of paclitaxel or docetaxel in the culture medium, CV787 replicates to the same degree of specificity in the non-PSA-producing human cell lines versus PSA^+ cells. Cell viability assays with MTT further indicate that CV787 in combination with taxane remains fully selective.

The antitumor effects of combination therapy with CV787 and taxane were also evaluated *in vivo*. Previous studies have demonstrated that tumors were eliminated within 6 weeks by a single intravenous administration of CV787 at a dose of 1×10^{11} particles [22]. Clinically, docetaxel is administered intravenously at 12.5 mg/kg once per week for 6 weeks. A single cycle of docetaxel is not toxic, whereas the full course of six cycles is highly toxic. Combination treatment of CV787 and taxane (three courses of 5 mg/kg) eliminated tumors within 4 weeks, whereas CV787 alone at the same dose of 1×10^{10} particles per animal could only slow down tumor growth (Fig. 3A). The experiments described below are with a single cycle of docetaxel. In combination with the higher clinical dose of 12.5 mg/kg docetaxel, 1×10^9 particles and of 1×10^8 particles of CV787 led to a complete elimination of tumors within 4 weeks. Thus, the dose of CV787 required for complete remission has been reduced by 1000 times from 1×10^{11} particles to 1×10^8. The LD_0 and LD_{100} single bolus dose of CV787 for Balb/c, *nu/nu* is 1.0×10^{11} particles and 2.5×10^{11} particles, respectively. Thus, the single-dose combination of docetaxel with CV787 has increased the potential curative therapeutic window, from 1 to 1000. Furthermore, healthier animals, characterized by body weight, were observed in the combination treatment group as compared to groups treated with either agent alone (data not shown).

The mechanism(s) of synergistic activity in the combination of taxane with CV787 is unknown at this time; however, our experiments suggest a few hypotheses. First, taxane at the synergistic dose may be augmenting viral replication. It has been previously shown that a low concentration of paclitaxel (1–14 nM) increased the number of cells transduced by recombinant adenovirus 3–35% in a dose-dependent manner [89]. Recently, it was found that taxane increases intracellular receptor trafficking. For example, more Coxsackie and adenovirus receptor (CAR) moved to outer membrane in the paclitaxel-treated cells (Nielsen Loretta, pers. commun.). It is suggested that adenovirus transduction efficiency may increase after the cells are treated with taxane. Indeed, our data show that although the synergistic dose of paclitaxel



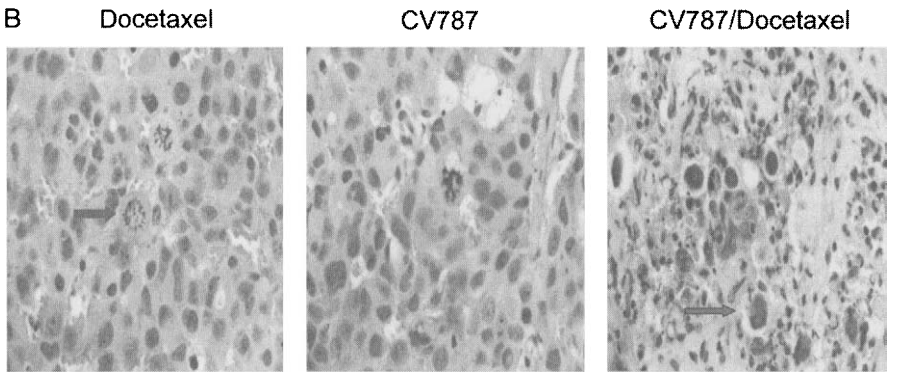


Figure 3 *In vivo* efficacy of intravenously administered CV787 and taxane against nude mouse LNCaP xenografts. (A) LNCaP tumor volume in mice treated with either vehicle, CV787 (1×10^{10} 1×10^{11} particles per animal, iv), docetaxel (5 mg/kg at day 2, 5 and 8, iv), or CV787 (1×10^{10} particles per animal) and docetaxel (5 mg/kg at days 2, 5, and 8, iv), ($n = 6$). Tumor volumes measured weekly. Error bars represent the standard error of the mean. (B) Histological features (H&E staining) of LNCaP tumors treated with either docetaxel (12.5 mg/kg) (left), CV787 (1×10^{10} particles per animal) (middle), and CV787 (1×10^{10} particles per animal) plus docetaxel (12.5 mg/kg) (right) at × 400 original magnification. LNCaP xenografts were harvested 14 days after treatment. The open arrow indicates cells with abnormal mitosis, the filled arrow indicates necrotic cells.

or docetaxel did not alter virus replication kinetics, the chemotherapy drugs slightly increased the burst size of CV787 in LNCaP cells. Second, CV787 may be augmenting the anti-tumor activity of taxane. E1A gene expression has been shown to increase cellular sensitivity to chemotherapeutic agents [90] and this enhanced sensitivity is partially caused by the induction of p53-dependent apoptosis by the E1A-induced sensitization of the cells [91]. Recently, Ueno *et al.* found that human ovarian cancer cells that were originally resistant to paclitaxel, became paclitaxel sensitive in E1A downregulated HER-2/neu cells [66]. In CV787, the E1A gene is intact and may be overexpressed in PSA-producing LNCaP cells, which may enable significant interaction between E1A and taxane. In addition, E1A is a potent inducer of p53 protein expression in infected cells [92]. p53 levels may increase following infection, thereby increasing cell sensitivity to chemotherapy-induced apoptosis. This is consistent with the observation that more apoptotic cells were seen in the LNCaP tumors that received combination treatment than in tumors that received either agent alone (Fig. 3B). Interestingly, in the combination-treated tumors, most of the cells were empty and virtually devoid of cellular content. Finally, the actions of the two agents may be occurring at two distinct points in the same apoptotic pathway, analogous to the activity of cyclosporine A and rapamycin on two distinct points in the T-cell activation signal transcription pathway [93]. Further investigation of the possible mechanism(s) of synergistic activity in the combination of taxane with CV787 is under way.

IV. Toxicity of Intravenously Administered ARCAs in the Absence or Presence of Docetaxel

As a prerequisite for a Phase I trial, we evaluated the biodistribution, persistence, and toxicity of CV787 following intravenous administration. Cotton rats were chosen over the mouse for Cotton rats are semipermissive for adenoviral replication [32, 94]. The objective of the first study was to determine the toxicity of CV787, after a single tail vein intravenous administration into the male Cotton rat, followed by a 3-day observation period. CV787 was administered at 3×10^{10} particles, 1×10^{11} particles, and 3×10^{11} particles per animal. There were no deaths or treatment-related clinical observations over the study period. In addition, there were no effects of treatment noted on body weight, food consumption, or clinical pathology or at the macroscopic pathological examination at all dose levels. As 3×10^{11} particles per animal was the highest achievable dose, this dose was considered the no-effect level when administered as a single bolus intravenous injection to the Cotton rat.

To investigate the biodistribution and persistence of CV787 following intravenous administration, we conducted a 21-day study with Cotton rats.

Twenty-seven male Cotton rats received either vehicle, replication-defective Ad5 CV730, an E1A deleted adenovirus mutant, and CV787 at 3×10^{11} particles per animal in a single intravenous tail vein injection on day 1. Three animals from each group were sacrificed on days 3, 8, and 22. DNA was extracted from plasma, testes, brain, spleen, liver, heart, lungs, kidneys, and bone marrow. The DNA obtained from tissues was quantified by optical density measurement and 100 ng was subjected to quantitative PCR using a TaqMan procedure specific for the Ad5 packaging site present in both CV730 and CV787.

There were no ill effects seen upon intravenous delivery of 3×10^{11} particles of CV787 per Cotton rat. As similar distribution of adenovirus seen in other rodents [94], CV787 accumulated primarily in the liver and spleen, and to a lesser extent in the heart, kidney, lung, and bone marrow. Viral DNA levels were very low in the testes and undetectable in the brain. The tissue distribution was similar for Ad5 CV730, a replication-defective control virus that was detected primarily in the spleen, liver, and kidney. The potential for nonspecific replication of CV787 was found to be very low, resulting in no more than a two- to fivefold increase in viral copy number during the course of the study.

A third study was designed to compare the acute toxicity and inflammatory potential of CV787 and CV706 by subcutaneous and intradermal administration in immunocompetent mice, C57BL/6. Four groups of five mice each were treated with a single dose of 1×10^{11} particles of CV787 or CV706 by subcutaneous (sc) or intradermal (id) injection on day 1. Four control groups were treated either intradermally or subcutaneously with the vehicle for CV787 or the vehicle for CV706. Two additional groups were treated intravenously (iv) with either virus at the same dose to compare the acute liver toxicity between CV787 and CV706. All animals were scarified on day 4. Blood was collected for serum chemistry analysis. Macroscopic examination for signs of toxicity was performed at necropsy. Portions of the liver, lung, spleen draining lymph nodes, and sc or id injection sites were collected in buffered formalin and examined for histopathological effects.

All animals survived until the scheduled day of sacrifice. No signs of toxicity were observed after treatment or during macroscopic examination in any of the id or sc groups. Serum chemistry analysis showed no significant changes following subcutaneous or intradermal administration of either virus preparation. With both viruses, histopathological analysis revealed a mild chronic inflammation in the areas surrounding the subcutaneous injection site in some of the animals, but no effects on any of the body organs were observed. Some inflammation was observed in the dermas following intradermal administration with both viruses, but the inflammation remained localized to the injection sites and the overall effects were no more severe than those observed after subcutaneous treatment. As expected, intravenous

delivery of the virus at a dose of 1×10^{11} particles per animal resulted in significant liver toxicity, as evidenced by 5- to 100-fold increase of ALT and AST enzyme levels. The ALT and AST response was more pronounced for CV706 than for CV787. Intravenous treatment with CV706 also resulted in statistically significant effects on BUN, serum cholesterol, glucose, and calcium levels, which were not as apparent following the intravenous administration of CV787.

The subcutaneous and intradermal effects observed in this study suggest that CV787 poses no additional risk of inflammation over CV706. Results from intravenous treatment of C57BL/6 mice indicate that CV787 may show less systemic toxicity than CV706. Also, it does not appear that Cotton rats add any new information on toxicity compared to the use of more common and easier to use laboratory mouse strains such as C57BL/6.

A synergistic antitumor efficacy was observed when CV787 was combined with taxane in the prostate tumor xenograft [88]. To examine the toxicity of CV787 in combination with the chemotherapeutic agent docetaxel, we conducted a fourth toxicology study in C57BL/6. The 4-day and 28-day effects were evaluated in mice given a single daily dose of dexamethasone (Decadron) each day for 3 consecutive days, a single injection of CV787 at low (1×10^8 particles per animal), medium (3×10^9 particle per animal), or high (1×10^{11} particles per animal) dose, and a single dose of the chemotherapeutic agent, docetaxel (100 mg/m^2). Six different treatment groups were established using identical treatment regimens for animals in subgroups sacrificed at 4 days and at 28 days. Each treatment subgroup consisted of eight randomly assigned mice. The ARCA virus vehicle and all three doses of virus were given intravenously via tail vein injection. All virus injections were given on day 1 at 1–2 h prior to Decadron administration. Dexamethasone and docetaxel were both given separately by intraperitoneal injection. At the end of the 4-day and 28-day treatment periods, respectively, blood samples were taken for complete blood count (CBC), including platelet count, and serum chemistry analysis just prior to sacrifice. Tissues were taken for organ weights and histopathologic evaluation of liver, lung, spleen, kidney, brain, heart, mesenteric lymph nodes, bone marrow, any enlarged lymph nodes, and any gross lesions.

Acute virus-associated changes at day 4 were characterized by hepatocellular necrosis and elevated serum leakage (liver) enzymes (ALT, AST) seen in the groups who received a high dose of CV787 alone or high dose of CV787 in combination with docetaxel, as well as an increasing incidence of lymphoid hyperplasia from mid-dose (3×10^9 particles per animal) to high-dose (1×10^{11} particles per animal) virus subgroups. These findings indicate dose-related viral effects without evidence of augmentation by docetaxel in combination with dexamethasone. In contrast, the severity of the lymphoid hyperplasia in the high-dose virus subgroup may have been ameliorated some by the docetaxel combination. Evidence of acute (high-dose) virus-associated

hepatocellular toxicity was resolved by day 28 and replaced by a viral dose-dependent increase in mild multifocal hepatic mononuclear cell inflammatory infiltrates in subgroups who received CV787 at a dose of 3×10^9 particles per animal or 1×10^{11} particles per animal with or without docetaxel. These inflammatory changes are most likely to be associated with chronic effects of viral replication and antigen stimulation and do not appear to be ameliorated by docetaxel combination.

There was acute mild/moderate bone marrow necrosis at day 4 from high-dose virus plus docetaxel combination in two of eight mice, but not from high-dose virus alone or from lesser-dose virus plus docetaxel combination. There were no bone marrow lesions seen in any animals at day 28, so if the effect is real, it appears to be a reversible effect of the high-dose virus plus docetaxel combination. Acute mild to moderate bone marrow hypoplasia was found at day 4 in all eight mice only in the high-dose virus plus docetaxel combination. Hypoplasia was not seen at day 28. The occurrence of bone marrow necrosis (2/8) and hypoplasia (8/8) in the day 4 high-dose virus plus docetaxel combination subgroup constitutes evidence for an additive but reversible effect of the combination. The 25% bone marrow necrosis and 100% hypoplasia were the only additive toxic effects ascribed to the combination of docetaxel with virus. These toxicities appeared to be dose-dependent with respect to the virus and are reversible.

V. Effects of Preexisting Adenovirus Antibody on Antitumor Activity and Immunoapheresis for Human Therapy

Preexistent antibodies to adenoviruses are highly prevalent in the human population. By 7 months of age 23% of infants have circulating antibody titers to adenovirus [95], a percentage that increases with age to essentially 100%[96,97]. Adenovirus antibodies are readily divided into total antibodies (TAb) and neutralizing antibodies (NAb). Almost all adults have antibodies (TAb) to adenovirus type 5, but only 57% of adults have neutralizing antibodies [75, 98]. These antibodies are of significant interest in the design of therapeutic strategies based on adenoviruses.

Transient immunosuppression during initial adenovirus administration with cyclosporine [7, 99], FK506 [100–103], cyclophosphamide [104, 105], deoxyspergualin [105], IL-12 [106], CTLA4Ig [107, 108], anti-CD4 antibody [109–111], anti-CD40 ligand antibody [112, 113], and dexamethasone [113, 114] has been used to blunt the formation of anti-Ad Abs in animal models. This strategy has successfully allowed repeat adenovirus treatments in animals and should extrapolate extremely well to the clinic. Clinically, this strategy could prevent a rise in antibody titer, thereby maintaining preexistent

anti-Ad5 Ab titers at pretreatment levels. However, treatment in these animal models was initiated in immunologically naive animals, animals without preexistent circulating anti-Ad antibody [115–117]. This does not represent the situation in the clinic where many patients already have preexistent anti-Ad antibodies [118–120]. Transient immunosuppression would not be expected to suppress the levels of these preexistent circulating antibodies. Three approaches have been explored to overcome preexistent adenovirus antibody: inducing immunologic tolerance [120, 121], the use of another of the 51+ different adenovirus serotypes [122–124], or the use of polyethylene glycol (PEG) to block adenovirus virions from antibody binding [125–127]. All three approaches show promise, but all may also be encumbered by practical considerations. The induction of tolerance may be time-consuming, sporadic, and unpredictable. Changing serotypes changes the drug, implying separate costly drug approval pathways. The conjugation of PEG to purified virions to successfully block antibody interactions incurs questions of also blocking the virus from the interaction with the CAR [128,129] as well as virus penetration, internalization, and uncoating [127, 129]. In addition, large-scale purification of conjugates from nonreacted components during manufacturing will be required [130].

Surprisingly, preexistent adenovirus antibody has not impaired the therapeutic activity of adenoviruses directly injected into tissues in animal studies or in human clinical trials [118, 131–134]. Presumably, the therapeutic dose of viruses directly injected into tissues overwhelms the level of free adenovirus NAb available. We have been interested in systemic administration of adenovirus for the treatment of metastatic prostate cancer. Such treatment requires intravascular injection of adenovirus to achieve virion distribution to the multiple distant tumor sites. In the vascular compartment, adenovirus will be subject to the opsonizing activity of TAb and the neutralizing activity of NAb; both could adversely affect efficacy [115, 131]. There is considerable evidence that repeat administrations of adenovirus-based therapeutics in the face of mounting specific anti-adenovirus immune responses eliminates efficacy [98, 112, 115, 135].

A. Preexisting Adenovirus Antibodies Inhibit Systemic Toxicity and Antitumor Activity

In order to better understand the effect of preexisting antibody on ARCA-mediated toxicity and anti-tumor efficacy, we first developed an animal model controlling both the dose of the intravascularly administered adenovirus and the level of preexistent antibody while measuring the virus-induced toxicity and the anti-tumor activity. To this end we prepared hyperimmune sera to purified adenovirus in rabbits, purified the rabbit total IgG containing high titer adenovirus antibody, and passively injected the purified rabbit anti-Ad5

antibody into Balb/C *nu/nu* mice bearing LNCaP prostate cancer xenografts. This established a measurable humoral anti-Ad5 antibody titer. The prostate specific adenovirus, CV706, was injected into the tail vein (iv) of animals, and toxicity and anti-tumor activity was measured. First, preexisting neutralizing antibodies were found to be protective of nude mice from liver toxicity. Adenoviruses injected intravenously into mice have been shown to cause liver toxicity from a rapid induction of chemokines and neutrophils that lead to hepatic necrosis and apoptosis [44, 86, 136–138]. In the absence of preexistent antibody, the lethal dose (LD_{100}) was 2.5×10^{11} CV706 particles, whereas 1×10^{11} CV706 particles was not lethal. Yet, in the presence of a 1:80 preexistent titer of Ad5 NAb, iv injection of 5×10^{11} CV706 particles was no longer lethal. Serum ALT levels started to elevate 48 h after viral injection in the animals that had no or low titers of preexistent antibodies (1:10 or 1:20), and increased progressively, reaching peak levels within 72–96 h. However, the animals injected with the same lethal dose of CV706 but containing a 1:80 level of preexistent anti-Ad5 NAb exhibited normal or only slightly elevated ALT levels during the entire treatment period (Table II). In this study, a maximum tolerated dose of CV706 with a 1:80 pre-existent NAb titer was not found.

Secondly, no effect of preexistent antibodies on the uptake of CV706 in different organs was found in the animals after receiving virus [76]. Balb/C *nu/nu* mice, pretreated with purified IgG (NAb titer 1:80) or buffer (no neutralizing antibodies), were administered 2.5×10^{11} particles of CV706 by a single tail vein injection. As expected from prior observations [139], the majority of Ad DNA was accumulated in liver and a small percentage of DNA was found in spleen, whereas Ad DNA was undetectable in blood, lung, heart, or kidney. The level of Ad genome in liver was equivalent with or without preexistent anti-Ad5 Ab at 30 min and at 2 h after CV706 injection. However, at 24 h postinjection, Ad DNA was undetectable in the liver of mice with preexistent antibodies, whereas a significant amount of Ad DNA was still present in the liver of animals devoid of preexistent Ad5 Ab. No Ad DNA was detectable in liver for both treatment groups 72 h after virus administration. This observation suggests that the reduced amount of Ad DNA in liver of mice with preexistent antibodies at 24 h is not due to virus redistribution, but may be related to accelerated virus DNA degradation in the liver. Most likely, virus complexed with antibody remains in the endosomes and is subjected to lysosomal degradation processes, an outcome that may not trigger the adenovirus toxicity response.

Third, preexistent anti-Ad5 Ab was found to be critical to the outcome of CV706 anti-tumor efficacy. In our previous work, we have shown the curative antitumoral activity of a single intratumoral injection of CV706 [21]. In the same animal model following a single intravenous tail vein injection we showed CV706 could inhibit additional tumor growth. With our second generation virus, CV787, a single intravenous administration could eliminated distant

Table II
Preexisting Ad-Neutralizing Antibody Protects Animals from Liver Toxicity

Treatment (particle/mouse)	Preexisting Ab	Animal survival rate(%)	Clinical symptom	ALT (unit/L)				Infectious virus ($TCID_{50}$ unit/mL)	
				D1	2	3	4	4 h	24 h
2.5×10^{11}	0	0	Weakness, jaundice, loss of body weight	175	2700	19,150		3.7×10^5	7.2×10^2
2.5×10^{11}	1:10	0	Weakness, jaundice, loss of body weight	105	1955	13,450	14,600	1.2×10^4	3.8×10^2
2.5×10^{11}	1:20	50	Weakness, jaundice, loss of body weight	N/A	N/A	N/A	N/A	3.0×10^4	ND
2.5×10^{11}	1:80	100	Healthy	N/A	450	150	85	3.7×10^2	ND

preexistent tumors [22]. These reports showed that the nonspecific immunity, most notably the uptake of adenovirus by liver Kupffer cells [44, 139], does not eliminate the anti-tumor activity following intravascular administration of these viruses. Our *in vivo* studies demonstrated that 1×10^{11} particles of CV706 inhibited additional growth of LNCaP mouse tumor xenografts in the absence of preexistent anti-Ad5 NAb. This anti-tumor activity was also shown using the same dose but in the presence of 1:20 preexistent anti-Ad5 Ab. However, in the presence of preexistent antibodies (1:80), tumors in animals that were iv injected with 1×10^{11}particles CV706 grew progressively, a growth similar to the growth found in the group that received 1:80 antibody but no CV706. These results indicate that at a low preexistent anti-Ad5 antibody titer of 1:20, 1×10^{11} CV706 particles can overwhelm the available antibody and retain anti-tumor activity. However, higher preexistent anti-Ad5 Ab (1:80) can eliminate the anti-tumor activity of intravenously administered CV706. The anti-tumor activity of CV706 can be restored in the face of a preexistent 1:80 antibody titer by a threefold higher dose of CV706 (3×10^{11} particles/mouse). The amount of infectious CV706 in the circulation of mice showed an antibody titer-dependent pattern. In animals that had no preexistent antibodies or had low levels (1:10 or 1:20) of preexistent anti-Ad5 NAb and had received 1×10^{11} infectious CV706 particles, infectious virus was detectable in the circulation 4 h after administration of CV706. In contrast, no infectious CV706 was found in mice that received a higher level of neutralizing antibodies (1:80) but the same dose of CV706. Interestingly, infectious CV706 was once again found in mice with a preexistent antibody (1:80) following a threefold higher dose of CV706 (3×10^{11} particles). These results suggest that both the level of circulating neutralizing antibodies and the dose of administered viruses play significant roles in determining therapeutic toxicity and efficacy of intravenously administered CV706.

Clearly, preexistent humoral antibody to Ad5 could present a serious barrier to the clinical application of systemically administered adenovirus. However, our data also indicate that therapeutic efficacy could be achieved by administering a higher dose of viruses, or by decreasing the levels of circulating neutralizing antibodies.

The December 1999 RAC meeting, convened after the death in Philadelphia of Jesse Gelsinger, explored the then available data with adenovirus administered intravenously. It was apparent from that meeting that while investigators at the University of Pennsylvania, Onyx, Aventis, and Schering-Plough were monitoring for the existence of preexistent antibody to adenovirus, none were using preexistent antibody levels as a patient inclusion/exclusion criteria. The data presented above caused us to adopt a strategy we call SIAPA, for screening and immunoapheresis of preexistent antibody (described below). Unfortunately, the data also showed that not prescreening patients for preexistent antibody levels and proceeding with a dose escalation Phase I clinical

trial could lead to an uncontrolled clinical trial. Namely, if a patient with a high anti-Ad5 antibody received a given dose of adenovirus administered intravenously showed no efficacy and no toxicity, the clinical answer is to proceed to the next higher dose. However, if the next patient had a low or undetectable antibody titer, the next dose of virus could be lethal.

B. SIAPA: Screening and Immunoapheresis of Preexistent Antibody for Monitoring and Removing Preexistent Ad5 Antibodies from Blood

Based on the observation that toxicity and anti-tumor efficacy of intravascular CV706 treatment is inversely proportional to the titer of circulating anti-Ad5 antibodies, we became interested in the clinical use of an affinity column consisting of recombinant capsid proteins to specifically remove anti-adenovirus antibodies from human sera. Such an affinity column could be used in conjunction with apheresis to become immunoapheresis. During the apheresis procedure, the serum could pass through a column of Ad5 antigen, specifically removing anti-adenovirus antibodies. Clinically, patients could be screened for the titer of preexistent anti-Ad5 Ab with the rapid 5′ disposable immunoassay. Those patients with a preexistent antibody titer above a given cutoff value would be candidates for adenovirus antibody immunoapheresis. After several hours of immunoapheresis, the antibody screening test could be repeated, confirming the removal of anti-Ad5 antibodies. Since anti-Ad5 antibodies would be expected to repopulate the vascular compartment at the rate of 5% per hour, a temporal window of several hours for intravascular adenovirus therapy could be created [140, 141].

The relevant preexistent anti-Ad5 antibodies are directed against the three principal capsid protein components: hexon, penton, and fiber [142, 143]. The Ad5 fiber, hexon and penton genes were cloned into the bacterial expression vector pQE-30. The recombinant proteins were expressed in *E. coli* M15 cells with IPTG induction and visualized as 120 kDa (hexon), 82 kDa (penton), and 62 kDa (fiber) polypeptide proteins by denaturing SDS–PAGE and Western blotting. These results agreed well with sizes of hexon, fiber, and penton proteins derived from the purified virions. To test the feasibility of removing anti-Ad antibodies from serum with the recombinant Ad5 antigens, we built three types of column by individually coupling the purified hexon, penton, or fiber proteins to the Ni-NTAmatrix. A human clinical serum sample collected during the CV706 clinical trial at Johns Hopkins Oncology Center was used to test the ability of the columns to remove anti-Ad5 antibody. The patient serum collected 15 days after administration of CV706 had an anti-Ad5 NAb titer of 1:3200. The patient serum was passed through each column separately or sequentially. The processed serum was tested for the efficiency and specificity of removing anti-Ad5 antibodies and found that total anti-Ad5

Table III
Efficiency of Ad Antigen Column to Remove Ad Antibodies from Plasma

Patient sera	NAb tier		TAb (μg/ml)	
	Before	After	Before	After
Serum-1	1:400	<1:10	15.6	0
Serum-2	1:1600	<1:10	75.6	3.7
Serum-3	1:400	<1:10	158.5	9.6
Serum-4	1:1600	<1:10	498.4	8.7
Serum-5	1:400	<1:10	604.4	19.8
Serum-6	1:400	<1:10	65.1	0.56

IgG was significantly reduced after passing through the columns. Table III showed that after passage through all three columns, neutralizing antibodies in sera were completely eliminated in all six samples and total anti-Ad IgG was reduced to 0.5 to 5% of initial levels (Table III). Further study demonstrated that the depletion of anti-Ad antibodies by an Ad antigen column was specific to Ad capsid proteins. This immunoapheresis column for specific removal of Ad antibody is currently under development at Calydon.

VI. Clinical Development of CV706 and CV787

CV706 and CV787 are novel therapeutic agents with a novel mechanism of action. As of this writing, a phase I/II clinical trial of CV706 for recurrent local prostate cancer has been completed and CV787 has entered a multicenter Phase I/II clinical trial for metastatic, hormonal refractory prostate cancer. A brief summary for CV706 trial result and factors impacting clinical efficacy and safety are discussed.

A. CV706 Phase I/II Trial for Locally Recurrent Prostate Cancer

A Phase I trial of CV706 was initiated in 1998 at the Brady Urological Institute of the Johns Hopkins Oncology Center under the direction of Jonathan Simons, MD, and Ted DeWeese, MD. The patient population consists of men with locally recurrent prostate cancer with rising PSA levels following definitive external beam irradiation. Men in this category are usually left untreated or receive androgen ablation therapy as serum PSA levels rise significantly above 10 ng/mL. On average, these men have a life expectancy of 3 years. The virus was administered under spinal anesthesia using the brachytherapy template

and ultrasound 3D imaging using the MMS Terapac Plus 6.6 B3DTUI (Charlottesville, VA) treatment-planning software for implantation of radioactive seeds. Virus was initially administered with 0.1 mL aliquots from up to 40 brachytherapy needles. PSA levels were determined and biopsies obtained.

Systemic toxicity was minimal and limited to brief Grade 1 fever with or without an associated chill. These episodes were self-limited, responded to routine anti-pyretics, and no patient required antibiotics. This phenomenon is consistent with previously reported series using intratumoral injections of replication-competent adenovirus (Onyx-015) [144, 145] and likely represents cytokine release (e.g., IL-1, IL-6, TNF-α) in response to the adenovirus [146, 147]. A transient non-clinically significant lymphopenia confined to the normal range was noted in a majority of patients (95%) within 24 h of viral instillation. Full recovery of cell counts occurred within 4 to 7 days posttreatment. As with the transient fever, the timing of this decrement combined with the quick recovery are consistent with an acute-phase reaction mediated by transient cytokine release, as occurs with a variety of agents including bisphosphonates, and are not consistent with viral induced bone marrow suppression [148, 149]. Importantly, treatment with CV706 was not associated with significant hepatic or coagulation abnormalities. No patient experienced ≥Grade 2 elevation of liver transaminase levels and no patient had evidence of alteration in PT or PTT or a decrement in fibrinogen. This safety was evident even at the highest dose level of 1×10^{13} viral particles and with viral shedding into the blood as documented in this study. Taken together, these data reveal a high degree of safety and tolerability of CV706 when administered by intraprostatic injection.

The analysis of secondary study end points provided compelling evidence of CV706 activity. Serum PSA is well known to be a marker of both disease activity as well as disease burden and the use of serum PSA as a marker of therapeutic efficacy has become increasingly well defined. Several investigators have correlated declines in serum PSA of greater than 50% with prolongation of survival in men with hormone refractory prostate cancer [150, 151]. In addition, other investigators have found that a slowly rising PSA following definitive management with radiation or surgery is associated with an increased time to clinically evident metastatic disease when compared to patients with a more rapid PSA doubling time [152, 153].

Moreover, there was a statistically significant reduction in the PSA velocity following treatment with CV706, most pronounced for patients in dose levels 4 and 5, again suggestive of a dose–response relationship. In the final two dose levels, 50% of treated patients achieved a PSA partial remission (PR). It also appears that treatment with CV706 resulted in a prolongation of the time required for the serum PSA to double, suggesting a slowing of cancer growth within the prostate even among individuals not achieving a PR as defined by the protocol. It is well known that biopsy of the prostate results in significant elevations in serum PSA for 2 weeks [154, 155]. It is

likely that while the design of this study, with frequent posttreatment biopsies, aided in the documentation of viral replication, these same invasive procedures prevented a full analysis of the PSA response to therapy with CV706. Thus, it is possible that significant reductions in serum PSA could have been obscured by these frequent prostatic manipulations. Despite this possibility, the evidence gathered on PSA levels following treatment with CV706 are encouraging and suggest that at the higher dose levels, a clinically meaningful treatment effect may be achievable.

This treatment effect at higher doses is associated with histologic evidence of viral replication. The viral inclusions seen on electron microscopy are consistent with viral replication in prostate epithelial cells. The positive staining for hexon protein seen on immuohistochemistry from day 4 biopsy materials is also confined to prostatic epithelial cells, is greatest in the highest dose levels, and, like the electron microscopy, is highly suggestive of intraprostatic replication of CV706 in these patients. Therefore, there is appropriate rationale for optimism given these findings in men treated with CV706, particularly at the high dose level. We believe these data to be significant and warrant CV706 evaluation in a Phase II study.

Importantly, we were able to rigorously document viral circulation in the blood following intraprostatic delivery of CV706 without significant associated clinical sequale. The quantitative PCR assay is very specific for CV706 and is capable of detecting 1300 copies per milliliter of plasma. These results confirm that a small but significant amount of the intraprostatically administered virus reached the circulation. The amount of virus released in the first "peak" varied between patients and did not appear to be related to the dose level or neutralizing antibody titer. The highest total amount of virus detected was in two patients (patients 12 and 14) with an estimate of less than 2% of the dose being detected. Circulating virus then became undetectable analogous to a virus "eclipse." A significant secondary "peak" of circulating CV706 was observed in most patients within 3 days of treatment, suggestive of viral replication in these patients. The appearance and size of the secondary "peak" seemed to correlate best inversely with the anti-Ad5 antibody titer at the time of treatment. These data are consistent with those derived from electron microscopy and immunohistochemistry, and are highly suggestive of CV706 replication in the human prostate.

Response of PSA to CV706 delivered directly into the prostate was not correlated with the presence of preexisting Ad5 neutralizing antibodies. As expected, following CV706 administration, most patients developed Ad5-neutralizing antibodies. However, development of neutralizing antibodies failed to correlate with response to treatment. Moreover, our data also reveal that the presence of preexisting anti-Ad5 antibodies was not correlated with treatment-related toxicity. These data extend the previously reported

work on intratumoral delivery of adenovirus by revealing a lack of association between preexisting neutralizing antibodies and treatment efficacy and toxicity [118, 133].

While circulating anti-Ad5 antibody may significantly impact on the efficacy and toxicity of systemically administered adenovirus [75, 86], it is not clear that these antibodies have the same access to the tumor-bearing prostate and thus may have a limited impact on direct intratumoral injections [156].

In summary, these data reveal that CV706 is safe and not associated with irreversible serious short- or long-term side effects when delivered by intratumoral injection using a planned, stereotactic approach. These data also suggest that CV706 replicates selectively in prostatic epithelial cells, *i.e.*, those prostate cells that make PSA, and does so in a time frame consistent with an adenovirus replication cycle. These data suggest that CV706 has significant biologic activity as evidenced by significant durable dose-related decreases in patient serum PSA. Indeed, three of five men treated with 1×10^{13} particles of CV706 experienced a PSA partial response. Thus, CV706 delivered by brachytherapy is an excellent candidate for the treatment of organ-confined prostate cancer.

B. Factors Impacting Clinical Efficacy and Safety

The pathogenesis of adenoviral infections is influenced by a large number of factors, some pertaining to the virus and others pertaining to the host defenses of the virus. Important issues for the virus include the route of infection, the size of the virus inoculum, the tropism of the virus for different-cell types, and whether the virus spreads directly from cell to cell or through extracellular fluid. Clearly, the vascularization of tumors, the leakiness of capillaries to virus, and the physical size of virus particle will affect intratumoral virus distribution. In the replication efficiency of the virus in prostate tumor cells, both the time of the replication cycle and the burst size are also important. Host defenses include mechanical defenses (epithelia, mucosal, liver Kupffer cells, or the blood–brain barrier), nonspecific immune defenses (interferons, recognition of infected cells by natural killer cells, release of cytokines, macrophage recruitment and activation, and triggering of complement and kinin cascades), and specific immune defenses (humoral immunity, mostly IgM and IgG but also IgA, IgD, and IgE, and finally cell-mediated immunity) [152].

In adenovirus-mediated prostate cancer therapy, the virus can be either injected directly into the tumor or administered by intravenous injection. In either case, the dose of virus is massive (10^{11}–10^{15} particles) compared to natural, vaccine-induced adenovirus infections (10^{0}–10^{6} particles) [153, 157, 158], or the clinical trials with wild-type adenovirus (10^{7}–10^{9} particles) [14]. Very little is known about the human host response to large doses of adenoviruses [14, 159] and nothing is known about the human host response

to using the intravenous route of administration of large doses of replicating adenoviruses. Liver toxicity of virion proteins may be limiting at these high doses.

Therapeutic antibody studies have indicated that antibodies do not effectively penetrate the core of a solid tumor; extravasation is limited to the tumor periphery. This suggests that the accessibility of replicating virus to antibody binding should be minimal following direct intratumoral injection [98, 131]. Cell-mediated immunity directed toward infected tumor cells may actually enhance the efficacy of replicating viruses in cancer patients if enough replication and spread occur initially. However, a systemically delivered replicating adenovirus is going to face several potential hurdles: (1) the nonspecific removal of adenovirus by liver Kupffer cells, (2) the inactivation of virus by preexisting circulating antibodies to adenovirus, (3) a limitation of viral replication mediated by a vigorous CTL response to virally infected cells, and (4) a limitation of the efficacy of repeat dosage by primary or secondary induction of humoral immunity.

Incorporation of the Ad5 genome into germ cells has been expressed as a concern but has not been found for any of the Ad5 gene therapy constructs. Indeed, adenovirus gene expression is characterized as transient in nature due to a lack of viral DNA integration. Virus shedding has been expressed as a concern but it has not been detected in any Ad5 clinical trial to date. In our clinical trial, virus replication was detected after 2–8 days but was undetectable after 2 weeks. It is difficult to estimate the increased cytolytic activity in humans of CV787 compared to CV706. However, replicating adenoviruses containing hepatitis B surface antigen (HbsAg), with and without the E3 region, have been tested in chimpanzees, a system permissive for infection by human adenoviruses [160]. In this study, the addition of the E3 region resulted in 10- to 100-fold increase in virus shedding and a 10- to 100-fold increase in titer to HBsAg. However, one should not lose sight of the fact that adenoviruses are ubiquitous. Twenty-three percent of normal healthy infants are seropositive for adenoviruses by 7 months of age [95] and CV787 is attenuated 10,000:1 compared to the wild-type virus. We believe the therapeutic use of CV787 will be safe; the major question is whether or not there is sufficient efficacy to be medically useful.

VII. Summary

Safety of administrating wild-type Ad5 either intratumorally and intravenously was demonstrated at intermediate doses (10^7 to 10^9 particles) over 40 years ago [14]. None of the treated patients had significant side-effects. Safety and efficacy will be the major issues as adenovirus doses escalate from 10^{11} to 10^{15}particles. CV787 is a replication-competent adenovirus attenuated 10,000:1 compared to the wild-type virus in PSA^- cells. This is an

unprecedented therapeutic index for a cytotoxic agent as measured *in vitro*. CV787 is capable of eliminating distant mouse xenograft tumors with a single intravenous injection. Synergy of prostate cancer-specific adenovirus variants has been demonstrated when combined with conventional therapies including radiotherapy and chemotherapy. Synergy of CV787 and docetaxel has opened a single-dose curative therapeutic window in excess of 1,000:1 *in vivo*. This progress, coupled with screening and immunoapheresis to control and remove preexistent antibody to adenovirus [75, 161], may overcome the hurdles and achieve clinical benefits with intravenous administration of adenovirus-based therapeutics. Ongoing clinical trials of agents such as CV787 will resolve the issues of safety and efficacy and hopefully point to a new mode of cancer chemotherapy, one that includes the use of targeted cytolytic adenoviruses.

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CHAPTER 11

Replication-Selective Oncolytic Adenovirus E1-Region Mutants: Virotherapy for Cancer

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I. Introduction

Chemotherapy for metastatic solid tumors generally fails due to an insufficient therapeutic index and/or insufficient antitumoral potency. Standard agents target a variety of different structures within cancer cells, but almost all of them are thought to kill cancer cells through the induction of apoptosis. As a result, apoptosis-resistant clones develop following standard therapies, even if numerous high-dose chemotherapeutic agents are used in combination. New treatment approaches should therefore have not only greater potency and greater selectivity than currently available treatments, they should also have novel mechanisms of action that will not be subject to cross-resistance with standard treatments (i.e., efficacy should not be dependent on apoptosis induction in cancer cells exclusively).

Replication-selective oncolytic adenoviruses appear to have these characteristics. These viruses have evolved to infect cells, replicate, induce cell death, release viral particles, and finally spread in human tissues. Replication in tumor tissue leads to amplification of the input dose, while a block in replication in normal tissues can lead to efficient clearance and reduced toxicity (Fig. 1). Selective replication within tumor tissue can theoretically increase the therapeutic index of these agents dramatically. In addition, viruses kill cells by a number of unique mechanisms. In addition to direct lysis at the conclusion of the replicative cycle, viruses can kill cells through expression of

toxic proteins, induction of both inflammatory cytokines and T-cell-mediated immunity, and enhancement of cellular-sensitivity to their effects. Therefore, cross-resistance with standard chemotherapeutics or radiotherapy should be much less likely.

Remarkable advances in molecular biology and genetics have led to a new understanding of both (1) the replication and pathogenicity of viruses and (2) carcinogenesis. The resulting techniques and knowledge have allowed novel agents to be engineered to enhance their safety and/or their antitumoral potency. Genetically engineered viruses in development over the past decade have included adenoviruses, herpesviruses, and vaccinia. Viruses with inherent tumor-selectivity have been evaluated and include reovirus, autonomous parvoviruses, Newcastle disease virus, measles virus strains, and vesicular stomatitis virus [1]. Each of these agents has shown tumor selectivity *in vitro* and/or *in vivo*, and efficacy has been demonstrated in murine tumor models with many of these agents following intratumoral, intraperitoneal, and/or intravenous routes of administration.

Preclinical data reported with these agents has been encouraging, but a number of important questions have awaited results from clinical trials. Viral agents like adenovirus have complex biologies, potentially including

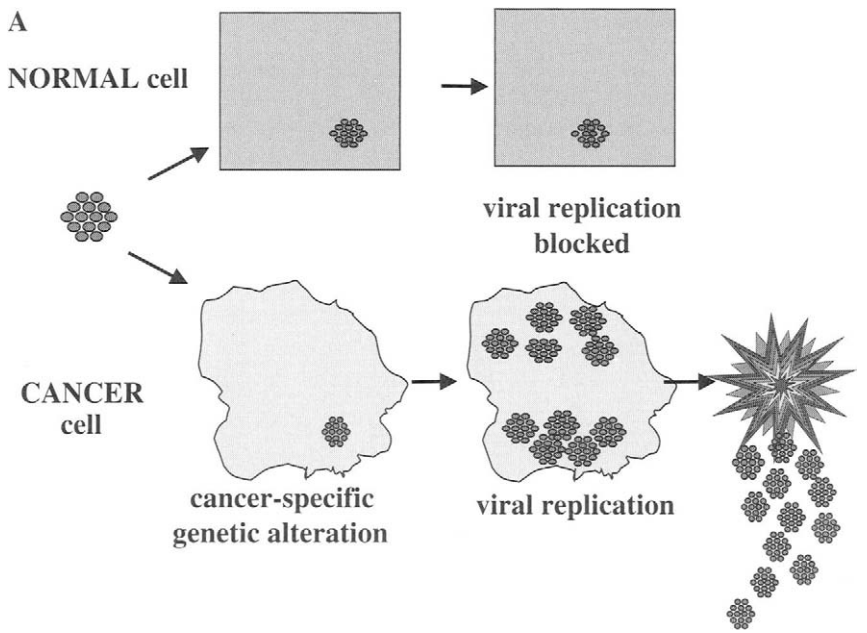

Figure 1 Schematic representation of tumor-selective viral replication and cell killing (A) and tumor-selective tissue necrosis (B). Reprinted with permission from *Journal of Clinical Investigation*.

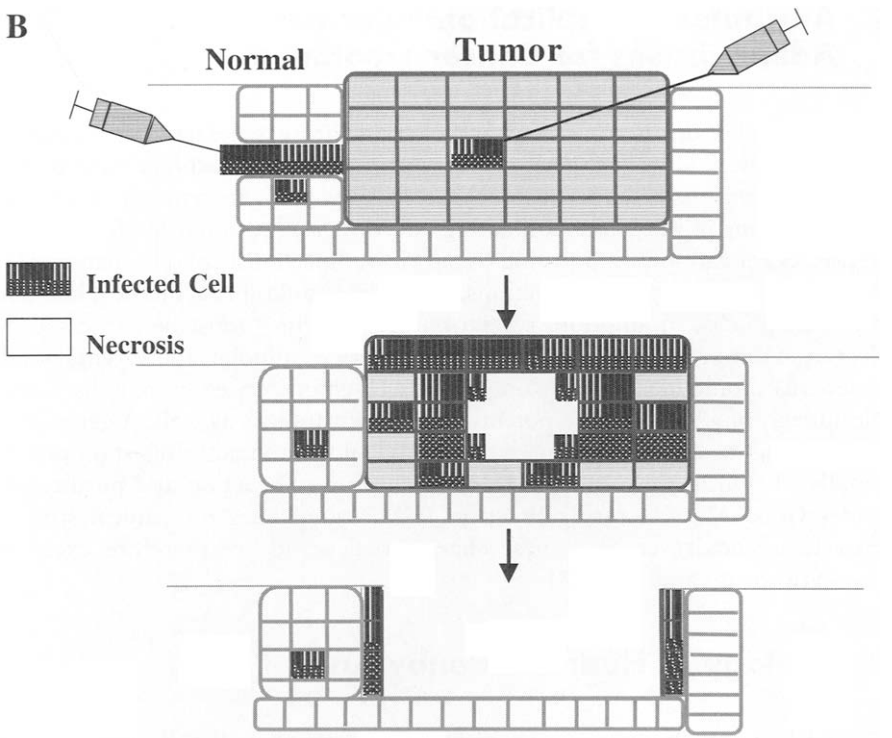

Figure 1 *(continued)*

species-specific interactions with host cell machinery and/or immune response effectors [2, 3]. Antitumoral efficacy and safety studies with these viruses have generally been performed in rodent or primate models. All published animal tumor model data with replication-selective adenoviruses has come from immunodeficient mouse–human tumor xenograft models [4–6]. Data from cancer patients has therefore been eagerly awaited. Now, after more than 5 years of clinical research with *dl*1520 (Onyx-015 or CI-1042, Pfizer Corp., Groton, CT), approximately 15 clinical trials have been completed and recently analyzed involving approximately 250 patients.

This chapter reviews the discovery and development of replication-selective oncolytic adenoviruses, with an emphasis on recently acquired data from phase I and II clinical trials with *dl*1520. The goal will be to summarize (1) the genetic targets and mechanisms of selectivity for these agents; (2) clinical trial data and what it has taught us to date about the promise but also the potential hurdles to be overcome with this approach; (3) future approaches to overcome these hurdles.

II. Attributes of Replication-Selective Adenoviruses for Cancer Treatment

A number of efficacy, safety, and manufacturing issues need to be assessed when considering a virus species for development as an oncolytic therapy [1]. First, by definition the virus must replicate in and destroy human tumor cells. An understanding of the genes modulating infection, replication or pathogenesis is necessary for rational engineering of the virus. Since most solid human tumors have relatively low growth fractions, the virus should infect both cycling and noncycling cells. In addition, receptors for viral entry must be expressed on the target tumor(s) in patients [7]. From a safety standpoint, the parental wild-type virus should ideally cause only mild, well-characterized human disease(s). Nonintegrating viruses have potential safety advantages as well. A genetically stable virus is desirable from both safety and manufacturing standpoints. Finally, the virus must be amenable to high-titer production and purification under Good Manufacturing Practices (GMP) guidelines for clinical studies. Human adenoviruses have these characteristics and are therefore excellent oncolytic virus candidates [8].

III. Biology of Human Adenovirus

Adenovirus biology is reviewed in detail elsewhere [9]. Roughly 50 different serotypes of human adenovirus have been discovered; the two most commonly studied are types 2 and 5 (group C). Adenoviruses have linear, double-stranded DNA genomes of approximately 38 kb. The capsid is nonenveloped and is composed of the structural proteins hexon, fiber (binds Coxsackie and adenovirus receptor (CAR)), and penton (binds $\alpha_V\beta_{3,5}$ integrins for virus internalization). The adenovirus life-cycle includes the following steps: (1) virus entry into the cell following CAR and integrin binding, (2) release from the endosome and subsequent entry into the nucleus, (3) expression of early region gene products, (4) cell entry into S-phase, (5) prevention of p53-dependent and -independent apoptosis, (6) shut-off of host cell protein synthesis, (7) viral DNA replication, (8) viral structural protein synthesis, (9) virion assembly in the nucleus, (10) cell death, and (11) virus release. The E3 region encodes a number of gene products responsible for immune response evasion [10, 11]. The gp 19-kDa protein inhibits MHC-class I expression on the cell surface (i.e. avoidance of cytotoxic T-lymphocyte-mediated killing) [12], and the E3 10.4/14.5-kDa (RID complex) and 14.7-kDa proteins inhibit apoptosis mediated by FasL or tumor necrosis factor (TNF) [11, 13].

IV. Mechanisms of Adenovirus-Mediated Cell Killing

Adenovirus replication within a target tumor cell can lead to cell destruction by several mechanisms (Table I). Viral proteins expressed late in the course of infection are directly cytotoxic, including the E3 11.6-kDa adenovirus death protein [14] and E4ORF4 [Branton, 1999 #1920]. Deletion of these gene products results in a significant delay in cell death. In addition, E1A expression early during the adenovirus life cycle induces cell sensitivity to TNF-mediated killing [15]. This effect is inhibited by the E3 proteins 10.4/14.5 and 14.7; deletion of these E3 proteins leads to an increase in TNF expression *in vivo* and enhanced cell sensitivity to TNF [3]. Finally, viral replication in and lysis of tumor cells has been shown to promote the induction of cell-mediated immunity to uninfected tumor cells in model systems with other viruses [16, 17]; whether this will occur in patients and with adenovirus remains to be determined.

V. Approaches to Optimizing Tumor-Selective Adenovirus Replication

Two broad approaches are currently being used to engineer tumor-selective adenovirus replication. One is to limit the expression of the E1A

Table I
Potential Mechanisms of Antitumoral Efficacy with Replication-Selective Adenoviruses

Mechanism	Examples of adenoviral genes modulating effect
I. Direct cytotoxicity due to viral proteins	• E3 11.6 kDa • E4ORF4
II. Augmentation of antitumoral immunity	
CTL infiltration, killing	• E3 gp 19 kDa[a]
Tumor cell death, antigen release	• E3 11.6 kDa
Immunostimulatory cytokine induction	• E3 10.4/14.5, 14.7 kDa[a]
Antitumoral cytokine induction (e.g., TNF)	• E3 10.4/14.5, 14.7 kDa[a]
Enhanced sensitivity to cytokines (e.g., TNF)	• E1A
	Unknown (? E1A, others)
III. Sensitization to chemotherapy	na
IV. Expression of exogenous therapeutic genes	

Note. CTL, cytotoxic T-lymphocyte; TNF, tumor necrosis factor; NA, not applicable.
[a]Viral protein inhibits antitumoral mechanism.

gene product to tumor tissues through the use of tumor- and/or tissue-specific promoters. E1A functions to stimulate S-phase entry and to transactivate both viral and cellular genes that are critical for a productive viral infection [18]. A second broad approach to optimizing tumor selectivity is to delete gene functions that are critical for efficient viral replication in normal cells but are expendable in tumor cells (described below).

Tissue- or tumor-specific promoters can replace endogenous viral sequences in order to restrict viral replication to a particular target tissue. For example, the prostate-specific antigen (PSA) promoter/enhancer element has been inserted upstream of the E1A gene; the result is that viral replication correlates with the level of PSA expression in a given cell [4]. This virus, CN706 (Calydon Pharmaceuticals, CA), is currently in a Phase I clinical trial of intratumoral injection for patients with locally recurrent prostate carcinoma. A second prostate-specific enhancer sequence has subsequently been inserted upstream of the E1B region [19]; the use of these two prostate-specific enhancer elements to drive separate early gene regions has led to improved selectivity over the first-generation virus [19]. A similar approach has been pursued by other groups using tissue-specific promoters to drive E1A expression selectively in specific carcinomas (e.g., alpha-fetoprotein, carcinoembryonic antigen, MUC-1) [20, 21].

A second general approach is to complement loss-of-function mutations in cancers with loss-of-function mutations within the adenovirus genome. Many of the same critical regulatory proteins that are inactivated by viral gene products during adenovirus replication are also inactivated during carcinogenesis [22–25]. Because of this convergence, the deletion of viral genes that inactivate these cellular regulatory proteins can be complemented by genetic inactivation of these proteins within cancer cells [26, 78]. The deletion approach was first described by Martuza *et al.* with herpesviruses; the thymidine kinase gene (*dlsptk*) [27] and subsequently the ribonucleotide reductase gene (G207) were deleted [28]. Two adenovirus deletion mutation approaches have subsequently been described (see below).

VI. E1A–CR2 Region Deletion Mutants

Mutants in the E1A conserved region 2 (CR2) are defective in pRB binding [29, 30] (Fig. 2). These viruses are being evaluated for use against tumors with pRB pathway abnormalities (e.g., loss of the G1-S checkpoint) [26, 31, 32]. The delta-24 E1A-CR2 mutant virus was able to efficiently replicate in tumor cell lines lacking functional pRB, while replication was significantly inhibited by reintroduction of wild-type RB protein into a tumor cell line lacking functional pRB; both *in vitro* and *in vivo* efficacy were demonstrated [32]. With *dl*922/947, a very similar E1A–CR2 mutant, S-phase induction and viral

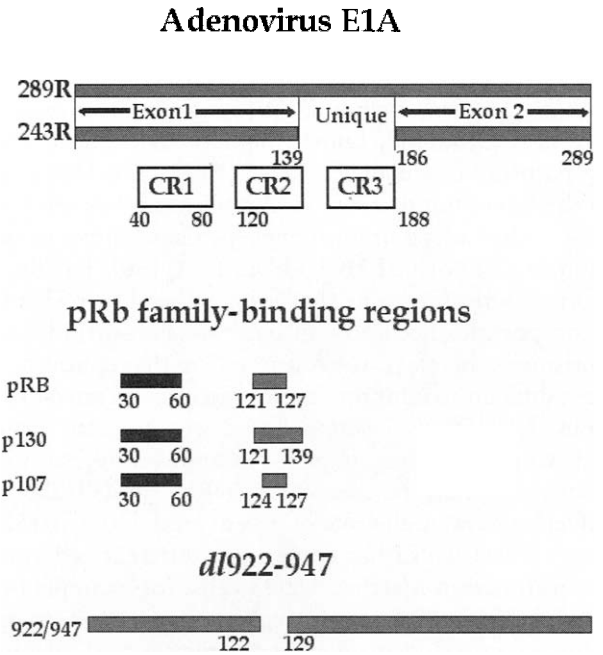

Figure 2 Diagram of the structure of the adenovirus E1A RNA (12S, 13S) and pRB protein family-binding regions showing deletion in *dl*922–947 mutant adenovirus.

replication are significantly inhibited in quiescent normal cells, whereas replication and cytopathic effects proceed efficiently in tumor cells; interestingly, *dl*922/947 demonstrates significantly greater potency than *dl*1520 both *in vitro* and *in vivo* [26, 31], and in a nude mouse–human tumor xenograft model, intravenously administered *dl*922/947 had significantly superior efficacy to even wild-type adenovirus [31]. The E1A mutant adenoviruses described by these two groups may in fact behave very similarly, although to date they have been tested in different fashions. Unlike the complete deletion of E1B 55 kDa in *dl*1520, these mutations in E1A are targeted to a single conserved region and may therefore leave intact other important functions of the gene product; therefore, viral potency is not attenuated.

VII. E1B 55-kDa Gene Deletion Mutant: *dl*1520

*dl*1520 (Onyx-015) was the first adenovirus described to mirror the gene deletion approach pioneered by Martuza with herpesvirus. McCormick *et al.*

hypothesized that an adenovirus with deletion of a gene encoding a p53-binding protein, E1B 55-kDa, would be selective for tumors that already had inhibited or lost p53 function. p53 function is lost in the majority of human cancers through mechanisms including gene mutation, overexpression of p53-binding inhibitors (e.g., mdm2, human papillomavirus E6), and loss of the p53-inhibitory pathway modulated by $p14^{ARF}$ [33–35]. However, the precise role of p53 in the inhibition of adenoviral replication has not been defined to date. In addition, other adenoviral proteins also have direct or indirect effects on p53 function (e.g., E4orf6, E1B 19-kDa, E1A) [36]. Finally, E1B 55-kDa itself has important viral functions that are unrelated to p53 inhibition (e.g., viral mRNA transport, host cell protein synthesis shut-off) [37] (Fig. 3).

Not surprisingly, therefore, the role of p53 in the replication-selectivity of *dl*1520 has been difficult to confirm despite extensive *in vitro* experimentation by many groups. E1B 55-kDa gene deletion was associated with decreased replication and cytopathogenicity in p53(+) tumor cells versus matched p53(-) tumor cells, relative to wild-type adenovirus, in RKO and H1299 cells [38–40]. However, conflicting data on the role of p53 in modulating *dl*1520 replication and/or cytopathic effect (CPE) has come from different cell systems; no p53 effect was demonstrated in matched U2OS cells, for example [40]. Although p53 can clearly inhibit *dl*1520 in many cell systems, it has become clear that many other cellular factors independent of p53 play critical roles in determining

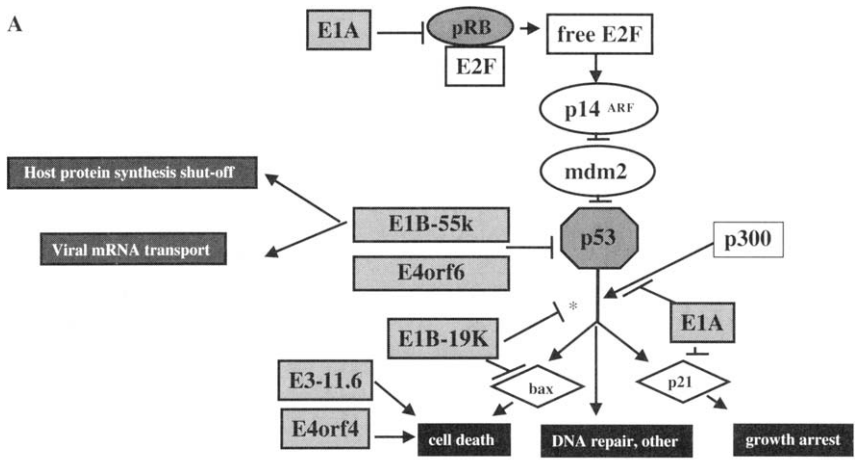

Figure 3 Diagram of both p53 pathway interactions with adenoviral gene products and functions of E1B 55 kDa: complexity of cancer cell and adenoviral biology. (A) Note that adenoviral proteins (light gray) target multiple components of this pathway at sites upstream of p53, downstream of p53 and at the level of p53 itself. Examples of p53-regulated cell functions are shown (black boxes). In addition, the known functions of E1B 55 kDa are shown (dark gray). (B) Graphic representation of the consequences of E1B 55-kDa gene deletion. In addition to the loss of p53 binding when E1B 55 kDa was deleted in *dl*1520 (Onyx-015), other important viral functions are also lost.

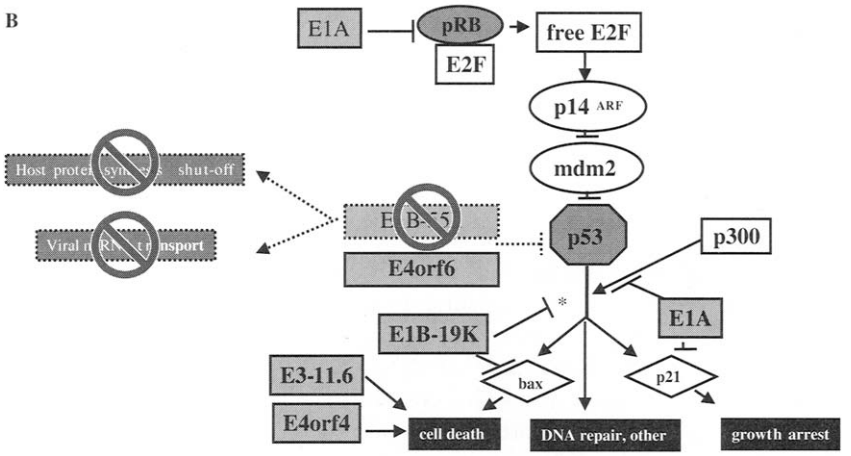

Figure 3 (*continued*)

the sensitivity of cells to *dl*1520 [39, 41–44]. Clinical trials were ultimately necessary to determine the selectivity and clinical utility of *dl*1520 (see below). Clinical trial data confirmed the tumor-selectivity of *dl*1520.

VIII. Clinical Trial Results with Replication-Competent Adenoviruses in Cancer Patients

A. Clinical Trial Results with Wild-Type Adenovirus

Over the past century a diverse array of viruses were injected into cancer patients by various routes, including adenovirus, Bunyamwara, Coxsackie, dengue, feline panleukemia, Ilheus, mumps, Newcastle disease virus, vaccinia, and West Nile [1, 45–47]. These studies illustrated both the promise and the hurdles to overcome with oncolytic viral therapy. Unfortunately, these previous clinical studies were not performed to current clinical research standards, and therefore none give interpretable and definitive results. At best, these studies are useful in generating hypotheses that can be tested in future trials.

Although suffering from many of the trial design flaws listed below, a trial with wild-type adenovirus is one of the most useful for hypothesis generation but also for illustrating how clinical trial design flaws severely curtail the utility of the study results. The knowledge that adenoviruses could eradicate a variety of tumor cells *in vitro* led to a clinical trial in the 1950s with wild-type adenovirus. Ten different serotypes were used to

treat 30 cervical cancer patients [47]. Forty total treatments were administered by either direct intratumoral injection ($n = 23$), injection into the artery perfusing the tumor ($n = 10$), treatment by both routes ($n = 6$), or intravenous administration ($n = 1$). Characterization of the material injected into patients was minimal. The volume of viral supernatant injected is reported, but actual viral titers/doses are not; injection volumes (and by extension doses) varied greatly. When possible, the patients were treated with a serotype to which they had no neutralizing antibodies present. Corticosteroids were administered as nonspecific immunosuppressive agents in roughly half of the cases. Therefore, no two patients were treated in identical fashion.

Nevertheless, the results are intriguing. No significant local or systemic toxicity was reported. This relative safety is notable given the lack of preexisting immunity to the serotype used and concomitant corticosteroid use in many patients. Some patients reported a relatively mild viral syndrome lasting 2–7 days (severity not defined); this viral syndrome resolved spontaneously. Infectious adenovirus was recovered from the tumor in two-thirds of the patients for up to 17 days postinoculation.

Two-thirds of the patients had a "marked to moderate local tumor response" with necrosis and ulceration of the tumor (definition of "response" not reported). None of the seven control patients treated with either virus-free tissue culture fluid or heat-inactivated virus had a local tumor response (statistical significance not reported). Therefore, clinically evident tumor necrosis was only reported with viable virus. Neutralizing antibodies increased within 7 days after administration. Although the clinical benefit to these patients is unclear, and all patients eventually had tumor progression and died, this study did demonstrate that wild-type adenoviruses can be safely administered to patients and that these viruses can replicate and cause necrosis in solid tumors despite a humoral immune response. The maximally tolerated dose, dose-limiting toxicity, objective response rate, and time to tumor progression, however, remain unknown for any of these serotypes by any route of administration.

B. A Novel Staged Approach to Clinical Research with Replication-Selective Viruses: The Example of *dl*1520 (Onyx-015)

For the first time since viruses were first conceived as agents to treat cancer over a century ago, we now have definitive data from numerous phase I and II clinical trials with a well-characterized and well–quantitated virus. *dl*1520 (Onyx-015, a.k.a., CI-1042, Pfizer, Inc.) is a novel agent with a novel mechanism of action. This virus was to become the first virus to be used in humans that had been genetically engineered for replication selectivity. We predicted that both toxicity and efficacy would be dependent on multiple

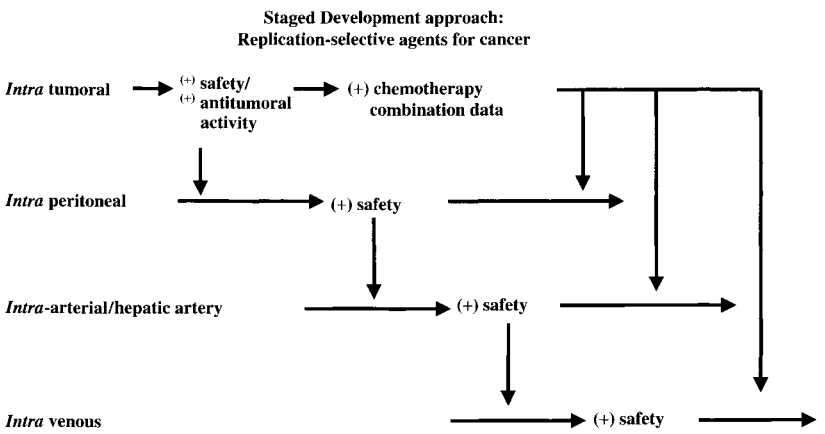

Figure 4 Staged clinical research and development approach used in research and development with *dl*1520 (Onyx-015). Once safety and biological activity was demonstrated by the intratumoral route, clinical trials were initiated sequentially to study intracavitary instillation (initially intraperitoneal), intraarterial infusion (hepatic artery) and eventually intravenous administration. Only patients with advanced and incurable cancers were enrolled on trials initially. Once safety was demonstrated in these patients, trials were initiated in patients with premalignant lesions. Finally, clinical trials of combinations with chemotherapy were initiated only after the safety of *dl*1520 as a single agent had been documented by the relevant route of administration. Reprinted with permission from *Gene Therapy*.

factors including (1) the inherent ability of a given tumor to replicate and shed the virus, (2) the location of the tumor to be treated (e.g., intracranial vs peripheral), and (3) the route of administration of the virus. In addition, we felt it would be critical to obtain biological data on viral replication, antiviral immune responses and their relationship to antitumoral efficacy in the earliest phases of clinical research.

We therefore designed and implemented a novel staged clinical research and development approach with this virus (Fig. 4). The goal of this approach was to sequentially increase systemic exposure to the virus only after safety with more localized delivery had been demonstrated. Following demonstration of safety and biological activity by the intratumoral route, trials were sequentially initiated to study intracavitary instillation (initially intraperitoneal), intraarterial infusion (initially hepatic artery), and eventually intravenous administration. In addition, only patients with advanced and incurable cancers were initially enrolled on trials. Only after safety had been demonstrated in terminal cancer patients were trials initiated for patients with premalignant conditions. Finally, clinical trials of combinations with chemotherapy were initiated only after the safety of *dl*1520 as a single agent had been documented by the relevant route of administration.

IX. Results from Clinical Trials with *dl*1520 (Onyx-015 or CI-1042)

A. Toxicity

No maximally tolerated dose or dose-limiting toxicities were identified at doses up to $2x10^{12}$ particles administered by intratumoral injection. Flu-like symptoms and injection-site pain were the most common associated toxicities [48]. This safety is remarkable given the daily or even twice-daily dosing that was repeated every 1–3 weeks in the head and neck region or pancreas [49].

Intraperitoneal, intraarterial, and intravenous administration were also remarkably well tolerated in general. Intraperitoneal administration was feasible at doses up to 10^{13} particles divided over 5 days [50]. The most common toxicites included fever, abdominal pain, nausea/vomiting, and bowel motility changes (diarrhea, constipation). The severity of the symptoms appeared to correlate with tumor burden. Patients with heavy tumor burdens reached a maximally tolerated dose at 10^{12} particles (dose-limiting toxicities were abdominal pain and diarrhea), whereas patients with a low tumor burden tolerated 10^{13} without significant toxicity.

No dose-limiting toxicities were reported following repeated intravascular injection at doses up to 2×10^{12} particles (hepatic artery) [51] or 2×10^{13} particles (intravenous) [52]. Fever, chills, and asthenia following intravascular injection were more common and more severe than after intratumoral injections (grade 2–3 fever and chills vs grade 1). Dose-related transaminitis was reported infrequently. The transaminitis was typically transient (<10 days) and low-grade (grade 1–2) and was not clinically relevant. Further dose escalation was limited by supply of the virus.

B. Viral Replication

Viral replication was documented at early time points after intratumoral injection in head and neck cancer patients [49, 53]. Roughly 70% of patients had evidence of replication on days 1–3 after their last treatment (Table II). In contrast, day 14–17 samples were uniformly negative. Patients with injected pancreatic tumors, in contrast, showed no evidence of viral replication by plasma PCR (indirect evidence) or fine-needle aspiration. Similarly, intraperitoneal *dl*1520 could not be shown to reproducibly infect ovarian carcinoma cells within the peritoneum. Therefore, different tumor types can vary dramatically in their permissiveness for viral infection and replication (Table II).

Proof-of-concept for tumor infection following intraarterial [51] or intravenous [52] administration with human adenovirus has also been achieved. Approximately half of the roughly 25 patients receiving hepatic artery infusions

Table II
Viral Replication Data from Phase I and II Trials of *dl*1520 (Onyx-015): Intratumoral, Intraperitoneal, Intraarterial, or Intravenous Injection

Route of administration	Tumor type	Phase	Dose/cycle (particles)	Regimen/ cycle frequency	Tumor biopsy *days post-treatment* # positive/ evaluable (% +)	Blood (quantitative PCR) # positive/ evaluable (% +)
Intratumoral	Head and neck	I	2×10^8–2×10^{12}	Single dose/ q 4 week	*Day 5*: 4 / 16 (25)	Not done
Intratumoral	Head and neck	II	10^{12}	Daily × 5/q 3 week	*Days 1–3*: 5/ 7 (71) *Days 7–10*: 2/ 4 (50) *Days 14–17*: 0/ 10 (0)	*Day 10*: 2/ 19 (11) *Day 17*: 0/19 (0)
Intratumoral	Gastrointestinal—primarily colorectal	I	2×10^9–2×10^{12}	Single dose/ q 4 week		*Days 5-10*: overall—7/ 19 (37%) high dose—5/ 6 (83%) *Day 15*: 0/19 (0)
Intratumoral	Pancreatic	I	2×10^8–2×10^{12}	Single dose/ q 4 week		*Day 5*: 0/ 22 (0) *Day 15*: 0/22 (0)
Intratumoral	Pancreatic	I/II	2×10^{11}	Single dose/ day 1, 5, 8, 15		Not done
Intraperitoneal	Ovarian	I	10^{11}–10^{13}	Daily × 5/q 3 week	*Days 1–4*: 0/12	Day 5: 0/16 (0) *Day 15*: 0/16 (0)
Intra-arterial (hepatic artery)	Gastrointestinal—primarily colorectal	I	2×10^9–2×10^{11}	Single dose/ q 1 week (2 weeks on, 2 off)		
		II	6×10^{11}–2×10^{12}			*Day 3* approx. 50% (on-going)
Intravenous	Metastatic carcinoma in lung	I	2×10^8–2×10^{12}	Single dose/ q 1 week (3 weeks on, 1 off)	*Day 4*: overall—1/ 9 (11) high dose—1/ 3 (33)	*Day 8* (low dose): 0 / 4 (0) *Day 8* (high dose): 3 / 5 (60)

of 2×10^{12} particles were positive by PCR 3–5 following treatment. Since input virus genomes are cleared to undetectable levels within 6–12 h, these late recurrent peaks of viral genomes are highly suggestive of viral replication and shedding into the blood. Three of four patients with metastatic carcinoma to the lung treated intravenously with $\geq 2 \times 10^{12}$ particles were positive for genomes in the blood on day 3 (± 1). Therefore, it appears to be feasible to infect distant tumor nodules following intravenous or intra-arterial administration.

C. Immune Response

Neutralizing antibody titers to the coat (Ad5) of *dl*1520 were positive but relatively low in roughly 50–60% of all clinical trial patients at baseline [49]. Antibody titers increased uniformly following administration of *dl*1520 by any of the routes tested, in some cases to levels >1:80,000. Antibody increases occurred regardless of evidence for replication or shedding into the bloodstream [49]. Acute inflammatory cytokine levels were determined prior to treatment (by hepatic artery infusion), 3 h posttreatment and 18 h posttreatment: IL-1, IL-6, IL-10, interferon-gamma, tumor necrosis factor. Significant increases were demonstrated within 3 h for IL-1, IL-6, tumor necrosis factor, and to a lesser extent interferon-gamma; all cytokines were back down to pretreatment levels by 18 h [54]. In contrast, IL-10 did not increase until 18 h. However, cytokine levels varied greatly from patient to patient and from treatment cycle to treatment cycle.

D. Efficacy with *dl*1520 (Onyx-015) as a Single Agent

Two Phase II trials enrolled a total of 40 patients with recurrent head and neck cancer [49, 53]. Tumors were treated very aggressively with 6–8 daily needle passes for 5 consecutive days (30–40 needle passes per 5-day cycle; $n = 30$) and 10–15 per day on a second trial (50–75 needle passes per cycle; $n = 10$). The median tumor volume on these studies was approximately 25 cm^3; an average[1] $cm^3$of tumor therefore received an estimated 4–5 needle passes per cycle. With this dose-intensive local treatment regimen, the unconfirmed response rate at the injected site was 14% and the confirmed local response rate was 7%. Interestingly, there was no correlation between evidence of antitumoral activity and neutralizing antibody levels at baseline or posttreatment [49]. No objective responses were demonstrated in patients with tumor types that could not be so aggressively injected (due to their deep locations), although some evidence of shrinkage or necrosis was obtained. In summary, single agent responses across all studies were uncommon, and therefore combinations with chemotherapy were explored.

E. Efficacy in Combination with Chemotherapy: Potential Synergy Discovered

Evidence for a potentially synergistic interaction between adenoviral therapy and chemotherapy has been obtained on multiple trials. Encouraging

clinical data has been obtained in patients with recurrent head and neck cancer treated with intratumoral *dl*1520 in combination with intravenous cispaltin and 5-fluorouracil [55]. Thirty-seven patients were treated and 19 responded (54%, intent-to-treat; 63%, evaluable); this compares favorably with response rates to chemotherapy alone in previous trials (30–40%, generally). The time-to-tumor progression was also superior to previously reported studies. However, comparisons to historical controls are unreliable. We therefore used patients as their own controls whenever possible ($n = 11$ patients). Patients with more than one tumor mass had a single tumor injected with *dl*1520 while the other mass(es) was left uninjected. Since both masses were exposed to chemotherapy, the effect of the addition of viral therapy to chemotherapy could be assessed. The *dl*1520-injected tumors were significantly more likely to respond ($P = 0.017$) and less likely to progress ($P = 0.06$) than were noninjected tumors. Noninjected control tumors that progressed on chemotherapy alone were subsequently treated with Onyx-015 in some cases; two of the four injected tumors underwent complete regressions. This data illustrates the potential of viral and chemotherapy combinations. The clinical utility of *dl*1520 in this indication will be definitively determined in a phase III randomized trial.

A phase I/II trial of *dl*1520 administered by hepatic artery infusion in combination with intravenous 5-fluorouracil and leukovorin was carried out ($n = 33$ total) [54]. Following phase I dose escalation, 15 patients with colorectal carcinoma who had previously failed the same chemotherapy were treated with combination therapy after failing approximately to respond to *dl*1520 alone; 1 patient underwent a partial response (following initial progression on virus alone) and 10 had stable disease (2–7+ months). Combination virus plus chemotherapy-induced responses in colorectal liver metastases was therefore possible via hepatic artery infusions, although the magnitude and frequency of this effect remains to be determined. In addition, the optimal combination regimen has not yet been defined. In contrast, data from a phase I/II trial studying the combination of *dl*1520 and gemcitabine chemotherapy were disappointing ($n = 21$); the combination resulted in only two responses, and these patients had not received prior gemcitabine [56]. Therefore, potential synergy was demonstrated with *dl*1520 and chemotherapy in two tumor types that supported presumed viral replication (head and neck, colorectal), but not in a tumor type that was apparently resistant to viral replication (pancreatic).

X. Clinical Trial Results with *dl*1520 (Onyx-015): Summary

*dl*1520 was well-tolerated at the highest practical doses that could be administered (2×10^{12}–2×10^{13} particles) by intratumoral, intraperitoneal, intraarterial, and intravenous routes. The lack of clinically significant toxicity in the liver or other organs was notable. Flu-like symptoms (fever, rigors,

asthenia) were the most common toxicities and were increased in patients receiving intravascular treatment. Acute inflammatory cytokines (especially IL-1 and IL-6) increased within 3 h following intraarterial infusion, although these changes were extremely variable. Neutralizing antibodies increased in all patients, regardless of dose, route, or tumor type. Viral replication was documented directly (by biopsy) or indirectly (by delayed viral genome peaks in blood) in head and neck and colorectal tumors following intratumoral or intraarterial administration. Neutralizing antibodies did not block antitumoral activity in head and neck cancer trials of intratumoral injection. Single agent antitumoral activity was minimal (≅15%) in head and neck cancers that could be repeatedly injected. No objective responses were documented with single agent therapy in phase I or I/II trials in patients with pancreatic, colorectal, or ovarian carcinomas. A favorable and potentially synergistic interaction with chemotherapy was discovered in some tumor types and by different routes of administration.

XI. Future Directions: Why Has *dl*1520 (Onyx-015) Failed to Date as a Single Agent?

Future improvements with this approach will be possible if the reasons for *dl*1520 failure as a single agent, and success in combination with chemotherapy, are elucidated. Factors that are specific to this particular adenoviral mutant, as well as factors that are generalizable to other adenoviruses, should be considered. Regarding this particular E1B 55-kDa gene mutant, it is important to remember that this virus is significantly attenuated relative to wild-type adenovirus in most tumor cell lines *in vitro* and *in vivo*, including even p53 mutant tumors [31, 39, 40, 43, 57]. This is an expected finding since this virus does not have critical E1B 55-kDa functions that are unrelated to p53, including viral mRNA transport and shut-off of host protein synthesis. This attenuated potency is not apparent with the adenovirus mutant *dl*922/947 [31].

The deletion in the E3 gene region of genes for the 10.4/14.5 complex is likely to make this virus more sensitive to the antiviral effects of tumor necrosis factor; an immunocompetent animal model will need to be identified in order to resolve this issue. Factors likely to be important for any adenovirus include barriers to intratumoral spread (e.g., fibrosis), antiviral immune responses and inadequate viral receptor expression (e.g., CAR, integrins). Viral coat modifications may be beneficial if inadequate CAR expression plays a role in the resistance of particular tumor types [58, 59].

XII. Improving the Efficacy of Replication-Selective Oncolytic Adenoviral Agents

Alterations within the adenoviral genome can be used to enhance selectivity and/or potency. The promising adenoviral E1A CR-2 mutant (*dl*922/947) has been described that demonstrates not only tumor selectivity (based on the G1-S checkpoint status of the cell) but also significantly greater antitumoral efficacy *in vivo* compared to *dl*1520 (all models tested) and even wild-type adenovirus (in a breast cancer metastasis model) [26]. A very similar E1A CR-2 mutant adenovirus has demonstrated replication and cytopathic effects based on the pRB status of the target cell, in addition to encouraging *in vivo* antitumoral efficacy [32]. Deletion of the E1B 19-kDa gene (antiapoptotic bcl-2 homolog) is known to result in a "large plaque" phenotype due to enhanced speed of cell killing [60]. This observation has now been extended to multiple tumor cell lines and primary tumor cell cultures [61, 62]. A similar phenotype resulted from overexpression of the E3 11.6-kDa adenovirus death protein [63]. It remains to be seen whether these *in vitro* observations are followed by evidence for improved efficacy *in vivo* over wild-type adenovirus.

"Arming" viruses with therapeutic genes can also result in improved potency (e.g., prodrug-activating enzymes and cytokines) [64–67]. Prodrug-activating enzyme conversion of nontoxic prodrugs to active cytotoxic agents within the tumor is an attractive strategy that has been pursued; however, this approach may result in virus inactivation and decreased viral oncolysis, in addition to beneficial "bystander effects." Viral coat modifications may be beneficial if inadequate CAR expression plays a role in the resistance of particular tumor types [58, 59]. Improved systemic delivery may require novel formulations or coat modifications, as well as suppression of the humoral immune response. Determination of the viral genes (e.g., E3 region) and immune response parameters mediating efficacy and toxicity will lead to immunomodulatory strategies. Finally, identification of the mechanisms leading to the potential synergy between replicating adenoviral therapy and chemotherapy may allow augmentation of this interaction [68].

XIII. Summary

Replication-selective oncolytic adenoviruses represent a novel cancer treatment platform. Clinical studies with *dl*1520 and now other viruses have demonstrated the safety and feasibility of this approach, including the delivery of adenovirus to tumors through the bloodstream [5, 51, 69]. The discovery of

the inherent capacity of replication-competent adenoviruses to sensitize tumor cells to chemotherapy led to chemosensitization strategies. Clinical research is anticipated with novel adenoviral agents, including constructs expressing exogenous therapeutic genes to enhance both local and systemic antitumoral activity [8, 64, 70]. In addition to adenovirus, other viral species are being developed, including herpesvirus, vaccinia, reovirus and measles virus [1, 17, 27, 71–75]. Since intratumoral spread also appears to be a substantial hurdle for viral agents, inherently motile agents such as bacteria may hold great promise for this field [76, 77]. Although data from *in vitro* cell-based assays and murine tumor model systems will be important for testing and generating hypotheses, it is vital that encouraging adenoviral agents are tested in well-designed clinical trials as soon as possible. Data from clinical trials must be used to guide future laboratory approaches, as well as the converse. This "iterative loop" between laboratory and clinic may result in major cancer treatment advances.

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CHAPTER 12

Innate Immune Responses to in Vivo Adenovirus Infection

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I. Overview: Components of Innate Immunity

Innate immunity is the first line of defense against infections. The phylogenetically ancient, phagocyte-based effector mechanisms of innate immunity exist in their mature state prior to microbial exposure and infection does not alter the intrinsic specificity of the system components. This is in sharp contrast to the more recently evolved mechanisms of adaptive immunity in which the microbial encounter leads to gene rearrangements that alter the intrinsic properties of the system components, e.g., following infection, microbe-stimulated antibody gene rearrangements lead to production of antibodies of greater affinity for the microbe. Mechanisms of innate immunity are either constitutively active or are activated very rapidly after infection (prior to development of adaptive immune responses) and serve three very important functions. First, as the initial host response, innate defenses limit or prevent infection by rapidly eliminating microbes (clearance). Second, the effector components of innate immunity interact and work together with components of adaptive immunity to synergistically augment microbial clearance. Third, innate immunity stimulates and can reprogram adaptive immune mechanisms to optimize clearance of specific types of microbes. Although viral clearance has been traditionally thought to be due to lymphocyte-mediated clearance of infected cells, recent data have demonstrated the important role for phagocyte-mediated clearance of recombinant, replication-deficient adenoviral vectors administered to the lung and systemic circulation. In this chapter we will review data regarding the interaction between adenovirus and host innate immune mechanisms and discuss the implications for use of recombinant adenoviral vectors for *in vivo*

ADENOVIRAL VECTORS FOR GENE THERAPY

gene transfer. The discussion will be focused on pulmonary innate immune responses because much of our information comes from preclinical and clinical studies done in an effort to an develop adenoviral vector-based *in vivo* gene therapy for the clinical manifestations of cystic fibrosis lung disease. A brief review of innate immunity is useful prior to discussing data related to adenovirus.

Components of the innate immune system are found, presumably, in all Mesozoic organisms in contrast to adaptive immunity which is present only in cartilaginous and bony fish, amphibians, reptiles, birds, and mammals. Coevolution during the 400 million years since appearance of lymphocyte-based adaptive immunity accounts for the close interconnection between innate and adaptive immune mechanisms. Innate immunity is composed of a set of constitutive and inducible components including physical and biochemical barriers, circulating or mobile effector cells and proteins, and proinflammatory and chemotactic cytokines (Table I). The principal effector components of innate immunity involved in clearance of microbes during *in vivo* infection include phagocytic and natural killer (NK) cells, cytokines, and complement.

Phagocytic cells are the primary effectors of microbial clearance *in vivo* and consist of tissue macrophages and circulating monocytes and neutrophils. Tissue macrophages are derived from circulating monocytes that enter the various organs and differentiate into morphologically, histochemically and functionally distinct phagocyte populations [1, 2]. Macrophages, which are normally resident in tissues and phagocytically competent prior to infection, provide a constitutive mechanism for primary clearance of microbes in tissues and organs. Neutrophils, normally abundant in blood and also phagocytically competent prior to infection, are not normally present in tissues but are recruited in response to localized release or generation of chemotactic factors at the site of infection. Thus, neutrophils provide a potent, inducible mechanism for secondary clearance of microbes in tissues and organs. Both macrophages and neutrophils are capable of internalizing and destroying a variety of microbial pathogens and secrete a number of potent inflammatory mediators in response to infection. NK cells normally constitute 5 to 20% of the blood mononuclear cells and are primarily involved in clearance of viruses and some other intracellular pathogens. NK cells are activated and lyse virally infected host cells by three incompletely understood mechanisms: (1) binding to microbe-specific antibody molecules attached to infected host cells, (2) binding to adhesion molecules whose expression is upregulated on infected host cells, and (3) binding by an unknown mechanism to host cells lacking class I major histocompatibility complex (MHC) molecules (reviewed in [3]).

Cytokines constitute a group of soluble protein components of innate immunity that serve critical proinflammatory and chemoattractive functions (reviewed in [4]). These functions include augmentation of inflammation (e.g., by TNFα, interleukin (IL)-1), induction of resistance to viral infection within

Table I
Components of Innate Immunity In the Lung

Class	Type	Effector	Principal function	Presence
Barriers	Physical	Epithelium	Block microbe entry	Constitutive
		Ciliary escalator	Eject trapped microbe	Constitutive
		Glycocalyx	Trap microbe	Constitutive
	Biochemical	Mucin	Trap microbe	Constitutive and inducible
Soluble proteins	Defensins	Multiple	Killing of microbe	Constitutive and inducible
	Proteases	Lysozyme, lactoferrin, elastase metalloproteases	Killing of microbe	Constitutive and inducible
	Collectins	Surfactant protein A (SP-A), SP-D, mannose binding protein	Opsonization of microbe and activation of complement	Constitutive
	Complement	Multiple	Killing of microbe and opsonization of microbe and leukocyte activation	Constitutive
	Pentraxins	C-reactive protein	Opsonization of microbe and activation of complement	Constitutive and inducible
	Coagulation factors	Multiple	Sequester microbe	Constitutive
	Cytokines	TNFα, IL-1	Inflammation	Inducible
		IFNα/β	Resistance to viral infection	Inducible
		IFNγ	Macrophage activation T_{H1}/T_{H2} cell development	Inducible
		IL-12/IL18	IFN production by NK cells	Inducible
		IL-15	NK cell proliferation	Inducible
		IL-10/TGFβ	Modulation of inflammation	Inducible
		IL8, MIP-2	Neutrophil recruitment	Inducible
		MCP-1	Monocyte recruitment	Inducible

(*continued*)

Table I
(continued)

Class	Type	Effector	Principal function	Presence
Cells	Phagocytes	Tissue macrophages	Primary microbe clearance and inflammatory signaling	Constitutive and inducible
		Neutrophils	Secondary microbe clearance and inflammatory signaling	Inducible
	NK cells		Lysis of infected cells and activation of macrophages	Constitutive
Cell surface receptors	Pattern recognition receptors	Mannose receptor, LBP/CD14, Toll homologs	Phagocytosis of microbe inflammatory signaling	Constitutive
		Scavenger receptors	Phagocytosis of microbe	Constitutive

other cells (e.g., by IFNα, IFNβ), macrophage activation (e.g., by IFNγ), neutrophil recruitment and activation (e.g., by IL-8, macrophage inflammatory protein (MIP)-2), monocyte recruitment (e.g., by MCP-1), stimulation of NK cell-mediated clearance (IL-12, IL-18), stimulation of adaptive immunity (TNFα, IL-2, IL-12, IL-18, others), and suppression of acute inflammation (e.g., by IL-10, TGFβ) (Table I). These and other cytokines elicited during *in vivo* microbial infection, together, form part of the complex and functionally overlapping molecular network of intercellular communications that orchestrate the ensuing inflammatory cascade and immune events. In so doing, these cytokines augment innate immune microbial clearance by activating resident phagocytes and by recruiting and activating other phagocytes and augment adaptive immune clearance by influencing the nature and strength of specific immune responses.

Complement (C) is a component of innate immunity composed of several soluble proteins capable of recognizing microbes, stimulating inflammation and augmenting microbial clearance. The complement system can be activated in three ways: (1) direct recognition of certain microbial surface structures (e.g., via the alternative pathway, a phylogenetically older system); (2) recognition of microbe-bound antibody by a complement component called C1q (e.g., via the classic pathway); and (3) direct cleavage of C3 by a protease associated with complexes of collectins bound to microbes (see below). Subsequent to

recognition, complement activation can neutralize viruses and other microbes by several mechanisms, including direct killing by microbial membrane perforation, agglutination of the microbes with a net loss in infectivity, and opsonization resulting in clearance by C3-receptor-bearing phagocytes. Activation of each of these three pathways (classical, alternative, and collectin) has been demonstrated in response to viral infection.

The soluble protein effector component of innate immunity also includes a heterogeneous collection of other members that serve opsonic and lytic roles in microbial clearance. Collectins are one such group present in blood and in tissues whose family members include conglutinin, mannose-binding protein (MBP), and the surfactant proteins A (SP-A) and D (SP-D) [5]. Members of this family share a common basic structure consisting of collagen-like and lectin domains separated by a short neck region. The lectin moiety binds to microbial surface carbohydrates in a calcium-dependent manner and thus, collectins share both structural and functional homology with C1q. Although surfactant proteins found in the lung (SP-A, SP-B, SP-C, SP-D) were initially regarded as functioning to maintain alveolar structural integrity and patency, emerging data has defined an important role for SP-A and SPD in innate immune mechanisms of lung host defense against bacterial and viral pathogens. Other soluble protein effectors include defensins (a family of proteins with bacteriocidal properties secreted from neutrophils and epithelial cells) lysozyme, elastase, cathepsin G, phospholipase A2, lactoferrin, and transferrin. At present, the potential role of these molecules in viral infection is not particularly clear. Finally, natural antibodies, which are constitutively expressed in the absence of microbial infection, recognize and bind components of the microbial surface and serve as opsonins and can activate complement. Natural antibodies are of fixed specificity because they are expressed from germline genes which do not undergo genetic rearrangements subsequent to microbial infection. Examples include antibodies directed against blood group antigens and those that recognize species-specific cell surface carbohydrate structures. Natural antibodies directed at species-specific murine cell surface carbohydrates present on retroviral vectors derived from murine producer cells are largely responsible for the rapid, complement-mediated lysis of these vectors by human serum.

