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George F. Vande Woude
George Klein

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Advances in CANCER RESEARCH

Volume 75

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Specificity within the *ets* Family of Transcription Factors

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I. INTRODUCTION

The *ets* genes encode regulatory transcription factors that share a highly conserved, ~85-residue DNA-binding domain, termed the ETS domain. The founding member of this gene family, *ets-1*, was discovered in the early 1980s as part of the tripartite oncogene of an avian retrovirus. In the following decade, many *ets-1* related genes were identified, and *ets* proteins were shown to be sequence-specific DNA-binding proteins that regulate transcription (see Table I). The discovery of *ets* proteins as transcription factors provided a framework for understanding the oncogenic potential of *ets* genes. In addition, *ets* proteins became a model system to study the molecular mechanisms of transcriptional control, including how transcription factors bind DNA, modulate promoter activity, and respond to signaling input. Previous reviews have covered these early discoveries and are recommended for more comprehensive reading (Papas *et al.*, 1989; Karim *et al.*, 1990; Macleod *et al.*, 1992; Janknecht and Nordheim, 1993; Wasylyk *et al.*, 1993; Crepieux *et al.*, 1994; Tenen *et al.*, 1997; Sharrocks *et al.*, 1997).

The existence of the *ets* gene family raises the issue of biological specificity. Although some functional redundancy within the family could exist, mechanisms undoubtedly have evolved that provide unique biological roles for individual *ets* proteins. The expected route to divergence is the evolution of distinct sets of target genes. However, DNA-binding activity is extremely similar among the *ets* proteins. The residues within the ETS domain that contact DNA are highly conserved. The DNA element recognized by all *ets* proteins contains a similar core motif, 5′-GGA(A/T)-3′. Lack of divergence of this crucial step defines the specificity problem. The cellular distribution pattern of *ets* proteins also contributes to this issue. In vertebrates, most *ets* proteins are found in several cell types, and some *ets* genes are even expressed ubiquitously (e.g., *ets-2*, *gabpα*). Other family members show restricted tissue distribution (see Table II). Consistent with these expression patterns, multiple *ets* genes are usually present in any particular cell type. These data also predict that there are both tissue-specific and ubiquitously expressed target genes. Indeed, this is the case. In T lymphocytes, which express at least five *ets* genes, putative target genes include the T cell receptor subunits and the interleukin-2 (IL-2) receptor. Ubiquitously expressed target genes such as

c-*fos* and the cytochrome oxidase genes also are expressed in this cell type. The question clearly emerges: how can an individual *ets* protein regulate a unique set of target genes? A full understanding of the role of *ets* proteins both in normal biological regulation and in oncogenesis requires a resolution of this specificity issue.

In this review, current knowledge of the *ets* proteins will be discussed within the framework of this critical issue. The review has two major parts. In the first part, we set the stage by illustrating the specificity problem. The high degree of sequence conservation among *ets* family members is presented in the form of a phylogenetic tree. Next, we document that individual *ets* proteins have unique biological properties by summarizing the recent reports of *ets* gene disruptions in the mouse and human as well as the genetic analysis of *ets* genes in the model organisms *Drosophila* and *Caenorhabditis elegans*. In the second part of this review, we present possible solutions to the specificity problem. We initially focus on how the highly conserved ETS domain binds DNA. Then, we discuss the functional domains involved in protein–protein interactions and transcriptional activation or repression. The divergence of these regions provides for gene-specific regulation. Next, the distinctive responses of *ets* proteins to signal transduction pathways are discussed. Finally, we illustrate how autoinhibitory sequences also contribute to specificity. There are many levels of control that can influence specificity. From the extensive characterization of a few family members, the picture emerges that each *ets* protein is regulated by a combination of mechanisms. This view of *ets* proteins suggests how transcriptional regulation can go awry and contribute to oncogenesis. We hope that this review can lead to a greater appreciation of the importance and complexity of the specificity issue and guide future investigations directed toward this problem.

II. SEQUENCE CONSERVATION

The *ets* gene family is noted for its wide distribution among metazoans. Multiple *ets* genes are present in all phyla that have been tested, including chordates and arthropods, as well as lower invertebrates represented by sponges, ctenophores, and flatworms. The *ets* genes in vertebrate genomes are best characterized. For example, there are currently 21 known homologs in the human genome. Family members also have been studied in invertebrates, including the fruit fly, *Drosophila melanogaster,* several sea urchin species, and the nematode, *C. elegans*. The *ets* gene family has not been found in plants, fungi, or any protozoan. No *ets* genes are found in the entire genome of the budding yeast *Saccharomyces cerevisiae*. Phylogenetic analysis of the *ets* gene family (Lautenberger *et al.,* 1992; Laudet *et al.,* 1993), as well as the identification of multiple *ets* genes in lower invertebrates

Table I ETS Genes in the Phylogenetic Tree[a]

Group[b]	Name in tree[c]	Other protein names[d]	Gene name	Organism	Accession number[f]
ELF	ELF-1	Elf-1*	*elf-1*	Human	P32519
	ELF-1	Elf-1*	*elf-1*	Mouse	U19617
	NERF	NERF-1,-2*,[e]	*nerf*	Human	U43189
	E74	E74A, B*,[e]	*E74*	*Drosophila*	P11536
ELG	GABPα*	NRF-2α	*gabpα*	Mouse	Q00422
	E4TF-1*	—	*e4tf1*	Human	Q06546
	ELG*	—	*elg*	*Drosophila*	Q04688
ELK	SAP-1	SAP-1a, b*,[e]	*sap-1*	Human	B42093
	SAP-1	SAP-1a, b*,[e]	*sap-1*	Mouse	P41158
	NET	Net,* SAP-2	*net*	Human	P41970
	NET	Net,* ERP	*erp*	Mouse	A56019
	ELK-1	Elk-1*	*elk-1*	Human	P19419
	ELK-1	Elk-1*	*elk-1*	Mouse	Z36939
	LIN-1	Lin-1*	*lin-1*	*Caenorhabditis elegans*	U38937
ERF	ERF*	—	*erf*	Human	U15655
	ERF*	—	*erf*	Mouse	U58533
	PE-1*	ETV3, PEP-1	*etv3*	Human	P41162
ERG	FLI-1	Fli-1,* ERG-B	*fli-1*	Human	A49015
	FLI-1	Fli-1*	*fli-1*	Mouse	P26323
	FLI-1	Fli-1*	*fli-1*	*Xenopus laevis*	P41157
	ERG*	—	*erg*	Human	P11308
	ERG	—	*erg*	Chicken	X77159
	ERG	—	*erg*	Mouse	A54617
	ERG	—	*erg*	Sea urchin	Q01414
	ETS-6	Ets6*	*ets-6*	*Drosophila*	P29776
ETS	ETS-1	Ets-1*	*ets-1*	Mouse	P27577
	ETS-1	Ets-1*	*ets-1*	Human	P14921
	ETS-1	Ets-1*	*ets-1*	Chicken	P15062
	ETS-1a	Ets-1*	*ets-1*	*Xenopus laevis*	P18755
	ETS-1b	Ets-1*	*ets-1*	*Xenopus laevis*	P18756
	ETS-1	Ets-1*	*ets-1*	Rat	P41156
	ETS-2	Ets-2*	*ets-2*	Mouse	P15037
	ETS-2	Ets-2*	*ets-2*	Human	P15036
	ETS-2	Ets-2*	*ets-2*	Chicken	P10157
	ETS-2a	Ets-2*	*ets-2*	*Xenopus laevis*	B53236
	ETS-2b	Ets-2*	*ets-2*	*Xenopus laevis*	A53236
	ETS-2	Ets-2*	*ets-2*	Sea urchin	L19541
	PNT	Pnt P1, P2*,[e]	*pointed, pnt*	*Drosophila*	S33167

(*continues*)

Table I (*continued*)

Group[b]	Name in tree[c]	Other protein names[d]	Gene name	Organism	Accession number[f]
PEA3	ER81*	ETV1	*etv1*	Human	X87175
	ER81	—	—	Mouse	P41164
	ERM*	ETV5	*etv5*	Human	P41161
	PEA3*	—	*pea3*	Mouse	P28322
	E1A-F*	ETV4	*etv4*	Human	S35534
SPI	PU.1*	SPI-1	*spi-1*	Human	P17947
	PU.1	SPI-1	*spi-1*	Mouse	A34693
	SPI-B*	SPI-2	*spi-B*	Human	Q01892
YAN	YAN	Yan*	*yan, pok, Aop*	*Drosophila*	Q01842
	TEL*	ETV6	*tel, etv6*	Human	P41212
No group	ER71*	ETV2	*etv2*	Mouse	P41163
No group	ETS-4	Ets-4*	*ets-4*	*Drosophila*	P29775

[a] The *ets* genes included in the phylogenetic tree were identified by a BLASTP search of public databases (performed in November, 1996) using the Ets-1 ETS domain as a probe. All of the genes have been reported in the literature. There is a sequence for the complete ETS domain of each included gene. More recently reported *ets* family members include MEF (Miyazaki *et al.*, 1996), ESX/ESE (Chang *et al.*, 1997; Oettgen *et al.*, 1997), and FEV (Peter *et al.*, 1997).

[b] Groups of closely related *ets* genes are listed alphabetically. Groupings are based on the phylogenetic tree (Fig. 1).

[c] Gene/protein names are given in uppercase letters in this column to match the format nomenclature in the phylogenetic tree. Asterisks denote protein names used in text.

[d] Other protein names (if different than names in the tree). Asterisks denote protein names used in text. The format most frequently used in the literature (e.g., Ets-1, Elk-1, GABP, and PEA3) was chosen. The ETV designations are recommended by the Human Genome Database Nomenclature Committee.

[e] Locus directs the synthesis of two functionally distinct isoforms of the *ets* protein, either by alternative splicing or promotor utilization.

[f] Accession numbers in public databases. In cases of multiple sequence submissions the most complete report was selected, otherwise one sequence was arbitrarily selected. Citations for the report of these genes accompany the sequence in the public databases.

(Degnan *et al.*, 1993), indicate that the *ets* genes in contemporary species are derived from the duplication of an ancestral gene early in metazoan evolution (Shenk and Steele, 1993). The amplification of such families of transcription factors is viewed as a critical step in the evolution of multicellular animals, including higher vertebrates (Degnan *et al.*, 1993; Shenk and Steele, 1993).

A. Phylogenetic Tree

To review the known *ets* genes in the context of their evolutionary relatedness, we have constructed a phylogenetic tree. The amino acid sequences

of the ETS domains from 49 *ets* genes were aligned, then the tree was constructed by distance methodology (Fig. 1). The alignment is simple and robust because sequence conservation within the ETS domain is quite high, with the most divergent members displaying at least 40% identity to the *ets-1* sequence. The length of the horizontal lines (branches) connecting two genes indicates relative similarity of their ETS domain sequences. Genes are clustered into groups of highly related homologs as indicated by short branch lengths. There are at least nine groups with multiple members, and these have been named arbitrarily for convenience (ELF, ELG, ELK, ERF, ERG, ETS, PEA3, SPI, and YAN; see Table I and Fig. 1). Two genes, *etv2* and *ets-4* (ER71 and ETS-4), cannot be assigned to any of these groups. Groups ETS, ERG, ELG, ELK, ELF, and YAN comprise both invertebrate and vertebrate genes, indicating that the family expanded by gene duplication prior to the evolution of vertebrates.

Homologous genes, which derive from a single ancestral gene, are classified as either orthologs or paralogs, depending on their derivation. Orthologs are derived during speciation. Paralogs coexist in one genome and are derived from gene duplications. Both orthologs and paralogs are present in the *ets* phylogenetic tree because more than one species is represented and multiple genes have been identified in each species.

Orthologs can be tentatively identified by sequence similarity; a gene is often more similar to its ortholog in a related species than it is to its paralogs within the same genome. Thus, orthologs are expected to be in close proximity on a phylogenetic tree. Numerous apparent orthologs between mouse and human *ets* genes have been identified. Pairwise sequence comparisons of 14 mouse and human genes show that these genes display between 82 and 97% identity over the entire protein sequence (see Fig. 2), with almost complete identity in the ETS domain (Fig. 1). This level of similarity is consistent with the recent divergence of the human and mouse lineages (Knoll, 1992).

Orthologous relationships between distantly related species are more difficult to establish due to the relatively low sequence similarity. For example, *Drosophila pnt, yan, ets-6,* and *E74* as well as *C. elegans lin-1* are found within groups that also contain vertebrate genes; however, it is not possible to confirm that these are orthologous to any of the vertebrate genes in these groups. As indicated by longer branch lengths, these *Drosophila* and *C. elegans* genes are the most dissimilar members of each group. This is even more dramatically indicated by comparisons of sequences not present in the ETS domain. For example, in the ELF group, *Drosophila* E74 and human Elf-1, which are 85% identical in the ETS domain, show no sequence similarity outside of this domain. Likewise, *Drosophila* Pnt is only 43% identical to human Ets-1, even though the sequence identity in the two ETS domains is 95%. *Caenorhabditis elegans* Lin-1 is 70% identical to human Elk-1 in the ETS domain; however, there is only 32% identity over the entire protein se-

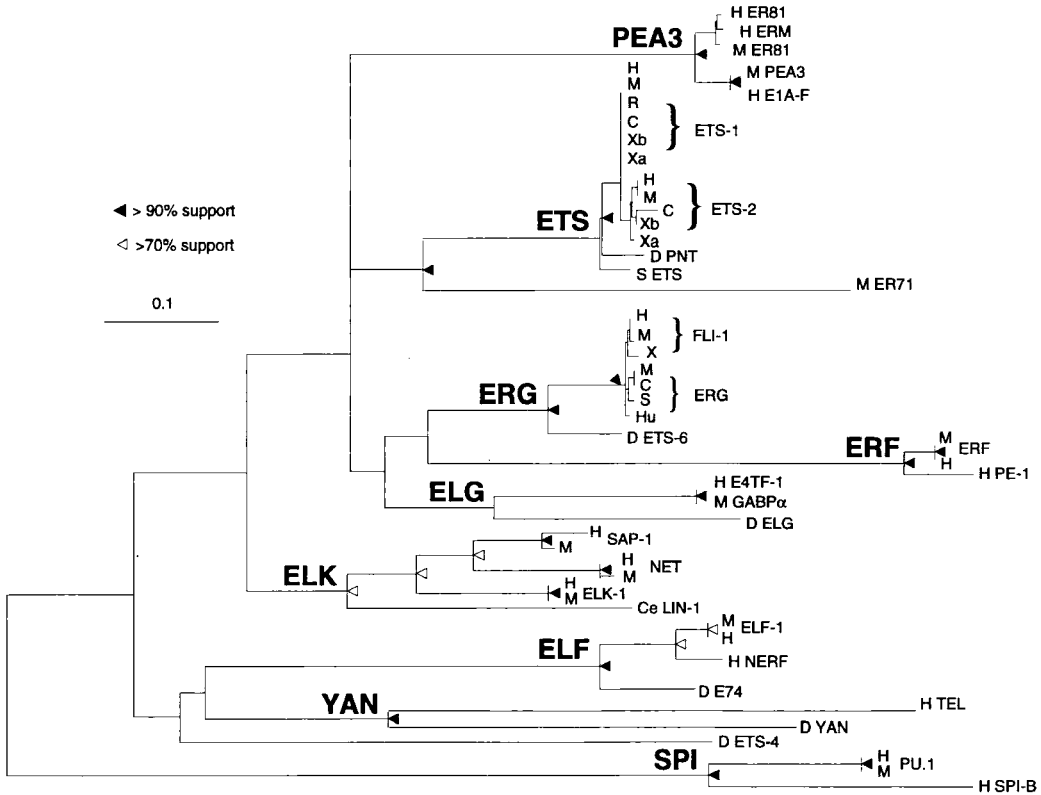
quence. There are two possible explanations for these differences in the regions outside of the ETS domain. These regions could have been allowed to diverge or were selected to diverge after a speciation event. Alternatively, new sequences could have entered into the family by gene rearrangements involving modules from non-*ets* genes.

All *ets* genes within a single genome are paralogs. For example, the *ets* family tree (Fig. 1) contains 18 human, 14 murine, and 6 *Drosophila* paralogs. None of these genomes has been completely sequenced, thus these numbers are likely to be underestimates. (Indeed, new family members have been reported since the construction of this tree; see Table I notes.) Highly related paralogs are found within groups based on the similarity of the ETS domain. The similarity between paralogous genes extends beyond the ETS domain but is less substantial than the similarity between orthologs. For example, human paralogs Ets-1 and Ets-2 are only 54% identical over the entire protein sequence (not shown). It has been proposed that *ets-1* and *ets-2* arose from a gene duplication in an ancestral vertebrate. The report of only one known *Drosophila* gene in the ETS group suggests that the gene duplication to generate the *ets-1* and *ets-2* paralogs may have occurred during vertebrate evolution (Laudet *et al.*, 1993; Albagli *et al.*, 1994). Another set of human paralogs, *etv1* and *etv5* within the PEA3 group, are 66% identical over the entire protein sequence (not shown). The *elk-1*, *sap-1*, and *net* paralogs within the ELK group show less similarity; nevertheless, sequence conservation can be identified over the entire length of the protein sequence with 55 invariant residues outside of the highly conserved ETS domain (not shown). These conserved sequences, which lie outside of the ETS domain, are found only in genes of the ELK group. The simplest explanation for sequence similarities outside of the ETS domain among paralogous genes is duplication followed by retention of sequences by selection.

B. ETS Domain

There is strong amino acid conservation over a region of 84 to 90 residues referred to as the ETS domain (Fig. 2). Conserved sequences extend beyond this region within subsets of *ets* genes. It is also remarkable that the ETS domain is never located at the extreme C terminus of an *ets* protein. Nevertheless, the ETS domain (shown in Fig. 2) can be produced as a stable protein fragment and is sufficient for DNA binding. Thus, it is both a structural and a functional domain.

Consensus residues correspond to positions important both for the maintenance of the folded protein structure and for contact with DNA. Highly conserved amino acids are found within secondary structure elements (α helices and β strands) of the ETS domain as determined for Fli-1, Ets-1, and


PEA3
H ER81
H ERM
M ER81
M PEA3
H E1A-F
H
M
R
C
Xb
Xa
ETS-1
ETS
H
M
C
Xb
Xa
ETS-2
D PNT
S ETS
M ER71
> 90% support
>70% support
0.1
H
M
X
FLI-1
ERG
M
C
S
Hu
ERG
D ETS-6
ERF
M
H
ERF
H PE-1
ELG
H E4TF-1
M GABPα
D ELG
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Ce LIN-1
M
H
ELF-1
ELF
H NERF
D E74
YAN
H TEL
D YAN
D ETS-4
SPI
H
M
PU.1
H SPI-B

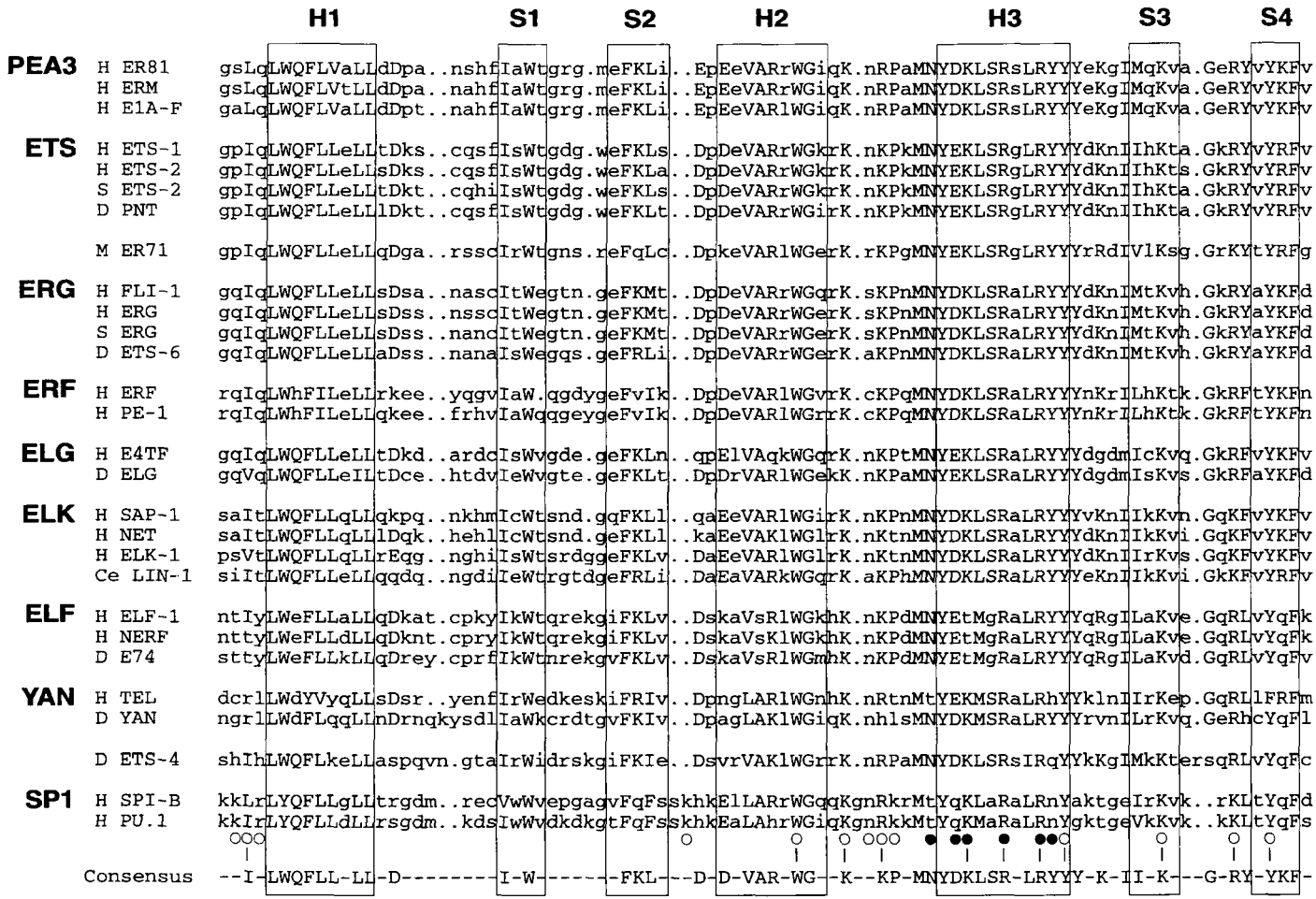
PU.1 (Fig. 2) (Donaldson *et al.*, 1994; Liang *et al.*, 1994b; Werner *et al.*, 1995; Donaldson *et al.*, 1996; Kodandapani *et al.*, 1996). Many of the residues that contact DNA in the PU.1–DNA complex (Kodandapani *et al.*, 1996; Pio *et al.*, 1996) are also highly conserved. Additional discussion of the functional implications of this conservation is presented in Section IV.

C. PNT Domain

A second highly conserved domain is found in a significant fraction of the known *ets* genes. Of the 49 genes in the phylogenetic tree, this domain is present in 21 of the 41 vertebrate *ets* genes plus one sea urchin and three *Drosophila* genes. Figure 3 shows the degree of conservation of this domain. The alignment extends in the amino-terminal direction to include a MAP kinase phosphorylation site that is present in Ets-1, Ets-2, and Pnt P2. However, the exact boundaries of this domain await additional structural studies and a better understanding of its function.

The name and function of this conserved domain are controversial. This region was first recognized on the initial discovery of the *ets* gene family, at which time the region was referred to as the "A" domain (Papas *et al.*, 1989). Description of the *Drosophila ets* family member, *pointed* (*pnt*), provided a

Fig. 1 Rooted phylogenetic tree relating ETS domain sequences. All genes listed in Table I were used to calculate this tree. Amino acid sequences of the ETS domain were aligned using PILEUP (Genetic Computer Group, University of Wisconsin) followed by minor manual adjustments in the regions of small gaps. Figure 2 shows a representative subset of genes in the alignment. The topology shown is the 50% majority-rule consensus of 1000 phylogenies generated by bootstrapping (Felsenstein, 1993); clades that appear in fewer than half the replicates are shown if they do not conflict with more frequent clades (Phylip: Consense). For each replicate, the neighbor-joining method [Phylip: Neighbor (Saitou and Nei, 1987)] was used to estimate a phylogeny from a pairwise distance matrix, which was then calculated from a sequence alignment created by resampling from the original alignment (Phylip: Seqboot). Matrices of pairwise divergence were generated by the Dayhoff PAM matrix [Phylip: Protdist (Dayhoff, 1978)]. Residues overlapping gaps were discarded in individual pairwise comparisons, but not from the entire analysis. Branch lengths on the tree shown were estimated using the least-squares method of Fitch and Margoliash [Phylip: Fitch (Fitch, 1981)] to fit pairwise distances (determined as above from the original alignment) to the consensus topology; the distances fit with an average percent standard deviation of 8.1%. The tree was then midpoint rooted (Phylip: Retree). The bar represents a branch length of 0.1 evolutionary distance units (the estimated number of changes per amino acid). The distance between two genes is indicated only by the horizontal length of branches. Nodes marked with closed and open triangles are present in >90% and >70% of bootstrap replicates, respectively. Nine groups (names in bold) were defined by highly resolved clades displaying significant nodes and were named arbitrarily. Abbreviations: H, human; M, mouse; R, rat; C, chicken; Xa, Xb, *Xenopus laevis* (due to genome ploidy this organism has duplicated copies of most genes); S, sea urchin; D, *Drosophila;* Ce, *Caenorhabditis elegans.*


H1
S1
S2
H2
H3
S3
S4
PEA3
H ER81 gsLqLWQFLVaLLdDpa..nshfIaWtgrg.meFKLi..EpEeVARrWGiqK.nRPaMNYDKLSRsLRYYYeKgIMqKva.GeRYvYKFv
H ERM gsLqLWQFLVtLLdDpa..nahfIaWtgrg.meFKLi..EpEeVARrWGiqK.nRPaMNYDKLSRsLRYYYeKgIMqKva.GeRYvYKFv
H E1A-F gaLqLWQFLVaLLdDpt..nahfIaWtgrg.meFKLi..EpEeVARlWGiqK.nRPaMNYDKLSRsLRYYYeKgIMqKva.GeRYvYKFv
ETS
H ETS-1 gpIqLWQFLLeLLtDks..cqsfIsWtgdg.weFKLs..DpDeVARrWGkrK.nKPkMNYEKLSRgLRYYYdKnIIhKta.GkRYvYRFv
H ETS-2 gpIqLWQFLLeLLsDks..cqsfIsWtgdg.weFKLa..DpDeVARrWGkrK.nKPkMNYEKLSRgLRYYYdKnIIhKts.GkRYvYRFv
S ETS-2 gpIqLWQFLLeLLtDkt..cqhiIsWtgdg.weFKLs..DpDeVARrWGkrK.nKPkMNYEKLSRgLRYYYdKnIIhKta.GkRYvYRFv
D PNT gpIqLWQFLLeLLlDkt..cqsfIsWtgdg.weFKLt..DpDeVARrWGirK.nKPkMNYEKLSRgLRYYYdKnIIhKta.GkRYvYRFv
M ER71 gpIqLWQFLLeLLqDga..rsscIrWtgns.reFqLc..DpkeVARlWGerK.rKPgMNYEKLSRgLRYYYrRdIVlKsg.GrKYtYRFg
ERG
H FLI-1 gqIqLWQFLLeLLsDsa..nascItWegtn.geFKMt..DpDeVARrWGqrK.sKPnMNYDKLSRaLRYYYdKnIMtKvh.GkRYaYKFd
H ERG gqIqLWQFLLeLLsDss..nsscItWegtn.geFKMt..DpDeVARrWGerK.sKPnMNYDKLSRaLRYYYdKnIMtKvh.GkRYaYKFd
S ERG gqIqLWQFLLeLLsDss..nancItWegtn.geFKMt..DpDeVARrWGerK.sKPnMNYDKLSRaLRYYYdKnIMtKvh.GkRYaYKFd
D ETS-6 gqIqLWQFLLeLLaDss..nanaIsWegqs.geFRLi..DpDeVARrWGerK.aKPnMNYDKLSRaLRYYYdKnIMtKvh.GkRYaYKFd
ERF
H ERF rqIqLWhFILeLLrkee..yqgvIaW.qgdygeFvIk..DpDeVARlWGvrK.cKPqMNYDKLSRaLRYYYnKrILhKtk.GkRFtYKFn
H PE-1 rqIqLWhFILeLLqkee..frhvIaWqqgeygeFvIk..DpDeVARlWGrrK.cKPqMNYDKLSRaLRYYYnKrILhKtk.GkRFtYKFn
ELG
H E4TF gqIqLWQFLLeLLtDkd..ardcIsWvgde.geFKLn..qpElVAqkWGqrK.nKPtMNYEKLSRaLRYYYdgdmIcKvq.GkRFvYKFv
D ELG gqVqLWQFLLeILtDce..htdvIeWvgte.geFKLt..DpDrVARlWGekK.nKPaMNYEKLSRaLRYYYdgdmIsKvs.GkRFaYKFd
ELK
H SAP-1 saItLWQFLLqLLqkpq..nkhmIcWtsnd.gqFKLl..qaEeVARlWGirK.nKPnMNYDKLSRaLRYYYvKnIIkKvn.GqKFvYKFv
H NET saItLWQFLLqLLlDqk..hehlIcWtsnd.geFKLl..kaEeVAKlWGlrK.nKtnMNYDKLSRaLRYYYdKnIIkKvi.GqKFvYKFv
H ELK-1 psVtLWQFLLqLLrEqg..nghiIsWtsrdggeFKLv..DaEeVARlWGlrK.nKtnMNYDKLSRaLRYYYdKnIIrKvs.GqKFvYKFv
Ce LIN-1 siItLWQFLLeLLqqdq..ngdiIeWtrgtdgeFRLi..DaEaVARkWGqrK.aKPhMNYDKLSRaLRYYYeKnIIkKvi.GkKFvYRFv
ELF
H ELF-1 ntIyLWeFLLaLLqDkat.cpkyIkWtqrekgiFKLv..DskaVsRlWGkhK.nKPdMNYEtMgRaLRYYYqRgILaKve.GqRLvYqFk
H NERF nttyLWeFLLdLLqDknt.cpryIkWtqrekgiFKLv..DskaVsKlWGkhK.nKPdMNYEtMgRaLRYYYqRgILaKve.GqRLvYqFk
D E74 sttyLWeFLLkLLqDrey.cprfIkWtnrekgvFKLv..DskaVsRlWGmhK.nKPdMNYEtMgRaLRYYYqRgILaKvd.GqRLvYqFv
YAN
H TEL dcrlLWdYVyqLLsDsr..yenfIrWedkeskiFRIv..DpngLARlWGnhK.nRtnMtYEKMSRaLRhYYklnIIrKep.GqRLlFRFm
D YAN ngrlLWdFLqqLLnDrnqkysdlIaWkcrdtgvFKIv..DpagLAKlWGiqK.nhlsMNYDKMSRaLRYYYrvnILrKvq.GeRhcYqFl
D ETS-4 shIhLWQFLkeLLaspqvn.gtaIrWidrskgiFKIe..DsvrVAKlWGrrK.nRPaMNYDKLSRsIRqYYkKgIMkKtersqRLvYqFc
SP1
H SPI-B kkLrLYQFLLgLLtrgdm..recVwWvepgagvFqFsskhkElLARrWGqqKgnRkrMtYqKLaRaLRnYaktgeIrKvk..rKLtYqFd
H PU.1 kkIrLYQFLLdLLrsgdm..kdsIwWvdkdkgtFqFsskhkEaLAhrWGiqKgnRkkMtYqKMaRaLRnYgktgeVkKvk..kKLtYqFs
Consensus --I-LWQFLL-LL-D--------I-W-------FKL---D-D-VAR-WG--K--KP-MNYDKLSR-LRYYY-K-II-K---G-RY-YKF-

more extensive lineup of *ets* genes and led to the term "POINTED domain" (Klambt, 1993). We have adopted this terminology and used the abbreviation PNT for brevity. This domain is proposed to function in protein–protein interactions. Several reports indicate that the PNT domain of TEL serves as a self-association domain (Carroll *et al.*, 1996; Golub *et al.*, 1996; McLean *et al.*, 1996; Jousset *et al.*, 1997). However, no biophysical data are yet available that characterize the oligomerization interface. Other family members subjected to assays for homotypic self-association (including ETS-1, Fli-1 and GABPα) do not display this property (Jousset *et al.*, 1997). For *ets* proteins other than TEL, heterotypic complexes involving two different *ets* proteins might form, such as those seen in the bZIP and bHLH classes of transcription factors. Alternatively, the PNT domain of some *ets* proteins could serve a different function, interacting with non-*ets* proteins. Possible associating proteins would include other DNA-binding transcription factors, coactivators, corepressors, or components of the basal transcriptional machinery. Some of these alternatives will be discussed in later sections of this review.

Additional support for the protein–protein interaction model comes from the recent alignment of the highly conserved core of the PNT domain with a region of conserved sequences within at least a dozen non-*ets* proteins (Bornemann *et al.*, 1996; Alkema *et al.*, 1997; Gunster *et al.*, 1997). The domain is termed SPM, SAM, or SEP in this context. The best-characterized proteins in this alignment, the *Polycomb* group proteins, assemble into multiprotein complexes to regulate gene expression without directly binding

Fig. 2 Sequence conservation within the ETS domain. Sequence alignment and group assignments were performed as described in Fig. 1. To derive a consensus for the ETS domain, all genes listed in Table I were included except the presumed vertebrate orthologs of the 14 human *ets* genes. These presumed mouse and human orthologs are similar at the amino acid level over the entire length of the gene: mouse Ets-1 is 97% identical to human Ets-1; PEA3 is 92% identical to E1A-F; GABP is 96% identical to E4TF-1; likewise, Ets-2, 92%; Net, 91%; ER81, 97%; Fli-1, 96%; ERG, 98%; ERF, 96%; Elk-1, 90%; SAP-1, 82%; Elf-1, 90%; and PU.1, 88%. (ERG and Net alignments were restricted to approximately half of the protein sequence because only fragments of the murine genes have been sequenced.) The use of only 28 ETS domains prevented a bias due to the highly similar vertebrate orthologs. The consensus on the bottom line was derived as a 21 of 28 plurality (PRETTY: Genetic Computer Group, University of Wisconsin). Consensus positions or conservative substitutions, as allowed by a Dayhoff PAM matrix (e.g., R = K; L = F, M, I; W = F, Y) are indicated by uppercase letters. Lowercase letters designate positions that do not match the consensus. Secondary structure elements (α helices, H1–H3, and β strands, S1–S4), as determined for Ets-1 (Donaldson *et al.*, 1994; Skalicky *et al.*, 1996), are enclosed by rectangular boxes. Identical placement of structural elements is reported for Fli-1 (Liang *et al.*, 1994a,b), and PU.1 (Kodandapani *et al.*, 1996; Pio *et al.*, 1996) except for variability in C termini of helices H2 and H3. Residues that contact DNA in the PU.1–DNA complex (Kodandapani *et al.*, 1996) are indicated (●, bp contacts; ○, phosphate contacts). Vertical lines indicate contact residues in PU.1 that match the ETS domain consensus defined here.

DNA. Preliminary studies indicate that the SPM domain plays a role in these protein–protein interactions in both *Drosophila* and vertebrates.

The evolutionary origin of the PNT domain is not clear. The conservation is noted exclusively within the ETS, ELG, ERG, and YAN groups. All genes within these groups, for which there is sequence information for the entire open reading frame, encode a PNT domain. It is not possible to determine whether the PNT domain was introduced early into one member of the family after the initial expansion of the family or whether it was present in the ancestral *ets* gene but lost at some later time. The former hypothesis predicts that all *ets* genes with a PNT domain should lie in a single clade (tree branches distal to a single node). This arrangement is not seen in the tree topology (Fig. 1). However, given the resolution of this tree, it is not in conflict with this hypothesis. Other clues to the origin of the PNT domain come from a comparison of the overall organization of conserved sequences. In all genes that encode both conserved domains, the sequences for the ETS domain are located 3′ to sequences for the PNT domain (Fig. 4). In the genes of the ELK group, the ETS domain is located in the extreme 5′ end of the open reading frame, and the genes do not have a PNT domain. This arrangement suggests that the PNT domain could have been lost in the founder of this group due to a chromosome rearrangement. The route to loss of the PNT domain in other groups is not clear.

The secondary structure of the Ets-1 PNT domain has been determined by nuclear magnetic resonance (NMR) spectroscopy (C. Slupsky, L. Gentile, L. Donaldson, B. Graves, and L. McIntosh, unpublished observations). Four α helices (H2–H5) are found within the most highly conserved region of the domain. The alignment of these structural elements to the PNT domain consensus indicates that conserved positions lie both within helices and between helical regions (Fig. 3). The amino-terminal region of Ets-1 that extends toward the consensus site for MAP kinase contains an α helix. The existence of this fifth helix in the other *ets* proteins cannot be predicted confidently due to the lower level of sequence conservation in this region. Early structural predictions and the self-association data for TEL led to the use of the name "HLH domain" for the PNT domain (Siddique *et al.*, 1993; Golub *et al.*, 1994). There was an expectation that the domain would resemble, both structurally and functionally, the helix–loop–helix dimerization domain of the bHLH class of transcription factors that is composed of two helices and a long loop. The finding of five helices and no extended loop indicates that this proposal is incorrect.

D. Conclusions

The evolutionary relatedness of the *ets* genes provides insight into function. Conserved regions indicate sequences and structural domains likely to

be important in the biological function of these proteins. The ETS domain identifies all *ets* proteins as sequence-specific DNA-binding proteins. The role of DNA-binding proteins in transcription is well-established; thus, each *ets* gene is expected to play a role in regulation of gene expression. All paralogs retained within a genome, even those retaining significant sequence similarity, are not expected to be redundant (Brookfield, 1992; Thomas, 1993). Thus, there is a prediction that each *ets* gene will have a unique biological role. To utilize fully the potential of this gene family, the high level of conservation of the ETS domain must be counterbalanced by regulatory pathways that can provide specificity of action of *ets* genes. The conserved regions outside of the ETS domain provide clues to these pathways. We have discussed only the PNT domain because it is prominent within the family. Discussion in later sections will highlight other regions of sequence conservation and divergence.

III. BIOLOGICAL SPECIFICITY

Different *ets* proteins are expected to regulate the expression of distinct target genes, thus generating biological specificity. Due to the difficulty in experimentally demonstrating this target gene selection, there are only tentative lists that link putative targets to *ets* regulators. Therefore, we will illustrate the biological specificity of the *ets* proteins by reviewing the genetic analysis of *ets* genes in both vertebrate and invertebrate systems. Organisms and cells with mutations in an *ets* locus display diverse phenotypes, strongly suggesting that individual *ets* proteins direct distinct biological processes, despite their overlapping cellular distribution and similar DNA-binding properties.

A. Aberrant Gene Expression in the Mouse

Genetic alterations that change the expression of a gene can provide information about its normal function. The expression of several *ets* genes is altered during retroviral infections of mice with nonacutely transforming viruses. More recently, transgenic mouse technology has allowed such aberrant expression to be engineered for additional *ets* genes. The observation of distinct phenotypes is consistent with the proposed biological specificity of *ets* family members.

Activation via retroviral insertion demonstrates that specific diseases are associated with aberrant expression of different *ets* genes (Table II). The *fli-1* and *spi-1* (PU.1) loci are targets for provirus integration of Friend murine leukemia virus and Friend spleen focus-forming virus, respectively. Activa-

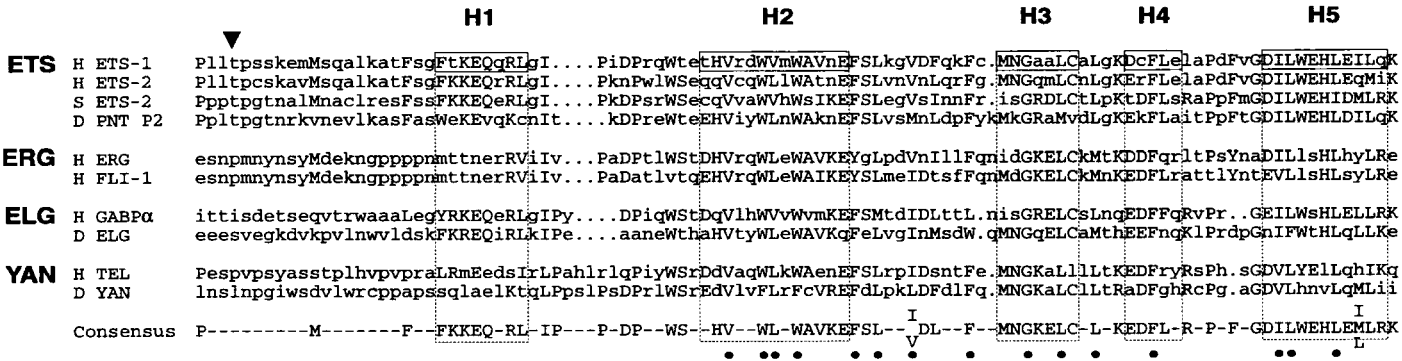


Fig. 3 Sequence conservation within the PNT domain. Amino acid sequences were aligned and gene groups were assigned as described in Figs. 1 and 2. Presumed vertebrate orthologs for six human *ets* genes were not included. The consensus sequence on the bottom line represents the conserved amino acids as determined by a 5 of 10 plurality. ●, Invariant positions with a 10 of 10 match to the consensus. Secondary structure elements (α helices, H1–H5) as determined for Ets-1 by NMR spectroscopy (C. Slupsky, L. Gentile, L. Donaldson, B. Graves, and L. McIntosh, unpublished observations) are depicted as solid rectangular boxes within the Ets-1 sequence and dashed rectangular boxes through the remainder of the sequences. Although sequence similarity among these genes strongly suggests that helices H2–H5 are present in all PNT domains, helix H1 may not be a conserved structural element. Note that many positions are invariant within the region that includes H2 through H5, whereas no positions are invariant in H1. In addition, gaps were necessary to align sequences in this region. The arrowhead marks the threonine residue that is modified by MAPK in Ets-1, Ets-2, and Pnt P2.

tion of the *fli-1* locus results in erythroleukemias (Ben-David *et al.*, 1991), whereas activation of the *spi-1* locus causes erythroid tumors (Moreau-Gachelin *et al.*, 1988; Ben-David *et al.*, 1991).

In mouse transgenic experiments, ectopic expression of the genes encoding Ets-2, PU.1, and Fli-1 leads to distinctive phenotypes (Table II). Mice with an *ets-2* transgene develop neurocranial, viscerocranial, and cervical skeletal abnormalities resembling those seen in humans with Down syndrome (Sumarsono *et al.*, 1996). This suggests that misregulation of *ets-2* may cause the skeletal abnormalities associated with Down syndrome. This proposal is supported by the location of *ets-2* on human chromosome 21. Mice with a PU.1 trangene develop erythroleukemia, consistent with the role of PU.1 in the differentiation of lymphoid and myeloid lineages (Moreau-Gachelin *et al.*, 1996). Transgenic mice aberrantly expressing *fli-1* display a high incidence of a progressive immunological renal disease and ultimately die of renal failure (Zhang *et al.*, 1995). Splenic B cells exhibit increased proliferation and prolonged survival in response to mitogens, suggesting that overexpression of *fli-1* perturbs normal B cell function, including programmed cell death.

A concern with overexpression experiments is whether the findings are relevant to the normal role of a particular gene. It is also difficult to make comparisons of experiments performed with different genes because the expression levels and the cellular distribution of the aberrantly produced protein can vary significantly. Regardless of these concerns, the variety of phenotypes associated with the aberrant expression of different *ets* genes in transgenic mice and retroviral-induced disease is consistent with the hypothesis that individual *ets* genes display distinctive biological functions.

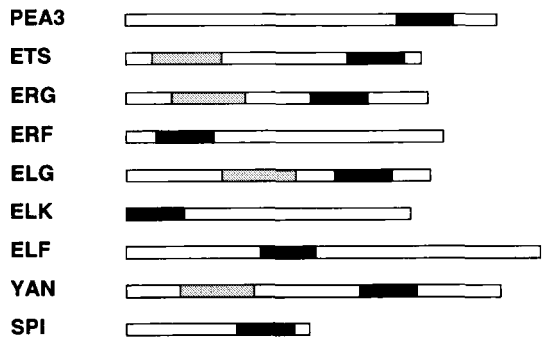


Fig. 4 Placement of the ETS and PNT domains. The representative locations of the ETS domain (black box) and the PNT domain (gray box) within *ets* protein groups defined in Fig. 1. The location of each domain is very similar for all members of a group.

B. Gene Disruptions in the Mouse

The development of gene targeting technology has facilitated manipulations of *ets* genes within the mouse genome. The genes that encode PU.1, Ets-1, Fli-1, PEA3, and TEL have been disrupted, and the phenotypes of the mutant mice are distinct (Table II). These experiments provide strong support for the proposal that the *ets* genes within the mouse genome are not functionally redundant.

The disruption of the gene encoding PU.1 was performed independently by two laboratories. In one case, the PU.1 gene disruption results in embryonic lethality (Scott *et al.*, 1994). Mutant embryos have no normal progenitors for B and T lymphocytes, monocytes, or granulocytes. In further experiments with PU.1-deficient cells, it was shown that PU.1 is required for terminal myeloid differentiation (Olson *et al.*, 1995; D. Tenen, personal communication). In the other disruption, mice are born alive but die of severe septicemia within 48 hr (McKercher *et al.*, 1996). Analyses reveal a lack of mature B and T lymphocytes, macrophages, and neutrophils. Interestingly, when these mice are maintained on antibiotics, normal T cells develop, although the number of cells is low. These findings suggest that development of T cells is normal in spite of the reduced numbers and the delayed onset of differentiation. Taken together, these two studies show that PU.1 is not essential for myeloid and lymphoid lineage cell commitment, but is required for normal differentiation of these lineages.

The *ets-1* gene has been disrupted in the lymphoid lineages by use of a *RAG-2*-deficient mouse (Bories *et al.*, 1995; Muthusamy *et al.*, 1995). Because mature T and B cells cannot develop in the absence of the *RAG-2* gene product, the lymphoid lineage must be derived from the embryonic stem cells that have the *ets-1* gene disruption and an unaltered *RAG-2* gene. This system allows assessment of the role of Ets-1 only in the lymphoid compartment and, thereby, avoids a possible embryonic phenotype. In this system, disruption of the *ets-1* gene leads to abnormal B and T cell development. The T cells are present in reduced numbers and are highly susceptible to apoptosis *in vitro*. The B cells are present in normal numbers; however, an unusually large proportion are IgM plasma cells. Thus, Ets-1 appears to be required for normal B and T cell populations.

Mice with a *fli-1* disruption are homozygous viable, but have a reduced number of total lymphocytes as well as fewer T cells in all subsets (Mélet *et al.*, 1996). In contrast to the *ets-1* gene disruption in T cells, the fli^{-}/fli^{-} T cells are not more susceptible to apoptosis, suggesting that these two *ets* genes play important, but distinct, roles in the development and function of B and T cell lineages.

Mice with a disruption in the gene encoding PEA3 are homozygous viable; however, males are "sterile" (J. Hassell, personal communication). Further

analysis is required to determine whether the phenotype is behavioral or due to a developmental defect in spermatogenesis. Nonetheless, these results demonstrate a specific function for PEA3.

Targeted gene disruption of the *tel* gene leads to embryonic lethality in 5.5-day-old embryos (Wang *et al.*, 1997). Lethality is due to defects in yolk sac angiogenesis. There is also marked apoptosis in cells that normally express *tel*, suggesting that TEL is essential for the survival of mesenchymal cells and neural tissues.

C. Chromosome Translocations in Humans

Disruptions of *ets* genes are found in a variety of human cancers. The genetic lesion, a chromosome translocation, fuses the *ets* gene-coding sequences in frame with the sequences of a second gene. The rearranged locus produces a fusion protein that contains a portion of two proteins. These genetic alterations are different from those engineered by gene targeting or transgenic technology. In each case a single functional domain of the *ets* protein is retained and plays an active part in generating the observed phenotype.

Chromosomal translocations involving the *ets* genes *fli-1*, *erg*, and *etv1* (Table II) retain the ETS domain. The t(11;22) translocation, present in 85% of Ewing's sarcomas, fuses the gene *ews* to *fli-1* (Delattre *et al.*, 1992; May *et al.*, 1993). The DNA-binding activity of the ETS domain is a critical function of the fusion protein. Two other translocations also are linked to Ewing's sarcoma: t(21;22), which fuses *ews* to *erg* (Sorensen *et al.*, 1994), and t(7;22), which fuses *ews* to *etv1* (Jeon *et al.*, 1995). The apparent interchangability of the ETS domains from three different *ets* proteins within the EWS fusion proteins highlights the redundancy of the ETS domain if allowed to function in isolation. Another translocation, t(16;21), links the ETS domain of *erg* to *fus*, a gene that displays sequence similarity to *ews*. This chromosomal abnormality causes a different disease, acute myeloid leukemia, suggesting that the non-*ets* portion of these fusion proteins can influence the disease specificity (Ichikawa *et al.*, 1994; Panagopoulos *et al.*, 1994). In all cases, these translocations are predicted to cause a loss of specificity of the ETS domain, stressing the importance of regulatory pathways that modulate DNA-binding activity.