Alveolar macrophages (AMs) play a central role in lung host defense by providing a critical primary barrier to microbial infection mediated by both intrinsic and extrinsic pathways of resistance [6, 7]. Intrinsic resistance mechanisms, which lie completely within the realm of innate immunity, include the ability of macrophages to internalize, degrade, and thus restrict the replication of microbial pathogens. Extrinsic resistance mechanisms include release of cytokine mediators that recruit and activate other inflammatory cells (e.g., neutrophils, NK cells) or stimulate antiviral resistance in neighboring cells (e.g., IFNα/IFNβ). Both of these mechanisms are also clearly components of innate

immunity. Extrinsic resistance pathways also include mechanisms by which innate immunity reprograms adaptive immune responses to alter the nature or strength of microbe-specific responses (e.g., cytokines that modulate T_{H1}/T_{H2} development and the balance of cellular and humoral immune responses. However, while macrophages help to provide a barrier against infection by some viruses (e.g., vesicular stomatitis, encephalomyocarditis virus and influenza virus), with other viruses (e.g., human immunodeficiency virus) they do not provide a barrier but instead serve as a reservoir of latent infection and facilitate recrudescent disease. Recent data from preclinical and clinical studies with replication-deficient recombinant adenoviral vectors in humans and various animal models strongly support the notion that alveolar macrophages provide a critical innate immune barrier to infection by adenovirus. These data will constitute the focus of the remainder of this chapter.

II. Distribution and Clearance of Adenovirus from the Respiratory Tract

A. Clinical Aspects of Natural Adenoviral Infection in Humans

Adenovirus is an important respiratory pathogen affecting individuals of all ages with an annual incidence of between 5 to 10 million in the United States. Infections can occur sporadically, epidemically and nosocomially but most individuals are infected at a young age; adenovirus accounts for 7 to 10% of all respiratory illnesses in infants and children [8, 9]. Although adenovirus frequently causes a mild, acute upper respiratory illness, e.g., the "common cold," respiratory infections occur as a broad spectrum of distinct clinical syndromes ranging from self-limited acute pharyngitis to fatal pneumonia [10–12]. Adenovirus has also been identified an etiological factor of exacerbations in individuals with chronic obstructive lung diseases and infections can be especially problematic in immunocompromised individuals. Examples of the latter include persistent bladder infections in individuals with chemotherapy-induced neutropenia, fatal pneumonia in neonates, and exacerbation of graft rejection and bronchiolitis obliterans in lung transplant recipients [13, 14].

Natural adenovirus infections are typically initiated by deposition of aerosol droplets containing adenovirus on the mucosal surface of the respiratory epithelium [15]. Some virions then diffuse to the cell surface and enter by receptor-mediated endocytosis. Once inside the cell nucleus, wild-type (replication competent) adenovirus DNA overtakes, reprograms, and eventually kills the infected cell ultimately releasing up to 10,000 virions per infected cell. Newly replicated and released virions then infect neighboring cells, repeating the process and thus spreading the infection through the epithelial sheet. The

ensuing clinical course is determined by the race between virus replication and spread and the successful mounting of host innate and adaptive immune responses.

B. Distribution of Recombinant, Replication-Deficient Adenoviral Vectors

In considering infection of the respiratory tract by recombinant adenoviruses, it is important to recognize two important and fundamental differences from natural infections by wild-type adenovirus. First, recombinant human adenoviral vectors used to date for *in vivo* gene transfer have generally been deleted of E1 region sequences and are thus relatively replication-deficient in human cells (reviewed in [16, 17]). Further, in mice and primates, two species most commonly used in preclinical gene therapy studies, human adenoviral vectors have a species-related host range restriction that prevents viral replication [15]. These restrictions on viral replication, however, do not affect the infection of a given cell by the vector, i.e., virion internalization and transgene expression proceed normally. Second, and very importantly, there is an enormous difference in the size of the infecting inoculum of adenovirus in the two scenarios. A typical natural infection is thought be caused by less than 1000 adenovirus virions. In contrast, human trials have been conducted in which the virus dose administered was up to one billion times higher ($\sim 10^{12}$ virions/individual).

Studies in rodent models utilizing transtracheal, liquid bolus administration of adenovirus demonstrated that: (1) gene transfer to and expression in airway epithelium was dependent on the dose of virus administered; (2) all cell types could be infected; and (3) gene transfer occurred throughout the bronchial tree but was patchy [18, 19]. Studies in nonhuman primates utilizing bronchoscopic or aerosol delivery also demonstrated gene transfer but confirmed the overall inefficiency of gene transfer [20–23]. Finally, both direct liquid-based or aerosol-based vector administration in humans also demonstrated only low level infection and transduction of the respiratory epithelium. *In vitro* studies demonstrated, unexpectedly, that while intact airway epithelium was poorly transduced, damaged epithelium, immature epithelial cells, or differentiating airway epithelium were all easily transduced. These findings were reconciled by studies utilizing a bronchial epithelial cell xenograft model which demonstrated that integrin $\alpha_v\beta_5$, a coreceptor required for adenoviral virion internalization, was not expressed on the apical membrane of mature airway epithelium and was expressed only on the basolateral surface of the cell [24]. In the context that physical access to the basolateral membrane of epithelial cells of intact epithelium is restricted by tight junctions which connect adjacent airway epithelial cells just below the apical (luminal) surface [25], this data provided early insight into one mechanism by which airway epithilium

present an innate immune barrier to adenovirus infection. The apical membrane surface glycocalyx represents another barrier [26]. Evaluation of various organs of cotton rats or monkeys following intrapulmonary adenovirus vector administration using a sensitive polymerase chain reaction technique demonstrated that vector does not escape from the lung [21, 27]. Recently, studies in rodents have shown that a large portion of adenovirus administered to the respiratory tract is distributed to alveolar macrophages rapidly after pulmonary administration (see below).

C. Kinetics and Mechanisms of Clearance of Adenovirus

The initial report of adenovirus-mediated, *in vivo* transfer of CFTR to the lungs of cotton rats demonstrated the presence of adenoviral vector DNA in the lung as late as 6 weeks after vector administration [28]. Subsequent studies carried out in various rodent models, nonhuman primates, and humans, however, have demonstrated that most of the adenoviral vector DNA initially administered to the respiratory tract is eliminated from the lung within several weeks in the context of an intact immune system (reviewed in [29]). Data showing that adenovirus-mediated pulmonary transgene expression in athymic mice lasted for more than 3 months implicated the cell-mediated adaptive immunity in pulmonary clearance of adenovirus [30]. This conclusion was supported by the demonstration of prolonged transgene expression in mice depleted of $CD4^+$ cells. The mechanism of this T lymphocyte-mediated clearance was shown to be direct lysis of adenovirus vector-transduced cells by cytotoxic lymphocytes (CTLs) directed at both adenoviral- and transgene-derived proteins. Adaptive immune CTL-mediated clearance is a delayed mechanism because CTL are detected only after several days and are most abundant for 7 to 14 days after infection.

The importance of innate immunity in clearance of adenovirus from the lung was first demonstrated by the finding that ~70% of the adenoviral DNA present immediately after pulmonary administration in mice was eliminated by degradation within 24 h [31] (Fig. 1). Since this "early phase" clearance was well before a significant adaptive immune response could have been mounted, an innate immune mechanism was sought. Similarity in the pattern of clearance in athymic and normal mice demonstrated independence from lymphocyte-based adaptive mechanisms and alveolar macrophages were postulated to be the mechanism of this early clearance. Several findings support this hypothesis. First, *in vitro* studies demonstrated that infection of human, rat, and murine alveolar macrophages led to loss of approximately two-thirds of the viral DNA within 24 h, whereas similar infection of epithelial cells resulted in no significant loss of viral DNA. Second, pretreatment of the lungs with clodronate-laden liposomes to deplete phagocytic cells significantly impaired the rapid clearance of adenovirus. However, because rapid and significant neutrophil influx occurs during the first 24 h of infection [32], thus overlapping

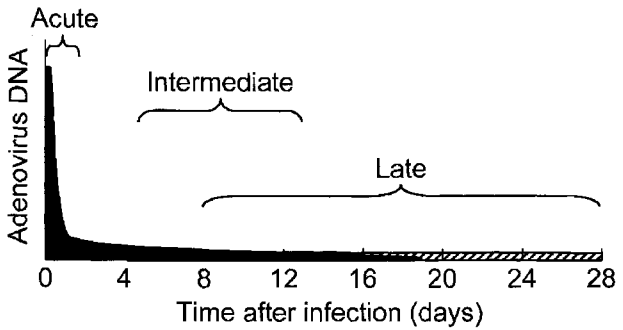

Figure 1 Clearance of viral DNA after adenovirus infection of the respiratory tract. Adenoviral vector DNA is cleared from the lung in a biphasic pattern. Most adenoviral DNA is cleared from the lung very early over the first 24 h after lung infection. The remainder is cleared more slowly over the following several weeks. Clearance during the acute phase is due to internalization and degradation within phagocytes, mostly resident alveolar macrophages (primary clearance). Clearance during the intermediate phase is probably mostly due to recruitment and activation of innate immune NK cells. Clearance during the late phase is principally mediated by the cytotoxic T-cell response. Thus, viral clearance is due to both innate (acute, intermediate) and adaptive (late) immune mechanisms.

the early phase of adenovirus elimination [31], neutrophil-mediated clearance cannot be excluded as an important mediator of viral clearance in these experiments. This concern is supported by the direct demonstration of the uptake of fluorescently labeled adenovirus by neutrophils recruited to the lung using confocal microscopy (Zsengeller and Trapnell, unpublished observations). Third, infectious, fluorescently labeled adenovirus is rapidly internalized by alveolar macrophages *in vivo* as early as 1 min following pulmonary administration in mice [33].

The mechanism by which alveolar macrophages internalize adenovirus *in vivo* is not known but may involve endocytosis and/or phagocytosis and may involve other factors within the local milieu. In order to better understand this mechanism, it is useful to first consider the mechanism of adenovirus infection in epithelial cells which has been well studied (reviewed in [34]). The virion is internalized by receptor-mediated endocytosis and can be summarized as follows: (i) high-affinity binding of the virion to the cell mediated by attachment of the adenovirus fiber knob to its 46-kDa cell surface receptor, CAR [35]; (ii) receptor clustering and rapid virion internalization via a clathrin-coated vesicle mediated by interaction of the adenovirus penton base with integrins $\alpha_v\beta_5$ or $\alpha_v\beta_3$ [36–40]; (iii) release of clathrin to generate an endocytotic vesicle; (iv) endosome acidification mediated by an endogenous vesicular membrane proton pump [41]; (v) penetration of the endosome membrane (endosome lysis) and release of the virion into the cytoplasm mediated by the TVD motif-containing cytoplasmic tail portion of integrin β_5 [42]; (vi) virion translocation

to the nuclear membrane mediated by microtubules [39, 43]; (vii) virion binging to the nuclear pore [44]; (viii) capsid disassembly (continued) at the nuclear pore [34]; and (ix) translocation of viral chromatin into the nucleus through the nuclear pore [44]. Adenovirus uptake by mononuclear phagocytes has been studied to some extent *in vitro*. In contrast to highly susceptible Coxsackie and adenovirus receptor $(CAR)^+$ epithelial cells, hematopoietic lineage cells including alveolar macrophages, monocytes, and related cell lines do not express CAR and internalize adenovirus about 100 to 1000-fold less well [45–48]. Internalization of adenovirus by these cells *in vitro* requires cell surface integrin α_v, similar to CAR^+ epithelial cells and upregulation of integrin $\alpha_v\beta_5$ and $\alpha_v\beta_3$ on human monocytes facilitates infection [45]. Studies using RAW264.7 murine macrophages have shown that the internalization of adenovirus by these cells is temperature-sensitive and calcium-dependent and requires phosphatidylinositol 3-OH kinase [33]. Data from administration of adenovirus to mice *in vivo* suggest the potential involvement of other factors or an alternative mechanism of uptake. For example, *in vivo* uptake of adenovirus by alveolar macrophages is reduced in mice deficient in surfactant protein A [49]. Mice deficient in GM-CSF due to targeted gene ablation are unable to clear adenovirus from the lung and alveolar macrophages in these mice are unable to internalize adenovirus efficiently [50] due to a generalized defect in phagocytosis/endocytosis [51]. In contrast, mice deficient in M-CSF (osteopetrotic mice) have no apparent defect in uptake of adenovirus (Zsengeller and Trapnell, unpublished observations). Thus, further studies will be required to determine the mechanism by which alveolar macrophages internalize and degrade adenovirus *in vitro* and *in vivo*.

A second important innate immune mechanism of clearance of adenovirus DNA has recently been demonstrated to be the clearance of virus-transduced cells by recruited NK cells [52]. Intravenous administration of adenovirus results in detectable levels of NK cells in infected tissues by 7–10 days and depletion of NK cells prolonged the duration of transgene expression. Interestingly, variation in transgene expression between different strains of mice was associated with significant differences in levels of IL-12 and IFNγ production and NK cell activation.

In summary, multiple innate immune barriers block adenovirus infection of the lung and several, redundant innate immune mechanisms of clearance contribute to elimination. Barriers to uptake include production of a mucous layer that traps virions, the ciliary escalator that ejects trapped virions, the epithelial cell glycocalyx that traps virions, and epithelial tight junctions that sequester required adenovirus cell surface receptors away from the luminal surface of the airway epithelial cells. Innate immune mechanisms of clearance include rapid phagocyte-mediated internalization and destruction of the virion (i.e., by primary alveolar macrophages and secondarily recruited neutrophils) and NK cells that destroy adenovirus-infected cells.

III. Molecular Mediators of Inflammation

Until recently, little was known about the molecular inflammatory responses triggered by adenovirus infection of the lung. In contrast, bacterial infection and sepsis has been extensively studied in humans and a variety of animal models and has provided a context for the evaluation of these responses to adenovirus infection. Recent studies of adenovirus pneumonia in children and a great number of studies in animal models and humans receiving replication-deficient adenoviral vectors have provided important details of the molecular signaling responses to adenovirus infection of the respiratory tract. The latter are the result of intense interest and efforts to develop adenovirus-based strategies for *in vivo* gene therapy for lung diseases such as cystic fibrosis (reviewed in [29]). Consequently, it is now known that pulmonary infection by either wild-type (replication competent) or replication-deficient adenovirus initiates expression of a complex cascade of cytokine mediators that accompanies cellular infiltration of the lung. The precise temporal relationship of expression of these mediators and their relationship to parenchymal infiltration by the various leukocyte populations are beginning to define their role in regulation of both innate and adaptive immune events (see below). Before discussing in detail the molecular responses to adenovirus, it is first useful to briefly review some general features of the principal cytokines involved. We will then discuss the cytokine responses during adenoviral pneumonia and the cytokine responses to pulmonary administration of replication-deficient adenoviral vectors in humans, nonhuman primates, and mice.

Cytokines are proteins secreted by cells of the innate and adaptive immune systems in response to microbes and antigens that mediate many of the functions of their cellular components [4]. For historical reasons, various terms including monokine, lymphokine, and IL have been used to refer to these molecules. For simplicity, we will use only the term "cytokine," which does not make reference to the cellular origin or target. Cytokine responses to microbes are generally brief, redundant, and often include pleiotropic local and systemic effects. The effects of cytokines are mediated by binding to specific cell surface receptors resulting in alteration of gene expression and acquisition of new target cell functions or cell proliferation. Cytokine responsiveness can be regulated by expression of its receptor on the target cell and cytokines often alter the expression or effects of other cytokines, i.e., some cytokines antagonize while others synergize the effects of another cytokine. Cytokines serve as important regulators of innate immunity (e.g., TNFα, IL-1, IL-12), adaptive immunity (e.g., IL-2, IL-4, IL-5), and hematopoiesis (e.g., granulocyte macrophage-colony stimulating factor (GM-CSF), M-CSF, IL-3, stem cell factor (SCF)). Other cytokines play an important role in both innate and adaptive immunity (e.g., IFNγ). While such functional grouping is useful, it is not absolute. For example, while GM-CSF is relevant to hematopoiesis, recent findings have

shown that it plays a critical role in innate immunity in the lung [53], but has only a noncritical or redundant role in hematopoiesis [54]. TNFα is a principal mediator of the acute inflammatory response to microbial infection and is responsible for many local and systemic effects of infection. The main function of TNFα is enhancement of neutrophil and monocyte recruitment to sites of infection and stimulation of their microbial clearance functions. TNFα is mainly produced by activated mononuclear phagocytes. The principal action and source of IL-1 is similar to that of TNFα. Chemokines (chemotactic cytokines) are a large family of small, structurally similar cytokines that stimulate migration of leukocytes from the blood to sites of local production in tissues (reviewed in [55]). Chemokines have been divided into four groups on the basis of cysteine motifs in their primary structure: C-chemokines (e.g., lymphotactin); CC-chemokines (e.g., MIP-1α); CXC-chemokines (e.g., IL-8, MIP-2); and CXXXC-chemokines (e.g., fractalkine). In terms of inflammatory cell recruitment, CXC chemokines mainly promote neutrophil chemotaxis while CC chemokines act on monocytes, lymphocytes, and eosinophils. MIP-1α also functions in an autocrine manner to enhance TNFα expression [56]. Other cytokines are primary mediators of innate immune responses and serve to stimulate adaptive immunity. For example, both IL-12 and IL-18 are released by macrophages and stimulate NK and T_{H1} cell release of IFNγ, a potent stimulator of cellular immunity. In contrast to the many cytokines that stimulate innate immune cell functions, IL-10 functions as an important inhibitor of macrophage inflammatory cytokine release (e.g., TNFα release), and thus functions as a homeostatic regulator of both innate and adaptive immune responses to microbial infection. IL-6 is a cytokine with pleiotropic functions affecting both innate and adaptive immunity. Among its diverse functions are the stimulation of acute-phase proteins by hepatocytes and stimulation of B lymphocyte proliferation and function. This background will serve as a brief context for considering the cytokine responses to adenovirus infection of the respiratory tract.

A. Clinical Adenovirus Infections in Humans

Cytokine responses have not been adequately studied in mild cases of natural adenovirus infection, however, a recent study of moderate to severe infections has provided important insights relating the severity of lung infection to the production of certain cytokines [57]. In this study of apparently healthy children, ages 3 weeks to 19 months, who were hospitalized for acute adenovirus pneumonia, the infection was mild in 10, moderate in 12, and fatal in 16. While neither IL-6 nor TNFα were detected in the serum of mild–moderate cases, both were detected in the serum of most fatal cases (13/16, 9/12, respectively). IL-8 was detected in all three groups with serum levels correlating with disease severity. The more severe cases were also noted to have reduced levels of complement, increased levels of circulating immune

complexes, and a decrease in IgG consistent with a consumptive process. IL-1 and IL-4 were rarely detected in any patients and all patients demonstrated by IgM and IgG directed against adenoviral epitopes. Finally, development of septic shock was associated with markedly increased serum levels of IL-6, IL-8, and TNFα. This study demonstrated a clear association between the level of severity of adenoviral infection of the lung, morbidity/mortality and production of certain proinflammatory cytokines.

Cytokine responses have also been studied in individuals with cystic fibrosis in which recombinant, replication-deficient, E1-,E3-region-deleted adenoviral vectors were administered to either nasal or bronchial epithelium or both. In one double-blind, vehicle-controlled, dose-escalation study of nasal administration, nostrils receiving the adenoviral vector had a greater increase in IL-1, IL-6, IL-8, and IL-10 than vehicle control nostrils in the same patient [58]. In another study of bronchoscopic delivery of a similar vector to the lungs of individuals with cystic fibrosis, IL-6 was increased in the serum 4 h after vector administration with values tapering over 8–24 h [59]. Further, the maximum increase in serum IL-6 levels was proportional to the dose of adenovirus administered. IL-6 was also detected in the bronchoalveolar lavage fluid of these individuals.

Taken together, these data show that infection of the respiratory tract by either replication-competent or replication-deficient adenovirus results in release of similar proinflammatory cytokines. Further, cytokines can be detected locally and systemically and appear to be released in proportion to the magnitude of the infection.

B. Adenovirus Infections in Animal Models

Adenovirus infection of the respiratory tract has been recently studied in a variety of animal models including mice [30, 32, 60], Cotton rats (Sigmodon Hispidus) [15, 19, 27, 61], and primates [20–23] as part of efforts to develop adenovirus-based vectors for human gene therapy for cystic fibrosis (reviewed in [29, 62]). In nonhuman primates receiving an E1-,E3-deleted vector by bronchoscopic delivery, IL-1β and IL-8 were both elevated in lung lavage fluid compared to animals receiving only vehicle control. IL-1β levels were mildly increased in the lungs of animals receiving 10^{10} plaque-forming units (pfus) at 3 days, but progressively higher levels were seen at 21 and 28 days. In contrast, IL-8 levels were consistently elevated at 3, 10, 21, and 28 days following vector administration. No increases were seen in monkeys receiving only low doses (10^6 pfu/animal). Thus, studies in nonhuman primates are consistent with human studies and demonstrate a dose-dependent increase in lung cytokine expression following adenovirus infection.

Cytokine response studies have been most thoroughly studied in mice. Inbred C57BL/6N mice infected intranasally with 10^{10} pfu of wild-type human serotype 5 adenovirus (Ad5) showed elevated lung levels of TNFα, IL-1, and

IL-6 24 h after infection. While TNFα and IL-1 were not elevated in serum at any time, IL-6 was elevated as early as 24 h after infection. TNFα levels were baseline in the lung by day 3 in contrast to IL-1 levels which rose progressively through 7 days. IL-6 levels peaked on day 1 and fell progressively but were still elevated at 7 days. Subsequent studies have evaluated cytokine responses in BALB/c mice receiving intratracheal administration of an E1-,E3-deleted adenoviral vector [32, 33]. These studies more completely defined the chronology and levels of cytokine responses and demonstrated that the different cytokines could be grouped in terms of patterns of expression at different times after infection: acute (elevated by 6 h), intermediate (not elevated at 6 h, but elevated at 24 h), and late (not elevated at 6 h, but progressively increasing thereafter through 72 h) (Fig. 2). The observation that these responses are similar in nude and control mice demonstrates that these early cytokine responses to adenovirus are independent of adaptive immunity.

C. Acute Cytokine Responses

Intratracheal administration of an E1-,E3-deleted adenoviral vector in BALB/c mice results in elevation of TNFα, IL-6, MIP-2, and MIP-1α levels in

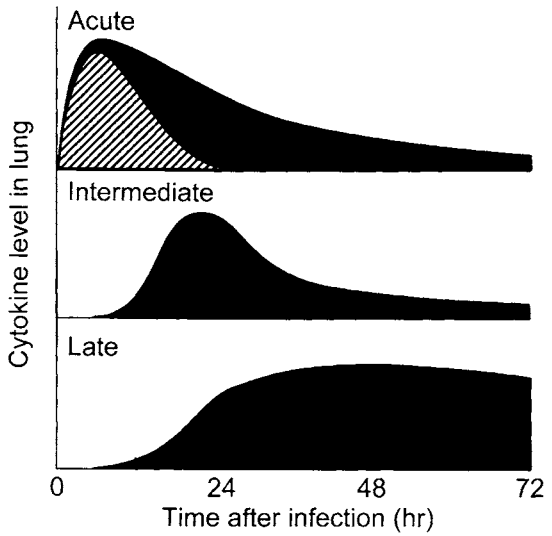

Figure 2 Patterns of innate immune cytokine responses in the lung after adenovirus infection of the respiratory tract. Patterns of cytokine responses to adenovirus lung infection can be grouped into "phases" of expression including an acute phase (e.g., TNFα, IL-6) or hyperacute (MIP-2, striped area), an intermediate phase (e.g., IL-1β and IFNγ), and a late phase (e.g., MCP-1). Specific cytokines elicited during these phases coordinate a number of innate immune defenses and initiate regulatory pathways that modulate the level of specific adaptive immune responses. See text for details.

bronchoalveolar lavage (BAL) 6 h after infection [32]. In another study TNFα mRNA was detected as early as 30 min after pulmonary infection, whereas TNFα protein was detected in lung by 3 h [33]. In the later study, *in situ* hybridization analysis demonstrated that adenovirus-induced TNFα mRNA expression was localized to alveolar macrophages; however, expression was not seen in either respiratory epithelium or vascular endothelium for the duration of the study (6 h). The importance of TNFα in adenoviral clearance was demonstrated using mice deficient in TNFα due to targeted gene ablation (TNFα-/-) [63]. Intravenous administration of an adenoviral vector expressing a chloramphenicol transgene resulted in prolonged transgene expression in TNFα-/- mice compared to controls. Abrogation of the adenovirus-induced TNFα response by coadministration of corticosteroids or the suppressive cytokine, IL-10, also increased the duration of adenoviral vector-mediated transgene expression. Blocking TNFα receptor function prolongs adenoviral vector-mediated transgene expression as demonstrated by prolonged transgene expression after coadministration of an adenoviral vector expressing a TNFα receptor decoy with one expressing a conventional marker [52]. Together, these studies support the concept that TNFα, which is derived primarily from alveolar macrophages, plays an important role in elimination of adenovirus following pulmonary infection. MIP-2, an important neutrophil chemoattractant, is elevated within 3 h after pulmonary administration of an E1-,E3-deleted adenoviral vector in mice. Levels peak at 6–8 h and then rapidly decline to baseline by 24 h. Consistent with this, MIP-2 mRNA levels are elevated by 30 min in both lung and alveolar macrophage total RNA of adenovirus-infected but not vehicle-exposed mice. These studies demonstrate that the alveolar macrophages can be a potent effector of neutrophil recruitment following adenovirus lung infection. MIP-1α expression is initiated with kinetics similar to MIP-2, but in contrast, expression remained elevated at 24 h. Mice deficient in MIP-1α due to targeted gene ablation also demonstrate substantially reduced inflammation and delayed viral clearance after Coxsackie virus or influenza virus infection [64]. IL-6 expression after adenoviral infection of the respiratory tract was similar to that of TNFα at both the mRNA and protein levels [33]. Similarly, *in situ* hybridization localized IL-6 mRNA to alveolar macrophages but not epithelium nor vascular endothelium after adenoviral vector infection of the lung [33]. Despite this chronological description of its response, the role of IL-6 in adenovirus infection of the lung is not currently known.

D. Intermediate Cytokine Responses

The "second wave" of cytokine responses includes IL-1β and IFNγ, both of which are at baseline 6 h after adenovirus lung infection, but are increased by 24 h. While much is known about the general functions of IL-1 and IFNγ in other systems, studies in IL-1 or IFNγ gene-ablated mice have not been done

and specific data regarding their role in adenoviral lung infection is lacking. Interestingly, coadministration of corticosteroids abrogated the IFNγ response to adenoviral lung infection [32] and was associated with increased adenoviral vector-mediated gene expression. However, since other cytokines were also altered by this treatment, the relevance of changes in IFNγ will require further evaluation. IFNγ production by NK and T_{H1} cells can be stimulated by both IL-12 and IL-18, which are both stimulated by adenovirus infection of the lung [65]. However, only IL-18 is necessary for optimal production of IFNγ, independent of IL-12. Further, T_{H1} cell development was unaffected by blocking both IL-12 and IL-18, demonstrating that other factors may be required for T_{H1} differentiation following adenovirus infection. Differences in IFNγ levels have been demonstrated between BALB/c and C57BL mice after adenovirus infection and have been related to differences in the duration of adenoviral vector transgene expression in these two mouse strains.

E. Late Cytokine Responses

MCP-1 was not elevated at 6 h after adenovirus lung infection but progressively increased thereafter and was still at the highest value 3 days after infection. This pattern of expression is different from the immediate cytokines because it is of substantially longer duration. In fact, a decline in expression after adenovirus lung infection has not been defined. MCP-1 is capable of recruiting multiple mononuclear cell subsets, however, its precise role in adenoviral infection is currently unclear. In other lung inflammation models (e.g., silicosis) a network of cytokine/ chemokine signaling has been observed linking TNFα expression to MCP-1 production and T-cell recruitment (Tamez and Shanley, unpublished observations). MCP-1 is also important in recruitment of monocytes. Thus, MCP-1 may have multiple roles in adenoviral lung infection. Interestingly, overexpression of MCP-1 in the lung results in skewing toward T_{H2} development with increased IL-10 and IL-13 levels and decreased IL-12 and IFNγ levels [66].

IV. Inflammatory Cell Recruitment

While the clinical manifestations of natural adenovirus infection in the respiratory tract range from inapparent infection to overwhelming pneumonia, sepsis, and death, the histological responses have been well-described in humans only in cases of fatal pneumonia which result in necrotizing bronchiolitis [67]. In human clinical trials with replication-deficient adenoviral vectors, virus administration to the nasal epithelium has also produced mixed results. In one study in which adenoviral vector was applied to one nostril and saline to the other, viral infection was associated with an increase in the percentage of

neutrophils recovered by swabbing compared to the saline-exposed nostril [68]. Neutrophilia resolved by 1 week in this study. Another study involving direct nasal administration of various doses of a similar adenoviral vector showed no differences in the number of inflammatory cells in epithilium or submucosa between biopsy specimens from adenovirus- or saline-treated nostrils [58]. Histological data are not available from clinical trials in which adenoviral vectors were administered to the lungs of patients. Histological responses to wild-type adenovirus have also been studied in cotton rats and mice in an effort to develop an animal model of human adenovirus diseases and more recently as part of preclinical studies in attempts to develop adenoviral vectors for human gene therapy. It was previously reported [61] that despite evaluation of multiple laboratory animals, clinical disease from wild-type human adenovirus infection of the respiratory tract was identified only in these cotton rats and mice. However, in recent studies, administration of large doses of replication-deficient E1-,E3-deleted adenoviral vectors has produced histological evidence of cellular inflammation in several animal models including Cotton rats, mice, nonhuman primates, sheep, and others. Data suggest that the histological responses in these different models are similar. Since they have been best characterized in rodent models we will focus on that data here.

Intranasal administration of Ad5 in C57BL/6N mice resulted in development of pneumonia characterized by perivascular, peribronchiolar, and intraalveolar leukocyte [60]. The infiltrate was present on day 1 and was still present on day 7 after infection and consisted primarily of mononuclear cells. The infiltrate was described as consisting of an early phase noted on days 2 through 5 and an overlapping delayed phase peaking on days 5 to 7. The early phase consisted of infiltration by monocytes/macrophages and lymphocytes with scattered neutrophils while the delayed phase consisted of a very prominent lymphocytic perivascular and peribronchial infiltration. In nude mice which lack thymus-derived T lymphocytes, the delayed lymphocytic phase was markedly reduced in all locations especially in the perivascular region [60]. In contrast, the early phase was not affected in athymic mice. Together, these data suggested that cytotoxic T cells were an important component of the delayed but not the early phase of cellular infiltration. Nasal administration of Ad5 in cotton rats, which are partially permissive for replication of Ad5, produced a similar histological response which was proportional to the dose of virus in the infecting inoculum [61]. Two phases were also noted: an early phase consisting of monocytes–macrophages, neutrophils, and occasional lymphocytes, and a delayed phase consisting almost exclusively of lymphocytes which was especially prominent in the peribronchiolar and perivascular space.

Histological responses to infection of the respiratory tract by replication-deficient adenoviral vectors has been fairly well studied in cotton rats and mice. In Cotton rats, direct comparison of histopathology following intratracheal administration of Ad5 and Ad5-derived E1-,E3-deleted replication deficient

vectors showed a qualitatively similar histopathologic pattern of virus dose-dependent leukocyte infiltration [27]. Quantitatively, however, the response to Ad5 was markedly more pronounced than the response to the replication-deficient vector. This study also showed a prominent neutrophilic component early after infection, including infiltration of airway epithelium as early as 24 h after infection. This neutrophilic component was evaluated more carefully in a subsequent study in mice infected by the replication-deficient adenoviral vector, which demonstrated a very rapid neutrophil infiltration which peaked at around 6 h and resolved over the ensuing 3 to 4 days. Interestingly, this coincided with expression of the major neutrophil chemoattractant MIP-2 in these mice [32].

Recently, NK cells have been demonstrated to be an important component of the delayed phase inflammatory cell infiltrate present in the liver after intravenous administration of a replication-deficient adenoviral vector in mice [52]. Increased NK cell numbers were apparent on days 7 and 10 after infection and were associated with increased levels of IL-12 and IFNγ. Differences in levels of NK cell accumulation in hepatic tissues and hepatocellular damage were strongly inversely correlated with the duration of transgene expression in BALB/c and C57BL/6 mice. These finding suggest that differences in NK cell responses may explain strain differences in the duration of transgene expression independent of CTL responses.

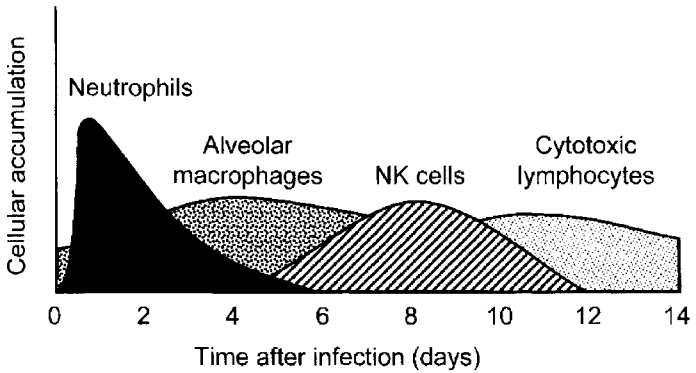

Figure 3 Pattern of inflammatory cell accumulation in the lung following adenovirus infection of the respiratory tract. Infiltration of the lungs following adenovirus infection has been characterized in several animal models and the general features are similar in each. Neutrophils appear very early and recede fairly quickly. An increase in the macrophage-monocyte population probably begins quite early but is apparent by 2–3 days and slowly recedes over the course of a week or so. NK cells have been shown to be present in increased numbers on about days 7–10 in infected liver tissues and is presumably similar in lung. Cytotoxic lymphocytes are seen late and are prominent on days 7– 14. These patterns of leukocyte recruitment can be correlated with specific patterns of chemokines known to be chemotactic for the specific leukocyte subsets. See text for details.

Taken together, these studies suggest that massive adenovirus infection of the respiratory tract as would be the case in current strategies for *in vivo* adenovirus-mediated gene transfer, elicits a histopathological response consisting of "acute," "intermediate," and "delayed" phases (Fig. 3). The acute phase begins within hours of infection, peaks within 6–24 h, and resolves thereafter. Both the peak and the duration of the inflammatory infiltrate, which consists primarily of neutrophils, are strongly dependent on the dose of infecting virus. Release of potent neutrophil chemoattractants (e.g., MIP-2 and MIP-1α) from alveolar macrophages are likely part of the molecular mechanism of this recruitment. The intermediate phase begins within 1–2 days, continues for upto 7–10 days, and consists of accumulation of monocytes–macrophages and NK cells. Increased levels of MIP-1α, MCP-1, IFNγ, and other factors are involved in recruitment and activation of cells during the intermediate phase, although more studies are required to delineate the specific role of these and other factors. The late phase is evident by 7 days, is still present on day 14 but probably subsides thereafter and consists primarily of adenovirus-specific lymphocytes. The cytokines responsible for this phase are not yet well-studied but multiple cytokines are likely to be involved.

V. Innate Immunity and Programming of Adaptive Responses

Phagocyte-based mechanisms of innate immunity are primarily responsible for clearing the bulk of adenovirus administered to the lungs during massive infections. However, clearance of adenovirus-infected cells is due to both NK and CTL-based mechanisms belonging to innate and adaptive cellular immunity, respectively. Further, successful reinfection by adenovirus is inversely proportional to the presence of neutralizing antibodies produced by the humoral adaptive immune system [19, 69]. While these topics are covered in detail in other chapters of this book, it is worth noting how innate immune responses to adenovirus infection *in vivo* regulate these adaptive immune responses. Cytokines, produced by cells of both innate and adaptive immunity have a profound effect on the nature and magnitude of specific adaptive immune responses.

TNFα, the principal mediator of the acute inflammatory response adenoviral infection of the lung, also has been shown to profoundly affect both cellular and humoral adaptive immune responses to adenovirus infection *in vivo*. Mice deficient in TNFα due to targeted gene ablation (TNFα -/-) had markedly reduced CTL infiltration of the liver compared to normal controls after intravenous administration of an E1-,E3-deleted adenoviral vector [63]. Importantly, the absence of TNFα expression was associated with prolonged expression of the viral transgene. These mice also had substantially reduced

levels of anti-adenoviral antibodies following infection compared to normal controls. Mice deficient in both TNFα and its related protein, lymphotoxin (TNFβ) had reduced inflammation and impaired production of anti-adenoviral IgG and IgA antibodies after intravenous adenovirus administration [70]. The impairment of humoral response in these TNFα/TNFβ-deficient mice was sufficient to permit successful reinfection by the vector. In a separate strategy targeted at the TNFα receptor, prior administration of soluble TNF receptor (type I) resulted in prolonged adenoviral vector-mediated gene expression [71]. In a similar approach, mice genetically deficient in both type I and type II TNF receptors demonstrated a significantly reduced humoral antibody response, but no significant prolongation of transgene expression [72]. In the latter study, adenoviral vector-mediated gene transfer of human IL-10, a potent deactivator of macrophages that suppresses TNFα synthesis, also abrogated the neutralizing antibody response to adenoviral vector infection, but had no effect on prolonging transgene expression. Together, these observations show that TNFα, a critical regulator of innate immunity to adenovirus, is also an important regulator of the adaptive immune responses to adenovirus.

Other cytokine mediators of innate immunity are also important in regulating the adaptive responses to adenovirus. For example, IFNγ is important in activation of alveolar macrophages and also directs T helper lymphocyte development toward T_{H1}-type responses. IL-18 has recently been defined as a critical component of adenovirus-induced IFNγ production after *in vivo* infection which is independent of IL-12-induced IFNγ expression [65]. Although compelling, these studies demonstrating that cytokines produced by innate immune cells regulate both the nature and strength of adaptive immune responses represent only the beginning of our understanding of the complex interplay between innate and adaptive immune responses to adenoviral infection.

VI. Innate Immunity and *in Vivo* Gene Therapy

Recent progress in our understanding of the significance of innate immune responses to adenovirus infection *in vivo* have important implications for strategies of *in vivo* gene therapy using adenovirus. First, the phagocyte-based immune system is present and active prior to vector administration in contrast to adaptive immune responses. Therefore, any strategy designed to deliver large amounts of vector to tissues must take into account the loss of vector due to phagocyte-mediated clearance. Second, clearance of adenovirus by macrophages results in potent inflammatory signals which profoundly influence both molecular and cellular inflammation and likely determine the extent of tissue damage and systemic effects due to adenoviral infection of the

lung. Consequently, if clearance of adenovirus cannot be prevented, uncoupling of phagocytic internalization from release of large amounts of proinflammatory mediators would be useful. Third, because release of inflammatory cytokines determines the level of clearance of adenovirally infected cells by NK cells (e.g., IL-12, IFNγ) and CTL (TNFα, TNFβ), innate immunity also has a major influence on the duration of transgene expression. Thus, immunosuppressive strategies should be aimed at both innate and adaptive clearance mechanisms or their regulation cytokines. Finally, innate immune barriers to infection of epithelials cells (mucous barrier, glycocalyx, sequestration of required receptors) must be overcome in order to achieve adequate initial levels of adenoviral vector-mediated gene transfer.

VII. Future Directions

Recent efforts to better characterize the *in vivo* host responses and barriers to adenovirus infection have defined a number of hurdles to the use of adenoviral vectors for *in vivo* human gene therapy. With this groundwork laid, systematic exploration of these barriers will hopefully lead to development of the means to circumvent them. This is already a very active area of research and is likely to continue to be productive. Adenoviral vectors also provide excellent tools with which to study various biological processes *in vivo* and to dissect the specific molecular and cellular innate immune responses to viral infection of the lung. Thus, separate from the utility with respect to development of adenovirus-based gene therapy, such studies are clearly increasing our knowledge regarding the basic biology of an important respiratory pathogen which infects most individuals by adulthood.

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CHAPTER 13

Humoral Immune Response

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I. Introduction

Replication-deficient adenovirus vectors are useful tools for the transfer and expression of therapeutic genes into various types of cells and tissues. They have rapidly emerged as the most efficient system of *in vivo* gene delivery and are widely used for gene therapy in basic research and clinical trials. It is now well recognized, however, that there are many limitations to the use of adenovirus vectors in gene therapy applications. In particular there is a growing body of evidence that suggests that immune responses against components of viral vectors limit gene expression and may induce immunopathological consequences. For the most part immune responses consist of conventional antigen-specific lymphocyte responses; in addition, however, there is a nonspecific and innate response inherent to cells such as macrophages, natural killer cells and organ parenchymal cells.

Antivector immune responses can be broadly divided into cellular and humoral. While the cellular immune response limits duration of transgene expression, the humoral response may reduce the therapeutic efficacy of repeated vector administration. It is also emerging that the humoral immune response to viral vectors is not limited to responses against the viral vector components but also includes anti-transgene responses that can affect the persistence of transgene expression.

If gene therapy is to be successful the host immune response to the viral vector needs to be overcome. In the case of adenoviral vectors there is a robust immune response following systemic or local administration of vector. Most notably there is a significant rise in neutralizing antibodies directed against the virus which can preclude repeat administration of the vector. This may be a disadvantage in definitively correcting genetic defects where repeated administration of vector would be required. An additional problem potentially

exists for human gene therapy applications in that most individuals have preexisting immunity to adenovirus; thus, even the first administration of an adenovirus vector to some patients is likely to be ineffective.

In this chapter the humoral immune response to adenovirus-based vectors is discussed, in particular the many strategies that have been developed to overcome this problem are reviewed. Such strategies include modifications to the viral vector in addition to approaches that modulate the host immune response of the recipient organism.

II. Adenovirus Structure and Serotype

A. Classification of Adenoviruses

The human adenoviruses (Ads) belong to the genus Mastadenovirus and to date 51 serotypes of human Ads have been recognized [1] and grouped into six groups (A to F) [2] on the basis of their hemagglutinating properties and biophysical and biochemical criteria. Genera of the adenovirus (Ad) family have been further subdivided into numerous serotypes (Table I). Serotype is defined on the basis of its immunological distinctiveness as determined by quantitative neutralization with animal antisera [3]. The most common serotypes used for gene therapy applications are Ad2 and Ad5 both belonging to group C. Virus is only neutralized by antibodies raised against antigens or virus of the same type. Thus for example, types 2 and 5 are in the same species group, but antibody to type 2 will only neutralize type 2 virus. Early studies on neutralization of adenovirus by antibodies demonstrated that although most of the antibody made is group-specific and can bind intact virus, only type-specific antibody can neutralize the virus [4, 5].

B. Adenoviral Structural Proteins and Type-Specific Epitopes

Adenoviruses share a common architecture consisting of an nonenveloped icosahedral capsid surrounding a linear dsDNA genome of approximately

Table I
Adenovirus Serotypes

Group	Serotypes
A	12, 18, 31
B	3, 7, 11, 14, 16, 21, 34, 35, 50
C	1, 2, 5, 6
D	8, 9, 10, 13, 15, 17, 19, 20, 22, 23, 24, 25, 26, 27, 28, 29, 30, 32, 33, 36, 37, 38, 39, 42, 43, 44, 45, 46, 47, 48, 49, 51
E	4
F	40, 41

36 kbp. The viral capsid is composed of three structural proteins: hexon, fibre, and penton base. Hexon is the major structural component, forming the 20 facets of the icosahedron, whereas pentons, being complexes of penton base with fibre, form the 12 vertices [6]. The Ad structural proteins, hexon, fibre and penton base, are the antigenic determinants for both group- and type-specific antibodies. The α determinant demonstrable on hexon is responsible for cross-reacting group antigen found on most of the known human and animal Ads, while the ε determinant of fibre give rise to type-specific neutralization antibodies. In addition, hexon possesses a complex arrangement of antigenic determinants, including those with genus, type, intersubgenus, and intrasubgenus specificities [7].

1. Hexon

Ad2 hexon and the closely related Ad5 have been extensively studied. Hexon was the first animal viral particle to be crystallized [8–11] and the Ad2 structure has been resolved by X-ray crystallography to 2.9 Å resolution [12, 13]. These studies revealed the Ad2 hexon trimer as a hexagonal "pedestal" base from which a "tower" region projects outward into the solvent. Three surface loops, L1, L2 and L4 from each monomer interdigitate to form the tower domain (Fig. 1a, see color insert). Sequencing studies completed on adenoviruses from groups A, B, and C have confirmed that there are common sequences within the hexon protein coding domain that are extensively shared. More than 99% of the variability between the hexons of different Ad serotypes is accounted for in seven hypervariable regions [14] that map to the exterior of the protein and include the serotype-specific epitopes [11]. In contrast, the sequences that code for the pedestal are highly conserved.

In the first reported X-ray crystallography of the structure of Ad2 hexon [13], there were some unexplained findings which have now been resolved by X-ray cyrstallographic analysis of Ad5 hexon [11]. In particular, the Ad5 hexon model provides new insights into the location of the type-specific epitopes; Ad2 and Ad5 hexon sequences are homologous (86% identity), so their molecular structures are closely related. The structure of the Ad5 hexon monomer (Fig. 1b, see color insert) is in general similar to the previously described Ad2 hexon structure [13]. However, the most significant difference is the localization of seven hypervariable regions (HVRs), five of which mapped to the surface of the Ad2 hexon structure and two of which were buried. The new model for Ad5 hexon clears up this anomaly, with all the HVRs locating to the top of the molecule [13].

In efforts to define the hexon residues responsible for type specificity, several analyses have been made of adenovirus hexon primary sequences and of anti-peptide sera that can neutralize adenovirus in a type-specific manner [12–19). Results from these studies suggest that adenovirus-type determinants are localized to sequences within DE1 and FG1 in the Ad5

hexon (Fig. 1b). For Ad2, type-specific domains have been mapped to unique sequences in loop 1(amino acids 281–292) of the hexon and loop 2 (amino acids 441–455) (Fig. 1a), by generating neutralizing antibodies to peptides from each of these regions [19].

2. Fibre

Pentons are the vertex structures of adenovirus capsids. They consist of a penton base protein which anchors a single fibre. The fibre is a trimeric protein [20–22] which allows virus attachment to cells [23], prior to penton base-mediated entry [24, 25]. The fibre has a tail, a slender shaft of variable length, and a globular head [26, 27]. The head domain facilitates attachment and there is appreciable variation in head domain amino acid sequences, with several amino acids fully conserved [28]. Variation in head domain sequences may explain differences in receptor specificity between serotypes. For instance human adenovirus serotypes 3 and 5 have been shown to bind to two different cellular receptors via their fibre knob domain [29]. Until recently little was known about the receptor responsible for Ad attachment. However, a 46-kDa protein, Coxsackievirus and adenovirus receptor (CAR), was identified as the receptor responsible for Coxsackie B virus infection of human and mouse cells. In addition, this protein also functions in adenovirus attachment and adenovirus-mediated gene delivery [30].

High-resolution X-ray crystallography of adenovirus type 5 (Ad5) head has revealed much information on the organization of this domain [31]. From these studies it was determined that strongly recognized antigenic determinants containing linear epitopes map to the outer loops or uppermost β-sheets in the fibre head or knob domain. Studies with peptide-based epitope mapping using polyclonal or monoclonal antibodies against both native Ad2 fibre, Ad2 or Ad5 head domain further support this observation [32–34] and demonstrate that the Ad2 knob domain contains a neutralizing type-specific epitope, a neutralizing group-specific epitope, and a nonneutralizing type-specific epitope [32].

3. Penton Base

Although hexon and fibre contain most of the epitopes recognized by neutralizing antibodies, there are epitopes on penton base that are also recognized by some neutralizing antibodies [35]. Recent studies on the identification of immunoreactive domains on penton base show that there are three major immunoreactive regions [36]. One is located at the N-terminal domain, whereas the two others are symmetrically displayed on both sides of a conserved RGD motif, one overlaps the fibre-binding site. Interestingly, MAbs against these immunoreactive domains are not neutralizing. However, the RGD motif of penton base, which is involved in binding to cell integrins and internalization of virus, is thought to escape neutralization due to steric hindrance by the fibre protein which prevents IgG binding to all RGD sites on penton base within the intact virus [37].

C. Chimeric Adenovirus Vectors

The value of the detailed structural studies on adenovirus hexon is of significance in the design of chimeric adenoviruses. Alteration of type-specific epitopes in hexon can be achieved by exchange of epitopes between adenovirus serotypes. The practical significance of changing the type determinants of a vector would be to allow readministration of a gene to a host with circulating neutralizing antibodies. Chimeric adenoviruses were created by replacing the entire hexon in Ad5 or just the hexon FG1 loop (Fig. 1b) with that from Ad2 [38], the rationale behind this being that type-determining epitopes are primarily associated with this loop. However, in spite of the serotype distinctiveness of the chimeric hexon viruses, epitope similarity between the vectors resulted in a low level of cross-reactive neutralizing antibody. Thus exchanging the Ad5 hexon with the Ad2 hexon protein did not allow readministration to animals that had previously been exposed to Ad5 virus and thus had anti-Ad5 neutralizing antibodies [38].

In contrast attempts to isolate Ad5/Ad7 chimeras failed, suggesting that it may be difficult to exchange hexons between adenoviruses of greater evolutionary distance [38]. However, a recent study demonstrates that it is indeed possible to package the Ad5 genome into virus particles that contain hexon proteins from other Ad serotypes [39]. Using viruses that represent subtypes B (Ad3), D (Ad9), and E (Ad4), it was shown that Ad5 DNA could be packaged into capsids that contain the hexons of more divergent subtypes. An alternative strategy was employed in the generation of an Ad5/Ad12 chimera where rather than replacing the entire Ad5 hexon, the external loops of hexon were just replaced [40]. With this approach an Ad5/Ad12 hexon chimera was created by replacing all four loop domains of Ad5 hexon with those of Ad12 hexon [40]. Ad12 is in subgroup A, which is evolutionary quite distant from the Ad5/Ad2 containing subgroup C [41]. This chimeric virus efficiently evaded host immunity in mice immunized previously with an Ad5 vector.

The alteration of the type-specific epitopes is not limited to the exchange of epitopes between adenovirus serotypes. For example, an eight-amino-acid sequence from the major antigenic site in the VP1 capsid protein of poliovirus type 3 was inserted into two regions of Ad2 hexon [42]. Antisera specific for the poliovirus sequence efficiently neutralized the modified adenovirus and antisera raised against the modified adenovirus recognized the VP1 capsid protein of poliovirus type 3.

The ability to switch hexons is most likely due to the considerable conservation of the amino acid sequence of hexon across subtypes [39]. As already discussed, more than 99% of the variability between hexons of different Ad serotypes is accounted for in seven hypervariable regions that map to the exterior of the protein and include the serotype specific epitopes (section II.B.1). Since hexon protein is a major antigenic determinant of the Ad capsid, this approach of switching capsids or replacing epitopes may prove

useful in reducing the antigenicity of therapeutic Ad vectors to allow repeated vector administrations. Thus modifications to the hexon structure can be used as a strategy to modulate the immune response to adenoviral vectors.

D. Influence of Serotypic Variations on Adenoviral Cell Interactions

Entry into and trafficking through target cells has been studied mainly with subgroup C viruses. Binding to target cells occurs via a high-affinity interaction between the fibre protein and the coxsackie-Ad receptor (CAR) on the cell surface [30]. Subgroup C adenovirus then rapidly enter cells by endocytosis through interaction of the penton base protein with vitronectin binding integrins on the cell surface, including $\alpha v\beta 3$, $\alpha v\beta 5$, $\alpha m\beta 2$, and $\alpha 5\beta 1$ integrins [24, 43–46]. Endosomal membranes are lysed by adenovirus, allowing the escape of capsids to the cytosol [24, 47, 48]. Finally, adenovirus translocates to the nucleus by using microtubules in the cytoplasm, binds to the nuclear envelope, and inserts its genome through nuclear pore complexes [49, 50].

There are three viral interactions with the host cell entry pathway which can be influenced by serotypic variations in the capsid proteins: (i) different serotypes of adenovirus bind through different cell surface receptors [29, 51], (ii) the secondary virus–cell interaction mediated through penton base varies by serotype [24, 25, 43], and (iii) escape from the endosome may involve distinct mechanisms of vesicle disruption, again in a serotype-dependent manner [46, 51].

At least two points along the pathway of gene transfer by adenovirus are susceptible to blockage by circulating antibodies, blocking the fibre protein–cellular receptor interaction (CAR) and escape from the endosome. Escape of the viral genome from the endosome can be inhibited by capsid-bound antibodies against both hexon, the major capsid component, and penton base [35]. Antihexon antibody is considered to be the dominant neutralizing antibody in response to adenovirus infection [35]. Neutralization by anti-hexon antibodies has single hit kinetics with an average of 1.4 antibody molecules bound per virion, and it is thought that bound antibody inhibits a low pH-induced conformational change that takes place in the acidic endosomes [35]. During this conformational change the N-terminal region of the protein is exposed and antibodies directed against an N-terminal 15 K proteolytic fragment can neutralize virus infectivity [52]. In contrast anti-penton base antibodies play a far less significant role in the neutralization of adenovirus compared to anti-hexon antibodies, inhibiting infection by only 50% [35]. As already mentioned (section II.B.3), this may be due to the architecture of the penton–base–fibre complex where steric hindrance by fiber may prevent IgG binding to penton base.

Although antifibre antibodies can neutralize adenovirus infection [35], there are reports claiming that fibre is not an important immunogen [4, 53]. Additionally, the immunogenicity of fibre varies; it is a relatively weak

immunogen if it is delivered as a purified protein and a relatively strong immunogen when delivered in the context of a whole virus particle [54]. It is thought that anti-fibre antibodies do not possess true neutralizing activity until present at very high titer [55] and only then do they prevent transduction following a systemic administration of a gene therapy vector [35].

Use of an Ad chimera, adenovirus type 5 with a fibre gene from Ad7A, [55] has helped define the contribution of the antifiber humoral immune response against adenovirus infection. The chimeric virus clearly demonstrated that under standard repeat administration conditions, in the rat, the antibody generated against the fibre gene is inconsequential with regard to neutralizing function, suggesting that the only capsid protein that is functioning as a dominant neutralizing epitope is the hexon trimer [55]. In addition, results from a human clinical trial following administration of an Ad vector suggest that anti-fibre antibodies are only neutralizing when acting in synergy with anti-penton base antibodies [56].

III. Host Response to Gene Therapy Vectors

Three phases have been described in the elimination of adenovirus after intravenous delivery [57]. Phase one involves innate immune mechanisms, which occurs within 24 h postinfection and accounts for the elimination of 90% of the adenoviral DNA. Phase two is mediated by the adaptive immune system and consists of a cytotoxic T lymphocyte (CTL) and/or antibody response to the transgene and/or to the viral proteins. Phase three is characterized by a slow and constant decrease of the transgene expression even in the absence of an immune response to the transgene and is thought to be the result of leaky expression of viral gene products which leads eventually to clearance of the transduced cells.

A. Innate Immune Response

Although immune-related mechanisms play a significant role in eliminating the recombinant Ad genome following *in vivo* administration of Ad vectors, it is not clear how the immune response to Ad vectors is initiated, i.e., for the immune system to be sensitized to Ad antigens. Studies by Worgall *et al.* [58] suggest that there is some initial destruction of the Ad vectors by innate immune mechanisms. In contrast to the antigen-specific, adaptive immune response, innate immune mechanisms comprise the immediate, nonantigen-specific events which include tissue macrophages, which act as scavengers to clear incoming pathogens. In addition tissue macrophages influence the initiation of the adaptive immune response. Innate pathways of virus clearance are also mediated by Kupffer cells within the liver [58]. Following the innate immune response a second phase of the immune response occurs, the adaptive immune response. This results in the generation of antibodies against viral

capsid proteins and transgene, i.e., the humoral immune response, in addition CTLs against viral proteins and transgene products ensue to generate the cellular immune response.

B. Adaptive Immune Response: B–T Cell Interactions

Activated T cells play a critical role in the generation of humoral and cellular immune responses. In general there are two major classes of T cells,

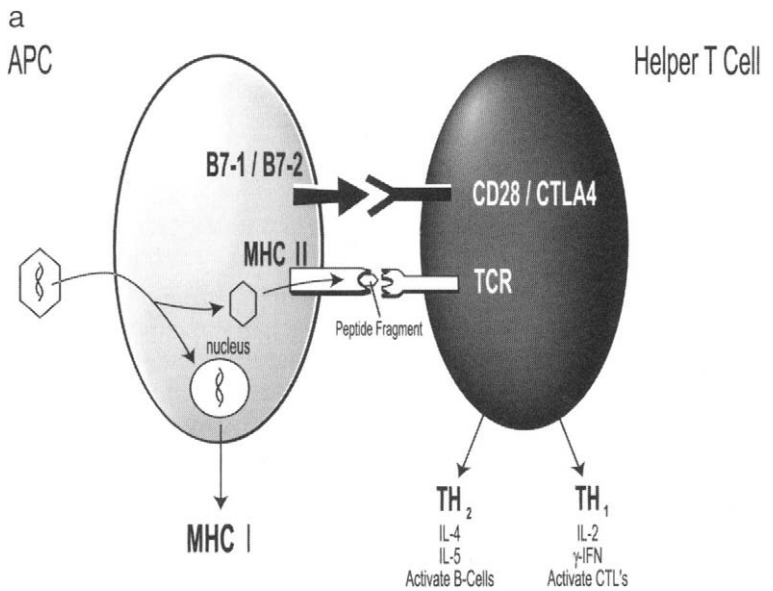

Figure 2 (a) Antigen-presenting cells (APCs) such as dendritic cells and macrophages ingest foreign proteins (viral particles) nonspecifically. Ingested antigens are processed intracellularly and presented as peptide fragments in the context of the MHC class II complex. Activation of the helper T cell occurs following recognition of the foreign peptide/MHC class II molecule by the T-cell receptor, TCR. In addition a second signal is required for helper T-cell activation which occurs when the plasma membrane bound signaling molecules B7-1/B7-2 are recognized by a coreceptor protein CD28/CTLA4-Ig which is present on the surface of the helper T cell. Cellular immune response: At the same time viral vectors can infect (APCs) and deposit their genomes into the nucleus. The genome encodes viral and transgene proteins that are expressed and presented by MHC class 1 molecules to $CD8^+$ cells or cytotoxic T lymphocytes (CTLs). (b) Foreign antigens are taken up from the extracellular fluid by receptor mediated endocytosis following binding to the B cell receptor. They are then degraded and recycled to the cell surface in the form of peptides bound to MHC class II molecules. Thus the helper T cell activates the B cell that displays the same antigen in the context of the MHC class II molecule as originally activated it. In addition, interaction of CD40 ligand with CD40 activates B cells to proliferate and mature into memory and antibody-secreting cells. In secondary antibody responses, memory B cells themselves may act as APCs and activate helper T cells as well as being the subsequent targets of the helper T cells.

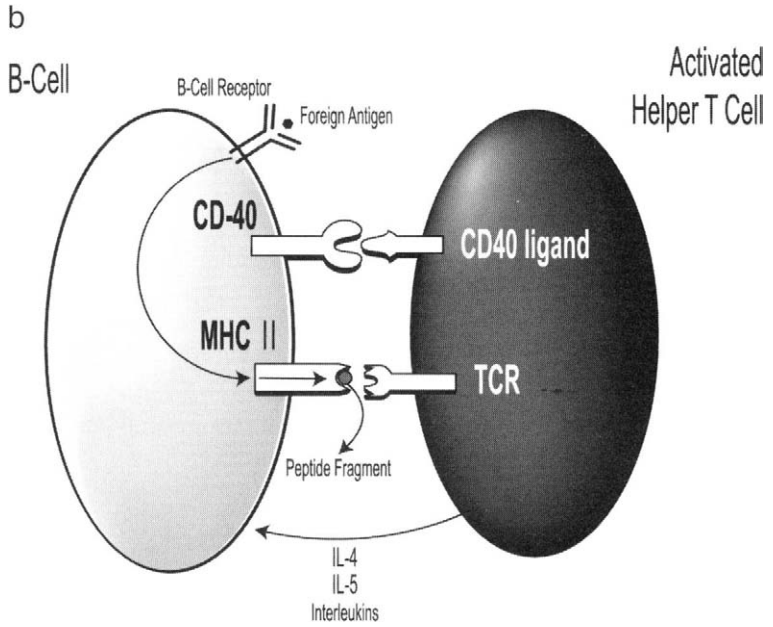

Figure 2 *(continued)*

cytotoxic $CD8^+$ T cells (CTLs) and helper T cells ($CD4^+$ T cells). In the context of a viral infection, CTLs eliminate virally infected cells expressing neoantigens such as viral proteins and the transgene product. In contrast, $CD4^+$ T cells help activate the responses to extracellular antigens by stimulating B cells to proliferate and secrete antibodies. Activation of antigen-presenting cells (APCs) and B cells by input viral capsid proteins underlies the mechanism responsible for the production of the humoral immune response to Ad vectors. Administration of UV-inactivated virus leads to a full humoral response without any CTL involvement, which is consistent with the role of exogenous viral capsid proteins in the activation of B cells and of endogenously produced antigens in the activation of primary $CD8^+$ cells [59, 59a]. However, activation of $CD4^+$ T cells by viral capsid proteins has been shown to contribute to CTL-mediated clearance of Ad-transduced cells in addition to stimulating B cells to produce neutralizing antibodies [59–60].

Activation of T cells by antigens requires a complex program of molecular interactions between the T cell and an APC, each of which could be a target for immune blockade in gene therapy. Initiation of a T-cell-mediated response requires APCs which present short peptides derived from ingested foreign antigens (e.g., a virus particle) in association with major histocompatability (MHC class II) molecules to interact with the T-cell receptor (Fig. 2a). In

addition, there are other signals that are needed for successful stimulation of the T-cell response. These include B7-1 (CD80) and B7-2 (CD86) ligands present on antigen presenting cells which bind to the CD28 / CTLA4 receptors on T cells and elicit a costimulatory response needed for this activation [61]. Such costimulatory responses result in cytokine secretion and full T-cell activation (Fig. 2a). There are two functionally distinct subclasses of helper T cells that can be distinguished by the interleukins that they secrete upon activation. Th1 cells secrete IL-2 and gammainterferon and are concerned mainly with helping cytotoxic T cells and macrophages, while Th2 cells secrete interleukin (IL)-4 and IL-5 and are concerned mainly with helping B cells (Fig. 2a).

Once activated, the helper T cell can stimulate a B cell that specifically displays the same complex of foreign antigen and MHC class II protein on its surface (Fig. 2b). T-cell dependent activation of B cells in turn leads to upregulation of CD40 ligand (CD40L) on the T cell, promoting the engagement of CD40 on the cognate B cell [62] (Fig. 2b).

IV. Strategies to Overcome the Humoral Immune Response

The humoral and cellular immune response to recombinant adenoviral vectors, as described in several animal models, result in extinction of transgene expression, severe local inflammation, and production of neutralizing antibodies that prevent readministration [59, 63, 64]. A direct correlation between neutralizing antibody and the block to readministration of vector has been established by passive transfer of immunity by sera from treated to naïve animals [59]. One approach to enhance adenoviral-mediated gene transfer is to modulate the host immune response by immunosuppression of the recipient organism.

A. General Immunosuppression

Chronic immune suppression with drugs such as cyclosporine and cyclophosphamide has improved the stability of adenovirus-encoded transgene expression in animal models of liver-, lung-, and muscle-directed gene therapy [64–66]. Cyclophosphamide is a commonly used immune suppressive agent for the treatment of autoimmune diseases and prevention of rejection following allograft organ transplantation [67]. It is activated by hepatic cytochrome p450 to metabolites that exhibit toxicity primarily to dividing cells, including activated T and B cells [68].

Administration of cyclophosphamide with intravenous infusion of adenoviral vector blocked activation of both CTL and T helper cells, resulting in prolonged transgene expression in the liver with reduced anti-Ad neutralizing antibody production [66]. A similar effect was seen in the lung; however, a

much lower dose of cyclophosphamide was needed to prevent neutralizing antibody formation. In contrast, stabilization of transgene expression was achieved only at a high dose. This difference may be a consequence of differences in the route of administration of the vector, which could result in differences in presentation of antigens. For example the intravenous route more likely deposits larger quantities of antigens to tissue enriched with antigen-presenting cells such as the spleen. In addition neutralization of virus in the lung is restricted to the Th2-dependent isotope, which is easier to ablate than ablation of both Th1 and Th2 subsets which contribute to formation of antiviral responses when vector is delivered systemically [66].

In contrast cyclosporin (CSA) alone failed to reduce the production of neutralizing antibodies to cFIX in hemophilia B dogs but was effective at prolonging gene expression of FIX [64, 65]. CSA reportedly inhibits early events in T-cell activation such as activation of interleukin-2 gene expression [69], which may explain why CSA most likely affected the cellular rather than the humoral immune response following adenovirus-mediated gene therapy in the hemophilia B dogs.

One of the main concerns with the protocols used in animal models for general immunosuppression is the high dose necessary to successfully obtain readministration of gene therapy vectors. This is substantially higher than approved doses for use in humans. Thus it remains to be established whether clinically acceptable doses (presumably lower doses) may indeed have the same effect on immunosuppression and allow readministration of gene therapy vectors in a clinical setting.

Bouvet *et al.* [70] report that etoposide at clinically acceptable doses suppresses the formation of neutralizing antibodies and CTLs to adenovirus and results in successful intratumor transgene expression in immunized mice. Etoposide is a semisynthetic derivative of podophyllotoxin that causes an arrest at G2 of the cell cycle. It inhibits DNA synthesis by interfering with the enzyme topoisomerase II and leads to cell death by apoptosis [71]. Thus repeated adenoviral-mediated gene therapy may be achievable in cancer patients who are concurrently undergoing treatment with chemotherapy.

Most of the immunosupressants discussed so far have the distinct disadvantage of causing general immunosuppression that may not be desirable in some clinical settings. An alternative immunosuppressant, deoxyspergualin, (DSG), with more selective properties has been shown to be useful in readministration of systemically delivered viral vectors expressing Factor IX [72] or lung-directed viral vectors expressing the human cystic fibrosis conductance regulator (hCFTR) [73]. Deoxyspergualin interferes with the differentiation of B and T cells and also with antigen processing. An important property of DSG is that it does not induce a general suppression of the immune system, but rather results in a selective lack of response to specific antigens presented at the time of drug treatment.

B. Transient Selective Immunosuppression

The central role of the $CD4^+$ T cell provides a strategy to prevent humoral and cellular responses to adenovirus vectors through a transient blockade of $CD4^+$ T-cell activation at the time of vector administration. The rationale for this approach is that chronic immune suppression should not be necessary if the primary stimulus for activation is the input capsid proteins. In support of this hypothesis, it has been shown that depletion of $CD4^+$ T cells with a monoclonal antibody (GK1.5) at the time of vector administration can effectively prevent CTL and B-cell responses in murine models of liver- and lung-directed gene therapy [74–76].

1. Cytokine Treatment

Selective inhibition of the TH2 subset of T helper cells by administration of the cytokine interleukin 12 or gamma interferon (IFNγ) with adenovirus vector has prevented the humoral immune response in mouse lung tissue [77]. The success of this approach, however, depends on the relative contribution that Th2-dependent immunoglobulin (Ig) isotypes play in virus neutralization, the profile of which may be affected by strain and species of animal as well as routes of vector administrations. Th2-specific ablation with IL-12 is an effective approach for lung-directed gene therapies in the mouse where IgA is the primary source of neutralizing antibodies. However, in the case of the mouse liver both Th1 and Th2 cells contribute to the production of virus-specific antibodies and although IL-12 reduced the total amount of neutralizing antibody in this organ it was not enough to allow effective readministration of the virus [75].

2. CTLA4 Ig

Interfering with the distal pathway of $CD4^+$ activation by administering CTLA4 immunoglobulin (Ig) with adenovirus vector improved the stability of recombinant gene expression in mouse liver but did not significantly impact neutralizing antibody production or allow systemic vector readministration [78]. muCTLA4Ig is a chimeric protein of murine immunoglobulin IgG2a fused to murine CTLA4 and is an inhibitor of the CD28-B7 pathway (Fig. 2a). In contrast in the lung huCTLA4-Ig treatment significantly blocked the formation of neutralizing antibodies allowing efficient readministration of virus, whereas transgene expression was only moderately prolonged [79].

Differences between the effects of this drug on humoral and cellular responses in lung versus liver are surprising. It is known that secretory IgA, which depends on Th2 cells, contributes to neutralization in the lung, whereas antibodies of other isotypes, such as IgG1 and IgG2a, neutralize virus administered systemically. As previously discussed, different routes of virus instillation may result in different mechanisms of antigen presentation, which could affect

inhibition of B- and T-cell activation by CTLA4Ig. For example, local injection of an adenovirus vector expressing CTLA4 Ig into the brain suppressed not only local cell infiltration in this tissue but also reduced the humoral immune response to adenovirus [80].

3. Anti-CD40 Ligand Antibody

The expression of CD40L by activated helper T cells (Th) triggers B cell cycling through binding to CD40 (Fig. 2b). CD40L is expressed transiently at high levels on activated $CD4^+$ T cells [81, 82]. The costimulation provided by CD40L with CD40 is essential for thymus-dependent humoral immunity [82, 83] and is also thought to play an important role in the generation of cellular immune responses through the production of helper cytokines [83, 84]. A transient block of costimulation between T cells and B cells and other antigen-presenting cells using a monoclonal antibody against CD40 ligand (MR-1) suppressed the development of antibodies against Ad delivered to mouse or nonhuman primate lung, in addition to decreasing the cellular immune response to the vector [85–87]. This in turn resulted in an increase in persistence of transgene expression. Furthermore, when MR1 was administered with a second dose of Ad vector to mice preimmunized against vector, it was able to interfere with the development of a secondary antibody response and allowed for high levels of transgene expression upon a third administration of vector to the mouse airway [86]. Similarly in the nonhuman primate lung administration of a humanized anti-CD40 ligand MAb (hu5C8) at the time of vector instillation, markedly suppressed adenovirus-induced lymphoproliferation and cytokine responses, in addition there was a marked suppression of IgA and neutralizing antibodies which permitted vector readministration [87].

In the case of systemic delivery of vector it is thought that only CD40 ligand blockade inhibits anti-Ad antibody generation sufficiently to allow redosing to the liver [88]. Thus a combination of anti-CD40 ligand and murine CTLA4Ig was necessary to allow transduction after secondary vector administration in mouse liver, whereas neither agent alone was sufficient [89].

C. Oral Tolerance

Orally administered antigens have been shown to induce systemic unresponsiveness to a subsequent exposure to the antigen. Oral tolerance primarily results in active suppression by regulatory T cells, clonal anergy, or clonal deletion. What activates one mechanism over the other is not altogether clear, although it is generally agreed that lower doses of antigen more likely lead to suppression and higher doses to clonal anergy or deletion [90]. In addition, the form of antigen and frequency of feeding influences the type of tolerance induced [91].

Long-term adenovirus-based gene expression was observed in the liver of rats with preexisting anti-adenovirus antibodies that were tolerized by feeding

viral proteins (11 × 1-mg dose) [92]. In the tolerized rats the anti-adenovirus humoral immune response was downregulated allowing systemic readministration of vector. Moreover, in the tolerized rats vector readministration did not lead to a secondary humoral immune response, suggesting that repeated adenovirus-directed gene transfer may be possible despite the presence of a residual antibody titer from a previous exposure to vector [92].

Similarly, this approach has been used to enhance gene transfer to the rat parotid gland which is within the mucosal immune system [93]. As it is more difficult to induce complete mucosal tolerance compared to systemic tolerance only partial tolerance to mucosally applied viral vectors was achieved. A different feeding regimen of adenovirus (5 × 50 μg dose) along with a different form of antigen, UV inactivated vector, in addition to a difference in route of viral challenge may explain the differences in the results from the two studies. Nonetheless, it is possible to induce some degree of tolerance to mucosally applied adenovirus by feeding animals the virus. In clinical situations, some concern may exist about tolerizing a host toward adenoviruses, which can be pathogenic in humans.

D. Serotype Switching

One strategy to circumvent Ad vector-specific neutralizing immunity is to switch the serotype of the Ad vector [94, 95]. As already discussed in section II.A, there are 51 serotypes that are classified on the basis of biological, chemical, immunological, and structural properties into six subgroups and then into serotypes based on neutralization by antisera to other Ad serotypes. Following an initial administration of adenovirus, serotype-specific antibodies are generated against the major viral capsid proteins (section II.B). Group C adenoviruses include Ad2 and Ad5, the more commonly used serotypes for gene therapy vectors. While the capsid proteins of the group C adenoviruses are highly conserved, viruses from a different subgroup have capsid proteins which are only weakly homologous to the group C viral capsid proteins. Thus the immune response against the non-group-C viruses in many instances does not block infection by a group C virus [94]. This was demonstrated in a study where Sprague–Dawley rats were injected intraperitoneally (ip) with wild-type Group B virus, WT Ad7, either alone or sequentially with WTAd4 (group E) prior to intracardial administration of an Ad5-based gene transfer vector [94]. Transgene expression in all animals that received non-group-C viruses prior to Ad5 was equivalent to naïve animals. In contrast, animals that received WT Ad5 prior to the Ad5-based gene transfer vector had greatly reduced levels of transgene expression compared to naïve animals.

Similarly, in the development of gene therapy vectors for the lung and the treatment of cystic fibrosis, it was shown that intratracheal administration of an immunizing does of wild-type Ad 4 (subgroup E) or Ad30 (subgroup D)

did not affect the subsequent expression of human CFTR from an Ad5 based gene transfer vector [95]. More importantly the alternate use of Ad vectors from different serotypes (Ad2, Ad5) within the same subgroup (C) can also circumvent anti-Ad humoral immunity and permit effective gene transfer to the lung [96] and to the liver [97] upon repeat administration. In the context of future clinical applications for this approach it is relevant that Ad2- and Ad5-based vectors can be administered alternately as these are the Ad serotypes that are in current use in human clinical trials.

E. Masking Neutralizing Epitopes

Alternative approaches have been developed for circumventing antibody neutralization of Ad vectors that are centered on modification of the Ad virion rather than on immunosuppressive treatment of recipient animals [98–101]. One such approach involves the covalent attachment of the polymer PEG (polyethylene glycol) to the surface of adenovirus [99, 100]. Covalent modification of Ad virions using chemically reactive PEG renders the virus less susceptible to neutralization, due to shielding of neutralizing epitopes on the surface of the virus by PEG molecules. The components of the capsid that elicit a neutralizing immune response, i.e., hexon, fibre, and penton base (see section II.B) are the main targets for PEGylation. Importantly the covalent attachment of a PEG polymer to the surface of the adenovirus can be achieved with retention of infectivity, while PEG-modified adenovirus have been shown to be protected from antibody neutralization in the lungs of mice with high antibody titers to adenovirus [99, 100].

Similarly, Beer *et al.* [98] demonstrated that adenovirus vectors could be formulated in a polymer preparation of PLGA (poly(lactic/glycolic acid) with retention of bioactivity. Mice immunized subcutaneously with encapsulated recombinant adenoviral vectors show a greater than 45–fold reduction in anti-adenovirus titers relative to nonencapsulated vectors. Although the authors do not show any *in vivo* data they postulate that the process of encapsulation of a vector in a polymer preparation may potentially mask the adenovirus from circulating antibodies. This is also based on the observation that encapsulated vectors are less susceptible to neutralization than nonencapsulated vectors *in vitro*. The possible disadvantages of this approach include the efficiency at which the Ad vector is released from the polymer and the true demonstration that this approach has any advantage *in vivo*.

More recently another nongenetic strategy to modify the surface of the virion has been described. This involves a covalent coating using a multivalent hydrophilic polymer based on poly-[*N*-(2-hydroxypropyl)methacrylamide] (pHPMA). Multivalent polymeric modification of adenovirus rendered the virus less susceptible to neutralization by anti-adenovirus antibodies. As with the studies with PLGA this approach was shown only to be effective *in vitro* and may not be applicable in an *in vivo* setting.

F. Immunoapheresis

Another novel approach to overcoming the problem of serum neutralizing antibodies was described by Chen *et al.* [102], and involves the principle of immunoapheresis. An affinity column consisting of cloned recombinant capsid proteins was generated to specifically remove anti-adenovirus antibodies from human clinical serum samples. The authors postulate that such an affinity column could be used in conjunction with apheresis in a technique called immunoapheresis. During the apheresis procedure, patients' serum could pass through this immunoaffinity column removing anti-adenovirus antibodies. Anti-adenovirus antibodies would be expected to repopulate the vascular compartment eventually but a temporal window of several hours for intravascular adenovirus therapy could be created [103].

V. Factors Modulating Host Responses to Gene Transfer Vectors

The immune response generated to adenoviral vectors can be broadly divided into cellular and humoral responses. The cellular immune response results in cytotoxic T cells against viral proteins and transgenes that can lead to the destruction of vector-transduced cells and reduced persistence of transgene expression. In addition, there is the humoral immune response against both capsid proteins and transgene. The design of the vector backbone has been shown to influence the persistence of transgene expression by modulating the cellular immune response. Similarly, the use of tissue-specific promoters to drive transgene expression can modulate the humoral immune responses to the transgene product.

A. Viral Vector Backbone

1. First-Generation Adenoviral Vectors

First-generation adenoviral vectors are typically deleted for E1 and sometimes for E3, which result in decreased expression of early and late genes and deficient replication of the virus (Fig. 3). Multiple studies performed with these vectors in mice, Cotton rats, and nonhuman primates have shown successful gene transfer in a variety of tissues with high levels of expression of transgenes [104–107]. However, a major shortcoming of these E1-deleted vectors is the general loss of transgene expression at 3–4 weeks after administration [59, 63, 108], although administration to neonatal or immune-deficient animals frequently results in more persistent expression [64, 63, 109]. The replication defect of E1-deleted viruses can be overcome in part because cellular factors complement the function of the E1 gene, leading to the expression of viral

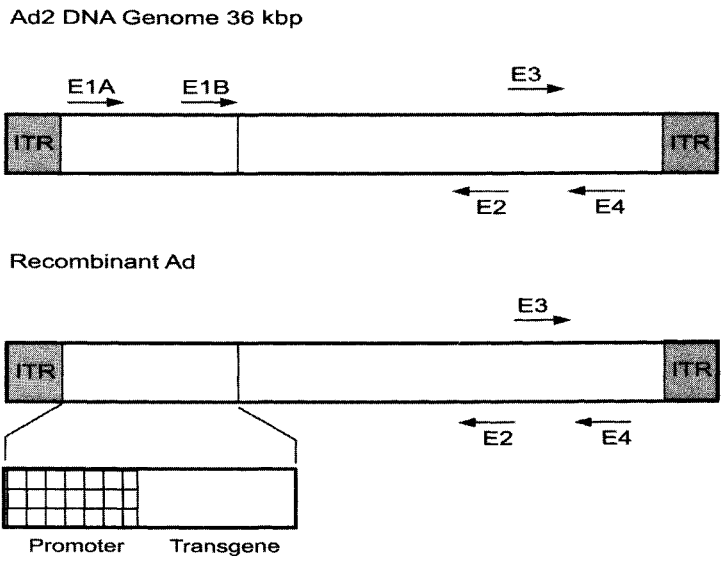

Figure 3 Schematic of adenovirus genome. The bar indicates the DNA genome of 36 kb. The black arrows indicate the transcription units: E1A immediate-early transcription unit and the delayed-early E1B, E2, E3, and E4 transcription units. The expression cassette replacing the E1 region is depicted. The cassette contains a promoter with transcriptional elements used to drive expression of the transgene. The transgene is the open reading frame in the recombinant transcript that translates the desired protein. First-generation adenoviruses are made by substituting an expression cassette for the E1 and/E3 regions. Second-generation adenovirus vectors are generated by the additional deletion of genes necessary for viral replication, e.g., E2a DNA-binding protein [114, 115]; E4 region [113]; the E2b-encoded terminal protein and viral DNA polymerase [120]. Third-generation vectors are vectors deleted for all viral genes, but retain the *cis*-acting sequences necessary for viral replication and packaging (see Chapter 15).

proteins that are presented in the context of MHC class1 molecules to elicit a cytotoxic T-cell response [63]. For these reasons additional changes have been introduced into the backbone of adenovirus vectors to render them more replication-defective and thus further reduce their potential for viral gene expression.

2. Second-Generation Adenoviral Vectors

Two regions of adenovirus, E2 and E4, which play critical roles in viral DNA synthesis and late gene expression have been targeted for deletion [110–113]. A temperature-sensitive mutation, ts125, and a deletion have been introduced into the E2a region [111, 114, 115], vectors containing this mutation showed prolonged transgene expression in CBA mice, Cotton rats, and nonhuman primates. However, contrary to these reports, a recent study using BALB/C mice and hemophilia B dogs demonstrates that this E2a mutation

is insufficient for achieving persistent expression [116]. In the case of E4-deleted vectors long-term gene expression is dependent on both the promoter used to control expression and the context of the E4 region [110]. Interpretation of the earlier work on deletions in the E2 and E4 regions were complicated by the immunogenic transgenes which were used in these studies [117, 118]. Another version of a second-generation vector was generated using a nonimmunogenic protein the hCFTR [119]. The vector contained wild-type E2 and E4 with a partial deletion in the E3 region and when instilled into the lungs of various strains of immunocompetent mice persistent transgene expression was measured in lung tissue up to 70 days. In this vector the persistence of transgene expression was attributed in part to the CMV enhancer–promoter used for transgene expression in conjunction with a wild-type E4 region [110, 119].

Other groups have also reported on the optimization of vectors deleted for E1 and DNA polymerase. This significantly modified vector expressing the highly immunogenic β-gal transgene was shown to persist in the livers of immunocompetent mice for up to 2 months [120]. Such a vector could have broad benefits for use in human gene therapy in which the encoded transgene may be seen as a neoantigen by the human immune system.

3. Helper-Dependent Vectors

More recently, vectors deleted for all viral coding sequences (helper-dependent or "gutless" Ad vectors) have been developed, so that leaky expression of viral protein is eliminated [121–124] (see Chapter 15). Such a helper-dependent vector has been generated which contains the entire human alpha1-anti-trypsin gene under the control of a tissue (liver)-specific promoter. This vector results in more than 1 year of stable expression, provides supraphysiological levels of hAAT in the mouse, and demonstrates less hepatotoxicity compared with first-generation vectors [125, 126]. Although many of the advantages of this gutless vector can be attributed to elimination of leaky viral gene expression it was later shown that the inclusion of a tissue-specific promoter also helped reduce the development of a host immune response to the transgene [127].

4. Tissue-Specific Promoters

The use of tissue-specific promoters may be helpful in avoiding host immune responses to transgenic proteins in human gene therapy, the rationale behind this being that expression in antigen-presenting cells would be greatly reduced. Thus the transcriptional unit responsible for expression of the transgene in an adenoviral vector could have a substantial effect on the nature of the ensuing immune response. Most experiments have used constitutively active promoters that may express efficiently in dendritic cells. A reduced immune response is seen with vectors that contain more specific promoters, which may not express efficiently in antigen presenting cells [127]. For example, when a helper-dependent Ad vector expressing the hAAT cDNA from a liver-specific

promoter was used to express hAAT in C3H/HeJ mice, anti-hAAT antibodies did not develop and long-term expression of hAAT resulted [128]. In contrast, use of a non-liver-specific promoter to drive expression of the same transgene resulted in antibody production to hAAT in the same mouse strain. Thus, vector-specific differences in transgene expression within APCs due to choice of promoter could explain some of the variation in immune responses that have characterized *in vivo* applications of gene therapy vectors.

5. E3 Region

In addition to making progressive deletions of the adenoviral backbone other groups have coexpressed the Ad early region (E3) with the transgene of interest [128]. Injection of Ad-overexpressing E3-encoded gene products leads to inhibition of cytotoxic activity toward Ad-infected cells in addition to marked down regulation of antibody formation to structural viral proteins. Genes encoded by the E3 region downregulate surface MHC class I expression, which in turn interferes with presentation of viral peptides and reduced CD8+ cytotoxic T lymphocytes. Thus the absence of Ad-specific CTLs in animals injected with this vector was expected. What was unexpected was the inhibition of a humoral antibody response to this E3 overexpressing vector. The authors suggest that transduction of an E3 containing Ad vector into liver cells in the absence of CTL or TNFα-induced cytolysis may result in poor antigen release, consequently there is little antigen presentation by APCs to initiate an antibody response. TNFα is one of the cytokines that controls dendritic cell maturation and migration; thus, early antigen presentation may be downregulated by inhibition of this cytokine by E3 proteins [128].

However, other studies using an Ad vector overexpressing the herpes simplex ICP47 gene suggest that use of any vector that downregulates MHC class I presentation should be assessed carefully [129]. Coexpression of ICP47 has a similar result to expression of E3 in that there is downregulation of MHC class I presentation. Administration of this vector to the lungs of rhesus monkeys inhibited the generation of Ad-specific CTLs. However, natural killer cell activity was enhanced, suggesting that strategies to protect the Ad-transduced cell without interfering with MHC class 1 expression should also be explored.

B. Species and Strain

To date the majority of animal studies evaluating the efficacy of adenovirus vectors have been performed with vectors expressing bacterial or human proteins. Clearly these proteins also constitute potential antigens recognized as foreign by the host immune response. Administration of vectors encoding the murine erythropoietin resulted in long-lasting elevated hematocrit levels in mice [130]. In contrast, injection of adenoviruses carrying the human erythropoietin induced a strong immune response directed against the human protein, which resulted in transient expression of the transgene.

Most *in vivo* studies are performed in inbred mouse strains of various MHC haplotypes whose immune systems might react differently to a given antigen. Inbred immunocompetent C57BL/6 mice have been a favored strain to study transgene expression of human blood coagulation Factor IX from viral vectors. This is in part because systemic expression of the secreted protein is not limited by antibody responses following intravenous (iv) injection of vector. Importantly iv injection of an Ad vector results in sustained expression of human FIX in normal or hemophilic C57Bl/6 mice, while antibodies against FIX develop in other strains [131, 132]. A similar observation was seen with an Ad vector encoding human Factor VIII under the control of a liver-specific promoter following treatment of hemophilic C57Bl/6 mice. High-level human FVIII expression was detected in the serum of the mice for over 5 months with no antibody production against the transgene [133]. In contrast treatment of FVIII-deficient hemophilic dogs with an Ad vector encoding human FVIII resulted in a strong antibody response directed to the human protein [133].

A reason for the difference in antibody response between different mouse strains was thought to be due to the MHC haplotype. C57BL/6 mice (haplotype H-2b) lack MHC class II allele IE, and may therefore have some deficiency in humoral immune responses. However, mice of another strain with the same haplotype, and therefore the same lack of the IE allele, did mount an immune response to human FIX following systemic administration of a similar adenoviral vector. This suggests that the data produced in studies based on C57BL/6 mice often cannot be extrapolated to other species. The mechanism of tolerance to FIX or FVIII by iv injection of adenoviral vector in C57BL/6 mice remains elusive, but illustrates the difficulty of extrapolation of results obtained in inbred strains of mice and highlights the importance of studies in other animal models [132].

Similarly, others have reported [109, 134] that intravenous administration of an E1-deleted adenovirus vector carrying the human alpha-antitrypsin (hAAT) cDNA leads to a strain-related variation in persistence of expression of transgene. Transient expression of hAAT was seen in C3H/HeJ and Balb C mice with longer persistence of expression seen in C57Bl/6 mice [109, 134]. Persistence was shown to correlate with poor anti-hAAT antibody formation in these mouse strains while Balb C and C3 H mice developed significant levels of anti-hAAT antibodies which resulted in a corresponding disappearance of hAAT in the serum [134]. In contrast, when a helper-dependent Ad vector expressing the hAAT cDNA from a liver-specific promoter was used C3H/HeJ mice failed to develop antibodies and demonstrated long-term expression of hAAT [127].

Careful identification and characterization of the host factors involved in the formation of anti-transgene antibody responses should provide insight into the development of useful gene therapy systems for the treatment of patients with genetic diseases involving null mutations. A good understanding

of these immune responses is critical to the appropriate interpretation of many previous gene therapy studies and to the design of future studies. Although new generations of adenoviral vectors may offer many advantages compared to first-generation vectors in terms of persistence of transgene expression, it is important to establish an improved experimental paradigm to evaluate the effect of vector modifications on transgene expression, especially in the context of a highly immunogenic transgene.

C. Route of Delivery

1. Intravenous versus Intraperitoneal

Studies by Gahery-Segard *et al.* [135] investigating the humoral immune response to Ad capsid components demonstrated that routes of immunization modulate virus-induced Ig subclass shifts. Two routes of immunization, intravenous (iv) and intraperitoneal (ip) were compared for the response induced against the adenovirus particle in particular the three major components of the viral capsid, hexon, penton base, and fiber. The molecular components of the viral capsid are differentially recognized depending on the route of administration. The sera from mice immunized ip recognized only the hexon protein and a preferential switch to the IgG2a subclass was obtained. The sera from mice immunized iv had a more complex response. At the beginning of the response an isotype bias toward the IgG2a subclass was observed, but the isotype distribution changed during the period of the response. Neutralizing activity was maximum 45 days after immunization by both routes. However, iv serum displayed a higher neutralizing activity than ip serum, while the two routes of immunization did not induce the same IgG isotypes to neutralize viral infectivity.