Several translocations involving the *tel* gene retain the PNT domain. A fusion protein composed of TEL and the PDGFβ receptor is produced by the translocation t(5;12). This chromosome abnormality is associated with chronic myelomonocytic leukemias (Golub *et al.*, 1994). The *tel* gene also is altered in the t(12;21) translocation that fuses the *tel* and *aml1* genes and mediates a form of acute lymphoblastic leukemia (Golub *et al.*, 1995). In

Table II Genetic Analyses of ETS Genes

Family member	Normal cellular distribution[a]	Genetic alteration	Phenotype	Ref.[c]
Fli-1	Thymus, ovary, bone marrow, spleen, heart (Ben-David *et al.*, 1991; Watson *et al.*, 1992; Klemsz *et al.*, 1993); developmentally regulated[b]	Friend murine leukemia virus insertion	Erythroleukemia (mouse)	Ben-David *et al.* (1991)
PU.1	Spleen, testis (Klemsz *et al.*, 1990; Galson *et al.*, 1993; Su *et al.*, 1996)	Friend spleen focus-forming virus insertion	Erythroleukemia (mouse)	Moreau-Gachelin *et al.* (1988)
PU.1	Spleen, testis (Klemsz *et al.*, 1990; Galson *et al.*, 1993; Su *et al.*, 1996)	Transgenic overexpression	Viable; erythroleukemia	Moreau-Gachelin *et al.* (1996)
Fli-1	Thymus, ovary, bone marrow, spleen, heart (Ben-David *et al.*, 1991; Watson *et al.*, 1992; Klemsz *et al.*, 1993); developmentally regulated[b]	Transgenic overexpression	Lethal; progressive immunological renal failure	Zhang *et al.* (1995)
Ets-2	Ubiquitous expression in all organs (Kola *et al.*, 1993; Maroulakou *et al.*, 1994)	Transgenic overexpression	Viable, skeletal abnormalities	Sumarsono *et al.* (1996)
PU.1	Spleen, testis (Klemsz *et al.*, 1990; Galson *et al.*, 1993; Su *et al.*, 1996)	Gene disruption	Lethal; no meyloid or lymphoid differentiation	Scott *et al.* (1994), McKercher *et al.* (1996)
Ets-1	Thymus, astrocytes, heart, lung, gut, spleen (Chen, 1985; Ghysdael *et al.*, 1986; Wernert *et al.*, 1992; Kola *et al.*, 1993; Maroulakou *et al.*, 1994; Fleischman *et al.*, 1995); developmentally regulated[b]	Gene disruption in lymphoid lineage	Viable; abnormal B and T cell development	Bories *et al.* (1995), Muthusamy *et al.* (1995)
Fli-1	Thymus, ovary, bone marrow, spleen, heart (Ben-David *et al.*, 1991; Watson *et al.*, 1992; Klemsz *et al.*, 1993); developmentally regulated[b]	Gene disruption	Viable; decreased lymphocytes	Mélet *et al.* (1996)
PEA3	Brain, testis (Xin *et al.*, 1992)	Gene disruption	Viable; "sterile" mates	J. Hassell (personal communication)

TEL	Heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas (Golub *et al.*, 1994)	Gene disruption	Lethal; yolk sac angiogenesis defect; abnormal apoptosis	Wang *et al.* (1997)
Fli-1	Thymus, ovary, bone marrow, spleen, heart (Ben-David *et al.*, 1991; Watson *et al.*, 1992; Klemsz *et al.*, 1993); developmentally regulated[b]	Translocation (11;22)	Ewing's sarcoma	Delattre *et al.* (1995), May *et al.* (1993)
ERG	Thymus (Dhordain *et al.*, 1995); developmentally regulated[b]	Translocation (21;22)	Ewing's sarcoma	Sorensen *et al.* (1994)
ERG	Thymus (Dhordain *et al.*, 1995); developmentally regulated[b]	Translocation (16;21)	Acute myeloid leukemia	Ichikawa *et al.* (1994), Panagopoulos *et al.* (1994)
ETV1/ ER81	Ubiquitous expression; absent from placenta and peripheral blood leukocytes (Brown and McKnight, 1992)	Translocation (7;22)	Ewing's sarcoma	Jeon *et al.* (1995)
TEL	Heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas (Golub *et al.*, 1994)	Translocation (12;22)	Myeloid and lymphoid leukemia	Buijs *et al.* (1995)
TEL	Heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas (Golub *et al.*, 1994)	Translocation (5;12)	Chronic myelomonocytic leukemia	Golub *et al.* (1994)
TEL	Heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas (Golub *et al.*, 1994)	Translocation (12;21)	Acute lymphoblastic leukemia	Golub *et al.* (1995)

[a] Cellular distribution of *ets* gene products is given for adult tissues.

[b] In developing embryos, Ets-1 is widely expressed in several tissues, including brain, lung, heart, thymus, liver, gut, kidney, skin, intersegmental arteries of the spine, and capillaries (Kola *et al.*, 1993; Maroulakou *et al.*, 1994). Fli-1 expression is restricted to mesodermal lineage early in embryogenesis. Following morphogenesis, Fli-1 expression is dramatically reduced. Expression in endothelial cells is transiently restricted to decidual blood vessels and newly formed embryonic endothelial cells (Mélet *et al.*, 1996). ERG is present in mesodermal derivatives and neural crest in chicken embryogenesis. There is an association with cartilage morphogenesis (Dhordain *et al.*, 1995).

[c] Reference for genetic alteration report.

these two cases, the PNT domain is proposed to function in self-association, leading to altered activity of either AML1 or the PDGFβ receptor. The chromosomal translocation t(12;22) directs production of two chimeric proteins, TEL-MN1 and MN1-TEL, and causes myeloid and lymphoid leukemias (Buijs *et al.*, 1995). In this case, it has not been determined which fusion gene is oncogenic.

Chromosomal translocation can alter many properties of a genetic locus, including the expression pattern and protein structure. The isolated domains appear to acquire new biological roles while retaining their molecular function. Thus, the phenotypes observed do not directly indicate the normal biological function of the unaltered *ets* gene. Instead, these naturally occurring mutations emphasize the role of the intact protein, not just isolated domains, in determining the biological function of a particular *ets* protein.

D. *Drosophila* and *Caenorhabditis elegans ets* Proteins

Genetic analyses of *ets* genes within the model organisms *C. elegans* and *Drosophila* also provide strong evidence for biological specificity. Three of the seven known *Drosophila ets* genes have been shown to be essential (*E74, pnt, elg*), and a distinct cellular function has been demonstrated for a fourth (*yan*). Mutation of the *E74* gene results in embryonic lethality. E74 functions in a regulatory hierarchy in the larval salivary gland (Burtis *et al.*, 1990), regulating the expression of over 30 ecdysone-responsive genes (Fletcher and Thummel, 1995). In addition, phenotypic analysis has demonstrated that E74 is required for pupation and metamorphosis (Fletcher *et al.*, 1995). Mutations in the *pnt* gene also result in larval lethality (Klambt, 1993). The *pnt* locus produces two isoforms of Pnt, P1 and P2, by alternate promoter utilization. Pnt P1 is required for proper development of midline glial cells as well as the tracheal system (Krasnow, 1996). Pnt P2 functions as a positive regulator of photoreceptor R7 cells (Brunner *et al.*, 1994). Mutations in the *elg* gene result in late pupal lethality. Elg is required for anterior–posterior patterning during embryogenesis and for egg chamber patterning during oogenesis (Schulz *et al.*, 1993; Schulz, 1995). Mutations in the *yan* gene result in a decrease of viability and fertility (Lai and Rubin, 1992). Yan is a negative regulator of photoreceptor development in *Drosophila*. More recent experiments demonstrate that Yan also acts as a general inhibitor of cell fate specification at multiple points during *Drosophila* development (Rebay and Rubin, 1995; Rogge *et al.*, 1995). Although a number of *ets* genes can be detected in the *C. elegans* genome by a database search using ETS domain sequences, only *lin-1* has been characterized. This *ets* gene regulates development of the vulva, being specifically expressed in vulval precursor cells (Beitel *et al.*, 1995).

E. Conclusions

The analysis of *ets* genes disrupted by chromosomal translocation and gene targeting or aberrantly expressed as a result of retroviral insertion or transgenic experiments has provided insight into the biological roles of these proteins. Clearly the most definitive findings come from the disruption of *ets* genes in the mouse, *Drosophila,* and *C. elegans.* The distinct phenotypes observed in these experiments demonstrate that extensive redundancy does not exist within the *ets* family. Instead, the emerging picture suggests strong biological specificity, presumably through the selection of distinct target genes.

One important step in the characterization of the biological roles of individual *ets* proteins is the identification of these target genes. This is an ongoing area of investigation by many laboratories. In most cases, the definitive identification of targets and their assignment to a particular *ets* protein await stronger experimental data. Evidence to link a specific target gene to a single *ets* protein requires aggressive application of genetic tools (available only for a few *ets* genes). The continued development of new technologies to look directly at transcription factors on genomic, chromatin-bound DNA also will help answer these important questions. The resolution of this question is more tractable in the model organisms *Drosophila* and *C. elegans* because there are more advanced genetic tools and fewer paralogs within their genomes. Nevertheless, it is in vertebrates, with the larger number of *ets* genes and the potential for a vast number of target genes, that the specificity issue is the most complex and critically important to resolve. We predict that there will be distinctive regulatory mechanisms to mediate specificity in the complex vertebrate systems.

IV. DNA BINDING

Specificity of action for transcription factors begins at the level of sequence-specific DNA recognition. Recognition of DNA is mediated by structural modules referred to as DNA-binding domains. High-resolution structural analyses demonstrate that a very large number of transcription factors display a relatively small set of structural motifs for DNA binding. Thus, families of transcription factors are often defined by the sequence conservation of their DNA-binding domains. Recent structural studies have elucidated the tertiary structure of the ETS domain, providing the first high-resolution picture of how *ets* proteins recognize their cognate DNA target sites. These structural data are complemented by a combination of genetic and biochemical findings and the stage is now set for understanding specificity of DNA binding for *ets* proteins.

A. Consensus Recognition Sequences

Due to the sequence conservation within the ETS domain, all *ets* proteins bind similar DNA sequences. One picture of a consensus sequence comes from a comparison of binding sites detected in promoters and enhancers. The core recognition motif 5′-GGA(A/T)-3′ is present in the vast majority of all biological sites. Binding-site selection is another approach that has been reported for at least 11 members of the *ets* family (Fig. 5). This experimental approach selects relatively high-affinity binding sites from a population of randomly specified DNA duplexes. Sequence-specific DNA recognition spans a region of 9 to 15 bp within each duplex. The invariant core sequence, 5′-GGA-3′, lies near the center of each selected sequence. There is also remarkable similarity among the *ets* proteins in the preferred nucleotides that flank the core GGA. Importantly, binding sites for *ets* proteins derived from promoters and enhancers fall within the selected consensus.

The selected consensus for each *ets* protein displays few invariant positions beyond the highly conserved GGA core. The significance of this promiscuity was analyzed in the case of Ets-1. The relative affinity of a set of selected sites was compared, and a binding site with 5′-ACCGGAACG-3′ was determined to have the highest affinity (Nye *et al.*, 1992). Interestingly, this site has high affinity for a wide variety of *ets* proteins (Gunther and Graves, 1994; Graves *et al.*, 1996). It is possible that most *ets* proteins prefer the same optimal site; however, as we discuss below, there may be discrimination as DNA sequences diverge from the most strongly preferred sites.

Several distinctive features are detected in the selected consensus sequences. One of the most striking differences is the identity of the nucleotide position immediately to the right of the GGA core. Binding-site selections performed with Elf-1, E74, and Fli-1 indicate an invariant preference for A at this nucleotide position. In contrast, Ets-1, SAP-1, GABPα, ER71, ER81, PU.1, Elk-1, and Spi-B select either a T or an A at this position. The importance of this position also is illustrated by an altered specificity mutant of Ets-1 that can no longer recognize a GGAT core (Bosselut *et al.*, 1993). This mutant is generated by changing a single amino acid within the Ets-1 ETS domain to match the homologous residue in Elf-1. Another feature that varies among the *ets* proteins is the number of selected nucleotides. PU.1 and Spi-B show sequence-specific recognition over 15 bp, Fli-1 selects 13 bp, and all others select 9–11 bp. Finally, the nucleotides selected to the left of the GGA core are the most variable among the family members. This region may be important for determining sequence selectivity of individual *ets* proteins.

It is likely that the binding-site selection approach does not detect the potential discrimination of *ets* proteins for relatively low-affinity sites that do not precisely match the selected consensus. In fact, such selectivity has been reported between pairs of *ets* proteins. PU.1 and Ets-1 discriminate sites in

GROUP	PROTEIN	SELECTED CONSENSUS			REFERENCE
ELF	D-E74	NNNNN AACCA T C	NNN GGA	NNN AGT	(Urness and Thummel, 1990)
	M-Elf-1	NNNNN AACCC ta	NNN GGA	NNNN AGTa g c	(John *et al.*, 1996)
ELG	M-GABPα	NNN GCC aga	NGA GGA	NNNN AGTN tac	(Brown and McKnight, 1992)
ELK	M-SAP-1	NNN ACC	NNN GGA	NNN AGT tac	(Shore and Sharrocks, 1995)
	M-Elk-1	NNN ACC	NNN GGA	NNNN AGTG A	(Shore and Sharrocks, 1995)
ERG	M-Fli-1	NNNNNN TNGACC g A	NNN GGA	NNNN AGTA acG	(Mao *et al.*, 1994)
	M-ER71	NNN GCC CGa	NGA GGA	NNNN TGTC Aac	(Brown and McKnight, 1992)
ETS	M-Ets-1	NNN ACC g a	GGA GGA	NNNN AGCN TaT	(Nye *et al.*, 1992)
PEA3	M-ER81	NNN GGC aca	NGA GGA	NNNN AGTN Tac	(Brown and McKnight, 1992)
SP1	M-PU.1	NNNNNNN AAAAAGA tcC G	NNN GGA	NNNNN AGTAG c GC	(Ray-Gallet *et al.*, 1995)
	M-Spi-B	NNNNNNN AAAAAGA tcc	NNN GGA	NNNNN AGTAN c T	(Ray-Gallet *et al.*, 1995)

Fig. 5 Selected DNA consensus sequences for *ets* proteins. Consensus sequences were selected using DNA duplexes with randomized sequences. Nucleotide positions randomized (N) or fixed (G or A) during the *in vitro* selections are indicated above each sequence. Repeated selections were used in all cases. Additional binding site selections for Ets-1 (Fisher *et al.*, 1991; Woods *et al.*, 1992), SAP-1 (Treisman *et al.*, 1992), Elf-1 (Davis and Roussel, 1996), and Fli-1 (Murakami *et al.*, 1993) show similar results. ELK group binding was studied in the presence of SRF. The highly conserved GGA core motif is boxed. Nucleotides in lowercase letters were less frequently selected. Protein groups are defined in Fig. 1.

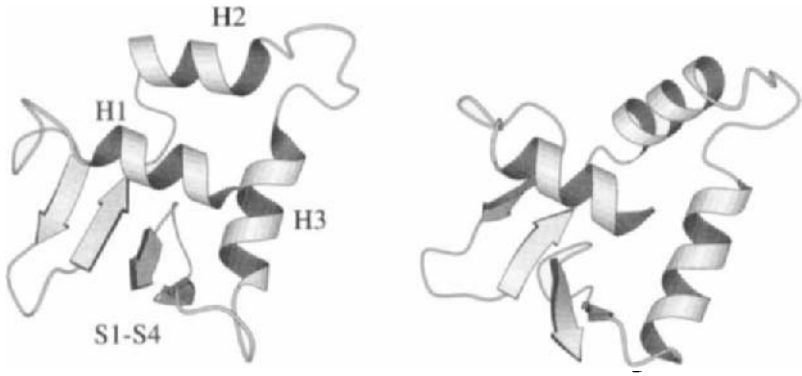


Fig. 6 Tertiary structure of the ETS domain. Three-dimensional structures of the Ets-1 (left) (Donaldson *et al.*, 1996), Fli-1 (center) (Liang *et al.*, 1994a), and PU.1 (right) (Kodandapani *et al.*, 1996) ETS domains are presented as ribbon diagrams. The α helices and β strands of Ets-1 are labeled for reference. The Ets-1 and Fli-1 structures were determined by NMR spectroscopy and the PU.1 structure was solved by crystallographic analysis. Regions flanking the ETS domain that were present in the reported structures were removed. Three helices (H1–H3) form a helix–turn–helix (HTH) motif that packs against a four-stranded, antiparallel β-sheet (S1–S4). The term "winged helix–turn–helix" is used to describe this domain and highlights the importance of both the HTH element and the β-sheet (wing) in the structural fold and in contacting DNA.

the immunoglobulin heavy-chain enhancer (Nelsen *et al.*, 1993) whereas Elf-1, but not Ets-1, binds a site within the enhancer of the IL-2 receptor (John *et al.*, 1996). In a more dramatic deviation from its consensus, PU.1 recognizes a unique core 5′-AGA-3′ DNA element within the immunoglobulin J chain enhancer (Shin and Koshland, 1993). In the binding-site selection for PU.1, only 2 out of 51 clones displayed an AGA core. Moreover, binding studies show that PU.1 binds only weakly to a site containing the AGA core in the context of the selected consensus (Ray-Gallet *et al.*, 1995). Thus, deviations from the consensus, high-affinity binding sites may play an important role in binding specificity of *ets* proteins.

B. Structure of the ETS Domain

The three-dimensional structure of the ETS domain has been described in both the absence and presence of DNA. Secondary structural analysis of Ets-1 and Fli-1 by NMR initially established that the ETS domain is composed of three α helices (H) and four β strands (S) arrayed linearly as H1-S1-S2-H2-H3-S3-S4 (Fig. 2) (Donaldson *et al.*, 1994; Liang *et al.*, 1994b). Tertiary structures of Fli-1, Ets-1, and PU.1 show that the three helices within the ETS domain fold into a helix–turn–helix (HTH) element that packs against a

Fig. 6 *(continued)*

four-stranded, antiparallel β-sheet (Fig. 6) (Liang *et al.*, 1994a; Werner *et al.*, 1995; Donaldson *et al.*, 1996; Kodandapani *et al.*, 1996). Inspection of the tertiary structure provides insights into the roles of highly conserved residues within the ETS domain. The majority of the highly conserved amino acids are found within the α helices and β strands (Fig. 2). Several of these highly conserved amino acids form the hydrophobic core of the ETS domain and must play an essential role in the proper folding of the domain (Donaldson *et al.*, 1996). In contrast, the regions with less sequence conservation map to the loops or turns.

The *ets* transcription factors have been grouped into a large structural class of DNA-binding proteins termed the "winged helix–turn–helix" (wHTH) proteins. The term "winged helix" is also used. All wHTH proteins have the same topological array of secondary structure elements that form a tertiary structure consisting of a three-helix bundle and a two- to four-stranded β-sheet. The β-sheet with its accompanying loops provides the "winged" image (Brennan, 1993; Clark *et al.*, 1993). The wHTH motif is found in a wide variety of DNA-binding proteins, including the prokaryotic transcription factors CAP (Schultz *et al.*, 1991), LexA (Fogh *et al.*, 1994), and BirA (Wilson *et al.*, 1992), as well as the replication terminator protein RTP (Bussiere *et al.*, 1995). There is also a growing number of eukaryotic wHTH proteins, including histone H5 (Ramakrishnan *et al.*, 1993), the HNF/*forked head* family of transcription factors (Clark *et al.*, 1993), heat-shock factor HSF (Harrison *et al.*, 1994), diphtheria toxin repressor DtxR (Qiu *et al.*, 1995), and topoisomerase II (Berger *et al.*, 1996). Due to the absence of significant sequence conservation, it cannot be determined whether this structural similarity of wHTH proteins reflects evolutionary relatedness.

Although the wHTH proteins share a common fold, there exists considerable variation in the fine structure and recognition modes utilized by the dis-

tinct members of this class of proteins. For example, crossing angles for the three helices and the length of the turn between helices 2 and 3 show considerable variation. In addition, the β-sheets display different numbers of strands and sizes of loops. The comparison of *ets* proteins to other wHTH proteins demonstrates the ability of a conserved protein fold to mediate a wide variety of DNA interactions. The wHTH proteins utilize distinct oligomerization states for recognizing DNA. For example, the *ets* proteins and HNF/*forkhead* proteins recognize DNA as monomers, CAP binds to DNA as a dimer, and HSF recognizes DNA as a trimer. The tertiary fold is only a scaffold for presenting amino acids to the DNA. There is considerable variability at this point among the wHTH proteins. For example, only the *ets* proteins recognize DNA sites with GGA core sequences. In the case of histone H5 and topoisomerase II, there is no sequence-specific recognition.

C. ETS Domain–DNA Complex

Three-dimensional models of PU.1 and Ets-1 bound to DNA provided the first high-resolution structural information on DNA recognition (Werner *et al.*, 1995; Kodandapani *et al.*, 1996). Surprisingly, these two complexes, as initially reported, differed with respect to the orientation of the ETS domain on DNA. An approximate 180° rotation was necessary to superimpose the two proteins with respect to the GGA core. A revised version of the Ets-1-DNA structure demonstrates that Ets-1 binds DNA with a mode similar to that determined for the PU.1–DNA complex (Werner *et al.*, 1995, 1997).

A high-resolution crystallographic analysis of a PU.1–DNA complex provides the most detailed model (Kodandapani *et al.*, 1996; Pio *et al.*, 1996). The structure is derived from a 112-residue fragment of PU.1, which spans the ETS domain, bound to a 16-bp DNA duplex. Four regions of the ETS domain make contact to DNA over 10 bp [Figs. 7 (see color plate) and 8A]. The HTH, composed of helices H1–H3, lies in the major groove. All of the base-specific contacts identified in the PU.1–DNA complex are mediated by residues within H3 and the turn immediately preceding it. Two arginines in H3 that are invariant in the *ets* family (Fig. 2) provide base contacts to the two guanine residues of the GGA core. Major DNA backbone contacts flank the GGA core and are mediated by three regions of the ETS domain. Amino acids in the β-sheet (S3 and S4) and the intervening loop contact phosphates to the left of the GGA core while residues within the turn of the HTH motif contact phosphates to the right of the GGA core. Additional backbone contacts to the right of the GGA core are mediated by amino acids preceding the amino terminus of H1. A subset of these base pair and phosphate contacts are mediated by water molecules in the crystal structure.

Biochemical and genetic data support the structural analysis of the ETS

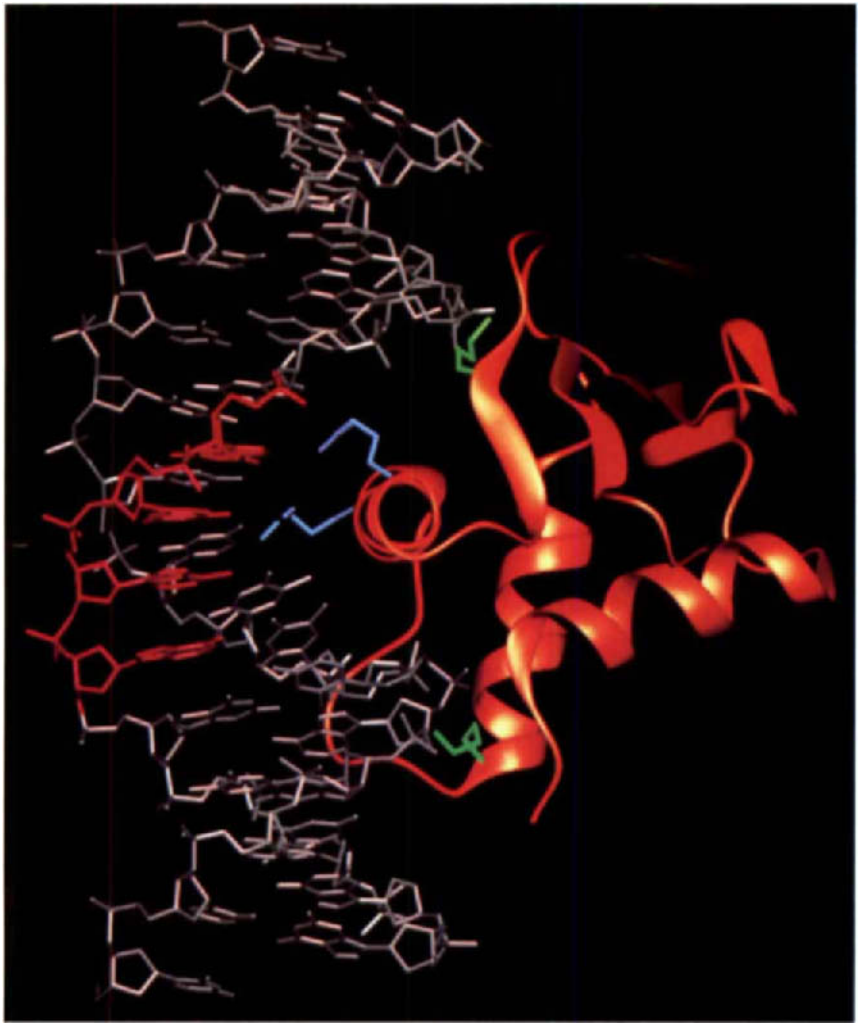


Fig. 7 Structure of PU.1–DNA complex. The three-dimensional structure of the PU.1 ETS domain bound to a 16-basepair DNA duplex (Kodandapani *et al.*, 1996). The core GGAA DNA element is highlighted in red. In this view, H3 of the ETS domain lies within the major groove perpendicular to the DNA backbone. The two highly conserved arginine residues (blue; also see Fig. 2) extend from H3, making base-specific contacts to the core GGA. Two key interactions to the phosphate backbone (green) are a lysine residue from the loop between S3 and S4 and a lysine residue within the turn between H2 and H3. See Fig. 8 for details of these DNA contacts. From Kodandapani *et al.* (1996).

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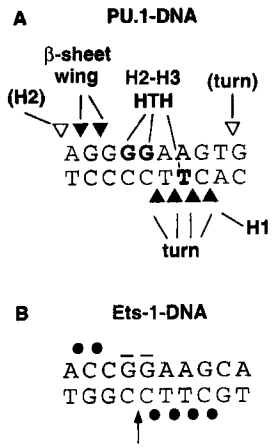

Fig. 8 DNA–protein interactions. (A) ETS domain–DNA contacts as determined by crystallographic analysis for the PU.1–DNA complex (Kodandapani *et al.*, 1996). Bases contacted by protein elements within H3 and H2 are shown in bold. Major phosphate contacts (▲, ▼) are made by the β-sheet (wing), the turn between H2 and H3, and residues near the amino terminus of H1. The phosphate contacts (▽) may be unique features of PU.1 because they are not made by conserved residues. (B) ETS domain–DNA contacts as determined by biochemical analyses of Ets-1. Phosphate contacts (●) were identified by ethylation interference analysis. Ethylation interference analyses of GABP, Fli-1, and Elf-1 yield similar data (Gunther and Graves, 1994). The bars over the nucleotides correspond to positions where methylation strongly interferes with DNA binding. The arrow marks the position of DNase I hypersensitivity detected in all ETS domain–DNA interactions (Gunther and Graves, 1994; Graves *et al.*, 1996).

domain–DNA complex. Base-specific and phosphate contacts determined within the PU.1–DNA complex structure correlate with detailed contact mapping analysis of Ets-1 bound to DNA (Fig. 8B). Methylation interference studies provide evidence for the recognition of the central GGA core triplet in the major groove. Methylation of either guanine shows strong interference (Nye *et al.*, 1992; Eisenbeis *et al.*, 1993; Galson *et al.*, 1993; Gunther and Graves, 1994). Ethylation interference experiments identify backbone contacts. A signature pattern of ethylation interference sites was detected in comparative studies of several *ets* proteins (Gunther and Graves, 1994). The two regions of interference correspond precisely to the phosphate contacts flanking the GGA core that are observed within the PU.1–DNA complex (Graves *et al.*, 1996; Kodandapani *et al.*, 1996; Rao *et al.*, 1997).