2. Lung Instillation

The delivery of adenoviral vectors to the lung has received much attention due to the concerted efforts of many groups to develop gene therapy vectors for the treatment of lung disorders such as cystic fibrosis. To correct the CFTR defect, CFTR cDNA needs to be delivered to the respiratory epithelium *in situ* and must direct gene expression independent of cell division. Many groups have shown that Ad vectors can deliver CFTR cDNA to airway epithelial cells, leading to protein expression [105, 108, 136] and correction of the CF phenotype *in vivo* and *in vitro* [137–139], although the efficiency of repeated Ad administrations is diminished by the development of serum and mucosal neutralizing antibodies to the Ad vector [140]. In a study with repeat administrations to the nonhuman primate lung both IgG and sIgA antibodies against Ad2 were detected and it was shown that sIgA alone can contribute to neutralization of the infectivity of Ad particles entering the lung [105, 140, 141].

3. Delivery to the Brain

Interestingly, injection of an E1-deleted adenovirus into the brain triggered a humoral immune response to the adenovirus and its gene products but no neutralizing antibody was detected thus repeat administration of the adenovirus was possible [142]. The authors claim that one reason for the lack of neutralizing activity may be due to the relative immunological privilege in the brain. Similarly, when adenovirus vectors are injected into the subretinal space, which is also considered to be immunologically privileged, they do not elicit humoral immune responses and repeated administration of adenovirus vectors is possible [143]. Thus there are regions within the body that may be more resistant to immune clearance on the basis of their anatomic structure. The retina for example, which is a derivative of the central nervous system, has the equivalent of a blood–brain barrier. In another report intranasal immunization of mice with wild-type adenovirus 1 month before intratumoral administration with an Ad vector of the same serotype did not efficiently inhibit repeat administration to the tumor [144]. It is likely that the structural integrity of the tumor or the extracellular matrix around the tumor presented a barrier to the neutralizing antibodies.

4. Intramuscular Delivery

More recently it was shown that effective repeat administration of adenovirus vectors to muscle was not hindered by the presence of neutralizing antibodies in the serum [145]. The authors reasoned that the concentration of adenovirus-specific neutralizing antibodies in the muscle may be considerably lower than in the serum, thus permitting effective multiple dosing to the muscle. The ability to repeat dose to the muscle has significant implications for cardiovascular gene therapy where it has been shown that intramuscular administration of adenovirus vectors expressing vascular endothelial growth factor (VEGF)-stimulated angiogenesis in hind-limb ischemia in rats [146]. Thus, the ability to repeat dose to the muscle for both peripheral and coronary vascular disease could significantly improve the efficacy of gene therapies for cardiovascular disorders.

VI. Immune Response to Adenoviral-Based Vectors in Humans

Host immune response can play a significant role in the outcome of *in vivo* gene therapy. Experiments with adenoviral vectors clearly demonstrate the development of neutralizing antibodies that block readministration and cellular responses that extinguish gene expression. However, most of the work described in this field relates to animal models that are naive to the virus. This will not be the case in humans, many of whom have been exposed

to Ad due to a naturally acquired infection. A study performed by Chirmule *et al.* [147] surveyed normal subjects and cystic fibrosis patients to demonstrate the relevance of pre-existing immunity to Ad to the outcome of *in vivo* gene therapy. They found that antibodies reactive to Ad capsid proteins were present in 97% of individuals; however, serum from only 55% of subjects actually neutralized Ad infection *in vitro*. Due to this discrepancy between seropositivity and neutralization of virus *in vitro*, the authors suggest that human trials should include all patients irrespective of *in vitro* measurements of preexisting immunity. This conclusion is also based on results from a Phase 1 gene therapy clinical trial for localized mesothelioma where it was shown that preexisting humoral immune responses did not preclude gene transfer [148].

Similarly, in human clinical trials where an adenovirus vector encoding hCFTR was repeatedly administered to the nasal epithelium of patients with cystic fibrosis [149], a complex immune response ensued which varied from patient to patient. Importantly, the pattern of the immune response did not differentiate patients with either large or absent correction of the CF defect following exposure to an Ad/CFTR vector [149].

Thus, what are the strengths and the limits of using experimental animals to predict human responses to gene transfer vectors? The intensity and the nature of the anti-Ad humoral immune response in experimental animals is dependent on the dose and on the route of administration of the vector. But is this the case in humans? To address this question a study was designed to determine the variability of human systemic humoral immune responses to adenovirus administered to different organs [150]. The study aimed to determine (a) if the administration of Ad vectors to humans always produced systemic anti-Ad neutralizing antibodies, (b) if the extent of the neutralizing antibody response depended on the route of administration, (c) if the systemic anti-Ad humoral response was dose dependent, and (d) how much preexisting anti-Ad antibodies influence the subsequent humoral response to Ad vector administrations.

Vectors were administered to the airway epithelium of individuals with cystic fibrosis, or individuals with liver metastatic tumors from colon cancer, or the skin of healthy normal individuals or the myocardium of individuals with coronary artery disease. Interestingly, the administration of the Ad vector to the bronchial epithelium of CF patients yielded the lowest antibody response, while direct injection of the Ad vector to colon carcinoma metastases in the liver resulted in the most vigorous antibody response. For those individuals that received Ad vector intradermally or intramyocardially there was a varying response, with some individuals having no increase in anti-Ad neutralizing antibody titer with others having a robust response. The most significant observation was the strong correlation between preexisting neutralizing antibodies and the likelihood that an individual would mount a higher titer following vector administration. Irrespective of route of administration and underlying disease

state individuals with higher baseline anti-Ad neutralizing antibodies mounted a higher neutralizing antibody response after exposure to an Ad vector.

How much does disease state modify the systemic humoral host response to Ad vectors? In the case of individuals with CF they yielded the lowest antibody response following intrabronchial administration of an Ad vector. The authors suggest that the minimal responses to bronchial administration of Ad in CF patients could be due to the fact that the airway epithelium of these individuals is covered by mucus, which can preclude efficient Ad vector infection of the airway epithelial cells.

To address this concern the same authors have just recently reported the results of a clinical trial using an E1–E3-deleted Ad vector, which was repeatedly administered to the lung airway of six normal individuals [151]. There were minimal to no systemic or local (epithelial lining fluid) anti-Ad neutralizing antibodies in the normal individuals following vector administration. This is a significant observation as in this situation there are no adverse conditions in the normal lung unlike the CF lung, that might affect host immune responses. Thus, in contrast to experimental animals where it has been shown that there is a robust anti-Ad response following repeated vector administration to the lung, it appears that for Ad-based vectors for use in human lung gene therapy this maybe less of a problem.

VII. Conclusion

There are several important conclusions on host responses to adenovirus-based vectors that can be made on the basis of extensive studies in experimental animals and some limited studies in humans. In particular, genetic composition of the Ad vector, dosing regimen, and routes of administration all modulate the humoral immune response generated to an adenoviral vector. In addition, factors other than the Ad-specific adaptive immune responses play a significant role in modulating gene expression following *in vivo* Ad transduction. These factors are related to host species and the immediate innate host responses following vector administration. Importantly, responses in experimental animals are not always predictive of responses in a human and care should be taken in extrapolating data from animal studies to predict outcomes in human clinical trials.

Several strategies that may be used to overcome host humoral immune responses to Ad-based vectors have been developed. Administration of different-serotype Ad vectors may circumvent the local host immunity elicited by a first vector administration. Vectors with capsid components modified at specific capsid sites known to be targets for the anti-Ad-neutralizing antibodies might be useful in overcoming preexisting, serotype-specific anti-Ad immunity. In addition strategies to suppress humoral immune response in the host such as cytokine treatment, use of monoclonal antibodies, T-cell depletion strategies, immunosuppressive drugs have all shown efficacy as being effective in allowing

repeat administration of Ad vectors. To date, all of these strategies have been evaluated only in experimental animals, while their efficacy and safety have yet to be tested in humans.

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CHAPTER 14

Novel Methods to Eliminate the Immune Response to Adenovirus Gene Therapy

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I. Introduction

The immune response to adenovirus (Ad) vectors or their transgene is a limiting factor in the successful application of gene therapies [1–3] (Fig. 1). Components of the adenovirus are recognized for their ability to elicit either a strong antigenic response or to subvert the immune response. Early proteins (E), which are produced continuously by adenovirus, can both enhance and inhibit the immune response. E1 (including E1A and E1B) and E2 are necessary for Ad replication but also evoke a strong immune response. E4 is a p70 antigen that promotes early and late transportation of viral mRNA and is highly immunogenic. E3 can be immunogenic but also produces a 19 K product that inhibits major histocompatibility complex-I (MHC-I) transportation to the surface of antigen-presenting cells (APCs) and therefore subverts the immune response [4, 5]. Adenovirus late proteins include the penton base which is in a cryptic position but can evoke an immune response. The neutralizing antibody response is formed against the fiber knob which is also a strong antigen on the surface of adenovirus. For applications to gene therapy, the transgene expressed by the Ad vector can also evoke an immune response, especially when combined in the environment of viable transduced adenovirus.

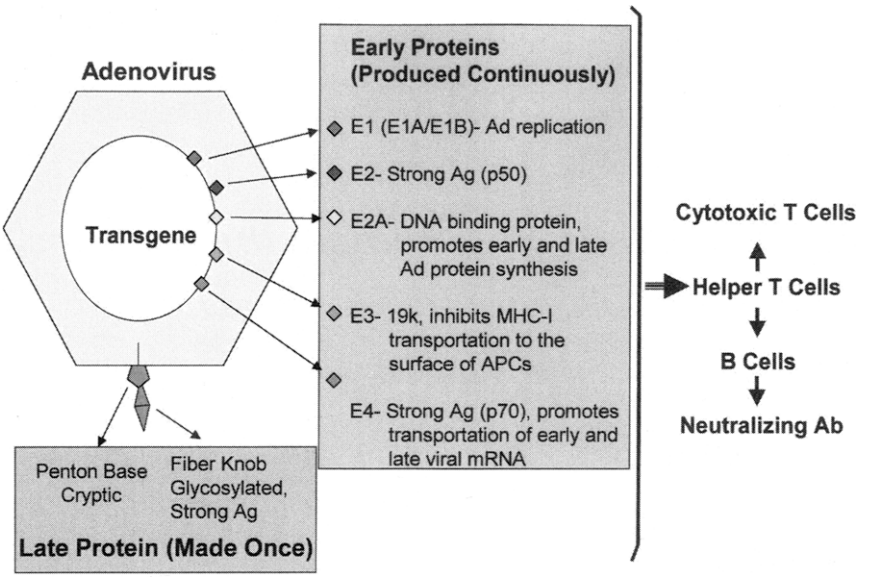

Figure 1 Components of adenovirus that can evoke an immune response. Adenovirus expresses early proteins including E1, E2, E3, and E4, most of which can provoke a strong immune response. E3 is known to inhibit MHC class I transportation of antigens to the surface of antigen-presenting cells (APCs), and minimizes the immune response to adenovirus. Late proteins include the penton base and the fiber-knob of the adenovirus. The fiber knob can elicit a strong antigenic response and promote production of neutralizing antibody against the fiber knob. The gene therapy transgene can also evoke an immune response.

II. Immune Suppression

Both cellular and humoral immune responses have been implicated in the shortening of the time span of transgene expression. Transgene expression is limited by eradication of the transfected cells. Induction of anti-Ad neutralizing antibodies precludes the opportunity to readminister the gene therapy. Immunosuppressive drugs including cyclophosphamide [6], cyclosporine [7], and FK506 [8–10], have reduced the T-cell immune response. Lochmuller *et al.* [8] used FK506, resulting in prolonged expression of adenovirus-mediated dystrophin gene transfer in *mdx* adult mice for at least 2 months, even though the FK506 treatment was discontinued after 1 month. There was a marked reduction in inflammation and reduced levels of nitric oxide synthesase in macrophages in the muscles of such treated animals. Howell *et al.* [9] used the dystrophin-deficient golden retriever dog model and showed that cyclosporine significantly prolonged transgene expression after Ad-mediated expression of a truncated human dystrophin gene. Ilan *et al.* [10] showed that combined immunotherapy in humans resulted in prolonged transgene expression.

Combined therapy with cyclosporine, azathioprin, and prednisone has been shown to reduce the immune response to adenovirus-mediated gene therapy and prolonged transgene expression.

Other strategies reported to control the immune response include reduction of T-cell response by oral tolerance [11], thymic tolerance, anti-T-cell therapy, and anti-CD4 monoclonal antibody therapy [12–15]. Modulation of T-cell subset development after adenovirus therapy has successfully been attempted using recombinant interleukin 12 (IL-12). IL-12 activates Th-1 cells to secrete gamma interferon (IFNγ) which diminishes Th-2 T-cell formation and reduces formation of neutralizing antibodies [16].

III. Immune Modulation

Other specific strategies include reduction of the costimulatory signaling activity using the CTLA4 immunoglobulin Fc (CTLA4-Ig) and blocking CD40–CD40L interaction (Fig. 2). Interaction of APC with processed adenovirus antigens or adenovirus transgene antigens with T cells through the MHC-T-cell receptor (TCR) ligation constitutes signal 1, which is an incomplete signal and can induce T-cell nonresponsiveness or anergy. A second signal can be provided by interaction with B7-1 or B7-2 (CD80/CD86) on the APC with CTLA4 (CD152) or CD28 on the T cell (Fig. 2). A soluble form of CTLA4-Ig (sCTLA4-Ig) can bind to B7-1 and B7-2 and block interactions of this molecule with membrane bound CTLA4 on T cells. The anti-adenovirus response can be decreased and there is prolonged expression of the adenovirus transgene in the presence of either sCTLA4-Ig or in the presence of an adenovirus that expresses CTLA4-Ig (AdsCTLA4-Ig).

Kay *et al.* [17, 18] demonstrated that systemic coadministration of recombinant adenovirus with sCTLA4-Ig leads to persistent adenovirus gene expression in mice without long-term immunosuppression. Ideguchi *et al.* [19] utilized local administration of adenovirus that expressed β-galactosidase as well as CTLA4-Ig (AdsCTLA4-Ig) in the central nervous system and showed that this combination decreased T-cell infiltration and also decreased the anti-adenovirus antibody titer. Expression of β-galactosidase at the injection site in the striatum and corpus callosum peaked at day 6 and remained until day 60 in both control and treated groups at about the same level despite suppression of the inflammatory response. Guibinga *et al.* [20] showed that the combination of CTLA4-Ig plus FK506 resulted in prolonged adenovirus vector-mediated production of dystrophin compared to treatment with either immunosuppressive alone. Schowalter *et al.* [21] demonstrated that murine CLTA4-Ig markedly prolonged adenovirus transgene expression in the liver and diminished formation of neutralizing antibodies as well as decreasing the proliferative response without causing irreversible immune suppression. Ali *et al.* [22] used AdsCTLA4-Ig administration in association with intraocular administration

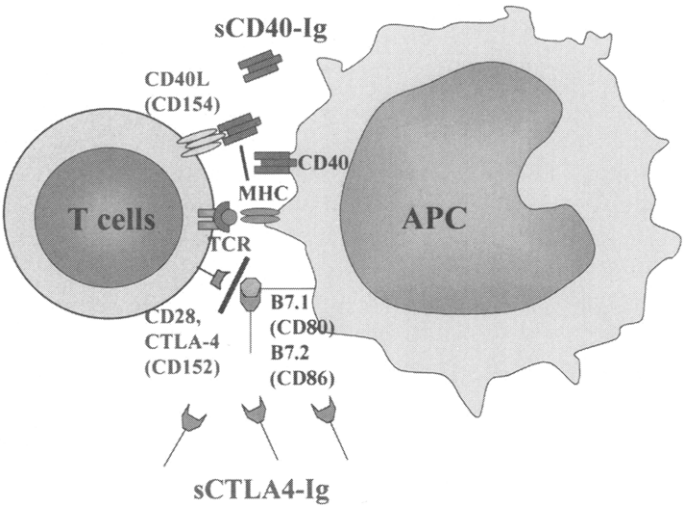

Figure 2 Role of costimulatory molecules to induce cytotoxic or helper T-cell response and promote anti-Ad antibody production. Signal 1 consists of the processed antigen presented by the MHC expressed by APCs stimulation of T cells. These antigens are recognized by specific T-cell receptors (TCRs) on T cells. This interaction is also assisted by CD8 expressed on cytotoxic T cells or CD4 expressed on helper T cells that interact with MHC. In addition to this central, specific T-cell–APC interaction, costimulatory molecules are necessary for optimal T-cell response. These consist of CD28 and CTLA4 (CD152) expressed on T cells that interact with B7.1 and B7.2 (CD80 and CD86), expressed on APCs. This interaction can be blocked by soluble CTLA4-Ig (sCTLA4-Ig) that tightly binds to B7.1 and B7.2 and prevents the interaction of this molecule expressed on APCs with CD28/CTLA-4 expressed on T cells. A second costimulatory molecule pathway consists of CD40 ligand (CD154) expressed on T cells that interact with CD40 expressed on APCs. This interaction can be blocked by administration of a soluble CD40-Ig (sCD40-Ig) which binds with the CD40 ligand and prevents the interaction between CD40 and CD40 ligand.

of adenovirus encoding β-galactosidase and demonstrated reduced immune response to adenovirus, as well as prolonged expression of the transgene in retinal cells. Kay *et al.* [17] showed that combined treatment with sCTLA4-Ig and anti-CD40 ligand resulted in prolonged adenovirus-mediated gene expression for up to 1 year in the liver and the ability to readminister adenovirus in 50% of mice. Following readministration, there was persistent secondary gene expression lasting 200–300 days, and diminished spleen proliferative response, tumor necrosis factor (TNF)-α and IFNγ production and decreased production of neutralizing antibodies. Chirmule *et al.* [23] showed that despite the absence of CD40–CD40 ligand interaction in CD40 ligand knockout mice, after administration of LacZ into the mouse lung, these mice developed a functional humoral response to the vector evidenced by germinal center formation and anti-adenovirus IgG1 and IgA that resulted in effective neutralization of virus and prevented effective readministration of the virus.

Wilson *et al.* [24, 25] used combined treatment with an adenovirus vector expressing sCTLA4-Ig to block CD28 stimulation and a monoclonal antibody against CD40 ligand to demonstrate prolonged adenovirus transgene expression after intratracheal administration. In addition, secondary administration and transgene expression after secondary administration was prolonged in the lung, but there was increased reaction from the liver. These results indicate that the mechanisms limiting transgene expression in the airways and the alveoli are different to those of the liver. Stein *et al.* [26] have shown that combined treatment of Ad-human factor IX (FIX) with an anti-CD40 ligand antibody MR-1 as well as depletion of macrophage liposomes resulted in prolonged expression of AdFIX as well as higher levels of plasma FIX. This persistence was accompanied by inhibition of anti-adenovirus IgG, and decreased IL-10 and IFN-γ production from spleen lymphocytes following reexposure to virus particles *in vitro*. This treatment regimen also enabled secondary and tertiary infusions of AdFIX which was superior to treatment with CD40 ligand blockade alone. Kuzmin *et al.* [27] utilized macrophage depletion in combination with blockade of CD40 ligation to demonstrate the prolonged expression of transgene after administration of E1-deleted adenovirus. This resulted in a decreased cellular and humoral response as well as the induction of transgene tolerance in the animals. Animals that were rendered immunologically unresponsive to vector and transgene antigens regained their ability to mount a productive immune response against the vector after recovery of immune function but remained unresponsive to the transgene product. Stein *et al.* [28] used an anti-CD40 ligand (anti-CD154) in combination with adenovirus-mediated low-density-lipoprotein receptor (LDLR) gene transfer in LDLR-deficient mice to demonstrate that these mice express LDLR on hepatocytes and maintain cholesterol levels below or within the normal range for at least 92 days.

It has been more difficult to eliminate B-cell responses. B-cell production of neutralizing antibodies is decreased after treatment with anti-CD40 or soluble CD40 [29] and deoxyspergualin [30–32]. Readministration of adenovirus vector has been achieved in the lungs of nonhuman primate by blocking of CD40–CD40 ligand interactions. A humanized anti-CD40 ligand antibody hu5C8 was used to treat primates in the presence of administration of adenovirus vectors [23]. These animals produced IgM but did not develop secretory IgA or neutralizing antibodies. This is significant since this is the first demonstration that anti-Ad neutralizing antibodies could be inhibited in a primate system by inhibiting the CD40 interaction with CD40 ligand.

A third approach includes modification of the adenovirus vector to reduce the immune response [33–36]. A more universal strategy for decreasing response to adenovirus vectors includes production of the "gutless" adenovirus which greatly reduces the immune response to Ad and its transgene [37–40]. It was initially demonstrated that constitutive expression of the

immune modulatory gp19 K protein in adenovirus vectors reduced the cytotoxic response. Further refinement of vectors including the removal of E4 also resulted in prolonged transgene expression [34]. A gutless Ad that was depleted of all adenovirus genes, showed prolonged expression of β-galactosidase in muscle. This prolonged expression correlated with a decrease in the infiltration of $CD4^+$ and $CD8^+$ lymphocytes. However, in LacZ transgenic mice, which was predicted to result in immunologic tolerance to β-galactosidase expression, there was prolonged expression of the vector DNA, indicating that the immune response to this "gutless" Ad was primarily against β-galactosidase and that the response to the adenovirus vector lacking all genes was minimal [37]. Gene therapy expression using adenovirus vectors with deletions of the E1, E2A, E3, and E4 regions could be prolonged when combined with immunosuppressive drugs including cyclophosphamide and FK506.

One limitation of immunomodulating therapy has been that it is not specific for the adenovirus or transgene. The present review will focus on our attempts to reduce the immune response mediated by TNFα and other cytokines. A second strategy to prolong gene therapy expression is to ablate the immune response to cells that are the target of gene therapy. Such apoptosis-inducing factors include TNFα but also Fas ligand and TNF receptor apoptosis-inducing ligand (TRAIL). We have also developed methods to specifically reduce the T-cell response to Ad and also new methods to prevent B cell responses including blocking of the TNF receptor (TNFR) homolog transmembrane activator and calcium modulator and cyclophilin ligand (CAML) interactor (TACI) signal in B cells. These strategies strongly suggest that it will be possible to develop strategies to ablate the immune response to adenovirus including the cytotoxic response that leads to the loss of cells carrying the transgene.

IV. Treatment with Soluble TNFR1 to Eliminate Ad Inflammation in Lung and Liver

The rationale for use of soluble TNF receptor as a modulator of adenovirus inflammation stems from the observation that TNFα is one of the principal mediators of inflammation after adenovirus gene therapy [41–43]. Neutralization of TNFα with TNFα inhibitors, such as soluble TNFR1 (sTNFR1) greatly reduces tissue injury and cell death after endotoxin and other inflammatory agents. This is a rational approach, since adenovirus takes advantage of TNFα as an immune mediator to promote expression of several immunosubversive proteins supporting its escape from immunosurveillance [5]. The interaction of TNFα with its receptor is a strong virulence factor for inflammation and elimination of virus infection. The E3-gp19 K protein not only prevents CTL recognition of Ad-infected fibroblasts by sequestering

MHC class I proteins in the endoplasmic reticulum, but also E3 proteins 10.4 K, 14.5 K, and 14.7 K function to protect infected cells from TNFα cytolysis. Transgenic mice that express the E3 gene encoding these proteins have been shown to exhibit decreased pulmonary infiltration after intranasal inoculation [42]. Peng *et al.* [43] showed that adenovirus gene transfer of an sTNFR results in effective blockade of tumor necrosis factor activity and also prolongs the gene therapy. Therefore, neutralization of TNFα is a rational approach to decrease chronic inflammation as well as prolonged transgene expression.

Certain cytokines such as TNFα can result in rapid clearance of adenovirus or viral therapy. Administration of anti-inflammatory cytokines, such as IL-10, can reduce inflammation and prolong gene therapy [44]. We have evaluated the effect of the treatment with a novel TNF-binding protein (TNF-bp), a polyethylene-glycol (PEG)-linked dimer of sTNFR1, on inflammation of the lung and viral clearance after intranasal administration of AdCMVLacZ (1×10^{10} pfu) [45, 46] (Fig. 3, see color insert). Three days after intranasal administration, there was a moderate inflammatory infiltrate in the lungs of control (CT)-treated C57BL/6-+/+ mice, which peaked at day 7 and was nearly resolved by day 30 (Fig. 3A). In contrast, 3 days after administration of AdCMVLacZ, there was no evidence of an inflammatory infiltrate in the lungs of TNF-bp-treated C57BL/6-+/+ mice and only minimal evidence of infiltration was observed from day 3 through day 30. We next determined the expression of LacZ adenovirus gene-therapy product. The results indicate that the expression of β-galactosidase (β-Gal) in control-treated C57BL/6-+/+ mice was high at day 3, but was considerably reduced by day 30 (Fig. 3B). The expression of the β-Gal in TNF-bp-treated C57BL/6-+/+ mice was equivalent at day 3 but, in contrast to the control-treated mice, the expression of β-Gal remained high in the lung through day 30. These results indicate that there is greatly decreased inflammatory disease and prolonged gene expression in AdCMVLacZ-infected mice treated with TNF-bp compared to vehicle-treated mice. The results also indicate that TNFα is a key factor in the pathogenesis of inflammation in AdCMVLacZ-virus-infected mice. Thus, the TNF-bp PEG-linked dimer may be therapeutically useful in reducing the inflammatory response to adenovirus gene therapy.

V. Inhibition of Cell Cytolysis Which Combines Treatment with Soluble DR5, Soluble Fas, and Soluble TNFR1

A major factor limiting prolonged transgene expression is due to the elimination of the cells infected by the adenovirus gene therapy that expresses the gene therapy product. Elimination of cells by either nonspecific "bystander" mediators or specific induction of cell death by T cells and other inflammatory

cells is carried out by the process of either necrosis or apoptosis. Necrosis results in lysis of the cell and is mediated by TNFα as well as other cytokines. Apoptosis results in elimination of the cell by triggering programmed cell death followed by phagocytosis of the cell into nearby reticulo-endothelial cells. The primary molecules that contain an intracellular death domain include Fas, TNF receptor (TNFR), and death domain receptor (DR3, -4, and -5) mediated by TRAIL (Fig. 4).

The relative contribution of TNFα-mediated necrosis compared to Fas ligand (FasL) or TRAIL-mediated apoptosis was investigated using systemic treatment or adenovirus that expressed soluble forms of receptors capable of neutralizing these factors. We have previously shown that Fas-Fc is capable of neutralizing Fas ligand and preventing Fas ligand-mediated apoptosis [47]. Similarly, a soluble form of the death domain receptor 5 (sDR5-Fc) can neutralize TRAIL and inhibit TRAIL-mediated apoptosis [48]. Adenoviruses were constructed that expressed soluble forms of DR5-Fc (sDR5), AdsDR5, and soluble forms of Fas-Fc (sFas), AdsFas. The pretreatment with AdsFas can prevent liver cell apoptosis after iv administration of anti-Fas Jo-2 [49]. AdsDR5 was shown to protect TRAIL-mediated apoptosis of Jurkat T cells.

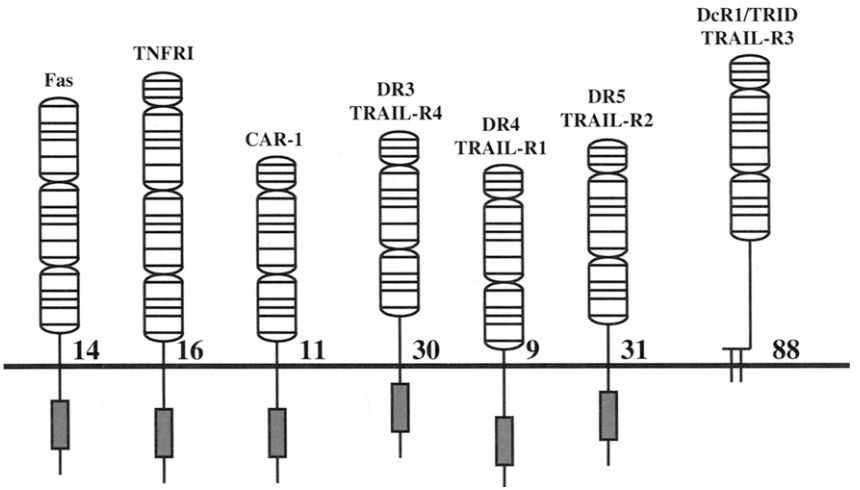

Figure 4 Death domain receptor family. Death domain family members are exemplified by Fas and TNF receptor 1 (TNFR1). These have three and four extracellular cystine-rich repeat domains, respectively. There is an 9- to 31-amino-acid linker between the extracellular domain and the transmembrane domain. Both molecules have a homologous intracellular death domain represented by a rectangle. Other members include cytotoxic apoptosis receptor 1 (CAR-1) and also death domain receptors that bind TNF-related apoptosis-inducing ligand (TRAIL). These receptors include death domain receptor 3 (DR3), DR4, and DR5. Also, there is a decoy receptor (DcRI) that can bind TRAIL but lacks the intracellular death domain and therefore binds to TRAIL but does not introduce apoptosis.

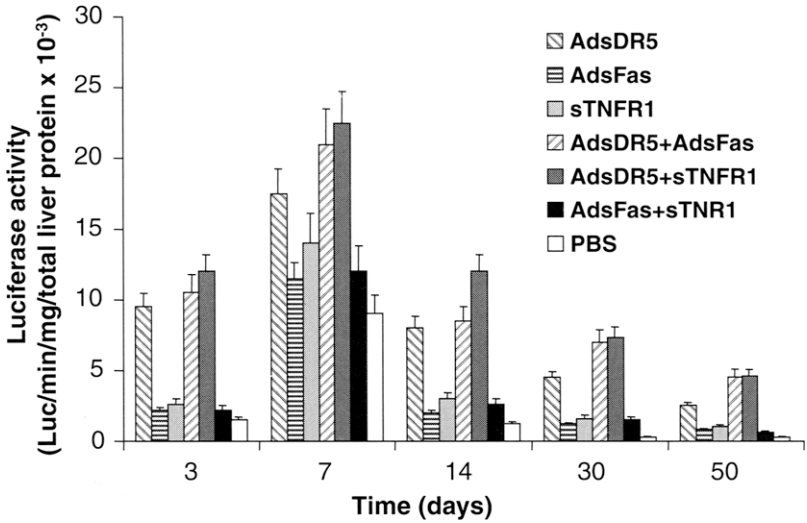

Figure 5 Inhibition of liver apoptosis by soluble TNFR1, soluble Fas, and soluble DR5. Mice were treated with adenovirus-expressing soluble Fas (AdsFas) and soluble DR5 (AdsDR5) as well as soluble TNFR1 (100 μg/mouse, iv). Mice were also given an Ad-luciferase. The luciferase activity was measured at 3, 7, 14, 30, and 50 days in mice treated with AdsDR5, AdsFas, sTNFR1 protein, AdsDR5 plus AdsFas, AdsDR5 plus sTNFR1, or AdsFas plus sTNFR1. As a control, mice were given Ad-luciferase plus PBS.

To determine if adenovirus gene expression can be prolonged by protecting liver cells from apoptosis, mice were pretreated with either AdsDR5, AdsFas, or sTNFR1, or different combinations of these cytoprotective therapies. Pretreatment with AdsDR5 alone resulted in a greatly prolonged expression by the liver after subsequent administration of an Ad vector expressing luciferase (AdLuc) (Fig. 5). Consistent with our previous results, the second most effective molecule to prolong luciferase expression was treatment with sTNFR1. Pretreatment with AdsFas provided only modest prolongation of the luciferase gene expression. Combined treatment with AdsDR5 and sTNFR1 provided the greatest protective effect after administration of AdLuc and the greatest prolongation of gene expression. These results indicate that liver gene therapy is limited primarily by expression of TRAIL by either infected liver cells or the immune response to this adenovirus gene therapy and TNF and FasL play a lesser role.

VI. Immune Privilege

One physiologic mechanism for induction or maintenance of tolerance to self-antigens in the body is for the antigens to be present in an immune privileged site [50–52]. Although anatomical barriers and soluble mediators

have been implicated in immune privilege, it appears that apoptotic cell death of Fas-positive cells by tissue-associated FasL is an important component. Constitutive expression of FasL occurs in immune privilege sites including the retina, ciliary body, iris and cornea of the eye, and the testes, which have been known to be an immune privilege site. T cells that interact with antigens in immune privilege site upregulate Fas and enable Fas apoptosis signaling and are killed by FasL present in these sites. This prevents inflammation of these sites since the mouse cornea expresses abundant FasL and immune privilege has been implicated in the success of these corneal transplants. The ability of the eye to kill invading inflammatory cells helps maintain immune privilege and minimize bystander tissue damage, while tolerance regulates dangerous inflammatory reactions to prevent autoimmunity. Adenovirus delivered to the eye fails to elicit an immune response [53–55].

The immune privilege site has been proposed to prevent reactivity in other tissues including the thyroid gland and pancreatic β-islet cells. Belgreu *et al.* [50] demonstrated that FasL expression by testicular Sertoli cells protected β-islet from rejection when transplanted heterotopically into the kidney capsule. Similarly, Griffith *et al.* [51, 52], were able to prevent rejection of allogeneic pancreatic islet cells by cotransplantation with FasL positive myoblast which were also transplanted to the kidney capsule. These observations were presumably the result of induction of apoptotic cell death of Fas-positive cells invading the graft from the Fas-positive graft tissue. The implication of these studies is that manipulation of the Fas–FasL system might provide a mechanism to prevent local inflammation.

We have utilized the immune privilege concept to prolong gene therapy expression in muscle [56, 57]. For this purpose, we have produced a binary adenovirus system consisting of an AdLoxpFasL plus an AxCANCre [56]. AdLoxpFasL can be grown to high titer in 293 cells and does not produce Fas ligand in the absence of AxCANCre. Therefore, these viruses can be grown to high titers separately. However, when transfected into the same cell line, this leads to high-titer production of FasL. To create a local immune privilege site, we used gene therapy to reproduce the immune privilege site created by muscle cells as described above [56, 57]. BALB/c mice were injected intraglossally with either AdLoxpFasL plus AxCANCre plus AdCMVLuciferase or with AdLoxpFasL plus AdCMVTK (thymidine kinase) plus AdCMVLuciferase. As a control, mice were injected with luciferase. The mice were analyzed at days 7, 21, 35, and 50 postinjection and luciferase was determined in the harvested tissue. There was no increase in the prolongation of the expression of the vector-encoded transgene by attempting to produce an immune privilege site in muscle and diminish the immune eradication of these vector-transduced cells. This brings up several issues related to the use of FasL for prolongation of gene therapy. First, it is possible that, in the attempt to produce an immune privilege site, ectopic expression of FasL can actually elicit an inflammatory response.

Second, the coexpression of Fas with an ectopic FasL can induce an autocrine loop which might induce target cell apoptosis. In addition, the magnitude and temporal pattern of FasL expression may be critical to determine its efficacy in creation of an immune privilege site. For these reasons, creation of a local immune privilege site in muscle by FasL does not result in prolonged transgene expression.

VII. APC-AdFasL Prolongs Transgene Expression and Specifically Minimizes T-Cell Response

Adenovirus gene therapy is limited by induction of an immune response to the virus or gene therapy protein product. APCs lead to antigen processing and presentation of T cells which can be highly immunogenic or tolerogenic, depending on costimulatory molecules and production of other cytokines, such as FasL. T-cell tolerance after an immune response to adenovirus is also maintained by activation-induced cell death (AICD) of T cells mediated by Fas/FasL interactions. We surmise that APCs such as macrophages, which express FasL, might directly induce apoptosis of T cells that express Fas as cell therapy resulting in an adenovirus-specific T-cell tolerance without toxic effects of FasL (Fig. 6). The AdLoxpFasL can be used to infect APCs from *lpr/lpr* mice and does not kill these APCs since these APCs lack Fas expression [58, 59]. These AdLoxpFasL plus AxCANCre-infected APCs (APC-AdFasL) result in high production of FasL capable of lysing A20 target cells. The macrophages infected by the adenovirus exhibited at least a 50- to 100-fold higher FasL titer compared to PMA-activated T cells. High levels of FasL expression by the macrophages were sustained for at least 7 days of *in vitro* culture. These results indicate that adenovirus can deliver FasL into the primary-culture macrophages from Fas mutant *lpr/lpr* mice, and this leads to a high level of FasL expression by the macrophages without toxicity to the macrophages.

To determine if APC-AdFasL could inhibit an immune response to Ad, mice were pretreated with APC-AdFasL every 3 days for five doses. After 7 days, the mice were inoculated intravenously with 10^{10} pfu of AdCMVLacZ. β-Gal staining was determined up to 50 days later (Fig. 7) [58]. The levels of LacZ gene expression in the liver of control-treated mice decreased rapidly after pretreatment with APC-Ad control. In contrast, in mice treated with APC-AdFasL, the levels of LacZ expression did not decrease but were sustained for at least 50 days after infection. These results indicate that pretreatment with APC-AdFasL significantly prolonged AdCMVLacZ transgene expression. To determine if the APC-AdFasL therapy resulted in a preferential specific deletion of Ad-reactive T cells, but not T cells reactive with other viruses, wild-type C57BL/6-+/+ mice were treated with APCs, APC-Ad control, or APC-AdFasL

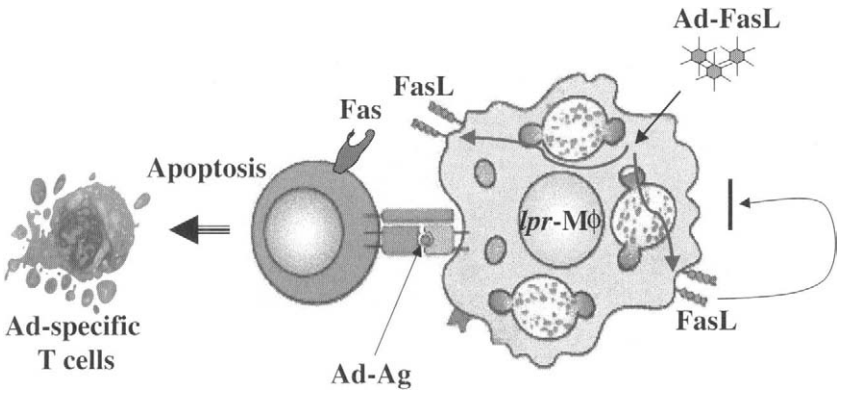

Figure 6 Specific apoptosis of T cells by APC-Ad-FasL therapy. Macrophages are infected with an AdFas ligand (Ad-FasL) gene expression system. This results in high expression of FasL on the surface of macrophages. Macrophages do not undergo autocrine apoptosis since they are derived either from *lpr* mice (Fas mutant) or express an anti-apoptosis gene. In addition, macrophages will process and present adenovirus antigens (Ad-Ag) specifically to T cells. Activation by processing Ad-Ag by macrophage upregulates Fas expression and also Fas apoptosis signaling, facilitating apoptosis of Ad-specific T cells in the presence of FasL produced by the macrophage.

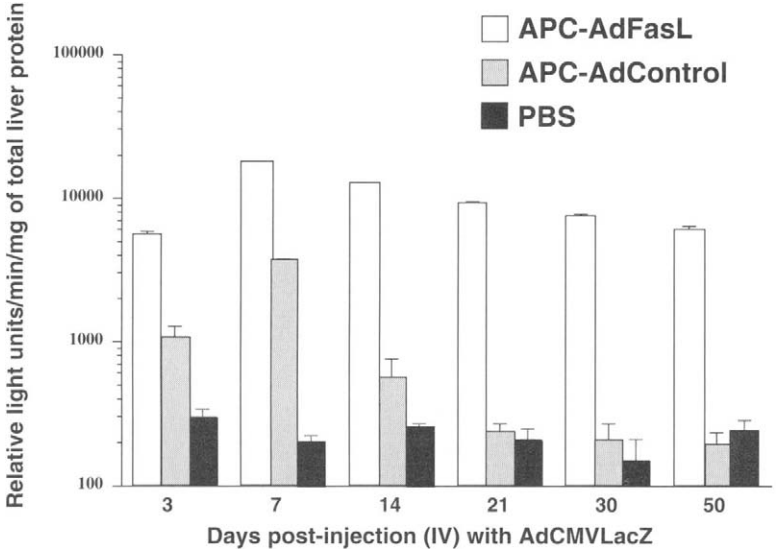

Figure 7 AdLuc transgene expression after APC-AdFasL followed by AdCMVLacZ. Wild-type C57BL/6-+/+ mice were treated with 1×10^6 of APCs cotransfected with AdLoxpFasL plus AxCAN-Cre (APC-AdFasL), or APCs cotransfected with AdLoxpFasL plus AdCMVLUC (APC-AdControl), or phosphate-buffered saline (PBS) every 3 days until five doses were given. After 7 days, the mice were inoculated iv with 1×10^{10} pfu of AdCMVLacZ and β-Gal was determined up to 50 days later. The error bars represent the mean ± standard error of the mean (SEM) for three mice analyzed separately in triplicates.

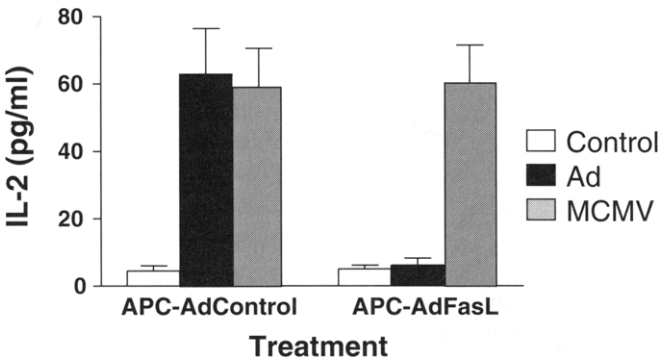

Figure 8 IL-2 production by T cells stimulated with APC plus MCMV after APC-AdFasL. Wild-type mice were treated with either APC-AdFasL or APC-Ad Control. Seven days later, mice were challenged *in vitro* with either AdCMVLacZ or MCMV. Seven days after splenic T cells were stimulated *in vitro* with either APCs, AdCMVLacZ transfected APCs, or MCMV-infected APCs. IL-2 production in the supernatants was determined by ELISA.

every 3 days until five doses as above. To determine whether the T-cell tolerance induced by APC-AdFasL was specific, the T-cell response of APC-AdFasL- and APC-Ad control-tolerized mice to murine cytomegalovirus (MCMV) infection was evaluated. C57BL/6-+/+ mice were treated as described above and then challenged 7 days later with either adenovirus or MCMV. Although there was a reduction in the T-cell response through adenovirus vector, the T-cell response to MCMV was not impaired as demonstrated by comparable levels of IL-2 produced by T cells from both APC-Ad control- and APC-AdFasL-treated mice (Fig. 8). These results indicate that inhibition of the T-cell response in APC-AdFasL-tolerized mice is specific for the adenovirus vector.

VIII. Production of AdsTACI Prolongs Gene Expression and Minimizes B-Cell Response

The TNF receptor family includes apoptosis-signaling molecules as described above including TNFR1, TNFR2, Fas, CD40, DR4, and DR5 [60–64] (Fig. 4]). In addition, the TNF receptor family contains factors related to B-cell growth including TNF- and apoptosis ligand-related lymphocyte-expressed ligand 1 (TALL-1)/B lymphocyte stimulator (BlyS) factor that belongs to the TNF family. TALL-1 is a potent B cell costimulatory factor and acts by direct binding and activating its cell surface receptor on B cells, referred to as transmembrane activator and calcium modulator and cyclophilin ligand (CAML) interactor (TACI). The interaction between TALL-1 and TACI

is important for regulation of B-cell growth and humoral immunity. Stimulation through this pathway promotes production of antibody and autoantibodies. We surmise that blocking this pathway with a soluble TACI-Fc (sTACI-Fc) that binds to TALL-1 would inhibit the B-cell response to adenovirus gene therapy. We therefore constructed an adenovirus expressing sTACI-Fc in the E1A site of adenovirus.

C57BL/6-+/+ mice were injected on day 0 with AdsTACI (5×10^9 pfu, iv) and AdLacZ (5×10^9 pfu iv). On day 3, the mice were either untreated or treated with APC-AdFasL to modulate the T-cell response to adenovirus. On days 7, 14, 30, and 50, the antibody production to Ad was determined using an Ad neutralization assay. The Ad neutralization assay was carried out using two-fold dilution of sera, which was then incubated with an Ad vector expressing green fluorescent protein (AdGFP) for 1 h (Fig. 9). The AdGFP was then inoculated with 293 cells for an additional hour and the unbound AdGFP was washed with PBS. The infected 293 cells were incubated at 37°C for another 3 days, and the percent of GFP-positive cells was assayed as an indicator of the level of adenovirusneutralizing antibody. In control mice, there were high levels of anti-Ad IgG immunoglobulin that reached a maximum titer at day 30 in

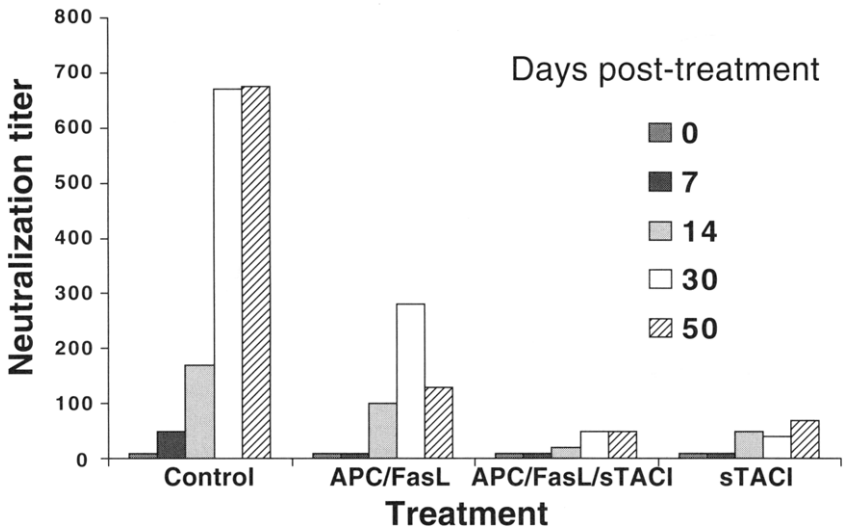

Figure 9 Reduction of anti-adenovirus antibody response by treatment with AdsTACI. C57BL/6 mice were injected on day 0 with AdsTACI (5×10^9 pfu, iv) and AdLacZ (5×10^9 pfu, iv). On day 3, the mice were either untreated or treated with AdAPC-FasL to modulate the T-cell response to adenovirus. The Ad neutralization assay was carried out using a twofold solution of sera which was incubated with AdGFP for 1 h. Sera were collected on days 0, 7, 14, 30, and 50 after administration of AdLacZ or AdsTACI.

the absence of any immunosuppressive treatment. In the presence of AdsTACI administered on day 0, the IgG anti-Ad neutralization titers remained very low throughout the time course of the study. APC-AdFasL therapy reduced the peak titer especially on days 30 and 50, but the neutralization titer at these time points was higher than AdsTACI treatment alone. The combined treatment with AdsTACI and APC-AdFas ligand was similar to treatment with AdsTACI alone. These results indicate that treatment with APC-AdFasL or blocking B cells signaling with AdsTACI can greatly inhibit the peak anti-Ad immunoglobulin production and also prevent long-term antibody production against adenovirus.

IX. Summary

Suppression of the T-cell main response to adenovirus, to date, has generally been achieved using the same paradigms to reduce the cellular immune response as applied in other systems. This includes reducing the T-cell response either by inhibiting T-cell activation at the surface by blocking costimulatory molecules such as the CD28/CTLA4 or B7.1/B7.2 ligand pathway or by blocking intracellular signaling using cyclosporine or FK506. More general immunosuppressants such as prednisone and cyclophosphamide also suppress the immune response and are synergistic with more specific T-cell surface or intracellular signaling pathways. Similarly, more general immunosuppressants such as TNF receptor Fc or sTNF receptor can block proinflammatory cytokines after stimulation of an immune response by adenovirus and are synergistic with direct immunosuppressants of the T cells. Methods to eliminate these cells rather than to suppress them include treatment with anti-CD4 or anti-CD3, or more specific cell gene therapy methods using APC-AdFasL which kill T cells that interact with APCs that express Ad virus or transgene. All of these three approaches can be used together to exhibit synergistic effects to reduce the number of Ad-reactive T cells, the activation of Ad-specific T cells, and the inflammatory mediators produced by these Ad-specific T cells. A second approach is to reduce the antigenicity of the adenovirus or the transgene. Such methods are analogous to, for example, decreasing the cellular immune response to organ transplant by tissue typing. In the case of adenovirus, certain components of the virus are known to be more antigenic than others and can be eliminated to produce replication-deficient Ad. The ultimate result is the "gutless" adenovirus that only contains the transgene with the Ad inverted terminal repeats (ITRs). The immune response to the transgene is similar to the immune response to any biological administered reagent such as factor IX or soluble TNF receptor-Fc. These responses lead to neutralizing antibodies to the transgene or administered biologic protein and mechanisms to eliminate antibody response can also be successfully reduced or eliminated.

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CHAPTER 15

High-Capacity "Gutless" Adenoviral Vectors: Technical Aspects and Applications

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I. Introduction

Successful somatic gene therapy fundamentally depends on the availability of vectors that allow the efficient and nontoxic delivery of nucleic acids into the appropriate target cells. E1-deleted first-generation adenoviral vectors have been used in a number of clinical trials for the treatment of neoplastic and inherited disorders. So far these vectors have been based on adenovirus serotypes 2 and 5 and have usually been produced in the E1-complementing 293 cell line [1]. These vectors may still find an application in the treatment of cancer diseases or for vaccination in which "immunostimulation" by viral functions may act as a beneficial adjuvant to the function of the transgene. However, it is unlikely that in the future these vectors will still be used for the treatment of inherited recessive or dominant disorders that would require durable expression of the therapeutic gene. Immune responses to viral proteins expressed from the vector have been observed in various systems. Immediate toxic effects following gene transfer with high vector doses have been attributed both to the viral capsid and to viral gene expression. Chronic toxicity, apparently unrelated to a specific antiviral immune response has been noted [2]. A DNA capacity of 7–8 kb allows the expression of many cDNAs

but not of all. However, the main reason why first-generation vectors should not be used for the treatment of inherited disorders in which long-term gene expression is required lies in the realistic appreciation that our knowledge of potential interactions between functions from different viruses from the same or different species or between viral functions and exogenous factors is very slim. As has been discussed before [3], it is likely that there could and would be interactions between viral functions of the gene transfer vector and of other virus species. Because the consequences of such interactions are currently largely unpredictable, gene transfer studies in humans that are based on vectors that still carry viral genes have to be subjected to extremely careful risk–benefit considerations.

In an attempt to address several of the disadvantages of first and second-generation adenoviral vectors, a new vector has been developed [4–11] that has been variably named "high-capacity (HC)," "gutless," "gutted," "mini," "deleted," "third-generation," "delta (Δ)," or "helper-dependent (HD)" adenoviral vector. For simplicity the term HC-Ad vector is used throughout this text.

This chapter is divided into two parts. In the first part, technical aspects are discussed as they relate to the production and the design of HC-Ad vectors. In the second part the results of gene transfer experiments are summarized.

II. Technical Aspects

A. Vector Production

The production of first-generation adenovirus vectors is relatively simple: only the E1 functions that are absent from the vector have to be complemented in a producer cell line. The 293 cell line [1] has been extremely valuable in serving the production needs for gene transfer vectors for many years. Vectors with additional mutations in the E2 and/or E4 genes (second-generation vectors) can still be relatively easily complemented by cell lines that provide the missing functions in *trans*. The production is considerably more complicated with vector genomes that have increasingly large deletions. The successful completion of a productive infection cycle and the generation of a large number of infectious particles during production require the precise coordination of a complex viral transcription and replication program. The current production of HC-Ad vectors is based on earlier studies in which the accidental generation of hybrid vector genomes was observed. These consisted of both adenovirus and human or SV40 DNA, respectively, and were dependent on the presence of a wild-type helper virus [12, 13]. Based on these studies, several research groups successfully rescued recombinant adenoviral vector particles that did not contain any viral coding sequences and expressed different transgenes [4, 5, 8, 14]. The

first vector that expressed a reporter gene and that was deleted in L1, L2, VAI+II, and pTP was produced by using wild-type adenovirus type 2 as helper virus [4]. It was possible to rescue, serially propagate, and partially purify a recombinant, although not fully deleted adenoviral vector. In one system [4, 15] a replication-deficient helper virus was engineered to carry a partially defective packaging signal in order to impair the packaging of the helper virus and thereby to enhance vector production. The packaging signal of Ad5 has been characterized in a series of elegant studies that involved the generation and analysis of a large number of packaging-deficient adenoviral mutants [16–19]. The adenoviral packaging element is located between nucleotides 230 and 370 and consists of seven elements, the so-called consensus A repeats AI–AVII. The detailed molecular mechanism of adenoviral DNA packaging is still not clear [20]. To attenuate the packaging capability of the helper virus, 91 bp of the packaging element involving AII–AV were deleted [5] using the packaging impaired adenoviral mutant dl309-267/358 as a template [17]. Compared to wild-type Ad5, this mutant can be grown in cell culture with about 90-fold reduced titer. Using this mutated packaging signal in an E1-deleted helper virus it was possible to serially propagate a plaque isolate containing the vector and helper virus genome on 293 cells, and to separate helper virus and vector particles by CsCl equilibrium centrifugation. The HC-Ad vector was obtained in fairly high titers with a 1% contamination by helper virus. In these early studies an HC-Ad vector was generated that contained the 13.8-kb full-length murine dystrophin cDNA under the control of a 6.5-kb muscle-specific promoter and a lacZ reporter gene. The only viral elements retained on the vector genome are the inverted terminal repeats (ITRs), which is the viral origin of replication, and the packaging signal. Using a comparable strategy, an HC-Ad vector that expressed the human Factor VIII gene was generated [15]. In these systems the recombinant vector DNA with the wild-type packaging signal is preferentially packaged into capsids. Because of differences in the densities of the particles it is possible to separate vector particles from helper virus by CsCl equilibrium centrifugation so that contamination of the vector with the helper virus is around 1%. However, our own experiences indicate that by using this production system it would be very difficult if not impossible to produce clinical grade material in large amounts. A different and improved production scheme, which takes advantage of the Cre–loxP recombination system of bacteriophage PI [21] considerably increased the ease of vector production and resulted in an increased vector yield and purity. This system utilizes a helper virus with the packaging signal being flanked by two loxP-recognition sites [9, 10]. As in the earlier production system the helper virus is E1 deficient and corresponds, therefore, to a replication-defective first-generation adenovirus vector. The recombinant vector carrying the transgene is produced in 293 cells constitutively expressing Cre recombinase [22]. Following infection of producer cells the packaging signal of the helper virus is excised with high

efficiency without affecting viral replication. Having lost the packaging signal by Cre-mediated excision, the helper virus is excluded from the capsids while the recombinant vector is efficiently packaged. Although the Cre-mediated removal of the packaging signal was shown to efficiently suppress helper virus contamination, sometimes overgrowth of the helper virus during amplification has been observed [23]. The helper virus could escape suppression if one of the loxP sites flanking the packaging signal was lost by an intermolecular recombination event that occurred between the two identical packaging signals that are present on the helper virus and on the vector genome. Exchanging the DNA sequences between the consensus A repeats reduced the chances of homologous recombination between vector and helper virus genomes [23]. In a modification of the original Cre–loxP system, additional gene functions (DNA polymerase and preterminal protein) were deleted from the helper virus genome and supplied in *trans* in a packaging cell line [24, 25].

For clinical-grade production of HC-Ad vectors it is likely that Cre-recombinase-expressing cell lines will be used that are not based on 293 cells. As with first-generation adenovirus vectors there is sequence overlap between the E1 region in 293 cells and the helper virus genome. Therefore, a generation of replication-competent adenovirus (RCA) by homologous recombination is expected to be likely, especially if large amounts of vector are produced. Cell lines that exclude the generation of RCA have been developed [26, 27]. When Cre recombinase is expressed in these cells, efficient production of HC-Ad vectors with low helper virus contamination is possible [G.S., unpublished observation].

For construction of HC-Ad vector genomes, plasmid cloning strategies have been implemented. Most plasmids for HC-Ad vector construction harbor the left and right adenoviral ITRs, including the packaging signal, and different sizes of stuffer DNAs to accommodate different insert sizes. In these plasmids the ITRs are flanked by unique restriction sites that are used to release the plasmid backbone prior to the rescue of vector in the producer cell line. Following transfection of the vector plasmid into E1 and Cre expressing producer cells that are coinfected with a loxP helper virus, the vector titer is increased through four to six serial amplifications. In every amplification the cells are coinfected with loxP helper virus. Although excision of the packaging signal of the helper virus is not 100% efficient, the final helper virus contamination in this system can be less than 0.1%. The vector yield per cell can be as high as 1000–2000 infectious units [G.S., unpublished observation].

B. Stuffer DNA

Earlier experiences with Ad5-SV40 hybrid vectors had suggested that the lower size limit for efficient production of adenoviral genomes is about 25 kb [66]. However, most expression cassettes that are currently in use for gene transfer are of much smaller sizes. Rearrangements and/or amplifications

of the vector genome resulting in concatamerization of a starting monomer has been the rule in several studies in which small expression cassettes were rescued as deleted adenoviral vectors. The vector preparations consisted of viral particles that contained both monomeric and dimeric genomes and frequently were mixtures of particles with head-to-head, head-to-tail, or tail-to-tail DNA concatemers [6, 14, 28, 29]. This size-dependence of stable vector production was confirmed with the loxP production system that was used to rescue and propagate HC-Ad vectors with differently sized vector genomes as starting material. Only vectors with genome sizes of at least 27 kb allowed efficient and stable vector amplification [30]. Thus, "stuffer" DNA has to be added to the therapeutic gene cassette to bring the total vector genome size to at least 27 kb. Some practical considerations are outlined here. Since approximately 30-kb plasmids are typically used for vector construction, the stuffer DNA should not contribute to instability during plasmid propagation in *Escherichia coli.* Larger stretches of repetitive elements might increase the likelihood of vector instability during cloning and production procedures and therefore should be avoided. Likewise, stuffer DNA should support stability and growth during viral vector amplification. Stuffer DNA should not interfere with transgene expression *in vivo* and should be transcriptionally silent. Other elements like matrix or scaffold attachment regions (MARs or SARs) may have positive influences on vector stability in the transduced cells. Stuffer DNA has the potential to promote recombination between HC-Ad vectors and the recipient cell genome since these vectors may share large stretches of homology with the genomic DNA of the target cell. This could increase the possibility of vector integration by homologous recombination. However, experimental evidence from *in vitro* studies suggests that, compared to first-generation adenoviral vectors, integration frequencies might be somewhat increased but are unlikely to be high [31].

There is some evidence that the source of the stuffer sequences may have an impact on the levels of transgene expression from the vector. An HC-Ad vector containing CpG-rich stuffer DNA derived from phage lambda resulted in significantly reduced and only short-term hepatic expression of a lacZ transgene when compared to an HC-Ad vector containing noncoding stuffer DNA from the human hypoxanthine–guanine phosphoribosyltransferase (HPRT) locus [32]. One explanation that could account for this observation was a possible inadvertent expression of phage lambda genes in eukaryotic cells resulting in toxicity or immunogenicity. In addition, lambda DNA harbors a high number of immunostimulatory CpG motifs which could contribute to the immunogenicity of the transgene protein. Human DNA sequence is probably the best source of stuffer DNA. Various HC-Ad vectors carrying human stuffer DNA have demonstrated high and long-lasting transgene expression *in vivo* [2, 29, 33–36]. These vectors either contained noncoding stuffer DNA from the human HPRT locus and/or from the human cosmid C346. Even though these

stuffer DNAs were stable through cloning and amplification in most HC-Ad vectors, some reports have indicated instability of the stuffer DNA that was derived from the HPRT gene [23]. In these analyses, HC-Ad vectors containing stuffer DNAs from other human genomic loci seemed to have some growth advantages during amplification and also showed improved expression levels when compared to vectors containing HPRT stuffer DNAs. Future experiments will likely add to the understanding of the impact of human stuffer DNA on expression levels and stability.

C. Vector Capsid Modification

Experimental strategies directed toward the improvement of efficacy and safety of adenoviral, including HC-Ad vector-mediated, gene transfer by modification of the vector capsid involve three different aspects: first, attempts to circumvent the neutralizing immune response raised within the recipient following the initial vector delivery and preventing repeated administration; second, efforts to abolish the native adenoviral tropism in order to minimize transduction of nontarget tissues; and third, introduction of new ligands or binding domains to target the vector to specific cell types. The capsid of HC-Ad vectors, whose protein components are encoded by the helper virus genome, is not different from that of first-generation vectors. Therefore, neutralizing antibodies produced as a consequence of the first vector delivery still represent a significant problem in readministration schemes. Based on the observation that neutralizing antibodies are Ad type-specific [37], Parks *et al.* recently demonstrated that this problem can be overcome by the sequential use of HC-Ad vectors of alternative serotypes [38]. In addition to the Ad5-based helper virus originally used in the Cre/loxP system [9], a new helper virus was constructed that was based on serotype 2. An HC-Ad vector with an Ad2 capsid was injected into mice, followed 3 months later by administration of a HC-Ad vector that had either an Ad2 capsid or an Ad5 capsid. The repeat administration of the HC-Ad vector of the same serotype resulted in a 30- to 100-fold reduction in reporter gene expression in the liver, compared with unimmunized animals, whereas no decrease in transgene expression was observed when the second HC-Ad vector was of the different serotype. No Ad5-cross-reactive antibodies were produced in mice immunized with the Ad2-based vector [38]. Similarly, successful repeat vector delivery was achieved in baboons by sequential administration of Ad5- and Ad2-based first-generation Ad vectors [39]. These data indicate that such an approach, taking advantage of the availability of different adenoviral serotypes, might allow repeated gene transfer in immunocompetent individuals.

With respect to strategies that aim at a modification of the tropism of adenoviral vectors, HC-Ad vector technology will build on the experience and results collected with first-generation vectors. Considerable progress has

recently been made to develop infectious vector particles with reduced or no affinity for the native coxsackie-and-adenovirus receptor, CAR, by site-directed mutagenesis of the CAR-binding region in the fiber knob domain [40, 41], by the design of knobless vector particles [42], or by production of completely fiberless particles in specialized producer cells [43]. These modifications, when applied to HC-Ad vectors in the future, could further add to their targeting efficiency and safety by reducing undesired infection of nontarget cells and increasing vector concentration at target sites *in vivo*. Retargeting of first-generation vectors to cell surface molecules of interest has been achieved by the use of bispecific "adapter" molecules like bispecific recombinant antibodies [44] or CAR fusion proteins [45], by chemical cross-linking of binding moieties to the vector capsid [46] or by genetic insertion of ligands either into the fiber knob protein [47] or the hexon protein [48]. (For comprehensive description of these strategies, see Chapter 8 in this volume). For retargeting of HC-Ad vectors, we have recently constructed a new Ad5-based helper virus containing two unique restriction sites in the fiber gene which facilitate insertion of binding ligands into the fiber knob HI loop. In one line of experiments, an RGD peptide motif was inserted into this reengineered HI loop site for redirecting vectors to αv integrins. HC-Ad reporter vectors produced using this helper virus transduced ovarian carcinoma cells as well as primary endothelial and smooth muscle cells with a 2- to 20-fold higher efficiency, depending on the cell type, than unmodified vectors [49], providing proof-of-concept experiments for the powerful combination of HC-Ad vector technology and retargeting strategies.

III. Applications

A. Liver Gene Transfer

The liver possesses a variety of characteristics that make this organ very attractive for gene therapy. Because of the fenestrated structure of its endothelium, the liver parenchymal cells are readily accessible to large particles such as viruses present in the blood. With respect to blood circulation, the liver can serve as a secretory organ for the systemic delivery of many therapeutic proteins. In addition, in many inborn errors of metabolism the liver is the mainly affected organ. Adenoviral vectors gained considerable interest for liver gene therapy owing to their capacity to very efficiently transduce quiescent hepatocytes *in vivo*. In fact, upon intravenous injection into the tail vein of mice, a large proportion of adenovirus particles preferentially localizes to the liver. However, in immunocompetent animals and with first-generation adenoviral vectors, transgene expression in general is transient both due to the loss of transduced hepatocytes and to promoter inactivation. Immunological

and toxic effects in transduced cells due to viral gene expression significantly limit the use of first-generation vectors for hepatic gene transfer *in vivo*.

An HC-Ad vector expressing the human α1-antitrypsin gene was used in several instructive experiments. Using the loxP helper virus production system, an HC-Ad vector was generated containing the 19-kb genomic human α1-antitrypsin locus that included both the macrophage and liver-specific promoters, all exons and introns, and the natural polyadenylation signal [2]. α1-Antitrypsin antagonizes neutrophilic elastase and is abundantly expressed in hepatocytes and at a lower level in macrophages. Expression in the two cell types is regulated by different tissue-specific promoters. Currently, α1-antitrypsin-deficient patients have a shortened life expectancy due to emphysema. Patients are treated with weekly injections of human α1-antitrypsin purified from human plasma.

Gene transfer of 2×10^{10} particles of this vector in immunocompetent C57BL/6J mice resulted in tissue-specific and stable gene expression for longer than 1 year. Transcription of the human α1-antitrypsin RNA in the liver of transduced animals was initiated from the liver-specific promoter, but not from the macrophage-specific promoter. Gene transfer with increasing vector doses resulted in high and stable α1-antitrypsin levels in serum. Significantly, with increasing vector doses, serum levels of α1-antitrypsin were obtained that would be considered supraphysiological in humans. Even these very high vector doses were not accompanied by liver toxicity. Mice that received the same dose of a first-generation vector carrying the human α1-antitrypsin cDNA under the control of the murine phosphogylcerate kinase (PGK) promoter experienced liver damage as documented by histological abnormalities and elevated liver enzymes detected in the serum of transduced mice [50]. Gene transfer of this vector in baboons resulted in relatively stable transgene expression for longer than 16 months in two of three baboons [39]. In these animals only a slow decline was observed to 19% and 8% of peak levels at 16 and 24 months, respectively. This was not surprising for two reasons. First, hepatocytes are not postmitotic and there is a regular, albeit slow, turnover in this cell type. Second, the animals were young and still growing when they were injected. Therefore, a decline of α1-antitrypsin levels correlated with animal growth. In a third baboon, the generation of anti-α1-antitrypsin antibodies was associated with a short duration of expression of only 2 months. Transgene expression in all three animals injected with a first-generation vector was limited to 3 to 6 months. The lack of anti-α1-antitrypsin antibodies in these animals and further immunological studies suggested that cellular immune responses against viral proteins might have resulted in the elimination of vector-transduced hepatocytes. In summary, these studies demonstrated the main advantages of HC-Ad vectors: increased capacity allowing the incorporation of large DNA fragments and even some genes in the genomic context, improved levels and persistence of transgene expression, and significantly reduced toxicity.

Improved expression and decreased liver toxicity has also been observed following gene transfer with an HC-Ad vector expressing the murine leptin cDNA from the human cytomegalovirus (HCMV) promoter [29]. Leptin is a potent modulator of weight and food intake. In leptin-deficient *ob/ob* mice, daily delivery of recombinant leptin protein suppresses appetite, induces weight reduction, and decreases blood insulin and glucose levels. Results from gene transfer experiments with a first-generation vector suggested that delivery of the leptin cDNA might provide therapeutic benefit equivalent to daily leptin protein treatment. However, the effects were only transient in both lean and *ob/ob* mice due to the loss of DNA and due to significant inflammatory changes in liver. Using an HC-Ad vector carrying the same expression cassette, leptin expression and physiological consequences were analyzed following gene transfer. In lean mice, tail vein injection of $1-2 \times 10^{11}$ particles of the HC-Ad vector resulted in long-term leptin expression. Gene expression in *ob/ob* mice (which are leptin-deficient and therefore not tolerant to leptin) following gene transfer with the same dose of an HC-Ad vector was improved, prolonged, and associated with increased weight loss. However, even in HC-Ad vector transduced *ob/ob* mice leptin serum levels declined and finally disappeared due to the generation of anti-leptin antibodies.

Relatively realistic disease targets for HC-Ad vectors are the clotting disorders hemophilias A and B. The hemophilias are characterized by spontaneous and prolonged bleeding into joints, muscle, and internal organs. Current treatment of the hemophilias, which are often life-threatening and frequently associated with disabling arthropathy due to recurring joint bleeding, consists of protein-replacement therapy with infusion of plasma-derived or recombinant factor VIII (FVIII) or factor IX (FIX). The hemophilias are attractive candidates for gene therapy since they are due to single gene defects. A significant advantage is the fact that the therapeutic window is relatively broad. In addition, tissue-specific expression and precise control of the transgene expression is probably not required. Importantly, even moderate increases of FVIII or FIX levels would be sufficient to convert a severe hemophilia to a milder form. Intravenous injection of first-generation adenoviral vectors expressing the human or canine B-domain-deleted FVIIII cDNA in normal or hemophilic mice and dogs resulted in therapeutic and physiological levels of biologically active FVIII that was accompanied by a correction of bleeding tendency. However, both in hemophilic mice and dogs FVIII levels gradually declined, resulting in only short-term phenotypic correction. In mice transduced with a first-generation adenoviral vector expressing the human FVIII gene, anti-FVIII antibodies were not detectable. However, in hemophilic dogs, neutralizing FVIII antibodies were generated upon gene transfer of first generation vectors expressing either the human or canine FVIII cDNA [for review see 51].

Recently, an HC-Ad vector that carries the full-length human FVIII cDNA under the control of the 12.5-kb albumin promoter was injected into

hemophilic mice, resulting in efficient hepatic gene transfer and therapeutic FVIII expression which led to the correction of the phenotype. However, FVIII levels declined, possibly due to the generation of inhibitory antibodies to the human FVIII protein. Histopathological findings of vector-induced toxicity were not observed [52]. Therapeutic expression levels could only be observed with relatively high vector doses (2×10^{11} viral particles per mouse). With a 10-fold lower vector dose FVIII could not be detected in the serum. These results suggested a nonlinear "threshold" effect which also has been observed with first-generation vectors [53].

Two further examples of liver gene transfer by HC-Ad vectors are mentioned since they point to additional advantages of this new vector type. In one instance an HC-Ad vector was generated to express murine erythropoetin (mEPO), a glycoprotein regulating erythropoiesis [35]. EPO is mainly secreted by kidney peritubular cells in response to hypoxia and promotes late erythroid precursor proliferation and terminal differentiation of erythrocytes. Patients suffering from chronic renal failure show anemia as a major complication resulting from the destruction of EPO-secreting cells. These patients are treated with administration of recombinant human EPO protein. As an alternative treatment, delivery of the human EPO gene via an HC-Ad vector was tested and compared to a first-generation adenovirus vector with the same expression cassette. Relatively low amounts of an HC-Ad vector (3×10^5 infectious units or 3×10^7 particles per mouse) were sufficient to elevate hematocrit levels significantly, although with varying efficiencies, in different immunocompetent mouse strains. In this system the HC-Ad vector was at least 100-fold more efficient than a first generation vector. Because the low vector doses did not initiate any detectable neutralizing antibody response, intravenous readministration of the vector was possible without a need for immunosuppression. In contrast, a second injection of a first-generation virus into mice that had been previously transduced with the same vector induced a much smaller and only transient hematocrit increase.

A second example concerns the use of the mifepristone inducible gene expression system within the HC-Ad vector context [34]. In this system a chimeric *trans*-activator was used consisting of a mutated progesterone receptor ligand-binding domain, part of the activation domain of the human p65 subunit of the NF-κB complex, and a GAL4 DNA-binding domain. Expression of the *trans*-activator was under the transcriptional control of the liver-specific transthyretin (TTR) promoter. A second expression cassette was located on the same vector and consisted of a 17-mer GAL4-binding site just upstream of a minimal TATA box containing the promoter and cDNA of human growth hormone (hGH). In the presence of the progesterone antagonist mifepristone the transactivator dimerizes, binds to the Gal4 DNA binding site and induces hGH expression. *In vitro* studies in HepG2 cells and *in vivo* experiments in mice demonstrated extremely tight control of gene expression and very strong

induction of hGH expression upon administration of mifepristone. Following liver gene transfer, repetitive induction was possible for longer than 1 year [34, and unpublished data].

B. Gene Transfer into Skeletal Muscle

The first *in vivo* application of HC-Ad vectors was for gene transfer studies toward a treatment for Duchenne muscular dystrophy (DMD), an inherited muscular dystrophy caused by mutations in the dystrophin gene. The dystrophin cDNA is 14-kb in length; thus, only shortened versions of this cDNA could be accommodated by first-generation or second-generation adenoviral vectors. Therefore, HC-Ad vectors provided the potential to deliver the full-length dystrophin cDNA with an adenoviral vector. DMD is the most common form of muscular dystrophy with an incidence of 1:3500 male births. Mutations in the dystrophin gene result in the absence of the cytoskeletal dystrophin protein that is normally located at the cytoplasmic face of the cell membrane in skeletal and cardiac muscle. In normal muscle, dystrophin serves as a link in a network of proteins that span from actin within the muscle cell to laminin in the extracellular matrix. The absence of dystrophin results in a secondary loss of dystrophin-associated proteins, increased fragility of the muscle membrane, and cycles of degeneration followed by regeneration. Ultimately, the regenerative process fails and muscle fibers are replaced with fibrosis.

HC-Ad vectors encoding the dystrophin cDNA were developed by several groups [5, 8, 14]. Two groups incorporated a muscle-specific muscle creatine kinase (MCK) promoter [5, 8], allowing demonstration of striated muscle-specific expression of dystrophin from the vector. Direct intramuscular injection of these dystrophin-encoding HC-Ad vectors in the dystrophin-deficient *mdx* mouse model resulted in expression of recombinant dystrophin that properly localized to the muscle sarcolemma [7, 14]. Furthermore, dystrophin-associated proteins, which are lost in DMD and *mdx* muscle secondary to the primary absence of dystrophin, were restored in muscle fibers expressing HC-Ad vector-delivered dystrophin [54]. The prevention of dystrophic morphologic changes in muscle of *mdx* mice receiving an intramuscular injection of dystrophin-encoding HC-Ad vector was a second indicator of normal function provided by the recombinant dystrophin that was expressed from the HC-Ad vector [7].

One HC-Ad vector encoding a MCK-driven murine dystrophin cDNA and an HCMV-controlled lacZ gene, called AdDYSβgal, resulted in a profound cellular infiltrate composed primarily of $CD4^+$ and $CD8^+$ T cells when injected intramuscularly in nondystrophic, normal mice, even when gene delivery was performed during the neonatal period [54]. Expression of β-galactosidase was identified as the principal cause of the observed cellular immune response

by performing parallel intramuscular injections of AdDYSβgal in neonatal mice with a germline lacZ transgene on the same genetic background. LacZ-transgenic mice did not develop a cellular infiltrate in skeletal muscle at any time point after intramuscular AdDYSβgal delivery [54]. Further studies demonstrated that dystrophin expression from AdDYSβgal in skeletal muscle of *mdx* mice also could induce at least an antibody-mediated immune response to dystrophin antigens (P.R.C., unpublished observations). When immunity to the vector was largely eliminated in direct muscle gene transfer studies, the AdDYSβgal vector DNA was stably maintained in skeletal muscle for at least 5 months [33]. Furthermore, the integrity of vector DNA remained intact [33]. This provided assurance that HC-Ad vector DNA could remain as a stable episome in transduced muscle cells.

These studies clearly show the utility of HC-Ad vectors for muscle gene transfer. An important issue to address in future studies is the nature of immunity induced by transgene proteins and adenoviral capsid antigens in the context of specific disease applications. It is likely that the underlying pathology of a muscle disorder will influence immunity induced or augmented by HC-Ad vector-mediated gene delivery. The low efficiency and extent of gene delivery to muscle is a second issue that currently prevents clinical applications of HC-Ad vectors. Targeting of HC-Ad vectors for muscle gene delivery may permit systemic administration that could result in transduction of muscle tissue widespread throughout the body.

C. Gene Transfer into the Eye and into the CNS

Adenoviral vectors have successfully been used for transgene delivery to different anatomic compartments and cell types of the eye, *in vitro* and *in vivo*. Several groups have demonstrated efficient transduction of retinal cells with first-generation adenoviral vectors expressing reporter or therapeutic genes [see for example 55–61]. The eye is considered a site of immune privilege, which is immunologically tolerant to foreign antigens similar to the testis, ovary, and uterus [62]. However, following adenoviral-mediated gene transfer into different ocular cell types, gene expression has always been transient. The short duration of gene expression obtained, together with the limited insertion capacity of first-generation Ad vectors, recently prompted studies that aimed at developing HC-Ad vectors for somatic gene therapy of human retinal degenerative diseases. R. Kumar-Singh *et al.* constructed an "encapsidated adenovirus mini-chromosome" containing a full-length murine cDNA encoding the β-subunit of the guanosine 3′,5′-monophosphate (cyclic GMP) phosphodiesterase (β-PDE) under control of a human β-PDE promoter which is transcriptionally active in photoreceptor cells of the neuronal retina [28, 63]. This vector was prepared by cotransfection of 293 cells with helper virus DNA and a circular plasmid with head-to-head-oriented adenoviral ITRs generating linear adenoviral "mini-chromosomes" following rescue in 293 cells. The

vector particles contained either monomers of the 13-kb starting material, or dimers in a head-to-head, head-to-tail, or tail-to-tail configuration [28, 63]. The β-PDE HC-Ad vector was delivered to the subretinal space of homozygous *rd* mice. These mice, which show a similar retinal phenotype as retinitis pigmentosa patients, suffer from an early-age onset of degeneration of retinal photoreceptors due to a loss-of-function mutation in the β-PDE gene. Expression of β-PDE in transgenic *rd* mice is known to rescue photoreceptor degeneration in this model [64]. In the β-PDE HC-Ad vector-treated animals, expression of the transgene in the neuronal retina was demonstrated by RT-PCR, Western blot analysis and functional enzymatic assays [28, 63]. When the thickness of the outer nuclear layer, as a marker of photoreceptor cell rescue, was evaluated at 2-week intervals, significant differences were observed between mice injected with the β-PDE HC-Ad vector and control vector up to 12 weeks postinfection [28, 63]. Despite these encouraging results the expression of the β-PDE Ad vector was transient and loss of expression was complete at 120 days following subretinal injection. Whether the loss of expression was due to an immune response directed against contaminating first-generation helper virus or against the transgenic protein, to promoter shutdown, or simply to instability of the vector DNA is not clear at the time of this writing. Since quiescent cells of the CNS allow efficient gene transfer by adenoviral vectors, glial and neuronal cells are very interesting target cells for HC-Ad vectors. In an *in vitro* study primary neuronal cells isolated from the cerebellum of 8- to 9-day-old mice were transduced with either a first-generation or an HC-Ad vector expressing *E. coli* β-galactosidase [65]. Compared to gene transfer with a first-generation vector, transduction of these primary cells with the HC-Ad vector resulted in a marked decline in vector-mediated toxicity as assessed by morphological and metabolic studies. In particular, this was evident at moderate vector doses, corresponding to up to 50 multiplicities of infection (m.o.i.), a vector dose that resulted in an 85% transduction rate. However, at very high doses, the HC-Ad vector exhibited cytotoxicity, though not as severe as could be observed with a first-generation vector control.

A problem of clinical significance that has been rarely addressed concerns the fate of a viral vector following the superinfection by a virus of the same or a closely related serotype. Stereotactic injection in rats into the striatum of the brain of both a first-generation and an HC-Ad vector expressing lacZ resulted in stable gene expression over at least 60 days with both vectors [36]. However, challenge by peripheral subcutanous injection of a first-generation vector expressing an immunologically unrelated transgene resulted in a strong inflammatory response in the brain of rats that had received the first-generation vector but not the HC-Ad vector. Gene expression was completely abolished in rats that were injected with the first-generation vector while expression from the HC-Ad vector was stable. This experimental setup is mirrored by a

clinical situation in which therapeutic gene transfer is followed at a later time by infection with a virus of the same or a closely related serotype.

IV. Conclusion

Studies to date convincingly demonstrate the utility of HC-Ad vectors for gene transfer into different tissues. Safety and expression features of HC-Ad vectors are improved over earlier-generation adenoviral vectors. The increased capacity may allow coexpression of different therapeutic genes and improved control of gene expression. A critical issue that stands between the current status of HC-Ad vector development and clinically useful applications for human patients is at the level of vector production. It is felt, however, that with recently improved producer cell lines, vectors, and helper viruses the production under GMP will be possible in the near future. Retargeting strategies will increase the spectrum of therapeutic applications and will further add to the safety of HC-Ad vectors.

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CHAPTER 16

Xenogenic Adenoviral Vectors

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I. Impetus and Rationale

Although human adenoviruses (HAdVs) have been extensively studied over the past four decades, it is only in the past 10 years or so that studies on animal adenoviruses have begun to approach the same level of molecular analysis. This was partly driven by the desire to characterize viruses that clearly had very different properties and host ranges compared with HAdV, but it was also recognized that natural infection of human populations would very likely induce a level of immunity that might curtail the effective use of HAdV vectors. Molecular studies of xenogenic AdVs have substantially expanded our knowledge. Understanding their biology will ultimately lead to an increased choice of gene delivery vectors, providing more options in therapeutic strategy and design.

II. Classification of Adenoviruses

Adenoviruses were classified originally on the basis of serological tests and hemagglutination ability (reviewed in [1, 2] but the availability of genetic data has enhanced the ability to assess viral relatedness. The great majority of AdVs are classified as members of the mastadenovirus genus. This group includes all known human and many AdVs of animal origin. Bovine, porcine, canine, murine, equine, simian, and ovine viruses are all represented, some as multiple serotypes [3]. The genus aviadenovirus has also been known for many years. This group consists exclusively of viruses of avian origin, as the name suggests. Again, multiple serotypes of fowl AdV occur, with the prototype virus being the FAdV1 isolate known as CELO. A third group,

proposed as a new genus called the atadenoviruses [4–7], comprises viruses from bovine, ovine, and avian species and, tentatively, viruses from goats, deer, and possum (B. Harrach and D. Thomson and H. Lehmkuhl, pers. commun.). The OAdV7 isolate 287 has been proposed as the prototype of this group [5, 8]. Turkey hemorrhagic enteritis virus (HEV) and frog virus (FrAdV1) may constitute a fourth genus [9]. To assist in defining the potential uses of each vector it is important to understand the host range and biology of each virus. Although some information has been gleaned from genetic data, for the nonmastadenoviruses especially, few studies of the nonstructural viral gene products have been done.

III. Factors Affecting Vector Design and Utility

A. Host Range and Pathogenicity

A driving force behind the development of HAdV vectors was the knowledge that they are not associated with significant disease in healthy individuals [1]. The production of defective vectors in complementing cell lines has provided an additional margin of safety [10]. Several of the xenogenic AdVs reviewed here are being adapted for use as vaccine vectors in the homologous host. Thus, it is important that wild-type BAdV3, PAdV3, CAdV2, FAdV1, and OAdV7 cause only mild or subclinical symptoms upon experimental infection of the species from which they were isolated [11–15].

When considering viral host range it is important to distinguish between host range defined by viral replication and host range defined by the ability to transduce cells. Transduction is influenced largely by the interaction between the fiber protein and a primary cellular receptor. Some avi- and mastadenoviruses have a second fiber protein [16, 17]. The major primary receptor for HAdVs has been identified as Coxsackie and adenovirus receptor (CAR) [18, 19]. This is probably also used by SAdVs (Table I) because they grow well in human cells and were propagated in human embryonic kidney 293 cells [20]. For most other xenogenic AdVs no primary receptor has been characterized, nor is it clear whether secondary receptors such as integrins [21] are involved in virus uptake. Indeed, xenogenic AdVs lack identifiable or functionally confirmed integrin-binding sequences in their penton proteins [22–27]. For fiber, the coiled coil, trimeric structure of the stalk [28] is conserved, but the distinct sequences of the cell binding domains for the avi and atadenoviruses suggest that they utilize primary receptors that are distinct from CAR. Consistent with this, although HAdV5 and OAdV7 can both infect CSL503 ovine lung cells, they do not compete with each other for entry [29]. CAdV2 is the only xenogenic mastadenovirus that has been examined with respect to cell binding and uptake. Despite the differences between the HAdV5 and CAdV2 capsids

Table I
Complete Nucleotide Sequences of Xenogenic Adenovirus Vectors

Virus type	Isolate	GenBank Accession No.
Canine adenovirus CAdV1	RI261	NC_001734
Canine adenovirus CAdV2	Toronto A26/61	U77082
Bovine adenovirus BAdV3	WBR-1	AF030154
Porcine adenovirus PAdV3	6618	AF083132
Murine adenovirus MAdV1		NC_000942
Simian adenovirus SAdV21 SAdV25	C1 C68	US patent 6,083,176
Fowl adenovirus FAdV1	CELO; Phelps (ATCC VR-432)	U46933
Fowl adenovirus FAdV8	ATCC strain A-2A	AF083975
Ovine adenovirus OAdV7	287	U40839
Duck adenovirus DAdV1	EDS strain 127	Y09598
Frog adenovirus FrAdV1	VR-896	AF224336
Turkey adenovirus TAdV3	Hemorrhagic enteritis virus	AF074946

the kinetics of uptake and trafficking of the two viruses in dog kidney cells was surprisingly similar [30]. CAdV2 shares some features of AdV2/5 tropism but also exhibits distinct characteristics. For example, CAdV2-infected Chinese hamster ovary (CHO) cells that expressed human or mouse CAR but it did not bind to human dendritic cells that were efficiently infected by HAdV5. Uptake of CAdV2 in susceptible cells must be augmented principally by CAR because the Arg-Gly-Asp (RGD) motif that binds to $\alpha_v\beta_5$ integrin is absent from the CAdV2 penton. However, CAdV2 also appears capable of binding to other cell surface proteins [31]. Identifying the receptors for xenogenic adenoviruses and defining the mechanisms of virus uptake is important as it will allow target and nontarget cells to be identified, thus suggesting potential uses for each vector.

However, it is possible that amino acid variation between a natural viral receptor and its counterpart on heterologous cells may alter virus binding affinity.

B. Neutralization

HAdVs are ubiquitous in the human population. As a result of natural infection most individuals develop immunity to adenoviruses by the time they reach maturity. Antibodies against multiple serotypes are common [32] and a substantial portion have neutralizing activity [33]. Nonneutralizing antibodies can also bind to virus particles, leading to their indirect inactivation via the complement system [34]. In addition, individuals commonly develop a long-lived CD4+ T-cell response against multiple serotypes of human adenovirus [35] which may mitigate the strategy of using human adenoviruses from alternative serotypes as vectors [36, 37]. Apart from preexisting immunity, administration of a HAdV at high dose can elicit an inflammatory response [38]. The vector may also induce an immune response that can reduce the efficacy of subsequent doses, although the extent of this effect may vary with the route of administration [39, 40]. A variety of methods have been used to overcome these problems, including transient immunosuppression, blocking of antibodies with agents such as polyethylene glycol and removal of antibodies from serum by immunoapheresis [41, and references therein].

The use of xenogenic adenovirus vectors is expected to avoid neutralization due to preexisting immunity to HAdVs. To investigate this, random human sera were examined for the presence of antibodies that neutralized OAdV7 or CAdV2. Of a panel of 57 sera, most of which neutralized HAdV5 to high titer, only three also neutralized CAdV2 [42, 43]. Similarly, 13 individual and two pools of human sera that neutralized HAdV5 did not neutralize OAdV7 [44]. SAdVs were also not neutralized by antisera that neutralized HAdVs [45]. These data suggest that xenogenic adenoviruses will provide an advantage upon initial administration although it is not expected that the vector will be immunologically silent. However, whether vector is given locally or systemically may determine whether it is possible to administer more than a single dose [39, 40].

C. Genome Structure and Function

Of the xenogenic mastadenoviruses, complete nucleotide sequences have been determined for bovine (BAdV2 and 3) [24], porcine (PAdV3) [46], murine (MAdV1) [27], canine viruses (CAdV1 and 2) [26], and simian viruses [20] (Table I). For the aviadenoviruses, FAdV1 [23] was the first genome sequenced but FAdV8 [47] is now also completed. Among the atadenoviruses, ovine (OAdV7) [22], bovine (BAdV4) (B. Harrach, pers. commun.), and duck (DAdV1) [48] genomes are sequenced. The turkey (TAdV1) [49] and frog (FrAdV1) genomes [9] have also been characterized. All of these viruses

are potential vectors for gene delivery because they can now be rationally engineered, but not all are being developed as vectors at this stage.

The viruses described above represent the extreme ranges of genome size, the largest being ~43.8 and 45 kb for FAdV1 and FAdV8, respectively, and the smallest being ~26.3 kb for TAdV1 and ~29.5 kb for OAdV7. The Mastadenovirus genomes range in size from ~30.9 (MAdV1) to 34.4 kb (BAdV3).

1. Central Core

In comparing the nucleotide sequence for prototype viruses in each genus it is apparent that there is a central core in each genome bounded by the pVIII and IVa_2 genes (Fig. 1). This codes for the DNA replication, structural proteins, and accessory polypeptides required for their assembly. Most capsid proteins have homologs in each genus but proteins V and IX are unique to mastadenoviruses. Instead, OAdV7 has a gene for the structural

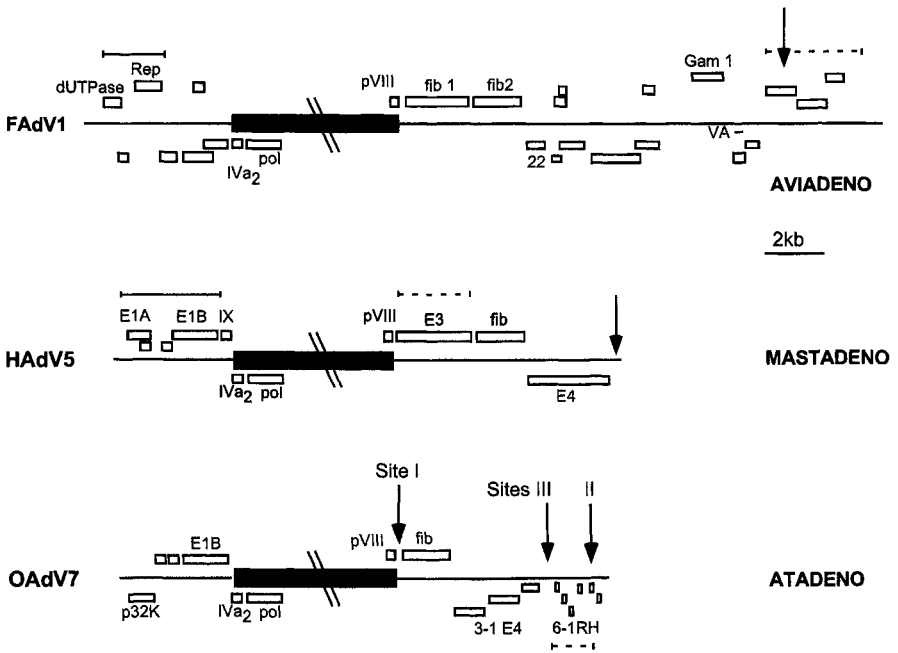

Figure 1 Comparison of the genome structures of prototype viruses from the avi-, mast-, and atadenoviruses. The central core of each genome (filled rectangle) flanked by the IVa_2 and pVIII genes is essentially conserved in arrangement and is truncated for simplicity. Other major open reading frames are indicated by open rectangles. Arrows indicate sites for insertion of foreign gene cassettes. The solid and broken lines indicate regions that can be provided in *trans* and regions that can be deleted, respectively. Note that E4 and E2 sequences have also been deleted in HAdV5 and SAdV vectors but this has not been demonstrated for other xenogenic mastadenoviruses.

protein, p32 K, that lies at the extreme left end of the genome (Fig. 1). This capsid protein complement correlates with the observation that FAdV1 and OAdV7 are more heat-stable than the mastadenoviruses [50, 51]. It will be of interest to determine whether a functionally equivalent protein exists for the aviadenoviruses. Also in the central core of HAdVs are one or two copies of VA RNA genes [52]. Except for PAdV3 [46] and SAdVs [53], these are not present in xenogenic mastadenoviruses or OAdV7 [54], but a single copy is present near the right-hand end of FAdV1 [55] and DAdV1 (Fig. 1) [23, 48].

2. Right-End Sequences

To the right of the central core the genomes vary greatly in structure and gene complement. In the mastadenoviruses the E3 region varies in size and complexity but is located between the pVIII and fiber genes (Fig. 1). HAdV2 and −5 have an E3 region of ~2.5 kb that codes for numerous polypeptides, many of which interact with components of the immune system [56]. For the xenogenic mastadenoviruses the least complex E3 region from MAdV1 appears to encode a single reading frame that may be variably spliced [57, 58]. BAdV1, −2, and −3, CAdV2, and PAdV3 and −5 have E3 regions of intermediate complexity, ranging in size from ~1.2 to 2.3 kb. These code for a variable number of putative proteins that show some homology within a species and occasionally across species [59–63]. The BAdV3 E3 codes for a 284-residue glycoprotein and a 14.7-kDa polypeptide that appears to be the homolog of the HAdV5 14.7-kDa protein. The BAdV3 gene can functionally substitute for the human gene to protect cells against tumor necrosis factor (TNF)-induced lysis [62, 64].

E3 sequences are nonessential for replication *in vitro* [65] and were some of the first sequences deliberately deleted in the construction of recombinant HAdVs [66]. However, it was shown that retention of E3 sequences in a HAdV5 vector dampened the immune response in a rat model, thus extending the time of transgene expression [67]. Consistent with this, a HAdV5 virus in which E3 sequences were deleted showed an enhanced inflammatory response in a Cotton rat model [68]. It remains to be determined whether these results will translate to xenogenic vectors with less complex E3 regions. However, the timing and duration of gene expression that is required is a factor to be considered in vector design.

In contrast to the mastadenoviruses, the avi- and atadenoviruses lack E3 regions between pVIII and fiber and instead have small intergenic regions of ~200 and ~400 bp, respectively, that contain signals for transcription termination and splicing of fiber RNA [69].

To the right of the fiber gene in mastadenoviruses lies the E4 region. Like HAdV2/5, a single promoter in BAdV3 and MAdV1 produces seven transcripts that encode multiple polypeptides, some of which are homologous to HAdV proteins [70, 71]. In particular, homologs of HAdV5 E4 ORF6 carry a short

amino acid motif that is highly conserved in many adenoviruses. Based on the conservation of this motif in OAdV7, where it was first recognized [22], the proposed E4 region in the atadenoviruses is penultimate to the right end of the genome (Fig. 1). Two promoters apparently control the expression of three open reading frames (ORFs), two of which contain the motif [48, 72]. No E4 region has been identified in the right-hand portion of aviadenoviruses. Indeed, the function of most reading frames in the right hand ~25% of the genome remains to be determined. In FAdV1, the products of GAM-1 and ORF22 (Fig. 1) have been identified as proteins that interact with pRb [73]. However, in comparing the related FAdV1 and FAdV8 genomes, 5 of 13 unassigned ORFs are unique to FAdV8 [74]. At the extreme right hand end of FAdV1 are 3 ORFs that can be deleted and replaced with a luciferase reporter gene cassette without affecting virus viability [51].

The extreme right ends of the avi- and atadenovirus genomes carry genes that are species specific. For example, DAdV1 has numerous ORFs of unknown function that have no counterpart in OAdV7 [22, 23, 48, 75]. Within the right-hand-end region of OAdV7 lies a series of six short reading frames (RH1 to RH6) (Fig. 1), four of which (RH1, −2, −4, and −6) are closely related to each other. This is surprising in a compact genome of only ~29.6 kb. In DAdV1 there are two ORFs that are related to each other and to those in OAdV7 [72, 76]. For OAdV7 only two transcripts from the region were detected by RT-PCR and these were spliced such that RH1 and RH6 were the only ORFs that could be translated. The apparent redundancy of these ORFs was confirmed by the fact that the reading frames RH2 to RH5 could be deleted without seriously affecting virus viability [75]. The function of these ORFs remains to be determined.

3. Left End Sequences

Left of the central core the genome structures also differ significantly (Fig. 1). For the xenogenic mastadenoviruses there are three ORFs at the left end that show homology with HAdVs [77–80]. The genome packaging signal is also present within the first ~500 nucleotides of the HAdV5 genome [81], but until recently this had not been defined for any xenogenic virus. For CAdV2, however, it was shown that the packaging region consists of a ~200-bp region that contains redundant, but not functionally equivalent sequences. The consensus sequence for HAdVs [81] is present only once and is of minor importance [82].

For the avi and atadenoviruses, some ORFs at the left end are unique to individual viruses or have homologs only within the genus. Two ORFs from the atadenoviruses show some homology with the HAdV5 E1B 19- and 55-kDa genes, suggesting that these functions are conserved. However, no homolog of the E1A gene was identified [22, 48]. An additional ORF that could encode a ~9.6-kDa protein is present in OAdV7 and BAdV4 (B. Harrach, pers.

commun.) but is missing from DAdV1 [48]. The gene for the p32 K structural protein is also present near the left end. The promoter for ORFs LH1 and LH2 is also on the opposite strand within this gene [72]. The packaging signal for atadenoviruses has not been defined but it may incorporate the ~160-bp region between the C-terminus of p32 K and the ITR. For the aviadenoviruses, ORFs with homologies to dUTPase and the REP protein of adeno-associated virus have been found [23] but there are distinct differences between FAdV1 and FAdV8 with three of eight ORFs in the left end being unique to FAdV8 [74]. It was also reported recently [83] that the cysteines and several other residues in the conserved sequence motif of E4 ORF6 are conserved in FAdV1 ORF14, which lies near the left end of the genome [23].

4. Transcription Maps

The determination of transcription maps for some xenogenic viruses has assisted vector design by complementing the data on genome structure. The major transcription units have been described for BAdV3 and PAdV3 [24, 46, 71], FAdV1 [84] and OAdV7 [72]. No transcription map has been reported for CAdV2. More detailed data is available for the E1, E3, and E4 regions of MAdV1 [58, 70, 85] and for the BAdV3 E1 [79, 86] and E3 regions [60]. For BAdV3 and PAdV3, there are minor differences in the splicing pattern within some transcription units but on a broader scale the basic units described for AdV2/5 are completely conserved. Studies of FAdV1 identified many transcripts for ORFs in the genome and a major transcription unit that is controlled by the MLP. However, at the left and right ends of the genome there are 5 and 15 kb, respectively, for which the promoters and transcriptional organization is undefined [84]. In the OAdV7 genome, the left (LH1 to LH3)- and right-hand ends (RH1 to RH6), E2 and the proposed E4 region (E4.1 to E4.3), as well as the structural protein genes constitute individual transcription units. The IVa2 and p32 K ORFs also appear to be transcribed from their own promoters. The LH and E4 regions each appear to be regulated by two promoters [72]. The identification of promoter regions and transcription termination sites has identified possible sites for gene insertion that are less likely to interfere with viral functions.

D. Transforming Ability

Many AdVs are known to carry oncogenes. Members of the mastadenoviruses readily transform cells in culture [11, 87–89], although these viruses differ in their ability to induce tumor formation in animals. Among HAdVs, the group A viruses such as AdV12 are highly oncogenic, while group C (including HAdV5) and E viruses are not known to be tumourigenic (reviewed in [2, 65, 90]). BAdV3 can induce tumor formation in hamsters [91] but there are no reports of tumor induction by other animal mastadenoviruses. FAdV1

also transforms cell *in vitro* [92, 93] and rapidly induces tumors in newborn rodents [94, 95]. For the atadenoviruses there are conflicting reports of tumor induction in hamsters. In one study, tumor formation was reported in hamsters inoculated with BAdV8 [96]. In a second study, none of BAdV4 to -10 produced tumors [97]. More recent studies showed that OAdV7 was unable to transform cells that were transformed by HAdV5 [98]. Primary rat embryo cells were infected with HAdV5 or OAdV7 but only the former produced colonies with a transformed phenotype. Similarly, baby rat kidney cells were transformed by HAdV5 E1A/B sequences but not by the nonstructural genes of OAdV7. The apparent absence of oncogenes in the OAdV7 genome suggests that the virus interacts with the cell cycle machinery in a way that differs from the mast and aviadenoviruses, although this is yet to be defined.

The presence of oncogenes in vector genomes has important implications for vector design in that it is customary to delete these sequences for safety reasons. Continuous cell lines that express the deleted genes in *trans* are established to permit virus propagation. The transforming properties of the mastadenoviruses reside primarily in the E1A and E1B genes at the left end of the genome (reviewed in [2, 65, 90]). The E1A products bind to proteins of the cellular retinoblastoma (pRb) protein family [99], thereby releasing E2F transcription factors that regulate cell cycle progression into S phase [100]. The E1B 55-kDa protein binds to the tumor suppressor protein, p53, and blocks p53-mediated apoptosis [101]. The E1B 19-kDa protein is also anti-apoptotic [102]. Thus, animal adenoviruses typified by BAdV3, PAdV3, CAdV2, SAdV, and MAdV1 have E1A and E1B homologs that have similar transforming and oncogenic potential. The E4 ORF3 and ORF6 products of HAdV5 can also augment the transforming activity of the E1A and E1B genes [103–106]. However, the E4 regions of human and animal mastadenoviruses vary in sequence and complexity. Homology with HAdV5 ORF6 is always evident, especially in a cysteine-rich motif [22] that is thought to mediate ORF6/p53 interaction [83]. Furthermore, a complex between the E4 ORF6 and E1B 55-kDa proteins promotes the selective nuclear export of late viral transcripts [107] and references therein). This ORF may be therefore be conserved as it provides a core function for replication in all adenoviruses. However, other E4 ORFs in the xenogenic viruses are unique [22, 26, 108–110] and their function/transforming potential is not clear.

In FAdV1 there are no identifiable E1A/B or E4 regions in the genome [23], but recently two proteins, GAM-1 and ORF22, that interact with pRb were identified [73]. In addition, GAM-1 has been identified as an anti-apoptotic protein [111] and one that can activate the cellular heat-shock response, the latter being required for viral replication. The Hsp40 gene is a primary target [112]. GAM-1 may also functionally substitute for the E1B 19 kDa [111]. FAdV1 therefore appears to share with the mastadenoviruses an ability to disrupt complexes between pRb and the E2F transcription

factors to modulate the cell cycle, albeit via different effector proteins [99, 113]. In contrast, OAdV7, the prototype atadenovirus, lacks an identifiable E1A homolog, although it appears to carry E1B 19- and 55-kDa genes. Penultimate to the right end is a transcription unit that contains a unique ORF (E4.1) of unknown function and two ORFs (E4.2 and E4.3) which contain the conserved ORF6 cysteine-rich motif mentioned above [22, 72, 98]. These ORFs otherwise appear unrelated. Similar features are found in the DAdV1 and BAdV4 genomes [48 and B. Harrach, pers. commun.]. However, OAdV7 so far lacks oncogenic activity as the complete OAdV7 genome did not transform primary rodent cells under conditions where transformation was achieved with control HAdV5 sequences [98]. These findings invite the hypothesis that OAdV7 lacks the ability to activate the cell cycle in quiescent cells, instead taking advantage of the cycle as it progresses.

The presence or absence of transforming sequences strongly influences the design of xenogenic adenovirus vectors for gene delivery. Based on HAdV2 and −5, vectors derived from BAdV3, PAdV3, SAdV, and CAdV2 were designed such that the potentially oncogenic E1A/B homologs were deleted [20, 42, 43, 114, 115]. A similar approach could be applied to MAdV1 [116]. Such vectors are replication-defective in cells lines that do not express the deleted genes [42, 43], but in some cases, homologs from HAdV5 can substitute [114, 115]. Some vectors derived from OAdV7, avian, and PAdV3 viruses retain potential transforming genes and carry foreign DNA inserts in nonessential regions of the genome [51, 75, 117–120]. This strategy may be acceptable for vectors that are intended for gene delivery in the homologous animal or avian host but is unlikely to be acceptable for gene therapy purposes, except perhaps in the case of OAdV7, where the vector apparently lacks transforming genes.

E. Cell Lines

Successful rescue of a virus requires a cell line that can be transfected with high efficiency to initiate infection. The cells should also have abundant copies of the primary and secondary receptors to facilitate spread and the production of high titers of virus. Depending on the recombinant, the cells may or may not carry viral sequences to complement a deletion in the viral genome.

1. Primary Cell Lines

The general strategy has been to identify a cell line that is permissive for the wild-type virus and then adapt it for more specialized purposes. For propagation of BAdV3, MDBK, buffalo lung, primary kidney, and bovine cornea endothelial cells have all been tried, with MDBK cells being preferred [114, 121]. CAdV2 was grown in MDCK, dog kidney (ATCC CRL6247) or greyhound kidney [43, 121], MAdV1 in mouse 3T6 [116] and PAdV3 in swine testis cells [115]. FAdV1 recombinants were rescued in leghorn male hepatoma (LMH) cells [51]. FAdV1 can be grown in embryonic chicken kidney

cells but, for reasons of cost, is often grown in embryonated chicken eggs [23]. OAdV7 has a narrow host range and failed to grow in several ovine cell types [15]. However, it grew to high titre in CSL503 cells, a primary ovine fetal lung cell line [122] and a fetal ovine skin fibroblast line HVO-156 (C. Hofmann and P. Loser, pers. commun.).

2. Transformed Cell Lines

Primary cells are adequate for growing replication-competent recombinants. However, there was a need to produce cell lines that would complement genomic deletions and an expectation that transformed cell lines would ensure a continuous supply of cells. This encouraged attempts to develop lines equivalent to 293 cells [123]. Note that SAdVs grow in 293 cells [20]. Based on this and similar precedents [124], the E1A/B sequences of BAdV3 were used to stably transfect MDBK cells [114, 125, 126]. These grew poorly and expressed undetectable amounts of the BAdV3 E1 proteins [114] but nevertheless complemented the growth of an E1A-deleted HAdV5/lacZ recombinant [114, 125]. Attempts were also made, unsuccessfully, to transfect foetal bovine retinal cells (FBRCs) with BAdV3 E1A/B sequences. Because BAdV3 complemented HAdV5/E1A-defective replication [125], it was expected that HAdV5 E1A/B sequences would complement BAdV3/E1 deleted vectors. Transfection of FBRCs with HAdV5 E1A/B sequences in which E1A and E1B were controlled by the mouse PGK and E1B promoters, respectively, produced morphologically distinct clones, one of which was single-cell cloned and characterized as the VIDO R2 line. These cells expressed detectable levels of E1A and E1B 19-kDa, but not E1B 55-kDa protein, supported plaque formation by BAdV3 and HAdV5, and were transfected more efficiently than MDBK cells. Transfection of E1-deleted recombinant genomes into VIDO R2 cells resulted in the rescue of several viruses that carried expression cassettes [114]. For propagation of PAdV3 vectors a transformed fetal porcine retinal cell line (VIDO R1) was also produced by transfection of swine testis cells with HAdV5 E1 sequences [115]. These cells were also morphologically distinct from the parental cells. E1A and E1B 19-kDa proteins were produced, as shown by Western blots, but E1B 55-kDa protein was not detected. While PAdV3 grew well in these cells, for reasons that are not understood, an E1/E3-deleted vector and a similar virus that carried a GFP cassette in E1 grew two logs less efficiently [115].

Similarly, the E1A/B region of CAdV2 was used to transform MDBK and DK cells [42]. Again, low levels of E1A transcripts were produced and E1B transcripts were not reliably detected. Nevertheless, the cells were morphologically and phenotypically distinct from parental MDCK cells. A second series of clones was produced by transfecting DK cells with CAdV2 sequences in which E1A and E1B were controlled by the HCMV and E1B promoters, respectively. Cells produced in this way expressed detectable E1A and E1B transcripts and E1B 19-kDa protein [42] and allowed the rescue and propagation of E1A/B-deleted CAdV2 vectors [43].

Attempts were also made to produce a transformed derivative of CSL503 cells, which are permissive for OAdV7, using the left end (~4 kb) of the OAdV7 genome [98]. The sequences used incorporated the proposed E1B homologs of OAdV7 and a 9.6-kDa ORF of unknown function. No E1A homolog was identified [72, 98]. Only two clones that grew well enough to prepare frozen stocks were obtained and these were morphologically similar to the parental cells. In contrast, transfection of CSL503 cells with HAdV5 E1A/B sequences produced morphologically distinct clones. Growth of OAdV7 in these cell lines appeared to be retarded compared with its growth in wild type CSL503 cells (Xu and Both, unpublished results).

F. Strategies for Vector Construction and Rescue

A huge amount of work carried out over some 30 years on HAdV2 and −5 has defined viral promoters, transcripts and their splice sites and genes that could be deleted or that would function in *trans* (reviewed in [2, 65]). The packaging capacity of the viral capsid was also shown to be ~105% of the viral genome [127]. The strategic design of bovine, canine, porcine, simian, and murine adenovirus vectors, although based on new genetic information, has drawn extensively on historic precedents.

As precedents did not exist for the avi- and atadenoviruses it was necessary to identify intergenic regions within genomic sequences and to use mutagenesis to identify nonessential reading frames for the insertion of gene cassettes. Vector design and virus rescue was also confounded initially by the absence of transcription maps and the lack of knowledge concerning the packaging capacity of these viruses.

Construction of xenogenic adenovirus vectors first required the identification of an insertion site(s) that could stably accommodate a gene cassette without affecting virus growth. For the mastadenoviruses, vector construction strategies followed those for human Ad vectors. Genes were inserted into the nonessential E3 region of BAdV3 [126, 128] or PAdV3 [118] or between the E4 promoter and the right ITR of PAdV3 [118, 120] to generate viruses that were replication competent in noncomplementing cells. More recently, E1A/B region replacements that generated replication-deficient viruses were produced for BAdV3 [114, 121], PAdV3 [115], SAdV [20], and CAdV2 [42, 43]. It is likely that a similar strategy would be successful for MAdV1 where an infectious clone is now available [116]. For the aviadenoviruses, a mutation strategy was used to identify nonessential regions of the genome or regions that could be complemented in *trans* [51]. Deletions between nucleotides 938 and 2900 were complemented by cotransfection of a plasmid that carried the left hand ~5.5 kb of the genome. Deletion of three ORFs adjacent to the right end of the FAdV1 genome did not require transcomplementation, identifying these genes as nonessential for replication *in vitro*. Similarly, replication-competent FAdV8

vectors were constructed by inserting a gene cassette into sites near the right end of the genome (Fig. 1) [119]. For the atadenovirus, OAdV7, genes were initially inserted at site I (Fig. 1) in the pVIII and fiber intergenic region [44, 50, 75, 117, 129], but additional sites were subsequently identified by a mutation strategy. It was found that foreign DNA could be inserted into a unique *Sal*I site (Site II) within ORF RH2, ~1 kb from the right end, and that ORFs in the vicinity could be deleted [75]. In addition, unique cloning sites were tolerated between the right-hand end and E4 transcription units (Site III) [72, 117].

The identification of permissible insertion sites in the genome required the construction of plasmids that enabled the rescue of infectious viruses. The first BAdV3 recombinant was constructed by recombination between a plasmid that carried BAdV3 sequences flanking the luciferase gene inserted into E3 and BAdV3 genomic DNA that had been cut with *Pvu*I to reduce background. DNAs were transfected into MDBK cells that also expressed BAdV3 E1 sequences. However, this method was inefficient and produced relatively few plaques [126]. Similarly, a CAdV2 recombinant that expressed the lacZ gene was produced by recombination between the CAdV2 (Manhattan strain) genome and a plasmid that carried the expression cassette. However, this recombinant was contaminated by wild-type CAdV2 that could not be eliminated [42]. A more favorable approach was to construct a plasmid in which sequences required for the propagation of plasmid DNA in *Escherichia coli* were cloned into a unique restriction enzyme site that linked the ITRs of the viral genome [117]. There was one precedent for this approach [130], although others had reported that perfect palindromes longer than 30 bp were often unstable in *E. coli* [131] and plasmids with large palindromes based on HAdV5 were subject to rearrangement [132]. Unique restriction sites were also introduced into appropriate locations in the OAdV7 genome to allow cloning of gene cassettes. This plasmid design allowed the genome to be released intact by restriction enzyme digestion prior to its transfection into susceptible cells for virus rescue [75, 117]. Subsequently, it was discovered that such plasmids could be constructed using recombination in *E. coli* [133]. Infectious recombinant clones have now been constructed for BAdV3 [71, 114, 134], PAdV3 [118], CAdV2 (Toronto strain) [43], MAdV1 [116], FAdV1 [51], and OAdV7 [44]. The specific infectivity of these naked DNAs in the permissive cell line is usually low (often only a few plaques per microgram) and depends on the transfection efficiency of the cells. However, a significant advantage of this approach is that transfection of purified plasmid DNA almost invariably yields the corresponding virus without the need for extensive plaque purification that may accompany other approaches where background viruses can be generated.

Many xenogenic recombinant viruses have now been rescued. New viruses that first appeared with the formation of plaques or a cytopathic effect in the appropriate transfected cell line were amplified on fresh permissive cells to produce an infectious stock. Viruses were then characterized by restriction

enzyme, Southern blot [75, 115, 118, 126], or PCR analysis [43, 51] to confirm the integrity of the genome and expression cassette. For the vectors where an insertion strategy was pursued, it was particularly important to check the genome integrity because the packaging capacity of the new vectors was undefined. Mastadenoviruses can package 105 to ~107% of the wild-type genome [43, 120, 127], OAdV7 has a capacity of 114%, presumably because of its smaller genome and similar capsid volume [75], while the capacity of aviadenoviruses is undefined. Despite the increased packaging capacity of OAdV7, some viral genomes in which expression cassettes (ranging from 1.8 to 3.1 kb) were inserted into site I of the genome proved to be unstable upon passaging. By passage three, the genomic *Bam*HI profile of viruses that combined the HCMV promoter with a reporter gene sometimes displayed smaller fragments [50]. In contrast, a virus that carried 4.3 kb of "stuffer" DNA was successfully rescued [75] and with the RSV promoter, two viruses with site I cassettes in opposite orientations were stable to at least passage four [44; unpublished results]. Site I instability appears to vary with sequence and possibly orientation and may reflect the need to produce adequate amounts of fiber transcript and protein. Events that lead to transgene deletion with improved fiber production may generate viruses that have a growth advantage. The stability after passage of genomes for other xenogenic recombinant vectors has not been adequately reported.

The propagation of a mixed population of CAdV2 wild-type and deleted vector [42] illustrated the potential for producing gutless vectors based on xenogenic AdVs. The principles established with HAdV5 [135, 136] will further assist this process. It will be necessary to define the packaging signal [82] and a minimum permissible genome size for a particular virus, provide a suitable a helper virus for propagation, and devise a means to purify defective particles. The benefits may be greater safety and more efficient gene delivery in a naive host and prolonged transgene gene expression.

IV. Utility of Xenogenic Vectors

Xenogenic AdV vectors can potentially be used as gene delivery vectors for a range of purposes. However, it is necessary to understand the advantages and disadvantages of vectors in particular situations so as to identify their most appropriate uses. The next section discusses the first attempts to determine the safety and utility of xenogenic vectors for vaccination or gene delivery. The following section reviews the properties and behavior of vectors in heterologous situations.

A. Veterinary Studies

Within the limits of the testing done so far, the viruses discussed in this review are of low pathogenicity in the host from which they were isolated [11–15]. Vectors designed for use in those hosts are often replication

competent to facilitate vaccination by a live viral vector. In the first studies, carried out with BAdV3, the luciferase reporter was inserted directly into the E3 region where a small deletion had been introduced. Expression did not require an exogenous promoter and the vector remained replication competent in bovine cells, although its titer was reduced 10-fold [126]. In contrast to HAdV5 vectors that lacked part of the E3 region [68], this BAdV3 recombinant did not show increased pathogenicity in a Cotton rat model compared with the wild-type virus [137]. Similar replication-competent viruses that carried various forms of the bovine herpesvirus gD gene were shown to express the antigen [71] in an immunogenic form [128]. Intranasal vaccination of calves with these viruses induced gD-specific neutralizing antibodies, primed a cellular immune response and protected against viral challenge, despite the presence of preexisting serum antibodies to BAdV3 [138,139]. E1/E3-deleted replication-defective BAdV3 vectors that carried gD in the E1 region were also constructed [114]. These viruses allowed the parameters for vaccination of cattle by replicating and nonreplicating vectors to be compared. Administration of each vector at the same dose twice via the intra tracheal route and once subcutaneously showed that the replication-competent vector induced superior levels of serum IgG antibodies against gD. Partial protection against challenge was obtained with the replication-competent vector. However, with the replication-defective vector challenge with BHV1 dramatically boosted the levels of serum IgG and IgA antibodies, suggesting that animals had been primed for gD-specific antibody responses [140]. Similar BAdV3 recombinants were constructed in which the bovine diarrhea virus E2 glycoprotein linked to the BHV1 gD signal peptide was expressed from the BAdV3 E3/MLP [141]. The 53-kDa protein that was expressed formed dimers and was recognized by E2 specific monoclonal antibodies. Intranasal immunization of Cotton rats with the recombinant induced E2-specific IgA and IgG responses at mucosal surfaces and in the serum. In contrast, attempts to construct vectors that expressed the bovine coronavirus hemagglutinin esterase gene from the E3 region using the strategy for the BHV1 gD gene were unsuccessful. The addition of exogenous control elements comprising an intron and the HCMV or SV40 promoter increased the level of expression but altered the kinetics. The recombinant virus also replicated less efficiently than wild-type BAdV3 [142].

Replication-competent PAdV3 vectors that express the pseudorabies gD protein or the classical swine fever virus (CSFV) gp55 protein were also constructed. The gD gene was inserted into a partially deleted E3 region without flanking sequences. In contrast to similar BAdV3 vectors, expression of gD was observed at early but not late times pi [118]. The gp55 gene linked to the PAdV3 MLP and tripartite leader sequence (TLS) was inserted at the right end between the ITR and E4 promoter. Vaccination of outbred pigs with a single dose of recombinant virus induced complete protection from lethal challenge with CSFV [120].

A FAdV8 recombinant that expressed chicken gamma interferon from the viral MLP/TLS sequences was also constructed by inserting the cassette at sites near the right end [119]. Depending on the insertion site, the recombinants displayed differing growth characteristics in chicken kidney monolayers. Insertion of the cassette adjacent to FAdV8 ORF7, about 7.2 kb from the right end, produced a recombinant with wild-type growth characteristics. In contrast to the FAdV1 viruses discussed below, deletion of the FAdV1 36-kDa homolog in FAdV8 caused a significant reduction in growth. Interferon was produced in supernatants as early as 24 h pi in proportion to the growth characteristics of each virus *in vitro*. Interferon levels peaked at 48 h and were maintained for at least 10 days. Chickens treated with the recombinant showed increased weight gains compared to controls and suffered reduced weight loss when challenged with a coccidial parasite [119].

An OAdV7 vector was constructed in which the 45 W antigen of *Taenia ovis* was expressed from the viral MLP/TLS elements [143], the cassette being inserted at site I (Fig. 1) [75]. This vector was used alone, or in tandem with DNA or purified 45W protein to vaccinate sheep. Prime/boost strategies where vaccination was initiated with protein or DNA and boosted with the OAdV7 vector were effective in stimulating an immune response that protected animals against challenge with the parasite [144].

The above examples illustrate that with further refinement, xenogenic vectors may have utility for vaccination and gene delivery in their respective hosts.

B. Vector Biology

Ideally, vectors for gene transfer into human cells should be capable of transgene expression without replication or detrimental expression of viral genes. Infection of human cell lines with intact xenogenic adenoviruses established the principle that these viruses are replication defective at the inputs tested [42, 51, 76, 116, 118, 121, 126], although the molecular basis for defective replication is not understood. Studies in animal models have also allowed biodistribution profiles to be determined for some viruses.

1. Transduction of Cells

Selected cell lines have been used to examine viral transduction. However, it is sometimes difficult to compare data from different laboratories because, especially in early studies, the input virus was not characterized with respect to both particle number and infectivity. BAdV3 recombinants in which a HCMV/lacZ or HCMV/GFP gene cassette was expressed from the E1 or E3 region, respectively [114, 121], were used to infect human and other cell types. The GFP recombinant replicated in cells of bovine origin and in Cotton rat lung fibroblasts, but not in cells from other species. When cells were infected with more than 5 pfu/cell of BAdV3/GFP, some GFP expression was observed at

3 days pi in 293 and HeLa but not in A549 or HepG2 cells [114]. In contrast, others found that at an m.o.i of 10 pfu/cell, at 65 h pi A549 and MRC5 cells were efficiently transduced by a BAdV3/lacZ recombinant while HeLa and 293 and primary human muscle cells were transduced with lower efficiency [121]. Since both studies used the HCMV promoter and a similar multiplicity of infection, the reason for the difference with A549 cells is unclear.

The host range of CAdV2 vectors was also investigated. Human 293, HeLa, primary myocyte, and HIB cells were infected with 10^5 transduction units of CAdV2/RSVlacZ in the presence of wild-type CAdV2. All cell types showed β-gal expression when examined at 1 to 2 days pi [42]. In addition, replication-deficient CAdV2 vectors expressing GFP or lacZ from the HCMV and RSV promoters, respectively, were tested for their ability to transduce a range of human cell types in comparison with HAdV5/HCMV/GFP [43]. At 2 days pi HeLa, A172, and HT 1080 cells were transduced with similar efficiency by both viruses. *In vivo*, the CAdV2 vectors also transduced mouse airway epithelia cells with similar efficiency to a comparable HAdV5 vector. Similarly, a replication-deficient PAdV3 recombinant carrying a HCMV/GFP cassette in E1 was used to determine the ability of this vector to infect human and animal cells *in vitro*. At a m.o.i. of 1 pfu/cell PAdV3 apparently entered, but did not replicate in canine kidney, ovine skin fibroblasts, bovine (MDBK), and human (293, A549) cells [115].

Although an infectious clone of MAdV1 now exists, recombinant viruses have not yet been made. However, it was demonstrated by RT PCR that human 293 and primary umbilical endothelial cells were infected, the latter at low efficiency [116].

Replication-competent aviadenovirus vectors that express luciferase from the HCMV promoter [51] were constructed by inserting cassettes at the right end of FAdV1 to replace nonessential ORFs. Vectors replicated in LMH cells with kinetics similar to wild-type FAdV1. When compared to a HAdV5/luciferase recombinant for its ability to transduce human cell types, the FAdV1 recombinant showed a similar ability to express luciferase in HepG2, A549 and primary human fibroblasts [51].

Several recombinants that carried reporter genes at site I of the genome (Fig. 1) were used to investigate the host range of OAdV7 [29, 44, 50, 76, 129]. These studies showed that OAdV7 can infect, but not replicate in a variety of human cell types, including breast (MCF7, T47D2) and prostate cancer (PC3), liver carcinoma (HepG2), and retinal (911), foreskin (HFF), and lung (MRC5) fibroblasts [76]. Reporter gene expression increased proportionally with the m.o.i. Monkey (COS) and mouse prostate (RM1) cells were also infected efficiently *in vitro* [50 and unpublished results].

Considering the quite broad host range of OAdV7, it will be of considerable interest to identify the receptor(s) that mediates infection. In principle it is also possible to redirect the vector via an alternative receptor as was done for

HAdVs [36, 37]. It was shown [29] that the cell-binding domain of OAdV7 fiber protein could be replaced with the equivalent binding domain from HAdV5. This was the only change in the viral capsid but it profoundly altered the cell tropism of OAdV7, apparently independent of any integrin/penton RGD interaction, since this motif is absent from OAdV7 [22]. Although the hybrid virus grew less well, this result confirmed that the two viruses use distinct receptors and demonstrated that targeting of xenogenic viruses may be possible.

2. Abortive Replication *in Vitro*

Abortive replication of xenogenic adenoviruses probably reflects viral promoter function in human cell types. The function of early and late BAdV3 promoters in human cells was examined by RT PCR and Southern blotting [121]. In A549 and 293 cells E1A transcripts were detectable for at least 5 days. At very high m.o.i. hexon mRNA was detectable at day 3 in primary human muscle, MRC5 human lung fibroblasts, and nasal septum epithelial cells. It was also shown that CAdV2 replicated to a limited extent in some human cells, as judged by higher virus output compared with input and some expression of capsid proteins. However, this was observed only at the first passage [121]. For human cells infected with OAdV7 at m.o.i. 20 pfu/cell the situation was polarized, depending on the cell type. On the one hand, in MRC5 cells, all early promoters in the genome that were examined were active, as monitored by RT PCR amplification of selected transcripts. On the other hand, in HepG2 liver carcinoma cells, none of the early promoters had detectable activity. In most other cell types, e.g., MCF7 and T47D2 breast cancer and PC3 prostate cells, some promoters, typically E2, were active. Interestingly, in all human cell types tested, and even when the early promoters were active, transcripts from the OAdV7 major late promoter (MLP) could not be detected [72, 76]. This may be related to key events that occur in the transition from early to late protein synthesis. For HAdV2, accumulation of early gene products is not sufficient for MLP activity. DNA replication is also required for late gene expression. High-level transcription from the MLP is further dependent on a *cis*-acting change in the viral chromatin [145]. In addition, HAdV2 MLP activity is stimulated by *trans*-activating factors DBP and IVa_2 [146–148]. At a gross level there is little or no DNA replication in OAdV7 infected human, compared with permissive ovine cells. However, the OAdV7 E2 promoter was active in several human cell types and large amounts of DBP transcript (and presumably, transcripts for DNA polymerase and Terminal protein) were produced [76]. Cellular factors also cooperate with viral proteins during genome replication (reviewed in [2]). The apparent absence of DNA replication may be due to the incompatibility of one or more human cell factors with binding sites on the OAdV7 ITR sequences or with other viral proteins involved in the process. There are significant differences

in putative binding sites for transcription factors between the ITRs of human and xenogenic viruses [149]. The inactivity of the OAdV7 MLP could further be due to a missing *trans*-activating factor, such as IVa_2, whose expression in human cells has not been investigated. Such abortive replication makes it unlikely that conditionally replication-competent vectors [150, 151] based on xenogenic vectors will be developed in the near future.

3. Biodistribution and Persistence *in Vivo*

Few studies on the biodistribution and persistence of xenogenic AdVs *in vivo* have been reported, but some have been carried out with MAdV1 and OAdV7. In the homologous situation, mice were injected intraperitoneally (ip) or intranasally with 10^3 pfu of MAdV1 and the localization of virus was monitored histologically during acute infection [152]. Endothelial cells of the brain and spinal cord showed extensive evidence of infection. Endothelial cells in lungs, kidneys, and other organs gave a positive signal, indicating a widespread involvement of the systemic circulation. Some lymphoid tissues were also positive. In experiments that examined persistence of OAdV7 it was found that 5×10^9 pfu of a recombinant OAdV7 vector injected intravenously (iv) into SCID mice produced hAAT expression that persisted for at least 60 days. However, the same vector dose in BALB/c mice was cleared by 20 days. Thus, the vector did not persist in the normal host and a substantial dose of virus (2×10^{11} particles) did not cause significant toxicity in normal or immunocompromised animals.

The distinct nature of the OAdV7 receptor was reflected in the biodistribution of OAdV7 following iv or ip administration of the vector to mice. OAdV7 was evenly distributed between liver, heart, spleen, and kidney [44], whereas HAdV5 vectors given iv concentrated predominantly in the liver [153]. OAdV7 given via the intraportal vein led to a greater accumulation of vector in the liver, but the vector was still found in all tissues examined [50]. In addition, when virus was injected directly into mouse skeletal muscle, cells were transduced and high levels of hAAT reporter protein were secreted *in vivo* [129]. By judicious adjustment of the first dose of vector it was shown that a second dose that resulted in substantial reporter gene expression could be given, raising the prospect that the vector may be suitable for prime/boost vaccination strategies. The vector was not detected in liver and spleen, indicating that it did not spread via the circulation. Expression, however, was transient and the vector DNA had essentially disappeared by day 14. Clearance occurred in the absence of detectable OAdV7 gene expression as assayed by RT PCR. As proposed for HAdV5 vectors [154] clearance may occur via presentation of antigen using an MHC class I independent mechanism.

Experiments utilizing HAdV5 and OAdV7 recombinants demonstrated a perceived advantage of xenogenic AdV, showing that OAdV7 could deliver a reporter gene *in vivo* in the face of preexisting antibodies against human

HAdV5 [44]. This result was encouraging from a clinical viewpoint and should be mimicked by other xenogenic AdV. It may be possible eventually to use different vectors in tandem to deliver multiple doses of the same gene [155].

C. Gene Therapy Studies

To date no gene therapy applications have been reported for xenogenic AdV. However, work is in progress in this laboratory to assess OAdV7 as a gene delivery vector for prostate cancer. The strategy is based on gene-directed enzyme prodrug therapy (GDEPT). This is a two-component cell killing system: a gene that encodes an enzyme not present in mammalian cells and a nontoxic prodrug that is converted to a toxic product by cells that produce the enzyme. Although there are several GDEPT systems [156], in this case purine nucleoside phosphorylase (PNP), an *E. coli* enzyme, and the prodrug fludarabine are being used [157]. OAdV7 vectors that express the PNP gene under the control of the constitutive RSV, or a tissue-specific prostate promoter, were constructed and tested for cell killing *in vitro* and in mouse models of prostate cancer. Viruses were injected directly into human PC3 or LN3 tumors grown subcutaneously in nude mice or into mouse RM1 tumors grown subcutaneously (sc) in immunocompetent animals. Prodrug was given systemically [158 and Voeks *et al* (in preparation)]. Evidence of tumor shrinkage and prolongation of mouse survival indicate that this vector and GDEPT system has potential for prostate cancer therapy.

This work has also highlighted other important issues that must be addressed for OAdV7, and for xenogenic vectors in general, if they are to be developed for clinical application. These especially include biosafety and vector growth, purification, and scaleup.

V. Biosafety

Most work with xenogenic vectors is still firmly based in the laboratory and while this is appropriate to demonstrate the utility of a vector the amount of work required for eventual exploitation of a vector in the clinic should not be underestimated.

A. Complementation and Recombination

Although the xenogenic AdV undergo abortive replication in human cells, one hypothetical situation concerning the clinical application of these vectors is their potential for interaction with opportunistic, replication-competent human adenoviruses in a patient. This may involve complementation of a replication-deficient virus or recombination between genomes to create a hybrid with

undesirable properties. *A priori*, such events seem more likely to occur between viruses that are closely related, particularly if they share a common receptor to facilitate coinfection. Evidence was sought for interaction between HAdV5 and CAdV2. However, coinfection of HeLa or A549 cells with CAdV2 (m.o.i. 10) and HAdV5 (m.o.i. 2) had no effect on the production of CAdV2 over five passages, compared with CAdV2 infection alone. DNA extracted from the cells was also digested and analyzed by Southern hybridization using a whole genome CAdV2 probe to track the DNA and look for the appearance of hybrid genomes. In coinfected HeLa and A549 cells CAdV2 DNA disappeared after one to three passages. HAdV5 DNA became visible by passage four and its restriction enzyme profile was identical to HAdV5 alone. No CAdV2 sequences were detected in these samples by hybridization [121].

Similar experiments have been done to determine whether any productive interaction occurred between OAdV7 and HAdV5, a typical human adenovirus. No complementation of OAdV7 replication was detected in the presence of wild-type HAdV5 in MCF 7 cells, although both viruses infect these cells [76] and HAdV5 replicated with high efficiency. Similarly, when DNA from several passages of cells that were coinfected by OAdV7 and HAdV5 was analyzed by Southern blot using whole genome OAdV7 or HAdV5 probes, no hybrid genomes were detected [158a]. Considering the differences in genome structure between the two viruses (Fig. 2), the apparent lack of viable hybrid

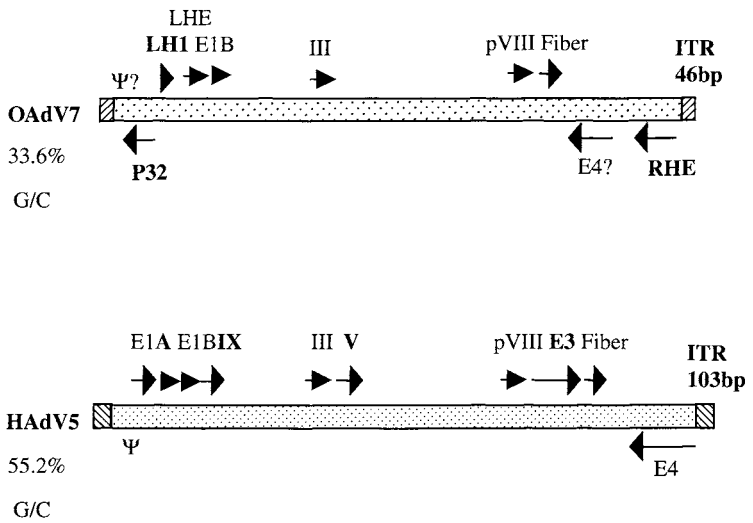

Figure 2 Difference map between HAdV2/5 and OAdV7. The stippled rectangles indicate the genomes with distinct G/C content, striped boxes at each end show the ITRs of different length, and sequence and ORFs with bold type are unique. The packaging signal is shown in (Ψ).

virus formation and the absence of complementation was not surprising. First, the G/C content of the two genomes is vastly different, indicating low nucleotide sequence homology. Next, the ITRs of each genome differ in length and sequence, suggesting that neither would be compatible with the DNA replication machinery of the other. Third, each virus has a distinct complement of capsid proteins, including unique proteins and distinct fibers as well as non-structural genes (Fig. 1). In addition, the packaging signals for each genome are likely to be incompatible. Thus, vectors such as OAdV7 may offer a greater margin of safety over those that are more closely related to HAdVs, such as SAdVs, with respect to potential for unwanted interactions. It is significant, therefore, that no human atadenoviruses have yet been described.

B. Oncogenes in Viral and Cellular DNA

As discussed above, replication-deficient E1-deleted vectors are rescued and propagated in continuous cell lines that were derived from primary cell lines by transformation with adenovirus E1A/B genes [42, 43, 114, 115]. While this is an advantage for cell growth and virus production it is a disadvantage for downstream processing and purification. Regulatory agencies impose strict limits on the permissible levels of contaminating DNA (10 ng/dose) in purified vector preparations [159]. A rigorous purification process is therefore required to remove potentially oncogenic DNA. Thus, an advantage of OAdV7 vectors is that they grow in a primary fetal ovine lung cell line. The trade-off is that the cells grow more slowly and have a life span of 50 to 70 doublings [122].

Oncogenes must also be removed from the vector genome. This may be more straightforward for the mastadenoviruses, where precedents exist from HAdV2/5 studies, but within this genus some viruses are more oncogenic than others [2, 65] and some ORFs exhibit unexpected transforming properties [160–162]. Progress toward oncogene identification in FAdV1 has also been made [73, 111], but others may exist. Ultimately, the regulatory authorities will require tests to be conducted on the residual oncogenicity of xenogenic vectors prior to clinical application. The apparent lack of transforming ability of OAdV7 in systems that have been used as a benchmark for such assays was therefore encouraging [98].

C. Virus/Cell Interactions

Adenoviruses undergo a lytic infection cycle in permissive cells. The mechanism behind cell lysis is not well defined in all cases but for HAdV5 it is due to the production of a "death protein" late in the infectious cycle [163]. Other mechanisms that may be involved in selective killing of tumor cells are being investigated [164]. These observations highlight the potential for interactions between a virus and a cell that may be undesirable in the context of extended gene expression or from a biosafety perspective.

Despite the inactivity of its MLP, in some cell types, typified by MRC5 lung fibroblasts, OAdV7 produced an apparent cytopathic effect (CPE) that was limited by the m.o.i. CPE was not due to viral replication because virus passaged twice on MRC5 cells failed to produce CPE in permissive CSL503 cells [76]. Thus, the effect is likely to involve an early gene product. This is currently under investigation. In this regard it is intriguing that the induction of rapid cell death following infection by certain HAdVs appears to be due to an interaction between p53 and the E1B 55-kDa product [164]. The response was abrogated by the absence of either protein due to mutation or lack of expression. Given the many genes of unknown function that exist in the expanding range of xenogenic AdVs the potential to discover other unwanted interactions exists. It may prove necessary to engineer vectors to remove deleterious genes and to grow them in complementing cell lines, but that raises complementation risks.

D. Replication Competent Viruses

A significant problem with the production of HAdV5 vectors has been the emergence of replication-competent viruses from cells that were designed to prevent their formation. Sequence overlap between the viral vector and integrated genes and subsequent recombination between them has generally been the cause [165]. Thus, PERC6 cells and matching vectors in which sequence overlaps were eliminated were specifically designed to overcome the problem [166]. An advantage offered by the xenogenic vectors is that all of them are replication-deficient in all human cell lines that have been tested. Additional work with particular vectors and cell types to understand the molecular basis for abortive replication would be very helpful in assessing the safety of new vectors.

VI. Vector Production and Purification

For vector production at the laboratory level the availability of a cell line or egg system [23, 48] for virus rescue and propagation is sufficient. Virus can be purified using methods based on cell lysis and CsCl centrifugation similar to those described for HAdV5 [10, 15, 43, 49, 137]. However, increasing success with a vector brings increasingly stringent requirements as work proceeds toward production for veterinary applications or a clinical trial. Strategic decisions taken early to facilitate subsequent steps in vector development and exploitation could save substantial time and effort later on. A key requirement for vector production is the availability of a cell line that, having been expanded and laid down as master and working cell banks (MCB/WCB), is tested and shown to be free of adventitious agents. Attention to detail in the creation and

documentation of such a cell line would pay dividends in the long term. A master virus seed stock also needs to be established. This dictates that the viral genome, including the transgene, must be stable upon serial passage such that biological activity and potency are maintained. This stock must also be free of other agents.

The issue of vector yield from the WCB should also be considered. For veterinary applications where the vector may be replication competent in the host, low yields may be less important. However, if gene therapy is being considered as an application a purified virus yield of $>10^4$ particles per cell is probably required for cost effective production of a vector.

For clinical applications in particular, a robust scheme for vector purification is required. While this might involve CsCl gradient centrifugation to produce quantities of vector for preclinical and perhaps phase I studies, such methodology is unlikely to be appropriate for producing larger amounts of vector. Methods involving chromatography may be more advantageous [167]. It is recognized that the above provides a very brief summary of issues that might be substantial for particular vector systems. However, the intention is to alert the reader contemplating the use of a new vector system to the many challenges that lie ahead in the process of chaperoning it through production and regulatory processes. The correct strategic decisions taken early can facilitate subsequent steps in vector development and exploitation.

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CHAPTER 17

Hybrid Adenoviral Vectors

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I. Introduction

The characterization of disease at the genetic level facilitates potential genotypic and/or phenotypic correction by gene therapy. Although the concept of gene therapy has been extensively established over the past two decades, the development of effective clinical protocols to facilitate efficacious reversal of disease has proven highly problematic. The development of an effective gene delivery system to the site of therapeutic significance has proven to be the major hurdle to the advancement of gene therapies. Many questions currently remain unanswered and these raise major debates over the best vector systems to treat a specific clinical disorder, and, at a more fundamental level, the choice of gene to be applied. The ultimate goal of a gene therapy protocol is efficient targeted delivery of a therapeutic transgene, whose expression can be sufficiently regulated, in a defective tissue. Vector delivery would ideally involve a single, lifetime treatment by a simple, noninvasive, and safe protocol, which can be incorporated into clinical practice. The vast array of diseases, for which gene therapy presents clinical promise, demands a multitude of different requirements for a vector system to meet.

Ideologies for gene therapy vectors will differ considerably among different disorders. The treatment of severely disabling genetic disorders such as Duchenne muscular dystrophy would require lifelong genetic complementation of the defective gene in an immense amount of both skeletal and smooth muscular tissues, as well as brain tissues to correct cognitive functions. Whereas somatic gene therapy for hemophilia B holds out greater potential for treatment; only a few percent of normal, reversed-phenotype cells would be sufficient to provide a constant level of factor IX in plasma, offering patients significant clinical improvements. In contrast to the aim of preservation of host

Table I
Comparison of the Ideologies of a Gene Therapy Vector for Genetic Disorders and Cancer

Genetic disorder
Aim: Cell preservation
Targeting diseased tissues
Efficient transduction of affected cells
Therapeutic levels of transgene expression
Adequate maintenance of gene expression levels
Long term stable transgene expression
Minimal vector toxicity
Cancer
Aim: Cell eradication
Targeting diseased tissues
Efficient transduction of tumor cells
Therapeutic levels of transgene expression
Transient vector expression for tumor clearance
Vector toxicity—danger signals attack tumor cells

physiology for inherited disorders, gene therapy for cancer focuses on efficient cell killing (Table I). Hence, genetic cancer therapies require different vector functions, requiring initial high local transduction of primary tumor masses to effect clinical removal, followed by subsequent systemic vector surveillance to eliminate metastatic disease. In essence, ideological concepts are rarely fully achieved and the current minimal aim of gene therapy is reversal of clinical phenotypes to the extent of easy maintenance, facilitating improvements in standards of life for patients.

Despite the development of increasingly complex nonviral gene delivery systems, it is virally derived vector systems which still offer most promise to the clinic. Viruses have throughout evolution developed highly skilled methods of entering cells, evading the host immune defense, and delivering their viral payloads. Hence, phenomenal amounts of research have been directed at harnessing the finely tuned transduction functions and obligate parasite lifestyles of viruses. A plethora of genetically modified viral vector systems has now been reported, all ingeniously subverting the parasitic viral life cycles for the presentation of therapeutic transgenes aimed at reversal of disease phenotype. The development of viruses as clinical vectors will revolutionize the medical world, providing an invaluable new tool for the treatment of disease. Our present understanding of the molecular genetics of many viruses renders possible their manipulation as cloning vectors for gene transfer both in cell culture and directly in patients. As the major objective is usually long-lasting

gene transfer, deletion of the key regulatory viral genes was deemed essential to manipulate the genetic program of the virus and to ensure that infection of the target cell does not lead to cell death. Conversely, for the treatment of cancer, more recent strategies have reversed this thinking and selectively retain the replicative functions of the virus to enhance tumor cell killing. Viruses have thus been designed with predictable biological properties, retaining the beneficial targeting/infectivity properties, while dissociating them from the major virulent determinants of pathology in normal tissues.

Currently, four classes of viral vector have presented most promise as gene delivery vehicles: retroviruses (RVs), adenoviruses (Ads), adeno-associated viruses (AAVs) and herpes-simplex-based viruses (HSVs). Although retroviruses embodied the pioneering vector when the concept of gene therapy began to emerge as a reality in the early 1980s, adenoviruses have since become the major vector choice in the clinic. More recent advances in the production technologies of HSV- and AAV-based vectors have greatly increased their clinical potentials. Additionally, the lentiviral (LV) subclass of retroviral vectors, with distinct biological properties, has emerged with great potential and has gained individual acclaim from the rest of the group. The major properties of each viral vector are presented in Table II, as well as being briefly discussed below.

A. Retroviral Vectors

Retroviruses are enveloped RNA viruses, whose genomes consist of three core genetic units termed *gag*, *pol*, and *env* (Fig. 1A) [1]. Retroviruses stably transduce cells by integrating their genomes into the host-cell chromosomes and subsequently release progeny virus by continuously budding viral particles from the cell membrane. The *gag* gene encodes proteins which form the viral core, while the *pol* gene encodes reverse transcriptase (RT), the viral integrase (INT), and a viral protease which acts on the *gag* gene products. The *env* gene encodes the glycosylated envelope proteins that determine the tropism of the virus. These genetic elements are flanked by the long-terminal repeat (LTR) sequences and a packaging signal (ψ) which directs the assembly of the genome into the viral particles (Fig. 1A) [1]. The LTR sequences contain the *cis*-acting elements required to regulate viral genome replication and transcription and mediate stable integration into the host genome [1]. Retroviral vectors have been principally based on the well-studied Moloney murine leukaemia virus (MoMuLV). Recombinant MoMuLV vectors are engineered by replacing the *gag*, *pol*, and *env*-coding units with a transgene of interest, while retaining the LTRs and packaging *cis*-acting sequences. Producer cell lines stably transformed with independent *gag/pol* and *env* expression cassettes are used to fully complement the viral polypeptides for packaging of the vector proviruses [2, 3]. Hence by transfecting these packaging cell lines with plasmid-based LTR-flanked retroviral cassettes, retroviral particles efficiently bud from the host

Table II
Properties of the Main Gene Therapy Viral Vectors

	Adenovirus Ad2/Ad5	Retrovirus MoMuLV	AAV AAV2	HSV HSV-1	Lentivirus HIV-1
Nuclear status	Episomal	Integrating	Episomal/integrating	Episomal	Integrating
Genome	dsDNA	RNA	ssDNA	dsDNA	RNA
Structure	Encapsidated	Enveloped	Encapsidated	Enveloped/ encapsidated	Enveloped
Genome size	36 kb	10 kb	4.7 kb	152 kb	10 kb
Nuclear targeting	Yes	No	Yes	Yes	Yes
Titers (iv/ml)	10^{11}	10^8–10^9	10^{11}	10^8–10^9	10^8–10^9
Insert capacity	7–8 kb (E1/3-) to 36 kb HD	9–10 kb	4.5–5 kb	25–150 kb HD	9–10 kb
Immunogenicity	High	Low	Low	Low (HD)	Low
Genetics	Early: E1–E4- transduction Late: Multiple structural	*Gag*: Capsid *Pol*: RT, INT, PR *Env*: Envelope proteins	*Rep*: Replication/Integration *Cap*: capsid	Multiple	*Gag*, *Pol*, and *Env* as retrovirus plus accessory genes

Pros	• High transduction efficiencies • Stable particles • High titers • Broad host range • Infect dividing/nondividing cells	• Low IR • Integrating • Potential long-term expression	• High transduction efficiencies • Stable particles • High titers • Targeted integration (+*Rep*) • Broad host range • Infect dividing/nondividing cells • Limited IR	• Wide tropism • Large insert capacities • Minimal IR to virion particles • High tropism for neuronal tissues • Large genome copy number in amplicon concatemers	• Integrating • Nuclear localization • Easily pseudotyped with MoMuLV
Cons	• Transient • High IR to viral proteins • Humoral IR • Cytotoxic IR • Innate IR • Leaky expression of viral genes (1st generation) • Infect dividing/nondividing cells • RCA	• Relatively low titers • Low transduction efficiencies *in vivo* • Virions relatively unstable • Complement-inactivation • No nuclear targeting • Transduction dep. on cell division • Random integration • Endogenous RVs recombination	• Low insert capacity • *Rep* toxicity • Helper contamination	• Transient • Low titers • No stable maintenance mechanism • Helper contamination • High IR • Unpredictable biology of concatemers in amplicon	• Transient • Comparatively low titers • Significant pathology of native virus • RCL • Limited host range • Virions relatively unstable • Endogenous RV recombination

Note. IR, immune response; RCA, replication-competent adenoviruses; RCL, replication-competent lentiviruses.

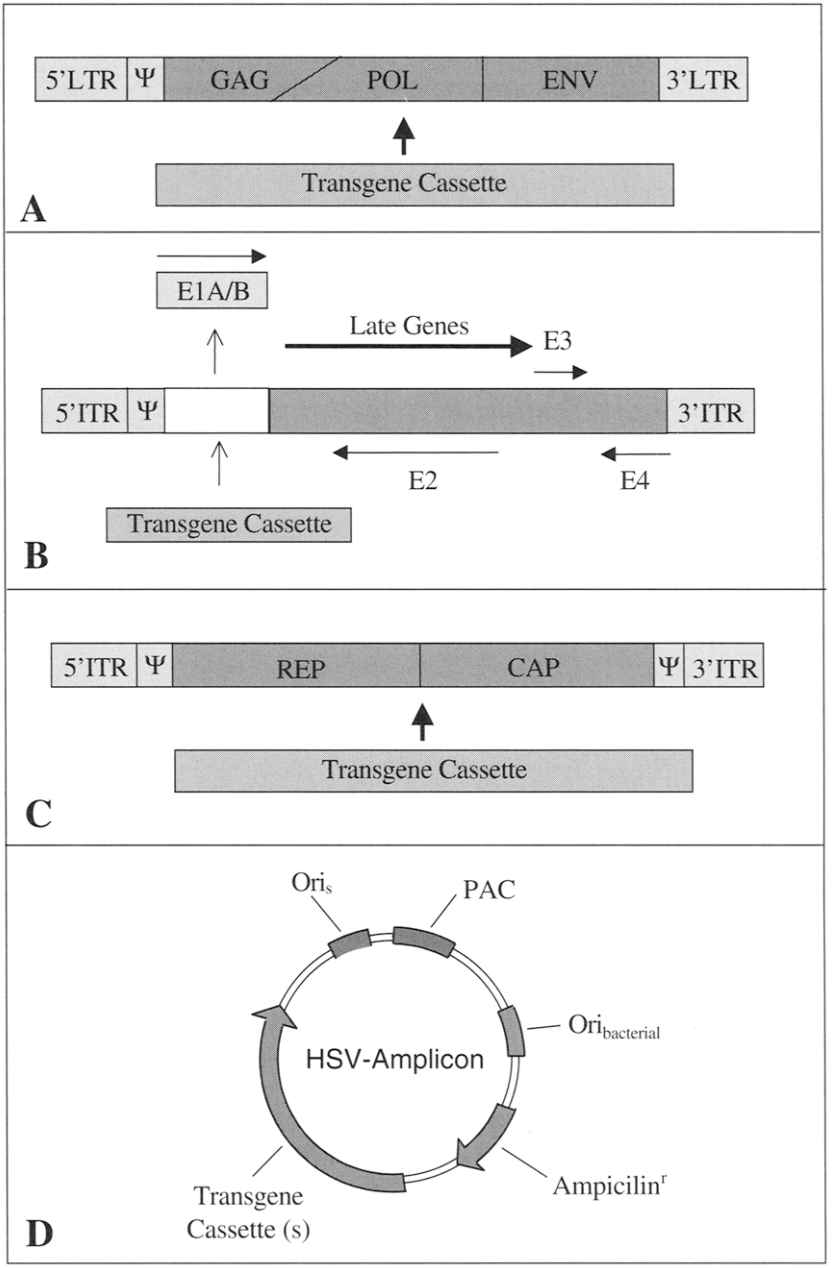

Figure 1 Vector genome structures. The wild-type viral genomes and the strategy of transgene substitution are presented for (A) retrovirus, (B) adenovirus, and (C) adeno-associated virus. (D) The minimal structure of the HSV-1-based amplicon vector.

cells containing the recombinant retroviral genome. These retroviral particles are capable of infecting cells and directing the expression of the transgene of interest, but cannot replicate or generate progeny virus.

B. Adenoviral Vectors

Adenoviral particles consist of lipid-free "spiked" regular icosahedra of 60–90 nm in diameter, consisting of three main structural proteins, termed hexon, penton base, and fiber [4]. The genome consists of a double-stranded linear DNA molecule of approximately 36 kb in length, functionally divided into two major noncontiguous overlapping regions, early and late, defined by the onset of transcription after infection (Fig. 1B) [5]. There are five distinct early regions (E1A, E1B, E2, E3, and E4) and one major late region (MLR) with five principal coding units (L1 to L5), plus several minor intermediate and/or late regions. At the extremities of the viral chromosome are the inverted terminal repeats (ITRs) and the encapsidation signal (ψ), encompassing the *cis* elements necessary for viral DNA replication and packaging [5].

Recombinant Ad vectors are constructed by deleting the essential early genes *E1A* and *E1B*, whose expression enables transformation of the host cell and *trans*-activates expression of the other early viral genes, as well as some host factors [6]. Transgenes are inserted into this deleted region (Fig. 1B) and can be assembled into infectious adenoviral particles in cell lines which *trans*-complement the E1A/B functions [7]. Additional deletions in the nonessential E3 region are also often performed to increase cloning capacities [8]. Thus infection of cells with the Ad vector enables expression of the transgene in the absence of expression of viral proteins. Further incapacitation of the Ad vector genomes, limiting leaky expression of viral proteins by further deletions in the E2 or E4, has also proved advantageous, further enhancing the cloning capacities, but requiring further complementation functions in packaging cell lines [9–12]. The development of so-called "gutless" or helper-dependent (HD) adenoviral vectors has also greatly expanded the potential of Ad vectors. These vectors retain just the terminal ITRs and ψ required for replication and packaging of adenoviral genomes, greatly increasing the cloning capacity [13].

C. Adeno-associated Viruses

Adeno-associated viruses have recently become attractive candidates for gene transfer. AAVs belong to the family parvoviridae and consist of nonenveloped icosahedral virions of 18–26 nm diameter, with linear single-stranded DNA genomes of 4680 nucleotides for the most characterized AAV2 strain [14, 15]. The genome consists of two coding regions, *cap* and *rep*, which are flanked by ITRs and encapsidation signals (ψ) at either end of the genome (Fig. 1C). The *cap* gene encodes the capsid (coat) proteins and *rep* encodes proteins involved in replication and integration functions [15].

After infection, AAV genomes can persist extrachromosomally in an episomal form [16, 17] or integrate into the cellular genome [18, 19]. AAV has been demonstrated to preferentially integrate into human chromosome 19 at site *q*13.4 (AAVS1), directed by the *rep* genes, facilitating latent infection for the life of the cell [20]. AAV is, however, naturally replication-incompetent and requires additional genes from a helper virus infection, which in nature is generally complemented by Ad or HSV coinfection [21]. AAV-based vectors generally involve replacement of the *rep* and *cap* genes with a transgene of interest (Fig. 1C), retaining the terminal repeats and packaging sequences essential to direct replication and packaging of the genome [15]. These AAV vectors can be packaged into infectious AAV particles upon complementation of the *rep/cap* genes and Ad/HSV helper functions in *trans*. Deletion of the *rep* genes, however, eliminates targeted integration of the AAV cassettes at AAVS1. The nonpathogenic nature of AAV, having not been associated with any disease or tumor in humans, makes it a potentially powerful clinical vector.

D. Herpes Simplex Viruses

Herpes simplex virus belongs to the herpesvirus family, a diverse family of large DNA viruses, all of which have the potential to establish lifelong latent infection [22, 23]. HSV consists of 110-nm-diameter particles comprising an icosahedral nucleocapsid, surrounded by a protein matrix, the tegument, which in turn is surrounded by a glycolipid-containing envelope [24]. The HSV-1 genome consists of a giant linear double-stranded DNA molecule of 152 kb encoding 81 known genes, 38 of which are essential for virus production *in vitro* [24]. First-generation HSV-based vectors involve replacement of one or more of the seven immediate-early (IE) genes whose functions are *trans*-complemented by packaging cell lines [24]. Second-generation HSV-amplicon vectors consist of plasmids containing just the HSV-1 origin of replication (Ori_S) for replication in packaging cell lines by the rolling circle mechanism, and the cleavage/packaging signal (pac) (Fig. 1D). These amplicon vectors can accommodate inserts of up to 15 kb, enabling the assembly of concatemer structures of up to 10 genomes, reconstituting the packaging size of 150 kb [25]. The future construction of "full-size" gutless HSV vectors could accommodate up to 150 kb of insert DNA [26].

E. Lentiviral Vectors

Lentiviruses are a subclass of retroviral vectors which have become infamous in world affairs by the HIV family members. LV vectors are characterized by the presence of additional accessory genes to the *gag/pol/env*-based genomes [27]. These accessory genes extend the functions of the viruses, with

the major gene therapy focus being on their ability to infect nondividing as well dividing cells, in distinct contrast to other retroviral family members such as MoMuLV. These karyotropic properties of lentiviruses provide a promising tool to direct retrovirus-mediated gene therapies to nondividing cells [28]. LV vectors are constructed by an analogous mechanism to conventional MoMuLV vectors.

F. The Choice of Gene Therapy Vector

No single vector system can presently provide the necessary flexibility for all the possible clinical applications of gene therapy. Vast variabilities exist in vector host range and uptake potentials for the many tissues of the human body, which together with the many biological barriers to reaching the target tissues make a universal vector unlikely. Thus disease-specific gene targeting strategies are likely to be required, involving the development of multiple gene delivery systems. Hence the technology of gene therapy stands to benefit from the vast range of clinical vectors being designed, each system having distinct properties which can complement each other in the clinic.

Extensive research has focused on the potential of adenoviruses as transducing viruses for use in gene therapy. The translation of laboratory-derived viral vectors as practical pharmaceutical tools is a major determinant of gene therapy interest in the clinic. In essence, the ease of generating Ad vectors, the efficiency of purification, and the superior titers which can be obtained ($>10^{11}$ pfu/mL) have made Ad the vector of choice for many applications of *in vivo* gene therapy [4]. The rapid technical advances in the construction and purification of alternative viral vector systems has, however, expanded clinical interests. The vastly improved techniques of helper-free AAV production have significantly increased the potential of these vectors. Titers of AAV vectors equivalent to those of Ad vectors are now routinely achievable, which are free of the once problematic helper virus contamination [29]. The production procedures, however, are still relatively laborious and problematic. The comparatively low titers of the MoMuLV-, HSV-, and LV-based vectors, generally greater than 2 logs lower stable titers, limit the effectiveness of these vector systems especially upon translation to the clinic. However, current immune system barriers preclude the beneficial attributes of administration of Ad vectors at their maximal titers, with significant safety concerns apparent with the maximal doses of Ad vectors in the clinic [30].

The generation of large-scale, high-titer vector preparations with stable shelf lives is essential for clinical applications. The stable pharmaceutical properties of Ad virions, as well as the similarly encapsidated AAV and HSV virions, present significant advantages over the much less stable enveloped retrovirus-based vectors. The integrative functions of retroviral vectors, however, confer on them the potential of long-term stable expression, fulfilling an additional

highly desirable vector property. These integrative functions, together with rapidly advancing methods of enhancing viral titers using concentration procedures [31], maintain major clinical interest in retroviral vectors. The integrative functions of AAV vectors are also highly desirable, specifically the chromosomal targeting mechanism in the presence of the Rep protein [32]. The absence of any cellular retention mechanisms for Ad and HSV vectors presents a distinct disadvantage to many gene therapy applications. In the context of tumor eradication, however, high-titer vector transduction is unlikely to require long-term maintenance of vectors.

In deciding the most appropriate vector for treatment of a clinical disorder, the main selection criterion for vector choice comes in the ability of a specific vector to efficiently transduce the target tissue. Ad vectors have a wide distribution of their target receptors dispersed throughout the body tissues. AAV and HSV have similar diverse tropism to most cells in the human body, with HSV-1 vectors having a major selective tropism for neuronal tissues. MoMuLV viruses are, however, severely limited by their dependence on host cell mitosis to enable stable transduction of a cell, limiting their efficacy in quiescent cell populations [33]. These cell cycle restrictions are not apparent with the LV subclass of retroviral vectors, which possess the additional nuclear targeting functions [34]. The additional nuclear targeting property of LV vectors, together with the integration functions, has significantly raised the clinical interest in respect of gene therapy. Additionally, the ability of MoMuLV vectors to infect only dividing cells can be deemed an advantage in targeting actively dividing tumor cells which are surrounded by nondividing normal tissues. The ideal vector system is thus very much dependent on the diseased tissue to be treated.

The extent of genetic material that is required to be delivered to a specific tissue is also a major influence on the vector system. Ad vectors offer a wide range of insert potentials from 7 to 8-kb insert capacities for first-generation vectors and up to 36-kb inserts in the "gutless" HD vector system [6, 35]. Whereas the relatively small packageable genome sizes of retroviral vectors (~8 kb), but more significantly of AAV vectors (~4.5 kb), severely limit their applications to some gene therapy protocols [15], specifically where the delivery of multiple genes or the insertion of large regulatory elements is deemed essential. It is, however, the HSV-1-based vectors that offer the superior transgene delivery potentials with inserts of up to 150 kb feasible in a gutless vector [36]. Additionally, in the alternative HSV-1 amplicon vector system, as well as providing an insert capacity of up to 15 kb, the assembly of concatemers vastly increases the copy number of transgene cassettes being delivered to target cells [25].

The immune system is a perpetual barrier to viral transduction. The compromised state of many diseases would be severely stressed by further immunological effects/inflammation induced by a "therapeutic" vector

challenge. The exception again is cancer gene therapy where activation of local immune responses can be advantageous in tumor recognition and possibly aid in breaking immune tolerance [37]. Viral vectors are designed to exploit specific biological properties of viruses, such as recognition of cell receptors for entry and mechanisms of host genome integration, that have evolved over time in relationship with the host. The natural response of the host has, however, also developed to eliminate disease-inducing viral pathogens. Current strategies of viral vector design are working to engineer viruses with predictable biological properties, maintaining the biological advantages of the virus that have been selected by nature while reducing the immunogenicity of the viral components. The majority of Ad vector-derived immunogenicity was deemed to be due to the leaky expression of retained viral transcripts in the vector genome [30, 38]. For AAV and HSV amplicon vectors, contaminating helper virus was also deemed highly immunogenic. The more recent improvements in Ad vector design [9–12] and generation of "helper-free" packaging systems for AAV and HSV amplicon vectors [29, 39] has stunted this immunogenicity to some extent. However, the immune system still stands as a major barrier to gene-therapy efficacy. The mere physical presence of the virus can induce significant cytopathology. The current requirement of repeated administration to boost expression levels further augments the immune memory responses to the presence of the virus until eventual complete immunity is developed to the applied vector [40]. The power of the immune system is emphasized by practically 95% of Ad virions being eliminated by the natural nonspecific innate immune response on each administration [41].

G. How to Maintain Stable Transgene Expression

The transient natures of Ad and HSV-1 vectors, as well as the rapid loss of transgene expression upon stable integration of AAV and RV vectors due to nuclear effects on the transgene cassettes, have dramatically limited the efficacy of each vector system. Hence the question remains: how do we maintain stable transgene expression following recombinant viral vector transduction? One solution may come from looking closer at the wild-type mechanisms of preservation evolved by the parental viruses.

Viruses have developed diverse mechanisms of self-preservation and maintenance to enable them to infect cells and direct self-replication and propagation. Mechanisms of maintenance vary according to the life cycle of the virus. Viruses such as retroviruses have developed life cycles that live in harmony with the host cell. They utilize the host cellular machinery to enable continuous shedding of the virus and thus require stable preservation of the viral genetic material. Retroviruses facilitate this function by stable integration into the host genome, permitting continuous replication/maintenance of the viral genome in the context of host cell replication [1]. Conversely, lytic viruses

such as adenoviruses subvert the host's cellular functions solely for their own preservation. Infected cells become short-term factories of virus production, amassing viral particles until host cell saturation is achieved and cell lysis occurs in less than 36 h [5]. The short-term association of virus and host does not therefore necessitate mechanisms for long-term persistence of the viral genome. The Ad genome is thus maintained extrachromosomally with a very efficient mechanism of replication to enable large-scale genome packaging into the vast numbers of viral particles generated. Herpes viruses, such as HSV, Epstein–Barr virus (EBV), and cytomegalovirus (CMV), have developed more complex mechanisms of self-preservation [42]. Upon infection, a lysogenic life cycle enables the virus to live in harmony with the cell, maintaining the genome in an extrachromosomal state, where methylation and histone binding to the viral genome keep viral gene expression essentially quiescent [22]. The switch of the life cycle from the quiescent latent state to the major virulent lytic phase, upon signals of cell stress, rapidly reveals the viral presence. This terminal lytic stage of rapid viral genome reproduction and mass assembly of virions enables the virus to rapidly multiply and abandon the host. The AAV life cycle is a further intriguing evolutionary mechanism, being naturally dependent on helper Ad or HSV coinfection to effect lytic AAV virion assembly and viral progeny release. In the absence of such helper functions, AAV remains lysogenic by either stable integration into the host genome or independent episomal replication in the infected cell [14].

II. Hybrid Viral Vectors

The inadequacies of each viral vector system are illustrated in Table II. The negative attributes of one vector, however, generally emphasize the positive attributes of another. Thus most of the criteria defined for a hypothetical perfect gene therapy might actually be met by considering defined properties of the currently available vectors defined in Table II. Hence, although at present no individual virus system alone can meet all the criteria, current research is focusing on combining individual viral properties into single vector constructs, termed "hybrid" or "chimeric" vectors.

Adenoviral vectors are currently the major vector choice for a variety of clinical disorders, despite the limited efficacy due to the transient nature of the vector. Mechanisms of enhancing the pharmaceutical properties of Ad vectors are thus highly desirable. The incorporation of other viral vector functions that could enhance the duration of Ad-directed transgene expression and/or target the vectors to a specific disease tissue would be extremely beneficial. In essence, whether the aim is to kill or cure the target cell, a vector encompassing the advantageous properties of high titer, broad host range, and infectivity of an Ad vector, together with the low immunogenicity and potential for long-term

stable expression of a retrovirus, AAV, or EBV vector would be extremely useful for gene therapy for a wide range of genetic and acquired disorders. Hence the main focus of this chapter is to review the properties of other viral vectors which have been utilized to generate hybrid adenoviral vectors in the aim of enhancing vector efficacy in the clinic.

A. Are Hybrid Vectors Truly New Technology?

The formation of hybrid adenoviruses is not a new technology and has been extensively reported to occur naturally in nature. Adenoviral/simian virus 40 (SV40) hybrids have been documented to occur in nature [43, 44]. Although human adenoviruses do not normally replicate in primate cells, upon coinfection with SV40, Ad genomes acquired sequences from the SV40 genomes (large T antigen) which permitted replication and assembly of hybrid genomes into wild-type Ad capsid particles [43]. Additionally it may be that the helper-dependent AAV genome represents a segment of an extinct or undiscovered virus that was selected upon coinfection with an Ad or an HSV. Perhaps the parental virus was too virulent to coexist in a human host, thereby explaining the nonpathogenic nature of the dependovirus.

The development of hybrid viral vectors is fundamentally not a new technology in gene therapy. Since the dawn of gene therapy, scientists have utilized alternative *cis*-acting sequences from other viruses, specifically promoters and enhancers, to drive transgene expression. Most significantly, the cytomegalovirus (CMV) immediate-early promoter and enhancer has been utilized in almost every viral vector reported to date and is well characterized as an extremely strong constitutive promoter in most tissues [45, 46]. Other well utilized viral promoters have included the Rous sarcoma virus (RSV) LTR promoter, the SV40 early promoter, hepatitis B virus (HBV), and the EBV promoter [45, 46]. Additionally, application of the picornaviral functions of "cap-independent" initiation of translation has also been extensively exploited in viral vectors. These translational regulatory elements, termed internal ribosomal entry site (IRES) sequences, enable bicistronic expression from a single mRNA transcript [47]. The application of these elements greatly complemented the limited insert capacities of viral vectors, thereby negating the need for separate promoters to drive two transgene cassettes.

Retroviral vectors have been studied in hybrid vector systems since the early 1980s, "pseudotyping" them with functions from other retroviral vectors. Specifically heterotropic viral glycoproteins from other retroviral *env* genes have been stably incorporated into MoMuLV vector particles. The incorporation of vesicular somatic virus G (VSV-G) glycoprotein [48], gibbon ape leukemia virus (GALV) and HIV-1 glycoproteins [49] into murine leukemia virus particles has been reported. These hybrid MoMuLV virions attain the tropism of the pseudotyped env proteins, retargeting or broadening the host

range of the MoMuLV vector. Additionally, incorporation of VSV-G env has been demonstrated to increase the stability of the virions, enabling higher titer-yielding purification techniques to be applied [50, 51]. Hybrid retroviral vectors have also been constructed, incorporating different *cis*-acting elements contained in the U3 region of the LTR, which direct the transcriptional activity of the virus. Replacement of these U3 regulatory elements can impart tissue-specific transcriptional activity on the RV vector [52, 53]. Hence the concept of hybrid vectors is not a new technology, but the new strategies proposed could vastly expand the repertoire of viral vectors available to the clinic.

III. Hybrid Adenoviral Vector Systems

A number of hybrid adenoviral vector systems have been reported in the literature, combining the properties of RV, AAV, and EBV vectors, as well as elements of other Ad serotypes, to enhance the therapeutic efficacy of Ad vectors *in vivo*. The principal aim of these new hybrid vectors is to overcome the limitations of transient Ad vector retention in infected cells. In addition to the well-documented limitations of Ad vectors (Table II), some initially perceived advantageous properties of Ad vectors do actually limit their effectiveness toward therapy for some diseases. The broad host range of Ad vectors induces significant disadvantages when tissue targeting is required and compromises systemic administration. Additionally, the low pathogenicity of adenoviruses in humans has resulted in many serotypes, including the conventional vector strains of Ad2 and Ad5, being endemic. Hence a potent natural anti-adenoviral immunity is fashioned generally at a very early age. The highly immunogenic nature of the proteinous Ad virion further confounds the system, with a rapid and highly effective host humoral response being developed to the Ad vector. Research is thus being channelled into both retargeting Ad vectors to specific tissues and silencing the structural immune stimuli to facilitate enhanced Ad vector transduction.

A. Pseudotyping and Retargeting Adenoviral Vectors

As targeting and humoral immunity are connected in essence to the same surface moieties of the Ad particles, both disciplines are fundamentally interlinked. Methods applied to limit the humoral responses have focused on two main strategies: application of alternative "immune silent" Ad serotypes or display of alternative ligands on the surface of the virions, which is also the major strategy for retargeting the vector.

The use of alternative serotypes enables the consecutive application of immunologically distinct Ad particles, enabling avoidance of specific humoral responses to previously applied vectors [54, 55]. This system has presented some success *in vivo* [56], although the presence of cross-reacting antibodies

is problematic due to the evolutionary similarities of Ad serotypes. The application of alternative Ad serotypes with different surface markers also provides a mechanism of alternative targeting, as different serotypes possess tropism for different tissues in the human body. For instance, the conventional gene therapy subtypes Ad2 and Ad5 have natural tropism for the gut epithelial layer. Hence, in terms of gene therapy for cystic fibrosis, initial vectors proved disappointing due to their low infectivity of the airway epithelia. To overcome this restriction, Zabner and colleagues investigated other Ad serotypes for airway epithelia tropism [57]. A number of other Ad serotypes, specifically Ad17, were found to infect the airway epithelia with increased efficiency to wtAd2 [57]. They therefore proceeded to generate Ad2 hybrid vectors pseudotyped with the Ad17 fiber, where the endogenous Ad2 fiber gene was replaced with the Ad17 fiber gene. The resultant chimeric vector displayed increased efficiency of binding and gene transfer to well differentiated human epithelial cells. A similar study by Croyle and colleagues demonstrated that wild-type Ad41 had enhanced transduction properties in intestines compared to Ad5 [58]. These studies emphasize the potential of alternative Ad serotypes with tropism for different tissues in the human body. Pseudotyping also provides an invaluable mechanism of integrating alternative serotype fiber (and/or penton base) genes from other Ad serotypes into the currently well-researched Ad vectors, without having to reconstruct the vector backbones. The use of nonhuman adenoviruses as vectors for gene therapy is also under investigation, with bovine, ovine, canine, feline, and avian adenoviruses being researched [59–62]. As well as being potentially unexposed to the immune system, they may also have specific tropism for selective tissues in humans. The potential of pseudotyping nonhuman Ad vector components with conventional human Ad vectors is therefore of interest.

The use of targeted viral vectors to localize gene therapy to specific cell types introduces significant advances over vectors with conventional natural tropism. As well as the safety aspects of reduced immunogenicity and toxicity, the reduced uptake by nontargeted cell types may enable application of systemic delivery with feasible viral titers and loads. In order to retarget Ad vectors, first the natural tropism of the virus must be removed and, second, novel, tissue-specific ligands introduced [63]. Two main mechanisms have been used to retarget Ad vectors. First, the use of external molecules with affinities for both the Ad surface structural moieties as well as a cell-type-specific surface ligand. These bispecific molecules act as bridges between the virions and the cell. A neutralizing antibody or high-affinity peptide for the fiber or penton base can act as the Ad-binding moiety, which can be covalently linked to a high-affinity ligand for a tissue-specific receptor [63]. A drawback of the bridging molecule approach is that native receptor binding is never 100% blocked. To truly block native Ad binding to its cognitive receptor, removal of the intrinsic receptor binding domains is required.

A second approach involves creation of hybrid Ad vectors, pseudotyped with novel receptor recognition functions. Genetic modification of the Ad genome by incorporating targeting ligands inside the genome, while deleting or ablating sequences of the penton and fiber involved in receptor recognition, has been reported. High-affinity peptide motifs have been subsequently demonstrated to be functionally incorporated into Ad particles. These "proof-of-concept" studies focused on the incorporation of ligands without ablating natural receptor interactions and resulted in expanding the vector tropism, which has proved beneficial *in vivo* in transducing both vascular smooth muscle and some tumor types [64–66]. Future studies will focus on honing the targeting functions to specific cell types. High-affinity ligands have been stably inserted into the HI loop or on the C-terminus of the fiber or into the integrin-binding RGD domain of the penton base [63]. However, the size, location, and type of ligand to be inserted are currently under debate and remain to be determined. Wickham and colleagues demonstrated 10- to 1000-fold reductions in transduction of cells expressing the coxsackievirus and adenovirus receptor (CAR) with CAR-ablated vectors [63], the residual transduction being penton-base-mediated, emphasizing the requirement for additional ablation of penton base binding [63]. The further requirement of novel packaging cell lines to facilitate infection and propagation of the CAR/integrin-binding ablated particles also remains an issue.

B. Adenoviral/Retroviral Hybrid Vector Technologies

A hybrid vector system incorporating the advantageous long-term stable integrative functions of retroviral vectors into adenoviral vectors could provide a major clinical advancement to gene therapy. Hybrid vector systems are thus being investigated, incorporating retroviral components into the backbones of adenoviral vectors. Initial studies have focused on utilizing adenoviral vectors as directors of retroviral vector production, delivering the *gag*, *pol*, and *env* genes as well as retroviral LTR cassettes to cell populations both *in vitro* and *in vivo*.

Conventional retroviral packaging cell lines are stably transformed with *gag*, *pol*, and *env* functions and release retroviral particles upon plasmid transfection of a retroviral LTR transgene cassette [2]. High-titer retroviral stocks of greater than 10^7 infectious units (iu)/mL can now be obtained from conventional stable producer cell lines [3]. To achieve the highest vector titer, it is necessary to select clones of vector-transduced cells individually due to the varying titers of producer cell clones [67]. Direct injection of retroviral vectors *in vivo* has, however, yielded limited efficiencies due to the limited transducing titers and poor infectivity. Application of retroviral vectors in the clinic has thus focused on *ex vivo* protocols. This involves the removal of patient tissues, which can be cultured for a brief period in the laboratory, transduction with the RV vector, and reimplantation back into the patient. The *ex vivo* approach has

yielded some success, although the procedure is cumbersome and costly, and in most cases, it can only transduce a small fraction of the target cells [68, 69]. The establishment of retroviral producer cells *in situ* provides a further mechanism of enhancing the efficacy of retroviral gene therapy. Transient transfection of target cells *in vivo* with the retroviral vector and packaging plasmids, previously used to generate producer cell lines *in vitro*, by direct DNA injection has been reported [70]. Although stable integration of subsequently generated retroviral particle genomes could be detected, the efficiency was very low. The implantation of retroviral producer cell lines into patients has presented a far greater potential for the *in situ* production of retroviral vectors. Gene therapy using MoMuLV-based producer cells to treat brain tumors [71] has been carried out in a clinical trial, but no clear clinical benefit has been reported to date.

The infectivity of Ad vectors both *in vitro* and *in vivo* provides great potential in increasing the efficiencies of retroviral production technology. The group of David Curiel pioneered the development of hybrid retroviral/adenoviral vectors by using the infectivity of adenoviral vectors to efficiently deliver the requisite retroviral packaging and vector functions to target cells *in vivo*, thereby rendering them retroviral producer cells *in situ* (Fig. 2). The subsequent release of high local concentrations of retroviral particles *in situ* would enable stable transduction of neighboring tissues, for the transient period of adenovirus transduction. The Ad/RV hybrid system reported by Feng and colleagues utilized a two-adenovirus delivery strategy [72]. The first adenovirus contained an LTR-flanked retroviral vector cassette encompassing the GFP marker and neomycin resistance genes: Ad/RV-vector. The second adenovirus contained the replication-defective retroviral helper machinery, carrying the *gag*, *pol*, and *env* genes of MoMuLV: Ad-*gag/pol/env*. High-titer adenoviral vectors could be generated containing the RV cassettes, which could efficiently direct the *in vitro* packaging of RV particles at titers similar to conventional packaging cell lines [72, 67]. These studies clearly demonstrate the compatibility of both the adenoviral and the retroviral life cycles in the context of a hybrid vector configuration.