The NMR studies of Fli-1 and Ets-1 provide support for these general conclusions and additional insight. The analysis of Fli-1 was done in the presence of DNA even though the DNA was not included in the structure calculations. A single intermolecular contact was detected between a residue of H3 and DNA within the GGAA core (Liang *et al.*, 1994a). The structure of

Ets-1 was solved in both the absence (Donaldson *et al.*, 1996) and presence of DNA (Werner *et al.*, 1995). Comparison of these structures indicates that no dramatic changes occur within the protein structure on DNA binding. This is consistent with protease sensitivity and circular dichroism studies that also detect no dramatic change in the secondary structure of the Ets-1 ETS domain on DNA binding (Petersen *et al.*, 1995). The turn between H2 and H3, as well as the loop between S3 and S4, are poorly defined in both analyses. This could reflect inherent flexibility in these regions or simply limited experimental NMR data for these portions of the ETS domain. Thus, there is not sufficient data to determine whether these regions undergo slight changes, or even adopt a more rigid structure, on DNA binding.

The structure of the DNA is an important feature of any co-crystal. Within the PU.1–DNA complex, there is no significant deviation from B-form DNA (Fig. 7). The DNA is uniformly curved over the entire length of the 16-bp duplex with a net change in the helical axis of only 8°. There is a small widening (~2–3 Å) of the minor groove occurring at the midpoint of the DNA complex. This subtly altered DNA conformation may account for the unusual DNase I hypersensitive site within the center of the binding site of all *ets* proteins (Urness and Thummel, 1990; Gunther and Graves, 1994; Graves *et al.*, 1996; Rao *et al.*, 1997). Two studies have reported biochemical analysis of the conformation of DNA when bound by *ets* proteins. A permuted binding site approach suggests that PU.1 bends DNA by 24° (Nikolajczyk *et al.*, 1996) whereas similar studies on Elk-1 detected no altered DNA conformation (Shore *et al.*, 1995). Resolution of this controversy awaits more biochemical analysis as well as the determination of the structures of additional ETS domains on a variety of DNA duplexes.

The PU.1–DNA complex provides insight into how the highly conserved ETS domain recognizes DNA in a sequence-specific manner. First, conserved residues contact DNA. Nineteen water-mediated or direct contacts to the bases or phosphate backbone were identified in the PU.1 complex. Approximately half of these contacts are made by either highly conserved or invariant amino acids within the ETS domain (Fig. 2). The bases of the conserved GGA core are contacted directly by invariant amino acids of the ETS domain. Thus, it is not surprising that *ets* proteins recognize similar DNA-binding sites. Second, the PU.1–DNA structure indicates that phosphate contacts may be utilized for sequence-specific DNA recognition. The sequence-specific position that flank the GGA core are contacted predominantly on the DNA backbone. In this type of DNA recognition, referred to as indirect readout, the DNA sequence determines the conformation of the phosphate backbone. The structure is recognized by amino acid contacts to backbone positions rather than by direct bonding to functional groups on the bases. The regions of the ETS domain that contact the DNA backbone show more variability than the recognition helix (H3) of the HTH. The length and sequence

of the loop between S3 and S4, as well as the turn between H2 and H3, differ among the *ets* proteins. Additionally, the amino acid sequences immediately preceding the amino terminus of H1 diverge. Thus, these regions of *ets* proteins may provide discriminating potential within the binding site via the mechanism of indirect readout.

D. Conclusions

This section has documented the high level of similarity in the mode of DNA binding by *ets* proteins. The remarkable concordance of consensus binding sites is explained by the strong conservation of DNA contact residues within the ETS domain. DNA-binding specificity among *ets* proteins will come only from subtle differences in sequence preference. Nonconsensus binding sites provide the best route to such discrimination. The required framework is now in place to begin to investigate possible divergent modes of binding. In spite of these possibilities, it is expected that levels of control, other than sequence-specific DNA binding, will be required to mediate the highly specific action of *ets* proteins.

V. PROTEIN PARTNERSHIPS

A general theme in the regulation of gene expression is combinatorial control. A complex array of transcription factors regulates every promoter or enhancer. Recognition of regulatory elements is governed by both protein–protein and protein–DNA interactions. These macromolecular interactions help dictate the subset of transcription factors that function on a given promoter or enhancer. The importance of combinatorial control has been well-documented with the *ets* family of transcription factors. The *ets* proteins act synergistically with a variety of other transcription factors to regulate many cellular and viral promoters and enhancers. Synergy in this context describes the phenomenon in which the combined transcriptional activity of multiple factors is greater than additive. Here, we focus on combinatorial control pathways in which DNA binding cooperativity or direct protein–protein interactions have been detected between the *ets* protein and a DNA-binding partner.

There are at least two general classes of *ets* protein interactions with DNA-binding partners. In one subset of the known partnerships only a single *ets* protein (or evolutionarily related members of a group) participates (e.g., Elk-1 and SAP-1 with SRF; PU.1 with Pip). This class of interaction typically requires sequences that lie outside of the ETS domain. In other interactions,

multiple members of the *ets* family can interact with a regulatory partner (e.g., *ets* proteins with Pax-5). At present, these less discriminating interactions appear to be mediated by the ETS domain. As these apparently less-specific interactions are better understood, additional restrictions or preferences may be discovered.

We have not comprehensively reviewed all of the *ets* partnerships, but rather focused on the best-characterized examples that illustrate the different classes of interactions. Additional partnerships are mentioned in Section VII. The discussion of the regulation of autoinhibition by protein–protein interactions complements this section. There also are other *ets*-associated proteins that do not bind DNA, some of which will be discussed in Section VI.

A. Partnerships Restricted to a Single *ets* Protein or Group

Ternary complex formation on the c-*fos* promoter provides the best example of an *ets* partnership (for review, see Treisman, 1994). Induction of c-*fos* expression by treatment of cells with serum requires the serum response element (SRE), which is composed of binding sites for the serum response factor (SRF) and an *ets* protein. There is substantial evidence that this *ets* binding site is bound by Elk-1 or SAP-1, such that a ternary complex with SRF is formed on the SRE (Shaw *et al.*, 1989). Thus, these mammalian ELK group proteins often are termed ternary complex factors, TCFs. SRF enhances the binding of the TCFs to the SRE because neither Elk-1 nor SAP-1 efficiently recognize the SRE element alone (Dalton and Treisman, 1992; Rao and Reddy, 1992; Janknecht *et al.*, 1994). (See Section VIII for more discussion of this partnership.)

The B box, a conserved region among the TCF proteins, is sufficient to mediate ternary complex formation (Hill *et al.*, 1993). A 30-amino acid peptide encompassing the Elk-1 B box physically interacts with SRF in the absence of DNA (Shore and Sharrocks, 1994) and single amino acid substitutions within the B box can disrupt the interaction of this domain with SRF (Ling *et al.*, 1997). Although there is no high-resolution structural model of the ternary complex, solution studies indicate that the B box forms an α helix that presents hydrophobic residues for interaction with SRF (Ling *et al.*, 1997). These studies illustrate how short protein–protein interaction motifs provide the specificity for partnerships.

DNA determinants for SRE ternary complex formation include the recognition sites for SRF and the TCF. Surprisingly, the spacing between the two binding sites can vary over 5 to 30 bp (Treisman *et al.*, 1992). Indeed, SRE elements in a variety of promoters show considerable diversity in the arrangement of SRF and *ets* binding sites. This degree of flexibility is possi-

ble because the B box is separated from the ETS domain by 50 amino acids. It is proposed that this region of the TCF is sufficiently flexible to accommodate the different spacing needs of SRE elements.

The partnership of PU.1 and Pip (PU.1-interacting protein, previously known as NF-EM5) represents another well-characterized interaction. Both proteins are required for the activity of several immunoglobulin light-chain gene enhancers, including $E_{\kappa 3'}$, $E_{\lambda 2-4}$, and $E_{\lambda 3-2}$ (Judde and Max, 1992; Pongubala *et al.*, 1992). All three enhancers have juxtaposed binding sites for PU.1 and Pip. DNA recognition by Pip is completely dependent on the partnership with PU.1 due to the presence of an autoinhibitory region. Phosphorylation of PU.1 is required for ternary complex formation (Pongubala *et al.*, 1993; Eisenbeis *et al.*, 1995; Brass *et al.*, 1996). The region of PU.1 involved in the interaction includes the sites of phosphorylation and lies N-terminal to the ETS domain (Pongubala *et al.*, 1992). Although PU.1 can function in isolation on the J chain enhancer (Shin and Koshland, 1993), the interaction with Pip is clearly important for its specific role on a variety of the immunoglobulin light-chain enhancers.

A partnership between Ets-1 and members of the small *cbf* gene family (Speck and Stacy, 1995) has been studied on several viral and cellular enhancers. Transcription assays indicate that Ets-1 and CBFα2 (also known as AML1) synergistically activate expression of the T cell receptor α and β subunits enhancers, as well as the Moloney murine leukemia virus enhancer (Wotton *et al.*, 1994; Giese *et al.*, 1995; Sun *et al.*, 1995; Mayall *et al.*, 1997). DNA binding assays demonstrate that Ets-1 and CBF bind cooperatively to these composite elements (Wotton *et al.*, 1994; Giese *et al.*, 1995). Ets-1 interacts with CBF even in the absence of DNA (Giese *et al.*, 1995). The interaction domain includes a portion of the PNT domain and a nonconserved region located N-terminal to the ETS domain. The specificity of this interaction is questioned by two sets of results. Transient expression studies indicate that both GABPα and Ets-2 also can function with CBF on these composite elements (Sun *et al.*, 1995). Consistent with this finding, the disruption of the *ets-1* gene in T lymphocytes did not alter production of the T cell receptor α subunit (Bories *et al.*, 1995) (see Section III). Resolution of this issue will require additional mutational and structural analyses.

B. Partnerships Available to Multiple *ets* Proteins

Multiple members of the *ets* family associate with the transcription factor, Pax-5, a paired box protein (Fitzsimmons *et al.*, 1996). Binding sites for Pax-5 and *ets* proteins are essential for high levels of transcription from the B-cell-specific *mb-1* promoter. DNA binding analysis demonstrates that B cell nuclear extracts contain complexes of Pax-5 and either Fli-1, Ets-1, or GAB-

Pα. In addition, Net, Elk-1, Ets-1, and Fli-1, but not SAP-1, can be recruited to form ternary complexes with Pax-5. The DNA-binding domains of both Pax-5 and the *ets* proteins are sufficient to mediate cooperative complex formation. An amino acid conserved in the ETS domains of Elk-1, Net, Fli-1, Ets-1, and GABPα, but not SAP-1, appears critical for the interaction. SAP-1 with a substitution to the more conserved residue can efficiently form ternary complexes with Pax-5. Thus, multiple members of the *ets* family use the ETS domain to interact with the Pax-5 regulatory partner. Which *ets* protein mediates this function in B cells remains to be resolved.

AP-1 transcription factors also interact with a variety of *ets* proteins. Initially, Ets-1 and Jun/Fos synergy was discovered on the polyomavirus enhancer; however, no DNA binding cooperativity was reported (Wasylyk *et al.*, 1990). More recently this partnership has been characterized on the promoter of the tissue inhibitor of metalloproteinases-1 gene (Logan *et al.*, 1996). *In vitro* binding studies demonstrate a direct interaction between Jun/Fos and full-length Ets-1. ERG also functions in combination with Jun and Fos. This combination of factors synergistically activates the collagenase-1 promoter (Butticé *et al.*, 1996). Furthermore, a direct interaction between ERG and the Jun/Fos heterodimer is detected in the absence of DNA. In these studies, the regions of Ets-1 and ERG that interact with the Jun/Fos heterodimer have not been mapped; however, in several other cases, AP-1 proteins have been shown to interact with the ETS domain. ERM binds Jun, and the region of ERM required for the interaction includes the ETS domain and the C-terminal extension (Nakae *et al.*, 1995). Binding studies performed in the absence of DNA have detected direct protein–protein interactions between Jun and the ETS domain of Ets-1, Elf-1, PU.1, and Fli-1; however the cellular significance of these *in vitro* interactions remains to be confirmed within the context of a transcription assay (Bassuk and Leiden, 1995).

There is surprising promiscuity in the *ets* partnerships with Pax-5 and AP-1 proteins. We also noted some question about the specificity of the Ets-1–CBF interaction. Further investigation will be necessary to determine whether this redundancy is utilized within a biological context.

C. Oligomerization of *ets* Proteins

Oligomerization of *ets* proteins provides another pathway to promoter-specific gene regulation. Many biochemical studies indicate that the *ets* proteins bind the GGA recognition sequence as a monomer. An interesting variation of this mode of binding is observed with GABP, a heterotetramer composed of two α and two β subunits (LaMarco *et al.*, 1991; Thompson

et al., 1991; de la Brousse *et al.*, 1994). The α subunit contains the ETS domain and binds DNA, whereas a leucine zipper motif within the β subunit mediates oligomerization. This subunit configuration directs GABP to recognize promoters or enhancers with repeated GGA motifs.

Several reports open the possibility that oligomerization of *ets* proteins may be possible even in the absence of a second subunit. Elk-1 self-association on the c-*fos* SRE has been reported (Gille *et al.*, 1996). A c-*fos* SRE quaternary complex composed of two SRF molecules and two Elk-1 proteins forms when Elk-1 is phosphorylated. Intermolecular association of Elk-1 occurs via the C-terminal region, which contains the critical site of phosphorylation. The formation of this quaternary complex appears to correlate with efficient activation of the c-*fos* promoter *in vivo*. TEL has been shown to self-associate via the conserved PNT domain (see Section II). However, there is no evidence that TEL self-association plays a role in DNA binding. If the PNT domain can direct DNA binding of dimers, oligomerization could influence DNA binding of all *ets* proteins with this domain. The possibility of frequent oligomerization of *ets* proteins is intriguing because several promoters and enhancers display multiple binding sites for *ets* proteins (Gitlin *et al.*, 1991; Wasylyk *et al.*, 1991; Seth *et al.*, 1993; Coffer *et al.*, 1994; Seth *et al.*, 1994; Villena *et al.*, 1994).

D. Higher Order Complexes

Enhancers and promoters contain multiple protein complexes. DNA sequences directing these arrays can span distances of hundreds of nucleotides. These assemblages require that intermolecular interactions link together proteins that lie at nonadjacent sites. The role of *ets* transcription factors within these higher order complexes has been documented in several cases.

A multiprotein complex assembles on the HIV-1 enhancer (Sheridan *et al.*, 1995). The enhancer element contains binding sites for LEF-1, Ets-1, TFE-3, and NF-κB whereas the HIV-1 promoter displays an Sp1-binding site. These elements are distributed over a region of ~130 bp. Optimal activity requires a combination of Sp1 and the enhancer-binding proteins. These activities have been analyzed on chromatin-assembled templates, demonstrating a role for Ets-1 in chromatin derepression.

The T cell receptor α subunit enhancer contains DNA-binding sites for Ets-1, ATF2/CREB, LEF-1, and CBF (Giese *et al.*, 1995; Mayall *et al.*, 1997). Within this enhancer, the binding sites for Ets-1 and CBF lie adjacent to one another whereas the binding sites for Ets-1 and ATF2/CREB are separated by approximately 60 nucleotides. The binding site for LEF-1, an HMG protein known to bend DNA by 130°, lies in between the Ets-1 and ATF2/CREB

binding sites. Direct contacts between ATF2/CREB and Ets-1 have been detected *in vitro*. Moreover, DNase I footprinting assays show that LEF-1 and ATF2/CREB stabilize the Ets-1/CBF ternary complex. These data together provide a model in which LEF-1 functions to bend DNA, thereby facilitating interactions between proteins at nonadjacent sites.

A similar model also has been proposed for the mitogenic stimulation of the IL-2 receptor α subunit gene in T lymphocytes (John *et al.*, 1995). Two mitogen-responsive elements bind the proteins NF-κB, HMG-1(Y), SRF, and Elf-1. Deletion or mutation of either the HMG-1(Y) or Elf-1 binding sites greatly diminishes promoter activity. *In vitro* binding studies demonstrate that Elf-1 physically interacts with HMG-1(Y) as well as the NF-κB subunits, p50 and c-Rel, in the absence of DNA. The Elf-1 and NF-κB binding sites are separated by hundreds of nucleotides. Binding sites for HMG-1(Y) are located in the intervening sequences. Thus, it is proposed that formation of a stereospecific complex requires HMG-1(Y) to bend DNA and bring Elf-1 in close proximity to NF-κB.

E. Conclusions

Cooperative DNA binding within a protein partnership enhances the promoter specificity of transcription factors. In the case of *ets* proteins, a single ETS domain can recognize 9 to 15 bp of specific DNA sequence. Although there may be little discrimination between *ets* proteins at this primary step in promoter recognition, a protein partnership can expand the specificity of promoter recognition in two ways. First, the binding site of the partner protein likely spans 10 to 20 bp of specific sequence; thus, recognition of the ternary complex would require a significantly larger DNA element. Second, a protein interaction domain involving even a small structural element such as an α helix or extended arm can provide additional stabilizing contacts for the *ets* protein. These protein–protein contacts could enhance significantly the affinity of the *ets* protein for the promoter sites.

Although numerous partnerships have been identified, the protein interaction domains used by the *ets* proteins have not been fully characterized. Conserved regions such as the ETS domain are implicated; however, other interaction domains show little sequence conservation. High-resolution structural data with accompanying mutational and biochemical analyses are needed to map definitively contact residues and establish the relative affinity and specificity of any proposed protein–protein interaction. As in the case of DNA–protein interactions, this level of resolution of protein–protein interactions will provide a framework for understanding the specificity of these important macromolecular interactions.

VI. TRANSCRIPTIONAL ACTIVATION AND REPRESSION

Transcription factors can either activate or repress gene expression. There are two general classes of mechanistic models for these activities in eukaryotic systems. First, regulatory transcription factors can interact with the general transcriptional machinery (e.g., TBP, TFIIA, TFIIB, or the RNA polymerase holoenzyme). These interactions can be direct or mediated by adaptors, termed coactivators or corepressors. Second, regulatory transcription factors can affect the repressive effects of chromatin. Again, this mechanism can employ either coactivators or corepressors. Regulatory transcription factors have independent domains that mediate these functions [e.g., transactivation domains (TADs)]. It is generally assumed that these domains function by contacting the basal machinery or in binding cofactors.

The sequence-specific DNA-binding properties of *ets* proteins strongly suggest that these proteins regulate transcription. This premise is supported by the common occurrence of binding sites for *ets* proteins within the promoters and enhancers of a wide assortment of genes. Furthermore, *ets* proteins act as transcriptional activators or repressors in a variety of assays. Transcriptional activity provides several avenues to generate specificity among *ets* proteins. First, there are both transcriptional activators and repressors in the family. Furthermore, these activation and repression domains are not highly conserved among *ets* proteins. Thus, it is possible that different *ets* proteins will use distinct coactivators or corepressors and mediate gene expression by different mechanisms.

A. Activators

Activation function has been studied in almost a dozen *ets* family members. Transcription activation domains must be identified functionally because there are no sequence motifs that definitively delineate such a domain. Nevertheless, TADs are often characterized by the abundance of a particular amino acid. There are proline-rich and glutamine-rich domains as well as ones with a preponderance of acidic residues. Furthermore, hydrophobic residues have been implicated as critical features within all classes of activation domains. The TADs of *ets* proteins bear hallmarks of well-characterized activation domains. For example, two separate domains of PU.1 function in activation (Hagemeier *et al.*, 1993; Shin and Koshland, 1993; Kominato *et al.*, 1995; Klemsz and Maki, 1996). One is rich in acidic residues and the other is marked by a set of glutamine residues.

There is no overall sequence conservation among the TADs of the *ets* fam-

ily; nevertheless, these functional domains show some similarity among the highly related *ets* proteins. Two TADs are present in ERM (Laget *et al.*, 1996). One region lies at the extreme N terminus in an acidic region that is conserved within the PEA3 group. Likewise, the TAD of the vertebrate ELK group proteins maps to a conserved C-terminal region, termed the C box (Bhattacharya *et al.*, 1993; Marais *et al.*, 1993; Janknecht *et al.*, 1994). TADs within the ETS and ERG group proteins map to a central location within the gene structure. A TAD, rich in acidic residues, lies between the PNT domain and ETS domain in both Ets-1 and Ets-2 of the ETS group (Schneikert *et al.*, 1992). The ERG group proteins, Erg and Fli-1, have a TAD in the analogous central region (Rao *et al.*, 1993; Siddique *et al.*, 1993). In each case, the conserved positioning and sequence conservation of these TADs are consistent with the evolutionary relatedness of these genes. There is, however, minimal sequence conservation. This sequence variation could reflect the lack of strict sequence requirements for activation domains. Alternatively, divergence could result from functional distinctions. Additional studies are necessary to determine whether activation mechanisms are conserved within these groups.

The mechanism of activation has been investigated in only a few *ets* proteins. PU.1 has been shown to interact directly with TBP (Hagemeier *et al.*, 1993). CBP and p300 are coactivators that display histone acetylase activity and are proposed to mediate chromatin remodeling (Bannister and Kouzarides, 1996). These coactivators function within a growing number of transcription factors (Janknecht and Hunter, 1996) and are expected to assist at least a subset of the *ets* proteins. Indeed, the coactivator CBP has been shown to work with SAP-1a (Janknecht and Nordheim, 1996). The TBP-associated factors (TAFs) function as coactivators for specific classes of TADs, such as the glutamine-rich activation domains (Verrijzer *et al.*, 1995). Thus, these general coactivators are expected to function with some of the *ets* proteins. In an unusual case, GABPα activates transcription by using a gene-specific coactivator. The TAD lies in the β subunit of GABP, not in GABPα, which bears the ETS domain (Gugneja *et al.*, 1995, 1996). Thus, the activation function of the *ets* protein GABPα is specifically controlled by the availability of a second protein, GABPβ. In conclusion, there could be a variety of mechanisms available for mediating transcriptional activation within the *ets* family. Further work is necessary to characterize this level of regulation. The emerging picture suggests that there is definitively potential for divergence among family members.

B. Repressors

A few *ets* proteins have been characterized as transcriptional repressors. In several cases the repression function of one *ets* protein has been proposed

to counter the activation activity of a second *ets* protein. A simple interference mechanism could be operating in which the two *ets* proteins (one with a TAD and one without) compete for binding to the same promoter element. More active repression models require the recruitment of corepressors and predict the existence of domains on the *ets* proteins that interact with corepressors. A combination of these two mechanisms also could be functioning as illustrated in the case of ERF. This *ets* protein functions as a repressor of a variety of promoters and enhancers in transient expression assays (Sgouras *et al.*, 1995). In this assay system, ERF also can compete with *ets* proteins that are activators. A repression domain has been mapped to lie at the carboxyl terminus of ERF.

Drosophila Yan negatively regulates photoreceptor cell differentiation (Lai and Rubin, 1992; O'Neill *et al.*, 1994; Rebay and Rubin, 1995). Yan is proposed to act as a transcriptional repressor to counteract the activator Pnt P2. These opposing activities have been reconstituted in transient expression assays. The reciprocal expression of these *ets* genes in photoreceptor cells supports a model in which genes required for differentiation are first repressed by Yan, then activated by Pnt P2.

Alternate utilization of *ets* proteins in repression and activation also is observed in the case of the two isoforms of *Drosophila* E74. E74B is truncated in comparison to E74A and retains only the ETS domain (Burtis *et al.*, 1990). Production of E74A and E74B is directed by two different promoters. Expression of the E74 locus is controlled by the steroid hormone ecdysone; the E74B promoter responds to low levels of ecdysone whereas the E74A promoter is activated only at high levels of ecdysone (Karim and Thummel, 1991). As a consequence, E74B synthesis precedes E74A production during the rise in ecdysone that directs metamorphosis. This expression pattern suggests that these two E74 proteins play different roles in the control of *Drosophila* metamorphosis. Indeed, ectopic expression experiments and mutant studies suggest that E74B represses the same genes that E74 activates (Fletcher and Thummel, 1995).

C. Conclusions

One of the first steps in understanding the mechanistic basis of transcriptional activation and repression is the identification of regulatory transcription factors that mediate these processes. The *ets* family provides examples of both transcriptional activators and repressors. The *ets* proteins that function in activation have domains that are similar to the TADs characterized in other transcription factors. Although *ets* proteins that function in repression are less well-studied, an interesting regulatory strategy has been detected in which *ets* repressors function as antagonists of *ets* activators. Addi-

tional novel insights are expected as this area of investigation receives more attention.

To function as transcription factors the *ets* proteins must retain both a structural motif that binds DNA and also domains that mediate activation or repression. Although DNA binding requires a highly-conserved domain, more divergence is tolerated in the sequences that function in transcription. Activation and repression domains lie outside of the ETS domain and no sequences are conserved among all *ets* family members. Although we have noted that there is some conservation of sequence and position of activation domains within groups of highly related family members, the general picture is one of divergence. Thus, in the case of both activators and repressors, distinct mechanistic routes may be used by different *ets* proteins, suggesting that transcription function can provide some solution to the specificity problem.

VII. SIGNAL TRANSDUCTION

Numerous transcription factors are regulated by extracellular signals. This type of regulation is commonly mediated by posttranslational modifications, with phosphorylation being the best documented. Signal transduction pathways, whose components include cell surface receptors and kinase cascades, are being intensively investigated in many systems; however, complete pathways connecting receptors to nuclear factors are known in only a few cases. Signaling cascades modulate the availability or activity of a particular transcription factor by a variety of mechanisms. For example, phosphorylation can affect DNA binding, protein–protein interactions, and the function of transcription activation domains. The control of nuclear import and export of transcription factors also can be regulated by phosphorylation.

Signaling regulates the activity of a variety of *ets* proteins. Indeed, the *ets* family of transcription factors is remarkable for its frequent appearance in signal transduction pathways. We will focus on the *ets* proteins that have been placed in well-characterized pathways. In all of these cases, a MAP kinase (MAPK) modifies the *ets* protein. MAP kinases are the final step in signaling pathways that initiate with a receptor tyrosine kinase, then utilize GTPases (e.g., Ras) and serine/threonine kinases (e.g., RAF and MEK) to transduce the signal from the cell surface to a nuclear target. Members of the ETS, YAN, and ELK groups, from both invertebrates and vertebrates, are targets of MAPKs.

A. ELK Group Regulation

Members of the ELK group in mammals [Elk-1 and SAP-1a; also known as ternary complex factors (TCFs)] are regulated by MAP kinase signal-

ing pathways (for review, see Treisman, 1994). These proteins function in combination with the serum response factor (SRF) to mediate the responsiveness to growth factors. SRF forms a ternary complex with any of the TCFs on serum response elements (SREs) (see Section V for additional discussion). The activity of an SRE is stimulated by whole serum or specific growth factors, as well as pharmacologically by TPA. The best-characterized target gene is the early growth response gene c-*fos*. The TCFs show high sequence similarity in three regions: the ETS domain; the B box, which mediates interacting with SRF; and the C box, which includes the transactivation domain. Growth factor stimulation leads to phosphorylation within the C box, and the major effect of this modification is enhanced transcription activity.

Substantial data support this general picture for Elk-1 and SAP-1a (for review, see Treisman, 1994; also Hipskind *et al.*, 1994; Hill and Treisman, 1995; Janknecht *et al.*, 1995; Price *et al.*, 1995; Whitmarsh *et al.*, 1995); *In vitro* experiments show that the MAP kinase ERK phosphorylates the same sites modified *in vivo* in response to extracellular stimuli. Constitutively activated components of the Ras/MAPK signaling pathway (e.g., v-ras, ΔNraf, ERK^{D319N}) can stimulate Elk-1 and SAP-1a. Point mutations that disrupt sites of phosphorylation interfere with activation. Because *in vivo* footprinting data indicate that a ternary complex is present at the SRE in unstimulated cells, it was proposed that phosphorylation would affect only transactivation activity. Indeed, augmentation of transcription activation, independent of DNA binding, is clearly demonstrated by the study of chimeric transcription factors. The Elk-1 transactivation domain fused to a heterologous DNA-binding domain can respond to MAPK signaling. In this context the C box is modified and displays enhanced transcription activity (Hill *et al.*, 1993). In the case of Elk-1, phosphorylation also stimulates DNA binding in both the presence and the absence of SRF (Kortenjann *et al.*, 1994; Gille *et al.*, 1995, 1996; Shore *et al.*, 1996).

Interestingly, SAP-2/Net is distinct from Elk-1 and SAP-1a in its response to serum stimulation and ERK-dependent phosphorylation (Giovane *et al.*, 1994; Lopez *et al.*, 1994; Price *et al.*, 1995; Maira *et al.*, 1996). DNA-binding activity and ternary complex formation on the SRE is much reduced relative to ELK-1 and SAP-1. Phosphorylation-dependent activation of the Net C box is below the level observed with Elk-1 and SAP-1. In fact, whole serum fails to stimulate the TAD of Net when it is fused to a heterologous DNA-binding domain. It remains to be resolved whether a different regulatory pathway controls Net function.

The linkage of a kinase to its substrate is difficult in vertebrate systems. For example, multiple MAP kinases have overlapping substrate specificity. The issue of specificity among MAP kinases, p38, ERK, and JNK, and *ets* proteins Elk-1 and SAP-1a, has been explored (Cavigelli *et al.*, 1995; Gille *et al.*, 1995; Price *et al.*, 1995; Strahl *et al.*, 1996; Whitmarsh *et al.*, 1997).

Although all three kinases activate Elk-1, under most conditions only ERK and p38 activate SAP-1a; however, in one case it is reported that JNK can stimulate SAP-1a (Janknecht and Hunter, 1997).

The Ras/MAP kinase signaling pathway in *C. elegans* also targets an ELK group protein, Lin-1 (Beitel *et al.*, 1995). This pathway is important for many different developmental processes in *C. elegans;* however, Lin-1 is uniquely required in vulva cell lineage differentiation. At present no target for Lin-1 transcriptional activity is known and there is no molecular analysis of possible phosphorylation sites. Nevertheless, the genetic tools available in *C. elegans* should assist in addressing these issues and completing the signal transduction pathway from the receptor to the target gene.

B. ETS and YAN Group Regulation

Two *Drosophila ets* proteins, Pnt P2 and Yan, are targets of the Ras/MAP kinase pathway. These two transcription factors, as well as the other components of the Ras/MAPK cascade, were identified by genetic analysis of *Drosophila* photoreceptor cell differentiation (Lai and Rubin, 1992; Klambt, 1993; Brunner *et al.*, 1994; O'Neill *et al.*, 1994; Wassarman *et al.*, 1995). Both Pnt P2 and a truncated version of Yan are phosphorylated *in vitro* by a MAP kinase (Brunner *et al.*, 1994). A single MAPK site lies just N-terminal to the PNT domain in Pnt P2 (Fig. 3). There are nine potential MAPK sites in Yan; however, mutational analysis has indicated that only one site is crucial for regulation.

Genetic studies indicate that Yan represses photoreceptor differentiation and that Ras/MAPK signaling inactivates this repression. Both genetic and molecular analyses support a model in which phosphorylation causes the export of Yan out of the nucleus (Rebay and Rubin, 1995; Treier *et al.*, 1995). Subsequent degradation of Yan also may be regulated by the MAPK signaling. Genetic analyses indicate that Pnt P2 positively regulates *Drosophila* photoreceptor differentiation through the Ras/MAPK signaling pathway (O'Neill *et al.*, 1994). Pnt P2, the isoform of the *Pointed* locus, which retains the MAPK site and nearby PNT domain, is responsive to signaling cues; however, the mechanism of activation of Pnt P2 is not known. Pnt P2 cooperates with a second transcription factor, D-Jun, and a potential target gene for this cooperative partnership is *phyllopod* (Treier *et al.*, 1995). Thus, this *Drosophila* system provides a complete signaling pathway, with events at the cell surface being linked to specific events in the nucleus.

MAP kinase-dependent signaling pathways also regulate the mammalian ETS group proteins, Ets-1 and Ets-2. As in the case of Pnt P2 (Fig. 3), the consensus sequence for a MAP kinase phosphorylation site lies near the PNT domain of Ets-1 and Ets-2. This site in Ets-1 can be phosphorylated *in vitro*

with the MAP kinase ERK2 (Rabault *et al.*, 1996). Ras activation of intracellular signaling leads to an increase in transcriptional activity of Ets-1 and Ets-2 in transient expression assays. Both the super activation and *in vivo* phosphorylation are dependent on the integrity of the MAPK phosphorylation site (Yang *et al.*, 1996). Activated RAF also stimulates Ets-2 transcription activity. In this context MAPKs p42 and p44 specifically participate in Ets-2 phosphorylation (McCarthy *et al.*, 1997).

Similar to the case of Pnt P2, the mechanism by which phosphorylation activates Ets-1 and Ets-2 is not known. Phosphorylation could affect the transactivation activity of Ets-1 or Ets-2 because the site maps near the TAD of both proteins. However, effects on DNA binding or protein–protein interactions cannot be eliminated. Finally, it is not known which specific receptor tyrosine kinase initiates the signaling cascade that is responsible for the activation of Ets-1 and Ets-2 during normal control of growth and differentiation.

C. Ras-Responsive Elements

Ectopic expression of constitutively active Ras can stimulate expression of a diverse group of genes. Ras-responsive elements (RREs) within promoters and enhancers contain *ets* binding sites, suggesting that *ets* proteins play a role in *ras*-mediated transformation. Similar to the SRE, these RREs are usually composite elements. In one class of RRE, the *ets* binding site is flanked by an AP-1 site. This composite element is found in several transcriptional control regions, including the enhancer of the polyomavirus (Wasylyk *et al.*, 1990; Bruder *et al.*, 1992), the promoter of several metalloprotease genes [e.g., urokinase plasminogen activator (Stacey *et al.*, 1995) and collagenase (Gutman and Wasylyk, 1990)], as well as in the promoters of the macrophage scavenger receptor gene (Wu *et al.*, 1994) and the heparin-binding epidermal growth factor gene (McCarthy *et al.*, 1997). In contrast, the RRE of the prolactin promoter is composed of binding sites for *ets* proteins and Pit-1/GHF-1 (Bradford *et al.*, 1995, 1996; Howard and Maurer, 1995). Although the synergy between Ets-1 and GHF-1 does not require the MAPK phosphorylation site in Ets-1 (Bradford *et al.*, 1997), Ras stimulates the transcription activity of this partnership. A variety of other *ets* proteins that are not in the ETS, ELK, or YAN groups also have been implicated in Ras/MAPK pathways by preliminary studies [e.g., ERM (Janknecht *et al.*, 1996), GAB-Pα (Flory *et al.*, 1996), ER81 (Janknecht, 1996), and ERF (Sgouras *et al.*, 1995)]. Thus, it remains unresolved precisely which *ets* proteins function on RREs and participate in *ras*-mediated transformation.

Additional phosphorylation events are discussed in other sections of this review. These include the modulation of the PU.1–Pip partnership by phos-

phorylation of PU.1 (see Section V) and the modulation of the DNA binding of Ets-1 by calcium-dependent signaling pathways (see Section VIII). The growing list of *ets* proteins that respond to signaling strongly indicates that this is a major regulatory pathway for the *ets* family.

D. Conclusions

We have summarized evidence that the Ras/MAPK signaling pathways modulate the activity of *ets* proteins. The data are most extensive for members of the ELK, ETS, and YAN groups; nevertheless, members of other groups are likely to be modulated by signaling input. Posttranslational modifications can augment the specificity of individual *ets* family members. Although multiple *ets* proteins may be expressed in a particular cell type, their specific activities may be differentially regulated by the responsiveness of the cell to signaling cues. Because sites of phosphorylation lie outside of the most highly conserved ETS domain, the responsiveness to a particular signaling cascade can be distinct among the different *ets* proteins. We described an extreme example of this differential regulation in the case of Pnt P2 and Yan in which phosphorylation inactivates one factor and stimulates the activity of another. Thus, the link of *ets* proteins to signal transduction pathways provides a route to enhanced specificity.

The regulation of *ets* proteins by signaling pathways is an exciting and fast-moving direction with many unanswered questions. It will be interesting to determine whether other signaling pathways also regulate members of the *ets* family and whether *ets* protein activities are modulated by phosphorylation in novel ways. The available biochemical and genetic tools should bring excellent progress in this area within the next few years.

VIII. AUTOINHIBITION

Transcription factors are modular proteins with individual domains for DNA binding, transactivation, and subunit association. Additional regions exist within transcription factors that negatively regulate these functional domains. Such inhibitory regions are defined by their absence; the activity of a domain is enhanced when the inhibitory sequences are removed. In this way, individual domains of transcription factors can be repressed, most likely through intramolecular interactions, until a regulatory pathway inactivates the autoinhibition.

The *ets* family has emerged as a model system to study the phenomenon of intramolecular inhibition. Both DNA binding and transactivation are neg-

atively regulated by an autoinhibitory mechanism in a variety of *ets* proteins. Protein–protein interactions between cooperative DNA-binding partners, as well as posttranslational modifications, have been implicated in derepression. Thus, autoinhibition is linked to other control pathways that regulate the specificity of *ets* proteins.

A. DNA Binding

In at least six members of the *ets* family, the intrinsic DNA binding activity of the ETS domain is repressed by inhibitory sequences. Deletion and partial proteolysis experiments demonstrate that the *ets* proteins SAP-1, Elk-1, Net, Ets-1, Ets-2, and ERM contain regions that negatively regulate DNA binding (Dalton and Treisman, 1992; Hagman and Grosschedl, 1992; Lim *et al.*, 1992; Rao and Reddy, 1992; Wasylyk *et al.*, 1992; Fisher *et al.*, 1994; Giovane *et al.*, 1994; Janknecht *et al.*, 1994; Lopez *et al.*, 1994; Petersen *et al.*, 1995; Price *et al.*, 1995; Jonsen *et al.*, 1996; Laget *et al.*, 1996; Maira *et al.*, 1996). Although inhibitory sequences that negatively regulate DNA binding and transactivation have been discovered in a significant number of other transcription factors, including p53 (Hupp *et al.*, 1992), HSF (Green *et al.*, 1995), and C/EBPβ (Williams *et al.*, 1995), no other family of transcription factors has been shown to use autoinhibition as such a prominent mechanism for regulating DNA binding activity.

The most complete picture of how autoinhibition can regulate DNA binding comes from molecular and structural studies of Ets-1. Two regions of Ets-1, N- and C-terminal to the ETS domain, act together to decrease the DNA-binding affinity 10- to 20-fold (Hagman and Grosschedl, 1992; Lim *et al.*, 1992; Wasylyk *et al.*, 1992; Fisher *et al.*, 1994; Petersen *et al.*, 1995; Jonsen *et al.*, 1996). Three α helices (HI-1, HI-2, and H4) that are located in the N- and C-terminal inhibitory regions interact with the first helix of the ETS domain (H1) to form an inhibitory module (Fig. 9) (Donaldson *et al.*, 1996; Skalicky *et al.*, 1996). Thus, the two inhibitory regions are both structurally and functionally linked. Insight into the mechanism of inhibition came from the finding that an α helix within the N-terminal inhibitory region unfolds on DNA binding (Petersen *et al.*, 1995). These data indicate that DNA binding leads to disruption of the inhibitory domain. Thus, DNA binding and the integrity of the inhibitory module appear to be mutually exclusive; DNA binding disrupts the inhibitory domain and reestablishment of the inhibitory module destabilizes DNA binding. The recent finding that helix H1 of the ETS domain is directly involved in DNA binding provides a possible mechanism for this model. The DNA contacts made by H1 may preclude intramolecular contacts between this helix and the inhibitory helices (HI-1, HI-2, and H4).

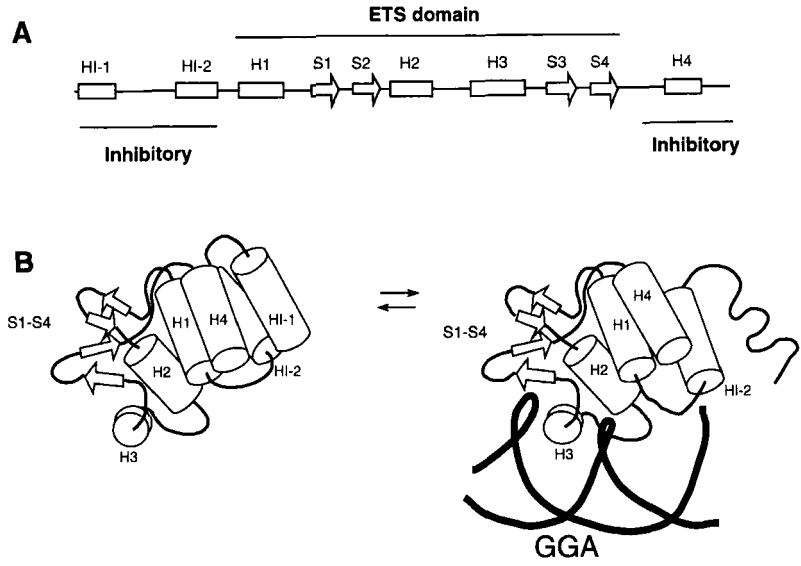

Fig. 9 Model of autoinhibition within the Ets-1 protein. (A) Secondary structure elements of the Ets-1 ETS domain and autoinhibitory sequences (Donaldson *et al.*, 1996; Skalicky *et al.*, 1996). The α helices are depicted as rectangles and the β strands as arrows. (B) Tertiary structure of the Ets-1 inhibitory module and the ETS domain in the presence and absence of DNA as based on NMR-derived structural data and biochemical analyses. Orientation on DNA is modeled from structural analyses of PU.1–DNA (Kodandapani *et al.*, 1996) and Ets-1–DNA complexes (Werner *et al.*, 1995). In the absence of DNA (left panel), the inhibitory module exists as a four-helix-like bundle structure composed of the N- and C-terminal inhibitory helices (HI-1, HI-2, and H4) as well as H1. Note that H1 is also a component of the ETS domain, thus structurally coupling inhibition and DNA binding. On binding DNA (right panel), disruption of the inhibitory module occurs and HI-1 unfolds (Petersen *et al.*, 1995; Jonsen *et al.*, 1996).

Figure 10 shows the location of inhibitory sequences that autoregulate DNA binding. The number and positions of the autoinhibitory regions vary among the family members. These variations may reflect differences in the mechanisms of autoinhibition. Indeed, in contrast to Ets-1, the flanking inhibitory regions within ERM do not function cooperativly to mediate inhibition (Laget *et al.*, 1996). Furthermore, the autoinhibitory domain within Net is distinct from the other ELK group proteins, Elk-1 and SAP-1 (Price *et al.*, 1995; Maira *et al.*, 1996). Moreover, sequences responsible for mediating inhibition are only conserved within highly-related *ets* genes. The inhibitory sequences of SAP-1 and Elk-1 lie in the conserved B box. Likewise, the inhibitory regions of Ets-1 and Ets-2 display sequence similarity. In contrast, there is no sequence conservation among inhibitory regions from the different gene groups, PEA3 (ERM), ELK (Elk-1, SAP-1 and Net), and ETS (Ets-1 and Ets-2).

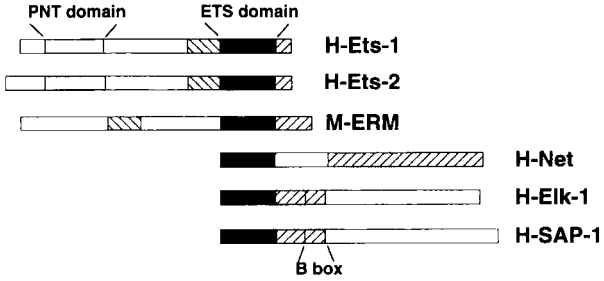

Fig. 10 Autoinhibitory sequences within *ets* proteins. Sequences that mediate repression of DNA binding are indicated by the stippled areas (Dalton and Treisman, 1992; Petersen *et al.*, 1995; Price *et al.*, 1995; Jonsen *et al.*, 1996; Laget *et al.*, 1996; Skalicky *et al.*, 1996). The ETS domain is solid black. The PNT domain is indicated in gray. Note the differences in numbers of inhibitory regions and their positioning with respect to the ETS domain. Note also that the B box in Elk-1 and SAP-1 mediates both DNA binding autoinhibition and protein–protein interactions with SRF. Additional mapping of Ets-1, Ets-2, Elk-1, and Net autoinhibitory sequences has been performed and yields results similar to those illustrated here (Hagman and Grosschedl, 1992; Lim *et al.*, 1992; Rao and Reddy, 1992; Wasylyk *et al.*, 1992; Fisher *et al.*, 1994; Giovane *et al.*, 1994; Janknecht *et al.*, 1994; Lopez *et al.*, 1994; Maira *et al.*, 1996).

The frequent repression of DNA binding within the *ets* family is intriguing. It will be interesting to determine whether a conserved feature of the ETS domain is targeted in each case. Perhaps the high degree of sequence conservation found within the ETS domain and the high affinity of DNA binding of the isolated ETS domain require this additional level of regulation. Consistent with this idea is the absence of autoinhibitory regions in PU.1, one of the most highly divergent vertebrate member of the *ets* family.

B. Activation

Transcriptional activation is also regulated by autoinhibition. ERM is the best-studied case (Laget *et al.*, 1996). To map an ERM transactivation domain, a heterologous DNA-binding domain from the yeast protein GAL4 was fused to ERM. On deletion of the ERM ETS domain, transactivation by the fusion protein increases ~20-fold. Disruption of the DNA-binding activity of the ETS domain has no effect on the repression. Thus, the ETS domain acts as a negative regulator of ERM transcriptional activity by a mechanism that is independent of DNA binding. Autoinhibition of the Elk-1 TAD is also detectable, although at a more modest level. An Elk–GAL4 fusion protein has threefold higher activation activity when the ETS domain is deleted (Janknecht *et al.*, 1994). Similar findings are reported for Ets-1 and Ets-2

(Schneikert *et al.*, 1992; Chumakov *et al.*, 1993). This suggests that within the *ets* proteins ERM, Elk-1, Ets-1, and Ets-2, intramolecular coupling of the TAD and DNA-binding domains occurs to regulate transactivation. It will be interesting to investigate whether a conformational change is part of this inhibitory mechanism.

C. Derepression of Inhibition

Intramolecular inhibition provides a pathway for regulation of *ets* proteins. For some members of the *ets* family, derepression of DNA binding inhibition is directly coupled to intermolecular protein–protein interactions. The full-length proteins, SAP-1a and Elk-1, bind the SRE weakly or not at all (Dalton and Treisman, 1992; Rao and Reddy, 1992; Janknecht *et al.*, 1994; Price *et al.*, 1995). Deletion of the inhibitory sequence element, the B box, activates SAP-1a and Elk-1 autonomous binding. The full-length SAP-1a and Elk-1 proteins also have high affinity for the SRE in the presence of SRF. These findings indicate that release of inhibition occurs on ternary complex formation with SRF (see Section V). Interestingly, the B box containing the inhibitory domain also mediates ternary complex formation with SRF. Thus, the B box participates in two different protein–protein interactions: intramolecular interactions that mediate autoinhibition and intermolecular interactions that are required for cooperative DNA binding with SRF. Mutation of amino acids required for SRF interaction does not release autoinhibition, implying that the two functions require different residues of the B box (Ling *et al.*, 1997).

Derepression of transactivation autoinhibition within ERM also may be coupled to intermolecular protein–protein interactions. Jun has been shown to enhance the transactivation activity of ERM in assays utilizing the ERM–GAL4 fusion proteins (Nakae *et al.*, 1995). Enhancement requires both the transactivation domain and the ETS domain. An interaction between Jun and ERM has been demonstrated and mapped to the carboxyl-terminal portion of ERM, which contains the ETS domain. In a model similar to that proposed for Elk-1 and SRF, intermolecular interactions between Jun and the ERM inhibitory domain release autoinhibition.

Posttranslational modifications also provide a mechanism for derepression of autoinhibition. In the case of Elk-1, effects of phosphorylation on inhibition are superimposed on the regulation of autoinhibition by SRF interaction. As discussed above, Elk-1 is phosphorylated via a MAP kinase pathway. Phosphorylation of Elk-1 enhances ternary complex formation with SRF on the c-*fos* SRE (Gille *et al.*, 1995). A calcium-dependent signaling pathway appears to regulate the DNA binding activity of Ets-1. However, in this case modification lowers the DNA binding activity (Rabault and Ghys-

dael, 1994). Importantly, the sites of phosphorylation are clustered near and within the N-terminal inhibitory region (Fig. 9). Therefore, phosphorylation could be a mechanism to stabilize the inhibitory module and reinforce repression. Due to this added repression, regulatory pathways that activate DNA binding may be very important to Ets-1 function.

D. Conclusions

Many routes to specificity of *ets* proteins converge at the level of autoinhibition. Two major functions, DNA binding and transactivation, are inhibited. By adding a layer of regulation to these vital functions, the activities of *ets* proteins can be differentially modulated. In the characterized cases, inhibitory sequences lie outside of the highly conserved ETS domain; thus, unique pathways for derepression are easily envisioned. Protein partnerships and signaling cascades play roles in derepression. Autoinhibition strengthens the requirement for a protein partner. The low DNA binding activity of the repressed protein may prevent any biological activity until a partner is present. Regulation of inhibition by signaling pathways provides a mechanism by which protein modifications can affect functions such as DNA binding and transactivation. Linkage of protein modifications to autoinhibition provides regulatory pathways that can be unique for each *ets* protein. Thus, autoinhibition can contribute to the biological specificity of *ets* proteins.

IX. PERSPECTIVES: *ets* PROTEINS AND CANCER

The review of *ets* proteins as transcription factors provides a framework for understanding their role in human cancer. The route to oncogenesis by a transcription factor is likely to be indirect, functioning through a change in expression of target genes. Although we do not yet have a list of what these target genes may be for the *ets* family, we can predict the routes to misregulation. We have described how the accurate control of gene expression by *ets* proteins is dependent on a large number of regulatory steps. Specificity for a particular target gene relies first on the DNA–protein interaction, which is set both by the affinity and the specificity of the interaction as well as the availability of active molecules in the nucleus. Additional layers of regulation, including protein partnerships, modifications directed by signaling pathways, and autoinhibitory sequences further limit the range of function of *ets* proteins. To summarize, we use Elk-1, which illustrates all of these levels of control (Fig. 11). Functional domains of Elk-1 include the ETS domain for DNA binding, the B box for protein–protein interactions, and the C box

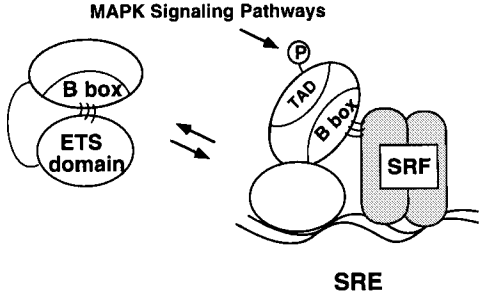

Fig. 11 Multiple levels of control of Elk-1. Regions of Elk-1, including the DNA-binding domain (ETS domain), autoinhibitory sequences (B box), a protein partner interaction domain (B box), and the transcriptional activation domain (C box), regulate Elk-1 activity at the serum response element (SRE). Phosphorylation adds an additional level of control. In the model, autonomous DNA binding of Elk-1 is negatively regulated by the autoinhibitory sequences within the B box. For simplicity, we have depicted the B box interacting with the ETS domain. Cooperative DNA binding with the serum response factor (SRF) to the SRE releases autoinhibition; direct protein–protein contacts occur between the Elk-1 B box and the SRF to mediate this derepression. Phosphorylation of the C box directed by the Ras/MAPK signaling pathway increases the interaction between SRF and Elk-1 and also stimulates Elk-1 transcriptional activity. The conformational change that accompanies Elk-1 binding to the SRE is based on data from Ets-1 showing that a conformational change occurs within the inhibitory domain on DNA binding. TAD, Transactivation domain.

for transactivation. DNA binding is negatively regulated by an autoinhibitory pathway that requires the B box and the ETS domain such that Elk-1 cannot efficiently bind DNA in the absence of a protein partner. Derepression involves cooperative DNA binding with the protein partner SRF; the B box is necessary for this protein–protein interaction. Phosphorylation of the C box in response to Ras/MAPK signaling stimulates the activation function of Elk-1 and also increases the efficiency of the SRF interaction and DNA binding.