Upon infection of cells *in vitro* with the Ad/RV vector alone, high initial levels of GFP expression were observed but gradual loss of expression was documented over a period of 60 days as the nonintegrated adenovirus was lost from dividing target cells. Conversely, upon application of both adenoviruses to cells *in vitro*, GFP expression was persistent for extended periods of time. However, the persistent level of gene expression was reduced beyond the time at which expression could be solely attributed to the Ad/RV vector. The stable integration of the retroviral cassette in surrounding cells was believed to be responsible for this extended expression. The longer term GFP-expressing cells in cultures transduced with both Ad vectors, were present in clustered outgrowths, suggesting local retroviral spreading and/or clonal origin. Subsequent demonstration of proviral integration was confirmed by the

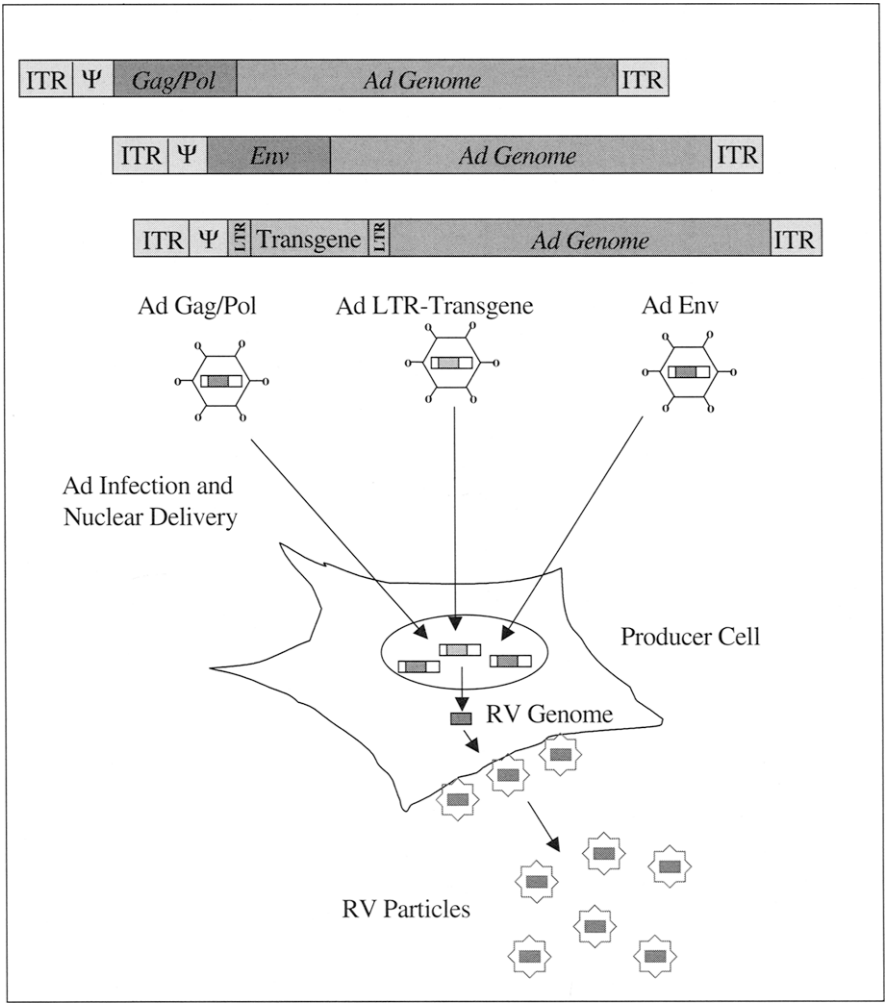

Figure 2 Hybrid-Ad/RV vector-mediated production of RV particles. Hybrid adenoviral vectors expressing the *gag/pol* and *env* RV genes either together or on split adenoviral constructs (as shown) are coinfected with the Ad-LTR transgene vector into cells *in vitro* or *in vivo*. Subsequent expression of the *gag/pol* and *env* genes in the cells establishes *in situ* retroviral producer cells which direct the packaging of the expressed retroviral genomes. RV particles expressing the transgene cassettes subsequently bud from the cells and are released into the surrounding environment.

presence of retroviral transgene sequences in high-molecular-weight cellular DNA [72]. No replication-competenet retroviruses (RCRs) were detected with the Ad/RV chimera, despite the large genome copy numbers associated with adenovirus production *in vitro* [72].

The *ex vivo* efficacy of the Ad/RV hybrid vector system was investigated by transducing the ovarian carcinoma cell line SKOV3 *in vitro* with the Ad/RV vector alone or in combination with the Ad-*gag/pol/env* vector at an m.o.i. of 50 pfu/cell. The infected cells were then mixed at a ratio of 3:1 with uninfected SKOV3 cells and subcutaneously implanted in athymic nude mice to allow tumor formation. Tumors were assessed 20 days posttransplantation for GFP expression. Large expansive clusters of GFP-expressing cells were observed only in tumors treated with both vectors. Further *in vivo* studies involved direct intraperitoneal injection of 5-day-old established SKOV3 tumors in nude mice (1×10^7 cells) with the single- or two-adenoviral strategy (1×10^9 pfu/mouse). Sixteen days post-Ad infection, the two-virus-treated tumors were observed to have islands of GFP-positive cells (10–15% transduction), consistent with secondary retroviral transduction. In contrast, single virus-treated tumors revealed very limited (<1%) GFP-positive cells. This pioneering study thus established the great potential of hybrid Ad/RV vectors, whose pros and cons are presented in Table III and discussed further in the concluding remarks of this chapter.

Following the initial proof of concept, a number of other laboratories have further investigated the concept of Ad-mediated establishment of retroviral producer cell lines *in situ*. Duisit and colleagues in collaboration

Table III
Pros and Cons of Hybrid Adeno-/Retroviral Vectors for Gene Therapy

Pros

- Exploit high-efficiency adenoviral infection to deliver retroviral assembly machinery
- Utilize stable high titer adenoviral vectors
- Increase the duration of biological activity of delivered transgene
- Avoid initial limitations of retroviral infectivity to nondividing tissues
- Utilize adenoviral vector tropism
- Therapeutic gene expressed by retroviral cassette will still be expressed in context of sole delivery of the Ad-RV cassette vector
- Initial burst of transgene expression can be converted to a stable lower level expression
- Delivery deep into cell layers

Cons

- Progeny retroviral vector can still only infect dividing cells
- In situ released retroviral vector limited according to RV infectivity and tropism
- Requires codelivery of two or more adenoviral vectors
- Risk of RCR
- Safety?
- Rescue of endogenous retroviral elements
- Interactions with host cell functions?
- Diffusion may still be very limited around the initial needle tract
- *In situ* titers of retroviral particles may be limited
- Different cells have different intrinsic potentials for retrovirus production

with François-Loic Cosset, reported on an extension of the hybrid vector system [67]. These studies further restricted the potential for RCR by separating the *gag/pol* core particle-expressing elements from the *env* surface glycoprotein gene, which they supply on a separate Ad vector (Ad-*gag/pol* and Ad-*env*; Fig. 2), to minimize retroviral sequence overlaps. Additionally, in the context of pseudotyping retroviral vectors, they replaced the natural MoMuLV *env* gene with the gibbon ape leukemia virus (GALV) *env* gene. In small-scale pilot experiments, TE671 cells simultaneously infected with the three Ad vectors efficiently released helper-free retroviral particles at titers of up to 5×10^6 iu/mL for at least 3 days following infection [67].

The further separation of the key retroviral elements facilitated the individual characterization of each retroviral function in terms of variable copy load on complementing retroviral cell lines. The results helped to shed light on the factors currently limiting retroviral vector production and allowed an investigation of particular cell type-specific features of the producer cells. The availability of packageable RNAs of the retroviral genome itself was not found to be rate limiting, with Ad-mediated overexpression resulting in increasing, nonsaturatable retroviral titers [67]. The results indicated that high expression of Gag-Pol and Env proteins through the introduction of high copy numbers of their genes was not required to achieve an efficient retroviral production and that there is probably a limit to the number of particles that a given cell may release. Increased GALV *env* copy number resulted in augmented glycoprotein synthesis, with RV particle production plateauing between m.o.i.s of 10 and 50. At higher m.o.i.s titers decreased, possibly by a break of tolerance by the cell to efficient RV particle assembly or budding. It was observed that Pr65 gag precursor expression saturated with Ad-*gag/pol* m.o.i.s of greater than 5 [67]. At higher titers, premature maturation of Pr65 transcripts became apparent, which normally occurs at maturation of the retroviral particles [73]. However, despite these observations, reported titers were equivalent to those generated on high titer-generating stable packaging cells [67]. A detailed analysis of the release of noninfectious/incorrectly processed budding particles may be of interest. Additionally, despite the expression of similar levels of Gag precursors and premature forms, a wide variability was observed in the capacity of different cell types examined to assemble and release retroviral particles [67]. In the context of the hybrid adenoviral/retroviral vector system, when applied at optimal m.o.i.s, a critical limiting factor for the production of retrovirus is the ability to avoid premature activation and convert the bulk of Gag and Gag-Pol precursors in nascent viral infectious particles [67]. Despite the high copy number of all three retroviral units introduced into cells, no RCR could be detected. These Ad/RV hybrid vector studies go some way in aiding the elucidation of the limiting factors involved in retroviral production in packaging cell lines and they indicate that the careful selection of packaging cell type is crucial. This observation is highly significant to therapeutic applications of the

hybrid vector system where different tissues of the body will be more suited to RV complementation than others. The hybrid Ad/RV system can also facilitate the rapid screening of various primate cells for their retroviral production potentials and allows simple substitution/pseudotyping of components in the system.

1. Tetracycline-Inducible Env Pseudotyping of Ad/RV Hybrid Vectors

Pseudotyping the VSV-G retroviral envelope in the MoMuLV back bone, as discussed earlier, enhances the stability and tropism of the native virus [31]. This enhanced stability enables higher titer preparation to be prepared by centrifugation. Generating Ad vectors expressing the VSV-G envelope glycoprotein has, however, proven technically difficult due to the cytotoxic nature of the protein product. Yang and colleagues demonstrated that the VSV-G gene could be effectively controlled under the tetracycline inducible system [74] in packaging cell lines obtaining unconcentrated titers of 10^5 to 10^6 iu/mL [75]. Yoshida and colleagues extended these studies by applying the tetracycline-inducible system in the context of an Ad vector [76]. Ad vectors were generated carrying VSV-G and MoMuLV *gag/pol* genes, both under the control of the tetracycline-controllable promoter. Hence, only upon the supply of doxycycline (a tetracycline homolog) efficient expression would proceed from the *gag*, *pol*, and *env* genes. Minimum "leaky" expression of cytotoxic VSV-G under the control of the inducible promoter remained low enough to allow Ad propagation to titers of 4×10^9 pfu/mL. The drawback of this system is the necessity to provide a further Ad construct containing the tetracycline transcriptional regulator (Ad-rtTA), expanding the system to a four-adenovirus transduction strategy, together with Ad-TetGag/Pol, Ad-TetEnv, and the Ad/RV vector expressing neomycin resistance. Application of the four viruses *in vitro* generated retroviral transgene titers of up to 5×10^5 iu/mL, which were further purified to titers of $>10^7$ iu/mL following simple centrifuge concentration of the virus from culture fluids at 50–80% recovery efficiency [76]. Caplen and colleagues extended these studies in two tumor model systems *in vivo* by subcutaneous injection of 9L glioma tumors in rat or human A735 melanoma xenografts in nude mice [77]. Only upon application of all four viruses in the 9L rat model were neomycin-resistant cell cultures established from harvested tumor tissues. Molecular analysis of genomic DNA extracted from neomycin expressing 9L rat cultures, derived both *in vitro* and *in vivo*, showed the appropriate integration of the retroviral transgene cassette [77].

The human-xenograft nude mouse model system meant that Ad was not cleared in the time frame examined (4 weeks); hence efficacy was assessed as increases in G418R cells compared to single-hybrid Ad/RV-vector transgene transduction [77]. In the human-xenograft mouse model system, tumors harvested at 1, 3, and 4 weeks posttransduction displayed increased numbers

of neomycin-resistant colonies with time only upon transduction of the full complement of adeno-retroviral constructs. At 4 weeks, up to 7.2% of xenografted cells were retrovirally transduced. Transduction of tumors with Ad/RV vector alone yielded no increase in the number of neomycin-resistant clones. DNA extracted from the xenograft tumors, as for the rat model, only showed the presence of integrated proviral sequences when transduced with the full complement of adeno-retroviruses.

Titers of retrovirus particles generated from the 9L rat glioma cells *in vitro* were dependent on the input m.o.i. of the adenoviruses, with maximum titers of up to 1×10^5 iu/mL generated at m.o.i.s in the range of 200–300 for each virus [77]. Under these optimal conditions the presence of doxycycline (1 μM) enhanced the titers by a factor of 2000-fold [77]. Interestingly, *in vivo* similar numbers of clones were observed after the four-adenovirus transduction strategy in the presence or absence of doxycycline: 30 and 20 colonies per 10^6 cells plated. Less than one colony per 10^6 plated cells was observed with the Ad/RV vector alone. These low transduction titers do, however, indicate the current inefficiencies of the system, which are reduced compared to other reports [72]. But the inefficiencies can to some extent be explained by the application of four separate Ad vectors for the system to function, significantly increasing the kinetic complexity of the generation of retroviral vector producer cells *in vivo*. Additionally, the poor efficiency of transduction of the rodent cells by Ad is emphasized by the required m.oi.s applied (>200) to generate optimum titers [77].

2. Cooperative Adenoviral/Retroviral Vector Delivery

Other mechanisms of combining the advantageous properties of adenoviral and retroviral vectors have involved combinatory application of the separate vectors. Delivery of the retroviral genome in the context of a retroviral particle (RV vector) coinfected with an adenovirus expressing the *gag*, *pol*, and *env* genes (Ad-*gag/pol/env*) has been reported [78]. Coinfection of the vectors into NIH 3T3 cells generated retroviral titers $>10^5$ iu/mL. The advantages of this system over the hybrid Ad/RV-vector delivery of the transgene cassette are questionable, specifically from a cell-targeting aspect.

Several groups have also recently demonstrated stable ecotropic retrovirus-mediated gene transduction of human cells using preinfection of Ad or AAV vectors expressing an ecotropic receptor [79–81]. In order to target retroviruses specifically to malignant hepatic tissues, an adenovirus expressing the ecotropic receptor under the control of a hepatoma-specific promoter [82, 83] was constructed. Although tissue-specific expression of the retroviral ecotropic receptor and subsequent tissue-specific targeting of ecotropic enveloped retroviral vectors was demonstrated *in vitro* [84], the clinical benefits of this system are limited. In essence, direct application of the tissue-specific promoter to expression of a therapeutic transgene in the adenoviral vector would be more beneficial.

3. Nonspecific Integration of RV LTR Cassettes in the Context of Ad Vectors?

The studies presented above all emphasize the requirement of all the *gag/pol* and *env* components to derive stable integration of a RV LTR-flanked transgene cassette. However, a controversial report printed in *Nature Biotechnology* challenged that doctrine. Zheng and colleagues reported that an RV LTR-flanked cassette contained in an Ad vector (Ad/RV-vector) could integrate efficiently in the absence of the retroviral enzymatic proteins [85]. The group studied a conventional MoMuLV LTR-flanked luciferase reporter gene cloned in the E1-deleted region of a first-generation Ad vector (AdLTR-Luc), analogous to previous hybrid Ad/RV-vectors. A variety of cells and tissues permissive to Ad infection (epithelial cells, macrophages, and hippocampal cells) were transduced *in vitro* and *in vivo* by the hybrid AdLTR-Luc, and compared with transduction by AdCMV-Luc, a vector containing the CMV promoter in place of the LTRs. The AdLTR-Luc vector was demonstrated to direct sustained luciferase expression compared to the CMV promoter-driven vector. Despite probable well-documented CMV promoter inactivation events, the authors present evidence for stable integration of the LTR-Luc cassette at sites within the LTR elements by a mechanism independent of classical retroviral integration.

Fluorescence *in situ* hybridization (FISH) analysis using probes for the 5' LTR and the luciferase gene revealed integration of the AdLTR-Luc vector with an apparent frequency of 10–15% *in vitro* and 5% *in vivo*. Southern blot analysis also implied integration of the 5' LTR of the hybrid vector, which was subsequently supported by sequencing of the region adjacent to the 5' LTR integration site. No integration of the AdCMV-Luc was reported. The frequency of spontaneous Ad integration has previously been reported at much lower frequencies (10^{-3} to 10^{-5}) [86], suggesting the presence of the retroviral LTR elements in the AdLTR-Luc somehow potentiates integration. The major question is whether an endogenous retrovirus is present in these cells; however, the authors reported negative results for reverse transcriptase activity. Additionally, the integration events reported are not classic retroviral integration as integration does not proceed at a conserved terminal position and results in the random loss of substantial terminal LTR sequence. *In vivo* studies involved injection of rat submandibular glands by retrograde ductal instillation of 1×10^9 pfu/gland. After initial high luciferase expression, the levels plunged to near zero for AdCMV-Luc after 9 weeks but stabilized with AdLTR-Luc after 2 weeks, although at significantly reduced levels. Although these findings are consistent with low-level integration of the LTR cassette, no specific mechanism of integration has been proposed and alternative interpretations of nonspecific LTR-independent mechanisms are probable. As no drug selection gene was present in the vectors, clonal populations were derived on the basis of sustained luciferase expression. Hence, considering first the

well-documented *in situ* inactivation of CMV promoters, specifically in the context of integration, luciferase expression would be absent in long-term cultures transduced with AdCMV-Luc. Therefore, the studies with AdLTR-Luc may have inadvertently selected for random integration events within the 5' LTR that maintained luciferase expression. The probability of such an event is fairly significant considering the limited extent of genetic material upstream of the LTR cassette in the Ad vector (ITR and ψ). Additionally, with the high m.o.i.s applied, selection of high copy number-transduced cells is highly probable, under which conditions spontaneous integration of the Ad vector is more probable. The sequencing data presented also demonstrate integration occurring at sites within the U3 region of the 5' LTR for several clones that would ablate LTR promoter activity. The selection of these luciferase-expressing cells would more probably be due to multiple integration events, which were clearly demonstrated by FISH analysis [85], rather than integration site promoter effects.

Integrated proviral sequences were not reported in animals that received the Ad/RV vectors alone in other similar studies [72, 77]. However these reports were looking specifically for retrovirus-mediated integration events and not the proposed alternative mechanisms reported by Zheng and colleagues [85]. The report by Caplen and colleagues [77] investigated the integration event based on the retroviral mechanism of reproducing the 3' LTR sequences to the 5' LTR structures [1]. The specific duplication of a nucleotide restriction site upon retroviral replication was used as a marker of integration in Southern blot analyses. Analysis of a pooled population of neomycin-resistant colonies revealed efficient band size switching, indicative of the duplication event, and thus retroviral replication. However, consistent with the observation of Zheng [85], randomly integrated Ad-RV transgene cassette fragments could be seen in context of generalized hybridization of the probe to high-molecular-weight DNA from single-vector-transduced animals [77]. These bands were, however, weak and consistent with random integration. Further studies are therefore merited to evaluate the efficacy of integration of LTR-flanked cassettes in the context of an Ad vector to determine whether a specific mechanism does exist and whether it could be further refined for vector use.

4. Integration of Closed Circle Retroviral Cassettes Delivered by Adenoviral Vectors

Following retroviral infection, reverse transcribed proviral DNA serves as a substrate for an integration reaction catalyzed by the retroviral integrase (Int) protein, which, along with viral Gag proteins, forms the preintegration complex [87]. This complex brings the 5' end of the 5' LTR (the U3 region) into close juxtaposition with the 3' end of the 3' LTR (the U5 region) [87]. The direct substrate for Int is most likely a linear, double-stranded molecule with blunt ends [88]. Int-mediated integration then occurs by a very precise mechanism

in which the terminal two base pairs of each LTR are lost prior to integration into the target cell genome [87, 89]. However, closed circular molecules have also been detected in the nuclei of retrovirally infected cells, which contain 2 LTRs joined covalently together at the so-called circle junction [87,90, 91]. Although there is considerable evidence that MoMuLV probably does not use a 2-LTR circle as the principal integration intermediate [87, 89, 92], it was hypothesized that it may still be possible for Int to use such a molecule as a template for integration if it were the only, or predominant, species delivered into the nucleus [87]. This hypothesis is supported by the existence of the 2-LTR circles in MoMuLV-infected cells [90] and evidence from the spleen necrosis virus (SNV) system that the LTR junction fragment can be an effective substrate for integration [91].

We investigated whether a 5' LTR–3' LTR junction fragment, in a closed circular DNA molecule excised from an incoming plasmid by Cre recombinase and in the absence of the preferred, linear viral DNA molecules, could be recognized by the retroviral integration machinery (Fig. 3). A fused LTR

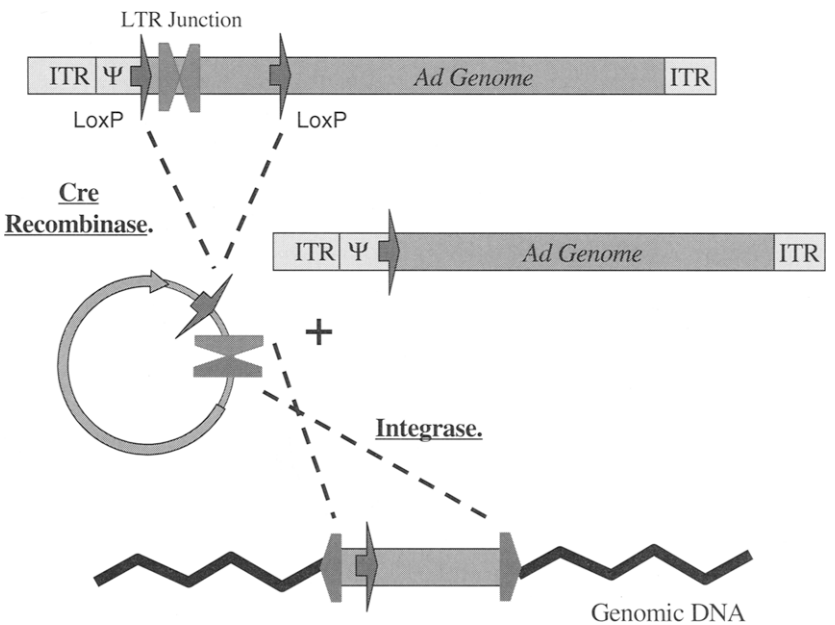

Figure 3 Genomic integration of an adenovirally delivered retroviral circular provirus cassette. A LoxP-flanked cassette containing a fused terminal LTR junction and transgenes of interest was inserted into an adenoviral vector. Upon infection of cells expressing Cre recombinase, this cassette is efficiently excised as a closed circular molecule. The fused LTR junction contained in this circular proviral molecule is subsequently recognized by retroviral Integrase, directing integration into the host chromosome.

junction fragment was thus cloned, containing the entire 3' LTR and just 28 bp of the U3 region of the 5' LTR (Fig. 4A). This LTR junction together with the puromycin resistance gene was flanked by LoxP sites and was demonstrated to efficiently excise a circular proviral intermediate *in vitro* upon supply of Cre recombinase in *trans* [93]. Further studies in cell lines *trans*-complementing *gag/pol* gene functions, together with Cre recombinase, generated long-term neomycin-resistant clones. Genomic DNA extracted from stable clones was used to investigate the proviral integration structures by utilizing a panel of diagnostic PCR primers. The PCR demonstrated that integration following plasmid transfection, Cre excision, and puromycin selection for >1 month can produce a very specific molecular structure which is distinct from that produced by random plasmid integration. PCR results demonstrated that the 5' and 3' LTRs, which are adjoining in the plasmid backbone, become separated by the intervening sequences of the retroviral vector genome (between the loxP sites). A molecule is thus generated in which the proviral genome is now bounded by the LTRs in a manner typical of Int-mediated integration (Fig. 3). Critically, the terminal two base pairs of both the 5' LTR (U3 region) and the 3' LTR (U5 region) were lost (Fig. 4B). Hence these studies confirm that a circular retroviral genome with terminally fused LTR structures can indeed serve as a substrate for the retroviral machinery.

From this initial proof of concept, the LoxP cassette was subsequently assembled into an E1-deleted Ad vector. The Ad virus is used to deliver the LTR junction fragment into the nuclei of cells; the proviral-like intermediate can then be excised from the Ad genome by the Cre/lox system and forms a template for Int-mediated integration. This hybrid Ad/RV system thus has the high transient titer of Ad vectors, does not depend upon cell division for infection, and leads to long-term gene expression via integration of a proviral transgene cassette. Delivery of the Lox-Puro-Junc.-Lox cassette in an Ad vector, in the presence of Cre and Gag and Pol allowed cloning of cells which are resistant to puromycin for long periods in culture. Without Cre, such clones were impossible to obtain. Moreover, these clones contain a molecular structure consistent with proviral integration by PCR and contain integration sites which, for the majority of the clones (seven of nine), are typical of Int-mediated, rather than random, integration processes (Fig. 4B). Codelivery of three separate Ad vectors, Ad-Gag/Pol, Ad-Cre, and Ad.LTR.Junc, was also able to produce long-term integrants. Therefore, we are currently optimizing the design and use of this novel hybrid vector system into a single, or double, Ad delivery system. Recent experiments have shown that Pol-expressed Int alone is sufficient to drive the integration of the Cre-excised proviral form *in vitro* without the need for additional Pol or Gag proteins. An Ad vector was thus cloned incorporating the Int gene in the same cassette as the transgene cassette to enable a two-vector transduction strategy, which is currently under investigation in our laboratory. This novel hybrid vector system presents great

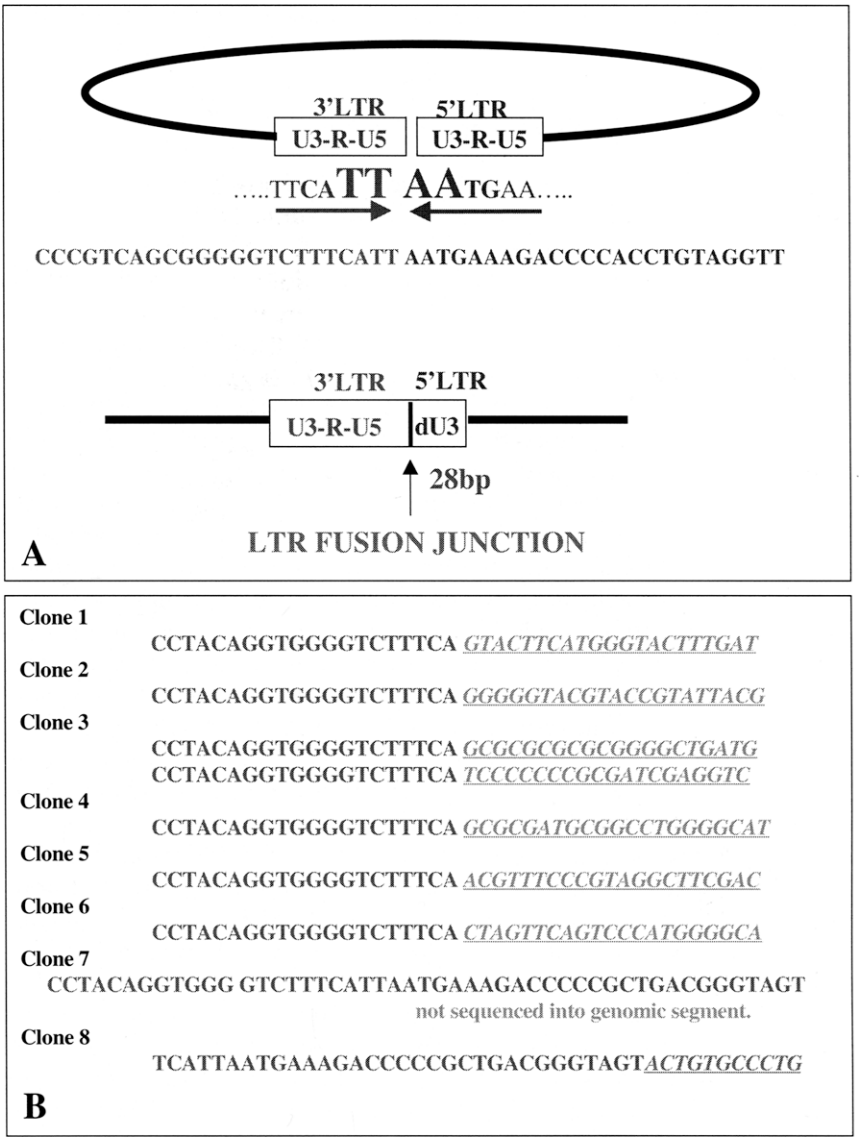

Figure 4 Sequencing of the integration junctions of the circular RV proviral cassette. (A) Schematic representation of the RV genome conformation in the noncovalently linked circular preintegration complex and the subsequently cloned fused LTR junction. (B) A human cell line expressing the retroviral *gag/pol* genes and Cre recombinase (TelCre) were infected with the Ad/RV hybrid vector expressing puromycin resistance. Colonies were selected which had stably integrated the RV proviral cassettes and the genomic DNA was extracted. The integration junctions were subsequently cloned by PCR amplification of religated restriction-digested fragments containing the integration site [93], which were subsequently sequenced through the integration site.

potential in enabling the stable transduction of all cells primarily infected by the Ad vectors.

C. Adenoviral/Epstein–Barr Virus Hybrid Vectors

An alternative application of the Cre/LoxP recombinase system of excising a circular proviral molecule from an Ad vector [93] has replaced the retroviral component with the genetic stability of the EBV replicon system [94, 95]. This hybrid Ad/EBV vector system utilizes Ad-mediated nuclear delivery of a Cre-excisable EBV replicon which can be stably maintained as an EBV episome [96]. EBV episomes contain the EBV latent origin of replication (Ori_p) and the EBV nuclear antigen-1 (EBNA-1) which acts on Ori_p, driving episomal replication (Fig. 5A). Previous studies have demonstrated that EBV nuclear episomes are stably maintained through multiple cell divisions in primate and canine cells, replicating once during S phase and segregating to both daughter cells with approximately 95% efficiency [97]. Tan and colleagues flanked Ori_p and EBNA-1, together with the puromycin resistance gene, with LoxP sites and cloned them into an E1-deleted Ad vector [94]. However, multiple attempts to make an adenovirus failed due to suspected inhibition of Ad replication upon binding of ENBA-1 to Ori_p. Hence a vector was assembled which only brought EBNA-1 upstream of its promoter following Cre excision of the proviral cassette (Fig. 5B), thus silencing its expression in the absence of Cre recombinase [94]. The resultant Ad/EBV hybrid vector stably transformed 37% of surviving canine D-17 cells to puromycin resistance following coinfection with AdCre. The circular EBV replicons were maintained in daughter cells for 14 weeks, ~110 cell generations. Surprisingly, the puromycin resistance gene was also discovered in an integrated form, in the cellular chromosomal DNA [94]. Integration of EBV episomes has not been reported previously in human cells, although a differential function in the canine cells might be involved. One major limitation of the hybrid system was, however, that a large cell fatality was observed upon transduction with the Ad vector into the D-17 cells at the optimum transduction conditions (m.o.i. 30). This was discussed by the authors as a function of leaky Ad gene expression from the first-generation vector in the canine cells and not related to EBNA-1 toxicity [94]. Reports have previously shown that EBNA-1 does not elicit a cytotoxic T-cell response, due to the presence of a series of glycine-alanine repeats [98]. These repeats act in *cis* to prevent MHC class I presentation by inhibiting antigen processing by the ubiquitous processing pathway [98].

A very similar Ad/EBV replicon vector has also been described in the context of an E4-deleted, second-generation Ad vector [99]. Coinfection of human cells with this vector, together with AdCre (also E4-deleted), resulted in efficient delivery and excision of the replicon in the absence of vector-induced toxicity. The replicons were maintained following successive cell divisions both

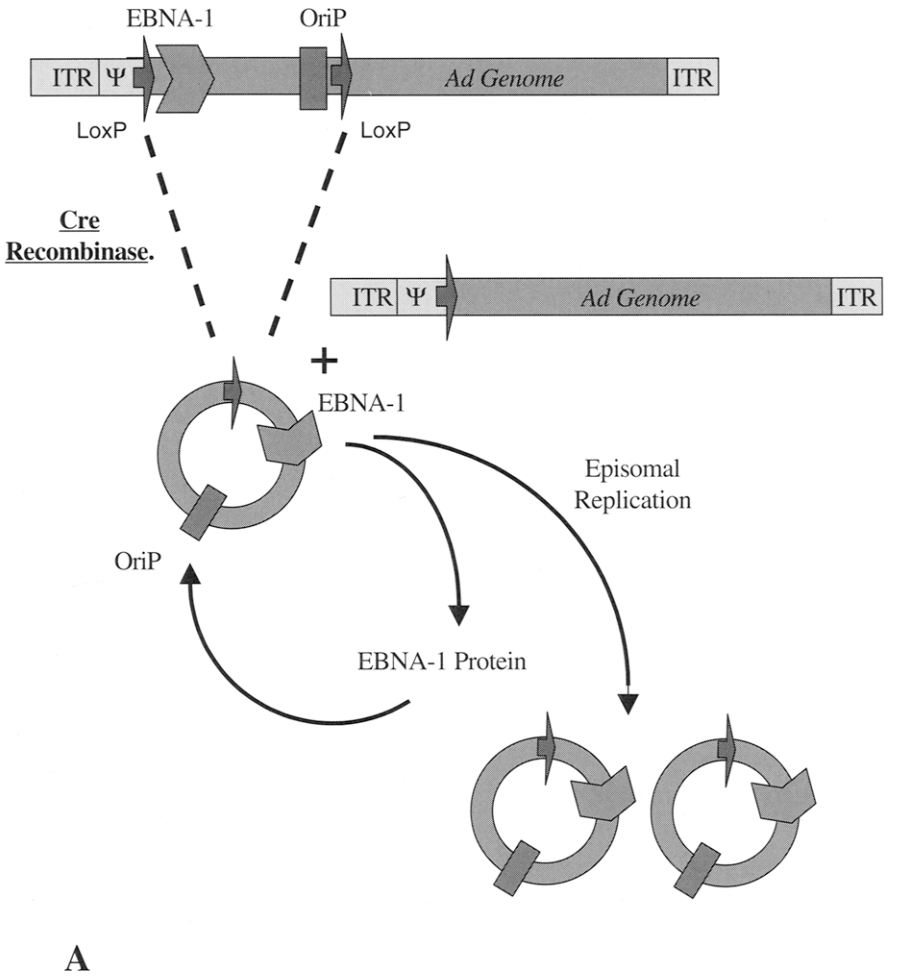

Figure 5 Episomal replication and maintenance of a Cre-excised EBV replicon from an adenoviral vector. (A) A LoxP-flanked cassette containing the EBV origin of replication (Ori_P) nuclear antigen (EBNA-1) and transgenes of interest was inserted into an adenoviral vector. Upon infection of cells expressing Cre recombinase, this cassette is efficiently excised as a closed circular molecule. Upon action of EBNA-1 on Ori_P, the circular cassette is efficiently replicated by the rolling circle mechanism, facilitating maintenance of the cassette in the infected cells. (B) In order to control the expression of the EBNA-1 genes, the promoter and gene are separated in the adenoviral cassette and become productively in line in the excised replicon form only following Cre-mediated excision. (C) By inserting the left LoxP site between the Ad LTR and Ψ, leaky excision of Cre recombinase and subsequent premature excision of the LoxP cassette would render the resulting Ad vector nonpackageable due to elimination of Ψ. This strategy eliminates contamination of the excised adenoviral form upon propagation of the hybrid Ad/EBV vector.

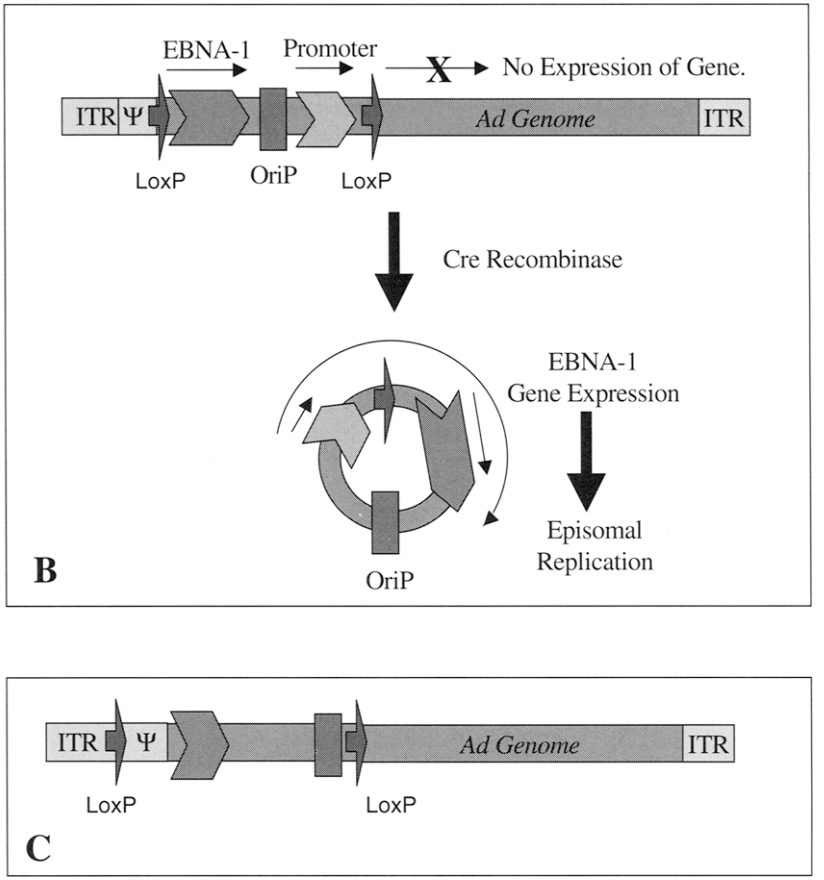

Figure 5 (*continued*)

in vitro and *in vivo*, suggesting efficient extrachromosomal replication as well as nuclear retention of the episome. The residual Ad backbones were, however, progressively lost by a dilution mechanism occurring in the absence of DNA replication [99].

As for all gene therapy vector systems, incorporation of all the components into a single vector would simplify delivery and therapeutic efficacy. As for the previously described Cre-excisable RV provirus strategy [93], combination of the vector elements for the Ad/EBV has its limitations. Any expression of Cre recombinase would result in premature excision of the EBV replicon, specifically upon initial Ad propagation. Wang and colleagues, however, enabled incorporation of all the components into a gutless helper-dependent (HD) Ad vector by use of a tissue-specific promoter to control Cre expression [100].

Placing *Cre* under the control of a synthetic promoter (HCR12), consisting of hepatic locus control elements from the human ApoE/C locus fused to the first intron of the human EF1α gene, allowed adequate suppression of expression in 293 cells while permitting recombination and subsequent gene expression in the target tissue. However, promoter activity was not completely extinguished in all nonhepatic cells. In order to limit the effects of leaky Cre excision of the LoxP cassette, the Ad packaging signal was included in the excisable cassette (Fig. 5C). The placing of a LoxP site between the LTR and ψ has been demonstrated not to inhibit Ad expression extensively [13]. Thus leaky excision would remove ψ from the Ad vector backbone, rendering the Ad genome nonpackageable and hence preventing contamination in the final viral stocks. Additionally, removal of ψ from the cassette also eliminates the E1 enhancer elements, which are interlinked with the packaging elements, which have been reported to additionally limit leaky viral expression events from the adenoviral tripartite leader sequence (TPL) [100].

D. Hybrid Retroviruses Trafficking to the Nucleus

While previously described strategies have focused on combining the advantageous properties of retroviruses into adenoviral vectors, research has also investigated the reverse scenario. An interesting study by Lieber and colleagues investigated the potential of inserting the nuclear localization functions of an adenovirus into a retrovirus [101]. The failure of MoMuLV to cross the nuclear membrane in the absence of cell division has limited retroviral vectors. Large proteins or complexes (>40–60 kDa), such as the retroviral preintegration complex, are too large to pass into the nuclear membrane by simple diffusion and require nuclear localization signals (NLSs). NLSs interact with cytoplasmic receptors initiating an energy-dependent multistep translocation into the nucleus. The efficient nuclear targeting properties of Ad vectors have made them ideal gene delivery vehicles. It is generally believed that NLSs in the preterminal protein (pTP) and the core protein V play a crucial role in directing the Ad genome complex to the nucleus. The Ad pTP binds alone or in a complex with the Ad polymerase to specific sequences at the termini of the adenoviral ITRs. Lieber and colleagues-investigated whether coexpression of pTP with retroviral DNA carrying pTP-binding sites would facilitate nuclear import of the preintegration complex and transduction of quiescent cells. Preliminary experiments demonstrated successful nuclear import of plasmid DNA via the karyotypic pTP (in the presence or absence of Ad polymerase) into the nuclei of growth-arrested cells [101].

The pTP binding motif was initially established by engineering two head-to-head adenoviral ITRs, but was later reduced to an 18-bp terminal fragment of the ITR, deemed the minimum required unit [101]. Interestingly, attempts to introduce the full Ad ITR fragment into retrovirus vectors resulted in viruses

with very low titers ($<10^2$ iu/mL), indicating adverse effects on retroviral replication. The minimal ITR 18mer oligonucleotide, however, allowed high-titer retrovirus production. The pTP binding site was placed in the center of the recombinant vector between hAAT and neo in order to avoid potential interference of pTP binding on preintegration complex stoichiometry. Results demonstrated that the incorporation of the pTP karyotypic machinery in the context of the retroviral backbone could indeed efficiently translocate the RV genome across the nuclear divide. pTP-mediated transduction was, however, always less than in proliferating cells, possibly indicating weak binding to the viral DNA, which is supported by AdPol increasing nuclear import and transduction. Alternatively the nuclear matrix binding properties of pTP could interfere with the retroviral transduction functions. Disappointingly, however, pTP nuclear import of MoMuLV DNA in nondividing cells was found not to be sufficient for stable transduction. Undetermined additional cellular factors activating during S phase and/or DNA repair are required for efficient retroviral integration [101].

E. Hybrid Adenoviral/Adeno-Associated Virus Vectors

Incorporation of AAV nuclear retention functions into hybrid Ad vectors has also become a great interest in gene therapy. AAV vectors have emerged strongly as candidates for gene therapy, being nonpathogenic and presenting a mechanism of stable integration into a specific locus of the human host chromosome. The terminal ITR structures contain all the *cis*-acting elements required to drive episomal replication, host genome integration and packaging into infectious AAV particles [102–105]. The *rep* gene products mediate the amplification of the AAV genome and facilitate site-specific integration into the human chromosome 19*q*13.3, termed AAVS1 [106, 107]. In the context of double-stranded circular DNA plasmid vectors, the presence of the two AAV ITRs was demonstrated to be sufficient to rescue an AAV genome from the plasmid backbone and to mediate its integration into host DNA [108, 102]. These findings paved the way to the development of AAV vectors and initiated the application of AAV genomes in hybrid vector systems. The ITR-flanked AAV cassettes were subsequently demonstrated to also be efficiently rescued from the backbones of other viral vectors. In cultured cells, AAV integrates into the host chromosome with a relatively low frequency of 1×10^{-4} to 3×10^{-4} genomes per cell, with alternative episomal replication of its genome permitting long-term persistent expression in cells [109]. However, the integration efficiency can be enhanced by stimulation of the host DNA repair machinery by gamma irradiation or topoisomerase inhibitors [110, 111]. The only requirements for AAV integration and episomal concatemerization appear to be the presence of AAV ITRs and as-yet-undetermined cellular factors [102, 103, 108].

Following the hybrid Ad/RV studies, AAV ITR-flanked transgene cassettes have been similarly applied in the context of an Ad backbone. AAV ITR cassettes can be efficiently rescued from Ad genomes and assembled into AAV particles upon the supply of *rep* and *cap* functions in *trans* [15]. In the absence of the *cap* genes, Ad-mediated delivery of the AAV ITR cassettes can result in its stable integration into the host genome, in the presence or absence of the *rep* genes (Fig. 6). However, studies on the relationship between Ad and AAV demonstrate a strong interference of AAV on the Ad life cycle [112]. Although the precise mechanism is undetermined, *rep* expression is sufficient to suppress the maturation of Ad replication centers [113]. Hence the major complication in the union of the Ad/AAV hybrid vector system has been strategies to facilitate *rep* expression in the context of an adenoviral vector.

The AAV *rep* gene encodes four proteins that are expressed from independent promoters (Fig. 7A). The Rep68 and Rep78 differentially spliced products are expressed from the P5 promoter and individually are capable of catalyzing AAV genome integration [114, 115]. The poorly characterized Rep40 and Rep52 proteins are expressed from the P19 promoter and, although having

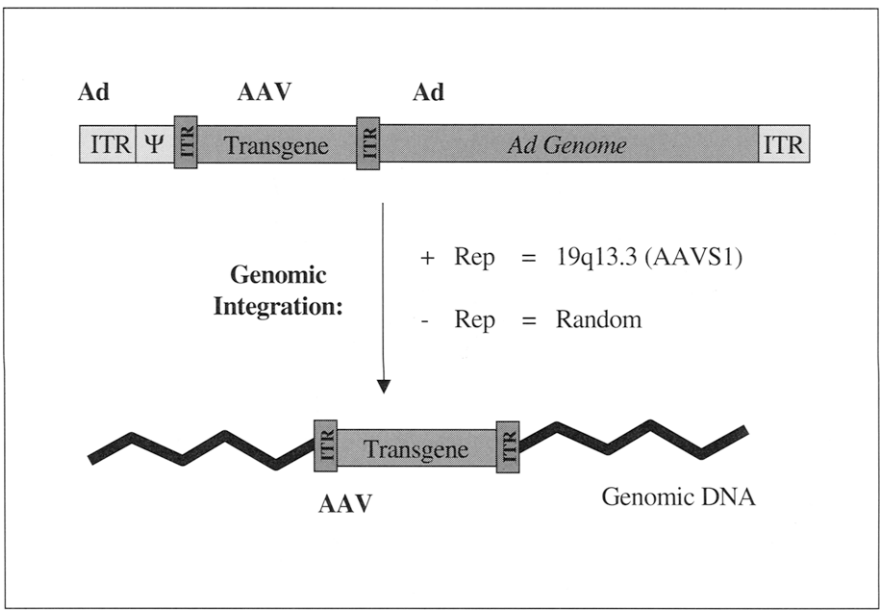

Figure 6 Hybrid-Ad/AAV vector-mediated integration of the AAV ITR cassettes. Infection of cells with the hybrid Ad/AAV vector enables precise excision of the ITR-flanked cassette from the adenoviral genome, which can subsequently be integrated into the host genome. This mechanism can occur in the absence or presence of the AAV Rep proteins. In the presence of Rep the cassette is predictably integrated into the AAVS1 locus on human chromosome 19.

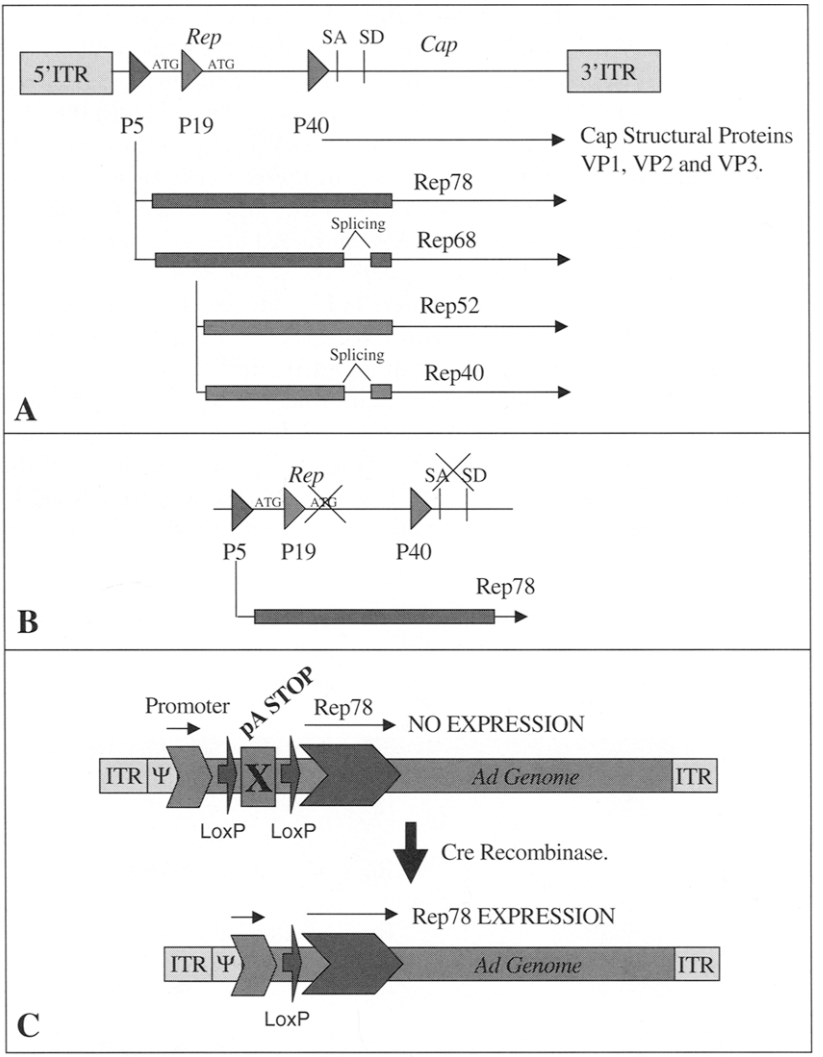

Figure 7 Control of the expression of the AAV Rep proteins in the context of their inhibitory effects on Ad production. (A). Schematic representation of the AAV genome. Three promoters are contained in the AAV genome: P5 and p19 control expression of the alternatively spliced Rep68/78 and Rep40/52 transcripts, respectively, while P40 controls expression of the Cap gene products. (B) In order to restrict expression from the Rep cassette to just the Rep78 form, a point mutation was introduced into the P19 promoter's ATG start site, preventing Rep40/52 expression, and a similar mutation in the P5 transcript's splice site eliminated Rep68 expression. (C) In order to further restrict Rep78 expression from the Ad vector, a LoxP cassette flanking a poly(A) stop site was cloned between the Rep78 gene and its promoter. This cassette completely silences Rep78 by preventing translation by premature termination at the introduced poly(A) site. Cre-mediated excision removes this cassette, permitting Rep78 expression to proceed.

similar catalytic properties to the other proteins, their function is undetermined and believed to be distinct from Rep68/78 [116]. Recchia and colleagues investigated amplification conditions that were likely to minimize Rep inhibition of vector production [117]. Specifically, P19 promoter expression of Rep52 and Rep40 was reported to impose significant inhibitory functions to Ad replication, although Rep68 and Rep78 functions were also apparent. To minimize the complications of Rep-mediated interference of Ad production, the expression of Rep proteins was restricted to just the Rep78 isotype, by inactivating the Rep52 and 40 transcripts by ATG mutation and preventing Rep68 splicing by similar point mutations at the splice site (Fig. 7B). Rep79 was placed under the control of either the T7 promoter, a promoter previously applied to the production of adenoviruses expressing toxic genes, or the α1-antitrypsin (α1at) liver-specific promoter, to additionally minimize any interference. The AdT7-Rep78 shuttle vector was successfully recombined in 293 cells to generate the Ad vector, whereas shuttle vectors containing the wild-type Rep, or only expressing the Rep52 and −40 isotypes, did not yield any viral plaques. The functionality of the AdT7-Rep78 vector was demonstrated in an AAV rescue study [117].

As expression from the α1at promoter restricts expression to hepatic tissues, Ueno and colleagues applied an alternative Cre/LoxP bacteriophage P1 system as a switch to regulate Rep expression from an Ad vector [118]. A LoxP-flanked cassette containing a transcriptional silencing sequence (SV40 poly(A)) was cloned between the *Rep78* gene and its CAG promoter (Fig. 7C). Hence upon Cre recombinase expression the LoxP cassette is excised, uniting promoter and transgene and allowing transcription to proceed. The authors failed to yield any virus with *Rep78* driven by the CAG promoter in the absence of the Lox-stop cassette. The vector system thus required a third Ad expressing Cre (AdCre) to be coexpressed with AdLoxP-Rep78 and the Ad/AAV hybrid vector. Only upon application of all three vectors to HeLa cells (m.o.i.s of 10 to 20) was site-specific integration into AAVS1 detected by PCR analysis of genomic DNA [118]. As with the previous systems, incorporation of all the vector components into a single gutless vector is a major aim. The application of Cre recombinase would thus, as with the previous Ad/EBV replicon system [94], require tightly controlled expression to be incorporated into the same helper-dependent (HD) vector as the LoxP cassette.

1. Helper-Dependent Ad/AAV

Recchia and colleagues furthered the studies of Ad/AAV hybridology by incorporating the system into HD Ad vectors [117]. The system applied a similar two-vector strategy with the AAV ITR transgene cassette and *Rep78* genes on separate gutless vectors, HD-AAV and HDα1at-Rep78, respectively. The gutless Ad constructs consisted of the terminal *cis*-acting regions of the Ad genome (ITRs and ψ) together with the transgene cassette(s), as well as

additional inert stuffer sequences, to bring the vector genome size above the efficient packaging size threshold (>27 kb) [35]. Large-scale production of HDα1at-Rep78 generated titers of 3×10^9 iu from 5×10^7 cells, indicating 50–100 Rep-expressing viruses per cell could be produced. This HDα1at-Rep78 virus expressed Rep78 selectively in hepatic cells (Hep3B). Rescue of the AAV-ITR transgene cassette from HD-AAV into infectious AAV particles was observed upon coinfection of Hep3B cells with HDα1at-Rep78 and wild-type Ad2 helper. No AAV rescue was detected upon elimination of any of the three vector components, demonstrating the functionality of each component. Coinfection of HD-AAV with HDα1at-Rep78 into a number of cell lines of hepatic origin showed stable integration of the AAV transgene cassettes into the host cell genome specifically at AAVS1, by nested PCR analysis, Southern blotting, and integration site junction sequencing [117]. FISH studies on HepG2 cells infected with both vectors demonstrated targeted integration to AAVS1 in 14 of 39 (35%) metaphases analyzed. In the absence of the Rep78 vector only one integration in chromosome 19 was observed in 34 metaphases analyzed (3%). Hence, the study by Recchia and colleagues demonstrates that Rep78 expression increases the targeted integration of AAV-ITR-flanked DNA without affecting the overall integration frequency in cells of hepatic origin [117]. In contrast to other studies on 293 cells [114, 119, 120], however, Rep78 did not increase the stable transduction efficiency on the hepatic cell lines investigated, which was believed to be due to cell-type effects [117]. The next advance in this study will be incorporation of both cassettes into a single HD Ad vector. This will be much more complex than originally perceived considering the action of Rep78 on the AAV cassette, especially in the high copy number context of adenovirus production. Additionally, considering the Rep-independent processing of the AAV ITR cassette, the fate of the cassette at high copy number in the producer cells would be of great interest.

2. Generation of Mini-Ad/AAV Hybrid Vectors by *in Vitro* Hybridization

Inverted repeat (IR) sequences inserted into first generation Ad vector genomes were recently reported to mediate precise genomic rearrangements resulting in vector genomes devoid of all viral genes but which were efficiently packaged into functional Ad virions [121]. These genomes were generated by a *trans*-recombination between two Ad genomes exchanging sequences either side of the IR regions. Hence two species are generated. Firstly, a small genome containing only the transgene cassette flanked on both sides by precisely duplicated IRs, Ad packaging signals (ψ), and Ad ITRs (Fig. 8). Second, a larger genome is generated containing the transgene cassette flanked by the IRs and also the rest of the Ad genome (Fig. 8). The presence of the Ad packaging signal only in the mini-genome product meant that only this form could be packaged, whereas the larger genome just facilitated helper

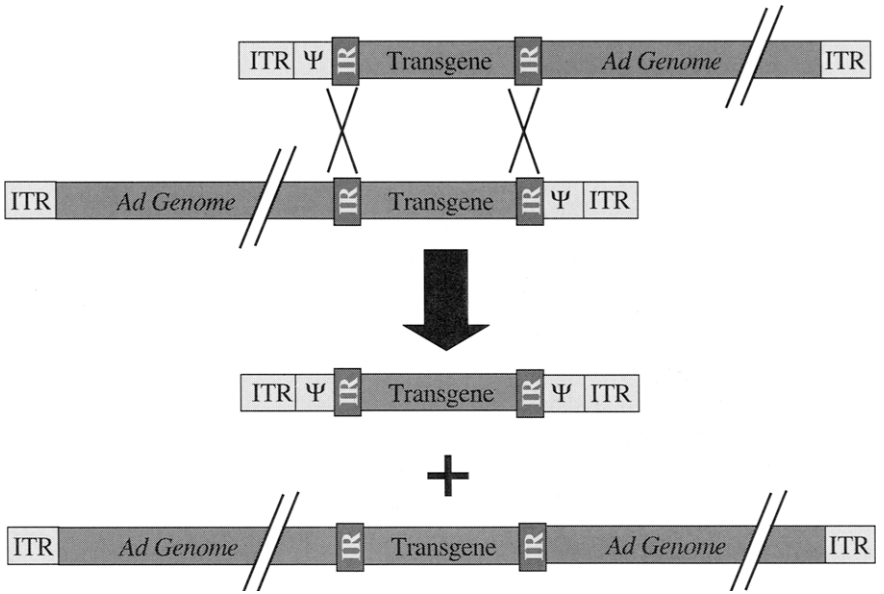

Figure 8 Generation of mini-Ad genomes by recombination between inverted repeat (IR) regions. The presence of IR regions in adenoviral cassettes enables precise recombinations between different Ad genomes within the IR regions. This recombination generates mini-Ad genomes with the precisely replicated IR cassettes being flanked at either end by Ad ITR and Ψ sequences. A second, much larger, recombinant species is also generated which also contains a precisely replicated IR cassette but is flanked on either side by the rest of the adenoviral genome. This larger recombinant, as well as being of a size nonpackageable into an adenoviral virion, lacks the Ad Ψ and hence is not packaged. In contrast, the smaller mini-Ad genomes can be efficiently assembled into adenoviral particles assembled by the larger genome's helper functions.

functions. Application of this precise recombination mechanism to generate mini-Ad genomes deleted of all viral genes could minimize the immunogenicity apparent with first-generation vectors. By modifying the IR regions to increase the efficiency of recombination, further selection for the recombinant mini-genomes could be achieved [121]. The mini-Ad virions could be efficiently separated on CsCl gradients by buoyant density, with great resolution from the larger helper viral genomes enabling efficient purification.

The generation of the recombinant mini-Ad genomes was very efficient ($\sim 5 \times 10^4$ genomes per cell) and did not depend on the sequences within or adjacent to the IRs [121]. The mini-Ad vectors efficiently infected cultured cells with the same efficiency as first-generation vectors. However, in the absence of any vector selection in the cell (episomal replication or integration), transgene expression was only transient (~7 days) due to the instability of the deleted genomes within transduced cells.

Lieber and colleagues further developed the system to incorporate AAV cassettes into a hybrid vector system [121]. The AAV ITRs flanking the transgene cassette were used as the IRs to mediate the recombination event, as well as stimulating transgene integration into the host genome of transduced cells. The Ad-AAV vectors efficiently generated mini-genomes by IR recombination as by-products of first generation Ad-AAV vector amplification. The mini-genomes containing only the transgene flanked by AAV ITRs, Ad ψs, and Ad ITRs could be efficiently assembled in Ad capsids and purified to high titers and purity. The mini-Ad-AAV hybrid vectors transduced cells with efficiencies comparable to AAV, but were less efficient than conventional Ad vectors due to elevated particle to infectious unit ratios [121]. Since the hybrid mini-vectors contained no cytotoxic viral genes, the hybrid virus could be applied at very high m.o.i.s to increase transduction rates. The AAV transgene cassettes randomly integrated into the host cell genomes as head-to-tail concatemers, as shown by Southern blot analysis and pulsed-field gel electrophoresis.

Amplification of Ad-AAV hybrid vectors in 293 cells routinely yielded final mini-Ad-AAV genome titers of 5×10^{12} genomes per milliliter, or $\sim 10^4$ packaged genomes per 293 cell, comprising 10% of the total number of adenoviral virions [121]. The 5.5 kDa mini-Ad-AAV hybrid vectors which contain two Ad packaging signals were, however, packaged approximately fivefold less efficiently than the corresponding full-length genomes [121]. These results are compatible with the published observations that Ad vector genomes of less than 27 kb package with much reduced potentials compared to full-length genomes [35]. Additionally, contamination of mini-Ad-AAV hybrid vector preparations with the parental Ad-AAV hybrid vector was less than 0.1%, consistent with conventional gutless Ad purification [13]. The efficiency of vector production measured on a genome-per-cell basis was reported to be comparable to or higher than the labor-intensive techniques for AAV production. The transducing titers expressed as neomycin-resistant colonies per milliliter were 9×10^5 for AAV and 2.5×10^8 for the mini-Ad-AAV hybrid vector [121]. These results present significant clinical promise for mini-Ad/AAV hybrid vectors in the clinic.

IV. Conclusion

The establishment of hybrid Ad vectors incorporating the advantageous properties of other viruses greatly expands their therapeutic potential. In the early 1990s, after the initial decade of proof-of-concept for Ad-mediated gene therapy, the main focus was on limiting the immunogenicity of the vectors to enhance transgene expression. Further restricting the expression of the highly immunogenic late viral transcripts by E4 deletions or by complete deletion of all viral genes in the gutless vectors notably enhanced transgene expression [11–13, 35]. However, complete ablation of immunogenicity is

restricted by the highly inflammatory nature of the Ad particles themselves in the absence of viral expression. While suppression of specific immune responses can counter these effects to some extent, in many disease states where the immune system is already compromised this rationale is not ideal. Pseudotyping the Ad vectors with alternative surface moieties does, however, offer great potential. First, novel surface structures can be introduced which can reduce the immunogenicity of the viral particles by either shielding or replacing the highly immunogenic wild-type structures. Future research may permit the complete replacement of the viral external domains with immune-tolerated surface structures. Second, the introduction of new targeting ligands will enable selected infection of desired tissue populations, limiting the required vector doses. Additionally, the avoidance of infection of nontargeted tissues, specifically cells of the immune system, will negate potentially immunogenic signalling which the vectors can initialize upon receptor docking.

It is now well accepted that the immune system is not the major limiting factor in the transient expression attained from Ad vectors. The absence of a specific mechanism of long-term retention of the viral genomes in infected cells is critical. As presented at the start of this chapter, the rapid lytic life cycle of wild-type adenoviruses does not require long-term persistence of the genomes. Adenovirus infection, genome replication, virion packaging, and lysis of the host cell are generally completed within 48 h. While these properties have proved highly beneficial in the area of vector production, they do not aid *in vivo* stability. By combining the long-term stable persistence mechanisms of other viral systems into the Ad vectors, the efficacy of Ad-mediated gene therapy has been significantly enhanced.

A number of mechanisms have been presented in this chapter for the combination of adenoviral and retroviral vectors. Initial applications utilized Ad vectors to deliver RV packaging functions to producer cells *in vitro*, in attempts to increase the efficiency of RV production. From these initial studies, the potential of the hybrid Ad/RV vectors for the establishment of RV producer cell *in vivo* was realized. As producers of RV packaging cells *in vitro*, the Ad/RV hybrid vector system has a number of advantages over conventional packaging cell lines. RV titers generated from transient plasmid-transfected producer cells are generally several orders of magnitude lower than the best stable clones [3]. Therefore, as the Ad/RV hybrid system has been demonstrated to generate RV titers of the same orders of magnitude as conventional producer cells, it bypasses the need to isolate clonal populations and makes scaling up production more manageable. The requirement of GMP screening for replication-competent adenoviruses (RCA), as well as replication-competent retroviruses (RCR), would, however, be a concern. The separation of the different retroviral components on separate viruses, as well as limiting the potential of RCR, makes pseudotyping very simple. For instance, in the treatment of specific tissues, an envelope gene best suited for tropism to that

tissue can be easily incorporated into the vector system. The separation also enables characterization of individual RV components. Application of the individual Ad/RV hybrid vectors at varying m.o.i.s enables study of the RV production at varying copy load. Additionally, as different target tissues have been well documented to have different potentials as RV producer cells, the hybrid Ad/RV system enables the rapid screening of tissues for their suitability as RV producer cells. However, this measure of suitability is also influenced by the susceptibility of the cells to Ad transduction. Understanding the effects of saturating RV components could allow us to determine what factors need to be further regulated in future hybrid vectors to enable enhanced RV production both *in vitro* and *in vivo*. Elucidation of host factors vital to efficient assembly of RV particles would be very valuable. In future vectors, these host factors could be codelivered or upregulated to enhance RV titers.

One major question is: what advantages does the hybrid Ad/RV vector system have over conventional Ad or RV delivery? The ability to establish RV producer cells *in vivo* following Ad infection is a major step forward in gene therapy. Previous methods of *ex vivo* transduction with retroviral vectors and reimplantation are laborious and inefficient. Vector spread is limited by the restricted migratory properties of the reimplanted cells. Application of the hybrid Ad/RV vector enables a noninvasive therapy with enhanced distribution and infectivity in target tissues. The subsequent local release of retroviral particles following adenoviral transduction also tackles the problem of inserting high levels of vector deep into the middle of tissue or tumor masses, rather than to just the peripheral layers. The major advantage over conventional Ad vectors is the establishment of a stable population of transgene-expressing cells in the surrounding tissues, through RV integration, following the initial transient Ad transduction. This permanency of therapeutic transgene has major implications in the clinic, specifically for the treatment of inherited diseases. The separation of the RV genes, as well as the introduction of additional regulatory elements carried on separate rAds, instills a multiple-vector transduction strategy. Vector systems involving more than one vector are limited by codelivery kinetics. The greater the number of individual vectors, the lower the probability that a cell will receive the full vector repertoire to allow retroviral production to occur. Therapeutic transgene expression can, however, proceed from the adenoviral vector itself, initiating an initial boost of gene expression, followed by a secondary level of sustained expression in RV transduced cells. Currently, however, the secondary phase of RV expression is much reduced compared to the initial Ad-mediated expression. This would minimize the sustained therapeutic effect of the vector system. Nevertheless, this hybrid Ad/RV vector system has great potential for the treatment of genetic disorders.

The dual transduction properties of the Ad/RV hybrid vector also present the possibility of combinatory gene therapy where the Ad and RV portions

of the vector provide different therapeutic effects. This mechanism could have specific advantages to the treatment of cancer. The initial Ad transduction could act to initially immunostimulate the tumor mass, aiding a break in tolerance by drawing in immune effector cells and initiating a "danger signal." The secondary RV transduction could deliver a cytoreductive transgene aimed at tumor cell killing, to eliminate tumor tissue and further immunostimulate the tumor environment. A major limitation of the system is the requirement of active cell division in neighboring cell populations to enable RV transduction. Hence, inserting a gene in the Ad vector, separate from the retroviral cassette, could trigger cell division of neighboring tissues so that they become fully receptive to the subsequently available retrovirus. An alternative strategic context of application could be applied to tumors, where the actively dividing tumor tissue is generally surrounded by virtually quiescent normal tissue. Utilizing a highly regulated cytotoxic gene, under the control of an inducible or tissue-specific promoter system, the primary Ad infection would enable production of retroviral particles which in theory would selectively infect dividing tumor cells. Subsequent cytotoxic gene expression could, to some extent, restrict cell killing to tumor cells.

Ad-mediated delivery of an excisable closed circular RV cassette that can subsequently be integrated into the host genome would be of great value to gene therapy in the clinic. The system provides the potential to direct stable transgene expression in each primarily Ad-infected cell. This would be a significant advance on the previous Ad/RV hybrid system, where the secondary RV transduction is extensively reduced compared to the primary Ad transduction. Although the closed circular form is not the primary substrate for retrovirus integration, in the absence of the wild-type substrate, Int has been demonstrated to integrate such structures into the host genome. While the efficiency of such a system has still to be addressed, further elaboration of the integration mechanism could enable increased affinity of the RV machinery for closed-circle LTR proviral forms. The system would also have the potential of combinatory gene therapy by the inclusion of transgene cassettes within or outside the integrating RV cassette. Transgenes outside the excisable cassette would provide transient expression for the duration of the Ad genome retention in infected cells. The integrated cassette could provide stable expression for the lifetime of the cell. The major advance of this system will come from the development of highly regulated expression systems that can completely silence Cre expression. Silencing of Cre recombinase expression would enable assembly of all the vector components into a single gutless HD Ad vector. Although the system proposed by Wang and colleagues [122] goes some way to prevent expression, the system is still leaky and restricts therapeutic application to hepatic tissue.

The alternative strategy of maintaining a Cre-excised circular molecule by utilizing the EBV episomal replication system provides another potentially

powerful gene therapy vector, providing many of the advantageous properties detailed above. The Ad/EBV hybrid system would again require absolute control of Cre expression to combine all the components into a single vector. One limitation of this system is that EBNA-1 gene expression would have to be permanently maintained in the host cell, which could involve long-term cell regulatory or immunological problems *in vivo*. Conversely, the integration mechanism requires only transient expression of integrase to facilitate integration, and the transgene cassette is then maintained in the context of host cell chromosome replication. While the EBV replicon has the advantage of avoiding integration-related shutdown of transgene expression, other cellular factors are believed to be involved in the eventual loss transgene expression. EBV retention in human cells has been deemed limited and lost with time [123, 124]. Without drug selection, plasmids carrying the EBV elements are lost from human cells at rates of between 1 and 5% per generation [125].

The Ad/AAV hybrid vector system provides a powerful mechanism of maintained transgene expression by integration or episomal replication. The system also provides the potential of predictable integration at a specific locus in the human genome in the presence of Rep78. The establishment of targeted integration strategies introduces valuable safety features into a gene-therapy protocol. This advantageous property of integration also carries with it the potential hazard of insertional mutagenesis and the risk of activating cancer oncogenes *in vivo*. Although there are limited literature reports on the impact of such phenomena in a gene therapy protocol, as vector technology increases and the efficiencies of integration in human tissues are potentiated, these effects could become more significant. However, even in the context of the targeted integration of AAV, the exact phenotype of integration at chromosome 19*q*13.4, as well as the activity of genes integrated at such a locus are still to be determined.

The generation of the mini-Ad/AAV hybrid vectors enables the high-titer purification of adenoviral particles deleted of all the Ad genes, analogous to the HD rationale. The mechanism of preparation and purification, however, appear to be simpler. The Ad/AAV hybrid vector is applied to the producer cells as an Ad, which also supplies the helper functions. This bypasses the necessity of HD plasmid transfection and subsequent serial passage to enhance titers to enable purification from the contaminating helper virus. The extensive size difference of the derived mini-Ad/AAV genomes, from the parental Ad/AAV genomes, also enables more efficient purification by buoyant density on CsCl gradients. Additionally, any contaminating parental vector will be a functional Ad/AAV hybrid vector. The biological stability of these mini-adenoviruses, in terms of both particle stability outside the cell and genome stability within the cell, still needs to be addressed. Nevertheless, considering the integration of the ITR AAV cassettes, the mini-Ad genome stability is not as important. Additionally although the transduction efficiency of the mini-Ad particles is

similar to AAV, the ratio of total particles to infectious virions is enhanced, limiting their efficiency compared to conventional Ad vectors. The vector is also limited in terms of codelivery of the *rep* gene, which unlike the HD vectors cannot be easily incorporated into the same vector.

HSV-based hybrid vectors have also been well reported in literature, presenting a number of advantageous properties over the described Ad-based systems. The development of the HSV-1 amplicon technology and helper-free packaging systems has made HSV-based vectors very promising clinical tools for gene therapy [39]. The large insert capacity of the amplicons (150 kb) and the concatemer-styled packaging, with up to 10 genome copies per virion introduces powerful features to gene therapy vectorology [25]. HSV vectors, like Ads, have tropism for most cells in the human body, but have particular affinity for neuronal tissues. The HSV-1-based amplicons do not, however, retain the episomal maintenance functions of the parental herpes viruses and thus, as with Ad vectors, the genomes are rapidly lost in dividing cells. Hence, hybrid technology has been investigated to enhance the expression from HSV amplicon vectors. As with the Ad/AAV hybrid system, the presence of an AAV ITR-flanked cassette in the HSV amplicon vector can promote both extrachromosomal amplification and integration of the transgene cassette into the host genome. The HSV/AAV hybrid vector system has been demonstrated to stably transform dividing cells for over 25 passages in culture [126]. Hepatic transduction *in vivo* with an HSV/AAV hybrid vector supported gene expression *in vivo* for considerably longer periods than traditional HSV-1 amplicons, with minimal toxicity and immunogenicity [119]. An additional feature of the HSV/AAV studies was the placement of the *rep* gene under the control of its own promoter, as literature has reported potential down-regulation feedback inhibition of transcription when Rep levels increase [127]. Thus the natural expression machinery of Rep is utilized to regulate its expression. Compared to Ad vectors, the HSV amplicon vector titers are limited (10^7 to 10^8 iu/mL). Increased copy number can compensate for reduced titers in some fields of gene therapy, although for many corrective genetic therapies higher transduction efficiencies from higher titer viral applications may prove more efficacious. The reduced immunogenicity of the HSV-1 amplicons is, however, a major advantage over Ad vectors. Other hybrid vectors have also combined RV and EBV functions within the HSV-1 amplicons, which also have great potential as gene delivery vectors [123, 124, 128]

The development of hybrid viral vector systems has thus revolutionized the way gene therapy vectors are conceived. The combination of the advantageous properties of different vectors goes some way to establishing a vector system approaching the ideologies of a perfect gene transfer vehicle. The technologies are, however, in their infancy and many factors need to be elucidated before the full potentials of the vectors can be achieved. In essence, further detailed elucidation of the viral life cycles and their interactions with

host cell factors is necessary. Understanding these factors will allow vectors to be developed which can interact with the host cellular machinery to facilitate long-term stable gene expression. Future "hybrid" vectors will be developed quite distinct from the currently perceived parental vectors. Virtually synthetic viral vectors will be established with predictable biological properties, which can effect desired clinical functions. The vast array of clinical phenotypes and biological properties of target tissues involved in human disease will, however, require a wide spectrum of clinical vectors, fashioned to specific disorders. Nevertheless, the current advances in gene therapy technology will ensure that hybrid viral vectors will play a major role in future clinical protocols.

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CHAPTER 18

Utility of Adenoviral Vectors in Animal Models of Human Disease I: Cancer

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I. Introduction

The development of molecular therapeutics for the treatment of human disease has a rational and predictable course. Because these therapeutics are generally derived from an understanding of molecular mechanisms underlying a disease process, the treatment strategies are hypothesis-driven and specifically targeted toward a pathway underlying the molecular and cellular pathogenesis. Accordingly, in addition to establishing therapeutic efficacy, the evaluation of a molecular therapeutic also confirms the importance of a specific genetic or biological pathway in the *pathogenesis* of a disease process. The evaluation of a molecular therapeutic typically begins by providing a molecular/cellular proof of concept *in vitro*, followed by an expansion of therapeutic principles and toxicological analyses of the intervention in animal models, and finally a systematic sequence of safety and clinical efficacy trials in human subjects.

Logically, gene therapy paradigms using adenoviral (Ad) vectors can be expected to proceed along this course in order to be considered for the treatment of human disease. This chapter will focus on the use of animal

models in the process of evaluating adenoviral gene transfer strategies for the treatment of human cancer. In this respect, we will offer a personal perspective, concentrated on outlining principles rather than cataloging individual examples. Because the focus is on principles, the review will not be an inventory of the various experimental therapeutic strategies for cancer that utilize the adenoviral vector, although specific examples may be cited. Rather, we will use our background and experience to illustrate the problems inherent in testing experimental hypotheses in animal models of cancer, with the confidence that themes particular to our research may have broader applicability. Last, because the authors have an interest in utilizing Ad-gene transfer techniques for the treatment of lung and prostrate cancer, respectively, this chapter will emphasize experimental designs relevant to those clinical entities.

A primary goal of *in vivo*/animal experimentation is to build on an *in vitro* proof-of-concept and to strengthen the rationale for clinical testing of an experimental therapeutic intervention. To justify animal studies, there should already be an existent pathophysiological rationale and/or *in vitro* experimental data suggesting that a strategy is likely to be effective. At this juncture, the investigator is faced with the formidable challenge of approximating a human disease in an animal model. Although animal models cannot be exact replicas of the human disease, they should, at the very least, provide useful molecular and cellular similarities to the pathogenesis and clinical manifestations of the target disease [1]. For a variety of reasons (low expense for breeding and maintenance, susceptibility to tumorigenesis, well-defined immunosuppressive states, feasible duration of experimental studies, etc.) mice are considered to be the prototypic animal model for experimentation. Ideally, a mouse model would mimic the target human disease in its etiology, genetics, clinical presentation, and progression. To model human lung cancer, for example, the ideal mouse model would systematically (in defined pathological stages) develop lung cancer from exposure to cigarette smoke, and the disease could be characterized by sequential gene defects that culminate in the clinical progression that typifies the human disease.

Second, in designing the experimental approach, the investigator must also take into consideration the "pharmacological intervention or drug" (the Ad vector here) that is being tested. Because the ideal drug should have reliable delivery, specific targeted distribution and mechanism of action, and predictable elimination, the challenge to test an adenoviral vector based therapeutic in an apt animal model becomes particularly daunting for a gene therapist. Thus, to test whether an Ad-based therapeutic will have efficacy for the treatment of human cancer, we must (1) model the complex human disease *in vivo* and then (2) test a multifaceted biological compound with ill-defined pharmacokinetic and pharmacodynamic properties that are likely

unique to the host and/or the disease state. In order to overcome the inherent complexity of the problem, we have adopted an approach that uses a combination of models to overcome specific deficiencies that accompany each individually. Consequently, we utilize xenogeneic models (engraftment of heterologous tissue derived from donors of a different species, typically into an immunodeficient host) to study the therapeutic-gene effects and Ad vector–target cell interactions. Syngeneic (engraftment of tissue from genetically identical donors) and allogeneic (engraftment of tissues from a genetically dissimilar member of the same species) models are used to study host–tumor interactions in terms of immunological parameters and metastasis. To further discern the specific immunologic parameters important for tumor rejection in mice, specific knockout (targeted gene disruption) mice are utilized. Last, we extensively utilize transgenic (in this context, referring to the tissue-specific expression of a transforming oncogene) models to study the effects of a molecular therapeutic in the setting of established orthotopic (referring to organ- or site-specific) malignancy. We believe that integrating the results of these individual approaches will enable us to meet the goals of *in vivo* experimentation for advancing adenoviral gene therapy.

II. Animal Models of Lung Cancer

A. Human Lung Cancer

Lung cancer is the leading cause of cancer-associated mortality in *both* men and women. Although susceptibility to environmental carcinogens may be predetermined and follow a pattern of autosomal dominant Mendelian inheritance [2, 3], lung cancer results from an accumulation of acquired genetic mutations [4–6]. In fact, it is suggested that 10–20 genetic mutations may be necessary for the development of lung cancer [7], although the discrete steps for the progression of a hyperplastic bronchial lesion to metaplasia and anaplasia have not been uncovered. Tobacco use is the strongest epidemiologic risk for the development of lung cancer and it is anticipated that approximately 10% of all smokers will develop lung cancer over their lifetime [8]. Current paradigms predict that lung cancer results from the widespread exposure of the carcinogen, leading to a process of "field cancerization," whereby the entire aerodigestive track is exposed to the offending agents and leads to the occurrence of synchronous and metachronous tumors [9]. The tobacco carcinogens apparently invoke the multiple clonal chromosomal abnormalities found throughout the airways and alveoli of smokers [10, 11]. Following, the series of genetic mutations likely results in patterned aberrancies in signal transduction and cell-cycle pathways, eventuating in malignant and metastatic phenotypes [12]. The general pattern of genetic changes are characteristic but

not specific for pathologic subtypes of lung cancer (see below). Overall, *K-ras* mutations are observed in 20–50% [13], *p53* mutations are present in 50% [14], 60% exhibit reduced expression of *p16-ink4a* [15, 16], and 30% show deletion of *Rb*. Small-cell lung cancers (SCLC) display a greater proclivity to c-*myc*-amplification and a greater degree of *p53* (80%) and *rb* mutations (90%). Chromosome 3*p* deletions, occurring at a chromosomal fragile site that includes the *FHIT* locus, are found in 50% of non-small-cell lung cancers (NSCLC) and in 90% of SCLC primary tumors [17]. Overexpression of the tyrosine growth factor receptor *erbB2-neu* is seen in 10–30% and overexpression of *bcl-2* [18] in 10–25% of NSCLC tumors [19].

Clinically, lung cancer is discriminated into SCLC and NSCLC categories by histopathology or cytopatholgy and by their characteristic clinical presentations and divergent responses to conventional cytoreductive therapies. NSCLC may be further subclassifed pathologically into squamous cell (SCCa), adenocarcinoma, broncho-alveolar cell carcinoma (BAC), adenosquamous (mixed pathology), or large-cell carcinoma. As noted above, the progression of lung cancer from a premalignant state to the clinical/pathological entity that is diagnosed in the vast majority of patients is unknown. This is because although the disease is prevalent, it is typically diagnosed when it has already spread outside the lungs and is pathologically advanced. Not surprisingly, because of the late stage of diagnosis, progressive genetic instability confers *marked genetic and phenotypic heterogeneity* within lung cancers, even in individual patients. The late stage of diagnosis also results in an absolute lack of premalignant material, making it difficult to assign specific roles for the genetic mutations in the systematic progression of lung cancer. Recently, however, some of the characteristic genetic mutations of lung cancer (e.g., loss of heterozygosity at chromosome 3*p*, *p53* mutations) are being identified in microdissected dysplastic epithelium [20]. Similar observations are implicating the characteristic *K-ras* abnormalities in lung cancer as a correlate of mucinous differentiation [21]. A precursor to lung adenocarcinoma, a lesion pathologically termed alveolar atypical hyperplasia or AAH, is being advanced. AAH is described by increased cellular proliferation when compared to adjacent normal parenchyma and by immunohistochemical evidence of *p53* stabilization, *K-ras* mutations, and c-*erb-B2* overexpression [22–24]. The presence of these mutations in AAH may explain why such mutations may be detectable in sputum cytology specimens that predate the onset of clinical lung cancer [25]. Identification of these early events are a particular focus of study because they may serve as genetic markers for malignant progression, or as targets of specific genetic or chemopreventative approaches. More relevant to this discussion, perhaps, these early events may be better modeled in murine models than late stage lung cancer (see below). Thus, there exists an inherent complexity in human lung cancer, and to precisely recapitulate the disease process in animals is not practical.

B. Animal Models of Human Lung Cancer

1. Murine Lung Cancer and Transplantable Allografts

Due to time of model development, ease of experimentation, and cost restraints, murine models of disease are the accepted standards. However, there are generic shortcomings in this approach. For example, cigarette smoke, which is a strong epidemiological risk for the development of human lung cancer and is proximally responsible for approximately 85–90% of lung cancer cases in humans [26], is only weakly carcinogenic in mice [27, 28]. In addition, although both mouse and human lung adenocarcinomas may share common molecular defects [27], the histopathological repertoire of spontaneous or induced tumors in mice is very limited [29, 30], and morphologically, nearly all mouse lung tumors bear structural similarities only to BAC or well-differentiated adenocarcinomas. Consequently, whereas humans typically die from lung cancer of "late stage" metastatic disease, mice succumb to respiratory failure following the diffuse involvement of their lungs by "early stage" carcinoma *in situ* [1].

Spontaneous lung cancer develops in 3% of wild mice [31, 32] with strain-dependent sensitivity. Clones have been isolated from spontaneously arising tumors, and established as cultures *in vitro*. These cultures now serve as a readily available source for the generation of transplantable allografts. Many investigators, including our group [33–38] have extensively utilized line 1 alveolar carcinoma (L1C2), a murine lung cancer cell line that is syngeneic to BALB/c, and 3LL (Lewis lung cancer), which is syngeneic to C57Bl/6. Usually, these cell lines are utilized to generate transplantable heterotopic (referring to a location outside of the organ of origin, typically subcutaneous) tumors in syngeneic mice. Our group has utilized these models to investigate, in general, the interplay between the immune system and the host. Both L1C2 and 3LL tumors are relatively "nonimmunogenic," as is human lung cancer, and immunogenetic strategies that modulate the immune system to generate an anti-tumor immune response can be systematically investigated in these models. However, other lung tumor-allografts, especially when cells are selected to express "marker antigens" to enable their easy detection in culture systems, may indeed become immunogenic. Notably, the transplantable allograft system is artificial, and all recipient hosts have a "stress" response to the implanted tumor that cannot be recapitulated in control animals. In addition, extrapolating anti-tumor responses in mice to humans is not a straightforward proposition, and many therapies that reliably "cure" tumors in inbred strains of mice are not as effective in humans. In part, these differences may be attributable to differences in immune responses in the two hosts. For example, cluster determinants (CD-antigens) in murine strains may not have homologous or functional cellular analogs in the human host.

Laboratory animals used for medical experimentation are genetically inbred strains with reliable phenotypic characteristics. Although this feature

imposes a generic limitation on the extrapolation of results in lab animal studies to outbred populations, and thus, human disease, there are significant advantages that need to be considered. The inbred nature of laboratory animals enable investigators to reliably establish disease in an animal host, and subsequently to study that disease process in controlled subsets. With respect to tumorigenesis, murine-A/J and SWR strains are the most sensitive, BALB/c is of intermediate sensitivity, and DBA and C57BL/6 are the most resistant. Crosses between susceptible and resistant inbred mouse strains may allow for the mapping of modifier loci for the development of lung cancer [39]. For example, it is reported that the propensity of strains to develop lung tumors correlates with a polymorphism in the second intron of *K-ras* [40]. Practical experience suggests that there are common genetic alterations affecting known tumor suppressor genes and proto-oncogenes occur during mouse lung carcinogenesis. Molecular abnormalities may also be shared with human lung cancer, and *K-ras* activation is a conspicuous example [41]. Human adenocarcinomas commonly carry *K-ras* mutations; most of these mutations are in codon 12 and are transversions of GGT to either TGT or GTT. It is postulated that these mutations occur early in lung cancer pathogenesis since they can be detected in sputum samples of smokers prior to the clinical diagnosis of lung cancer. Analogously, 80 to 90% of both spontaneous and chemically induced murine lung tumors contain *K-ras* mutations. Moreover, *K-ras* mutations also occur early in murine lung tumorigenesis, and remarkably, codon 12 is the site of genetic change induced by many chemical carcinogens [1]. Furthermore, a consistent loss of mouse chromosome region 4, an area that contains the mouse homolog of the human *p16-ink4a* [42, 43], has been described to result in an allelic loss of the *p16-ink4a* seen in 50% of mouse adenocarcinomas. Similarly, *p53* mutations are found, albeit infrequently [44], although mouse chromosomal regions containing *p53* and *Rb* more commonly exhibit LOH [43]. Reduced expression of *Rb* and *p16* and increased *c-myc* expression [39] have also been reported. These commonalties have suggested some to conclude that mouse and human lung carcinomas are sufficiently similar for the murine model to be informative [1], and have formed the rationale for the testing of chemopreventative strategies [39] in mice. Analogously, these commonalties may be advanced to form the basis for the testing of genetic therapies in murine tumors as well.

Mice strains also vary with respect to inducible-tumorigenesis. Generally, mice that are sensitive to the development of spontaneous lung tumors are also at the highest risk for chemically induced tumors [31] and form the basis for the quantitative carcinogenecity bioassays. Although a variety of agents, including urethane, metals, and concentrated components of tobacco smoke such as polyaromatic hydrocarbons and nitrosoamines [45, 46], can induce lung cancer in mice, tobacco smoke per se is only weakly carcinogenic [28]. Murine lung

tumors histologically resemble early lesions that originate peripherally (from type 2 alveolar cells or Clara cells) and simulate papillary or bronchioloalveolar cell cancer (BAC). In contrast, the bulk of human tumors are bronchogenic (arise in the airways) and, as described above, display a broad histopathologic variation. In fact, individual human lung cancers may be histologically heterogeneous; i.e., they often display mixed morphologies within the same tumor specimen. So how does one reconcile these differences between murine lung cancer and human lung cancer, and moreover, can one generalize observations and results from one species to another, or even from one human being to another? When considered in the context of adenoviral gene delivery, there is a limiting paucity of *in vivo* data to generate any broad conclusion. On the contrary, our observations *in vitro* suggest that gene transfer into subtypes of human lung cancer is highly variable, and strategies directed toward achieving intratumoral gene transfer may require patient or disease-specific vector formulations [47].

The biological heterogeneity of human lung cancer drives our investigations along specified pathways, utilizing many different models and strategies to come up with viable treatment approaches. For instance, we believe that a systematic assessment of the efficiency and optimal route of adenoviral gene delivery *in vivo* into murine lung tumors and transplanted human xenografts needs to be performed. Researchers are beginning to identify the Ad-cellular attachment receptor (termed the Coxackievirus-adenovirus receptor (CAR) [48]) as a major determinant underlying efficient transduction [49]. Along these lines, the scope and "polarity" of CAR expression in tumors *in vivo* needs to be defined. Thus, one focus of our program is to systematically evaluate gene transfer into these model systems using conventional and retargeted adenoviral vectors with the aim of optimizing a vector system and a mode of delivery. This focus evolves from the premise supported by our *in vitro* data that the histological heterogeneity of lung tumors may be a harbinger of variable responsiveness to both adenoviral entry and/or the efficacy of adenoviral gene therapy [47]. Because uniform targeting of tumor *in vivo* may be unattainable, we have also generated protocols in which the Ad-vector is used in precisely controlled *ex vivo* "dosing" approaches to genetically modify antigen-presenting cells (APCs) or tumor cells to vaccinate the host against their tumor [37].

2. Murine Models That Spontaneously Develop Lung Cancer

Murine models of lung cancer include strains susceptible to chemically induced tumors and transgenic strains that express viral and cellular oncogenes. The simian virus-40 large T-Ag (SV40-TAg) has been commonly used to produce tumors in transgenic mice [50, 51]. SV40-TAg binds and incapacitates the cell cycle checkpoint and DNA-binding capabilities of the *p53*

and *Rb* gene products, resulting in uncontrolled cellular proliferation [52]. To develop a murine model of lung cancer, Wikenheiser and colleagues chose to express the SV40-TAg under the transcriptional control of the lung-specific human surfactant protein C (SP-C) promoter in transgenic mice [53, 54]. They demonstrated that these mice consistently developed multifocal lung adenocarcinomas that had pathological features similar to some human lung adenocarcinomas, and that the mice succumbed to respiratory distress by age 4–5 months. As expected, the transgenic animals developed no tumors in any other organ systems, although some nonmalignant tissue also expressed the transgene [53]. Within the lungs, tumors consistently involved the bronchiolar and alveolar regions of the lung while sparing the large airways. The tumors of these mice also varied with respect to the expression of the large TAg, suggesting perhaps that SV-40 TAg may contribute to transformation, but continued expression may not be necessary for tumor progression. Likewise, organ-specific expression of SV40-TAg using the regulatory regions of uteroglobin [55] and the Clara cell-specific M_r 10,000 protein (CC-10) also results in the induction of lung tumors [56]. Uteroglobin is a marker protein for the nonciliated epithelial Clara cells, the source of xenobiotic metabolism in the lung, lining the respiratory and terminal bronchioli of the lungs. In animals expressing SV40-TAg under the uteroglobin promoter control, the pulmonary epithelium was morphologically normal at 2 months, dysplastic by 4 months, and transgenic animals were described as developing multifocal pulmonary adenocarcinoma present in various stages of differentiation by 5 months of age. *In situ* hybridization studies suggested that tumors did not contain the transcripts of the uteroglobin gene, and again, late stage tumors lost expression of the large T-Ag. Tumors also formed in the urogenital tract where uteroglobin is also expressed.