The numerous layers of control that modulate *ets* protein specificity provide a variety of routes for misregulation and, thus, altered gene expression. Overexpression, as observed in the case of insertional activation by retroviruses, is a simple scenario in which the higher concentration of an *ets* protein leads to DNA binding at new sites or binding to sites independent of a partner protein. Loss of an autoinhibitory domain, as might happen in chromosome rearrangements, provides another route. Derepression of DNA binding or transactivation activity would then lead to misregulation of target genes. Finally, alteration of signaling cascades could affect *ets* protein activity. In this case an *ets* protein could play a role in oncogenesis without being genetically altered or expressed at abnormal levels. Recall the case in which mutation in *ras* lead to changes in gene expression of *ets* target genes

with Ras-responsive elements. In conclusion, all of the regulatory strategies that provide specificity for *ets* proteins could be altered such that target gene expression is affected. Thus, oncogenetic transformation by *ets* proteins likely involves the disruption of one of the many control mechanisms that generate specificity.

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NOTE ADDED IN PROOF: The crystallographic analysis of the GABP heterodimer in complex with DNA provides new insights into the topics covered in Sections IV, V, and VIII of this review (Batchelor *et al.*, 1998; Graves, 1998).

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Kaposi's Sarcoma-Associated Herpesvirus

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Kaposi's sarcoma (KS) is a vascular tumor predominantly found in the immunosuppressed. Epidemiologic studies suggest that an infective agent is the etiologic culprit. Kaposi's sarcoma-associated herpesvirus (KSHV), or human herpesvirus-8 (HHV-8), is a gamma human herpesvirus present in all epidemiologic forms of KS and also in a rare type of a B cell lymphoma, primary effusion lymphoma (PEL). In addition, this virus is present in most biopsies from human immunodeficiency virus (HIV)-associated multicentric Castleman's disease (MCD). MCD is a lymphoproliferative disorder with, like KS, a prominent microvasculature. The genome of KSHV contains the expected open reading frames (ORFs) encoding for enzymes and viral structural proteins found in other herpesviruses, but it also contains an unprecedented number of ORFs pirated during viral evolution from cellular genes. These include proteins that may alter cellular growth (e.g., Bcl-2 and cyclin homologs), induce angiogenesis (e.g., chemokine, chemokine receptor, and cytokine homologs), and regulate antiviral immunity (e.g., CD21 and interferon regulatory factor homologs). No ORF with sequence similarity to the Epstein–Barr

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nuclear antigens (EBNAs) and latent membrane proteins (LMPs) of Epstein–Barr virus (EBV) is present, but proteins analogous to these in structure and in latent expression are found [e.g., ORF 73 encoding for KSHV latent nuclear antigen (LNA-1) and K12 encoding for a possible latent membrane protein]. Current serologic assays confirm the strong association of infection with KSHV and risk of KS development. The mechanism of how this new virus may trigger the precipitation of KS is still unclear.

I. INTRODUCTION

A. History

For over 100 years, Kaposi's sarcoma remained a rare curiosity to clinicians and cancer researchers, until it shot to prominence as the sentinel of what we now call the acquired immune deficiency syndrome (AIDS). In 1872, the Hungarian dermatologist Moriz Kaposi published the case histories of five middle-aged and elderly male patients in Vienna with idiopathic multiple pigmented sarcomas of the skin (Kaposi, 1872). All these patients initially presented with purplish skin nodules on the lower extremities, and two patients died from disseminated disease. This form of the disease was eponymously designated Kaposi's sarcoma in 1891 at the suggestion of another prominent dermatologist of the time, Köbner, and is now called classic KS.

Classic KS occurs predominantly in elderly male patients of Southern European ancestry (Franceschi and Geddes, 1995). A high frequency is also seen in Israel and other Middle Eastern countries. This form of the disease is generally not as aggressive as the form originally described by Kaposi, for unknown, possible immunological, reasons.

In some equatorial countries of Africa, KS has existed for many decades, long preceding HIV (known as endemic KS) (Oettle, 1962). This form is found in younger patients as well as in the elderly; the male:female ratio is >3:1. It is generally a more aggressive disease than classic KS, though less so than African AIDS-associated KS (Bayley, 1984) (Wabinga *et al.*, 1993). In particular, endemic KS in African children is associated with predominant lymph node involvement with rarer skin lesions. The majority of these children die from the disease (Ziegler and Katongole Mbidde, 1996). In most of the African countries where KS occurs, malaria is also found, suggesting a possible immunological mechanism similar to that seen with malaria and Burkitt's lymphoma.

During the past 20 years, the incidence of KS among renal transplant recipients and other patients receiving immunosuppressive therapy has increased (known as posttransplant KS or iatrogenic KS). Patients of Mediterranean, Jewish, or Arabian ancestry are also clearly overrepresented among immunosuppressed patients who develop KS after a transplant (Franceschi

and Geddes, 1995), indicating that those born in countries where classic KS occurs continue to be at risk of developing KS even if they migrate to "low-risk" countries. These data suggest that there is a genetic predisposition or environmental factor (possibly an infectious agent) responsible for KS development.

In 1981, the U.S. Centers for Disease Control and Prevention (CDC) became aware of an increased occurrence of two rare diseases in young gay men from New York City and California (Service, 1981): Kaposi's sarcoma and *Pneumocystis carinii* pneumonia (PCP). This was the beginning of what is today known as the AIDS epidemic and AIDS-KS is today the most common form of KS. In HIV-infected individuals the underlying immunosuppression leads to a fulminant disease that starts with a few skin lesions, but without treatment often develops into disseminated disease affecting various organs, including lung, liver, gut, and spleen.

B. Kaposi's Sarcoma Histogenesis and Clonality

Histologically, KS is a complex lesion (Fig. 1, see color plate). In early KS lesions, which normally appear on the skin, there is a collection of small, irregular endothelial-lined spaces that surround normal dermal blood vessels and these are accompanied by a variable, inflammatory infiltrate of lymphocytes (patch stage). This stage is followed by the expansion of a spindle-celled vascular process throughout the dermis. These spindle cells form slit-like vascular channels containing erythrocytes (plaque stage). The later nodular-stage KS lesions are composed of sheets of spindle cells, some of which are undergoing mitosis, and slitlike vascular spaces with areas of hemosiderin pigmentation. The spindle cells form the bulk of established KS lesions and are therefore thought to be the neoplastic component, but there is still some controversy over the histogenesis of spindle cells. Although the majority of the spindle cells stain positive for endothelial cell markers, including factor VIII and CD34, some cells express proteins characteristic of smooth muscle cells, macrophages, or dendritic cells (Nickoloff and Griffiths, 1989; Stürzl *et al.*, 1992). Some spindle cells simultaneously express antigenic determinants characteristic of several different cell types, suggesting that KS spindle cells might be derived from a pluripotent mesenchymal progenitor cell or a mesenchymal cell experiencing dysregulated differentiation. Circulating KS-like spindle cells have been isolated and cultured from patients with AIDS-KS and from those thought for other reasons to be at risk of AIDS-KS (Browning *et al.*, 1994). These circulating cells have an adherent phenotype and express markers of both macrophage and endothelial cells (Sirianni *et al.*, 1997).

The nature of KS remains controversial regarding whether it is a neoplas-

tic lesion or a reactive process. The exact tumor cell type is still controversial, and especially in early lesions the "tumor cell" compartment makes up the minority of the tumor bulk, the majority of cells being inflammatory cells. Furthermore, the clinical presentation of multiple skin lesions in a defined distribution and the spontaneous remission of lesions also favor a reactive hyperplasia rather than a true malignancy.

A useful marker for clonality is the inactivation pattern of X chromosomes (Vogelstein *et al.*, 1985). In females, one of the two X chromosomes in each cell is inactivated by condensation and DNA methylation and the other one remains active. This inactivation occurs in an early stage of embryonic development at random, and the same methylation pattern is passed to daughter cells in somatic replication. Normal tissues in females are thus composed of cellular mosaics, differing only in which of the two X chromosomes has been inactivated. In neoplasms derived from a single cell, all of the tumor cells would retain the same X chromosome inactivation pattern. In polyclonal tissues, approximately half the cells have a methylated X chromosome from one parent, and half have a methylated X chromosome from the other parent.

Using this marker for clonality, Rabkin and colleagues were the first to show that individual KS lesions are probably clonal (Rabkin *et al.*, 1995). A further study, however, indicated in skin lesions, including four with nodular KS, where more than 70% of the cells were spindle cells, a polyclonal pattern of inactivation (Delabesse *et al.*, 1997). Most recently Rabkin *et al.* (1997) showed that multiple lesions in the same patient were monoclonal, indicating that KS is a disseminated monoclonal cancer and that the changes that permit the clonal outgrowth of spindle cells occur before the disease spreads. These studies need confirmation.

Early KS (patch stage) is probably a nonclonal proliferation of endothelial cells or endothelial precursors (e.g., angioblasts) (Risau, 1997) with a prominent inflammatory and angiogenic response, whereas advanced disease may sometimes develop as a true clonal malignancy with metastases of clonally derived spindle cells to different sites. This hypothesis is comparable to the scenario in immunodeficient individuals with EBV-driven polyclonal lymphoproliferation, which can progress to clonal B cell lymphomas.

C. Cytokines

All known tumors produce cytokines and their cells respond positively or negatively to cytokines in culture. KS is no exception and *in vitro* and *in vivo* high local levels of cytokines are produced, including interleukin, basic fibroblast growth factor, and tumor necrosis factor (IL-6, bFGF, TNF-α), oncostatin M, and γ-interferon (IFN-γ) (Ensoli *et al.*, 1989, 1994; Miles *et al.*, 1990; Nair *et al.*, 1992; Salahuddin *et al.*, 1988; Samaniego *et al.*, 1995). In

particular IL-6, bFGF, and IFN-γ have been shown to be angiogenic *in vitro* and in some *in vivo* assays. IL-6 is produced by KS spindle cells and exogenous IL-6 can also enhance the proliferation of KS cells in culture (Miles *et al.*, 1990). Because of the nature of KS lesions it has been suggested that such lesions are "cytokine driven."

The more aggressive nature of HIV-associated KS has led to speculation that HIV-encoded proteins may enhance KS growth (Ensoli *et al.*, 1994). The HIV-1 Tat protein transactivates HIV viral genes and also some host cell genes (Vaishnaw and Wong-Staal, 1991). Tat can be released by infected cells and can act extracellularly (Frankel and Pabo, 1988; Ensoli *et al.*, 1993). Tat can induce a functional program in endothelial cells related to angiogenesis and inflammation, including the migration, proliferation, and expression of plasminogen activator inhibitor-1 and E selectin (Albini *et al.*, 1995). Tat induces growth of KS spindle cells *in vitro* and is angiogenic *in vivo* and in transgenic mice (Vogel *et al.*, 1988; Ensoli *et al.*, 1993, 1994). The Tat basic domain contains an arginine- and lysine-rich sequence that is similar to that of other potent angiogenic growth factors, including vascular endothelial growth factor-A (VEGF-A) and bFGF (Albini *et al.*, 1996). Tat specifically binds and activates the Flk-1/kinase domain receptor (Flk-1/KDR), a VEGF-A tyrosine kinase receptor (Albini *et al.*, 1997). Tat-induced angiogenesis can be inhibited by agents blocking this receptor (Albini *et al.*, 1997). The RGD-containing region of Tat has also been postulated to have a role in the pathogenesis of AIDS-KS; however, baboons infected with HIV-2, whose Tat lacks an RGD sequence, can develop KS-like lesions, albeit of myofibroblast, rather than endothelial, origin (Ensoli *et al.*, 1994; Barnett *et al.*, 1994). AIDS-associated KS is frequently more aggressive than non-HIV-related KS and it is possible that the angiogenic properties of Tat contribute to this phenomenon.

D. An Infectious Cause?

Studies of AIDS case surveillance support the pre-AIDS data on the existence of a sexually transmissible KS cofactor: KS occurs predominantly in gay and bisexual men with AIDS, less commonly in those acquiring HIV through heterosexual contact, and rarely in AIDS patients with hemophilia or in intravenous drug users (Beral, 1991).

E. Viruses

A viral etiology for this tumor was suspected long before the onset of the AIDS epidemic (Oettle, 1962). In 1972, the herpesvirus-like particles found by electron microscopy in KS tumor cells were attributed to cytomegalovirus

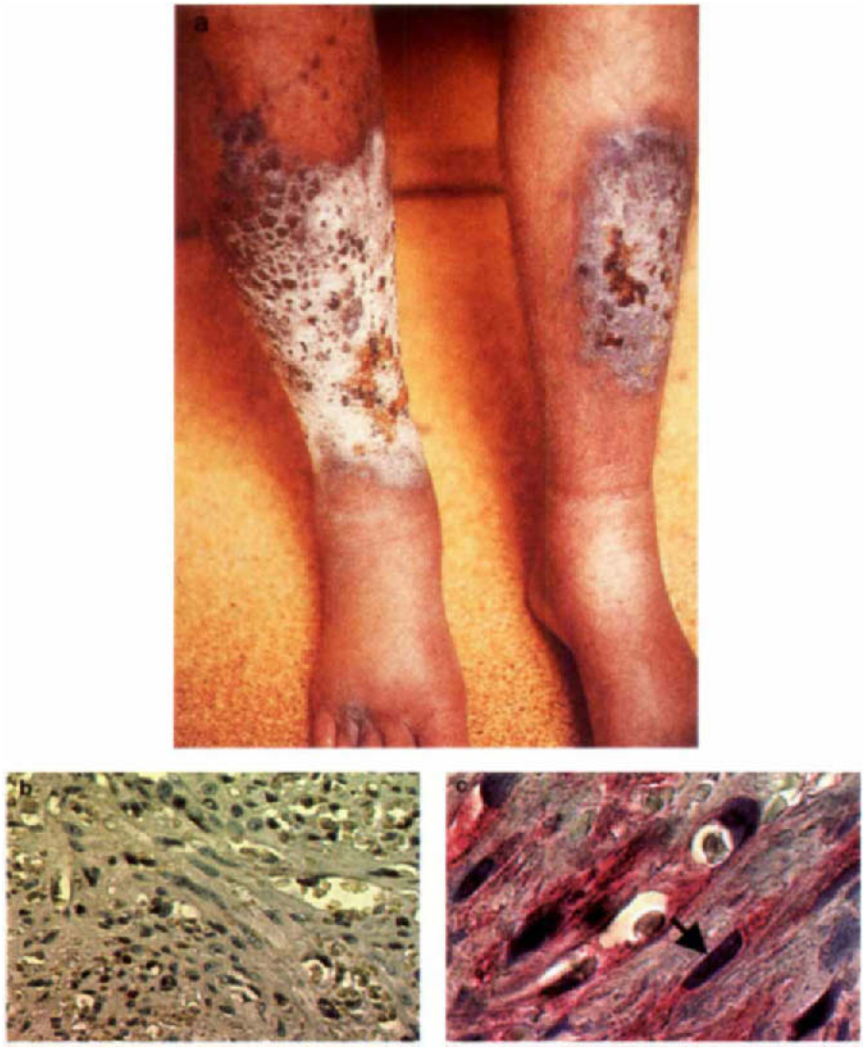

Fig. 1 (a) Kaposi's sarcoma lesions on the legs of a patient with classic KS; (b) nodular KS lesions showing numerous spindle-shaped cells, vascular spaces, and extravasated red blood cells; (c) KS spindle cells (arrow) stained for VEGF (magenta).

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(CMV) (Giraldo *et al.*, 1972, 1975). DNA sequences of CMV, human herpesvirus-6 (HHV-6), human papilloma viruses (HPV), and BK virus (human polyoma virus) and other viral (including retroviral) or bacterial pathogens have all been detected in KS lesions and put forward as the suspected agents of KS (Monini *et al.*, 1996b; Rappersberger *et al.*, 1990; Huang *et al.*, 1992). However, these agents, including CMV, HHV-6, and papilloma viruses are found only in some lesions, and BK virus is a ubiquitous agent present in many tumors and tumor cell lines (Monini *et al.*, 1996b; Kempf *et al.*, 1995; Huang *et al.*, 1992).

II. NEW HERPESVIRUS: KSHV/HHV-8

Chang *et al.* (1994) employed representational difference analysis (RDA) to identify sequences of a new herpesvirus (Kaposi's sarcoma-associated herpesvirus, or human herpesvirus-8) in AIDS-KS biopsies. RDA relies on cycles of subtractive hybridization and polymerase chain reaction (PCR) amplification to enrich and isolate rare DNA fragments that are present in only one of two otherwise identical populations of DNA. This powerful technique can detect small differences between complex genomes and has been used to identify DNA amplifications in tumor tissues and the lack of tumor suppressor genes in cancers (Lisitsyn *et al.*, 1993, 1995). This new virus is a gamma herpesvirus (Moore *et al.*, 1996a) (genus *Rhadinovirus*) with sequence similarity to the oncogenic viruses herpesvirus saimiri (HVS), Epstein–Barr virus (EBV), and murine herpesvirus 68 (MHV 68). These herpesviruses are transforming viruses capable of causing tumors in their natural hosts or in experimental animals (Table I).

Table I Gamma Herpesviruses Related to KSHV

Virus	Host	Associated tumors
Epstein–Barr virus (EBV)	Humans	Burkitt's lymphoma Hodgkin's disease Posttransplant lymphoproliferations Nasopharyngeal carcinoma
Herpesvirus saimiri (HVS)	New World monkeys	T cell lymphoproliferations Lymphosarcomas
Retroperitoneal fibrosis herpesvirus (RPHV)	Old World monkeys	Retroperitoneal fibrosis
Murine herpesvirus 68	Mice	Lymphoproliferations

A. Genomic Organization and Structure

The genome of KSHV was mapped with cosmid and phage genomic libraries from one of these PEL cell lines (BC-1, which also contains EBV) and was sequenced (Russo *et al.*, 1996). The BC-1 KSHV genome consists of an estimated 140.5-kb long unique coding region (LUR) flanked by approximately 800 bp of noncoding tandemly repeated units with an 85% G + C content. A total of 81 open reading frames have been identified, including 66 with sequence similarity to HVS ORFs. Novel ORFs not present in other herpesviruses are designated K1 to K14. Cellular homolog genes incorporated into the genome of KSHV are discussed in Section IV. In addition, ORFs encoding proteins that could be targets for antiherpesviral agents are present, including thymidylate synthase and thymidine kinase homologs (Moore *et al.*, 1996a).

Electron microscopy of KSHV particles have revealed classic herpes-type virions (Fig. 2).

B. Molecular Detection

The first indication that KSHV is indeed involved in KS pathogenesis was the detection of KSHV DNA by the polymerase chain reaction in all forms of the disease and in all fresh biopsies tested (and in the vast majority of paraffin-embedded material) (Table II) (Boshoff *et al.*, 1995a,b; Moore and Chang, 1995; Schalling *et al.*, 1995; Dupin *et al.*, 1995b). The virus is rarely detectable in non-KS tissues (except blood) from the same individual, indicating that viral load is highest in KS lesions. This raises the question of whether the cytokine-rich milieu of KS encourages KSHV replication or the proliferation of the cell type that harbors KSHV, in which case, KSHV might only be a "passenger" in these lesions and not necessarily the cause of them. However, KSHV is not present in other vascular tumors, including angiomas and angiosarcomas, or in granulomas containing angiogenic cells, and it is only rarely detectable in other forms of skin tumors (including squamous carcinomas and melanomas) in immunosuppressed patients (Adams *et al.*, 1995; Boshoff *et al.*, 1996; Uthman *et al.*, 1996). Furthermore the detection of KSHV DNA by PCR in the peripheral blood of HIV-positive individuals predicts who will subsequently develop KS (Moore *et al.*, 1996b; Whitby *et al.*, 1995), indicating that those at risk of KS have a higher viral load than those not at risk (Fig. 3).

Although one group reported the frequent detection of KSHV in the semen of healthy Italian donors (Monini *et al.*, 1996a), in North America and the United Kingdom KSHV is not detectable in semen donors and only rarely in semen of patients with KS (Ambroziak *et al.*, 1995; Tasaka *et al.*, 1996;

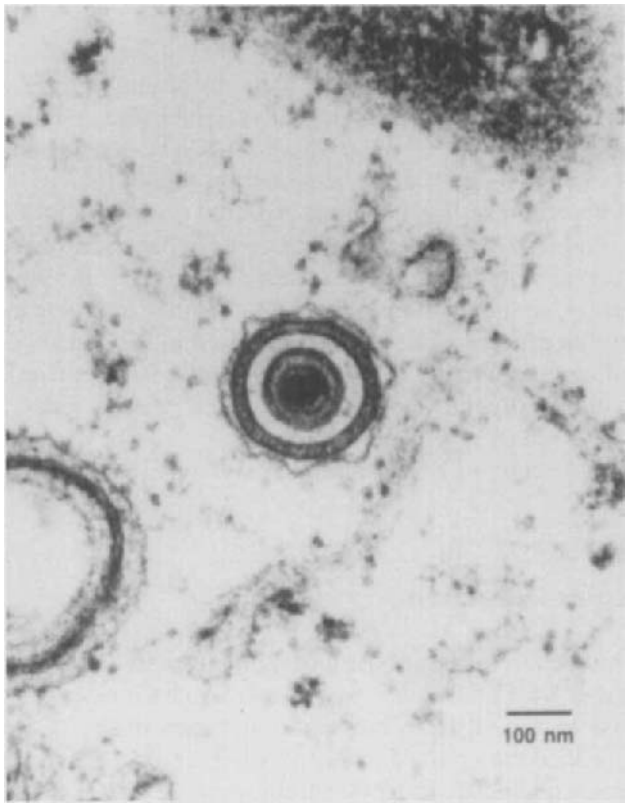

Fig. 2 A KSHV viral particle demonstrating the core, envelope, and tegument typically associated with herpesviruses. Kindly provided by D. V. Ablashi and J. E. Whitman, ABI Advanced Biotechnologies Incorporated.

Table II PCR Detection of KSHV DNA in Biopsies[a]

Type of KS lesion	Number positive	Number tested	Percentage positive
AIDS-KS	252	259	97%
Classic KS	160	175	91%
Iatrogenic KS	13	13	100%
African endemic	71	80	89%
HIV-negative gay men with KS	8	9	89%
Control tissues	14	743	1.8%

[a] Data compiled from Chang *et al.* (1994, 1996), Su *et al.* (1996), Huang *et al.* (1995), Dupin *et al.* (1995), Boshoff *et al.* (1995b), Ambroziak *et al.* (1995), Moore and Chang (1995), Lebbe *et al.* (1995, 1997), Schalling *et al.* (1995), Chuck *et al.* (1996), O'Neill *et al.* (1996), Buonaguro *et al.* (1996), Cathomas *et al.* (1996), Gaidano *et al.* (1996), Jin *et al.* (1996), Dictor *et al.* (1996), Marchioli *et al.* (1996), Luppi *et al.* (1996), McDonagh *et al.* (1996), and Corbellino *et al.* (1996).

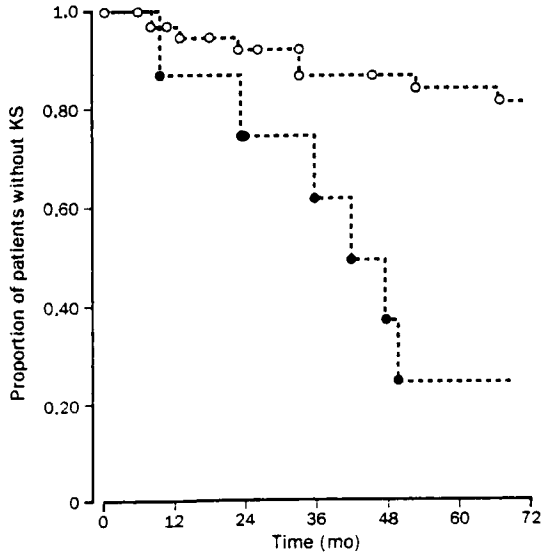

Fig. 3 Kaplan–Meyer curve showing that the presence of KSHV in peripheral blood mononuclear cells by PCR is associated with the subsequent development of KS in a cohort of HIV-positive gay men. Proportion of individuals, in whose peripheral blood KSHV was (●) or was not (○) detected, and who remained free of KS after indicated time of follow-up. From Whitby *et al.* (1995).

Howard *et al.*, 1997). Infectious virus is also found in the saliva of HIV-positive individuals (Koelle *et al.*, 1997). Although semen and sputa are therefore possible routes of viral transmission, their respective contribution to infection is still unknown.

C. Seroepidemiology

Assays utilizing immunofluorescence, Western blot, and enzyme-linked immunoassay (ELISA) to detect antibodies against latent and lytic KSHV antigens have been described. Most current serologic surveys are based on an immunofluorescent assay (IFA) of KSHV-infected B cells derived from lymphomas (Kedes *et al.*, 1996; Gao *et al.*, 1996; Lennette *et al.*, 1996; Simpson *et al.*, 1996). The IFA results for latent or lytic KSHV antigens indicate that those with KS (or at risk of developing KS) are far more likely to have detectable antibodies to KSHV in their sera than are those without KS or not at risk of developing the disease (Table III). ORF 73 of KSHV encodes for the immunodominant antigen recognized by patient sera with IFA (Fig. 4) (Rainbow *et al.*, 1997; Kellam *et al.*, 1997). In the majority of cases the nu-

Table III Detection of Antibodies to KSHV Latent and Lytic Antigens[a]

	Latent antigen assays (IFA)			Lytic antigen assays (IFA, WB, ORF65 ELISA)		
Sera	Number positive	Number tested	Percentage positve	Number positive	Number tested	Percentage positve
AIDS-KS	228	312	73%	156	244	64%
Classic KS	29	30	97%	17	18	94%
African KS	28	28	100%	42	45	93%
HIV-positive gay men, no KS	38	144	26%	106	218	49%
Sexually transmitted disease clinic attendees	40	357	11%	—	—	—
Controls						
United States/United Kingdom	6	413	1.5%	122	1207	10%
Africa	55	286	19%	184	290	63%
Greece/Italy	7	133	5%	3	26	12%

[a] Data from Miller *et al.* (1996), Gao *et al.* (1996), Kedes *et al.* (1996), Lennette *et al.* (1996), and Simpson *et al.* (1996).

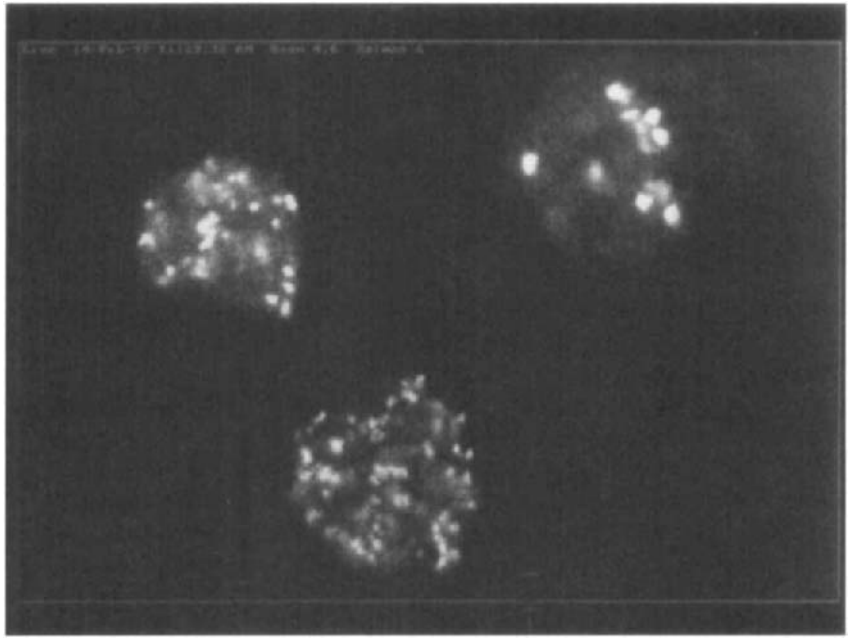

Fig. 4 The typical nuclear stippling pattern seen with KSHV-positive primary effusion lymphoma cells when reacting against KSHV-specific antibodies in patient sera.

clear stippling is caused by epitopes within the C terminus of ORF 73; however, some patient sera react against other latent nuclear antigens not yet identified (Kellam *et al.*, 1997). Recombinant proteins (i.e., ORF 65 and K8.1) have been used in ELISA and support the findings of IFA that infection in the West is not ubiquitous like that of most other herpesviral infections (Simpson *et al.*, 1996; Neipel and Fleckenstein, 1997). Infection in Northern Europe and North America is predominantly sexually acquired after puberty, whereas infection in Africa is common even in children. A study in South Africa indicated that all KSHV-positive children also had KSHV-positive mothers, whereas the children of KSHV-negative mothers are also all KSHV-negative. Such early studies suggest that vertical and horizontal transmissions from mother to children and among children in Africa play important roles in KSHV transmission. A further study from The Gambia confirmed a progressive increase in age-specific prevalence of KSHV antibodies in West Africa, again implying that transmission of this herpesvirus is not different from that of most other herpesviruses in Africa.

In Italy, there are marked variances in the incidence rates of classic KS in comparing the north, central, and southern areas of the country (Geddes *et*

al., 1995). Interestingly, the incidence of KSHV among blood donors, as determined by IFA serology, correlates well with this geographic distribution of KS (Whitby *et al.*, 1997). Furthermore, the geometric mean titer (GMT) of KSHV antibodies is highest in those blood donors from areas with the highest incidence rates of KS (Whitby *et al.*, 1997). Overall, the GMT of antibodies to KSHV in Italian blood donors is 1819; in United States donors, 617; and in United Kingdom donors, 119 (Whitby *et al.*, 1997). This was the first study to indicate that KSHV antibody titers may predict who is at highest risk to develop KS. This is reminiscent of the correlation between high EBV antibody titers and the onset of Burkitt's lymphoma in Africa and nasopharyngeal carcinoma in the Far East.

The main routes of transmission for KSHV between KS endemic areas and Northern Europe and the United States are clearly different, but the details need to be elucidated. However, no current assay consistently detects anti-KSHV antibodies in all those with KS. There is therefore no assay as yet sensitive enough to detect the true prevalence of KSHV in the general population, and it is therefore not possible to say whether KSHV infection in Western Europe and North America is truly confined to those at risk of developing KS, or whether this agent is more widespread. Using the molecular and serologic evidence, our impression is that KSHV is widespread in parts of Africa, reasonably common (i.e., ~20% of the population) in Mediterranean Europe where classic KS occurs, and infrequent in Northern Europe and the United States.

III. KSHV AND LYMPHOPROLIFERATION

A. Primary Effusion Lymphomas

The emergence of primary effusion lymphomas (PELs, also called body cavity-based lymphomas) as a new disease entity is an intriguing story linked to the identification of the new human herpesvirus KSHV. Two groups initially recognized the unique aspects of some effusion-based lymphomas in patients with AIDS (Knowles *et al.*, 1989; Walts *et al.*, 1990). The lymphoma cells in these cases were negative for most lineage-associated antigens, although gene rearrangement studies indicated a B cell origin with clonal rearrangement of the immunoglobulin (Ig) genes. Karcher *et al.* (1992) further demonstrated the distinctiveness of the syndrome, reporting a high prevalence of EBV yet absence of c-*myc* rearrangements. They also noted the tendency of the disease to remain confined to body cavities without further dissemination. Cesarman and colleagues (1995a) found that KSHV was ssociated specifically with PELs (body cavity-based lymphomas) but not with other high-grade AIDS-related lymphomas.

PELs posses a unique constellation of features distinguishing them from all other known lymphoproliferations: PEL presents predominantly as malignant effusions in the pleural, pericardial, or peritoneal cavities, usually without significant tumor mass or lymphadenopathy. These lymphomas occur predominantly in HIV-positive individuals with advanced stages of immunosuppression (Komanduri *et al.*, 1996), but are occasionally seen in HIV-negative patients (Nador *et al.*, 1996; Strauchen *et al.*, 1997; Said *et al.*, 1996). In addition, like KS, which often occurs in the same patient, it is seen primarily in gay men, but not in other HIV-positive risk groups (Jaffe, 1996; Nador *et al.*, 1996).

Most PELs do not express surface B cell antigens, but a B cell lineage is indicated by the presence of clonal immunoglobulin gene rearrangement and some plasma cell differentiation is seen (Ansari *et al.*, 1996; Cesarman *et al.*, 1996a,b). All PELs that lack c-*myc* rearrangements contain KSHV (Nador *et al.*, 1996). The majority of, but not all, PELs are coinfected with EBV (Cesarman *et al.*, 1995a,b; Otsuki *et al.*, 1996), suggesting that the two viruses may cooperate in neoplastic transformation. EBV monoclonality has been established in most cases (Cesarman *et al.*, 1996b; Nador *et al.*, 1996). Other lymphomas with effusion phenotypes, such as pyothorax-associated lymphoma (PAL), do not contain KSHV, but are frequently infected with EBV and also have c-*myc* rearrangements (Cesarman *et al.*, 1996a,b). PEL cells consistently lack molecular defects commonly associated with neoplasia of mature B cells, including activation of the protooncogenes Bcl-2, Bcl-6, n-*ras,* and k-*ras,* as well as mutations of p53 (Carbone *et al.*, 1996; Nador *et al.*, 1995).

Southern blot analysis of PEL cells shows the presence of KSHV sequences in very high copy number (50–150/cell), compared to that seen in KS. Cell lines from PELs have been established (Cesarman *et al.*, 1995b; Renne *et al.*, 1996; Ansari *et al.*, 1996; Arvanitakis *et al.*, 1996; Gaidano *et al.*, 1996). One cell line, KSHV-positive, EBV-negative, from the peripheral blood of a patient with PEL, has also been established (Boshoff *et al.*, 1998). Some lines are positive only for KSHV, others are coinfected with EBV. In the coinfected lines the expression of EBV latent proteins is restricted to EBNA-1 and LMP-2. EBNA-1 and KSHV may have the same transformation potential as EBNA-1 and c-*myc* translocations as seen in Burkitt's lymphoma cells (Boshoff *et al.*, 1998). Lines latently infected with KSHV can be induced with the phorbol ester PMA or butyrate to produce KSHV virions (Renne *et al.*, 1996; Moore *et al.*, 1996a). KSHV-positive, EBV-negative PEL lines have been proved very useful for initial seroepidemiologic studies based on immunofluorescence assays (Gao *et al.*, 1996; Kedes *et al.*, 1996).

It appears that KSHV-positive PEL cells lack many adhesion molecules and "homing markers" present on other diffuse lymphomas: this may contribute to the peculiar effusion phenotype of these lymphomas and the lack of macroscopic evidence of lymph node involvement (Boshoff *et al.*, 1998).

B. Castleman's Disease

Castleman's disease is a rare and usually polyclonal disorder of unknown etiology (Castleman *et al.*, 1956). Two distinct histopathological variants with different clinical characteristics have been described: the hyaline vascular type and the plasma cell variant. The more common hyaline vascular type usually presents with a solitary mass in the mediastinum or retroperitoneum and is usually surgically curable. The rarer plasma cell variant typically presents with generalized lymphadenopathy, B symptoms, and immunological abnormalities. The systemic variety is designated multicentric Castleman's disease (MCD) and is usually of the plasma cell type. Interestingly, patients with MCD are at an increased risk to develop KS and B cell lymphomas (Peterson, 1993).

Soulier and colleagues (1995) were the first to report the presence of KSHV in MCD biopsies. They found KSHV in all 14 lesions from HIV-positive French patients with MCD. These included plasma cell type, hyaline vascular, and mixed. Among HIV-negative cases, 7 of 17 lesions were positive for KSHV.

Various other investigators have since reported the presence of KSHV in Castleman's disease biopsies (Dupin *et al.*, 1995a; Gessain *et al.*, 1996; Barozzi *et al.*, 1996). KSHV is nearly universally present in HIV-positive individuals with MCD. Among MCD cases in immunocompetent hosts the presence of KSHV is restricted to about 40% of cases. However, from these studies there is no clear association with a specific type of Castleman's disease and KSHV emerging.

Immunoregulatory abnormalities probably contribute to these lymphoproliferations. It was previously hypothesized that a virus may act as a cofactor, perhaps as a stimulus for cytokine production (Peterson, 1993). The association between IL-6 and MCD is noticeable: IL-6 is present at high levels in Castleman's disease biopsies, and PBMCs of patients with MCD were shown to secrete high levels of IL-6. The role of KSHV-encoded IL-6 (vIL6) in MCD pathogenesis remains to be investigated.

IV. VIRAL PIRACY OF EUKARYOTIC GENES

A recognized feature of some DNA viruses, and in particular herpesviruses and poxviruses, is the incorporation of host cell genes into their viral genomes (Murphy, 1997; McGeoch and Davidson, 1995), rather as retroviruses have transduced oncogenes, except that some of the genes acquired from the host genome also play a role in viral replication. The number of genes transduced from the cellular host by KSHV is unprecedented among known viruses (Moore *et al.*, 1996c) (Table IV). In most herpesviruses a core

Table IV Potential Functions of Selected and Pirated KSHV Genes

Viral gene	Cellular homolog	Possible function
K1	—	Promote cellular growth
ORF 4	CD46	Complement regulation
K2	IL-6	Lymphocyte growth promotion
K4, K4.1, and K6	vMIP-I, -III	Paracrine growth and angiogenesis
ORF 16	Bcl-2	Prevents apoptosis
K9	IRF[a]	Promote growth, immunoregulatory
K12	—	? Transactivator
ORF 71	DED domain proteins	Prevent apoptosis
ORF 72	Cyclin D	Promote cellular growth
ORF 73 (LNA-1)	—	? Transactivator
ORF 74	IL-8 GPCR[b]	Promote cellular growth
K14	NCAM-like protein	Intercellular signaling

[a] Interferon regulatory family.
[b] G-protein-coupled receptor homolog.

set of genes with cellular homologs are responsible for central aspects of virus replication, e.g., DNA polymerases with sequence similarity to eukaryotic cellular DNA polymerases. Some KSHV pirated genes occur only sporadically in other herpesviruses or are not present at all. The core set of genes responsible for central aspects of replication and virion structure was probably present in an ancient progenitor of contemporary herpesviruses and this core set was derived from a nonviral source, whereas the sporadically occurring genes with cellular homologs are probably more recent acquisitions from the host genome or from other viral or genetic elements, and help the virus to function in a particular microenvironment or cell type (McGeoch and Davidson, 1995). These genes might be of less importance for viral replication. KSHV encodes homologs of human cyclin D (ORF 72) (Cesarman *et al.*, 1996b), an IL-8-like G-coupled protein receptor (vGPCR, ORF 74) (Cesarman *et al.*, 1996b), three chemokine homologs, vMIP-1, vMIP-II, and vMIP-1β (also called vMIP-III) (K6, K4, K4.1) (Moore *et al.*, 1996c; Neipel *et al.*, 1997a,b), a homolog of IL-6 (K2) (Moore *et al.*, 1996a–c; Neipel *et al.*, 1997a,b), a gene with some sequence similarity to interferon regulatory factors (vIRF, K9) (Moore *et al.*, 1996c), and a Bcl-2 homolog (ORF 16) (Cheng *et al.*, 1997; Sarid *et al.*, 1997; Nicholas *et al.*, 1997). In addition, KSHV also encodes genes similar to the complement-binding proteins CD21/CR2 (ORF 4) (Russo *et al.*, 1996) and an NCAM-like adhesion protein (ORF 14) (Russo *et al.*, 1996). There is a significant correlation between genes encoded by KSHV and the cellular genes induced by EBV (Moore *et al.*, 1996c), its closest known related human herpesvirus. The EBV EBNA and LMP proteins (genes with sequence similarity are absent in the KSHV

genome) are largely responsible for the induction of hIL-6 (Tanner *et al.*, 1996), cyclin D (Sinclair *et al.*, 1994), the IL-8-like receptor (a GPCR) EBI1 (Birkenbach *et al.*, 1993; Burgstahler *et al.*, 1995), cellular Bcl-2 (Henderson *et al.*, 1991), adhesion molecules, and the complement-controlling protein CR-2 (Larcher *et al.*, 1995). Therefore, it seems that KSHV and EBV have developed different strategies to attain the same objective: to overcome cell cycle arrest, apoptosis, and activation of cellular immunity, which are typical host cell responses to viral infection.

V. ANTI-"ANTIVIRAL" RESPONSES

When viruses enter cells, the cellular responses are comparable to that observed with the expression of some oncogenes: apoptosis, cell cycle arrest, and/or increased host cell immunity: e.g., the expression of oncogenic *ras* in primary human or rodent cells results in permanent G_1 arrest and is accompanied by accumulation of p53 and p16 (Serrano *et al.*, 1997). Viruses employ countermeasures to these cellular responses to overcome cellular defense mechanisms (Weinberg, 1997).

Classic examples are the cellular responses when cytomegalovirus or adenovirus enters a target cell: when CMV initiates its replication program, the infected cell starts to display viral antigenic peptides on its surface MHC class I molecules, trying to provoke an immune response; CMV then launches its anti-"antiviral" response by blocking the biosynthesis of class I molecules (Beersma *et al.*, 1993). When adenoviruses enter a cell, the cell triggers its apoptotic program: by killing itself, the cell would succeed in aborting the viral replicative cycle and subsequent viral spread to noninfected tissues. To counteract this cellular response adenovirus-encoded proteins are produced, allowing cells to survive long enough for viral progeny to be produced. Many viruses have evolved genes encoding proteins that effectively suppress or delay apoptosis long enough for the production of sufficient quantities of progeny. Although we are only starting to understand the function of some of the proteins encoded by KSHV, it is already clear that this virus is also well equipped to overcome cellular defense mechanisms. For clarity we shall discuss the cellular responses separately, although the *in vivo* situation is likely more complex with interactions between these various pathways.

A. Cellular Growth

A common strategy among oncogenic viruses is to provide their host cells with additional growth stimuli, thereby extending their proliferative capac-

ity. Viral oncogenes can deregulate cell cycle control mechanisms by interfering with receptor-mediated signal transduction pathways and the function of nuclear cell cycle regulatory proteins. Several DNA viruses, for example, encode proteins that specifically target and inhibit both the retinoblastoma protein (pRb) and the p53 tumor suppressor pathways involved in cell cycle regulation and apoptosis, respectively (Jansen-Durr, 1996). By overriding growth-suppressive signals, which control cell cycle progression in untransformed cells, viruses promote the progression into the S phase of the cell cycle (DNA synthesis), which is probably necessary for efficient replication of their viral genome (Jansen-Durr, 1996). A consequence of the deregulated progression through the cell cycle may be unlicensed cellular proliferation and ultimately transformation.

Cellular cyclins are components of cellular kinases, many of which regulate cell proliferation and cell cycle progression (Peters, 1994; Sherr, 1995). The G1 cyclins D and E accelerate transit through the check point in the G_1 phase of the cell cycle into the S phase and therefore commit cells to a further cycle of DNA synthesis. The aberrant expression of cellular D-type cyclins is strongly implicated in the pathogenesis of various malignancies (Bates and Peters, 1995; Bodrug *et al.*, 1994; Hunter and Pines, 1994; Jacks and Weinberg, 1996). All cyclins associate with their activating partners the cyclin-dependent kinases (cdks) (Morgan, 1995). When active, certain of these kinases phosphorylate and inactivate cell cycle checkpoint molecules and thereby facilitate progression of cells through the cell cycle. The cyclin encoded by KSHV (KSHV-cyclin) is part of a potential "oncogenic cluster" of genes in the KSHV genome (Fig. 5). The KSHV-cyclin (Cesarman *et al.*, 1996b) associates predominantly with cdk6, and this complex can phosphorylate, and therefore inactivate, pRB (Fig. 6) (Chang *et al.*, 1996; Godden-Kent *et al.*, 1997; Li *et al.*, 1997). This indicates that ectopic expression of the v-cyclin in cells could promote cell cycle progression, by a mechanism analogous to that of overexpressed cellular D-type cyclins. KSHV cyclin is expressed in latency in PEL cells (Kellam *et al.*, 1997) and also in most spindle cells in KS lesions, indicating a possible direct growth-promoting role in these tumors.

Although EBV does not encode a cyclin homolog, EBV EBNA-2 and EBNA-5 genes have been shown to induce the expression of cellular D-type cyclins in infected lymphocytes (Sinclair *et al.*, 1994). As discussed, this indicates that these two gamma herpesviruses have evolved different strategies to modify the same signal transduction pathway.

KSHV-encoded G-protein-coupled receptor (KSHV-GPCR) is fully active for downstream signaling in the absence of chemokine ligands (constitutively active) (Arvanitakis *et al.*, 1997) and is one of the potential oncogenes encoded by KSHV. Cellular GPCRs that are constantly stimulated or that become constitutively active by mutation can transform cells and are involved

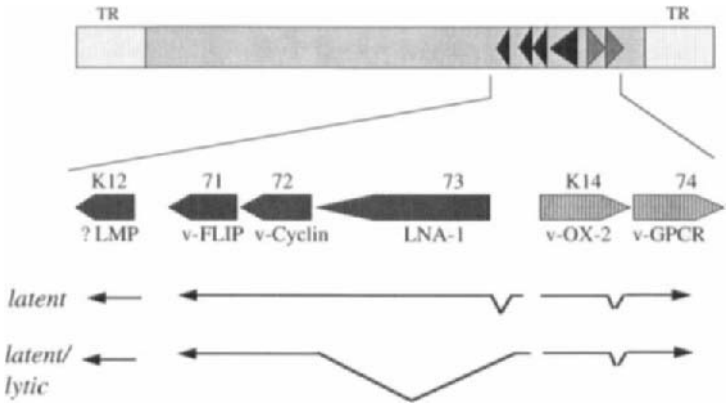

Fig. 5 An "oncogenic cluster" in the genome of KSHV encodes for the v-cyclin, v-FLIP, v-IRF, v-GPCR and v-OX2 homologs. LNA-1, the major immunogenic latent nuclear protein, analogous to the EBNAs of EBV, is part of a polycistronic transcript. K12 encodes for an abundantly transcribed latent membrane protein, which may act like an LMP of EBV.

in the pathogenesis of some human tumors (Alblas *et al.*, 1996; Coughlin, 1994; Julius *et al.*, 1989; Milano *et al.*, 1994). KSHV-GPCR shows the greatest sequence similarity to the human receptors for IL-8 (CXCR-1 and CXCR-2) (Cesarman *et al.*, 1996b; Guo *et al.*, 1997), an endothelial cell chemokine and angiogenic factor. KSHV-GPCR activates the phosphoinositide pathway (a mitogenic signaling pathway) in COS-1 cells, and *in vitro* transfection of rat fibroblasts with KSHV-GPCR leads to cell proliferation (Arvanitakis *et al.*, 1997). Of the chemokines tested, interleukin-8 (IL-8) binds to this GPCR with the highest affinity (Arvanitakis *et al.*, 1997). KSHV-GPCR is able to trigger signaling cascades, leading to activation of AP-1 (Arvanitakis *et al.*, 1997), which is a transcription factor involved in cell survival and proliferation (Karin *et al.*, 1997), and to activation of inflammatory and angiogenic growth factors (Kolch *et al.*, 1995). This suggests that KSHV-GPCR signaling is not only linked to cellular proliferation and possible transformation, but could also induce paracrine responses such as angiogenesis, which may be relevant to KS pathogenesis.

A further gene encoded by KSHV (ORF K9) has low but significant sequence similarity to the interferon-regulating factor (IRF) family of proteins. Two members of this pathway, IRF-1 and IRF-2, are antagonistic in their effector functions to each other (Taniguchi *et al.*, 1995). The oncogenic activities of IRF-2 in NIH 3T3 cells can be reversed by IRF-1 overexpression (Taniguchi *et al.*, 1995). It was recently shown that KSHV-IRF can also transform NIH 3T3 cells (Gao *et al.*, 1997) and this viral gene is therefore another potential oncogene involved in KSHV tumor formation. In PEL cells

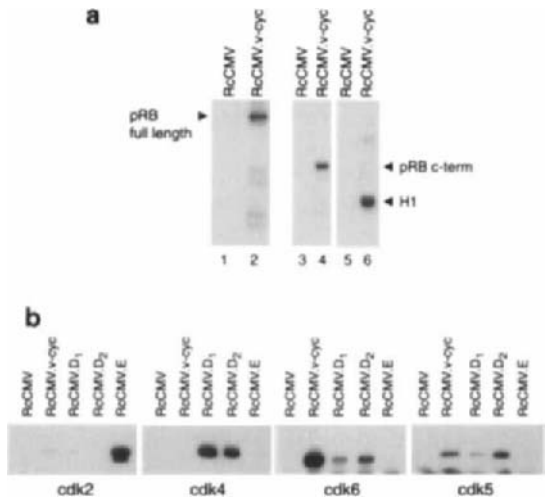

Fig. 6 The v-cyclin is expressed in most spindle cells in KS lesions and could therefore play a direct role in their proliferation. (a) Phosphorylation of pRb and histone H1 by immunopurified KSHV-cyc; (b) association of KSHV-cyc with cellular cdk subunits. v-Cyclin associates predominantly with cdk6, and this association seems resistant to the p16 and p21 of cdks. RcCMV.D1, RcCMV.D2 and RcCMV.E refer to vectors encoding human cyclin D1, human cyclin D2, and human cyclin E, respectively. From Godden-Kent *et al.* (1997).

KSHV-IRF is expressed at low levels during latency and may therefore be directly involved in viral-induced lymphomagenesis.

The IL-6 protein encoded by KSHV (vIL-6) is functional in preventing apoptosis of IL-6-dependent mouse myeloma cells, indicating that it can substitute for cellular IL-6 (Moore *et al.*, 1996c; Neipel *et al.*, 1997a). As previously mentioned, cellular IL-6 has been shown to stimulate the growth of KS spindle cells *in vitro* and to be expressed in KS lesions *in vivo*. vIL-6 is expressed by only a minority of cells in KS lesions; however, in patients with KS, vIL-6 is abundantly expressed in hematopoietic cells and in lymph nodes. It remains to be determined if vIL-6 stimulates the proliferation of KSHV-infected hemopoietic cells, including those associated with Castleman's disease.