Transgenic mice were also generated using the CC10 kDa promoter driving SV-40 large T-Ag [56], and it is in this model that we have chosen to test the immunomodulatory capacity of secondary lymphoid chemokine or *slc* [36]. In the 7736 mouse line, CC-10TAg-transgenic mice develop multifocal pulmonary adenocarcinomas and succumb to respiratory failure at 16–20 weeks of age. Pathology is localized to the lungs, and the tumors express the large T-Ag in normal Clara cells and in transformed tumor cells. Pathological progression is similar to that described above, with the lungs appearing morphologically normal at 2 months of age, a number of tumor foci are grossly discernable by 3 months, and the majority of the lung is replaced by coalesced nodules by 4 months of age. As tumor progresses, the expression of endogenous CC10 expression diminishes, and there is increased nuclear p53 expression, suggesting binding and stabilization of the protein by the large T-Ag [56]. From our standpoint, we have found that the reliable progression of lethal tumors in these transgenic mice enable us the test a number of hypotheses, dosing schemes, and dosing routes. Importantly, the effects of immunomodulation by

the gene transfer of specific cytokines and chemokines into tumor cells *in vivo* can be determined. Moreover, one can compare this direct-delivery strategy with alternative approaches, including *ex vivo* modification of autologous APCs using recombinant Ad-vectors. The subsequent reintroduction of gene-modified APCs back into the tumor environment overcomes the inability of dendritic cells to maturate in the presence of tumor *in vivo* [57] by providing functional APCs that are capable of processing and presenting tumor antigens to cytolytic T cells *in vivo* [6].

3. Murine Models with Transplantable Xenografts

Xenotransplantation of human tumors into immunocompromised mice began in the late 1960s [58] following the discovery of the nude mouse in 1962 and its characterization as an athymic mutant in 1968 [59]. The morphologic and karyotypic stability of tumors serially passaged in nude mice was described [60], and it was established that xenotransplanted tumors in nude mice often retained distinctive phenotypic and functional characteristics found in the human host [61]. However, the "tumor-take" rate for nude mouse xenotransplants is tumor-specific, and generally, carcinomas are more difficult to establish than melanomas or sarcomas [62]. Thus, progressive tumor growth from inoculated primary tumors (i.e., cultured directly from the patient) is observed in only 33% for lung cancers [61, 63] and is virtually nil for primary breast or prostate cancers. In addition to properties inherent to the tumor, nude-mouse-related factors also impact on tumor take. For example, mice infected with the mouse hepatitis virus do not accept xenotransplants, presumably because of enhanced NK-cell activity [64]. In this regard, it is important to recall that although nude mice lack functionally mature T cells, they are capable of mounting normal humoral responses to T-cell-independent antigens [65] and they exhibit high NK-cell activity [66], and these properties probably impact negatively on the tumor-take rate of xenotransplants. The high NK-cell activity also abrogates the metastatic potential of implanted tumors, and the incidence of metastasis is higher in mice with lower NK cell activity, e.g., young (3-week-old) syngeneic mice or the beige (bg^j/bg^j) mutants derived from the C57BL/6 mice [67, 68].

The discovery of a severe combined immunodeficiency in mice [69] offered yet another option for hosting human tumor xenografts. The scid/scid mice are characterized by the virtual absence of functional T and B lumphocytes due to aberrancies in the rearrangement of antigen receptor genes [70]. The first successful engraftment of human solid organ tumors into *scid* mice began with the subcutaneous inoculation using the A549 lung adenocarcinoma cell line [71]. Since that time, a variety of human solid-organ cancers, both from cell lines and primary tumor specimens, have been successfully engrafted [72]. The higher rates of successful engraftment, presumably because of the lack of residual B-cell function in *scid* mice, have led many investigators to prefer

scid/scid mice over *nu/nu* mice as the host recipients of human xenograft tumors. Xenografts are still impacted upon by the *scid* host's innate immunity, and NK and monocyte/macrophage activities can be upregulated in these hosts. For specific needs, selective breeding of other available mutants (beige mutants with reduced NK-cell activity and osteopetrosis with altered macrophage differentiation) enables the generation of strains that harbor overlapping defects in immune function [73]. Furthermore, genetic engineering and gene-targeting technology has helped create murine-mutants with exquisitely specific immune defects, including mice in which CD4 or CD8 T cells are deleted [74] and mice which lack β-2 microglobulin and thus do not express transplantation antigens [75].

Xenotransplants have many advantages, the primary being that they provide a replenishable source of human tumor. This enables the genetic characterization and gene discovery of tumor-specific phenotypes and, in rare occasions, the progression toward an advanced or metastatic phenotype of the tumor (e.g., from an androgen-dependent prostate tumor to one that is androgen independent, see below). Xenografts incorporating human tumor cells in immune-deficient mice are plentiful. For example, we have developed a novel animal model mimicking intrapleural malignancy that allows for a controlled, focal dosing of reagents and evaluation of therapeutic benefit [76]. The model is composed of 2.5-cm segments of rat intestine that is denuded, and then everted so that the serosal surface is converted into the lumenal surface of a tube. Lung cancer cells are instilled into the lumen via a polyethylene cannula on day -1, allowed to adhere to the serosal surface overnight, and this tubular xenograft is implanted into the interscapular subcutaneous tissue of a nude mouse on day 0 [76]. The graft simulates metastatic tumor growth on the pleural surface *basal lamina* both grossly and histopathologically and enables robust quantitation of tumor kinetics [76]. The appearance of tumor on this surface is nodular, and these nodules coalesce over time with intervening fibrous stroma. Neovascularization is evident on histological exam of the graft, and tumor growth is continuous with a variety of NSCLC cell lines. We have found this model to offer certain tangible advantages. For example, with respect to the transduction characteristics of tumor, the value of this model is evidenced by the following: (1) the cells are representative of human lung cancer; (2) the location of the tumor is precisely known and tumor is directly accessible; (3) the vast majority of cells that repopulate the graft are derived from those instilled (host leukocytes and fibrocytes comprise the remaining minority); (4) the mode of delivery of reagents (fluid inoculation rather than intratumoral injection) is designed so as to be clinically applicable for installation into pleural space; (5) the size of the xenograft enables quantitative assessments of transgene expression and morphometry simultaneously, containing human tumor into nude mice [76].

C. Gene Therapy of Lung Cancer Using Adenoviral Vectors

1. Gene-Based Therapies Targeting Molecular Transformation

Abnormalities at the cell surface (e.g., *erbB2*), signal transduction (e.g., *ras* -oncogene), gene regulation and cell cycle control (e.g., *p53*, *Rb*, *c-myc*-oncogene), or apoptosis (e.g., *p53*, *BCL-2*) are all implicated in the process of transformation and can serve as targets for rational therapeutic intervention. For example, to overcome the deficits due to mutated *p53*, one strategy for lung cancer gene therapy has opted to replace the mutated *p53* gene with a normal copy [77]. Restoring *p53* function in these cells has led to decreased tumorigenicity of human cancer cells *in vitro* and in animal models [78, 79]. Based on these preliminary studies, the first clinical gene therapy trial for human NSCLC also utilized a *p53*-gene transfer strategy [77]. In this study, nine patients with advanced NSCLC were treated with either bronchoscopic or percutaneous CT-guided injections with a retroviral *p53* expression vector (a genetically reengineered retrovirus that is designed to integrate into the cell genome and express the normal *p53*-protein). Of the seven patients evaluated, three showed evidence of tumor regression at the treatment site and six showed increased apoptosis of tumor cells on posttreatment biopsies. Importantly, there was no significant toxicity associated with the therapy, and *in situ* gene transfer was achieved. However, limited therapeutic efficacy was observed and the mechanisms responsible for the anti-tumor effects are still under study. For example, although it was originally believed that mutated *p53* function would have to be compensated in each and every cell for restoration of the normal apoptosis-program, the results suggested otherwise. Because there was substantive tumor regression despite poor *in situ* gene transfer, mechanisms for the observed "bystander effect" were hypothesized [80]. The term "bystander effect" refers to the ability of gene-modified tumor cells to mediate killing of neighboring nontransfected cells. One plausible explanation is that wild-type *p53* induces release of angiostatic factors, thus undermining the blood supply to the tumor [81]. In addition, the expression of *p53* may also contribute to an immune-mediated response [82, 83]. These issues have led to more mechanism-based bench and animal studies, as well as other phase 1 clinical trials using Ad vectors encoding the *p53* gene for a variety of cancers, including lung tumors [84].

Because of the high frequency of *p53* mutations, another strategy that uses *replication-competent viruses* has been hypothesized to be ideally suited for lung cancer. This approach employs adenoviruses (mutant dl1520 or ONYX-015) that are suggested to selectively replicate in *p53*-mutated (therefore, selectively in cancer) cells [85, 86]. Consequently, these mutant viruses are promoted as "magic bullets" that kill tumor cells and leave normal tissues intact. This particular approach has generated considerable controversy

both in terms of its reputed efficacy as well as its proposed mechanism of action [87–89]. In brief, its effectiveness in both *in vitro* and *in vivo* models of lung cancer needs to be confirmed. Nevertheless, the approach represents a prime example of a novel hypothesis-driven strategy that attempts to exploit the biology of a mutant virus to clinical advantage.

2. Immunogene Therapy

Effective immunotherapy has the potential for *systemic* eradication of disease, a payoff that is especially enticing for the treatment of lung cancer. Previous, largely unsuccessful immunomodulatory campaigns utilized nonspecific immune strategies (e.g., BCG adjuvants). Increasingly, the interest now is in developing *specific* immune interventions for lung cancer. The major obstacle for effective immunotherapy of lung cancer has been a meager understanding of the immunobiology of this disease. However, a better understanding of the reciprocal interaction between the tumor and the immune system is starting to emerge, lending itself to plausible hypotheses for intervention. We realize that an effective anti-tumor response may either provoke the immune system to recognize and attack the tumor, or conversely, it may serve to reduce the immunosuppression encumbered upon the host by the tumor.

Specific and effective anti-tumor immunity requires both *adequate tumor-antigen presentation* and the subsequent *generation of effector lymphocytes.* A variety of cytokines have been investigated to implement such a program *in situ* [90–97], and many of the studies have utilized the Ad-vector for gene delivery. For several reasons, our efforts have focused on IL-7, IL-12, and more recently on the chemokine *slc*, for the treatment of lung cancer. The rationale underlying the use of these particular cytokines and chemokines is that they all optimize conditions for tumor antigen processing and presentation by the host's APCs, and they help appropriately localize and sustain the effector lymphocytes response [36, 37, 97, 98].

Although the cellular infiltrates differ depending on the cytokine and model used, many studies indicate that tumor cells that have been transfected with cytokine genes can generate specific and systemic antitumor immunity *in vivo*. Based on these promising animal studies, what prevents these strategies from being translated into successful and curative human clinical trials? One major problem in human cancer patients may be that although lung cancers express tumor antigens [99], they are ineffective as APCs [100]. Tumor cells cannot function as APCs because (i) they lack costimulatory molecules, (ii) they are unable to adequately process Ag, and (iii) they secrete a variety of inhibitory peptides which promote a state of specific T-cell anergy. Thus, even for highly immunogenic tumors, professional APCs are required for antigen presentation [101]. As described above, local augmentation of IL-7 and IL-12 may help to overcome some of these defects [37]. In addition, one may bring into the tumor environment professional APCs to orchestrate a satisfactory

immune response against the tumor. In this regard, dendritic cells (DCs) are potent APCs that are ideal for interacting with and activating naive T cells to generate antigen-specific immunity [102, 103]. Recent advances in the isolation and *in vitro* propagation of DC has stimulated great interest in the use of these cells for clinical cancer therapy [104, 105]. In such approaches, DC may be envisioned to serve as vehicles for genes expressing antigens [106] or expressing cytokines in lung cancer gene therapy [33]. In addition, DC-based immunogenetic therapies may be used in combination with other strategies that have been optimized for Ag presentation [34, 37]. Importantly, of the various approaches tested to gene modify the DCs, our colleagues at UCLA have determined that the Ad-vector is best suited for DC-transduction [107].

3. Targeting Tumor Invasion and Angiogenesis

Overcoming metastatic disease is paramount for effective lung cancer therapy, and the biology underlying metastasis is gaining clarity. Metastasis is a process involving several complimentary yet distinct elements, including the capacity for tumor cells to invade and traverse the basement membrane, and to reestablish viable tumor foci in distant organs. Each step in this process may serve as a point for therapeutic intervention in lung cancer. As the molecular biology becomes better understood, the opportunity to incorporate specific genes into vector systems invariably materializes. The initial step, tumor invasion, requires proteolysis, which has been suggested to be mediated by an overexpression and secretion of matrix metalloproteinases (MMPs) by lung cancer cells [108–111]. Therapeutically, gene transfer strategies have incorporated tissue inhibitors of metalloproteinases (TIMP) to inhibit invasion and metastasis [112], or have utilized antisense abrogation of MMPs to inhibit tumorigenicity [113].

Similarly, angiogenesis (induced growth of blood vessels) is suspected to be critical for tumor survival and progression at each stage of metastasis [114]. Angiogenic progression in lung cancer is felt to be due to an imbalance of angiogenic and angiostatic factors, and the risk of metastasis in NSCLC directly correlates with the extent of tumor-derived angiogenesis [114]. Thus, strategies that inhibit of angiogenic mediators or restore angiostatic factors have potential utility for all stages of lung cancer [115–119]. The important mediators implicated in promoting or inhibiting angiogenesis lend themselves favorably for inclusion into gene therapy strategies. For example, recent studies indicate that vascular endothelial growth factor (VEGF) is an important angiogenic factor produced by a variety of tumors, including lung cancer. Lymph nodes with NSCLC metastases express significantly higher levels of VEGF than do normal, uninvolved nodes [120], consistent with the speculation that VEGF plays an important role in the metastasis of lung cancer. In addition to VEGF, recent studies have also implicated CXC chemokines in the abnormal angiogenic/angiostatic balance in NSCLC [121]. Members of

this family containing the ELR motif (e.g., IL-8) are angiogenic, whereas those that lack this motif (e.g., interferon-inducible protein 10; IP-10) are angiostatic. Accordingly, neutralizing antibodies to IL-8 reduce angiogenesis and consequently the growth of human lung tumors in *scid* mice [122].

Other molecular strategies to specifically target angiogenic vessels are also being developed. For example, the adhesion protein $\alpha v\beta_3$ is relatively specific for angiogenic vessels where it mediates endothelial cells interaction with extracellular matrix components [123] and enables cell motility [124]. Importantly, its blockade can promote tumor regression *in vivo* in lung cancer models by inducing apoptosis of tumor-associated blood vessels [125]. More recently, phage-display peptide libraries, which are used to screen the specific binding of a massive array of peptides, have isolated small peptides which selectively bind to receptors (including $\alpha v\beta_3$) on angiogenic vessels. Conjugating these peptides to chemotherapeutic agents have enabled investigators to specifically target tumor vasculature and abrogate tumor growth [126].

4. Adjuvants to Conventional Therapeutic Approaches for Lung Cancer

Conventional multimodality therapy for lung cancer incorporates surgery, radiation, and chemotherapy using a variety of clinical protocols dictated by the subtype and extent of disease. Theoretically, gene therapies may play important synergistic roles in augmenting the effectiveness of conventional approaches. For many such strategies, there already exists a scientific rationale to test them in combination with conventional multimodality therapy. For example, one may enhance the radiation-sensitivity or chemosensitivity of tumor cells (e.g., *p53* or IκBα gene therapy) [127, 128] or modify normal tissue susceptibility to cytoablative therapy (e.g., mucosal/tissue protection: by virtue of *MDR-1* or *bFGF* gene transfer). Examples of synergism with the suicide gene therapy approaches have also been studied. The HSV thymidine kinase gene/ganciclovir system induces radiation sensitivity into transduced tumor cells [129], suggesting that these two forms of therapy can be combined to potentiate antitumor responses [130]. Similarly, tumor cells transduced with the cytosine deaminase transgene exhibit enhanced radiation sensitivity following pretreatment with 5-fluorocytosine [131]. Because the loss of *p53* function can result in tumor resistance to ionizing radiation [132], restoring *p53* function may restore apoptotic pathways and promote effective radiation or chemotherapy. In fact, gene transfer of wild-type *p53* has been shown to enhance radiation sensitivity [133] and can act synergistically with *cis*-platinum-based chemotherapy to augment cytotoxicity [134].

Many of the approaches outlined above as being strategies for gene therapy of 'lung cancer" are generic; these approaches can be generalized to a variety of malignancies since transformed cells have in common the same aberrant growth regulatory and signal transduction pathways. The molecular

and cellular pathogenesis of tumor invasion and immune evasion are also similar between tumors originating in diverse organ systems. Unfortunately, this commonality may not confer a broad-based advantage when gene therapy strategies are advanced clinically. In this respect, vectors need to provide both efficient gene delivery as well as tumor specificity, and as a result, the gene transfer strategies have to become "disease specific." Targeted vectors (as discussed elsewhere in this compilation) have to incorporate features rendering them capable of selective cell surface adherence or entry or, alternatively, express their therapeutic transgenes under tumor-specific regulation. Unfortunately, a lung cancer-specific cell surface target (for transductional targeting) has not been identified, and one is left trying to use targets that are generally overexpressed in tumor cells or tumor-induced endothelium [135, 136]. Similarly, lung cancer also does not express a specific tumor marker. Thus, transcriptional targeting approaches largely utilize elements that are "tissue-specific" rather than "cancer-specific." Accordingly, constructs where transgene expression is regulated by tissue-specific promoters (e.g., SLPI, SP-A, CC-10) are being actively developed and tested.

III. Animal Models of Human Prostate Cancer

A. Human Prostate Cancer

After lung cancer, cancer of the prostate (CaP) is the second most common cause of cancer death in American males. A latent disease, many men have prostate cancer cells long before overt signs of the disease are apparent. The annual incidence of CaP is over 100,000 in the United States, of which over 40,000 will die of the disease. Nearly a third of patients present with locally advanced or metastatic disease, and androgen deprivation therapy forms the basis of conventional therapy for the majority of these patients. However, currently available approaches for advanced CaP are not curative [137], primarily because the cells lose their dependence on androgenic stimulation. The mechanisms of progression of CaP cells to hormone independence under androgen ablation therapy remain unclear. To investigate the factors and mechanisms that underlie the development of androgen resistance and metastasis, reliable *in vivo* models that mimic human CaP progression are essential. Moreover, it is critical that tumor models mirror the pathology and cellular and molecular characteristics of human CaP if it is to serve as a useful tool for basic research, drug screening, or the evaluation of new therapeutic strategies.

B. Spontaneous and Transgenic Models of Human Prostate Cancer

Currently, a single animal model cannot epitomize the multifaceted aspects of CaP pathogenesis and progression. Rodent models of prostate

carcinoma have been developed by hormone treatment [138], spontaneous development [139], transgenic prostate-specific oncogene expression [140], and knockout of CaP-tumor suppressor genes [141]. However, these models are largely inadequate in recapitulating the progression of human disease as bone metastasis, [142] the major cause of clinical morbidity attributable to CaP. Despite pitfalls, the mouse transgenic TRAMP model has been useful for studying the development and progression of prostatic adenocarcinoma. TRAMP mice, generated by expressing SV40-T antigen specifically in prostatic epithelium [140], develop prostatic intraepithelial neoplasia (PIN) by 10–12 weeks of age and eventually progress to adenocarcinoma with metastasis to lymph nodes and lungs [143]. As in human disease, androgen ablation therapy in these mice contributes to the emergence of androgen-independent disease with a poorly differentiated phenotype [144].

C. Xenograft Models of Human Prostate Cancer

As for lung cancer, investigators have chosen a number to utilize xenograft models of CaP. Unfortunately, CaP xenografts are far more fastidious than lung cancer xenografts, and the generation of models that are representative of typical human disease has only recently been accomplished. Until recently, the majority of research conducted for CaP relied on the cell lines PC-3, DU145, and LNCaP. Among these, only LNCaP cells exhibit androgen responsiveness and express the prostate-specific antigen (PSA) and androgen receptor (AR). Thus, the relevance of DU-145 and PC-3 cells to clinical CaP has been questioned. To overcome the shortage of representative models of human CaP, a number of investigators began establishing xenografts in immune-deficient *scid/scid* mice using samples obtained directly from patients [145–149]. These xenografts offered the following advantages: (1) the expansion of small amounts of starting clinical material, (2) the enrichment of relatively homogeneous cell populations from heterogeneous tumor cell populations, (3) the ability to investigate progression to metastasis and androgen independence [145, 146, 148], and (4) representative diversity that provided a more realistic picture of the heterogeneous nature of this disease. Investigators at UCLA established six distinct CaP xenografts from patients with locally advanced or metastatic diseases into *scid/scid* mice. Two of these xenografts, LAPC-4 and LAPC-9, have been maintained continuously for more than 2 years by serial passage in *scid/scid* mice [145, 146], and LAPC-4 has also been successfully established as a cell line in tissue culture to enable correlation with investigations performed *in vitro*. [145]. LAPC-4 and LAPC-9 offer several advantages over previous models; both express the wild-type androgen receptor (AR), both xenografts have intact AR-signal transduction pathways, and both secrete high levels of the androgen-dependent protein PSA. Accordingly, they grow as androgen-dependent cancers in male *scid* mice

and respond to androgen ablation treatment, but interestingly, they eventually progress to a hormone-refractory, androgen-independent state [145, 146]. LAPC-4 and LAPC-9 can be implanted subcutaneously, orthotopically into the mouse prostate, or intratibially. Orthotopic tumors metastasize reproducibly to regional lymph nodes and lung, providing an opportunity to study prostate cancer metastasis. Intratibial injection results in the formation of osteoblastic tumors typical of human CaP where bony metastasis is the major cause of morbidity.

From a research standpoint, the generation of these xenografts has provided significant dividends. Given the inability to culture CaP by other means, the xenografts have been used to identify chromosomal abnormalities and to pinpoint the genes important to the pathogenesis of CaP. For example, loss of chromosome 10*q* was a frequently observed genetic defect in prostate cancer. Recently, the PTEN/MMAC tumor suppressor gene was identified and mapped to chromosome 10*q*23.3 [150, 151]. PTEN encodes a protein/lipid phosphatase which has been clearly established to function as a negative regulator of the PI3-kinase/Akt signaling pathway [152–158]. Loss of PTEN leads to constitutive activation of PI3-kinase, and in turn the Akt-signaling pathway [158]. PI3-kinase is also a downstream target of several growth factors implicated in CaP pathogenesis including epidermal growth factor receptor (EGFR), insulin-like growth factor receptor (IGFR) and Her2/neu, and it is possible that deregulation of this pathway in PTEN-deficient cells may indeed be responsible for the cancer phenotype. Of note, knockout mice lacking PTEN as a consequence of targeted deletion develop multiple cancers, including prostatic hyperplasia and prostatic intraepithelial neoplasia [141, 159]. Correspondingly, 50–60% of all prostate cancer xenografts established contain deletions, mutations, or absent expression of PTEN [160, 161], making the xenografts a relevant and valuable source for biological and therapeutic discovery. Prostate cancer gene therapy approaches that specifically target this pathway are now underway in these models.

In addition to modeling the abnormalities of the PTEN/MMAC pathway, xenografts are important in delineating the role of androgens and androgen receptor (AR) signaling in CaP. Prostate epithelial cells utilize androgen as a growth and differentiation factor and are dependent on androgen for survival. Once transformed, androgen deprivation is associated with a transition of CaP cells through a range of diminishing androgen-dependence, and ultimately androgen independence. Although not well understood, this process likely involves perturbations in AR signaling of cellular growth control. Potential AR-related perturbations may involve (1) AR mutation or gene amplification, (2) cross-talk between AR and other signal pathways, and/or (3) alterations in transcriptional coregulators. Greater than 80% of clinical CaP specimens have confirmed AR expression, even in advanced androgen-independent diseases [162, 163]. Among these, AR-gene mutation or amplification has been

documented in 20–40% of CaP cases [164–166]. Both LNCaP and the CWR22 xenografts bear AR mutations that enable the receptor to be activated by nonandrogenic steroid hormones such as progesterone and estrogen. In addition, in a patient who had failed androgen ablation, it was recently demonstrated that his CaP-cells possessed a mutated AR with altered ligand affinity. Essentially, the mutant AR functioned as a high-affinity cortisol receptor, enabling the CaP cells to circumvent the androgen requirement for growth [167]. Another emergent theme is that some hormone refractory cancers have activated the AR signaling pathway through a ligand-independent mechanism. For example, in LAPC-4 cells expressing wild-type AR, the overexpression of *Her-2/neu* has been shown to activate AR [168]. Not surprisingly, the LAPC-4 xenograft progresses to androgen-independence after androgen ablation and differential gene expression studies reveal a consistent increase in *Her-2/neu* protein expression in androgen-independent tumors. Furthermore, forced overexpression of *Her-2/neu* in androgen-dependent CaP cells is sufficient to confer androgen-independent growth *in vitro* and to accelerate androgen-independent growth in castrated animals. Thus, *Her-2/neu* overexpression activates the AR signaling pathway in the absence of ligand and enhances the magnitude of AR response in the presence of low levels of androgen. Last, reconstitution experiments in a heterologous cell type expressing low levels of endogenous AR suggest that these effects of *Her-2/neu* on the AR pathway require AR-expression [168]. Although the point where *Her-2/neu* and AR pathway intersects is still undefined, nuclear receptor coactivators might be potential targets since amplification of steroid receptor coactivator, AIB1, is documented in breast and ovarian cancer [169]. Cross-talk between *Her-2/neu* and AR signaling pathways should provide a novel mechanistic insight into the development of androgen independence.

D. Gene Therapy Approaches with Adenovectors in Prostate Cancer

Recombinant Ad vectors are most commonly used for CaP because they have demonstrated the capacity to deliver genes intraprostatically in animal models [170]. Hence, several ongoing human CaP clinical gene therapy trials are using Ad [171, 172]. With respect to these applications, several groups are developing transcriptionally targeted prostate-specific Ad [172–175]. These strategies are beneficial in gene therapy applications in that they potentially restrict the expression of cytotoxic therapeutic genes to the malignant cells. Most commonly, the kallikrein-protease prostate specific antigen (PSA) gene regulatory regions have been used to direct prostate-specific expression because prostate epithelia, normal or malignant, specifically express the PSA [176]. Unfortunately, the transcriptional output from the native PSA enhancer and promoter (as from most highly regulated tissue-specific promoters) is much lower than from strong constitutive viral promoters such as CMV. For example,

our studies suggest that the native PSA enhancer and promoter inserted into Ad can direct tissue-specific and androgen-inducible expression in LNCaP cells, but the transcriptional activity is 50-fold lower than the constitutive CMV promoter [Wu *et al.*, unpublished data].

By exploiting the known properties of the native PSA control regions, we have improved the activity and specificity of the prostate-specific PSA enhancer (Wu *et al.* unpublished data). Previous studies had established that AR molecules bound cooperatively to AREs in the PSA enhancer core (−4326 to −3935) act synergistically with AR bound to the proximal promoter to regulate transcriptional output [177, 178]. To exploit the synergistic nature of AR action, we generated chimeric enhancer constructs by (1) insertion of a synthetic element containing four tandem copies of the proximal PSA promoter AREI (ARE4) element or (2) duplication of enhancer core and (3) removal of intervening sequences (−3744 to −2875) between the enhancer and the promoter. Each of these three strategies augments activity and androgen inducibility and retained a high degree of tissue discriminatory ability. As a result of these combined approaches, the two most active constructs are termed PSE-BC (duplication of core) and PSE-BAC (insertion of core and ARE4) are approximately 20-fold higher in activity than native PSA enhancer/promoter construct, PSE, composed of the PSA enhancer (−5322 to −2855) fused to the proximal promoter (−541 to +12). Most importantly, the enhanced activity and specificity of the new PSA-enhancer/promoter constructs are retained in an adenoviral vector. The recently developed human CaP xenografts should be excellent models to refine and evaluate this novel prostate-targeted gene therapy because their AR pathways are intact and their growth regulatory pathways bear close resemblance to clinical disease.

IV. Summary and Discussion

We have presented for discussion a broad-based review of the utility of adenoviral vectors in animal models of lung cancer. Since this entire compilation is devoted to Ad-gene therapy, we have particularly embellished the sections on "animal models" of disease, especially as they pertain to lung and prostate cancer. These examples illustrate that the development of our approaches may need to be disease specific, especially with respect to targeting and mode of delivery. From this review, it is evident that to realize the full potential of cancer gene therapy, advances need to be made on a number of fronts. Not only do we need to construct better Ad-vectors or more relevant animal models, we also need to incorporate emerging technologies to a useful purpose within the experimental design. For example, the pathway to human clinical trials may be better paved by an improved ability to gather interim surrogate measures of gene transfer and expression in animal models.

The implementation of a quantitative and noninvasive method capable of monitoring transgene expression in living animals repetitively would be useful toward validating the efficacy of any gene therapy strategy. In this respect, a number of investigators, including those at UCLA, are developing sensitive technologies for imaging transgene expression using positron emission tomography (PET) and optical measurements. PET is a noninvasive, tomographic imaging modality that already has clinical applications for the diagnosis and management of several diseases including cancer. Newer high-resolution animal microPET technology developed at UCLA, is allowing for the study of smaller animal systems (mice, rats, small primates) previously difficult to image with a resolution approaching 2 mm [179]. With relevance to gene therapy for cancer, the herpes simplex virus 1 thymidine kinase (HSV1-tk) gene has been demonstrated to be an excellent "PET reporter gene" by virtue of trapping positron-emitting 8-[18F] fluoroganciclovir (FGCV) specifically only in cells expressing HSV1-tk[180]. Using FGCV, repetitive PET imaging of adenovirus-directed hepatic expression of the HSV1-tk reporter gene in living mice has been achieved [180–182]. More importantly direct correlation between the retained PET reporter probe and the levels of HSV1-tk gene expression in the targeted organ have also been demonstrated [180–182]. Thus, PET is a sensitive and quantitative modality to image the location and magnitude of adenoviral vector-mediated gene expression in living animals which could be translated to clinical gene therapy application. Similarly, a charge-coupled device (CCD) camera is a highly sensitive camera for measuring photons. Advances in CCD technology can now enable investigators to quantitatively and reliably image low levels of luminescence (from the heterologous expression of the firefly luciferase gene) arising from within living animals [183]. Although tomographic images are not possible, and the signal is dependent on the depth of tissue from which the light source emanates, it is possible to get reproducible and semiquantitative images. The simplicity and minimal background signal of optical CCD luciferase approach may complement the detailed tomographic imaging of MicroPET and the newer confocal microscopy techniques and, ultimately, be more predictive of gene transfer strategies in the treatment of human disease.

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CHAPTER 19

Utility of Adenoviral Vectors in Animal Models of Human Disease II: Genetic Disease

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I. Introduction

A disease at the forefront of gene therapy research over the past decade is cystic fibrosis (CF). This hereditary, single-gene-defect disease, although affecting epithelial cells of multiple organs of the body, results most often in mortality due to complications associated with the lung. Cystic fibrosis lung disease has been considered as a prototypic disease state for "proof-of-concept" gene-therapy strategies. The lack of an alternative long-term treatment for the pulmonary manifestations of this disease, the accessibility of the lung via the airway lumen, and the fact that viruses known to infect the lung were being developed into nonreplicating gene transfer vectors led investigators to believe that administration of gene transfer vectors to the lung could potentially result in an effective treatment of this disease.

Shortly after the cloning of the gene responsible for CF pathophysiology, two groundbreaking observations made gene therapy for CF lung disease appear imminent. First, isolated epithelial cells cultured from the airway epithelium of CF patients could be phenotypically "corrected" by transferring into the cells the cDNA corresponding to the CF gene [1–5]. Second, adenoviral (Ad) vectors engineered to express the CF gene were administered to the airways of experimental animals and transgene expression observed in cells that were considered to require "correction" [6]. These initial observations produced a flurry of scientific activity and excitement in both the gene therapy and CF

scientific communities and within 3 years of these observations the first clinical trials describing successful Ad-mediated gene transfer to the airway epithelium of CF patients *in vivo* were reported [7].

These promising early observations have unfortunately not withstood further investigation. After approximately 20 gene therapy clinical trials for CF lung disease (of which greater than 70% utilized Ad) it has become apparent that gene transfer to airway epithelium *in vivo* is not a simple procedure. The difficulty lies in the evolution of the respiratory epithelium as an effective barrier to invading pathogens entering the lung (e.g., viruses). The epithelium achieves this "barrier function" by presentation of a host of innate and cell-mediated immune systems, which for gene transfer vectors culminate in reduced uptake and expression of the transgene. In this chapter, I describe the evidence that led investigators to believe that Ad would be useful in CF lung disease, why subsequently this simplistic approach failed, and how increasing knowledge of lung biology and viral bioengineering has and will allow novel strategies to be tested. In light of this emphasis on basic research, new strategies and models will need to be tested and successful demonstration of efficiency and safety will be required before we once again enter the clinic with Ad for CF lung disease gene therapy.

II. Pathophysiology of Cystic Fibrosis (CF) Lung Disease

Cystic fibrosis is a multifaceted disease with major morbidity and mortality resulting from chronic decline of lung function. This disease is the most common fatal inherited disease in Caucasians with 1 in 2500 live births affected [8]. Although CF is most devastating to the lung (accounting for 90% of mortality), resulting in chronic repetitive infections, chronic obstructive pulmonary disease, and respiratory failure, other tissues are also affected, including the liver, pancreas, the gastrointestinal tract, and the sweat glands. The abnormal CF gene (250 kb) encodes an mRNA of 6.5 kb which translates into an 180-kDa protein that has been extensively characterized as a cAMP-activated chloride ion channel, named the cystic fibrosis transmembrane conductance regulator (CFTR) [3, 9]. In the lung, CFTR is normally expressed in the respiratory epithelium and although the specific functions of CFTR are complex, is predominately involved in maintenance of ionic homeostasis in this tissue. Over 900 different mutations of CFTR have now been reported, resulting in a range of clinical manifestations and differing severity of the disease. However, 70% of these mutations are due to a three-base-pair deletion leading to the absence of phenylalanine (F) at position 508 (ΔF508) [10]. This particular mutation leads to misfolded CFTR being retained within the endoplasmic recticulum

of cells, so reducing CFTR function at the plasma membrane [11]. Currently, although the specific localization and functional capacity of ΔF508 CFTR in the different affected organs is a matter of controversial debate [12] and other mutations can display partial CFTR function, for CF patients, expression of abnormal CFTR in the airway epithelium generally results in reduced chloride ion secretion, hyperabsorption of sodium ions, increased viscosity of airway secretions, impaired mucociliary clearance, chronic bacterial infection, bronchiectasis, and premature death [8, 13]. Given that all of these effects are likely primary or secondary to loss of CFTR function, the most efficacious way to treat the broad range of effects would be to replace the defective CFTR gene with a normal copy. Gene therapy for CF lung disease therefore seeks to replace normal CFTR in the airway epithelial cells to hopefully "correct" lung epithelium function.

III. Trials and Tribulations with Adenoviral Vectors for CF Lung Disease

Clinical gene transfer trials with CF patients investigating the safety and efficacy of gene transfer vectors (predominately adenoviral and liposomal vectors) have been performed in both the United States and United Kingdom. Details of these trials and the background preclinical studies have been comprehensively reviewed in a recent review [14]. Although preclinical data have been largely promising for lung-directed gene transfer, the trials performed to date have shown, at best, only partial "correction" (<20%) of the CF bioelectrical defect [7, 15–18]. This relatively low degree of correction is most likely due to inefficient transfer of the CFTR cDNA to the airway epithelial cells, i.e., a low efficiency of gene transfer, and is most likely not sufficient to be of benefit to CF patients although long-term reversal of disease symptoms were not monitored in this studies.

The gene transfer efficiency required for physiological correction of CF lung disease has been a matter of recent debate. While Johnson and colleagues have shown that "correction" of ~10% of CF cells restores normal chloride secretory function to an epithelium, this degree of "correction" was insufficient to correct the hyperabsorption of sodium [19]. Since "correction" of the sodium defect is likely to be necessary for resolving CF lung disease, then transduction of a higher proportion of epithelial cells will be required [1, 20]. Indeed, it has been suggested that greater than 80% of epithelial cells will have to express CFTR to restore the normal sodium transporting capabilities of the epithelium [20]. With regards to efficiency of gene expression on a per cell basis, it appears that CFTR is normally expressed at levels as low as 10 copies per cell and heterozygotes for the CF gene although expressing only

50% of normal CFTR show no disease symptoms. This suggests that the level of expression per cell does not need to be high in order to correct function. On the other hand, overexpression of CFTR has been shown to have deleterious effects on cell function although the effects on polarized airway epithelial cells are not documented [21].

Issues of safety have arisen due to elicitation of inflammatory responses after Ad instillation in both animal and human experiments [22–29]. These effects have often been due to the large "loads" of vector that has been administered. A current hypothesis is that improvements in gene transfer efficiency may allow smaller quantities of Ad to be administered, possibly circumventing much of the inflammatory response.

IV. The Airway Epithelium: Cellular Targets for CF Gene Therapy

Airway epithelial cells are present throughout the conducting airways of the lung, including the nasal, tracheal, bronchial, and bronchiolar regions. In the upper airway, the surface epithelium lines these structures and is continuous with the tubulo-acinar submucosal mucus-secreting glands that invaginate from the airway surface. Airway epithelial cell-type composition is dependent both on the regional location and on the particular species studied and the reader is referred to comprehensive reviews that describe species-specific epithelial cell distribution in more detail [30, 31]. The epithelial cell types present in the lung are numerous and include ciliated cells, mucus-secreting cells (goblet), serous cells, clara cells, and basal cells. The cell types of the alveolar structures of the lung (alveolar Types I and II cells) are not thought to participate in the pathophysiology of CF lung disease. In human airways, the upper airway regions (nasal, tracheal, bronchial) are composed of a pseudostratified mucociliary epithelium in which ciliated cells predominate with interspersed mucus-secreting goblet cells. The columnar cells overlie intermediary differentiated cells and basal cell layers which interface with the basement membrane. In addition, the human upper airways contain numerous submucosal glands. In the human lower airways, the bronchioles are lined with a simple cubiodal ciliated epithelium containing few mucus-secreting cells, no basal cells, and an absence of submucosal glands. An important morphological difference between the upper airways of human and mice, the most common animal model for investigating airway administration of gene transfer vectors, is that for the mouse upper airway (excluding the nasal cavity epithelium) the columnar cells are roughly an equal distribution of ciliated and clara cells, compared to the predominance of ciliated cells in the human upper airway [32]. Clara cells are a nonciliated bronchiolar mucus-secreting cell type with distinct

properties from ciliated cells. Clara cells, although present in human airway, are located only in the distal airways and account for only a fraction of the cells present in that region [32].

The airway basal cells, or at least a subpopulation, are considered to be stem cell precursors for all other airway epithelial cells in the upper airway regions. Basal cells can differentiate into mucus or ciliated cell phenotypes [33]. Whereas mucus cells may also be able to differentiate into ciliated cells, the ciliated cell is considered as a terminally redifferentiated cell type.

An important observation with regard to experimental models of human airway epithelial cells is that isolation of upper airway epithelial cells for tissue culture purposes results initially in a predominately basal cell-like culture since isolated basal cells proliferate at a greater rate than isolated ciliated and mucus cells. Furthermore, for cells isolated from CF airways, the rate of proliferation of basal cells is even greater than that in normal airway, probably reflecting responses to ongoing inflammatory processes [34]. Therefore, morphological differences need to be considered when designing models to study the interactions of gene transfer vectors with airway cells that are presumed to represent the cells in the lung that are exposed to lumenally delivered vectors.

Although CF is a disease of the respiratory epithelium, the exact airway region where CF lung disease initiates is still a matter of debate. It does appear that the first signs of pathology occur in the distal airways with findings of bronchiolitis and mucus plugging in the small airways and although the exact nature of how the CFTR defect initiates the disease is not totally resolved, it does appear that hydration of the periciliary fluid layer in these regions may be a major cause [35, 36]. Currently, both the airway surface columnar cells lining the lumen of the small bronchiolar airways and the serous cells of the submucosal glands are candidates for the location for the onset of the disease. The cell type that is believed to be predominately involved in the onset of disease and therefore the specific target for gene transfer is the ciliated cell since these cells exhibit all of the ion and fluid transporting functions of CFTR and display abnormal function in patients with CF [37]. However, the submucosal gland serous cell is the highest CFTR-expressing cell type in the lung, suggesting that these cells may also be an important target for gene replacement [38].

Ultimately, it will be important to determine the location of disease initiation since it is likely that for a lumenal gene therapy to be successful, administration of vector will have to occur early in the life of a CF patient. Later in life, when the airways possess overwhelming mucus plugging and associated bacterial colonization and inflammation, delivery of genes to the target cells will likely become restricted. The current thrust for CF gene therapy strategies is to deliver transgenes to target cell types before such other barriers to treatment are present.

V. Adenoviral Vectors as Gene Transfer Vectors in the Lung

A. Animal Models for CF Airway Gene Transfer Studies

The generation of CF mouse models was an important step for understanding the physiology of CF disease. There have now been over 10 different mouse models produced displaying a range of CF-associated genetic mutations [39]. Although most of the models reflect the most common human mutation, either a complete gene knockout or a ΔF508 mutation of the mouse CFTR, other models with less common human mutations (e.g., G551D) have also been reported. The multiorgan pathophysiology associated with the different models has been recently reviewed [39]. Interestingly, although the gastrointestinal phenotype of CF mice is similar to that observed in CF patients, there is no CF-like pathology associated with the CF mouse lung. A comparison of bioelectrical measurements between CF human and CF murine airways has revealed that both species exhibit, relative to normals, hyperabsorption of sodium and an absent or reduced cAMP-induced chloride secretory response. However, it has been deduced that the ion transport defects in the CF mouse airway do not lead to CF-like lung pathology because CF murine airways compensate for the loss of CFTR activity by upregulating an alternative chloride secretory channel that is regulated by changes in intracellular calcium [40]. However, from a practical standpoint, the ability to measure the "bioelectrical defect" in CF mouse airways makes the model useful in terms of monitoring "bioelectrical correction" with gene transfer strategies, but the ability to monitor inhibition or reversal of CF-like pathology induced by transfer of normal CFTR is not possible in these current models. Therefore, the current gold standard for success in CF gene transfer to mouse lung *in vivo* is correction of the chloride (and sodium) ion transport defects.

Most gene delivery strategies to murine airways have focused on the epithelium of the nasal mucosa and trachea mainly because of accessibility to these regions but also because these regions are similar to those targeted for human CF gene-therapy trials. Unfortunately, baseline bioelectric measurements of murine trachea indicate that these tissues not display sodium hyperabsorption [41], a key indicator for the human disease, and one that will likely need to be corrected for a treatment to be successful. In contrast, the epithelium of the CF mouse nasal cavity and freshly isolated CF murine nasal mucosa both display sodium hyperabsorption and reduced cAMP-induced chloride secretory activity providing an ideal model for study [42]. A further difficulty with murine airways (excluding nasal epithelium) is the large proportion of clara cells that are present throughout the upper airway. The

distribution of this cell-type in the mouse may be misleading when comparing gene transfer efficiency between mouse and human upper airways (see below). The murine nasal mucosa, however, has few clara cells and exists as a pseudostratified mucociliary epithelium with a cell-type distribution similar to human nasal mucosa, again demonstrating the usefulness of this tissue for gene transfer studies.

Therefore, in conclusion, the CF murine models do not display spontaneous or induced pathological signs of human CF lung disease. However, CF murine airways do display bioelectric abnormalities associated with human CF and correction of these parameters by gene transfer can be measured both *in vitro* and *in vivo*. Given these considerations, since most clinical trials have focused on studying gene transfer to the nasal mucosa, the CF mouse nose is considered a good model for studying these strategies. In addition, since the epithelial cell-type distribution in human nose is similar to that of the human trachea and bronchus the nasal epithelium would appear to be a good model for a large proportion of the human airway epithelium.

B. Success and Limitations of Ad

1. Efficiency of Gene Transfer

a. Cell Types The major cell types that support wild-type Ad infection in the lung are the epithelial cells of the respiratory mucosa lining the airway passages. The tropism of Ad to the respiratory epithelium established this vector as an obvious candidate for delivering transgenes to the lung. Indeed, Ad-mediated gene transfer to airway epithelial cells grown under standard culture conditions *in vitro* is highly efficient [43, 44], with cellular transduction efficiencies of 90–100% and when the transgene is CFTR, full correction of the spectrum of CF bioelectrical defects is obtained [1]. In contrast, observations from *in vivo* epithelial cell models derived from cartilaginous (upper airway) regions of the airways of rodents and nonhuman and human primates show that transgenes are expressed after *in vivo* dosing in less than 20% of the surface epithelial cells, an efficiency unlikely to benefit to the defective physiology of a CF airway [43, 45]. Although the efficiency of gene transfer can be enhanced by prolonging the contact time of Ad with the epithelium for 12–24 h, it is difficult to envision this strategy as being practical in a clinical scenario [46, 47]. In the case of intralumenal delivery of Ad to the lower airways of rodents, gene transfer to 10–80% of the airway epithelial cells has been reported with apparently no cell-type-specific selectivity [48, 49], although, in a detailed study of Ad administration to murine airways, only the nonciliated bronchiolar epithelial cells (i.e., Clara cells) were observed to express transgenes [50]. Clara cells are not thought to require correction in the CF lung and this observation casts a shadow on the use of murine airway epithelium as a model for Ad-mediated gene transfer to the human airway epithelium where Clara cells

are less common. Therefore, it appears that lumenal-facing well-differentiated airway epithelial cells *in vivo*, at least in the upper airway regions, are resistant to efficient Ad-mediated gene transfer.

How can we envision that the airway epithelial cells facing the lumen of the airway are not transduced by Ad given the large body of clinical data that shows that these cells are targeted in wild-type infections? In a series of studies using human tracheal epithelium *ex vivo* and murine trachea *in vivo* it was discovered that injury to the epithelium by physical abrasion of the columnar cells revealed epithelial cell types that are susceptible to efficient Ad-transduction, as depicted in Fig. 1 [43, 51, 52].

This cell-type-specific variable efficiency led to the finding that underlying basal cell-like cells were efficiently transduced by Ad. These cells, as precursors to columnar cells could, once transduced, over time proliferate and differentiate into transgene-expressing columnar epithelial cells. Since the epithelial basal cells are probably stimulated to proliferate and differentiate upon injury these susceptible cells were described as "basal cell-like cells" or the poorly differentiated (PD) airway epithelial cells, i.e., injured or regenerating cells, and this cellular phenotype is similar to that displayed by airway epithelial cells grown on plastic that are also highly transducible by Ad [43, 44].

One consideration when comparing wild-type Ad infection to Ad vectors is that the latter rely on delivering many virus particles to a target tissue

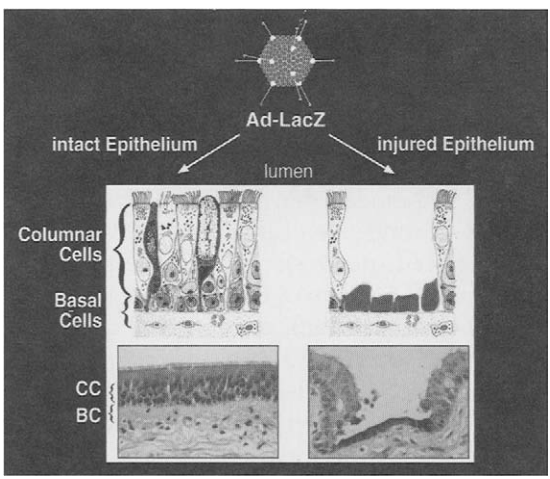

Figure 1 Increased susceptibility of injured epithelium to Ad-mediated gene transfer. Exposure of Ad vectors to intact pseudostratified columnar cells (CC) results in low gene transfer efficiency. Physical abrasion of columnar cells before Ad exposure results in efficient gene transfer to the underlying basal cells (BC). Upper figures show schematic of intact and injured pseudostratified columnar respiratory epithelium and lower figures are intact and abraded human tracheal epithelium exposed to AdLacZ *ex vivo*. Reprinted with permission from [20].

whereas wild-type Ad needs only access to a small number of cells from which Ad replication and spread can then occur. Therefore, wild-type Ad may be able to take advantage of regions of the airway in which epithelium integrity is compromised or injured. Initiation of wild-type infection in injured regions would then be able to spread as a "basal cellitus" effectively beneath the resistant superficial columnar cells.

b. Receptors The differences in the gene transfer efficiencies for the two cellular phenotypes of airway epithelial cells, the PD and well-differentiated (WD) columnar cells, suggests that an early step in the virus–cell interaction is deficient for the WD cells. Adenovirus enters epithelial cells by a two-step process: (1) initial attachment of the viral fiber-knob protein to a high-affinity receptor, the human Coxsackie B and adenovirus 2 and 5 receptor (hCAR)) [53, 54]; and (2) translocation of the virus into the cell cytoplasm via clathrin-coated pit internalization processes, in part mediated by an interaction of the viral penton base with $\alpha_v\beta_{3/5}$ integrins [55].

Since quantitative studies of the interactions of Ad with the airway epithelium *in vivo* are difficult and prone to considerable variation, specialized cell culture models have been generated to aid characterization of the interaction of Ad with both PD and WD cell types. These models have been shown by a number of groups to reproduce: (1) the well-differentiated (ciliated) and poorly differentiated cellular phenotypes and (2) the relative resistance of WD and permissiveness of PD cells to Ad-mediated gene transfer as observed *in vivo* [56, 57]. In addition, although these models were originally generated to ask specific questions regarding gene transfer strategies, they have subsequently become valuable in a whole series of studies where quantitative and qualitative measurement of events in the airway epithelium are difficult to perform *in vivo* [35, 36, 58–63].

Using these models of human airway epithelium, immunofluorescent and functional analyses of the interactions of Ad with human airway epithelial cells have shown that decreased gene transfer efficiency to WD compared to PD cultures is due to limited entry (penetration) of Ad across the apical membrane of WD cultures, which reflects a reduced specific Ad-attachment due to the absence of hCAR and $\alpha_v\beta_{3/5}$ integrins from the apical surface. Interestingly, columnar cells and basal cell-like cells express all the necessary receptors to efficiently allow Ad entry but for columnar cells these processes are segregated and limited to the basolateral membranes as depicted in Fig. 2. In these culture models systems, Ad has been shown to efficiently transduce epithelial cells when applied to the basolateral epithelial surfaces [56, 57, 64, 65].

It appears that the most significant Ad–cell interaction in determining efficiency is that of the Ad-hCAR interaction. Many cell types usually resistant to Ad infection have been shown to be efficiently transduced after heterologous expression of hCAR, although the status of integrin expression

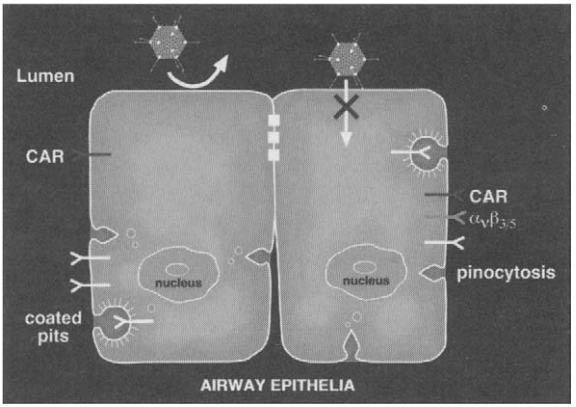

Figure 2 Schematic of polarized epithelial cells displaying resistance of the lumenal surface to adenovirus attachment and entry. The receptors required for Ad entry are located on basolateral membranes and excluded from the apical membrane by the tight junctional complexes. Reprinted with permission from [144].

in these cell-types is not always clear [56, 66]. Earlier observations had suggested that inefficient Ad-mediated gene transfer to a bronchial xenograft model of human *in vivo*-like ciliated airway epithelial cells reflected the absence of $\alpha_v\beta_{3/5}$ integrins from the lumenal membrane of the epithelium [65]. However, $\alpha_v\beta_{3/5}$ integrins may not alone account for decrements in gene transfer efficiency. In support of this hypothesis, Ad mutants lacking penton base RGD sequences (normally required for Ad–$\alpha_v\beta_{3/5}$ integrin interactions) are able to efficiently transduce human epithelial cells although the rate of internalization is reduced [67]. In addition, in a β_5 integrin-knockout mouse model, airway epithelial cells were equally susceptible to Ad-mediated gene transfer as were wild-type airway cells [68], again suggesting that $\alpha_v\beta_{3/5}$ integrins may be facilitative rather than necessary for efficient vector entry into the cell.

These observations are important for the design of targeted vectors that attempt to increase gene transfer efficiency to normally unsusceptible cell types [69, 70]. Retargeted vectors attached via nonspecific interactions or to noninternalizing receptors will probably depend on nonspecific uptake pathways to enter cells and while this approach is useful for PD cells *in vitro*, increasing Ad-attachment to WD cultures that do not exhibit these pathways is unlikely to improve gene transfer efficiency [56].

c. The Innate Immune System of the Lung Despite the progress on the cell biological aspects of vector–cell interactions, surprisingly little attention has been devoted to another fundamental component of innate airway defense that will almost certainly impact on the efficiency of lumenally

delivered vectors, the barrier/shielding function of epithelial surfaces by the carbohydrate-rich cell surface glycocalyx. Expression of hCAR, engineered to be expressed at the apical surface of polarized epithelia by incorporation of a glycosylphosphatidylinositol-linker (GPI-CAR), identified glycocalyx components as barriers for lumenally applied Ad, accessing these receptors as depicted in Fig. 3 [71]. Electron micrographs demonstrate a "fuzzy coat" on the cell surface [72, 73], termed the glycocalyx, and on epithelial cell apical surfaces it is composed of several families of carbohydrate-rich molecules, including glycoproteins (most notably the mucins), proteoglycans, and glycolipids. Glycoconjugates are variably modified by sialic acid and sulfate that impart a strong anionic charge to the cell surface. A major component of the airway glycocalyx will likely be the "tethered" mucins and the molecular biologic advances in the mucin field have revealed that the MUC1 and MUC4 are highly expressed in airway epithelium and have transmembrane anchoring (tethering) domains [74–82]. With respect to airway gene transfer, sialoglycoconjugates (including MUC1) comprising the glycocalyx on MDCK cells, appear to inhibit Ad gene transfer, presumably due, in part, to their negative charge since neuraminidase treatment to selectively remove sialic acid can circumvent the glycocalyx barrier in these cell types [83, 84]. Although apical surface mucins expressed on WD cells are also restrictive to Ad, neuraminidase alone is not sufficient to allow Ad permeation through the glycocalyx, and

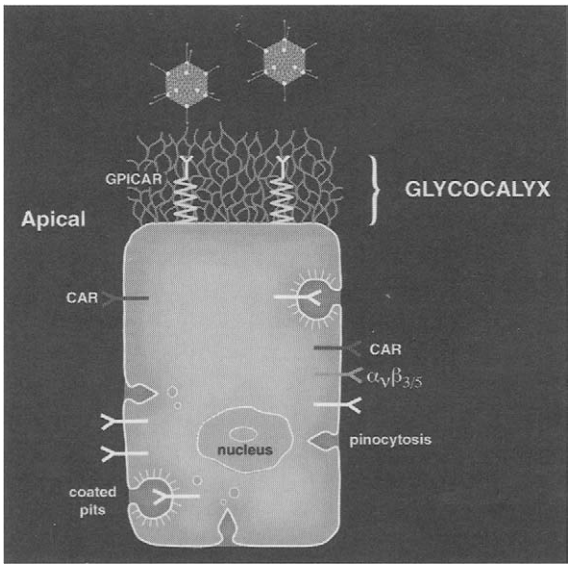

Figure 3 Schematic of polarized epithelial cell expressing reengineered Ad receptors at the apical surface. These studies revealed that the apical surface glycocalyx was an effective barrier to Ad accessing receptors located on the apical surface.

more stringent proteolytic treatments are required [116, 117]. Presumably, the mucins, including both tethered and secreted mucins, may also be present in the mucus layer in the airway and may act as false attachment sites for Ad, thus effectively reducing the amount of Ad that ultimately reaches the epithelial surface. The reported rheological properties of CF mucus producing a more viscous, more dehydrated and immobile barrier suggest that this obstacle to gene transfer will be even more pronounced in the CF lung.

Other components of the innate immune system, not studied in specialized cell culture models, may also have barrier effects on gene transfer efficiency. Ordinarily, such barriers occur in the lung as primary defense mechanisms and may be aggravated in the CF lung where airway lumens are inflamed. For example, alveolar macrophages have been reported to sequester up to 70% of Ad genomes within 24 h following tracheal administration to mouse airways [85]. In a mouse nasal model of CF lung bacterial colonization, Pseudomonas infection (PA01 strain) was shown to inhibit Ad gene transfer by 10-fold relative to noninfected control nasal airways [86].

In conclusion, there appear to be numerous potential barriers to Ad gene transfer in the lung especially in the CF lung that exhibits an overactive inflammatory milieu, and strategies to circumvent these barriers will likely need to be designed. However, even if all of these barriers are circumvented the major cause of low efficiency gene transfer is the lack of entry of Ad into the target cells. Strategies to improve the transduction efficiency will therefore be crucial to proving that the concept of gene transfer into the airway may actually be a feasible one.

In summary, human WD cultures are resistant to Ad-mediated gene transfer because of decreased specific attachment sites and reduced nonspecific entry paths that can internalize a fraction of a large vector load typical of CF gene therapy protocols using Ad. To circumvent the inefficiency of Ad-mediated gene transfer to the respiratory epithelium, either alterations of the host will be required, i.e., ability to access Ad receptors expressed on basolateral cell surfaces, or Ad will require retargeting to receptor types that are present in sufficient number on the airway epithelial lumenal surface which allow for efficient uptake of Ad into the cell.

2. Safety

Initial attempts to improve efficiency of Ad gene transfer to the airway epithelium *in vivo* have mostly involved delivery of greater doses of Ad to the lung. These doses can represent a relatively large protein load and the subsequent gene expression (even in nonepithelial cells) can produce an unusually high level of transgene in an organ that is designed for monitoring invading pathogen assaults. It is therefore not surprising that inflammatory and immune responses are observed when Ad is delivered to the lung and numerous studies have reported Ad-induced lung inflammation. In general, Ad induces

an acute nonspecific mixed cellular inflammatory response and a late specific, dose-related, lymphocyte-predominant, cell-mediated immune response in all species so far studied [22, 23, 25–27, 29, 87–90]. The acute response is nonspecific and likely induced by cytokine production in response to the protein load. It has also been suggested that neurogenic inflammation results after administration of Ad in rat airways, an effect shown to be partially due to vector gene expression but also to the viral proteins of the capsid coat [91]. The later, specific immune response to Ad is mediated by major histocompatibility complex (MHC) class I-restricted cytotoxic (CD8) T lymphocytes directed against viral gene products and transgene proteins in expressing cells. The subsequent destruction of these cells leads to loss of persistence of transgene expression and so reduces efficiency of gene transfer [28, 92, 93]. The use of second-generation and high-capacity "gutless" vectors aims to limit the amount of viral gene expression to decrease the effects of this late immune response and these approaches are the topics of other chapters [94, 95].

In addition to cellular immune responses, Ad also elicits humoral immune responses with the production of mucosal and neutralizing antibodies [25, 87, 90, 96–98]. These responses have been shown to be against the viral capsid proteins and are secondary to a helper ($CD4^+$) T lymphocyte response. The production of such an antibody response results in neutralization of subsequent readministration of Ad, resulting in loss of gene transfer, assuming that the same Ad serotype is used (see below).

Therefore, in addition to the innate immunity of the lung (receptor localization, glycocalyx, macrophages, mucus) reducing the efficiency of gene transfer, the cellular and humoral immune systems also respond to Ad delivery into the airway and as a result reduce the efficiency of gene transfer and the persistence of expression in the target epithelial cells.

C. Overcoming the Limitations of Ad

1. Efficiency

The localization of entry pathways for Ad to the basolateral surfaces of airway epithelial cells suggests that a delivery strategy to access these regions would be beneficial to improving gene transfer efficiency. This approach may also allow targeting of the epithelial stem cells (basal cells), resulting in transgene expression in the lung for the lifetime of the individual. This is an important consideration for gene transfer to the airway epithelium since fully differentiated lumenal facing cells (e.g., ciliated cells) have a relatively short lifetime, on the order of 40–100 days, and targeting these cell types specifically will require regular readministration of vectors.

Access to basal cells/basolateral surfaces may possibly be achieved by intravenous administration of vectors if penetration of the blood vessel wall, the connective tissue, and the basal lamina of the basement membrane were

achievable. Unfortunately, studies that have attempted intravenous delivery strategies have not been successful since vectors do not appear to gain access to sufficient lung epithelial cells to make this approach feasible [99–102]. Barriers functions provided by the blood vessel endothelial cells and connective tissue surrounding the airway passages seems unpenetratable by Ad. Indeed, the particle permeability of the basal lamina alone is thought to exclude inert particles of greater than 10 nm, which would certainly be restrictive to particles the size of Ad (100 nm). In an *in vivo* experimental mouse model where Ad was externally administered directly to the tracheal basement membrane, efficient gene transfer to the connective tissue fibroblasts adjacent to the basement membrane was observed without gene transfer to the epithelial cells of the juxtaposed epithelium [51].

To date, two main strategies to improve intralumenal delivery of Ad vectors have been focused on. One approach is to access the basolateral surfaces of the epithelial cells by disruption of the epithelial "tight" junctions, and the other is to retarget Ad vectors to nonviral receptors that are present on the apical surface of lumenal epithelial cells that allow for entry of Ad into these cell types. Retargeting has so far been achieved by chemically, immunologically, or genetically modifying the Ad capsid coat by incorporating new receptor ligands that can target candidate receptors.

a. Modification of the Host by Opening Tight Junctions Epithelial cell "tight" junctions (zonulae occludens) are collar-like structures composed of a diverse number of proteins that separate the apical and basolateral domains of the lumenal columnar epithelial cells. As well as functioning as a restrictive barrier to mixing of apical and basolateral membrane components, these intercellular junctions limit the transepithelial transport of solutes across the epithelium. A number of disease states have been shown to alter tight junction permeability (e.g., asthma) and reagents to increase the permeability of the junction are available. The key to successful disruption of tight junctions to allow Ad access to basolateral epithelial cell surfaces will be to use a reagent that opens tight junctions sufficiently for Ad to pass through but that is rapidly reversible to limit the passage of other lumenal contents (e.g., bacteria) or serosal fluid into the airway lumen.

A property exploited for this purpose is the calcium ion dependency of the structural integrity of the junction. Walters *et al.*, have successfully shown that treatment of the apical surface of human WD airway cells with the calcium chelator EGTA or hypotonic solutions (e.g., water) allow for improvements in Ad-mediated gene transfer presumably by allowing Ad access to basolateral receptors [64, 103]. The slow reversibility of this effect, however, is problematic; tight junction reformation takes a least a couple of hours, a time period that would be unacceptable in a clinical setting. *In vivo* studies in mouse airways have confirmed that these treatments improve gene transfer efficiency although parameters of safety were not assessed fully [104, 105].

More specific reagents are available for studying tight junction permeability and the effect on Ad-gene transfer. Parsons *et al.* used a detergent, polidocanol, in murine airways *in vivo* to enhance Ad-mediated gene transfer, an effect shown to be due to the ability of this reagent to transiently open tight junctions [86]. The short-chain fatty acid sodium caprate, has also been used to increase Ad-mediated gene transfer to human WD cultures and results in full correction of CF cultures when AdCFTR is subsequently applied to the apical surface. This result is exciting since the effect is rapidly reversible effect and has previously been used clinically for enhancing pharmaceutics absorption across the GI tract, again presumably by an effect on tight junctional permeability.

These studies although fraught with inherent safety issues are beginning to establish that this strategy for delivering transgenes to the lung may be a viable option. The possibility of targeting the basal stem cells by this procedure is reason enough to continue pursuing the usefulness of these strategies.

b. Targeted Ad to Increase Gene Transfer Efficiency Targeted Ad directed against specific receptors have been used to successfully transduce cell types that are usually refractory to Ad infection. The epidermal growth factor receptor, stem cell factor receptor, fibroblast growth factor receptor, α_V integrins, and T-cell receptors (CD 3), have all been used as surrogate receptors for Ad entry in a variety of cell types [106–109]. Given the lack of Ad receptors at the apical surface of lumenal airway epithelial cells, a retargeting strategy to receptors known to present on the airway lumen may allow for gene transfer efficiency to be improved. However, a successful targeting strategy to the lung epithelium will require the identification of target molecules that allow for attachment and internalization of AdV across the apical membrane of columnar airway epithelial cells.

The identification of target receptors to which to redirect Ad tropism on the lumen of airway epithelium is difficult because most receptors and entry mechanisms occur on the basolateral surfaces of the cells. Certain members of a specific seven-transmembrane-spanning G-protein-coupled receptor family (i.e., P2Y2-purinoceptors, B2-kinin receptors, and adenosine type 2b receptors) have been identified as putative utile target receptors for redirecting Ad tropism to the surface epithelium of the lung. These receptors have been shown to be present on the lumenal surface of human airway epithelium and internalized into clathrin-coated pits when activated by their respective agonists [110]. The utilization of clathrin-coated pit internalization pathways for native Ad receptors, suggests that the G-protein coupled receptors may provide an ideal surrogate entry pathway for Ad. The high potency of P2Y2 agonists (e.g., ATP, UTP) combined with the low affinity of these agonists for the receptor suggests that the P2Y2 purinoceptors are abundant in number on the lumenal surface of the human respiratory epithelium [111]. Since pharmacological

activation of airway epithelial P2Y2 receptors do not result in untoward effects in human airways, this receptor is an ideal target receptor to redirect Ad tropism. However, since the only available ligands for this receptor are low affinity, small organic molecules, certain technical difficulties are associated with conjugating these molecules to Ad. Other receptor types suitable for Ad retargeting exist on the airway, although specific retargeting data for Ad is lacking. The urokinase plasminogen activator receptor, uPA-R and the SEC-2 receptor have also been proposed as target receptors for Ad and AAV, respectively [112, 113].

i. Immunologically modified targeted vectors One immunological approach for targeting gene transfer vectors is using bispecific antibodies linking Ad directly to non-Ad-receptor-types present on the cell surface [108, 114]. For example, chemically conjugated antibodies, one of which is directed against an epitope-tagged Ad coat protein and the other against α_V integrin membrane proteins have been reported to increase gene transfer efficiency by seven- to ninefold compared to that of nonmodified Ad, indicating that increased Ad-attachment results in increased gene transfer efficiency [114]. In a similar approach, Ad was retargeted to nonviral receptor types in conjunction with ablation of the natural Ad tropism using an anti-fiber-knob protein antibody conjugated to folate [115]. Folate-conjugated antibody was the ligand of choice since the folate receptor is reported to be upregulated on the surface of malignant cells, thus providing a targeted vector for a variety of cancers. Retargeting Ad to cells expressing folate-receptors was shown to be specific and successful with significant increases in gene transfer efficiency.

As "proof of concept" studies, a hemagglutin (HA)-epitope-tagged P2Y2 receptor expressed at the apical surface of human WD cultures and targeted with bispecific antibodies consisting of antibodies to Ad fiber-knob protein/HA-tag has been shown to facilitate Ad entry into these cell types, shown schematically in Fig. 4 [116, 117]. This effect is enhanced by coadministration of exogenous ATP to activate the receptor, an effect that can be reduced by desensitization of the P2Y2 receptors prior to addition of targeted Ad. Importantly, the apical surfaces of the HA tagged-P2Y2 expressing cultures required a brief exposure to specific proteases before targeting was effective suggesting that the apical surface glycocalyx hindered access of the targeted vector to the target receptors [116]. This approach also relied on the expression of a HA-tagged receptor that may be overexpressed relative to the endogenously expressed P2Y2 receptors in the culture system. The number of target receptors and the affinity of the targeting ligand are both likely to be critical parameters for the success of such a targeting strategy.

ii. Chemically modified targeted vectors Since antibodies to the external domains of P2Y2 receptors are not currently available, a strategy to target Ad to the endogenous P2Y2 receptor was to chemically conjugate small molecule agonists (UTP) to the proteins of the Ad capsid coat. Using chemically reactive

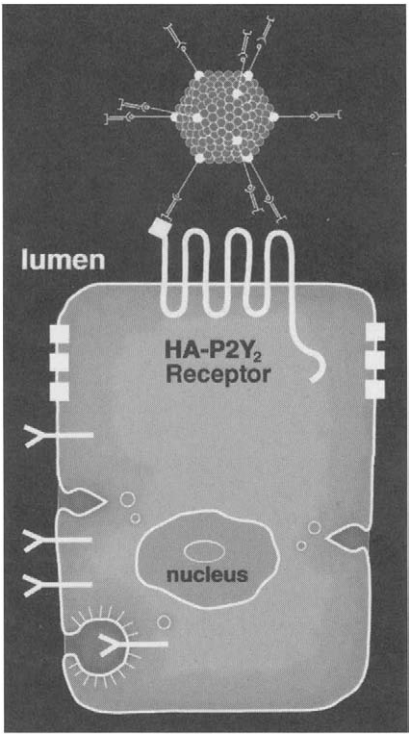

Figure 4 Schematic of targeting strategy used to redirect Ad tropism to P2Y2 receptors on the apical surface of human airway epithelial cells. Bispecific antibodies against the virus and the receptor were used as a targeting link and activation of the receptor results in receptor internalization and entry of Ad with subsequent gene transfer.

biotin derivatives, biotin was coupled to the Ad capsid coat predominately via hexon protein. This strategy is reported to couple 2–300 biotins to a single Ad particle and does not significantly alter the fiber-knob–hCAR interaction. By using commercially available biotin-linked UTP in combination with streptavidin as a "bridge" linking biotin–Ad to biotin–UTP, these molecular conjugates were shown to mediate gene transfer by an interaction specifically with endogenous P2Y2 receptors on the apical surface of WD cultures [110]. Again, the effectiveness of this approach was reduced by the presence of apical surface glycocalyx since gene transfer was only observed in cultures pretreated with agents that degrade this barrier. Regardless, gene transfer efficiency using these conjugates was still inefficient, probably due to the clumsiness of the "streptavidin bridge" and the low affinity of UTP for this receptor. Future experiments using this targeting strategy will require the identification of receptor agonists with higher affinity in addition to improved methods to directly couple the agonist ligands to the Ad capsid coat.

Another method for chemically conjugating receptor ligands to Ad is by the use of polyethylene glycol (PEG) that can be covalently linked directly to the Ad capsid coat. A number of groups have now shown that PEG conjugated viruses can be used to target Ad [112, 118]. For example, Ad conjugated to a 12-amino-acid peptide, identified from phage display assays on the apical surfaces of human WD cultures, resulted in a 10-fold increase in gene transfer efficiency to these cell types [118]. Similarly, Ad conjugated via PEG to a peptide that binds to uPA-R has been shown to target Ad to this receptor type and enhance gene transfer to polarized airway epithelia [112]. An additional bonus of using PEG-conjugated Ad is that these vectors appear to be less immunogenic that non-PEG-conjugated Ad. This effect is due to the masking of antigenic Ad capsid proteins (mainly hexon) from neutralizing antibodies ([119], see below).

iii. Genetically modified targeted vectors The ideal targeted vector would be one in which the target ligand could be incorporated into the capsid coat with minimal disruption of the physical and biological properties of Ad. For targeting strategies in which a peptide ligand is used, the most desirable method would be to generate an Ad vector genetically modified to express a functional peptide ligand on the viral surface. Such an approach for targeting vectors has been reported, where the Ad viral coat has been genetically modified to express multiple polylysine groups on the C-terminus of the Ad fiber-knob protein [70]. This redirects Ad tropism to heparan sulfate moieties that are present on the surfaces of most mammalian cells. With certain nonepithelial cell types, which lack hCAR, this modified vector has been shown to increase gene transfer efficiency 10- to 300-fold in comparison to nonmodified Ad. However, the modified vector will likely not be useful for gene transfer to the airway epithelium since heparan sulfate is not expressed at the apical surface of airway epithelial cells [120]. Targeted Ad in which the fiber-knob protein (responsible for Ad attachment to the hCAR) has been modified to express novel ligands that can interact with other receptor-types are being developed and the feasibility of this approach has now been reported by a number of groups [107, 121, 122]. A recent development in this type of approach was reported by Krasnykh *et al.* [123], who hypothesized that the HI loop region of the fiber-knob structure can withstand the insertion of heterologous peptide sequences without significantly compromising the tertiary structure of the fiber-knob protein or the production and infectivity of the modified Ad. These authors incorporated the FLAG octapeptide marker sequence into the HI loop region and were able to produce functional Ad. Importantly, they also showed that the sequence contained within intact virions was accessible to a FLAG-specific antibody, suggesting that sequences inserted into this region are capable of interacting with other target substrates such as cell-surface receptors.

A significant technical advance in Ad targeting strategies evolved from studies that deduced the viral sequences in fiber-knob protein that interact

with hCAR. Genetic ablation of these sequences from Ad vectors led to the generation of Ad that no longer binds to hCAR and no longer transduces cells that are permissive for normal Ad transduction [124]. The broad cellular tropism of Ad vectors can now be reduced, and by the addition of targeting moieties to these Ad vectors specific cell-type targeting is possible. Reduced Ad interactions with nontarget cells will lessen the potential for adverse effects with these vectors. In the lung however, the significance of natural tropism ablation is unclear since most of the epithelial cells targeted with delivery strategies do not express Ad receptors at the lumenal surface. However, the loss of transduction to other cell-types that may interact with Ad delivered to the lung (e.g., macrophages, dendritic cells) may benefit from the hCAR-binding ablation mutant.

Recent developments in immunologically, chemically, and genetically modified targeted Ad suggests that "designer" gene transfer vectors will one day be available. Although Ad vectors, in their present form, may not be ideal for a number of gene transfer target tissues, notably the lung epithelium, this vector clearly remains at the forefront of gene therapy research since it is still one of the most efficacious gene transfer vectors available, and will continue to be useful at least in proof-of-concept studies.

iv. Screening with other adenoviral subtypes Although over 51 different serotypes of wild-type Ad exist, the predominant serotypes used for gene transfer experiments are serotypes 2 and 5. The reason for this is largely historical since these two serotypes have been extensively studied over the past 30 years and understanding of the viral genome has allowed the manipulations necessary to evolve these viruses into gene transfer vectors. With regard to the airway epithelium, other serotypes have been suggested to be efficacious at delivering transgenes to human WD cultures. Serotypes 17 and 12 have been shown to bind/deliver transgenes 10-fold over Ad2 vectors [125]. However, as of yet no conclusive results have been presented that suggest that the improvements warrant future investigations with these vectors. One approach to determining if any of the other serotypes may be more efficacious in the lung epithelium could be envisioned using a recently reported system of generating an Ad5 capsid-expressing fiber proteins from the other serotypes [126]. This system was used to screen vascular endothelial and smooth muscle cells and the efficiency of gene transfer compared against the efficiency of gene transfer with Ad5. This screening procedure identified Ad5 with Ad16 fibers as being significantly more efficient at gene transfer than Ad5 in these particular cell types. It will be of interest to screen these serotypes on human WD cultures relative to Ad5 to determine whether other Ad serotypes may be of benefit to airway epithelial cell gene transfer. A serotype which may be of particular interest is Ad37, since it has been reported that Ad37 utilizes sialic acid residues that are present on the extracellular surfaces of most cells [127]. An abundance of sialic acid residues on the lumenal surface of airway epithelial

cells as components of glycoconjugates may allow for improved gene transfer. Whether attachment of Ad37 to sialic acid residues located on the airway lumen leads to efficient entry and gene transfer awaits further study.

v. Other methods to increase gene transfer efficiency Nonspecific methods to enhance Ad-mediated gene transfer to airway epithelial cells have been reported [128, 129]. Calcium phosphate coprecipitation has been used to precipitate aggregates of Ad and other vectors to increase gene transfer to airway epithelia both *in vitro* and *in vivo*. It has been suggested that *in vivo* these aggregates increase the rate of nonspecific endocytosis of Ad across the apical membrane of polarized epithelial cells. The possible effects of this technique on cellular and paracellular permeability have not been investigated.

Another method to both improve both the delivery and efficiency of Ad to the lung epithelium *in vivo* is using the inert perfluorochemicals (PFCs). These compounds are liquid in nature but due to high oxygen saturation capacities can be instilled into the lung for periods of time with maintenance of passive oxygen diffusion. Several studies have now shown that administration of gene transfer vectors (including Ad) with PFC results in increased gene transfer to rodent and nonhuman primate lungs [130–132]. The improvements in gene transfer are predominately localized to the alveolar regions with only modest improvements in the efficiency of gene transfer to the respiratory epithelium. The exact mechanism by which PFCs produce these effects remains to be determined, but may be due to prolonged contact time for the vector on the cells and reduced ingestion of Ad by macrophages and/or due to some nonspecific effect on the paracellular permeability. Nonetheless, this method provides an example of a new strategy to deliver transgenes to the lung without the need for direct instillation or aerosolization, which are both inefficient methods for airway epithelium delivery.

2. Safety

Strategies that improve gene transfer efficiency, as described above, will allow for lower doses of Ad to be administered to the lung. This achievement alone will be beneficial in reducing the inflammatory responses seen with Ad administration. However, attempts have also been made to reduce the inflammation produced by expression of viral genes that produce the cell-mediated immune responses described above. The identification of specific viral genes that initiate or amplify the immune response has led to the reengineering of Ad vectors to ablate the specific gene expression. For example, vectors deleted of E2a and E4 have been reported to display reduced immune responses and improve persistence of transgene expression [92, 93]. The ultimate vector is one that contains no viral genes and the high-capacity "gutless" vectors have been generated and appear to blunt the immune response considerably [133–135]. In contrast, several viral genes have been identified that have evolved to subvert the immune response and the inclusion of these genes into new vectors may be desirable (e.g., E3) [136].

Strategies to circumvent the humoral immune response have also been considered. Since this arm of the immune system results in the inability of readministration of specific Ad serotypes, serotype switching has been proposed as a method to allow repeat administration. Indeed, Ad5 administration but not Ad4 or Ad30 has been reported to prevent the gene transfer obtained with subsequent Ad5 administration the lung [97]. However, in addition to this being a somewhat limited procedure, it is not yet clear whether these different serotypes are as inefficient for gene transfer to the airway epithelium as Ad5. Transient immunosuppression has also been suggested to reduced the inhibitory effects of neutralizing antibodies. Intratracheal administration of immunosuppressive factors (IL-12, gamma interferon, antibodies to CD40, corticosteroids and cyclophosphamide) at the time of vector administration have all shown a reduction in generation of neutralizing antibodies [137–141]. The longer-term effects of administering these factors to lung have not been reported. Finally, covalent conjugation of PEG to the Ad capsid coat that permits addition of targeting moieties is also a strategy for the virus to elude neutralizing antibodies by masking capsid coat proteins, especially hexon protein. Although PEGylation of Ad leads to some loss of viral titer and aggregation the ability of this procedure to develop targeted vectors combined with reduction in immune response makes this a promising method for future study [119].

VI. Other Vectors

The focus of this review has been on Ad vectors for use in CF lung disease. However, a number of other vectors have been suggested as candidates for CF lung gene transfer vectors. Adeno-associated virus (AAV), retrovirus, lentivirus, and liposomal vectors have all shown promise in preclinical studies in the lung and some have been tested in clinical trials. The general observation is that all of these vectors, like Ad, do not appear to display the efficiency of gene transfer in WD airway epithelial cells as they do in nonpolarized cells, suggesting that these vectors confront similar barriers in the airways as do Ad vectors. Strategies to improve gene transfer efficiency for these other vectors have followed the progression of experiments with Ad, i.e., tight junction modulation, targeting, serotype switching, and immune response reduction, and all have been shown as for Ad to improve efficiency to some to degree. Whether efficiency can ever be improved to a point that shows efficacy in the lungs of CF patients remains to be determined. Meanwhile, other viruses (sendai virus [142] and lentiviruses pseudotyped with filovirus coat proteins [143]) may show promise for gene delivery to the airway and preliminary reports suggest that these viruses or components thereof may one day provide us with a method to deliver transgenes to the lung in an efficient and safe manner.

VII. Conclusion

It is clear that the evolution of gene therapy has been aided by many different aspects of basic biological and medical research efforts and the possibility of a gene therapy for CF lung disease will only take time and a continuation of these efforts. These findings will not only be beneficial to the treatment of CF lung disease but also other disease states, which are continually being brought closer to a treatment and perhaps a cure by this new and exciting biomedical technology.

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CHAPTER 20

Utility of Adenoviral Vectors in Animal Models of Human Disease III: Acquired Diseases

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I. Adenoviral Vectors for Infectious Disease

Recombinant adenoviral vectors for infectious diseases can generally be categorized into three general approaches. The first is the use of a vector-based vaccine where the vector encodes for proteins to achieve an immune response. In fact, adenoviruses have been used in the U.S. military for vaccines [1]. The second approach is to use adenoviral vectors, which encode immunostimulatory genes to achieve *in vivo* immunotherapy. Last, these vectors can be used to provide critical accessory molecules for T- or B-cell activation for patients that are deficient in these molecules or theoretically direct anti-infectious genes such as anti-bacterial peptides. These general paradigms hold true for most gene-therapy approaches with adenoviral-based vector systems regardless if the targets are infectious disease, an inherited immune deficiency sate, or cancer. In this chapter we focus on these paradigms in the context of specific disease entities that may be candidates for treatment with adenovirus-based vector systems.

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A. Tuberculosis

Mycobacterium tuberculosis the etiologic agent of tuberculosis (TB), is a facultative intracellular pathogen which remains the foremost cause of death from a single infectious agent among adults [2]. It has been estimated that approximately one-third of the world's population in 1990 (1.7 billion individuals) were infected with *M. tuberculosis*, affecting mostly people living in developing countries, and that with the global control measures in place at that time, 30 million people were expected to die due to tuberculosis by the year 2000 [2]. The most effective vaccine against tuberculosis in man is the BCG vaccine, an attenuated substrain of *Mycobacterium bovis*, which has been used for more than 50 years worldwide. However, this vaccine is very erratic in conferring protection, varying as much as 0–80% in separate clinical trials [3]. In countries with a lower incidence of tuberculosis, such as the United States, the emergence of multidrug-resistant strains threatens control measures with anti-mycobacterial drugs. It is apparent that current immunotherapeutic and chemotherapeutic approaches for the control of tuberculosis need to be improved.

After inhalation, the organism replicates within the lung macrophage. The protective response to infection with intracellular bacteria is cell-mediated [4]. Protective immunity in the mouse model of tuberculosis is mediated by T lymphocytes that secrete gamma interferon (IFNγ), which activates infected macrophages to control intracellular bacilli in a manner believed to be similar to the protective response in man. Several subpopulations of T lymphocytes contribute to the protective response in the lungs of infected mice [5]. Most of this protection is conferred by a short-lived population of rapidly dividing, IFNγ-secreting $CD4^+$ T lymphocytes [6] which peak within 3 weeks of infection, a time which correlates with the control of further mycobacterial growth in the host [7].

The pivotal role of IFNγ in protective immunity to *M. tuberculosis* was unequivocally demonstrated using IFNγ knockout (KO) mice. The single gene encoding IFNγ was disrupted, and these mice were originally shown to: (a) be incapable of IFNγ production, (b) poorly express class II MHC, (c) be deficient in the production of reactive oxygen and reactive nitrogen radicals, and (d) be very susceptible to *M. bovis* BCG [8]. IFNγ KO mice succumbed to infection with *M. tuberculosis* fairly rapidly whether the virulent bacilli were delivered intravenously at moderate [9] to high doses [10] or via a low-dose aerosol [9]. There is also an absolute requirement for interleukin (IL)-12 in the protective response against TB. This has been demonstrated using IL-12p40 KO mice. These mice do not produce the heavy chain of the IL-12 heterodimer and therefore do not make the bioactive p70 form of IL-12, which results in a poor cell-mediated response to antigen [11]. Recently, it was shown that IL-12p40 KO succumbed to an intravenous infection with *M. tuberculosis* within 50 days [12]. Whereas wild-type controls contained the infection and strongly

expressed genes encoding IFNγ, tumor necrosis factor (TNF), and inducible nitric oxide synthase (iNOS) in infected tissues, these KO mice produced no IFNγ message and delayed TNF and iNOS message.

As protective cytokines, which play a pivotal role in protection against tuberculosis, IFNγ and IL-12 represent attractive targets for cytokine-based therapy approaches designed to enhance protective cell-mediated immunity [13]. Recently, a replication-deficient adenoviral vector designed to deliver IFNγ (AdIFN) was delivered intratracheally into the lungs of BALB/c mice, which were subsequently challenged, with a sublethal aerosol of *M. tuberculosis*. Prior pharmacokinetic analyses of adenoviral-mediated expression of IFNγ in BALB/c mice had indicated that transfected mice expressed increased IFNγ in the lungs for as long as 21 days following delivery of the vector. Other mice were transfected with a control virus expressing lacZ (AdLacZ) shortly before the low-dose aerosol exposure to *M. tuberculosis*. AdIFN-treated mice initially contained the infection in the lungs much better than the control nontransfected mice or AdLacZ-treated mice (Fig. 1). The protective effect in the lungs paralleled the local production of IFNγ by the vector and, thus, was relatively short-lived, such that the load of viable bacilli in AdIFNγ-treated lungs reached levels similar to the controls by 30 days of infection. There was no protective effect on the control of mycobacterial dissemination or growth in other primary target organs. Similar AdIFNγ-mediated control of bacterial growth in the lungs was not seen in mice, which already had established chronic *M. tuberculosis* infection.

Based on these preclinical data, several clinical trial have been initiated for both multidrug-resistant *M. tuberculosis* and persistent mycobacteria avium

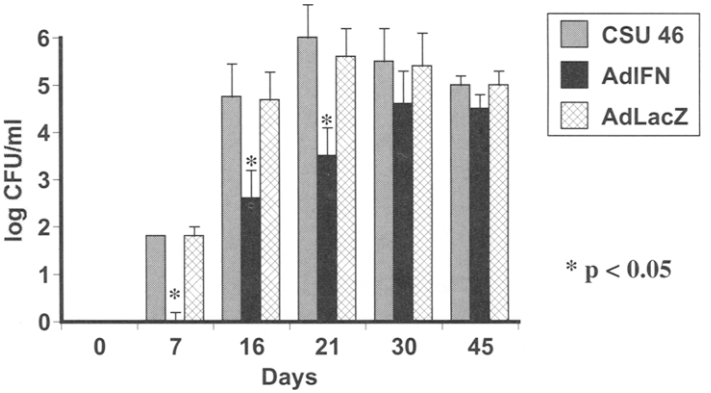

Figure 1 AdIFN reduces growth of *Mycobacterium tuberculosis* in the lung. Mice were pretreated with AdIFN, AdLacZ, or vehicle and then challenged with CSU 46, a clinical isolate of *M. tuberculosis*, and lung organism burden was quantified by quantitative organ culture serially after aerosol challenge (data provided by Dr. Elizabeth Rhoades and Dr. Ian Orme, Colorado State University).

complex (MAC) infection in non-HIV-infected hosts. Williams and colleagues, from our group, recently reported on a Phase I trial of aerosolized IFNγ to patients with persistent MAC infection [14]. All patients tolerated the aerosol well and three of eight had sputum acid-fast bacilli (AFB) smears convert to negative. Condos and colleagues have recently reported on five patients in New York City with multidrug-resistant tuberculosis who received 500 μg of IFNγ aerosolized three times a week for 1 month [15]. Again the aerosol form of the drug was well-tolerated and all patients had sputum smears for AFB convert to negative and the time to positive culture increased (from 17 to 24 days, not significant), suggesting a reduction in organism burden. Moreover, patient weight increased or stabilized, and there were objective decreases in the size of cavitary lesions in all patients 2 months after treatment had ended. It is important to note that data to date suggest that IFN, whether in protein or vector form needs to be provided for a relatively long time to control *M. tuberculosis* growth. Thus it is possible that newer generations of adenoviral-based or other vector systems may achieve longer-term control of infection.

B. Pneumonia

Pneumonia and influenza infection remain the sixth leading cause of death in the United States [16]. Drug-resistant organisms are increasingly isolated from infected patients, presumably due to the broad use of antibiotics. As mentioned above, several biological response modifiers such as granulocyte colony stimulating factor (G-CSF) and IFNγ have been investigated in patients as protein-based therapies. However, due to pharmacological advantages of adenovirus and other gene-based vector systems, gene therapy may provide an alternative approach for *in vivo* immunomodulation.

Adenoviral-mediated gene transfer of the murine IFN gene (AdIFN) results in dose-dependent increases in IFN in bronchoalveolar lavage fluid (BALF) in both mice and rats [17]. Recombinant protein expression occurs up to 28 days in Sprague–Dawley rats and up to 21 days in Balb/c mice [17]. Expression of IFN has a biological effect for at least 14 days in the lung as class II MHC is significantly upregulated in lavaged alveolar macrophages over this time [17]. Moreover, although AdIFN does not result in spontaneous release of TNF in the lung, a subsequent challenge with intratracheal endotoxin results in a greater than fivefold increase in peak TNF levels in BALF in AdIFN-transduced animals compared to control animals (Fig. 2). This enhanced TNF response is associated with increased neutrophil recruitment [17] and increased clearance of *Pseudomonas aeruginosa* up to 14 days after gene transfer [17]. Although the high levels of both IFN and TNF in the BALF were quite high, these cytokines were confined to the lung and remained essentially undetectable in the plasma (data not shown). Thus, these compartmentalized

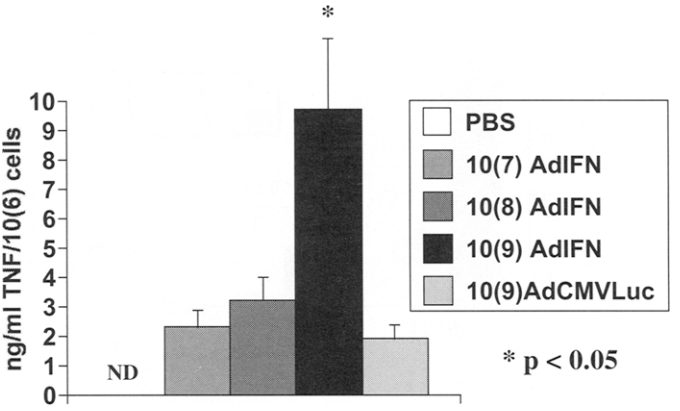

Figure 2 Dose-dependent increase of LPS-induced lung TNF production by AdIFN. Six- to 8-week-old BALB/c mice were pretreated with AdIFN, AdCMVLuc, or PBS 3 days prior to administration of intratracheal LPS. TNF was measure in bronchoalveolar lavage (BAL) fluid 3 h after LPS administration by ELISA and corrected for macrophage cell number in the BAL fluid. ND, none detected.

effects may offer cytokine gene transfer and advantage over systemically delivered protein-based therapies.

Alcohol abuse is a risk factor for bacterial pneumonia [18] as well as acute lung injury [19]. Alcohol intoxication increases the risk of aspiration and suppresses macrophage free-radical protection and bacterial killing [20, 21]. Moreover, alcohol can suppress the elaboration of alarm cytokines such as TNF [22, 23]. Alcohol-induced suppression of TNF production by macrophages can be reversed by IFN *in vitro*. To investigate whether IFN gene therapy could augment TNF and bacterial host defense *in vivo*, we administered AdIFN intratracheally to rats, followed 3 days later with an acutely intoxicating dose of ethanol (5.5 g/kg intraperitoneal). Thirty minutes later animals were challenged intratracheally with endotoxin (LPS) or live *Klebsiella pneumoniae* to measure LPS-induced TNF responses and lung neutrophil recruitment or bacterial clearance of *K. pneumoniae*, respectively. This dose of alcohol has previously been shown by our group to suppress LPS-induced lung macrophage production of TNF. AdIFN pretreatment prevented alcohol-induced TNF suppression as well as lung neutrophil recruitment (Figs. 3A and 3B). Moreover, we observed a significant increase in lung bacterial clearance of *K. pneumoniae* (Fig. 3C) [24].

Standiford and colleagues have shown that adenoviral gene transfer of functional IL-12 [25] produces the p70 heterodimer of IL-12 in the lung lavage fluid in a dose-dependent fashion for up to 7 days [26]. Mice pretreated with this vector, then challenged with 3×10^2 *K. pneumoniae*, had significantly improved survival, compared to AdCMVLacZ-treated or untreated

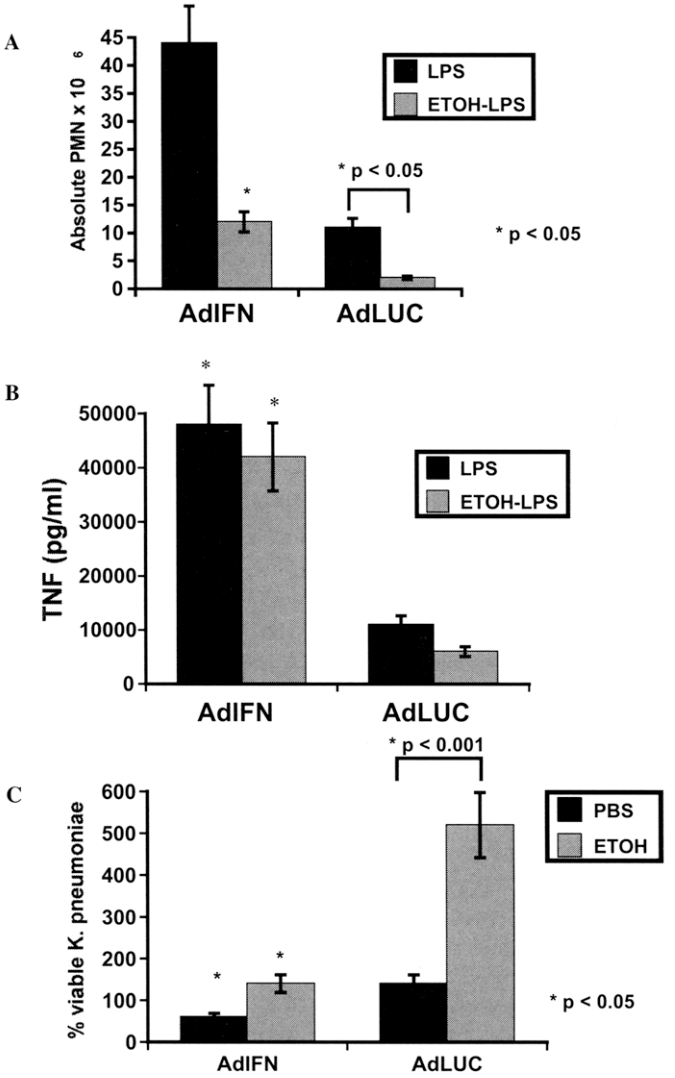

Figure 3 Enhancement of pulmonary host defense in an acute model of ethanol (ETOH) intoxication. Male Sprague–Dawley rats were treated with 10^9 pfu of AdIFN or AdLUC as a control. Three days later, rats were treated with intratracheal LPS or live *K. pneumoniae*. Animals receiving LPS were sacrificed 3 hours later to determine cell migration into or TNF concentration in the BAL fluid. Rats receiving *K. pneumoniae* were sacrificed immediately or 4 h after bacterial inoculation to determine bacterial clearance. (A) AdIFN reverses ETOH-induced suppression of lung neutrophil migration after LPS. (B) AdIFN enhances lung TNF release into BAL fluid after LPS in control and ETOH-treated animals. (C) AdIFN improves lung clearance of *K. pneumoniae* in a ETOH-treated rat model.

controls [26]. The beneficial effect of IL-12 overexpression was mediated by both tumor necrosis factor alpha (TNFα) and IFNγ, as survival in Ad5IL-12-treated mice was attenuated by concomitant neutralization of endogenous TNFα or IFNγ [26]. This same group demonstrated the feasibility of using TNFα, a critical proinflammatory cytokine in lung host defenses (27, 28), for *in vivo* immunomodulation of pulmonary host defense. To overexpress TNF compartmentally in the lung a recombinant adenovirus expressing TNF (AdmTNF) has been reported [29]. Concomitant bacterial challenge with *K. pneumoniae* and low-dose AdmTNF (10^8 pfu) resulted in improved host defenses against the organism. However, a higher dose of vector (5×10^8 pfu) was not beneficial in terms of bacterial clearance. Thus, understanding dose–response relationships in gene-based immunotherapies will be critical for this form of treatment to have an impact in clinical infections.

Crystal and colleagues have used an adenoviral vector encoding CD40 ligand (AdCD40L) in a vaccine approach to protect against *P. aeruginosa* (PA) lung infection [30]. CD40L is expressed on activated T cells and allows dendritic cells (DCs) which are specialized antigen-presenting cells, to interact directly with either $CD8^+$ cytotoxic T cells [31, 32] and B cells [33]. By transfecting DC, with AdCD40L, Kikuchi and colleagues demonstrated that gene-modified DCs pulsed with PA could stimulate naïve B cells to produce anti-PA antibodies. Moreover, if these pulsed, gene-modified DCs were administered *in vivo*, an *in vivo* anti-PA responses was achieved which protected the vaccinated mice against a subsequent challenge with PA [30]. To support the fact that B cells were critical to this response, passive transfer of either serum or B cells from vaccinated mice conferred protection to naïve mice subsequently challenged with PA.

C. Opportunistic Infections

In addition to its known effects on upregulating macrophage function and innate host defenses, IFNγ is also the prototypic TH1 cytokine that facilitates TH0 $CD4^+$ T-cell differentiation into TH1-expressing $CD4^+$ T cells [34]. Moreover, IFN can also modulate the cytokine expression of $CD8^+$ T cells to a Tc1 phenotype [35, 36]. As IFN is produced by activated $CD4^+$ T cells, a lack of IFN secretion could partly explain the pulmonary host defense defect associated with HIV infection. Among HIV-associated opportunistic infections, *Pneumocystis carinii* pneumonia remains a persistent complication of HIV infection. There is an inverse relationship between $CD4^+$ T-cell count and acquisition of this infection. Furthermore, IFNγ, in the form of recombinant protein given as an aerosol, has been shown to reduce the intensity of *P. carinii* infection in a mouse model [37]. Based on these data, our laboratory investigated whether adenoviral-mediated gene transfer of IFNγ to the lung would have a therapeutic effect in a mouse model of *P. carinii* pneumonia. To

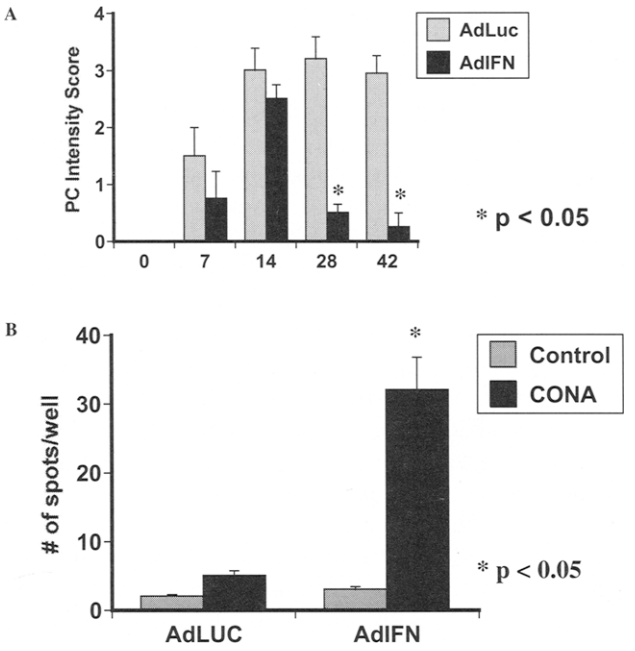

Figure 4 IFN-mediated clearance of *Pneumocystis carinii* in CD4 T-cell depleted mice. (A) Pretreatment with AdIFN resulted in significant clearance of *P. carinii* by 28 weeks. (B) Specific modulation of CD8 phenotype by AdIFN. Lung CD8 cells from AdIFN-treated mice show a significant higher precursor frequency of IFN-producing clones, as measure by Elispot, than AdLUC controls.

test this concept with gene delivery, we used the AdIFN model, which results in prolonged expression of IFN in the lungs of mice depleted of $CD4^+$ T cells [38]. AdIFN-transduced or control (AdLuc) animals were challenged with 2×10^5 *P. carinii* cysts and sacrificed at serial time points. There was similar growth of *P. carinii* in both AdIFN and control animals for the first 2 weeks of the infection. However, after this time point AdIFN-treated mice showed resolution of the infection over 4–6 weeks in spite of continued depletion of $CD4^+$ T cells (Fig. 4A). AdIFN-treated mice recruited greater numbers of T cells, which were largely $CD8^+$ [38]. There was also a significant increase in recruited natural killer (NK) cells in the AdIFN-treated mice [38]. AdIFN was ineffective in improving *P. carinii* infection in both *scid* mice (which have intact macrophages and NK cells) or in mice depleted of both $CD4^+$ and $CD8^+$ T cells, suggesting that $CD8^+$ T cells are required for the clearance effect imparted by AdIFN treatment. In further support of $CD8^+$ T cells having effector function is the fact that there is a greater precursor frequency of IFN-producing $CD8^+$ T cell clones in AdIFN-treated mice as measured by Elispot (Fig. 4B). Understanding effector function of $CD8^+$ T cells in the context of

P. carinii infection may have a significant impact in future therapies designed to support HIV-infected individuals against opportunistic infections.

D. Viral Hepatitis

Both hepatitis B and hepatitis C are important causes of chronic hepatitis and hepatitis B has been linked to hepatocellular cancer. Hepatitis C virus (HCV) is a positive-strand RNA virus and is the major infectious agent responsible for causing chronic hepatitis. Presently, there is no vaccine for HCV infection. There have been recent advances in drug therapy for this disease using a combination of Ribavirin with IFNα [39]; however, there is still need for improved sustained therapy. Lieber and colleagues have demonstrated that adenoviral-mediated gene transfer of hammerhead ribozymes directed against a conserved region of the plus strand and minus strand of the HCV genome were efficient at reducing or eliminating the respective plus- or minus-strand HCV RNAs expressed in cultured cells and from primary human hepatocytes obtained from chronic HCV-infected patients.

Another therapeutic approach has been to locally upregulate innate antiviral immunity. Toward this end Aurisicchio and colleagues demonstrated that adenoviral-mediated gene transfer of the IFNα2 under the control of a liver-specific promoter protected mice from a challenge with mouse hepatitis virus type 3 [40]. Lastly, another approach has been to construct dominant negative core mutants of hepatitis B and when these are expressed in hepatocytes cell lines in the context of a recombinant adenoviral vector, these molecules were capable of significantly suppressing viral replication [41].

II. Chronic Inflammatory Diseases

A. Inflammatory Bowel Disease

The gut has been proposed as a target for gene delivery for a variety of diseases including both metabolic diseases and primary diseases affecting the intestine, including the inflammatory bowel diseases, Chron's disease and ulcerative colitis [42, 43]. Toward this end Hogaboam and colleagues have shown that intraperitoneal delivery of adenovirus encoding interleukin-4 (AdIL-4), a prototypic TH2 cytokine, attenuates colitis induced by trinitrobenzene sulfonic acid (TNB) [44]. TNB-induced colitis is associated with an acute phase followed by an immunologically mediated phase, which is thought to be hapten-induced [45]. The attenuation as a result of AdIL-4 in colitis was associated with a reduction in colonic IFN levels and less induction of inducible nitric oxide synthase [44]. The same group has shown similar data for another TH2 cytokine, interleukin-10 in a similar model of colitis [46].

Adenovirus IL-10 treatment was again done by the intraperitoneal route and associated with a significant reduction in colonic myeloperoxidase activity and leukotriene levels, both markers of acute inflammation. What remains unclear from these studies is whether T-cell activation is modified and whether there is protection against a second bout of colitis. Last, the intraperitoneal approach is essentially a systemic form of therapy since IL-4 and IL-10 can be detected in the serum of these mice. Since the gut can be transduced directly with adenovirus vectors, this raises the possibility that local administration of vectors to inflamed intestine could be used to compartmentally upregulate an immunomodulatory gene that would prevent or attenuate existing colitis.

Toward this end, Wirtz and colleagues have investigated adenovirus-mediated gene transfer to the inflamed colon using intrarectal administration of Ad5-based vectors. These investigators observed significant gene transfer to colonic epithelium, whereas no colonic gene transfer was observed when the vector was given systemically (intravenous or intraperitoneally). Moreover, gene transfer was enhanced in the setting of TNB-induced inflammation. Last, the investigators investigated an Ad5-based vector with a lysine repeat engineered in the fiber gene, the protein responsible for initial interactions with the Coxsackie–adenovirus receptor. With this genetically modified vector, the investigators observed enhanced gene transfer to cells in the lamina propria and spleen, suggesting that antigen-specific T cells could be modified with this vector approach.

B. Arthritis

Like inflammatory bowel disease, rheumatoid arthritis (RA) is thought to be dominated by TH1-like inflammation (Fig. 5) [47, 48]. Among chronic inflammatory diseases, more has been published on gene therapy for arthritis than any other disease. This is likely due to the fact that (1) it is a common disease entity, (2) current treatment, although effective in many cases, can be improved upon, (3) there is a readily accessible site for gene transfer, (4) there are relevant clinical models of the disease, particularly RA, and (5) gene transfer can be accomplished locally to the synovial lining cells using adenovirus-based vectors [49]. The pathogenesis of RA is complex but data to date suggest that there exist alloreactive T cells that secrete TH-1-like cytokines such as TNFα, TNFβ, IL-2, and IFN, which drives inflammation. Accessory cells can also secrete TNF, IL-1β, and IL-18, which are also proinflammatory and can drive TH1 inflammation. This leads to an inflammatory synovial pannus, which mediates destruction of cartilage and joint erosion, which results in loss of joint function over time. A novel T-cell-derived cytokine, IL-17, has also been implicated in the pathogenesis of RA [50].

Since TH-1 inflammation can be downregulated by TH-2 cytokines, such as IL-4 or IL-10, these cytokines have been investigated as candidate genes

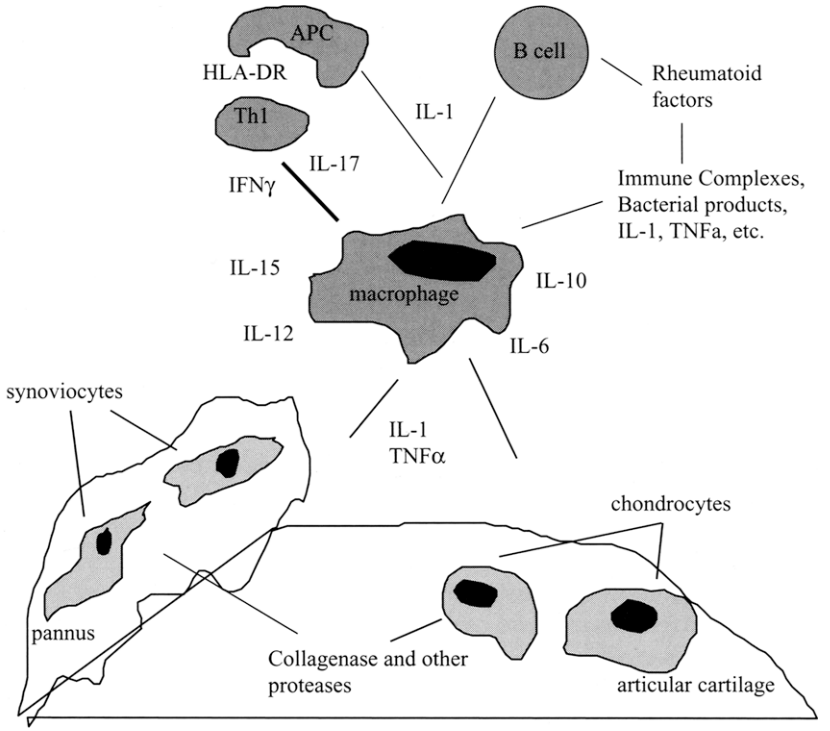

Figure 5 Schematic diagram of the pathogenesis of RA.

to modify RA inflammation. Woods and colleagues investigated adenoviral-mediated gene transfer of the human IL-4 gene into synovial explants from RA patients and demonstrated a significant reduction in IL-1β, TNFα, and IL-8 elaboration in the explant cultures treated with AdIL-4 [51]. In follow-up to this work, the same group demonstrated *in vivo* efficacy of intraarticular AdIL-4 treatment in adjuvant-induced arthritis in a rat model [52]. Of note was that AdIL-4 was effective in both a pretreatment and a posttreatment paradigm [52]. Similar to the *in vitro* findings in human explants, the *in vivo* treatment with AdIL-4 in the rat model was associated with lower TNFα and IL-1β levels [52]. Lubberts and colleagues have also shown efficacy of AdIL-4 in a murine model of collagen-induced arthritis (CIA) [53, 54]. Interestingly, in these studies IL-4 had less effect on the joint inflammation than it appeared to have on preservation of cartilage and in preventing bone erosion [53]. These later effects were associated with a reduction of mRNAs for IL-17, TNF, and IL-1β, as well as a decrease in metalloproteinase activity [53, 54]. These investigators also demonstrated that IL-4 can increase type I procollagen

synthesis and thus this may explain the joint-sparing/repair effect of IL-4 [53]. Last, Kim and colleagues demonstrated that both periarticular and systemic AdIL-4 were effective in a model of CIA [55].

Whalen and colleagues have investigated another TH2 cytokine, viral IL-10, encoded by an adenoviral vector (AdvIL-10) given by periarticular injection in the same model of CIA and found significant benefit in terms of development of arthritis and arthritis score. Moreover, the investigators showed that the injection of AdvIL-10 into one joint prevented arthritis in a second joint [56]. This may be due to *in vivo* T-cell immunomodulation by viral IL-10. In further support of a role for TH2 cytokine gene therapy in RA, Woods and colleagues have recently demonstrated that adenovirus-mediated gene transfer of interleukin-13, another TH2 cytokine, also suppresses TNF and IL-1β production in RA explant cultures [57].

In addition to the TH2 cytokine approach, the other approach of adenoviral gene transfer for arthritis has largely focused on the proinflammatory cytokines TNFα and IL-1β. Toward this end, our laboratory has created soluble type-1 receptors for both IL-1 [58] and TNFα [59] (Fig. 6). Both these molecules are dimerized by the addition of murine IgG Fc fragment and in the case of the TNF inhibitor, this molecule has been found to be more potent in TNF inhibition than monoclonal antibodies that only bind to one epitope [60]. Moreover, the proteins have longer half-lives *in vivo* than the monomeric soluble receptors [59]. Adenoviral-mediated gene transfer of either one of these constructs into the joint space in a rabbit model of arthritis showed less white blood cell infiltration as well as less joint swelling. However, the IL-1 inhibitor showed a better effect in preventing a reduction in cartilage matrix

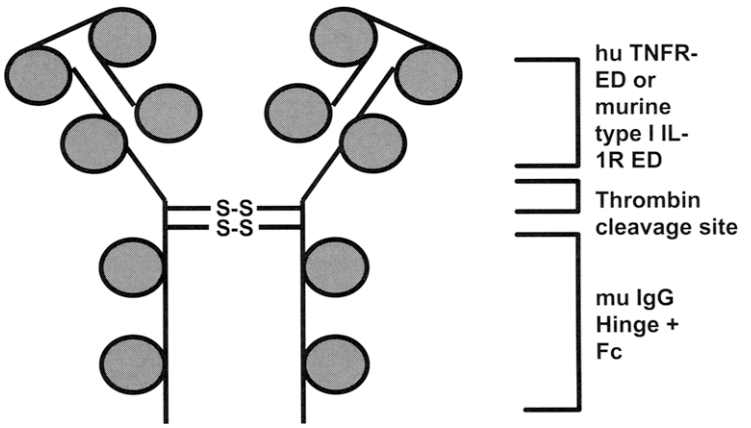

Figure 6 Schematic diagram of TNF and IL-1 receptor fusion proteins utilized in arthritis gene therapy.

degradation. Moreover, the two vectors together appeared to have an additive effect on white blood cell infiltration into the joint space. There was also an effect observed on contralateral joints in this study [58]. Le and colleagues also demonstrated efficacy of the TNF inhibitor gene in a rat model of CIA [61]. Interestingly, Quattrocchi have reported in a mouse model of CIA that there is an acute beneficial effect of the TNF inhibitor fusion protein; however, there is a subsequent rebound with enhanced inflammation despite continued circulating levels of the TNF inhibitor. The investigators speculated that this may be due to antibody formation against the extracellular domain of the receptor that the cross-linked endogenous TNF receptors in the joint [62]. It is important to note that these studies were performed with a chimeric fusion protein (mouse Fc/human p55 TNF receptor) and thus whether the exacerbation of arthritis would be seen with the mouse p55 TNF receptor remains to be seen. Last, Zhang and colleagues have shown that adenoviral-mediated gene transfer of a dominant negative form of inhibitory kappa-B, which facilitates nuclear translocation of nuclear factor-kappa-B, enhanced TNF-mediated apoptosis in synovial tissue [63].

C. Fibrotic Lung Disease

Idiopathic pulmonary fibrosis (IPF) is an insidious disorder that results in the deposition of collagen and fibrous tissue in the lung. The etiology of this disorder is unknown but several groups have reported decreased fibrinolytic activity [64, 65] and elevated tissue growth factor-beta-1 expression [66, 67] in the lungs of patients with IPF. Moreover, IFNγ has been shown in a pilot study to improve lung function in patients with IPF and this impairment was associated with decreased levels of messenger RNA for transforming growth factor-beta-1 and connective-tissue growth factor, the main growth factor product of transforming growth factor-beta stimulation [67]. To date this is the first compound to show improvement in lung function. Many trials have been performed with corticosteroids (prednisone) alone or in combination with cyclophosphamide [68]. However, these agents have not been shown to be effective in preserving lung function in randomized clinical trials, and moreover, their use is associated with significant side effects. Since IPF is associated with dysregulated growth factor gene expression, and a lack of definitive therapy, there is a rationale for gene therapy.

Simon and colleagues recently reported on enhancing fibrinolytic activity in the lung in an effort to ameliorate lung fibrotic injury in response to bleomycin, a chemotherapeutic agent that can cause lung fibrosis [69]. These investigators constructed a recombinant adenovirus encoding urokinase-type plasminogen activator (AduPA), a fibrinolytic activator protein. When expressed in the lung, AduPA resulted in a significant attenuation of bleomycin-

induced increases in hydroxyproline content, a measure of collagen deposition [69]. Furthermore, Nakao and colleagues have shown that adenovirus-mediated gene transfer of *smad7*, a downstream inhibitor of TGFβ signaling could also block bleomycin-induced fibrosis [70]. This finding was specific to *smad7* and not to *smad6*, which does not inhibit TGFβ signaling and thus the data suggest that the effect is through the downregulation of TGFβ signaling. Thus, other molecules such as dominant-negatives or soluble receptors for TGFβ may also be good candidate constructs for pulmonary fibrosis.

In addition to TGFβ other proinflammatory cytokines such as interleukin-1 and TNF have been implicated in pulmonary fibrosis [71]. Our lab reported several years ago on a soluble inhibitor of TNF that consists of the extracellular domain of the human p55 TNF receptor coupled to the murine CH2 and CH3 domains of mouse IgG1 (Fig. 6) [59]. This molecule forms as a dimer and is a potent inhibitor of TNF [60]. When expressed in the context of a recombinant adenovirus, after intravenous administration, the construct results in high circulating levels of TNF inhibitory activity [59]. In fact these mice provide a phenocopy of p55 TNF-receptor knockout mice in that they are resistant to mortality induced by endotoxin and d-galactosamine administration; however, they are susceptible to the intracellular pathogen *Listeria* monocytogenes [59]. However, this molecule also readily crosses into the lung [72] and inhibits TNF activity in this compartment. Moreover, this construct, by virtue of its ability to inhibit TNF in the lung (after systemic vector administration) attenuates the fibrotic response to intratracheal silica [73].

III. Conclusions

There are numerous acquired diseases in which adenoviral-mediated gene transfer has shown in proof-of-principle experiments a therapeutic benefit. The challenges for researchers in the field are to take these data and try to develop safe and effective therapies for these diseases. Toward this end, there will need to be advances in targeted vector therapy and regulated gene expression. One area, which may yield promising results in the near future, is in adenovirus-based vaccines either into somatic cells or professional antigen presenting cells such as dendritic cells or in compartmentalized chronic inflammation such as arthritis. In this case, precise gene expression is less likely and, thus, there are fewer technological hurdles to overcome.

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CHAPTER 21

Testing of Adenoviral Vector Gene Transfer Products: FDA Expectations

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I. Introduction

Adenovirus vectors that contain gene transfer products are biological products subject to Food and Drug Administration (FDA) regulation through the Center for Biologics Evaluation and Research (CBER) [1]. Sponsors of biologicals subject to FDA regulation that are not yet approved for marketing must file a "Notice of Claimed Investigational Exemption for a New Drug," which is abbreviated as "IND" for Investigational New Drug Application.

Adenoviral vector products have been studied in clinical trials under IND since 1993. As of December 2000, approximately 75 INDs involving administration of an adenoviral vector product have been filed with the FDA, with slightly more than 50% currently active. Each IND contains one or more clinical protocols. The vast majority (>90%) of the adenoviral gene therapy INDs target patients with cancer. The clinical studies contained in the remainder of the INDs target patients with vascular disease (coronary artery or peripheral) or genetic/metabolic diseases.

The sponsor is the entity or individual that holds and maintains the IND. The clinical investigator is the individual responsible for the care and welfare of the study participants at his or her site. Sponsors and investigators involved in FDA regulated research must be in compliance with federal regulations, described in the following sections of this chapter. In addition, investigators who receive federal funding for gene transfer clinical research or who conduct clinical studies at an institution that receives federal funds for recombinant DNA research must register the clinical protocol with the National Institute of

Health (NIH) Office of Biotechnology Activities (OBA) and be in compliance with the NIH Guidelines [2].

The FDA's assessment of safety and ultimately effectiveness of adenovirus-containing products involves thorough evaluation of the information contained in the IND, and any supporting information cross-referenced to another IND or drug master file [3]. The type of information contained in an IND is set forth in 21 CFR 312.20, subpart B.

The following sections describe many of the agency requirements and guidances regarding drug development of adenoviral-containing products.

II. Manufacturing Control and Product Characterization

A. Purity, Safety, and Potency

When an adenovirus-based vector is used for the first time in humans, a major goal of FDA oversight is to ensure the safety of patients who receive the investigational product. A crucial component of safety and effectiveness is careful attention to the details of manufacturing and product characterization. The extent and quality of this information allows an assessment of the purity of the final vector preparation that will be administered to patients. Assessment of purity involves biological and biochemical characterization of the vector preparation and assessment of how completely the formulated product conforms to expected characteristics. For adenovirus vectors assessment of purity includes structural and biochemical information about the vector itself as well as demonstration of freedom from unexpected and potentially harmful agents such as viruses, fungi, and bacteria or bacterial toxins.

Another important goal of product characterization is assessment of the potency of adenovirus vector preparations. Potency measurements are intended to determine the extent to which a particular vector preparation has a desired biological activity. A vector preparation with insufficient potency has little chance of behaving as desired in a clinical trial. Although infectious titer has been proposed as a measurement of potency, this is currently not considered sufficient since the correlation between *in vitro* infectious assays and biologic effects has not been established. While potency is related to safety and efficacy, it is also an indicator of product manufacturing consistency.

Direct measurement of potency for a new adenovirus vector product is often challenging due to lack of an appropriate *in vitro* or *in vivo* system to measure potency. Therefore, in initial phases of product development, demonstration of transgene activity by enzymatic means is often adequate for initiation of clinical trials. Development of a *bona fide* potency assay for vector lot release will be required before FDA can license an adenovirus product. It is generally expected that a potency assay will be in place before Phase 3. Thus,

as with all biological therapeutic products, assessment of the purity, safety, and potency of adenovirus vectors is a crucial part of product development.

B. Regulation of Process as Well as Product

The complexity of adenovirus vector manufacturing as well as inherent biological properties of the production system warrants oversight of the production process as well as the final product. Indeed, as with all complex biological products, in order to assure the purity, potency, and safety of adenovirus vectors, regulation of the manufacturing process is as important as characterization and testing of the final product. Therefore, there should be thorough characterization of starting materials and product intermediates in order to assure that the final vector product is acceptable for administration to humans.

Initial development of a new adenovirus vector involves manipulation and cloning of a transgene cassette with the desired gene and appropriate transcriptional regulatory elements. In a commonly used approach to vector production, an appropriate cell line is then cotransfected with the transgene cassette and a backbone shuttle vector that supplies the remaining components of the adenovirus genome. An appropriate cell line allows homologous recombination between the transgene and backbone plasmids and then supports synthesis of replication-defective adenovirus particles. It is the ability to mediate homologous recombination that allows assembly of the desired vector, but this ability also can lead to unintended structural changes. Thus it is crucial to select a vector clone that is fully characterized and has the intended structure. Since the same cell line is then also used to propagate vector for production of virus banks and for large-scale production, it is important to monitor the structure of the vector through several stages of manufacturing.

The cell lines used for production of adenovirus vectors add another complex, biological component to the manufacturing process. The characterization of the cell lines, including master cell banks and working cell banks is described in detail in section VI.

C. Current Good Manufacturing Practices

The principles of current Good Manufacturing Practice (cGMP) as per 21 CFR 210 and 211 apply to adenovirus gene transfer products. However, implementation of cGMPs may be staged according to the phase of product development, but there should always be appropriate documentation of manufacturing and of quality oversight. For Phase 1, this includes appropriate written protocols for each stage of product manufacturing and characterization. At later stages of product development, appropriate documentation of manufacturing should employ standard operating procedures (SOPs) and capture all important information relating to vector production. Quality oversight always involves quality control (QC) and quality assurance (QA) mechanisms,

regardless of where manufacturing is taking place. In essence, this means that the person(s) responsible for assurance that the production and characterization testing have all been performed properly and have met specified criteria (quality assurance) are separate from and not direct subordinates of the person(s) responsible for conducting these tests and filing these reports (quality control).

As product development moves from Phase 1 into later phases, cGMPs also stipulate development of validated assays that must be in place by product licensure. Data regarding assay performance (specificity, sensitivity, and reliability) should be submitted to the agency as part of the validation process.

III. Development of Recommendations for the Manufacture and Characterization of Adenoviral Vectors

Many factors contribute to development of FDA recommendations and requirements for characterization of adenovirus vectors. First are the regulations found in the various applicable parts of the Code of Federal Regulations (CFR). These include the regulatory requirements that biological products administered to humans must be sterile (21 CFR 610.12 or another valid alternative testing of equal sensitivity), be free of mycoplasma (21 CFR 610.30), and meet endotoxin limits (limulus amebocyte lysate [LAL] per 21 CFR 610.9 or pyrogenicity test 21 CFR 610.13(b)). These establish minimum criteria to assure that products administered to humans are not contaminated with microbial organisms or their toxic byproducts.

Next are FDA review staff who have accumulated experience from review of many adenovirus vector and other gene therapy products. Some reviewers maintain active research programs in areas related to adenovirus biology or have participated in such research in the past. CBER reviewers have regular internal meetings to discuss relevant issues and develop consistency in oversight of adenovirus vector products. A major effort in this regard was launched March 6, 2000, with the issuance of a letter to Gene Therapy Sponsors requesting comprehensive information on product, preclinical, clinical, and QA/QC areas (see section XVI). These data have been tremendously useful and will be used to refine CBER's recommendations regarding adenovirus and other vector products. The cumulative experience of FDA reviewers is also utilized to develop guidance documents, several of which are relevant to the manufacture of adenovirus gene transfer products [4–6]

The experience of the gene therapy community has also played a key role in development of FDA recommendations in regulation of adenovirus vector products. The experience of adenovirus vector manufacturers is communicated in meetings between the manufacturer and FDA staff, at presentations

at scientific meetings, and at presentations to the NIH Recombinant DNA Advisory Committee (RAC).

The NIH RAC has played an important role in the development of recommendations and it provides a public forum for discussion. Following the death of a patient in a gene therapy trial in late 1999, the RAC empanelled an ad hoc advisory group, the RAC Working Group on Adenoviral Vector Safety and Toxicity (Ad-SAT), to examine data from adenovirus gene transfer trials with the intent of formulating recommendations to improve the safety of these clinical trials. One important discussion centered on the accuracy of adenovirus vector titers in terms of both total particle and infectious particle titers [25]. Since toxic vector doses are just above doses with potential therapeutic effect, there was particular concern over lack of accuracy and comparability between titers determined for different product lots and between different clinical trials. This discussion highlighted the need for a reference standard that could be used to help standardize adenovirus vector titer measurements.

This public discussion helped stimulate a gene therapy community initiative to develop such standards. Several public meetings to develop consensus on the need for a standard, to discuss the nature of the reference material, and to discuss mechanisms for its development were held in late 2000 and early 2001 [7]. An Adenovirus Reference Materials Working Group (ARMWG) was formed under the auspices of the Williamsburg Bioprocessing Foundation (WBF), and an WBP/FDA partnership agreement was formulated that allowed participation of FDA staff in development of a reference stock of wild-type adenovirus type 5 which can be used to calibrate assays for particle number and infectivity. The role of FDA is to lend scientific and regulatory expertise in the form of recommendations to the ARMWG, which oversees the development of the reference material. Information on this initiative is available at the WBF website (www.wilbio.com) and the CBER website (www.cber.fda.gov). The information includes meeting minutes, transcripts from FDA cosponsored meetings, and explanations of the bid mechanisms by which participants volunteered donations of goods and services toward production and characterization of the reference material. This reference material will provide another mechanism for FDA to formulate recommendations for characterization of adenovirus-based gene transfer products.

FDA also seeks input from advisory committees such as the Biological Response Modifiers Advisory Committee (BRMAC) for recommendations regarding characterization of adenovirus vectors. BRMAC meetings allow FDA to obtain advice on scientific issues that impact gene transfer experiments in a public forum in which all interested parties are allowed to participate. Transcripts of these meeting are also available on the CBER website (www.cber.fda.gov). The BRMAC's advice on issues such as the amount and type of structural characterization of gene transfer vectors, discussed at two

recent committee meetings, has been valuable as CBER staff develop and update policy [8, 9]

In summary, the FDA receives input and feedback from a variety of sources in formulating recommendations regarding adenovirus manufacturing and characterization. The recommendations may change with advances in technology and through accumulating experience. FDA considers the potential risks and benefits of each vector product and each proposed clinical trial when making its recommendations. This case-by-case approach, which takes into account the severity of the disease and the proposed patient population, permits some flexibility in product manufacture and characterization.

IV. Considerations in Manufacturing Adenoviral Vectors

A. Components and Characterization

While the goal of adenovirus vector manufacturing is to produce a safe, pure, and efficacious vector, the complexity of the process necessitates careful control of the entire manufacturing procedure and of the components used. Raw materials can be a source of adventitious agents or toxic impurities that negatively impact safety of the final product. At early stages of product development, certificates of analysis (CoA) for many raw materials such as buffers, and basic tissue culture components should be part of the documentation demonstrating that these reagents are pure and free of adventitious agents. These CoAs should be kept in the manufacturer's records and sample CoAs should be submitted to the agency. At later stages of product development, development of testing and acceptance criteria for some of these materials may be required of the sponsor. As an example, current techniques for adenovirus vector production require mammalian cell substrates. Raw materials include a source of serum, usually fetal bovine serum (FBS), and enzymes such as porcine trypsin for cell culture. These reagents can be contaminated with adventitious virus. Trypsin has been identified as a potential source of porcine parvovirus while FBS can harbor several adventitious viruses. Therefore, FBS and porcine trypsin should come only from sources where appropriate testing is conducted and documented in a CoA. A manufacturer of adenovirus vectors should retain all such CoAs and submit sample copies to FDA. Also, bovine serum from geographic areas known to harbor endemic bovine spongiform encephalopathy agent (BSE) is considered inappropriate for use in manufacturing a biological for use in humans.

Since adenovirus vector production relies on cells that support replication of the vector, cell banking is an important aspect of production. Cell banks are cryopreserved stockpiles consisting of very well characterized cell populations that have been shown to be free of adventitious virus, are sterile, and have the

capacity to support production of the adenovirus vector. Ideally, cell banks are derived from early cell passages and assure that a reliable and consistent source of qualified cells is available for the foreseeable future production needs. Details of the necessary characterizations for cell banks are discussed below.

In similar fashion, virus banks are an important aspect of adenovirus production. Virus banks consist of frozen stocks of very well characterized molecular vector clones. Characterization includes structural, physical/biochemical, and functional assessments in addition to assessments of microbial sterility and freedom from adventitious viruses. Virus banks are derived as an early step in vector manufacturing and assure that a reliable source of infectious vector is available for foreseeable future production needs. Details of the necessary characterizations for virus banks are discussed below.

B. Protocols

The protocols used for each step of manufacture are important records which can demonstrate that the production process and the starting materials for vector production are of a quality sufficient to assure that the final product is pure and safe. Detailed descriptions of each step should be maintained and submitted to the FDA as part of an IND. Many protocols are an integral part of manufacturing and should be part of standard operating procedures (see below). Even though many protocols such as the molecular biology techniques used to assemble a vector are not repeated steps, detailed protocols for these stages are essential.

V. Process Controls

Control of the manufacturing process is obtained through testing and characterization of intermediates and final product in the production scheme. For adenovirus vectors this includes characterization of the cell substrate (master and working cell banks), the virus seed stock (master virus bank), the bulk vector preparation, and the final formulated product. Details of the testing are outlined below. The goal of process control is twofold; to ensure safe, pure, and efficacious vector products and to demonstrate that the production process is highly reproducible.

A. Standard Operating Procedures

Standard operating procedures are a mechanism to ensure that process controls and protocols for product manufacture and characterization are carried out in a reproducible and documented fashion for each stage of manufacturing and product testing. SOPs consist of detailed written documents describing each step of a process conducted in manufacturing. SOPs

can also refer to many different types of processes that impact adenovirus production, such as required training of personnel, acquisition and acceptance of raw materials, procedures for shipping and handling final product, and conduct of quality oversight. For early product development, SOPs should be developed for the manufacturing and testing steps discussed below. For later stages in product development, consultation with the FDA is advisable to assure comprehensive coverage of the manufacturing process by appropriate SOPs.

B. Quality Assurance and Quality Control Programs

Quality assurance and quality control programs are considered essential steps in assuring safe and high-quality adenovirus vector products. A key concept in a QA/QC program is that there should be separation of authority between the personnel responsible for conduct of testing and manufacturing and the personnel who examine and approve the test data and final product characterization. This can be accomplished in a variety of ways. For instance, separate QA and QC departments in the same institution can be used provided that the responsible personnel not be under direct supervision of one another.

An important topic that is often misunderstood is the division of responsibilities between an IND sponsor and a multiuse facility contracted to do some part of product manufacturing. When these facilities are used to produce more than one gene transfer vector, they are termed multiuse facilities. Many gene-therapy vectors are produced in multiuse facilities. IND sponsors often assume that the contract lab will provide all necessary QA/QC, manufacturing, and product testing information to the FDA and do not involve themselves sufficiently in designing the testing, examining the data, and/or answering FDA questions. Although the contract lab plays an important role, the responsibility for oversight of QA/QC and reporting lies with the sponsor. The sponsor must recognize that the FDA holds them accountable for oversight of production and testing conducted by a contract organization. An additional concern with multiuse facilities is the potential for cross-contamination of one product with a product made previously or concurrently. The multiuse facility should test for cross-contamination or validate the production and purification process to rule out cross-contamination.

The entire production process, from raw materials to oversight of testing and product release, is important in assuring that adenovirus and other gene transfer vectors are as safe and consistent as possible. The next sections describe in greater detail the characterization that should be done for each of the major components or intermediates as well as the final product in adenovirus vector production. These include the cell banks, the virus bank, the bulk virus preparation, and the final vector product.

VI. Characterization of Adenoviral Vector Production Intermediates

The necessity for and specifications for each of these characterizations is assessed on a case-by-case basis and can change depending on the phase of product development and as a result of feedback from the numerous sources discussed above. Therefore, the following material is intended to give the reader an overview of FDA expectations. Consultation with CBER at the pre-IND stage is strongly recommended.

A. Master Cell Bank

Testing of the cell banks used in adenovirus vector production is of two general types; safety testing and characterization. Table I is an overview of the recommended characterizations. The safety testing is intended to demonstrate that the cell bank is free of any detectable microbial contamination including bacterial, fungal, and viral. Sterility testing is a universal requirement for biologics and is set forth in 21 CFR 610.12. Alternative sterility assays validated to be of equal sensitivity may also be used. The basic premise is to apply the product, in this case cells from the master cell bank, to several growth media and to look for outgrowth of microbial contaminants over the course of 14 days. The specification for this test is no contaminants.

Mycoplasma testing is conducted by inoculation of both cells and cell supernatants into appropriate cultures and examining for growth of

Table I
Characterization of the Cell Banks[a]

Safety	Identity
Sterility	Morphology
Mycoplasma	Isoenzyme tests
Adventitious virus	Cell-specific identity test
• *In vitro* and *in vivo* virus	
• Bovine, porcine, canine viruses (ancillary product dependent 9CFR113.47)	
• Human viruses: EBV, HBV, HCV, CMV, HIV 1&2, HTLV 1&2, AAV, B19 (other cell substrate specific)	
Tumorigenicity	

[a]The necessity for and specifications for each of these characterizations is assessed on a case-by-case basis and can change depending on the phase of product development and as a result of feedback from the numerous sources discussed above. Therefore, this list is intended to give the reader an overview of FDA oversight. However, consultation with CBER is strongly recommended before submission of an IND.

mycoplasma. This testing is described in FDA guidelines [5]. Alternative tests such as PCR could be utilized following proper demonstration of the sensitivity and comparability to the culture-based assay.

Adventitious virus tests are also intended to show that the test material is free of a variety of viruses. The *in vitro* adventitious virus test is conducted by inoculating cell cultures with the test material, in this case supernatants from the master cell bank (MCB). Following 14 days in culture, cells are tested for their ability to mediate hemadsorption or hemagglutination with red blood cells from three different species. The cell lines are chosen for their ability to support replication and detection of many different viruses. A list of viruses that can be detected is given in Table II. The *in vitro* adventitious virus assay provides a nonspecific screen for many different viruses and can sometimes be used to identify certain viruses. The *in vivo* adventitious virus test is conducted by inoculating animals from several species with supernatant from the cell bank material. The species are chosen to optimize detection of possible contaminating adventitious viruses. The *in vivo* virus test is capable of detecting an array of viruses complimentary to those detected by the *in vitro* assay. A list of viruses that can be detected is given in Table II. For both types of adventitious virus tests, the acceptable specification is no detection of virus.

In addition to these nonspecific tests, a variety of specific tests for many different viruses may be required. As the current cell lines used to support adenovirus replication are of human origin, a variety of human virus tests are included. FDA-approved test kits should be used when available. Although the cell lines used to produce adenovirus are not generally thought to support replication of several of these viruses, experimental data to preclude this possibility do not exist. In addition, if sensitive cell-line-specific identity tests are not part of the MCB characterization, it is possible that other human cell lines could be present and may serve as a reservoir for some of these

Table II[a]
***In Vitro* and *In Vivo* Adventitious Virus Testing**

In vitro adventitious virus testing	*In vivo* adventitious virus testing
Picornaviruses: e.g., poliovirus, Coxsackie B, echovirus, rhinovirus	Picornaviruses: e.g., influenza, Coxsackie A and B, poliovirus
Togavirus: e.g., rubella	Bunyavirus: e.g., LCMV, hantavirus
Paramyxovirus: e.g., parainfluenza, mumps measles, RSV	Herpesvirus: e.g., HSV-1
Orthomyxovirus: e.g., influenza	Paramyxovirus: e.g., mumps
Adenovirus	Coronavirus
Herpesvirus	Flavivirus[b]

[a] "Fields Virology," Chap. 17 [33].
[b] "Fields Virology," Chap. 31 [34].

viruses. In addition, it is surprising that some viruses not thought to replicate in cell lines such as HEK 293 (human embryonic kidney fibroblasts) have been detected in adenovirus product lots. For the above reasons, these tests are currently recommended at various steps for all adenovirus vector production. Currently, the specific virus tests include Epstein–Barr virus (EBV), hepatitis B virus (HBV), hepatitis C virus (HCV), cytomegalovirus (CMV), human immunodeficiency viruses I and II (HIV 1 and 2), human parvovirus B19, human T-lymphotrophic viruses 1 and 2 (HTLV 1 and 2), and adeno-associated virus (AAV). The test methods, specifications and sensitivities for these tests should be submitted as part of the proposed acceptance criteria for cell banks.

Nonhuman cell lines could also be used to produce adenovirus vectors. In such cases additional testing may be necessary. For example, if rodent cells were used, the MCB should also be tested by the appropriate antibody production test: murine antibody production (MAP), rat antibody production (RAP), or hamster antibody production (HAP) [6].

Current adenovirus production methods commonly use fetal bovine serum (FBS) and porcine trypsin for propagation of producer cell lines. The use of FBS carries two types of risks; the potential for patient exposure to BSE and to adventitious bovine viruses. The use of porcine trypsin carries risk of patient exposure to porcine parvovirus. Producer cell lines with sufficient documentation may be usable without tests for bovine or porcine viruses or BSE. When FBS is used, sufficient documentation includes the following: certificates of analysis (CoA) showing that the FBS is not from one of the countries on the USDA list of countries where BSE is found and that the FBS has been tested for bovine viruses. For porcine trypsin, sufficient documentation includes CoAs showing that the trypsin is negative for porcine parvovirus. If documentation of viral testing is unavailable, the testing will be requested as per 9 CFR 113.47. Once an MCB is tested or shown to have an accepted history of nonexposure to these agents, these tests may be omitted in subsequent stages of production if CoAs of FBS and porcine trypsin contain the proper testing and come from approved geographic locations.

Although tumorigenicity testing has often been requested, it is acknowledged that the cell line used in adenovirus production may be tumorigenic in immunodeficient mouse strains. In later stages of product development, this test may be required. For products that are in Phase 1 of clinical testing, it may be possible to omit this test if there is sufficient testing of the product for cell substrate DNA (see below).

In addition to safety testing, characterization of MCBs should include tests for identity of the cell lines. Isoenzyme analysis can show the cell line is of the correct species. For most current adenovirus producer cells, this involves testing for human isoenzymes. Morphology is also assessed to show that the cell line retains the expected shape and size. Development of a cell-specific

identity test is currently recommended so that accidental contamination of the adenovirus vector producer cell line can be detected.

B. Working Cell Bank

Working cell banks are expanded cell populations derived from the MCB and are tested after a defined number of cell generations. The testing of WCBs is similar to that requested for MCBs and consists of the following safety tests: sterility, mycoplasma, and *in vitro* adventitious virus. Characterization includes morphology and isoenzyme analysis.

C. Master Virus Bank

A master virus bank (MVB) consists of a well-characterized stock of virus-based vector that serves as the inoculum for all subsequent large-scale vector production. It is sometimes referred to as a vector seed stock. Table III gives a summary of the types of characterization recommended by the FDA. Safety testing for a master virus bank is very similar to that done for a master cell bank. Thus a master virus bank is tested for sterility and mycoplasma, *in vitro* and *in vivo* adventitious virus, and specific viruses (EBV, HBV, HCV, CMV, HIV 1 and 2, AAV, B19, HTLV 1 and 2) if the cells used to produce the MVB were not fully characterized as described for the MCB. Depending on the degree of characterization of the FBS and porcine trypsin, a MVB may require testing for bovine viruses and porcine parvovirus.

Table III
Characterization of the Master Virus Bank[a]

Safety	Characterization
Sterility	Identity
Mycoplasma	• Sequence insert and flanking regions restriction map[b]
Adventitious Virus	Activity
• *In vitro* and *in vivo* virus	• Transgene specific protein expression
• Bovine, porcine, canine viruses (ancillary product-dependent 9CFR113.47)	• Other
• Human viruses: EBV, HBV, HCV, CMV, HIV 1&2, HTLV 1 & 2, AAV, B19	Titer
• Replication-competent adenovirus	• Infectious titer
	• Particle count

[a]The necessity for and specifications for each of these characterizations is assessed on a case-by-case basis and can change depending on the phase of product development and as a result of feedback from the numerous sources discussed above. Therefore, this list is intended to give the reader an overview of FDA oversight. However, consultation with CBER is strongly recommended before submission of an IND.

[b]BRMAC Advisory Committee Meeting, November 16, 2000: recommended entire sequence for vectors <40 kb [8].

In addition to the above testing for cell banks, an adenovirus MVB should be tested for replication-competent adenovirus (RCA). RCAs are a common byproduct of adenovirus vector production and are currently considered a safety risk. RCAs most often arise due to molecular recombination between the vector and endogenous elements of the producer cell line genome. Some of these recombinations restore the replication competence of a normally replication-defective vector and give rise to RCA. For example, in the HEK 293 cell-line, endogenous E1 sequences are required to allow replication of the E1 defective adenovirus vectors. The vectors can undergo homologous recombination with the endogenous E1, thus restoring their replication competence. This is a stochastic and unavoidable consequence of the biology of the certain producer cells. Development of producer cells with smaller or no regions of homology between vector and endogenous sequences may reduce homologous recombination but may still support nonhomologous recombination. For most replication defective adenovirus vectors, RCA testing is performed by inoculation of the test material onto a cell line that will support replication of a RCA but not of defective vector. Supernatant from this treatment is passaged to a second cell monolayer. Development of cytopathic effects (CPEs) or lysis indicates the presence of RCA. The current recommended specification for RCA is ≤ 1 RCA in a total of 3×10^{10} virus particles.

Characterization of the adenovirus vector MVB encompasses a variety of approaches to establish the physical, biochemical, and biological properties of the vector preparation. Identity is an important parameter and demonstrates that the intended product is the actual starting material for large-scale production. Current FDA recommendations for structural characterization of adenovirus vectors include determination of the nucleotide sequence of the transgene insert and flanking regions. The remainder of the structure can be demonstrated by techniques such as restriction mapping and PCR. In cases where extensive characterization of the transgene protein is available, no sequencing is necessary and restriction mapping of the vector would be sufficient. However, at a recent meeting of the BRMAC which addressed the issue of structural characterization, it was recommended that vectors ≤ 40 kb in length should be characterized by sequencing of the entire vector genome [8]. It is likely that the FDA will adopt this recommendation in the near future. For adenovirus vectors, the MVB would be the most appropriate material for this sequence analysis.

Another important characteristic of an adenovirus vector MVB is the activity of the transgene. Although this is not a potency assay per se, this parameter suggests that the therapeutic transgene will be functional in clinical trials and thereby justifies the risks of exposure to patients. Activity assays can include demonstration that the transgene-encoded protein is expressed and demonstration that the protein is functional in some biochemical assay. Assays that determine the expression and activity of the adenovirus vector should

be part of the acceptance criteria for each MVB. Methods and acceptable specifications for these assays should be part of IND submissions.

The number of adenovirus vector particles in a MVB is measured in two ways. One method is to determine the particle count. Most often this is determined by a measurement of the amount of DNA in a vector preparation which is then related to particle number by an agreed-upon conversion factor. Although this is a physical/chemical assay, the precision is affected by several factors including formulation of the vector preparation and nonviral nucleic acid content of the preparation. Cellular nucleic acids as well as differences in DNA sequence between vectors can affect the precision of this measurement, which can vary on the order of 10%.

A second measure of adenovirus vector quantity in a MVB is the infectious titer. This is an assessment of how many of the particles retain the capacity to interact with cell surface receptors and subsequently undergo internalization. This measure is an indication that the manufacturing process is gentle enough to preserve viral coat protein structure and will largely determine the ability of adenovirus preparations to infect patient cells and thereby introduce the desired genetic material. This assay is subject to much more variability than the particle number determination. In recent years, some sources of variability have been identified. The concentration and diffusion rate of adenovirus particles are two important parameters to consider [10]. Infectious titer assays utilize adherent cells sitting at the bottom of tissue culture dishes. Since adenovirus particles do not settle out of solution but instead randomly diffuse, the volume of material tested can have a profound impact on the apparent infectious titer.

A recent initiative to develop an adenovirus reference material should lead to increased accuracy in both particle and infectious particle determinations [11]. A reference material consisting of wild-type adenovirus 5 with a known particle and infectious titer will be produced and distributed. Comparisons between different adenovirus vector preparations within and between lots can be made using this reference material as an index to calibrate assays done in different places and at different times.

VII. Characterization of Adenoviral Vector Final Products

Testing of the final adenovirus vector product consists of safety testing and product characterization. Such testing involves physical/chemical and biological assessments. Table IV provides a summary of the currently recommended testing. Whereas production intermediates such as the MCB and MVB are subject to acceptance criteria, the final product characterization is subject to lot release and is recorded on a CoA with specified tests, methods, sensitivities, and results. Some of the safety testing is similar to that done for the

Table IV
Characterization of the Final Product[a]

Safety	Product characterization
Sterility	Identity
Mycoplasma	• Restriction map, structural characterization
Endotoxin	Activity
Adventitious virus[b]	• Transgene specific
• *In vitro* virus	Potency
• AAV	• Required by phase II/III
• Replication-competent adenovirus	Titer
General safety	• Particle count/infective particle ratio <30:1
• Required by time of licensure	Purity
	• Cell substrate DNA <10 ng/dose, <100–200 bp in size
	• Cell substrate protein
	• Ancillary products
	• Process residuals

[a]The necessity for and specifications for each of these characterizations is assessed on a case-by-case basis and can change depending on the phase of product development and as a result of feedback from the numerous sources discussed above. Therefore, this list is intended to give the reader an overview of FDA oversight. However, consultation with CBER is strongly recommended before submission of an IND.
[b]These tests should be done on the unpurified bulk in order to maximize sensitivity and not deplete final product.

MCB and MVB. Thus sterility and mycoplasma testing should be performed, as should testing for endotoxin levels in the final formulated product (LAL per 21 CFR 610.9 or pyrogenicity test 21 CFR 610.13(b)). Adventitious virus testing consists of the *in vitro* virus test, and tests for AAV and RCA. In general, an *in vivo* adventitious virus test is not recommended for final product. These adventitious virus tests should be performed on the unpurified bulk in order to maximize sensitivity and not deplete the final product. One other test that is required for licensed products is that of General Safety 21 CFR 610.11. The current recommendation for RCA is ≤ 1 RCA per 3×10^{10} virus particles.

Current recommendations for final product characterization are similar to those for MVB. However, identity (structural characterization) need not be done by DNA sequence analysis. Rather, other methods such as sensitive restriction mapping combined with Southern blot analysis or PCR mapping may be used to show that the final vector preparation is homogenous within the limits of the assays. The same activity assay used on the MVB can be used on the final vector preparation. Development of a potency assay that reflects the intended biological function of the vector preparation should commence as soon as possible during product development and should be in place by the end of Phase 2 or the beginning of Phase 3. Test methods, sensitivities,

and specifications for lot release should be submitted as part of an IND submission.

The number of particles and the infectious titer per unit volume should be measured and reported. Currently the recommendation is that the ratio of total particles to infectious particles in the final product should be no greater than 30:1. The previous recommendation of 100:1 was developed shortly after the first adenovirus vector trials were initiated and has remained constant until recently. However, review of data received in response to the March 6, 2000, letter to gene-therapy sponsors suggests that almost all adenovirus vector lots have a ratio of less than 30:1 particles to infectious particles. Advances in understanding of infectious titer assays and the development of an adenovirus reference material will be helpful in reassessing this recommendation in the near future.

Product characterization should also include assessments of potential impurities such as production cell DNA and protein. If the cell line used for production is tumorigenic, current FDA recommendations for adenovirus vector products are that no more than 10 ng/dose of cell substrate DNA be present. In addition, the DNA that is present should be degraded to a size less than 100–200 bp in length. If these criteria are met, the need for tumorigenicity assays of the cell substrate is less pressing. The current recommendation for cell substrate protein is that the sponsor should measure and report amounts present in order to set lot release acceptance criteria by Phase III. If cell substrate proteins are present, their potential for immunogenicity should be considered.

Other potential impurities should also be assessed in analysis of the final product. These include fetal bovine serum, other tissue culture reagents, antibiotics, process residuals such as CsCl, or column chromatography materials. Other tests that may be necessary include pH of the formulated final product, assessment of particulates, volume, and appearance. The necessity and extent of these tests should be discussed with FDA.

All lot-release testing of the product should be summarized in a certificate of analysis that accompanies the vector product.

A final consideration for product characterization is vector stability. Stability testing should be conducted on the final formulated, vialed product. In early phases of product development, stability testing should also assess procedures for shipping and handling of the final product. Stability testing should be initiated during Phase 1 and should be conducted according to a plan that has been discussed with the FDA.

VIII. Preclinical Testing of Adenoviral Vectors

In the development of a new adenoviral vector for gene transfer, the preclinical pharmacology and toxicology programs are typically conducted

in conjunction with the development of the product manufacturing. The overall purpose of preclinical animal and *in vitro* studies is to support the safety and rationale for use of the product in human subjects. Although not unique to gene therapy vectors in general, or more specifically, to adenoviral vector development, there are several basic goals to be achieved by preclinical testing which contribute to the design and conduct of the initial clinical trials. These include, but are not limited to, (i) identification of dose(s) which confer the desired biologic effect; (ii) definition of a safe starting dose and escalation scheme; (iii) identification of pharmacodynamic measures of biologic activity; (iv) identification of safety and toxicity parameters to monitor in the clinical trial; (v) definition of inclusion and/or exclusion criteria based on observed toxicities, and, finally, (vi) designated stopping rules for the clinical trial based on the toxicity profile observed in animals.

A. Pharmacologic Activity

Initially, the pharmacologic activity of a proposed vector system is evaluated either *in vitro* or *in vivo*, as demonstration of "proof of concept." These studies are designed to determine the feasibility and efficiency of the gene transfer, and whether the biologic activity in correcting the genetic defect or conferring that the desired response is observed (e.g., multidrug resistance in hematopoietic stem cells). When available, animal models which mimic the human disease, either through genetic or pharmacologic mechanisms may be used as "proof of principle," to demonstrate that transfer of the gene is actually able to correct the genetic defect, ameliorate or slow progression of the disease, or alleviate some of its clinical signs or symptoms. Based on the responses observed in the preclinical pharmacology program, a decision is made by the investigators to either further evaluate the candidate vector for safety with the intention of entering it into the clinic or to terminate the development of potentially unsuccessful products.

Preclinical pharmacology data are provided both to CBER in support of an IND application and to the NIH RAC in support of use of adenoviral vectors for gene transfer in several different clinical indications. Of the data which have been publicly reviewed and discussed, biologic activity of adenoviral vectors have been evaluated in murine tumor models and murine:human tumor xenografts, transgenic mouse models of human disease (e.g., ornithine transcarbamylase deficiency), human cell xenografts in immunodeficient rats and/or mice, and in pharmacologically induced disease states in rodents, monkeys, and dogs. Advantages of using adenoviral vectors are their ability to transduce a variety of different, nondividing cell types, high levels of gene expression for relatively short durations of time, and a large enough capacity to carry relatively large, transgene sequences.

IX. Toxicology Testing

A. Scope of Toxicity Testing

The next step in the preclinical program for a candidate gene transfer vector is the toxicology testing. Prior to initial entry of a new drug or biologic agent into humans, the basis for the determination of *in vivo* safety is the preclinical testing performed in animals. Toxicology studies to demonstrate safety of gene transfer vectors, including adenovirus, are intended to answer specific questions regarding the acceptable risk:benefit ratio to the patient, and provide an indication of what expected toxicities may occur on introduction of the product into humans.

Traditional drug development programs, evaluating the safety of small molecule or protein therapeutics typically conduct toxicology testing in normal animals, using a well-defined paradigm to establish the acute, subchronic, and cumulative toxicities of an agent prior to its first use in man. At least two animal species are used for the initial demonstration of safety; typically, testing is done both in rodents (i.e., mice, rats, or hamsters) and one nonrodent species (i.e., dog, pig, or nonhuman primate). The advantages of this approach are that a wide range of doses may be investigated to give high multiples of the expected human exposure, the metabolism and disposition profiles in the different species may be established as a basis for comparison for the clinical dosing, and the background incidence of any specific, adverse findings may be well-documented in that particular strain of animal being tested. The use of more than one species in traditional drug evaluation programs is encouraged to increase the chance of detecting any toxicity expected for the clinical trial.

Traditional toxicology programs, however, frequently are of little value in the determination of safety of gene transfer agents. For many of the vectors in development, the issues of species-specificity of the transgene product under study, as well as limitations in the doses that are feasible to administer and the interaction of the agent with its specific receptor must be taken into account in designing the safety program. In gene transfer research, demonstration of safety must also take into account toxicities due to both expression of the transgene, or the ultimate therapeutic agent, as well as any adverse effects associated with the vector, or delivery system used to introduce the foreign gene. Additionally, any underlying pathology associated with the disease being investigated may either exacerbate or confound any toxicity related to the gene transfer system. These points must be considered in designing a preclinical program to evaluate the safety and efficacy of a gene transfer agent.

The FDA recognizes that novel issues exist in designing and interpreting preclinical studies for gene transfer vectors, and has provided several guidance documents to assist investigators in developing their preclinical programs. CBER's recently published guidance document provides a framework for the

design of preclinical safety programs in gene therapy, based on the available data from both *in vitro* and *in vivo* efficacy models, as well any specific concerns for the clinical population planned for study [4]. The CBER document follows the guidance set forth by the International Conference on Harmonization S6 document, "Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals" (ICH S6). Although the ICH guidance does not directly address toxicology study design for gene transfer agents, many of the principles of this document apply [12]. In general, toxicity study design for gene transfer agents follows many of the principles set forth by ICH S6 regarding dose and species selection, route of administration, and study timing. Each of these points is addressed separately in the context of gene transfer, below.

To understand the safety of gene transfer vectors, the design of preclinical studies should take into consideration the following points: (i) the class of vector to be administered, (ii) the animal species, gender, age, and physiologic state most relevant for the clinical indication and product class, and (iii) the intended doses, route of administration, and treatment regimens planned for the clinical trial. With many of the gene transfer vectors, these considerations will be dependent, as the route of administration or the maximal feasible dose for the preclinical study may be influenced by the species selected for testing, and vice versa.

B. Species Selection

The recent death of a patient while participating in a clinical trial of adenovirus-mediated gene transfer, as well as the finding that data in Rhesus monkeys using the same class of vectors and route of administration predicted many of the toxicities observed in this subject have highlighted the importance of preclinical data, and the relevance of the animal model in determining a safety profile for these agents. CBER's recommendations for selection of species for safety evaluation of adenoviral vectors have generally followed the guidance set forth by the ICH S6 document, taking into account the limitations of the animal model being tested. Preclinical pharmacologic and safety testing of vectors for gene transfer should employ the most appropriate, pharmacologically relevant animal model available. In contrast to traditional drug development programs, for many biologic products including gene transfer vectors, safety evaluation and toxicology testing in a single, relevant species is permissible prior to Phase 1 studies in the clinic.

A relevant animal species would be one in which the biological response to the therapy would be expected to mimic the human response. Relevant animal species for safety evaluation may also be selected based on the clinical population intended for study and/or intended route of administration, or by the species-specificity of the transgene product. In some cases, the interaction of the transgene product with its specific receptor occurs only in humans

and nonhuman primates, necessitating toxicology testing in monkeys. In many cases, however, the toxicities observed are independent of the transgene product (e.g., inflammatory reactions in response to adenovirus capsid proteins) and may be tested in rodent species or other small, nonrodent laboratory species. In other cases, specific information regarding the safety of a gene transfer approach may be obtained only in an animal model of the disease, in which the underlying disease pathology can influence significantly the safety of the intervention. When evaluating the pharmacologic activity of a vector in an animal model of the clinical indication, it is recommended that safety data be gathered at the same time, in order to assess the contribution of disease-related changes in physiology or underlying pathology to the response to the vector.

C. Route of Administration

Most gene transfer studies, both in humans and in animals, are expected to involve either single administrations or a small number of repeat administrations over a short duration of time. CBER recommends that both the route of administration and the dosing schedule in animal studies mimic those intended for the clinical trial as closely as possible. However, there are issues specific to the gene transfer that need to be incorporated into the study design, for example, the persistence of gene expression following transduction of the target organ, which will impact upon the duration of the toxicity study. Another example would be the physical characteristics of the agent being studied (i.e., vector aggregation at high concentrations). The dose and the route of administration for the preclinical safety studies of cellular and gene therapies should mimic those intended for the clinical trial as closely as possible. It is understood, however, that some dosing techniques and/or regimens intended for the clinical trial may be difficult to achieve in a small animal species, such as a rodent. In these cases, a method of administration similar to that planned for use in the clinic is advised. For example, intrapulmonary instillation of adenoviral vectors by intranasal administration in Cotton rats or mice is an acceptable approach in lieu of direct intrapulmonary administration through a bronchoscope.

D. Selection of Dose

Current recommendations for dose selection for safety testing are based on those demonstrated in efficacy models to provide gene transfer sufficient for pharmacologic effect, as well as inclusion of doses with a likelihood of demonstrating toxicity. Dose selection should be based on preliminary activity data from studies both *in vitro* and *in vivo*. For the determination of safety, a no-observable adverse effect level dose (NOAEL), an overtly toxic dose, and several intermediate doses should be evaluated, to determine not only

the dose relationship of the toxicities to the amount of vector administered and/or transgene expression, but also to evaluate the shape and steepness of the dose–response curve. Preclinical safety studies should include one dose equivalent to, and at least one dose escalation level exceeding, those proposed for the clinical trial. The multiples of the human dose required to determine adequate safety margins may vary with each class of vector employed and the relevance of the animal model to humans.

Allometric scaling of doses based on either body weight or total body surface area as appropriate facilitates comparisons across species and allows determination (retrospectively) of whether an animal model was predictive of toxicities observed in the clinic. For example, adenoviral vectors used in cystic fibrosis demonstrated very similar toxicities after direct instillation into the lungs of Cotton rats, mice, hamsters, Rhesus monkeys, and baboons (Table V). These toxicities included dose-related, perivascular, and peribronchiolar inflammation, mononuclear inflammatory cell infiltrates, pulmonary edema, and interstitial pneumonia. When the NOAEL doses were calculated for each species after scaling by total body surface area, with the exception of Rhesus monkeys, it was discovered that these values were remarkably similar between the different species. Additionally, when scaled by total body surface area, the NOAEL doses in mice, Cotton rats, hamsters, and baboons for direct instillation of adenovirus into the lungs were approximately equivalent to the human dose of 2×10^9 IU, or 1.2×10^9 IU/m^2, which was the first dose in humans at which toxicity was observed, when scaled by body surface areas.

Table V
Allometric Scaling of Adenovirus Dose in Animals and Man

Species	Apparent NOAEL	NOAEL (pfu/m^2 surface area)
C57Bl/6 mouse	2.6×10^7 pfu/mouse	2.4×10^9 pfu/m^2
Hamster	3.6×10^7 pfu/hamster	1.7×10^9 pfu/m^2
Cotton rat	5×10^7 pfu/rat	1.9×10^9 pfu/m^2
Rhesus monkey	2×10^7 pfu/monkey[a]	8.2×10^7 pfu/m^2
Baboon	7×10^8 pfu/monkey	1.8×10^9 pfu/m^2
Human	2×10^7 pfu/patient	1.2×10^7 pfu/m^2[b]

Note. Cotton rats, mice, and hamsters were administered increasing doses of adenoviral vectors encoding the human CFTR gene by intranasal instillation. Baboons, Rhesus monkeys, and humans were treated with adenoviral vectors encoding CFTR via bronchoscopic instillation into an isolated lobe of the lung. Animals were sacrificed 3 to 5 days after vector administration, and histologic sections of the lung were examined microscopically for evidence of inflammation [15]. The human data were obtained via chest radiograms and CT scans of a patient in a phase 1 clinical trial [13].

[a]NOAEL not available; lowest dose tested with minimum pathology

[b]Toxic dose in humans, 2×10^9 IU, or 1.2×10^9 IU/m^2.

This finding allowed for a redesign of the clinical approach to gene therapy for cystic fibrosis, using smaller volumes for instillation of vector and a more targeted approach to deliver the adenovirus to the larger airway epithelial surfaces. To date, cystic fibrosis patients have been treated using two to three logs higher doses of adenovirus with this newer approach without the toxicities observed in the initial clinical trial [13].

In cases where gene transfer vectors may be in limited supply, or for vectors with inherently low toxicity, a maximum feasible dose may be administered as the highest level tested in the preclinical studies. In all studies, and especially when using animal models of the clinical indication, appropriate controls, such as naive or vehicle-treated animals should be included. This should allow determination of an adequate margin of safety for use of the vector in the clinical trial, as well as an acceptable dose-escalation scheme.

X. Biodistribution

One issue with direct administration of genetically modified cells or viral or other vectors is that the injected material may not stay where it is initially introduced. Therefore, localization studies designed to determine the distribution of the vector, or the trafficking of genetically modified cells after administration to the proposed site are incorporated into the toxicology testing. These studies have two purposes: (i) first, to identify potential distribution of the vector to sites other than the intended target site, where presence of the vector and/or aberrant expression of the transgene may lead to toxicity; and (ii) to evaluate potential distribution of vector to gonadal tissues and/or transfection of germ cells. In a discussion by the NIH RAC about the risk of potential, inadvertent gene transfer to germ cells, it was concluded that the risk of vertical transmission of the foreign gene was very small. A discussion by the RAC and several expert panelists in gene transfer or reproductive biology recommended that unless there were significant safety issues associated with either the vector or the transgene product, preclinical biodistribution studies in animals were not always required prior to initial Phase 1 trials. In addition, the panel concluded that in cases such as adenoviral vectors, where a large body of literature exists regarding their distribution and potential for toxicity, minor changes in the vector (e.g., substitution of a different transgene with no potential toxicity associated with it) did not require further preclinical distribution studies prior to initiating clinical trials [14]. Biodistribution studies, in which the disposition of the vector is detected after administration by the intended clinical route not only provide data regarding the potential for gonadal uptake and inadvertent germ-line gene transfer, but can also identify any target organs in which aberrant vector distribution or gene expression may be detrimental. CBER's current recommendation is that biodistribution studies of gene transfer agents are not always required prior to Phase 1 clinical trials; however, these

studies should be incorporated into the drug development plan so that data are available prior to commencing large-scale, pivotal studies in the clinic [14].

Dose levels selected for biodistribution studies should follow those used in the toxicity studies and include either vehicle or untreated control animals, and the route of administration should be relevant to that employed in the clinical trial. Transfer of the gene to normal, surrounding, and distal tissues as well as to the target site should be evaluated using the most sensitive detection methods possible and should include evaluation of gene persistence. When aberrant or unexpected localization is observed, studies should be conducted to determine whether the gene is expressed and whether its presence is associated with adverse effects. Additional groups of animals may be treated intravenously, as a "worst-case" scenario in cases where widespread vector dissemination may be expected to cause toxicities in organs other than the target site [15].

A. Good Laboratory Practices

Preclinical studies in support of use of gene transfer vectors including adenovirus, in clinical studies should be conducted in compliance with the regulations for Good Laboratory Practices (GLPs) as set forth in 21 CFR, part 58. Compliance with these regulations is intended to assure the quality and integrity of the animal safety data used in support of human research studies, as well as marketing approval.

There is often some confusion as to what types of studies need to be conducted under the GLP regulations. Preclinical pharmacology, "proof-of-concept," and efficacy studies in animals, as well as *in vitro* pharmacology studies are not expected to be conducted in full compliance with GLP. However, *in vitro* and animal toxicology studies, including single- and repeat-dose toxicity testing, reproductive toxicity and carcinogenicity studies, and, for gene transfer research, biodistribution studies are expected to follow the guidelines set forth by the regulation. Although studies for gene transfer vectors in early stages of clinical development need not be in full compliance with the GLP regulations (i.e., quality assurance audits, validation of test and other methodology may be omitted in early studies), CBER expects that any pivotal toxicology studies submitted to an IND or licensing application will be conducted under the auspices of GLP.

XI. Introduction to Clinical Testing

The goal of clinical testing is to provide information about the product's safety and effectiveness and, ultimately, allow new products to come to the marketplace. As discussed in the introduction to the preclinical section, the principles described below are neither unique to gene transfer vectors in general, nor to adenoviral vectors in particular.

A. Phases of Clinical Development

Premarket clinical testing proceeds in a stepwise fashion, often referred to as Phases 1, 2, and 3 of clinical development, although the phases are not always discrete. Phase 4 studies are those performed after marketing. Each phase of product clinical testing has its series of goals or objectives.

The primary goals of Phase 1 testing are to learn about the product's safety and pharmacokinetic profile and to identify a safe dose or doses for further study. Phase 1 studies involve small numbers of study participants who are closely monitored for the development of drug effects. A common Phase 1 design is a single dose, rising dose, cohort study. Escalation to the next dose cohort occurs after sufficient safety assessment of the proceeding cohort. The starting dose and dose escalation scheme employed depend on the data gleaned from product and preclinical testing, and other clinical data, if available (e.g., closely related products or same product studied in different populations). Dose escalation usually proceeds until a defined endpoint, such as a maximal tolerated dose, or an optimal biologic dose, is reached.

Phase 1 studies for some drugs may be conducted in healthy volunteers. This approach is common when anticipated side-effects of the product are expected to be minimal and transient and the target population (those with the disease or condition of interest) have high background rates of adverse events, making it difficult to tease out the safety profile of the product. However, for many classes of drugs and biologicals, including adenovirus gene transfer products, the potential short- and long-term adverse effects (see section XIII) generally makes their risks unacceptable for testing in healthy volunteers.

The next phases of clinical testing, Phases 2 and 3, build upon the information generated from the prior studies. The goal of Phase 2 testing is to gain preliminary evidence of the product's activity in the disease or condition of interest and to begin to characterize that activity. Phase 2 is the ideal time to optimize the dose and/or dosing regimen, the patient population, the response parameters that are most likely to reflect clinical benefit, as well to build upon the safety database. Phase 2 trials often are randomized, controlled, and conducted in multicenters.

Phase 3 of clinical testing includes clinical studies to establish the product's effectiveness. The number of efficacy trials, trial design(s), and size of the safety database necessary to determine net clinical benefit depend on a number of factors, including but not limited to the class of product under development, the condition or disease being studied, and the availability of other therapies.

Phase 4 of clinical testing are studies conducted after market approval. Their purpose is to address questions that arose during the premarketing investigations, or to evaluate the product in other related setttings, such as the elderly, or people with more advanced stages of the disease. The design of a postmarketing study (such as a randomized controlled clinical trial or a registry) depends on the questions to be addressed.

XII. Good Clinical Practices

Good Clinical Practices (GCPs) are a set of principles and procedures intended to preserve and protect the rights and confidentiality of human research subjects and to assure, to the extent possible, that the clinical research generates valid scientific data. The origins of a code of conduct to protect human subjects in clinical research date back to the Nuremberg war trials and the Declaration of Helsinki. In 1996, the FDA, under the auspices of the International Conference on Harmonization (ICH), published the guidance document entitled: "E6 Good Clinical Practice (GCP) Consolidated Guideline." Basic principles of GCP will be discussed below; the reader is referred to the CBER website http://www.fda.gov/cber/guidelines.htm for the full document [16].

A. Responsibilities of a Sponsor and Investigators

The sponsor oversees the IND and communicates with the FDA. As set forth in regulations at 21 CFR 312, subpart D, and in the ICH GCP guidelines, the oversight function includes selecting study investigators, reporting safety information to the FDA, and providing accurate and timely information to all investigators. In some cases, a sponsor may transfer all or some of its obligations to a contract research organization (CRO), although the sponsor retains ultimate responsibility for the IND.

Clinical investigators also have specific obligations, delineated in 21 CFR 312, subpart D, and in the ICH GCP guidelines. Investigators are responsible for selecting study participants based on eligibility requirements of the protocol and for obtaining the protocol-specified evaluations. The investigator is responsible for the welfare of the study subjects at his/her clinical site. This includes collecting safety data and reporting safety information to the IND sponsor. The investigator also must account for all investigational medical product, maintain accurate records, provide annual updates to the Institutional Review Board (IRB), and obtain consent from all study participants.

Where the sponsor and investigator are distinct, their separate roles, with the former overseeing the latter, incorporate the checks and balances that minimize bias and maximize patient safety and trial validity. These checks and balances may be lacking when the investigator is also the sponsor, and additional external oversight is advisable. Individual physicians who assume the role of sponsor, investigator, or sponsor/investigator should be familiar with guidances and federal regulations that set out the respective duties of the sponsor and the investigator.

B. Adverse Event Reporting

Adverse event collection and reporting is a fundamental aspect of drug development and of human subjects protection. The clinical investigator is the

individual who identifies, evaluates, and documents adverse events experienced by study participants at his or her site and who is responsible for updating the IND sponsor and the IRB as appropriate, as set forth in federal regulations (at 21 CFR 312.64).

The sponsor is responsible for submitting safety information to FDA. The timing and reporting format will depend on the nature of the adverse event. The sponsor must report to FDA in writing all serious and unexpected adverse event information associated with the use of the investigational product within 15 calendar days of receipt of the information. Any unexpected life-threatening or fatal event associated with the use of the investigational product must be reported by telephone (or facsimile) within 7 calendar days of receipt of the information (as per 21 CFR 312.32). The telephone and written reports constitute expedited reports. Although causality assessment is integral to expedited reporting, a determination that a given investigational product caused or was associated with an adverse event in the course of a clinical study is not always possible. The most reliable way to assess the contribution of a test article to an adverse event is by comparing adverse event rates and severity in treatment and control groups. Randomized controlled trials, however, are infrequent in early phases of clinical testing. Although one cannot always be certain that there is a relationship between the administration of the study product and the adverse event, the level of suspicion required for reporting is quite low. Except if there is no reasonable possibility that the product caused or contributed to an unexpected serious adverse event, that event must be reported to the FDA according to specified time frames.

The sponsor is also required to submit to the IND an annual report that includes a summary of the most frequent and the most serious adverse events (21 CFR 312). The ICH guideline entitled "E3: Structure and Content of Clinical Study Reports" describes the manner in which safety data for individual studies should be organized and presented to regulatory authorities in marketing applications [17]. A marketing application includes an integrated summary of the entire safety experience for the product. FDA, as part of the ICH process, is developing a guideline entitled "The Common Technical Document for the Registration of Pharmaceuticals for Human Use" that addresses, among other items, formatting of integrated safety data [18]. Once marketed, a passive surveillance system allows for the continued collection and reporting of safety information [19]. For some products, such as ones that pose unique long-term risks, a more active type of postmarketing follow-up will be required.

C. Consent and Vulnerable Populations

In general, prospective participants cannot be enrolled into a trial without their consent. Elements of the consent form and the consent process are set forth

in 21 CFR part 50. Before consenting, study participants must be informed of known and potential toxicities that may occur from participation in a trial of an investigational product, even if the likelihood of toxicity is remote. The IRB at each institution participating in a study must review and approve the consent form and the clinical research protocol before the study can be initiated at that institution. The composition and duties of the IRB are described in the ICH GCP guidelines and in 21 CFR part 56.

For some of the disorders that are targets of gene therapy, such as inborn errors of metabolism, the affected population will be pediatric subjects. Mechanisms exist to strengthen the human subject protections for study participants who may be particularly vulnerable, such as children, who cannot give valid consent [20]. When a child is to be enrolled in a research study, the parent or legal guardian consents (gives permission) for the child to be in the study. The FDA, as part of the ICH process, has published a guidance document that addresses clinical trials in children, including ethical issues [20].

In rare circumstances where it is not possible to obtain a participant's consent because of the nature of his or her illness or injury, and in which obtaining consent from a legally acceptable representative (e.g., next of kin) is not feasible, the FDA may permit the clinical trial to proceed with a waiver of consent, as set forth in 21 CFR 50.24.

D. Monitoring and Auditing

Monitoring and auditing are fundamental aspects of GCP. Although their purposes are similar (to assure appropriate trial conduct and data validity), the approaches differ. As stated in the ICH GCP document, monitoring is "the act of overseeing the progress of the clinical trial and ensuring that it is conducted, recorded, and reported in accordance with the protocol, standard operating procedures, GCP, and applicable regulatory requirements." Medical monitors, usually employees of the sponsor, perform on-site (and, if indicated, off-site) evaluations of trial-related activities. The extent and frequency of monitoring should be appropriate for the length, complexity, and other particulars of the trial. Among the functions of the monitor is identification of deviations in protocol conduct so that the sponsor may take appropriate corrective steps, e.g., retraining investigators, closing out certain sites, etc.

Auditing is defined in the ICH CGP document as "the systematic and independent examination of trial-related activities and documents." The audit is usually conducted at the conclusion of the trial. The sponsor may hire auditors who document findings in a written report to the sponsor. FDA field inspectors also conduct independent study audits. Traditionally, the purpose of the FDA audits has been to verify the data submitted to the FDA in support of a marketing application. However, the FDA and the sponsor may conduct "for cause" or directed audits at any stage of clinical investigation if there is reason to suspect a problem with trial conduct or data integrity.

The FDA has performed directed inspections at a few gene therapy clinical sites since 1999. The agency also audited approximately 70 gene transfer clinical sites selected at random to assess whether systemic problems with the conduct of such clinical studies existed. Inspectional findings will be discussed in more detail in section XVI.

An additional measure of human subject protection is use of a Data Monitoring Committee (DMC) to evaluate accumulating data from a clinical trial [22]. Generally, the sponsor establishes the DMC, including selecting the members and devising the charter. The DMC members should be independent of the sponsor and clinical investigators. The role of the DMC varies according to the charter and the nature of the study. The DMC is usually empowered to recommend study modifications to enhance safety of participants; in some cases, a DMC may recommend that a study be stopped if data indicate a major safety concern. Of note, DMCs review data submitted to them but do not visit sites to directly ensure that the data are accurate, the protocol is followed, consent is documented, etc. Thus, a DMC does not perform the functions of or obviate the need for study monitors. The FDA is in the process of developing guidance on DMCs.

XIII. Clinical Safety of Adenoviral Vector Products

Most of the completed and ongoing adenoviral vector clinical trials are early, uncontrolled trials. The absence of an internal control group limits the ability to draw definitive conclusions about the contribution of the adenovirus vector product to an adverse event. Despite this caveat regarding causality assessments, administration of replication defective adenovirus is associated with an acute cellular and cytokine mediated inflammatory response. Individuals have experienced systemic reactions such as fever, chills, hypotension, and laboratory findings consistent with disseminated intravascular coagulation, including thrombocytopenia. An overwhelming systemic inflammatory response, to which has been attributed, at least in part, the death of a volunteer in a trial of ornithine transcarbamylase (OTC) deficiency who received intrahepatic artery injection of a high dose of adenovirus-containing product, has not been observed in other clinical trials, including those that employ systemic administration of similar doses of adenovirus vector. See also discussion in section XVI.

The route of administration appears to play a key role in determining the type of and occurrence of adverse events. Toxicities have been particularly prominent in organs that are the sites of adenovirus injection, including the lung, brain, and liver [13, 23, 24]. In addition to route of administration, other variables associated with the clinical trial may influence the nature, frequency, and severity of an adverse event. Such factors include the adenovirus construct,

transgene, dose, and frequency of product administration, and host factors such as the underlying disease, other comorbidities, and use of concomitant medications. A committee of experts convened to discuss adenovirus safety in December 1999 questioned the role of the transgene in the toxicity profile and suggested employing null adenovirus vectors as controls when possible to tease out the relative toxicities of the transgene from the vector [25].

Preexisting antibody to adenovirus and/or the development of an antibody response following administration of an adenovirus-containing product may play a role in product safety, although a clear relationship has not been established [24]. The limited data available have not suggested a correlation between high baseline levels of neutralizing antibody and adenovirus toxicity (or activity). Moreover, in a study that involved repeat administration of an adenovirus-containing product, participants developed large spikes in serum levels of neutralizing antibody after the initial dose. However, the toxicity profiles of the first and subsequent doses were similar, again suggesting a lack of correlation. It is important that clinical investigators continue to characterize the immune status of study participants at baseline and following adenovirus vector administration, and attempt to correlate adverse events with levels of or changes in antibody titer. Ultimately, such information could be utilized in patient selection criteria or in clinical monitoring to enhance safety and effectiveness.

The long-term safety of gene transfer is under active discussion. Concerns about late adverse sequelae such as new malignancies occurring years or decades following administration of replication-competent, integrating viruses resulted in FDA guidance regarding testing for replication-competent retrovirus (RCR) in product and patient's serum and for lifelong clinical monitoring [26]. These recommendations are currently limited to retroviral vector INDs. Although adenovirus can become replication competent, the FDA had not previously recommended that patients exposed to this class of product be followed long term. Long-term follow-up of gene therapy products was discussed at recent meetings of the Biologics Response Modifier's Advisory Committee [8, 9, 9a]. FDA will revise the recommendations for long term follow up of recipients of gene transfer products including adenovirus-containing products, pending additional public discussions.

XIV. Bioactivity of Adenoviral Vector Products

A goal of Phase 2 testing is to determine if the adenovirus containing product is bioactive and, if so, to determine whether the observed activity findings, together with the safety profile, warrant further clinical testing. Bioactivity measures may be laboratory findings, clinical outcomes, or a combination of the two. One measure of bioactivity for gene therapy products is detection of gene transfer and gene expression. This may not be possible

where assays for the transgene are not yet developed or are insensitive to low levels of expression. Documentation of clinical or surrogate outcomes and/or alternative assessments (e.g., pharmacodynamic measurements), and correlations, if any, to levels of gene expression, are highly desirable in early product development. The extent to which the generation of such data will be feasible depends on, among other factors, the nature of the product, the clinical population in the study, and the state of the science regarding assays to detect the transgene.

The majority of the clinical investigations with adenoviral vectors to date target patients with cancer. In the oncology setting, studies that are in Phase 2 of development are usually designed to capture data on tumor responses (complete and partial response rates). The demonstration that the adenovirus gene therapy product results in a certain level of tumor response, and the characterization of those responses (rates of complete and partial responses, duration of response, etc.), along with an acceptable safety profile, will usually be sufficient evidence of activity to warrant efficacy trials.

Early studies of cystic fibrosis (CF) involved topical administration of the adenovirus product containing the cystic fibrosis transmembrane regulator (CFTR) protein gene to the nasal epithelium. Measures of product activity included gene transfer/gene expression and assessment of the potential difference across the nasal epithelium. Topical administration resulted in only low levels of gene transfer and limited pharmacodynamic affects. Gene transfer via aerosolized delivery systems appeared to be marginally improved over topical administration. Given the limited product bioactivity that has been seen, clinical development of adenovirus containing products for CF has largely been abandoned.

An evolving area of clinical research is use of adenoviral vector products that contain genes intended to promote vascular growth. Patients enrolled generally have vascular disease. Studies are ongoing in both cardiac and peripheral vascular disease settings. The activity measures can include laboratory measures such as myocardial perfusion, and measures of gene expression.

XV. Clinical Efficacy of Adenoviral Vector Products

FDA grants market approval for products that are shown to be safe and effective. The efficacy standard, applicable to all FDA-regulated products, as stated in section 505(d) of the Food, Drug, and Cosmetic Act, is *substantial evidence*, defined as "evidence consisting of adequate and well-controlled investigations, including clinical investigations, by experts qualified by scientific training and experience to evaluate the effectiveness of the drug involved, on the basis of which it could be fairly and responsibly concluded by such experts

that the drug will have the effect it purports or is represented to have under the conditions of use prescribed, recommended, or suggested in the labeling or proposed labeling thereof." The following paragraphs address the issues of the quality and quantity of clinical investigations that can provide "substantial evidence."

A. Choice of Control

An "adequate and well controlled" investigation is one whose design and execution produces valid scientific data. Clinical investigations intended to show efficacy must be controlled so that the effect(s) of the intervention can be distinguished from other influences, such as spontaneous change, placebo effect, or biased observation. In Phase 2 testing, controlled trials are helpful in teasing out adverse events and in assessing the magnitude of the effect relative to the control group. Such information will be useful for sample size calculations for the efficacy trial(s).

The choice of control (e.g., historical, active, placebo, etc.) depends on the clinical setting. The agency has approved products for market based on studies with various types of control groups. Each type of control has its advantages and limitations. The reader is referred to the ICH guidance entitled "E10 Choice of Control in Clinical Trials" for an extensive discussion on this topic [27].

A control for an adenoviral-containing gene transfer product could be the adenovirus vector without the transgene (i.e., containing a null vector) as discussed previously. Such a null vector control could help delineate safety and efficacy of the vector separately from the insert, as well as show that both vector and insert contribute to product effectiveness. A null vector control, if deemed appropriate, could be incorporated earlier in product development (rather than during Phase 3) as it might be beneficial to determine early on the contribution of and need for the transgene.

Adenovirus products are currently in Phase 3 testing in patients with malignancies. Most are designed as "add-on" trials, i.e., chemotherapy + gene product vs chemotherapy + placebo (or no additional treatment if a placebo is not feasible). If a trial is not blinded, such as would be the case if the control arm could not receive a placebo, it will be important to utilize objective outcome measures and to control use of concomitant therapies. If measures are not objective, blinded third party assessors may be useful.

B. Endpoint Selection

Trials intended to provide substantial evidence of efficacy must be "adequate" in addition to "well-controlled." They must be conducted according to GCPs (as discussed in section XII) to maximize human subject protection and data validity. They must also be designed with appropriate, relevant endpoints that either reflect clinical outcomes or are acceptable surrogate endpoints.

Surrogate endpoints are laboratory or other measurements not directly indicating clinical benefit but that are expected to correlate with or predict clinical benefit. Surrogate endpoints are usually easier to measure than clinical endpoints and occur earlier in the course of the disease, allowing for shorter, smaller, and, thus, less expensive studies. Their major disadvantage is the uncertainty surrounding whether and to what extent the surrogate reflects the true clinical benefit. Thus, if FDA bases important regulatory decisions regarding product licensure on a surrogate and the medical community bases practice decisions on data generated from trials using surrogates, it is critical that the surrogate be valid for the particular treatment and disease. Once a surrogate is validated for one treatment and disease using a particular product, the extent to which that validation applies to other products in the same class and across product classes could become important, particularly as one might define a product class in the context of adenoviral-containing products. In earlier phases of clinical testing, use of surrogate endpoints may serve useful and potentially less problematic roles. For instance, during product development, a surrogate may be used to assess dose–response and thus provide the rationale for dose selection for later trials, or they may be used as initial proof-of-concept to base decisions about further clinical development. Several excellent papers provide more in-depth discussions about surrogates and validation of surrogates [28, 29].

Where the disease is serious or life threatening and without acceptable alternatives treatments, it may be possible to establish efficacy and receive FDA approval based on trials employing a surrogate endpoint that is not yet validated but reasonably likely to predict clinical benefit. If a product is marketed based on an effect on such a surrogate endpoint, Phase 4 studies are required to verify the clinical benefit. These provisions are set forth in 21 CFR 601.40, subpart E. Oncology and AIDS are two areas where this provision has been used with some frequency.

The number of adequate and well-controlled trials that will be necessary to make a determination of substantial evidence of effectiveness has been discussed in FDA guidance [30]. Sponsors should meet with the agency at the end of Phase 2 and discuss and reach agreements about critical product development issues, such as the number and types of clinical trials and the size of a safety database considered necessary to file a marketing application.

XVI. How the Role of FDA Regulators Has Changed Since September 1999

In mid-September 1999, a participant in a clinical study of an adenoviral vector product for Ornithine transcarbamylase (OTC) deficiency became profoundly compromised and ultimately died 4 days after receiving the

experimental product by intrahepatic artery infusion. This event was the first death in a clinical gene transfer trial that was clearly directly attributable to the administration of a vector and resulted in a number of regulatory actions, as well as a commitment by the FDA to increase sponsor outreach programs to address issues related to the safety of all gene transfer vectors, including adenovirus. These efforts have included (i) safety symposia held in conjunction with the Office of Biotechnology Activities (OBA) at the National Institutes of Health (NIH); (ii) the FDA's issuance of a letter on March 6, 2000, to all gene therapy sponsors, requesting that they provide information regarding the oversight of their programs, including the manufacturing, animal data, and any ongoing or future clinical trials; (iii) targeted inspections of clinical sites for compliance with gene transfer protocols conducted at their site; and (iv) increased sponsor education and training in issues specific to gene transfer, as well as the conduct of clinical trials, in general.

A. Safety Symposia in Conjunction with OBA

Following the death of the study participant discussed above, the OTC trial was immediately placed on clinical hold, and the FDA initiated a search of its database to identify all protocols involving adenoviral vectors used for therapeutic intent. A total of 12 protocols were identified which used adenovirus administered by either systemic or intrahepatic artery infusion, or by direct injection into the liver. The sponsors of these protocols were informed of the death of the patient in the OTC trial and were asked to provide an assessment of the safety and toxicity of their adenovirus clinical studies, including the maximal dose of vector administered to date. After review of the information provided by these sponsors, one other clinical trial, using adenovirus encoding a tumor suppressor gene and administered by the same route of injection but at a higher dose than the OTC vector, was placed on clinical hold pending receipt and review of the safety data for that specific trial.

The NIH OBA issued a call for investigators to submit safety information from all adenoviral vector clinical and preclinical studies. On December 8 and 9, 1999, OBA held an open, public symposium whose purpose was to examine the available scientific, technical, and clinical data regarding adenoviral vectors in gene transfer, to identify specific safety issues that were unique to adenovirus, and to make recommendations to the gene transfer community where additional clinical or preclinical data should be required. Investigators from both industry and academic settings presented information regarding the biology, pathophysiology, and toxicities associated with adenovirus infection, both by the natural route of infection as well as by the different approaches used in the gene transfer research studies. Both preclinical study results and data from human subjects in adenovirus-vectored trials for cystic fibrosis, oncology, and metabolic disorders were discussed, with the majority of the clinical data coming from studies in the oncologic setting [25].

In general, comparison of the data across the different settings revealed that the toxicities associated with adenovirus, whether in animals or in human subjects were very similar, and consisted mainly of local, dose-related, and dose-limiting inflammatory responses and immune cell activation. These findings were consistent, whether the virus was administered by bronchoscopic instillation to the lungs, by direct injection into a localized tumor, or by systemic administration. Patients treated with adenovirus vectors at very high doses were found to exhibit some signs of clinical toxicity similar to those observed in the patient at University of Pennsylvania; however, there was no other incident of death attributable to the vector, even in the study where doses higher than that used in the OTC trial were administered by intrahepatic arterial infusion.

Based on the results presented and the discussion at this symposium, a working group on adenovirus safety and toxicity (Ad-SAT) was convened by OBA, composed of clinicians and scientists from FDA, industry and academia. The recommendations from this group were presented at the close of the safety symposium, and included the need for additional information regarding adenovirus vector standardization, biodistribution in human subjects as well as in preclinical studies, and the construction of a database which would include both preclinical data which could predict expected toxicities for the clinic, as well as data from human subjects which would allow comparison of the safety across a number of different settings. The findings and recommendations of the Ad-SAT and RAC were recently published [30a].

OBA and FDA have also cosponsored three additional safety symposia on clinical trials for gene transfer since the December 1999 meeting. These have included discussions of safety issues involved in development of helper-dependent adenoviral vectors and in clinical programs of gene transfer for cardiovascular disease, as well a recent discussion of the potential tumorigenicity of adeno-associated viral vectors in mouse models of human β-glucuronidase deficiency.

B. Results of FDA's Directed Inspections

In the weeks following the death of the patient in the OTC study, the FDA conducted a directed inspection of the clinical site and the Institutional Review Board at the University of Pennsylvania, as well as an inspection of the animal experiments conducted in support of the clinical program. All three inspections found deviations and deficiencies, including inadequate clinical monitoring and oversight of the clinical trial, inadequate reporting of adverse events, and failure to follow clinical and preclinical study protocols. As a result of these directed inspections, the FDA placed the remainder of the clinical studies under the same sponsorship on clinical hold and issued warning letters

to the sponsor, to all of the clinical investigators involved in the OTC trial, and to the director of the preclinical laboratory facility. The FDA also issued a Notice of Initiation of Disqualification Proceedings and Opportunity to Explain (NIDPOE) Letter to the principal investigator. Redacted versions of these letters are available at the CBER websites [31, 32].

A second clinical inspection of a different site, using a different class of vector for gene transfer in cardiac and peripheral vascular disease also found numerous discrepancies in the conduct of the clinical trials and compliance with the regulations governing investigational new agents [31]. As a result of these two inspections, the FDA determined that a more systematic review of procedures to ensure compliance with regulations was warranted. This was accomplished by two specific activities. In March of 2000, the FDA issued a letter to all Gene Therapy IND or Master File sponsors requesting information on the gene transfer product characterization, a review of the preclinical safety studies to ensure any findings that met the criteria requiring an expedited report as per 21 CFR 312.32-33 were submitted, and a summary of the procedures to ensure adequate monitoring and adequate oversight. A copy of the March 6, 2000, letter is available at http://www.fda.gov/cber/genetherapy/gtpubs.htm.

In April 2000, the FDA initiated a series of inspections of clinical sites conducting trials in gene transfer research. At the time, CBER had 211 active gene transfer IND submissions; a random sample of 30 INDs was taken and the principal investigators and clinical sites were identified. From these 30 INDs, 70 sites were identified for inspection to determine their level of compliance with the current regulations. A summary of the results of the March 6 letter and the additional site inspections is provided below.

C. Description of the March 6, 2000, Letter and Summary of Responses

The March 6, 2000, letter was sent to approximately 150 sponsors holding slightly less than 300 total active INDs or master files. Items 1–5 of the letter were questions regarding product testing and characterization data, test methods, specifications, information regarding other products produced in the facility, and quality control procedures. The goals were to: (i) ensure that all gene therapy products currently in clinical trials are adequately tested by contemporary standards, (ii) determine where testing requirements need to be made more stringent or relaxed, (iii) gather information to aid in development of additional guidance, (iv) gain information concerning product characterization and manufacturing processes and arrangements in order to move these products forward toward licensure, and (v) develop a mechanism to ensure that IND annual reports routinely contain updates of this information. In general, sponsors of adenovirus gene transfer trials have been in compliance with FDA recommendations and expectations regarding adenovirus vector product

characterization. In addition, review of the adenovirus vector lot information led to recent changes in recommendations regarding vector infectious particle and total particle measurements as well as a change in the recommendation regarding RCA.

In addition to requests for information on manufacturing practices, the March 6 letter also asked sponsors to provide a summary of the monitoring program for each clinical study conducted under their IND and documentation of their oversight function. The intent was to confirm or bring sponsors into compliance with GCP as required under 21 CFR 312, subpart D, and as described in the ICH GCP guidance. FDA review of the descriptions of the clinical monitoring programs found that the monitoring programs in general incorporated many of the activities and procedures in accordance with the ICH GCP guidance and the requirements listed 21 CFR 312, subpart D. However, some areas of deficiencies were noted, including but not limited to lack of procedures to correct or remove noncompliant investigators, ensuring reporting of protocol modifications to FDA, and ensuring safety reports are filed to the IND in a timely fashion.

The last question in the March 6 letter was intended to remind sponsors that certain findings from animal experiments, i.e., severe toxicities and/or deaths on study, also rise to the level of an expedited report. It asked the sponsors to verify that such data, if relevant, either had already been submitted as required under regulation, or, if not previously submitted, that the data be promptly submitted to the IND or master file. In general, most sponsors indicated they were already in compliance with reporting requirements for such data.

D. Results of Additional Inspections

The sites inspected were chosen at random. Specific questions regarding the background information on the product and the clinical study were developed by the inspection team, and focused on the conduct of the protocol, the reporting of adverse events, blinding of study medication where applicable, and whether the clinical end points were met. CBER field inspectors conducted the inspections between April and August 2000.

In general, these inspections found that most sponsors, both commercial and academic, as well as clinical investigators, were in compliance with the regulations. Of the 70 sites inspected, 11 had no current, active clinical trials or had never initiated their proposed studies, and 23 (33%) required no further action from FDA. Approximately half of the sites had objectionable conditions or practices identified by the inspection team; however, in 33 cases (47% of total sites), only voluntary action to correct the deficiencies was called for. Only three sites were identified where official regulatory action (*i.e.*, warning letters) was required. The most common deficiencies in all of these

cases were: (i) failure to follow the protocol; (ii) an inadequate consent form; (iii) lack of supporting data for case report form entries and/or discrepancies between the source documents and the case report forms; (iv) inadequate drug accountability records; and (v) the failure to notify the Institutional Review Board(s) of protocol changes, adverse events, or deaths.

In summary, the targeted inspections in gene transfer research clinical trials demonstrated, with a few exceptions, that studies were being conducted according to appropriate regulation and guidance. Where deviations were noted, they appeared to be similar to those found in routine inspections of Phase 3 studies of more traditional, biologic agents. The FDA will continue to conduct inspections of clinical, preclinical, and/or manufacturing sites involved in gene transfer research on "for cause" as well as routine bases as part of our role in protecting the safety of patients enrolled in these trials.

E. Sponsor Outreach and Education

CBER had routinely been involved in educational and training activities aimed at sponsors and investigators who are involved in gene transfer research. However, following the death of the patient in the OTC deficiency study, the agency recognized the need to inform potential sponsors of not only the issues specific to the conduct of gene transfer studies, but also on the issues involved in the design of a clinical program and the elements of GCP. Education sessions have taken place at various venues, including the Drug Information Association (DIA) annual meetings and a special satellite broadcast cosponsored by DIA and the FDA; the annual meetings of the Society of Toxicology, the American College of Toxicology, the International Society for Genetic Anticancer Agents, meetings of the Pharmaceutical Research and Manufacturer's Association, meetings of the RAC, and the annual American Society of Gene Therapy (ASGT) meetings. FDA will continue to participate in training courses held by ASGT, as well as other professional and scientific societies.

XVI. Summary

Adenovirus vectors are complex biologics. The FDA's recommendations and expectations for product manufacture and characterization, preclinical, and clinical testing incorporate the tremendous experience gained in the nearly 10 years since the first adenovirus gene transfer experiment, as well as from the experience with the entire field of gene transfer research. The FDA is cognizant of the need for flexibility in its recommendations and will consider many factors, including the intended target population, the seriousness of the disease under study, the potential benefits and risks from the investigational product, when advising sponsors about their adenovirus development program.

The agency will update and reassess recommendations for adenovirus vector production and testing based on the growing experience and on feedback from a variety of sources. The information in the above sections is intended to educate the reader about FDA processes and expectations and should be utilized in conjunction with consultation from FDA staff.

The FDA encourages new investigators to consult with FDA staff prior to submission of an IND. The formal process for FDA consultation is a pre-IND meeting. Sponsors may request information about the IND process in general through CBER's Office of Communication, Training, and Manufacturers Assistance (OCTMA) at 301-827-2000. A sponsor for a gene transfer product who is interested in meeting with the Agency should submit a written request (i.e., letter or fax) to the Director, Division of Application Review and Policy, Office of Therapeutics Research and Review, Center for Biologics Evaluation and Research. Requests for meetings should be submitted in triplicate to the following address: Center for Biologics Evaluation and Research, Attn: Office of Therapeutics Research and Review, HFM-99, Room 200N, 1401 Rockville Pike, Rockville, MD 20852-1448. Prior to submitting a written request for a meeting by fax, the sponsor should contact the Division of Application Review and Policy to determine to whom the fax should be directed and to arrange for confirmation of receipt of the fax.

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CHAPTER 22

Imaging Adenovirus-Mediated Gene Transfer

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I. Introduction

Gene therapy represents a new paradigm in the treatment of human disease. The future widespread application of gene therapy requires gene expression in the targeted cells or tissue. Gene expression means the successful accomplishment of gene delivery, which is most efficaciously accomplished with a gene-therapy vector. In their December 1995 report, Orkin and Motulsky identified shortcomings in all gene-therapy vectors, including a lack of quantitative assessment of gene transfer and expression [1]. Noninvasive imaging specifically addresses the latter issue and can advance the testing of enhanced gene-therapy vectors by providing information on the *in vivo* location of vector delivery, as well as the extent and magnitude of gene transfer and expression. In addition, the consequences of the gene expression can be evaluated, such as the production of specific enzymes or metabolites, induction of apoptosis, or measurement of tumor shrinkage. While imaging of gene transfer has not yet been approved for routine human applications, several groups have reported systems for detection of gene transfer in animal models. The purpose of this chapter is to provide an overview of current imaging technologies and their scientific bases, with emphasis on those technologies that are applicable to gene therapy. Finally, the current imaging literature will be reviewed with respect to imaging gene therapy vectors, especially adenoviral (Ad) vectors.

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II. What Information Is Provided by Imaging?

Noninvasive imaging technologies have become increasingly important over the past 20 years in the management of human diseases. Diagnostic radiology is the medical specialty that is responsible for imaging, providing critical information in three general areas, namely (i) anatomy/blood flow, (ii) metabolism, and (iii) receptor expression. The first area is the most widely applied in terms of the number of studies. This type of imaging affords an opportunity to detect the abnormality, since many conditions result in the disruption of normal anatomy, function, or blood flow. One example is the detection of a mass in an abnormal location on a chest radiograph, which, with further tests leads to a diagnosis of cancer. Another example is the identification of fractures following traumatic injury, or decreased bone density resulting from osteoporosis. These basic radiology techniques remain an important component of disease management. They are routinely accomplished by radiography and angiography, computed tomography (CT), magnetic resonance imaging (MRI), and ultrasonography (US). Less frequently, gamma-ray imaging (especially PET) studies assess blood flow.

Metabolism is the second general area that can be assessed by noninvasive imaging. This category includes the evaluation of organ function. Examples include noninvasive imaging to assess heart perfusion under stress, gastric emptying, ventilation/perfusion of the lung, renal and liver function, and blood flow to the brain. Metabolite imaging is a further example, since magnetic resonance spectroscopy (MRS) techniques now detect altered metabolites in disease processes. Another aspect of metabolism that can be assessed is energy utilization. The increased metabolic rate of cancerous tissue relative to normal tissue can be imaged using radioactive probes that accumulate in areas of higher metabolic activity. These studies are accomplished by administration of a radioactive drug; the increased uptake of the radioactive drug in the cancerous lesion is imaged with gamma-ray detection instruments. In a similar manner, the glucose or fatty acid metabolism in myocardium can be evaluated following ischemic injury.

The third area that can be assessed by noninvasive imaging is that of receptor expression. While receptors may potentially be assessed with MRI, the most success to date has been demonstrated using radioactive, gamma-emitting drugs followed by imaging with gamma-ray detection instruments as described above. This area represents the latest evolution in imaging; it is often described as "molecular imaging" since disease-specific receptors are detected. One example is the application of somatostatin receptor imaging for detection of neuroendocrine tumors. This recent capability is possible due to *in vivo* accumulation of a radiolabeled peptide with high affinity for somatostatin receptor expressed on the surface of the tumor cells. Another

example is detection and measurement of dopamine receptors, which become altered in Parkinson's disease. Molecular imaging represents a growth area for radiology, and promises to allow early detection and monitoring of disease response during therapeutic intervention.

III. Scientific Basis for Imaging

A. Electromagnetic Energy

In general, all imaging technologies require the use of electromagnetic energy. Each imaging modality exploits a different part of the electromagnetic energy spectrum. The spectrum includes gamma rays, X-rays, visible light, ultrasonic waves, and radiowaves. All of these waves are photons of energy, but they differ in their wavelength and, therefore, energy. Each imaging instrument is designed to detect a particular range of electromagnetic energy. Most often, the instrument also generates the requisite energy for the imaging application, and the detection occurs after interaction with the imaging subject. The exception is with gamma-ray imaging, where the requisite energy is provided by decay of an administered radioactive probe that is not part of the instrument.

B. Contrast

All imaging technologies require contrast in order for images to be produced. In the case of gamma-ray imaging, the contrast is provided by the localized accumulation of the radioactivity. In the case of radiography, the bone and soft tissues attenuate the X-rays to a different degree, which leads to contrast. Contrast is achieved in magnetic resonance imaging because the local environment of the proton is different in fat, water, and the soft tissues. With US, contrast is achieved because the reflectance of the ultrasonic wave is dependent on the tissue architecture or blood flow. Contrast for light-based imaging is provided by localized light emission or fluorescence. Contrast for radiography, CT, MRI, and US can be increased by administration of an exogenous contrast-enhancing agent. Angiography is always better with a contrast agent, whether done by fluoroscopy, CT, or MRI.

C. Gamma Rays and Detection

In the electromagnetic energy spectrum, the highest energy photons (shortest wavelength, highest frequency) are gamma rays. Gamma rays arise out of nuclear events during radioactive decay. For *in vivo* imaging purposes, the best gamma rays are of low energy, in the range of 100–511 keV. Gamma rays in this energy range can be efficiently stopped and therefore measured

by external detectors. Approximately 80–90% of nuclear medicine imaging is accomplished using radioactive ^{99m}Tc, which emits a 140-keV gamma ray during its radioactive decay. ^{99m}Tc has a 6-h half-life and is continuously available from regional nuclear pharmacies. It is the decay product of ^{99}Mo (half-life = 66 h) and is eluted daily from the ^{99}Mo/^{99m}Tc generator system and therefore available at very high specific activity and low cost. ^{99m}Tc is chelated (complexed) with various compounds that have different biological characteristics.

A typical mobile Anger gamma camera for planar imaging is shown in Fig. 1A. Gamma camera imaging requires the use of a collimator, a solidly constructed gamma-ray attenuator (usually made from lead) that is placed between the subject and the gamma-ray detector. There are various types of collimators, some more specific for low-energy gamma rays, while other are specific for higher ranges of gamma-ray energies. A pinhole collimator and parallel-hole collimator is shown in Figs. 1B and 1D, respectively. The pinhole collimator has a small round hole at the end (inset, Fig. 1C) that allows projection of the gamma rays onto the detector crystal, thus forming an image like a pinhole camera. In contrast, the parallel-hole collimator allows passage

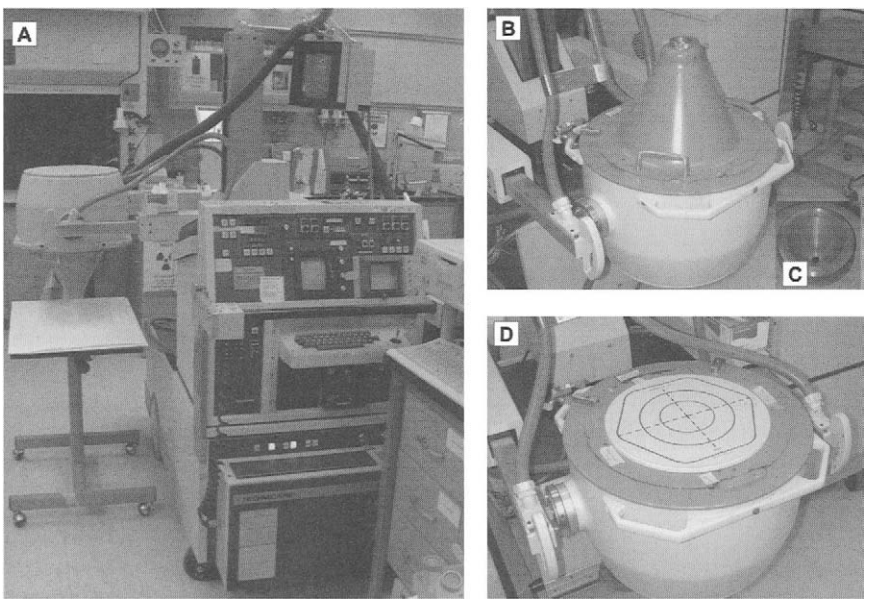

Figure 1 Gamma camera and collimators. (A) Anger 420/550 mobile radioisotope gamma camera (Technicare, Solon, OH); (B) gamma camera detector head with the pinhole collimator; (C) close-up of the pinhole; (D) gamma camera detector head with the high resolution parallel-hole collimator. The gamma camera has one detector head; the collimators are changed for the particular application.

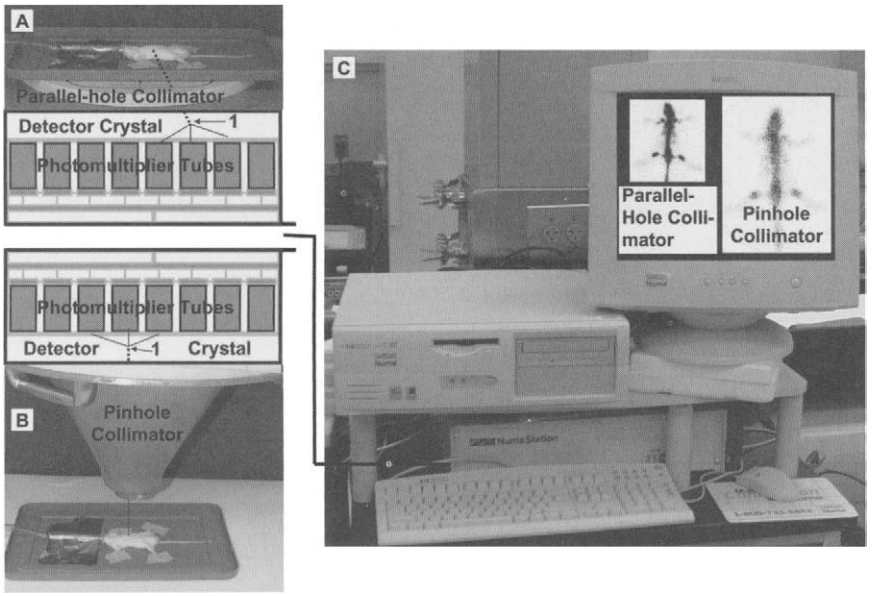

Figure 2 Diagram showing cross-section of the detector head with a mouse in position for imaging. (A) Imaging with a parallel hole collimator; (B) imaging with a pinhole collimator; (C) data acquisition computer (NumaStation, Amherst, NH) showing collected images. A gamma ray is depicted in A and B passing through the collimators and interacting with the detector crystal (1), leading to production of light that is detected by the photomultiplier tubes.

of gamma rays that are perpendicular to the plane of the collimator. Figure 2 presents a diagram illustrating a cross section of the gamma camera equipped with either a high-resolution parallel-hole collimator or a pinhole collimator. The mice are in position for imaging and were previously injected with a compound called methylene diphosphonate labeled with ^{99m}Tc (^{99m}Tc-MDP). This compound localized in areas of bone with high osteoblastic activity by 4 h after intravenous injection. The gamma rays emitted from the animal are stopped by the detector crystal (Fig. 2, "1") and visible light photons are emitted. These photons are captured by the photomultiplier tubes adjacent to the crystal and converted to a voltage pulse. The *X*, *Y* location of the interaction event is recorded, as well as the magnitude of the voltage pulse (*Z*, pulse height), which is proportional to the energy of the gamma ray that was stopped. The gamma camera in this example has an intrinsic spatial resolution of 3 mm; therefore, individual vertebra of the mouse were not detected separately. However, uptake in the spine and knee joints is clearly visualized.

Additional examples of ^{99m}Tc complexes for human imaging include ^{99m}Tc-MAG3 (mercaptoacetyltriglycine) for imaging renal function and

^{99m}Tc-HMPAO (hexamethylpropyleneamineoxime) for imaging blood flow in the brain. Peptides and antibodies radiolabeled with ^{99m}Tc are also approved for human imaging applications. Most often, the ^{99m}Tc is attached to protein with a bifunctional chelater. With this system one part of the chelator binds ^{99m}Tc in stable conformation, while a second part is used for attachment of the complex to the protein. Besides ^{99m}Tc, other radionuclides that are used for imaging include ^{67}Ga, ^{111}In, ^{123}I, and ^{131}I (see Table I). These radionuclides have different gamma-ray emissions; simultaneous imaging with ^{99m}Tc is possible. Multi-gamma-ray imaging is one feature that differentiates gamma camera imaging from PET. The latter is limited to detection of positron annihilation events, and therefore radionuclides lacking positron emission are not detected. The image presented in Fig. 2 is a planar image that represents a two-dimensional distribution of the ^{99m}Tc-MDP at 4 h after intravenous injection. Single photon emission computed tomography (SPECT) is also possible with specialized gamma cameras that are routinely available in nuclear medicine departments. SPECT is accomplished by collecting multiple images (or projections) at various angles around the subject; the gamma camera usually moves. A tomographic image of the distribution of the radioactivity is produced following reconstruction of these projections. The typical spatial

Table I
Radionuclides Commonly Used in Imaging

Radionuclide	Decay mode[a]	Half-life	Gamma-ray (keV) (Abundance)	Imaging application
^{11}C	β+	20.38 min	511	PET
^{13}N	β+	9.96 min	511	PET
^{15}O	β+	122 s	511	PET
^{18}F	β+	109.8 min	511	PET
^{64}Cu	EC 41%, β+ 19%, β− 40%	12.7 h	511 1345 (0.48%)	PET
^{67}Ga	EC	3.26 days	93 (37%), 184.6 (20.4%), 393.5 (4.6%)	Gamma camera
^{99m}Tc	IT	6 h	140 (89%)	Gamma camera
^{111}In	EC	2.83 days	171.3 (90.2%), 245.3 (94%)	Gamma camera
^{123}I	EC	13.2 h	159.1 (83%)	Gamma camera
^{124}I	β+ 25%, EC 40%	4.15 days	511, 602.7 (61%)	PET
^{131}I	β−	8.04 days	364.5 (81.2%)	Gamma camera

[a]β−, beta minus (electron emission); β+, beta plus (positron emission); EC, electron capture; IT, isomeric transition.

resolution provided by clinical SPECT imaging is on the order of 1 cm^3 [2, 3]. New methods for animal imaging are able to accomplish both planar and SPECT imaging at a spatial resolution of 1 mm^3 and better [4–6]. Many new generation gamma cameras also include solid-state detectors, which eliminates the need for the photomultiplier tubes.

PET is an alternate three-dimensional imaging technique for the indirect detection of positrons. Positrons are positively charged electrons that are emitted from a proton-rich nucleus during radioactive decay. The lifetime of positrons is relatively short since they undergo annihilation by combining with an electron, giving rise to two 511-keV gamma rays at opposite (180°) orientations. The 511-keV gamma rays are actually detected in PET, not the positrons. PET scanners have a circular array of detectors that are designed to operate in a coincidence mode. This means that a signal is generated only when two detectors at opposite orientations simultaneously detect the 511-keV gamma rays, arising from the positron annihilation event. Since various 511-keV pairs of photons strike different pairs of detectors, the location of the actual decay events can be determined when the image is reconstructed. PET scanners do not require collimators, since the coincidence circuitry accomplishes the same objective. In recent years, dual-head gamma cameras have been designed with coincidence circuitry, thereby enabling gamma cameras to conduct PET imaging studies. The volumetric resolution of clinical PET scanners is approximately 0.4 cm^3 [7, 8]. A new small animal PET scanner (MicroPET, Concorde Microsystems, Knoxville, TN) is reported to have a spatial resolution of 1.8 mm and volume resolution of 8 mm^3 for *in vivo* imaging [9]. This latter system represents a significant advancement in small animal imaging, since improved spatial was needed for detection of individual organs in mice.

Radionuclides that are used in PET imaging are proton rich and produced at cyclotrons using charged-particle reactions. A list of common PET radionuclides is included in Table I. Most PET radionuclides have short half-lives; therefore, production must be in close proximity to where imaging will be done. In addition, PET radionuclides such as ^{11}C, ^{13}N, and ^{15}O are suitable as intrinsic labels for many molecules, thereby enabling imaging studies of the actual molecule of interest. For example, fatty acid metabolism could be imaged with the ^{11}C-labeled fatty acid, where the ^{11}C replaced the normal ^{12}C in the molecular structure. Intrinsic labeling of this type cannot be accomplished with ^{99m}Tc, since the radionuclide is not part of the molecule. A bifunctional chelate would be required for the ^{99m}Tc to attach it to the fatty acid, and a ^{99m}Tc-labeled fatty acid might have different *in vivo* uptake and elimination characteristics than the natural fatty acid.

D. Light-Based Imaging and Detection

Visible light has a wavelength between 400 and 700 nm, while the near-infrared region is from 700 to 1000 nm. Light in the near infrared is better

for *in vivo* imaging, since it penetrates the tissue more readily. For light-based imaging, contrast is achieved by at least three mechanisms. A specific color can be produced due to transfer of an enzyme (e.g., β-galactosidase) that reacts with a substrate to produce a colored product. A second mechanism is provided by fluorescence, excitation at one wavelength, with simultaneous detection at another wavelength. Special filters are employed to block detection of the excitation wavelength, thereby reducing the background for better contrast. Spectral imaging [10] and hyperspectral imaging [11] are technologies recently developed, while data processing for optical imaging is also improving [12, 13]. New fluorescence techniques use quenched substrates, which become fluorescent (unquenched) after activation by a specific process. Finally, light emission by enzymatic reaction can be achieved following expression of the gene encoding for luciferase. *In vitro* light detection instruments include microscopes, fluorescence-activated cell sorters, and fluorescence-based plate readers. *In vivo* light-based imaging can be accomplished with fluorescent stereomicroscopes and light-tight boxes with CCD cameras. A commercial instrument for luciferase imaging is also available from Xenogen, Inc. (Alameda, CA).

E. Magnetic Resonance Imaging and Spectroscopy

The proton is the element responsible for the signal generation in proton MRI, and can be viewed as a minimagnet due to the spinning single electron. MRI utilizes two energies, a strong magnetic field and pulses of radio frequency (RF) electromagnetic energy. RF energy is not ionizing, and a trillion times less in magnitude than X-rays. The sensitivity of the technology is related to the large number of protons that are present in water and fat, the primary constituents of a human or animal. When protons are placed in a magnetic field, they become aligned with, or opposed to, the external field. Excitation with a precise resonance frequency (MHz) results in excited-state protons, all of which are in phase, but tilted away from the direction of the external magnetic field. The "in-phase" aspect is unique to the excited state, since ground-state protons are not in phase. Therefore, to summarize, absorption of a resonance RF energy by the proton results in an excited state, where all the excited-state protons are in phase. When the RF excitation is stopped, the excited protons "relax" and emit resonance RF energy in proportion to proton density. This is the MRI signal and is analogous to phosphorescence for light. The relaxation of the excited protons is also referred to as the spin-lattice relaxation, which is the time for the protons to realign with external magnetic field. The time course is described by an exponential equation that includes a constant (T1). The protons also "dephase" at an exponential rate, which is referred to as spin–spin relaxation. This decay of signal is also described by an equation that includes another constant (T2 or T2* time constant). The important fact

is that the magnitude of both T1 and T2 depend on the tissue (local proton environment) and therefore contrast is achieved in MRI due to this difference. MRI contrast agents exert their effects by modulating T1 and T2 in the local environment of the contrast agent. In other words, the enhanced contrast provided by an MRI contrast agent is not related to signal generated by the contrast agent, rather the effect of the contrast agent on the local protons. In this respect, the mechanism of the MRI contrast agent is different from an X-ray contrast agent. The latter causes contrast due to higher absorption of the X-rays. Overall, the *in vivo* spatial resolution of MRI is superb, reaching a voxel resolution of about 50 μm^3. Typical clinical MRI instruments have a magnetic field strength of 0.5–2 Tesla.

MRS represents a specialized capability of magnetic resonance technology. MRS is not concerned with T1 and T2 times constants, rather with accurate measurement of the chemical shifts associated with molecules that incorporate ^{1}H, ^{13}C, ^{19}F, and ^{31}P. Each particular molecule has a different signature, allowing for assignment of individual metabolites. This can be very important to the understanding of disease processes, or response during therapy. For example, brain phospholipid metabolites (such as free phosphate, phosphocholine, etc.) can be studied. MRS often requires magnets of high field strength in order to separate the overlapping signals of individual metabolites. An additional disadvantage of the MRS is the relatively low sensitivity for detecting the metabolites, which requires either very long imaging times or large voxel sizes (volume area). MRS methods have been reviewed recently [14–16].

IV. Imaging and Gene-Therapy Vectors

A. Gamma-Ray Imaging

Gamma-ray imaging has been applied in two different ways to evaluate gene-therapy vectors in animal models. First, the *in vivo* location of the administered vector can be imaged following dosing. Second, the vector-dependent location of transgene expression can be imaged. This is accomplished by detection of expressed genetic reporters encoded in the vector. Both methods yield different information and may, or may not be correlated. For example, following intravenous injection an Ad vector might immediately localize in the liver. However, the extent of liver gene expression would depend on the cell type infected, the type of promoter, and the time after dosing. The two methods are reviewed in the following sections.

1. Radiolabeling the Vector

Gamma camera imaging has been applied to evaluate directly labeled vectors. The *in vivo* distribution of ^{111}In-labeled herpes simplex virions

was imaged following intravenous dosing [17]. This method of nonspecific labeling with ^{111}In oxine showed no effect on viral infectivity; a maximum specific activity of 250 μCi/10^9 plague forming units (pfu) was achieved. ^{99m}Tc-labeled Ad was evaluated following aerosol administration to the lung [18] or following intravenous injection [19]. The former approach [18] used nonspecific labeling with SnF_2 as the reductant for the ^{99m}Tc ($[TcO_4]$-). The latter approach represented a new method for radiolabeling a recombinant Ad vector with ^{99m}Tc. A recombinant 6-His tag on the C-terminal knob was specifically targeting for labeling using Tc(I) carbonyl chemistry. This radiolabeling chemistry was originally described by Waibel [20]. Advantages of this system are related to the ease of radiolabeling and high stability over nonspecific methods, plus the specific attachment at the 6-His tag did not change the infectious characteristics of the Ad vector. It is anticipated that the technology will be used to image Ad vectors with modified tropism.

2. Imaging the Expression of Reporter Genes

One method to evaluate gene transfer is to construct a vector encoding a suitable gene that when expressed can be targeted with a radiolabeled probe. An Ad vector encoding the human type 2 somatostatin receptor (hSSTr2) was used to infect cells growing in cell culture plates, and the expressed hSSTr2 was detected by imaging accumulation of the ^{99m}Tc-labeled somatostatin analog P2045 [21]. An example of this approach is presented in Fig. 3. The *in vitro* method was further extended for detection of both hSSTr2 and herpes simplex virus thymidine kinase (TK) expression by simultaneously imaging "trapped" radiolabeled molecules specific for each expressed gene [22].

Imaging can also detect the location of gamma-ray emitting radiotracers that accumulate in target sites *in vivo*, due to localized expression of a reporter gene product. In this regard, the administered radiotracer must be cleared from the normal tissue so specific accumulation can be detected over background radioactivity. To date, this approach has been applied to image the *in vivo* expression of four different reporter genes, namely hSSTr2 [23–26], TK [27–39], the type 2 dopamine receptor (D_2R) [35, 37, 40–42], and the thyroid sodium/iodide symporter gene [43]. The expression of hSSTr2 has been imaged with radiolabeled peptide ligands including ^{99m}Tc-P829 [23], ^{188}Re-P829 [23], ^{99m}Tc-P2045 [24–26], and ^{111}In-octreotide [25]. Figure 4 presents images that compare two different ^{99m}Tc-labeled somatostatin analogs for imaging Ad-mediated expression of hSSTr2.

Several radiolabeled substrates have been applied for imaging TK expression, including ^{131}I-FIAU [27, 32, 37], ^{124}I-FIAU [23, 25, 30], 8-[^{18}F]fluoroganciclovir [29, 34, 35, 37], 8-[^{18}F]fluoropenciclovir [35, 37, 38, 40, 41], 9-[(3-[^{18}F]fluoro-1-hydroxy-2-propoxy)methyl]guanine [39], and others [37]. D_2R expression has been imaged with 3-(2′-[^{18}F]-fluoroethyl)spiperone [35, 37, 40, 41] and [^{11}C]raclopride [42]. The expressed

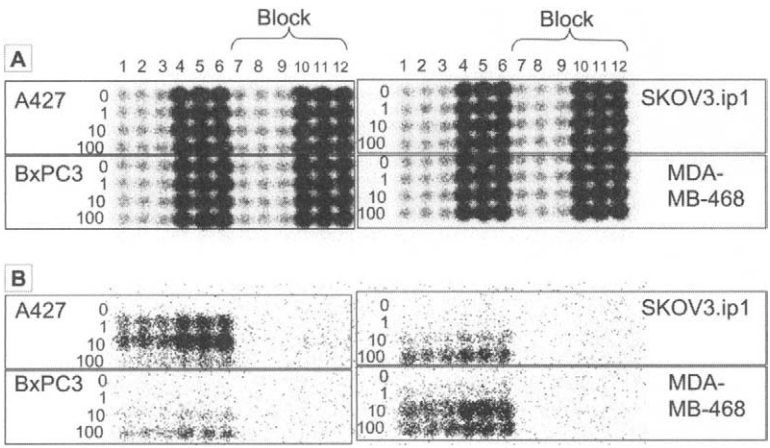

Figure 3 Imaging hSSTr2 gene transfer to tumor cell monolayers growing in 96-well plates by detection of internally bound ^{99m}Tc-labeled P2045 (a somatostatin analog). Two levels of ^{99m}Tc-P2045 (7 nM: wells 1–3, 7–9; 36 nM: wells 4–6, 10–12) were added to the two plates as shown in (A). Image (B) shows the quantity of internally bound ^{99m}Tc-P2045 was dependent on the cell line and amount of Ad-hSSTr2. Cells were incubated with ^{99m}Tc-P2045 for 3 h at 37°C and imaged following an acid wash to remove surface-bound activity. The cells were either uninfected (0) or infected with 1, 10, or 100 pfu/cell of Ad-hSSTr2 48 h earlier. Excess unlabeled P2045 (15 μM final concentration) was added to lanes 7–12 immediately prior to addition of ^{99m}Tc-P2045 in order to show that the internal binding was specific. Reprinted with permission from Zinn *et al.* [22].

reporters were imaged in xenograft tumors [23–28, 32, 34, 36–45], liver [29, 34–37, 39], and striatum [42]. In the majority of xenograft tumor studies, the reporter gene was transferred to the tumor cells prior to implantation in the animal [27, 28, 31, 36–38, 40, 41], or vector-producer cells were injected in an established tumor [27, 28, 31]. Transfer of the reporter gene by intratumor injection was accomplished with Ad-hSSTr2 for subcutaneous tumors [23–25, 45], Ad-TK for intrahepatic tumors [32], Ad-TK for subcutaneous tumors [39], and the Ad encoding the thyroid sodium/iodide symporter gene [43]. Transfer of the TK gene to rat striatum was accomplished by direct injection of Ad-TK [42]. Most recently, ^{124}I-FIAU and PET were applied to monitor TK expression resulting from replication and spread of a replication-conditional HSV-1 vector (encoding TK) in a subcutaneous tumor [44].

A biscistronic Ad vector encoding both TK and hSSTr2 was evaluated in a tumor model where the expressions of both TK and hSSTr2 were simultaneously imaged [24, 45]. The hSSTr2 system was more sensitive than TK and showed a dose–response relative to Ad dose, while the same was not observed for TK imaging. Additional advantages of the hSSTr2 system over other genetic reporters for imaging gene transfer in cancer, include the lower

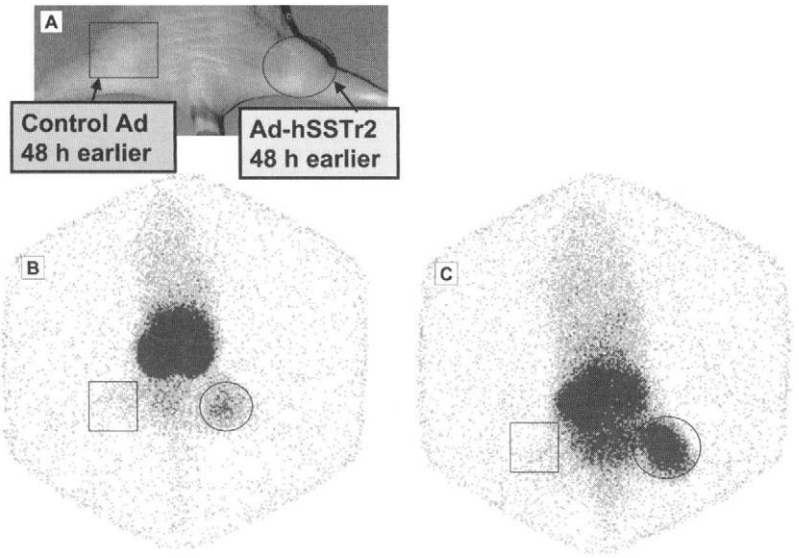

Figure 4 Imaging Ad-mediated gene transfer to subcutaneous tumors. (A) Picture of a mouse with two subcutaneous A-427 tumors (human lung cancer). The left tumor was injected 48 h earlier with a control Ad vector (1×10^9 pfu intratumor) while the right tumor was injected 48 h earlier with Ad-hSSTr2 (1×10^9 pfu intratumor). (B) Gamma camera image (pinhole collimator) at 5 h after intravenous dosing with ^{99m}Tc-P829 (NeoTect™, Diatide Research Laboratories, Londonderry, NH). (C) Gamma camera image (pinhole collimator) at 5 h after intravenous dosing with ^{99m}Tc-P2045, a new somatostatin analog (Diatide Research Laboratories, Londonderry, NH). The right tumors show uptake of the ^{99m}Tc-labeled somatostatin analog due to gene transfer and expression of hSSTr2.

cost of ^{99m}Tc relative to PET radionuclides, the wider availability of SPECT relative to PET, and the negative growth effect of hSSTr2 expression on cancer proliferation and metastasis [46–48].

B. Light-Based Imaging

Light-based genetic reporters are commonly used to detect *in vitro* gene transfer. Examples include β-galactosidase [49], green fluorescent protein (GFP) [50, 51], red-shifted GFP derivatives [52], blue- or yellow-shifted GFP derivatives [53], and luciferase [54]. Advantages include high spatial resolution and sensitivity. Light-based approaches for *in vivo* imaging can be applied to assess the broad physiology involving tumor growth, which includes biochemical processes, receptors, and enzymes [55–81]. Currently, two general approaches are applied for light-based imaging. The first approach uses fluorescent-based probes that specifically accumulate, or become activated, due to a tumor-specific process. This approach uses fluorescent probes

that target tumor-specific receptors or enzymes. One example reported by Jackson and group used light-based imaging to demonstrate accumulation of a tumor receptor-specific, single-chain Fv fragment labeled with Cy5 fluorescent dye in mice bearing melanoma xenografts [55]. Tumor uptake of antibodies conjugated with near infrared dyes was previously visualized by light-based technology [56, 57]. Optical imaging also detected tumor accumulation of a somatostatin-avid peptide conjugated with a near infrared fluorescent dye [58–60]. An alternate application was described by Weissleder *et al.* using Cy5.5 probes that were inactive (autoquenched) when injected in the mice, but became specifically activated by proteases expressed in breast xenograft tumors [61, 62]. A recent report described a quantitative light-based method for noninvasive imaging of human breast cancer [63]. In this study, the images were obtained using near-infrared diffuse optical tomography (DOT), with contrast enhancement provided by indocyanine green (ICG) that was administered to the patient. MRI was performed concurrently on the same patient and showed that ICG-enhanced optical images coregistered accurately with gadolinium-enhanced MRI, thereby validating the ability of DOT to image breast tumors. Others also used ICG and modified photodynamic agents in combination with frequency-domain photon migration techniques to detect spontaneous cancer in the canine mammary chain [64, 65].

The second general approach for light-based imaging uses reporter genes yielding protein products that achieve light emission. Luciferase is one example of a reporter gene, the expression of which can be imaged by detecting light emission following injection of luciferin [66–69]. Luciferase is normally absent in mammalian cells, but stable integration of a luciferase-containing plasmid was achieved in cancer cells [67, 68] which were implanted and detected by imaging. In a separate report, an adeno-associated viral vector was used to induce luciferase expression *in utero*, with detection by whole-body imaging [69]. One disadvantage to luciferase imaging is the requirement of luciferin substrate injection. However, the sensitivity is high and requires only short-term photon acquisition and integration to produce images of intact animals. Therefore, real-time studies and high-throughput *in vivo* screening of gene expression is possible, especially during therapeutic intervention.

Several investigators have applied GFP as a light-based reporter after stable integration of the GFP gene in cancer cells prior to implantation in mice [70–81]. Light emission was achieved following excitation of the GFP protein with blue light. Chishima *et al.* demonstrated that GFP-expressing tumor cells were visualized after tumor-bearing mice were dissected, and the metastasis of cancer was detected in many different organs [73–77]. This work was later extended by Yang *et al.* to include noninvasive imaging of GFP-positive melanoma metastasis in mice [78]. In a very recent article, Hoffman and his group reported imaging results from a study where an Ad vector encoding enhanced GFP was injected into different organs of nude mice.

Light-based *in vivo* imaging showed GFP expression in different organs [79]. Enhanced GFP is not cytotoxic and has stable fluorescence signal that can be readily detected. Therefore, it is a suitable reporter molecule for imaging gene expression in animal models [80, 81]. As an example, Fig. 5 presents a light-based image of GFP expression in a subcutaneous tumor following Ad-mediated gene transfer of GFP. Advantages of GFP imaging include the high sensitivity and tremendous spatial resolution that can be achieved. A further advantage is the fact that the protein is genetically encoded, without the need for exogenous substrates.

C. Magnetic Resonance Technologies

For cancer applications, MRI can indirectly assess the effect of gene therapy by measuring changes in tumor size, blood flow, or extracellular fluid volume [82–85]. Direct measurement of gene expression by MRI requires contrast-enhancement. This was achieved *in vitro* by stable induction of melanin, a protein with high affinity for metal ions [86, 87]. For *in vivo* imaging, cells expressing high levels of transferrin receptor were detected on T2-weighted images after injection of transferrin conjugated with paramagnetic iron particles [88, 89]. These monocrystalline iron oxide nanoparticles (also called MIONs) were improved with a cross-linked dextran coat and better conjugation chemistry for the transferrin attachment, leading to a 10-fold improvement in *in vitro* cellular uptake [90, 91]. These improved nanoparticles may be better suited for imaging the transferrin receptor following gene transfer *in vivo*. So far this remains a potential application.

An alternate strategy for imaging gene transfer was reported by Louie and Meade [92]. The basis of this approach was the construction of a MRI-contrast agent that was sensitive to β-galactosidase. Gadolinium was chelated in a manner that water was inaccessible, and therefore "inactive" as a contrast agent. However, β-galactosidase was capable of cleaving part of the molecular structure, rendering the gadolinium accessible to water, and "active" as a contrast agent on T1-weighted MRI images. For the approach to be applicable for *in vivo* imaging, the contrast agent must be delivered to the site of β-galactosidase expression. Therefore, the suitability of this strategy for imaging vector-mediated gene transfer remains to be proven.

There are three literature reports demonstrating the capability of MRS for noninvasive detection of enzyme-specific metabolites that are applicable to gene therapy [93–95]. The first report detected the metabolite [^{19}F]5-FU in cytosine deaminase-positive tumors following administration of 5-FC [93]. The subcutaneous tumor was established following implantation of HT28/yCD cells that stably expressed *Saccharomyces cerevisiae* cytosine deaminase. Imaging required a surface coil surrounding the tumor, and spatial information about cytosine deaminase expression in the tumor was not obtained. The second

report involved noninvasive monitoring of arginine kinase (AK) expression following Ad-mediated delivery to skeletal muscle [94]. MRS detected the metabolite [^{31}P]phosphoarginine in the muscle, the product of the AD enzyme. Imaging also required a surface coil surrounding the limb, and no spatial information about AK expression was obtained. The third report involved detection of [^{31}P]phosphocreatine in liver of mice following intravenous dosing of an Ad vector encoding creatine kinase [95]. The liver was surgically exposed for placement of a surface coil for MRS, in order for specific detection of this metabolite.

V. Gene-Therapy Vectors May Advance Molecular Imaging

While this chapter has focused on how imaging may advance gene therapy, the converse may also occur. Gene-therapy vectors may lead to better imaging approaches. One example relates to the need for a reliable method for early detection and monitoring of ovarian cancer. Ovarian cancer is the leading cause of death among gynecologic malignancies in United States [96, 97]. More than 23,000 new cases of ovarian cancer are diagnosed yearly, with 15,000 deaths annually [97, 98]. For gynecologists, ovarian cancer is extremely difficult to detect in early stage since no reliable screening method exists for this disease. This results in a poor prognosis. In the past decade, there was a 30% increase in the incidence of ovarian cancer and 20% increase in deaths from this disease [99]. Currently, surgical staging by histological examination and vigorous surgical debulking are routine practice for early detection and partial treatment of this disease [96]. Early detection of this disease would be the best way to improve survival. In that regard, the development of an accurate, noninvasive *in vivo* imaging modality with high sensitivity for the detection of small lesions is needed. Detection of small tumors using conventional techniques is difficult, thereby hindering both early diagnosis and effective therapeutic intervention.

Existing technologies do not meet the need for the early detection and monitoring of ovarian cancer. Imaging technologies have improved in their sensitivity to image ovarian, breast, and other cancers noninvasively by PET, CT, MRI, SPECT, and US [98–113]. However, these methods fall short in fulfilling the need for early and accurate diagnosis of neoplastic disease. According to Wahl, PET imaging with [^{18}F]FDG was able to detect primary breast lesions over 1 cm in diameter [108]. Similar findings were reported by Richter *et al.* [109]. Grab *et al.* concluded that a negative finding on PET or MRI would not exclude early ovarian neoplasia [110]. Kubik-Huch *et al.* reported in a comparative study that, PET, CT, and MRI were not a replacement for surgery in the detection of microscopic peritoneal

disease [111]. While PET imaging offered less accurate spatial assignment of small lesions compared with CT and MRI, the latter two modalities were less specific than [^{18}F]FDG PET [111]. In a separate report, Tempany *et al.* reported that CT and MR were equivocal for imaging advanced ovarian cancer [112]. Kurjak *et al.* reported that transvaginal color Doppler and three-dimensional power Doppler ultrasound imaging improved the ability to differentiate benign from malignant ovarian masses [113]. Taken together, all currently available imaging techniques are not satisfactory for the early and accurate detection of ovarian and breast tumors smaller than 1 cm in diameter.

Clinicians are searching for an effective screening method for the early detection of ovarian cancer. Our group has suggested that Ad vectors can be developed for this purpose [114]. The idea is to achieve GFP contrast in ovarian tumors and apply light-based imaging for detection (Fig. 6). For this to be realized, it will be necessary to induce GFP expression specifically in the tumors. This could be accomplished in two ways. First, it is likely that ovarian tumors express unique genes that are not found in normal tissues. The human genome project and DNA array research are likely to uncover these genes. Once a gene is discovered, the promoter element controlling the gene could be used to drive GFP expression. Intraperitoneal injection of an Ad vector encoding the GFP gene under control of the ovarian-specific promoter would enable visualization of GFP-expressing ovarian tumors. A second general method to cause GFP reporter gene expression specifically in the tumors would be to develop Ad vectors that specifically target tumor. Ad vectors have now been produced that lack native tropism [115]. Vector targeting continues to improve, and a clear demonstration of targeting to an artificial receptor has been realized [116]. While vectors are currently not available to target tumor-specific receptors and lead to specific tumor transduction, their future development is likely.

Thus, whether due to tumor-specific targeting or tumor-specific promoters, one can envision inducing GFP expression in tumors that could subsequently be detected by light-based technology. Fluorescent stereomicroscopy is adequate for noninvasive imaging in animal models, especially mice. However, this approach may not be possible in humans due to thickness of the abdominal wall. Detection of GFP expression could be accomplished with endoscopy or laparoscopy and enable physicians to detect the presence of tumor cells at an earliest stage without major surgery. This would be of great diagnostic value to gynecologists, especially in detecting intraperitoneal tumors at an early stage. In the clinical setting of second-look laparoscopy the patient could be injected 2 days earlier with an Ad vector that induced GFP expression in recurrent tumor. A fluorescent endoscope would detect GFP-positive tumors much smaller in size than what is currently detected through standard laparoscopy.

VI. Conclusion

Noninvasive imaging in its various forms represents an expanding endeavor that will positively impact gene therapy. Gamma-ray imaging modalities have an established track record for imaging gene transfer in animal models. Human studies with the same systems are likely to be the next evolution. At our institution two clinical trials are planned; both include the hSSTr2 reporter gene for imaging gene transfer.

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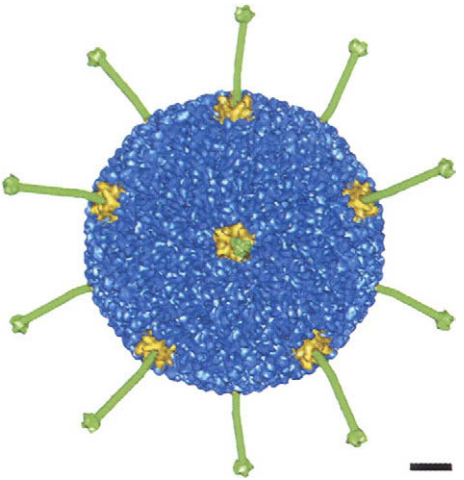
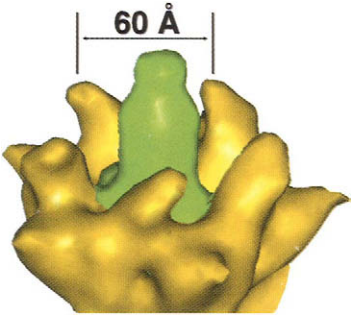
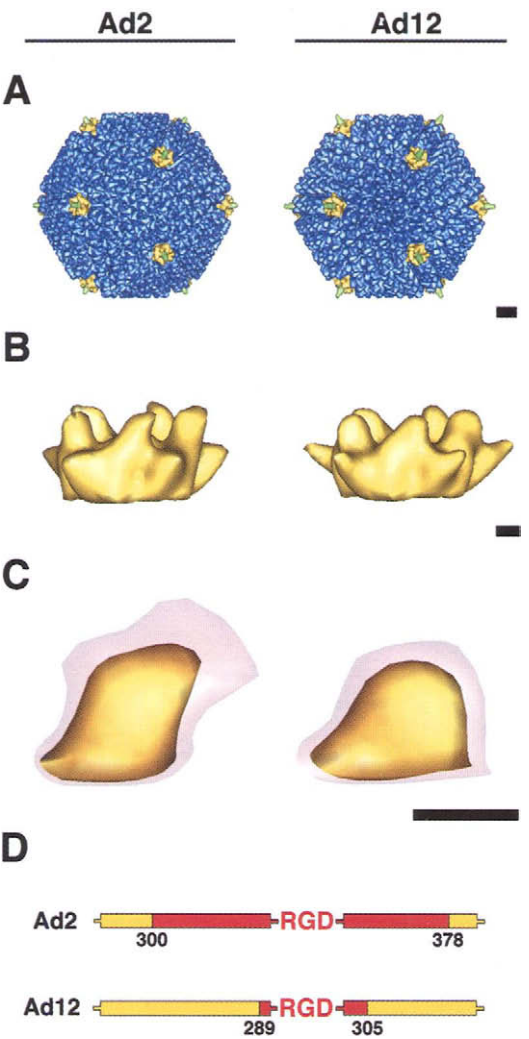


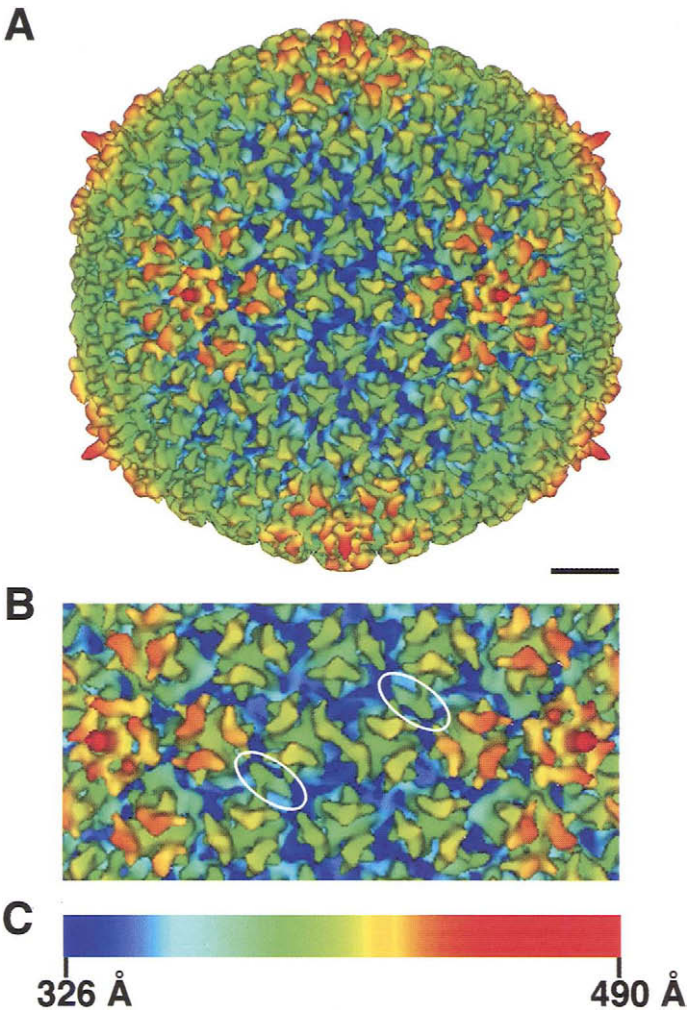
Chapter 1, Figure 1 A cryo-EM reconstruction of Ad2 (17 Å resolution) viewed along an icosahedral fivefold axis [32] and displayed with modeled full-length fibers. The penton base is shown in yellow, the fiber in green, and the rest of the capsid in blue. The fiber shafts are bent (5–15°) in random directions at a point ~90 Å beyond the penton base. The fiber knobs shown are filtered representations of the crystal structure [7]. The scale bar is 100 Å.



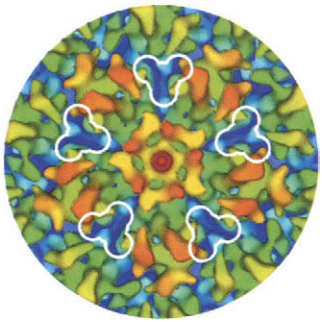
Chapter 1, Figure 3 The penton base (yellow) and reconstructed portion of the fiber (green) from a cryo-EM reconstruction of Ad2 (17 Å resolution) [32]. The boundaries chosen for the two proteins are arbitrary. Note the five protrusions of the penton base, which are spaced by 60 Å and surround the central fiber.



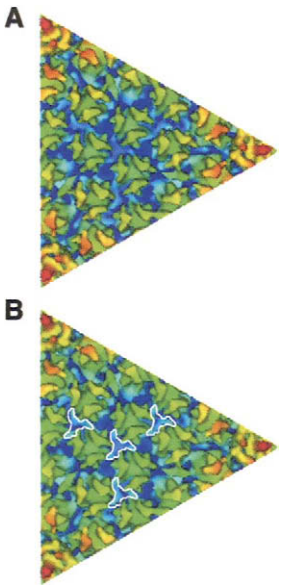
Chapter 1, Figure 4 Cryo-EM reconstructions of Ad2 (left) and Ad12 (right) at ~21 Å resolution. (A) Ad capsids viewed along an icosahedral threefold axis. The penton base proteins at the icosahedral vertices are shown in yellow, the reconstructed portion of the flexible fibers are in green, and the remaining capsid density is in blue. (B) Side views of the external portion of the penton base contoured at a level corresponding to the strong capsid density. (C) Enlargements of a single penton base protrusion at two isosurface levels, one just above the noise (transparent red) and the other showing well-defined density (yellow). (D) The lengths of the variable regions flanking the RGD sequence (red) in Ad2 and Ad12 are obtained from sequence alignment of five different Ad serotypes. The scale bars are 100 (A) and 25 (B and C) Å. Reproduced with permission from Chiu *et al.* [3].



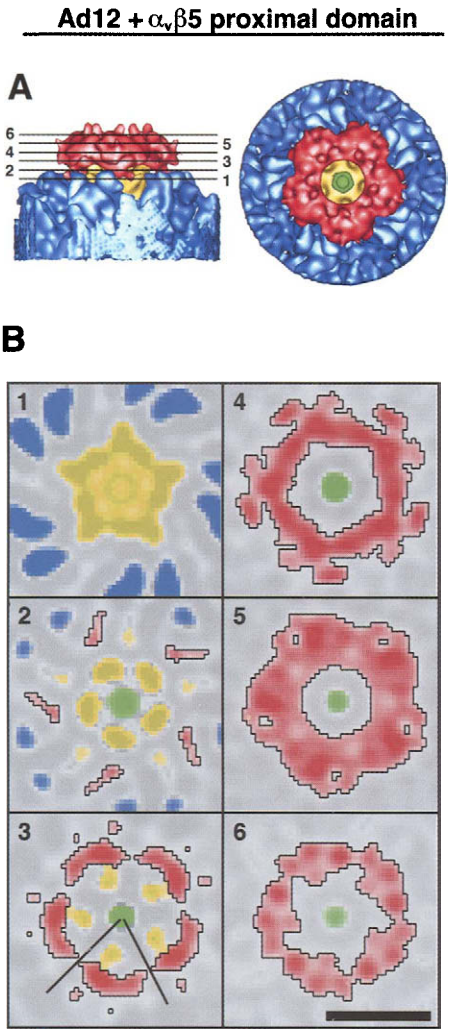
Chapter 1, Figure 6 A cryo-EM reconstruction of Ad2 (17 Å resolution) [32] shown color coded by radial height with respect to the center of the particle. (A) The full reconstruction viewed along an icosahedral twofold axis. The scale bar is 100 Å. (B) An enlarged rectangular section around the icosahedral twofold axis. The white ovals surround elongated density that has been assigned to polypeptide IIIa by cryo-EM difference mapping [61]. Note that the polypeptide IIIa density spans the full thickness of the capsid from the outer to the inner surface, but only the external portion is visible here. (C) The color scheme for the capsid density from blue at an inner radius of 326 Å to red at an outer radius of 490 Å.



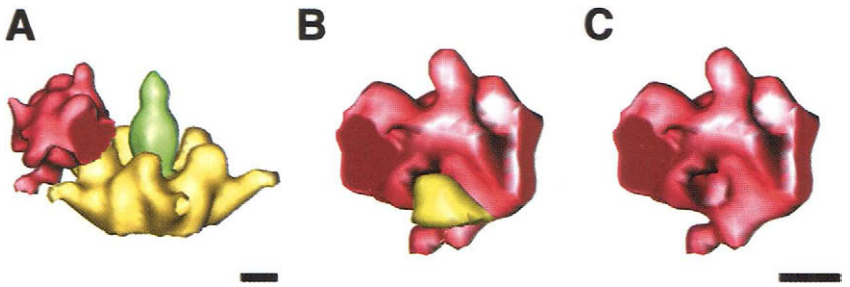
Chapter 1, Figure 7 An enlarged circular section of a cryo-EM reconstruction of Ad2 (17 Å resolution) [32] around an icosahedral fivefold axis. The white outlines indicate on the outer capsid surface the positions of trimeric density regions observed by cryo-EM difference mapping on the inner capsid surface [61]. This internal capsid density was assigned to polypeptide VI and is observed to bridge the bottoms of the five peripentonal hexons, the ring of hexons closest to the penton base. The color scheme is the same as in Fig. 6.



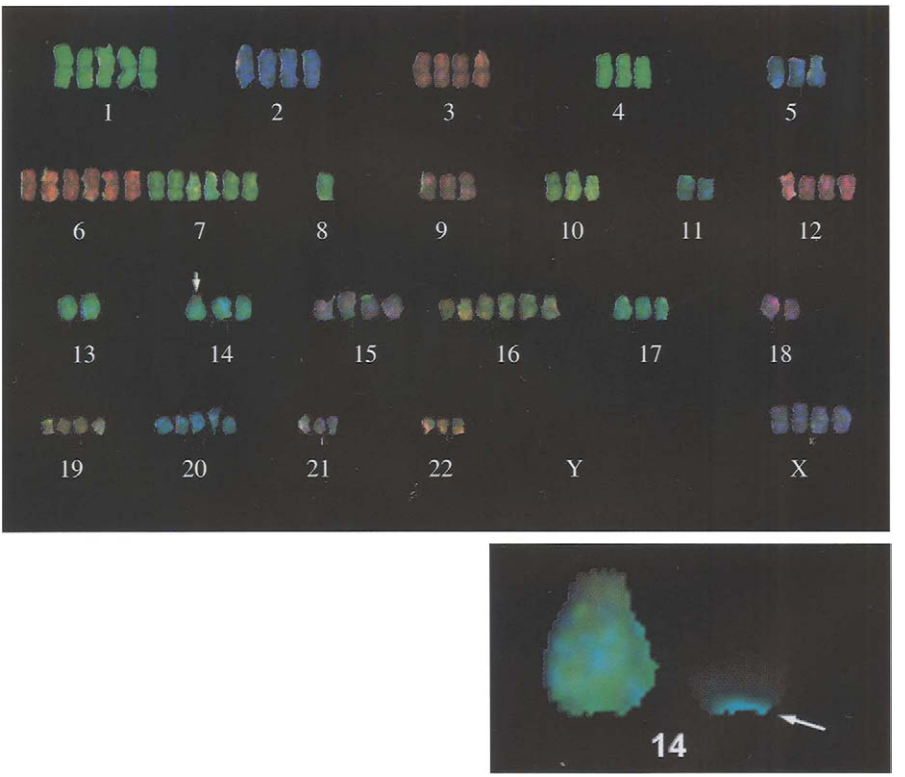
Chapter 1, Figure 8 An enlarged triangular section of a cryo-EM reconstruction of Ad2 (17 Å resolution) [32] around an icosahedral threefold axis. (A) The triangular section includes roughly one-fifth of three pentons at the corners, roughly half of four hexons along each edge, and six complete hexons in the center of the facet. (B) The same section shown in (A) with white outlines around the density assigned by cryo-EM difference mapping to polypeptide IX on the outer capsid surface [61]. The color scheme is the same as in Fig. 6.



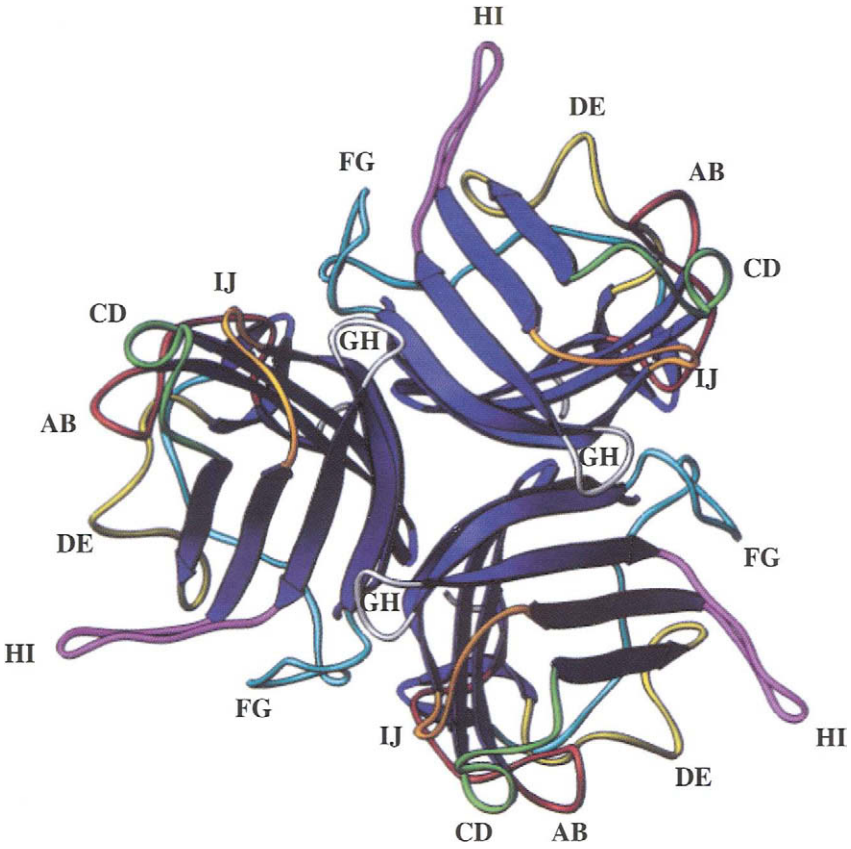
Chapter 2, Figure 2 Cryo-electron microscopic visualization of the αvβ5 integrin in association with adenovirus type 12 capsid. (A) Side- and top-surface views. (B) Slice planes through the integrin density perpendicular to an icosahedral fivefold symmetry axis. The heights of the slice planes are indicated by numbered lines in A. The color scheme for the individual proteins is as follows: blue (hexon), yellow (penton base), green (fiber), and red (integrin). Stronger density values are represented by darker shades, and weaker density values are represented by lighter shades. The black lines in slice 3 designate the boundary for the extracted model of one integrin proximal domain displayed in Fig. 3. The scale bars are 100 Å. Reprinted with permission from Chiu *et al.* [82].



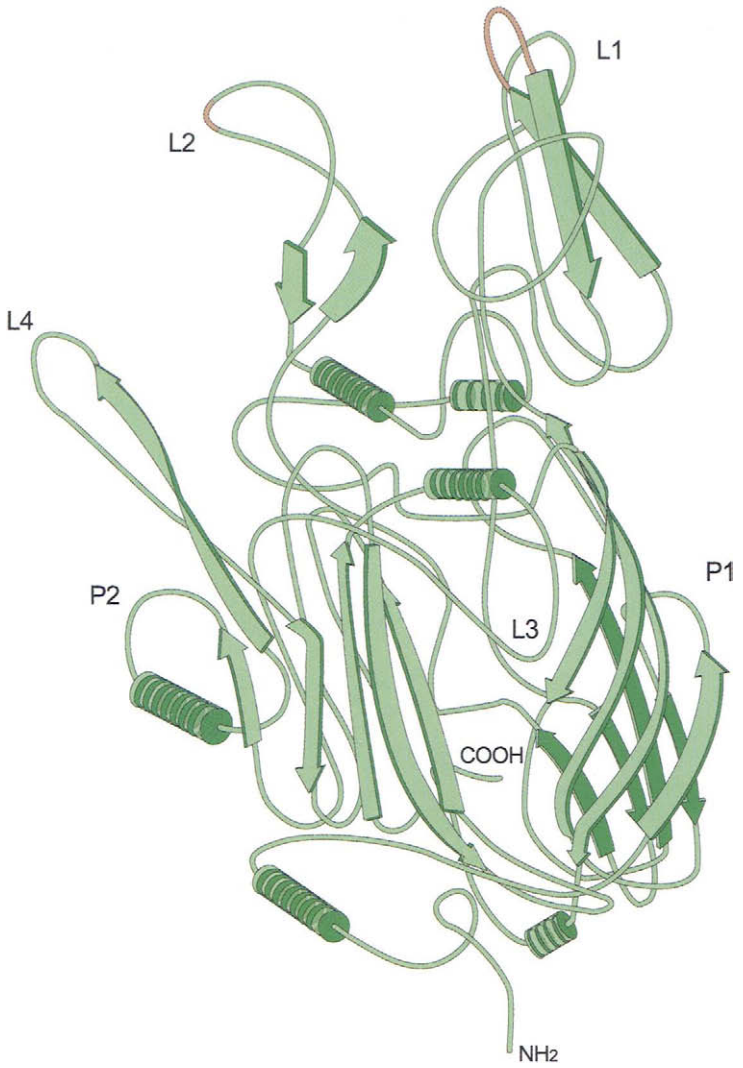
Chapter 2, Figure 3 Model for the interaction between the integrin proximal domain and the Ad12 penton base protein. One-fifth of the integrin ring density is shown extracted along estimated boundaries to model the proximal domain of a single αvβ5 heterodimer. (A) The modeled proximal domain shown in association with the penton base protein. (B) The modeled proximal domain rotated ~90° with respect to the view in A to show the interaction with a single penton base protrusion. (C) The same view as in B but with the protrusion removed to reveal the RGD-binding cleft on the inner surface. The scale bars are 25 Å. Reprinted with permission from Chiu *et al.* [82].



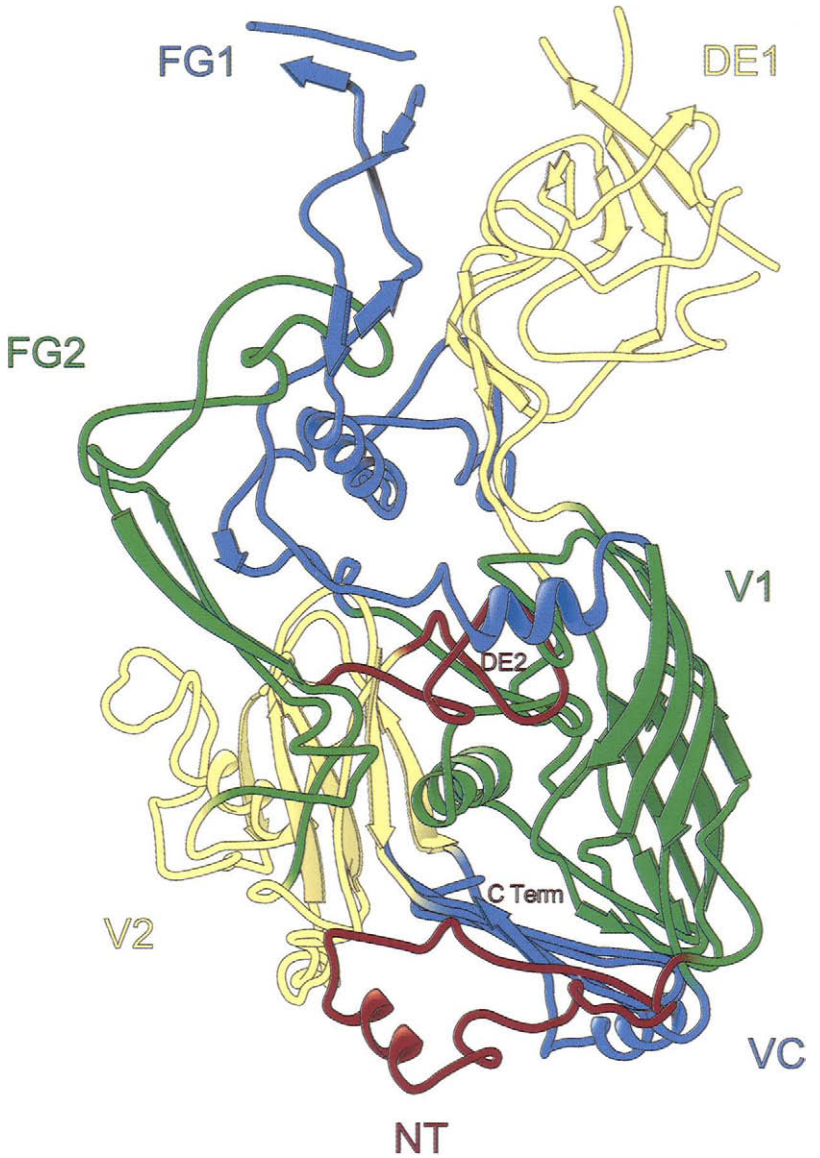
Chapter 6, Figure 9 Chromosomal localization of pIG.E1A.E1B in PER.C6 cells. Twenty-five-color COBRA-FISH with 24 human chromosome-specific painting probes combined with integrated plasmid probe DNA on PER.C6 metaphase chromosomes. One of three chromosomes 14 contains the integrated E1 construct; this chromosome is shown as an enlargement.



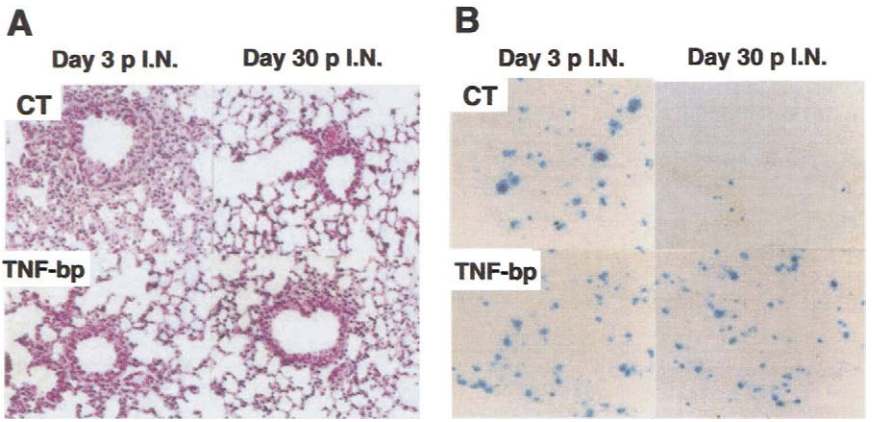
Chapter 8, Figure 2 Ad5 fiber knob trimer viewed along the threefold symmetry axis. Flexible loops localized on the surface of the molecule are indicated with two-letter codes, which specify the β-strands connected by a particular loop. The image was generated using X-ray crystallography data published by Xia *et al.* [89]. Reproduced with permission from [141].



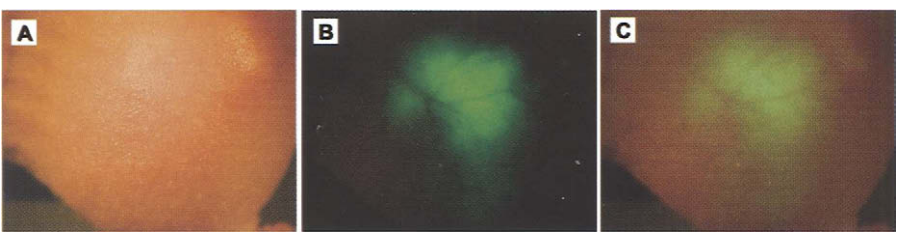
Chapter 13, Figure 1 (A) Schematic diagram of the three-dimensional structure of the Ad2 hexon monomer. Reprinted with permission from Roberts *et al.* [13]. P1 and P2 represent the two basal β-barrel domains. The surface tower domain consists of loops L1, L2, and L4. The yellow regions in loops L1 and L2 represent the variable regions exposed on the surface of the Ad2 hexon that encode type-specific neutralizing antigenic determinants [19].



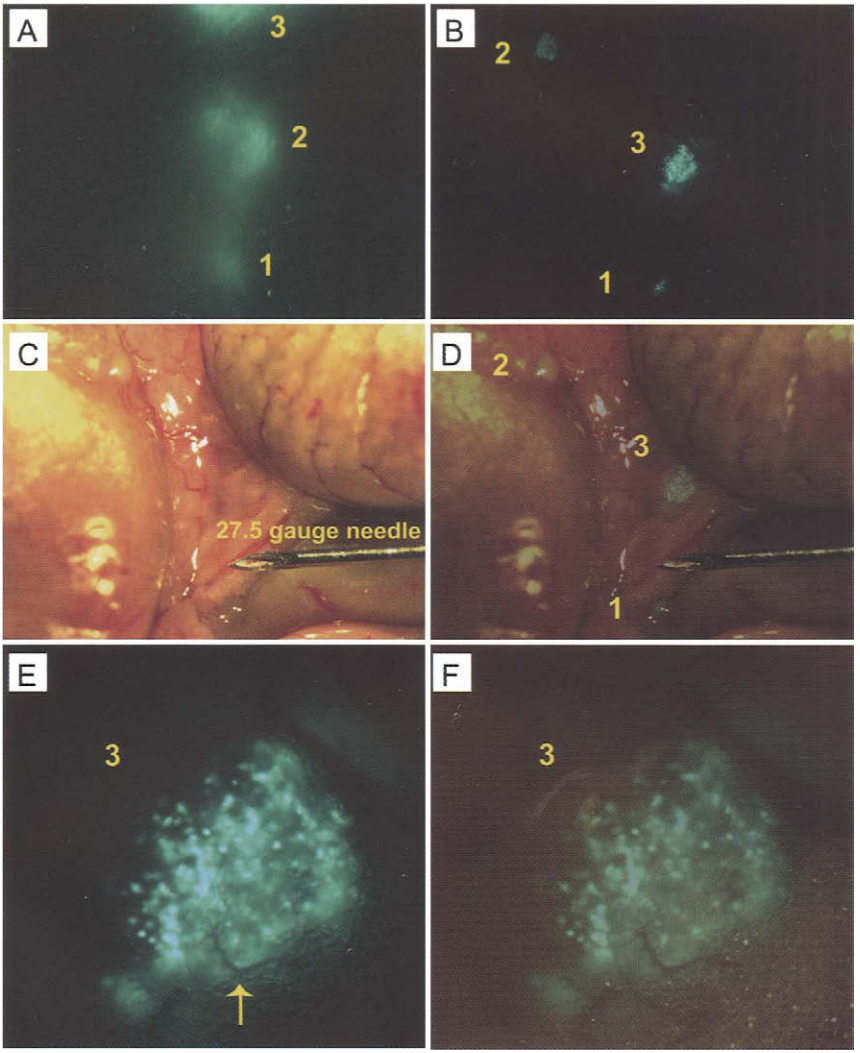
Chapter 13, Figure 1 (B) Schematic diagram of the three-dimensional structure of Ad5 hexon monomer. Reprinted with permission from Rux *et al.* [11]; DE1, FG1, and FG2 represent loop domains with type determinants located in DE1 and FG1. The hexon base contains a small loop domain DE2 in addition to two other domains V1 and V2, which are held apart by the VC or connector domain. NT represents the N terminus loop. Reprinted with permission from Academic Press.



Chapter 14, Figure 3 Decreased inflammatory response and prolonged β-galactosidase expression in the lung after administration of soluble sTNFR1. Lung tissue from vehicle-treated control (CT) C57BL/6-+/+ mice and from mice treated with TNF-bp was examined 3 days and 30 days after intranasal (in) administration of AdCMVLacZ (1×10^{10} pfu). (A) Tissue fixed and stained with hematoxylin and eosin. (original magnification, ×320). (B) Frozen section prepared for analysis of β-Gal staining.



Chapter 22, Figure 5 Light-based imaging of GFP expression following gene transfer with an Ad vector. (A) Bright-field image of a mouse with a subcutaneous tumor on the flank; (B) fluorescent image of the same field for detecting GFP expression; (C) fused image combining A and B. The tumor was injected with an Ad encoding GFP 48 h earlier. The mouse was imaged with a fluorescent stereomicroscope.



Chapter 22, Figure 6 *In vivo* imaging of GFP-positive xenografts. (A) *In vivo* fluorescent image from outside abdominal wall of nude mouse implanted with 1×10^6 GFP-positive SKOV3 cells 4 days earlier. Three tumors were visible, as labeled on the figures. (B) Fluorescence image after removal of abdominal wall and positioning to place all three ovarian tumors in the field; (C) bright field image of the same field shown in 6B; (D) fused fluorescence and bright field images of B and C; (E) higher magnification (×100) of tumor 3; (F) fused fluorescence and bright field image of tumor 3. New blood vessels are visible as indicated by the arrows. Reprinted with permission from Chaudhuri *et al.* [114].