Other KSHV-encoded proteins with possible cellular growth-promoting activities include ORF 73, which encodes the latent nuclear antigen (LNA-1, or LANA). KSHV latently infected cell lines have been established from PELs (Cesarman *et al.*, 1995b; Renne *et al.*, 1996). Patient sera have been tested in an indirect immunofluorescence assay against such PEL cells, with positive sera showing a distinct nuclear localizing stippling pattern (Kedes *et al.*, 1996; Gao *et al.*, 1996). The nuclear antigens detected in such assays are

formally analogous to the EBNAs of EBV (Kedes *et al.*, 1996), which show similar nuclear localizing patterns with IFA. We and others have employed this IFA to detect antibodies to latently infected KSHV-infected PEL cells in sera of nearly 95% of patients with KS (Kedes *et al.*, 1996; Gao *et al.*, 1996; Simpson *et al.*, 1996). ORF 73 encodes LNA-1, which is analogous to the EBNAs of EBV (Kellam *et al.*, 1997; Rainbow *et al.*, 1997). LNA-1 has many features reminiscent of several EBNAs: it is a very hydrophilic (38% charged residues) and proline-rich protein, with an extensive repetitive domain and a leucine zipper motif (Russo *et al.*, 1996). Potential nuclear localization signals consisting of runs of basic amino acids also occur in the C-terminal domain of LNA-1/ORF 73. EBV establishes a stable, latent infection of primary B lymphocytes (Klein, 1994). At least 11 EBV genes are expressed in latent infection (EBNAs 1–6, LMP1–3, and EBER 1 and 2), although only a subset of these are expressed in most EBV latently infected B cell lines (Liebowitz and Kieff, 1993). The EBNA and LMP proteins are required for B cell transformation (Klein, 1994). Each of the EBNAs have been shown by immune microscopy to give distinctive nuclear staining patterns (Liebowitz and Kieff, 1993), similar to those observed for LNA-1 in KSHV-infected PEL or ORF 73-transfected 293 cells (Kellam *et al.*, 1997).

Currently, it is not known whether LNA-1, by analogy to the EBNAs, plays a role in cellular transformation. Such a role might be direct or through the transactivation of cellular or other viral genes. LNA-1 forms part of a cluster of genes in the KSHV genome, all of which may have growth-regulating functions (Fig. 5). LNA-1 is transcribed with two other growth-promoting or antiapoptotic proteins: v-cyclin and K13 (Kellam *et al.*, 1997; Rainbow *et al.*, 1997). A complex splicing program controls the transcription of these proteins (Kellam *et al.*, 1997).

Two other ORFs with potential growth regulatory functions are K1 and K12 (Fig. 2). K1 shows no sequence similarity to the transforming genes encoded by this area of the genome of HVS (STP and Tip); however, K1, like STP (Lee *et al.*, 1997a), shows significant variability among different KSHV strains and it can transform rodent fibroblasts (Lee *et al.*, 1997b). In addition, a recombinant herpesvirus wherein HVS STP is replaced with KSHV K1 (the recombinant virus is called HVSΔSTP/K1) can transform human primary T lymphocytes and can induce lymphomas in some common marmosets (Lee *et al.*, 1997b). K1 nucleotide differences among different KSHV isolates have confirmed previous indications by sequencing ORF 26, showing that at least three different KSHV strains are present, i.e., A, B, and C. However, there is currently no indication that a specific KSHV subtype is associated with a specific pathology (e.g., KS or PEL) or geographic origin (C. Boshoff and P. Kellam, unpublished data). K12, the most abundant latent transcript, encodes an extremely hydrophobic small polypeptide that may behave like an EBV LMP-like protein (Ganem, 1997).

B. Apoptosis

The induction of apoptosis is a typical response of the host cell to virus infection. Deregulation of the host cell cycle machinery by viral-encoded proteins, leads to the up-regulation of the tumor suppressor p53, which in turn activates genes encoding for apoptosis-mediating proteins such as Bax, Bik, and other proteins of this family (Boyd *et al.*, 1995; Sato *et al.*, 1994). The p53 protein may also up-regulate expression of death receptors such as CD95 (Fas, Apo-1) together with its ligand CD95L (FasL), which signal a cell to apoptose in response to signals from cytotoxic T lymphocytes or via an autocrine mechanism induced by soluble CD95L produced by the infected cell (Thome *et al.*, 1997). KSHV encodes a gene (ORF 16) with sequence similarity to cellular Bcl-2, similarly to EBV and HVS (Cheng *et al.*, 1997; Sarid *et al.*, 1997). The heterodimerization of cellular Bcl-2 with bax is important in overcoming bax-mediated apoptosis (Sato *et al.*, 1994). Whether KSHV-Bcl-2 dimerizes with other Bcl-2 family members *in vivo* is not yet clear (Cheng *et al.*, 1997; Sarid *et al.*, 1997); however, KSHV-Bcl-2 can also overcome bax-mediated apoptosis. Interestingly, the herpesvirus Bcl-2 homologs (including KSHV-Bcl-2, BHRF1 of EBV, and ORF 16 of HVS) contain poorly conserved Bcl-2 homology 3 (BH3) domains compared to other mammalian Bcl-2 homologs, suggesting that BH3 might not be essential for the antiapoptotic function of these proteins (Cheng *et al.*, 1997).

The homology between KSHV ORF 16 and other members of the Bcl-2 family suggests that it might prolong the life span of virus-infected cells. Deregulated Bcl-2 expression has been shown to occur in some human malignancies, such as follicular lymphomas (Cleary *et al.*, 1986; Tsujimoto *et al.*, 1987), suggesting that ectopic expression of KSHV-Bcl-2 may also contribute to tumorigenesis through its antiapoptotic effect.

A newly identified family of viral inhibitors (vFLIPS, for FLICE inhibitory proteins) has been shown to interfere with apoptosis signaled through death receptors. vFLIPs are present in several gamma herpesviruses (including KSHV, K13/ORF 71), as well as in the oncogenic human molluscipoxvirus (Bertin *et al.*, 1997; Thome *et al.*, 1997). vFLIPs contain two death-effector domains that interact with the adaptor protein FADD (Boldin *et al.*, 1995; Chinnaiyan *et al.*, 1996) and inhibit the recruitment and activation of the protease FLICE (Boldin *et al.*, 1995; Muzio *et al.*, 1996) by the CD95 death receptor (Nagata, 1997). Recruitment of FLICE by the CD95 receptor in response to the binding of its ligand (CD95L) leads to the assembly of a receptor-associated death-inducing signaling complex (DISC) (Kischkel *et al.*, 1995). DISC-associated FLICE subsequently initiates proteolytic activation of other ICE protease family members, which in turn leads to apoptosis (Boldin *et al.*, 1995; Muzio *et al.*, 1996). Cells expressing vFLIPs are protected against apoptosis induced by CD95 or by TNF-R1 (Bertin *et*

al., 1997; Thome *et al.*, 1997). The HVS FLIP is detected late during the lytic replication cycle, suggesting that protection of virus-infected cells from death receptor-induced apoptosis may lead to higher virus production and contribute to the persistence and oncogenicity of FLIP-encoding viruses. It has been shown that the lack of responsiveness to CD95L-mediated apoptosis due to down-regulation of CD95 may contribute to the development of certain tumors, including melanomas and hepatomas (Hahne *et al.*, 1996; Strand *et al.*, 1996). Therefore, it is possible that vFLIPs may not only promote viral spread and persistence, but that they also contribute to the transforming capacity of herpesviruses such as KSHV.

Interestingly, all FLIP-encoding gamma herpesviruses also have a Bcl-2 homolog. The antiapoptotic Bcl-2 family members block cell death induced by growth factor deprivation, gamma irradiation, and cytotoxic drugs (Huang *et al.*, 1997; Yang and Korsmeyer, 1996). However, in contrast to the vFLIPs, these proteins have a less potent effect on CD95-mediated apoptosis of lymphoid cell lines. These viruses may therefore exploit two complementary antiapoptotic pathways provided by a Bcl-2 homolog and by a vFLIP.

C. Immune Responses

In common with all the herpesviruses, the ability of gamma herpesviruses to evade host immune defenses is critical to their survival. The mechanisms how KSHV-infected cells evade the host immune responses are not yet known.

VI. ROLE OF KSHV IN KS PATHOGENESIS: DIRECT OR INDIRECT?

Molecular epidemiology and current serological assays indicate that KSHV is central to the pathogenesis of KS. Endothelial cells and KS spindle cells are infected with KSHV *in vivo* (Boshoff *et al.*, 1995a). Initial studies suggested that most of these cells in KS lesions are latently infected (Zhong *et al.*, 1996; Staskus *et al.*, 1997; Decker *et al.*, 1996), indicating that KSHV may play a direct role in the growth promotion of spindle cells through the expression of latent growth-promoting viral proteins. However, it has become clear that a substantial minority of KS spindle cells produce infective viral particles (lytic infection) (Orenstein *et al.*, 1997). At this stage we do not know whether most spindle cells eventually enter lytic replication and whether lytic infection is necessary to drive lesion formation. We also do not know whether the latent/lytic program in B lymphocytes is the same as that

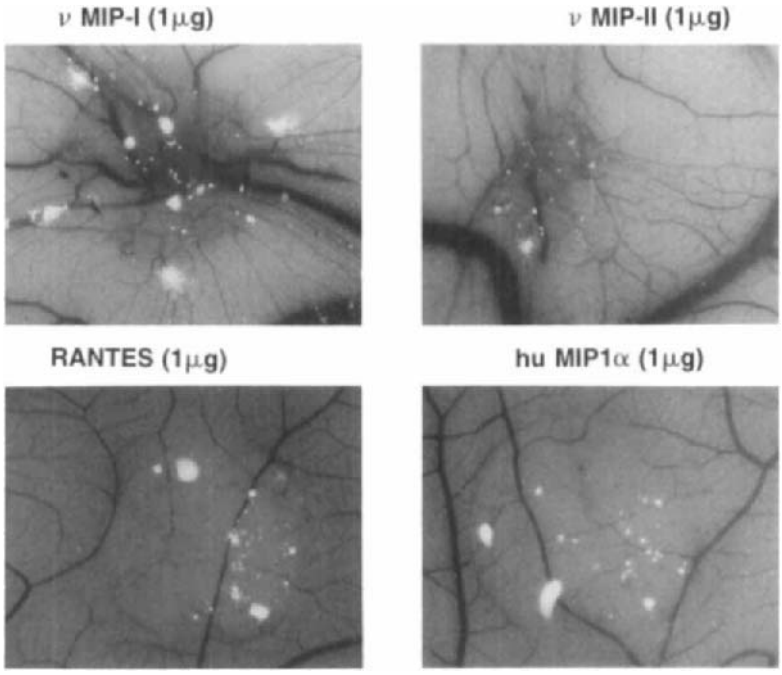

Fig. 7 KSHV MIP-I and MIP-II induce angiogenesis in the chick chorioallantoic membrane assay. However, cellular RANTES and MIP1α do not induce angiogenesis in this assay. From Boshoff *et al.* (1997).

in endothelial cells. Inflammatory cytokines could potentially stimulate the growth of KSHV-infected cells and such cells might also secrete the cytokines (viral or cellular) necessary for angiogenesis and the accumulation of inflammatory cells (Fig. 7). Loss of immunosurveillance (iatrogenic or acquired) might lead to the proliferation of KSHV-infected mesenchymal cells, analogous to the proliferation of B cells to form EBV-associated posttransplant or AIDS-associated lymphoproliferative diseases.

VII. THERAPEUTIC IMPLICATIONS

Currently, cytotoxic drugs (bleomycin, vinca alkaloids, anthracyclines) and radiotherapy are the standard treatment options for all forms of KS. In both HIV-infected individuals with KS and iatrogenic-immunosuppressed patients with KS, reversal of the immune deficiency would be the best long-term option. There are already reports of remission of KS lesions in HIV-pos-

itive individuals after treatment with aggressive antiretroviral therapy (e.g., triple therapy with two nucleosides and a protease inhibitor). Although KSHV also encodes a protease, KSHV replication is not inhibited *in vitro* by currently available protease inhibitors.

It was reported in one retrospective study, but not yet confirmed by other investigators, that the use of foscarnet to treat CMV infections in HIV patients reduced the incidence of KS development. *In vitro*, KSHV replication is insensitive to gancyclovir and acyclovir, but is moderately sensitive to foscarnet and sensitive to cidofovir. These agents target lytic viral genes (thymidine kinase) and if lytic infection is necessary to drive tumor formation or to recruit inflammatory cells to form KS lesions, these drugs might play a future role in the management of KS. Foscarnet and cidofovir, however, are associated with significant toxicity and would seem to be inappropriate therapy for most KS patients.

In posttransplant lymphoproliferative disorders adoptive immunotherapy with cytotoxic T cells (CTLs) against EBV-encoded proteins has become an attractive form of experimental therapy. The latent proteins of EBV (with the important exception of EBNA-1) evoke strong CTL responses. The fact that in posttransplant KS the lesions can regress when immunosuppressive therapy is stopped suggests that immunosurveillance plays an important role in the maintenance of these lesions. It remains to be seen whether adoptive immunotherapy with cytotoxic T cells directed against KSHV-encoded proteins will play a future role in the management of KSHV-associated tumors. We are currently investigating possible CTL responses against various KSHV-encoded proteins, including the immunogenic LNA-1, K12 (possible LMP analog), K1 (one of the most variable parts of the genome), and various other latently expressed viral proteins.

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Extracellular Matrix-Associated Transforming Growth Factor-β: Role in Cancer Cell Growth and Invasion

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Growth factors of the transforming growth factor-β (TGF-β) family inhibit the proliferation of epithelial, endothelial, and hematopoietic cells, and stimulate the synthesis of extracellular matrix components. TGF-βs are secreted from cells in high-molecular-mass protein complexes that are composed of three proteins, the mature TGF-β-dimer, the TGF-β propeptide dimer, or latency-associated protein (LAP), and the latent TGF-β binding protein (LTBP). Mature TGF-β is cleaved from its propeptide during secretion, but the proteins remain associated by noncovalent interactions. LTBP is required for efficient secretion and processing of latent TGF-β and it binds to LAP via disulfide bond(s). LTBP is a component of extracellular matrix microfibrils, and it targets the latent TGF-β complex to the extracellular matrix. TGF-β signaling is initiated by proteolytic cleavage of LTBP that results in the release of the latent TGF-β complex from the extracellular matrix. TGF-β is activated by dissociation of LAP from the mature TGF-β. Subsequent signaling involves binding of active TGF-β to its type II cell surface receptors, which phosphorylate and activate type I TGF-β receptors. Type I receptors, in turn, phosphorylate cytoplasmic transcriptional activator proteins Smad2 and Smad3, inducing their translocation to the nucleus. Recent evidence suggests that acquisition of resistance to TGF-β growth inhibition plays a major role in the progression of epithelial and hematopoietic cell malignancies. The role of secretion of TGF-β in tumorigenesis is more complex. The secretion of TGF-βs by tumor cells may contribute to autocrine growth inhibition, but on the other hand, it may also promote invasion, metastasis, angiogenesis, and even immunosuppression. Tumor cells may also fail to deposit LTBP:TGF-β complexes to the extracellular matrix. The elucidation of the mechanisms of the release of TGF-β from the matrix and its subsequent activation aids the understanding of the pathophysiologic roles of TGF-β in malignant growth, and allows the development of therapeutic agents that regulate the activity of TGF-β.

I. INTRODUCTION

Transforming growth factors were originally purified from the conditioned medium of murine sarcoma virus-transformed fibroblasts as sarcoma growth factor (SGF), a factor that promoted the anchorage-independent growth of normal rat kidney NRK-49F cells in soft agar (DeLarco and Todaro, 1978; see also Moses *et al.*, 1981; Roberts *et al.*, 1980; Assoian *et al.*, 1984). SGF was later found to be composed of two distinct factors, TGF-α and TGF-β (Anzano *et al.*, 1982, 1983). TGF-β was found in several mouse tissues, such as submaxillary gland, kidney, liver, muscle, heart, and brain (Roberts *et al.*, 1981). Subsequently, TGF-β was isolated also from human placenta (Frolik *et al.*, 1983) and platelets (Assoian and Sporn, 1986). Independent lines of study identified TGF-β-related factors as glioblastoma-derived T cell suppressory factor (Wrann *et al.*, 1987) and a growth inhibitor secreted by BSC-1 African green monkey kidney cells (Holley *et al.*, 1978; Tucker *et al.*, 1984a). Molecular cloning of TGF-β1 was accomplished in 1985 by Derynck *et al.* (1985). Growth inhibitors from glioblastoma and BSC-1 cells were cloned later, and found to be identical. They were assigned the name

TGF-β2 (de Martin *et al.*, 1987; Hanks *et al.*, 1988). Third, fourth, and fifth members of the family were cloned by low-stringency screening of cDNA libraries from human (TGF-β3) (Derynck *et al.*, 1988; ten Dijke *et al.*, 1988), chicken (TGF-β4) (Jakowlew *et al.*, 1988), and *Xenopus* (TGF-β5) (Kondaiah *et al.*, 1990) sources. Only three of these have been found in mammals, TGF-βs 1, 2, and 3 (Derynck *et al.*, 1985; de Martin *et al.*, 1987; ten Dijke *et al.*, 1988).

TGF-βs are growth inhibitory to most normal and tumor cell types (Tucker *et al.*, 1984a,b; Moses *et al.*, 1985). Epithelial and endothelial cells are particularly sensitive to TGF-β-mediated growth inhibition, whereas most cells of mesenchymal origin show little or no growth inhibition, or even growth stimulation (Moses *et al.*, 1985; Massagué, 1990). A characteristic feature of TGF-βs is their autocrine or juxtracrine action. In *Xenopus*, signaling by TGF-β requires that the producing and responding cells are juxtaposed (Reilly and Melton, 1996). In transgenic mice that overexpress active TGF-β in the mammary gland, only cells that are in very close contact with TGF-β overexpressing cells respond to the factor (Kordon *et al.*, 1995). An important way to target the effects of TGF-β is their deposition to the extracellular matrix in a latent form (Taipale *et al.*, 1994a; Taipale and Keski-Oja, 1997).

II. STRUCTURE OF TGF-βs

TGF-βs are secreted by cultured cells in a latent, inactive complex, containing two proteins, active TGF-β and its prodomain, TGF-β latency-associated protein (β-LAP) (Lawrence *et al.*, 1984, 1985; Gentry *et al.*, 1988). Early in the secretory pathway, two chains of pro-TGF-β associate to form a disulfide-bonded dimer (Gentry *et al.*, 1988; Gray and Mason, 1990; Miyazono *et al.*, 1991). Homodimeric forms are most common, but the heterodimers TGF-β1.2 and TGF-β2.3 have also been isolated (Cheifetz *et al.*, 1987; Ogawa *et al.*, 1992). In the secretory pathway, active TGF-β is cleaved from its propeptide by furinlike processing endoproteinase (Sha *et al.*, 1989; Dubois *et al.*, 1995). The propeptide remains associated with TGF-β by noncovalent interactions, conferring latency to the complex (Gentry *et al.*, 1988; Wakefield *et al.*, 1989; Gentry and Nash, 1990). Active TGF-β contains no carbohydrate, but the propeptide is glycosylated at multiple asparagine residues. Recombinant TGF-β1 produced in Chinese hamster ovary cells (CHO) contain mannose 6-phosphate (Purchio *et al.*, 1988), a lysosomal targeting signal (Kornfeld, 1992).

The latent TGF-β complex consisting of the propeptide β-LAP and mature TGF-β has been named small latent TGF-β. Most cell lines, however, secrete

large latent TGF-β complexes, containing additional high-molecular-weight proteins that associate with LAP (Miyazono *et al.*, 1988; Wakefield *et al.*, 1988; Olofsson *et al.*, 1992). Best characterized of these are latent TGF-β binding proteins (LTBPs), which bind to LAP by disulfide bond(s) (Miyazono *et al.*, 1988).

Four LTBP genes have been isolated thus far (encoding LTBPs 1–4) (Kanzaki *et al.*, 1990: Tsuji *et al.*, 1990; Morén *et al.*, 1994; Gibson *et al.*, 1995; Yin *et al.*, 1995; Giltay *et al.*, 1997; Saharinen *et al.*, 1998). The amino acid sequences of LTBPs are highly repetitive, containing 15–19 EGF-like repeats, three novel eight-cysteine repeats (Kanzaki *et al.*, 1990; Tsuji *et al.*, 1990), and one "hybrid domain," a repeat containing seven cysteines that shares homology with both EGF and the eight-cysteine repeats. EGF-like repeats are found in many extracellular proteins and they mediate protein–protein interactions (Handford *et al.*, 1995; Downing *et al.*, 1996). The function for the eight-cysteine repeats is still somewhat unknown, although the third eight-cysteine repeat of LTBP-1 was found to form the disulfide link to TGF-β1·LAP (Saharinen *et al.*, 1996). Eight-cysteine repeats seem to be able to fold in two different conformations, because no reduced cysteine–residues were found in a study of an eight-cysteine repeat by Reinhardt *et al.* (1995). Eight-cysteine repeats are also found in fibrillins (Maslen *et al.*, 1991; Zhang *et al.*, 1994). LTBPs and fibrillins 1 and 2 are highly homologous in overall domain structure (Fig. 1). Fibrillins are major constituents of connective tissue 10-nm microfibrillar structures (Rosenbloom *et al.*, 1993).

LTBPs have an important role in the processing and secretion of TGF-βs (Miyazono *et al.*, 1991). The small latent TGF-β complex is secreted very slowly, and a significant fraction of small latent TGF-β is retained in the cis-aspect of the Golgi apparatus (Miyazono *et al.*, 1992). LTBPs, in turn, are secreted rapidly, and association of small latent TGF-β with LTBP results in rapid secretion of the large latent complex (Miyazono *et al.*, 1991; see also Taipale *et al.*, 1994a; Saharinen *et al.*, 1996). LTBPs also have a role in the targeting of the latent TGF-β to the extracellular matrix (Taipale *et al.*, 1992, 1994a, 1996; Morén *et al.*, 1994; Olofsson *et al.*, 1995; Dallas *et al.*, 1995). LTBP-1 can be differentially spliced to long and short splice forms (denoted LTBP-1L and LTBP-1S) that differ in the absence or presence of an ~300-amino acid N-terminal sequence. The longer splice form associates more efficiently with the extracellular matrix when overexpressed in COS cells (Olofsson *et al.*, 1995). This is consistent with studies of the smaller splice form of LTBP-1, which was found to interact with the extracellular matrix (ECM) by its amino-terminal region (Saharinen *et al.*, 1996).

The expression of LTBP-1 is often coregulated with TGF-β1 (Miyazono *et al.*, 1991; Dallas *et al.*, 1994; Taipale *et al.*, 1994b). However, the major fraction (90–99%) of LTBPs secreted by cells do not contain TGF-β (Miyazono

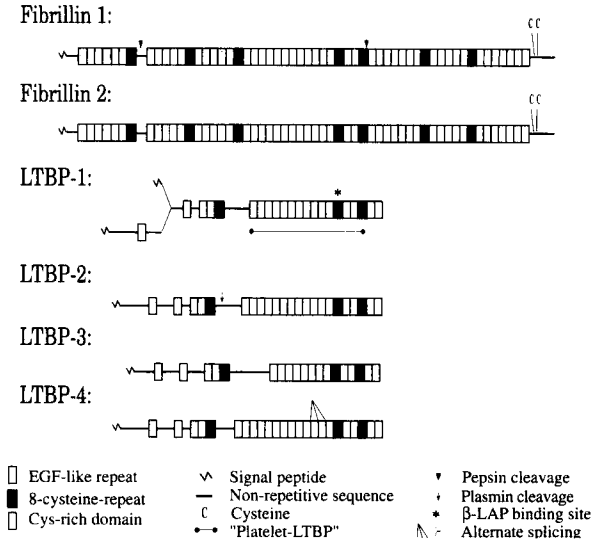


Fig. 1 Domain structures of LTBPs and fibrillins. LTBPs and fibrillins contain similar core regions of 8–13 EGF-like repeats flanked by LTBP-like (eight-cysteine) repeats. Sequenced pepsin cleavage sites in fibrillin 1 correspond to analogous sites cleaved in platelet LTBP (the first cleavage site is in the nonrepetitive, cysteine-free sequence and the second occurs after two EGF-like repeats). At least LTBP-1 appears to be alternatively spliced at the N terminus, generating two forms, LTBP-1L and LTBP-1S. The eight-cysteine repeat in LTBP-1 that associates with β1-LAP is also indicated (*). Adapted from Kanzaki *et al.* (1990), Tsuji *et al.* (1990), Rosenbloom (1993), Morén *et al.* (1994), Olofsson *et al.* (1995), Yin *et al.* (1995), Saharinen *et al.* (1996, 1998), and Hyytiäinen *et al.* (1998).

et al., 1991; Taipale *et al.*, 1994a, 1995, 1996). Thus, LTBPs probably serve a dual role as TGF-β carriers and as structural proteins of the extracellular matrix (Taipale *et al.*, 1994a; Dallas *et al.*, 1995; Gibson *et al.*, 1995). The three different LTBPs and three different TGF-β isoforms offer diverse ways to target the latent forms to specific sites, and to activate them.

TGF-β Superfamily

More than 30 genes with primary sequence homology to TGF-β1 have been isolated since 1985 (Kingsley, 1994; Massagué, 1990; Massagué *et al.*, 1994). TGF-β thus defines a large superfamily of growth factors. Traditionally, the TGF-β superfamily has been divided into four families, the TGF-β family, the bone morphogenic protein (BMP) family [= dpp-Vg-related (DVR) family], the inhibin/activin family, and the MIS family (Fig. 2). The

BMP family includes the bone morphogenetic proteins (BMPs 2–7), growth and differentiation factor-1 (GDF-1), and *Drosophila decapentaplegic (dpp), Xenopus* Vg1, and dorsalin-1 (Massagué, 1994; Basler *et al.*, 1993). Multiple members of the BMP family have key roles in bone morphogenesis and epithelial–mesenchymal interactions during embryonic pattern formation (Kingsley, 1994). The activin family consists of activin A and activin B. Activins regulate the secretion of pituitary follicle-stimulating hormone (FSH). The MIS family includes Müllerian inhibiting substance, which mediates Müllerian duct regression in male embryos. The rather specific functions of the TGF-β superfamily proteins are reflective of the purification and cloning strategies used, and a large number of other effects are likely to be assigned to many of the individual members.

Multiple new TGF-β superfamily members have been cloned that are difficult to assign to the above subfamilies. Therefore, a more open classification has been suggested based on a continuum of homologous factors, forming defined clusters with close homologs (Massagué *et al.*, 1994) (Fig. 2).

After the tertiary structure of active TGF-β2 was determined (Schlunegger and Grütter, 1992; Daopin *et al.*, 1992), it became evident that in addition

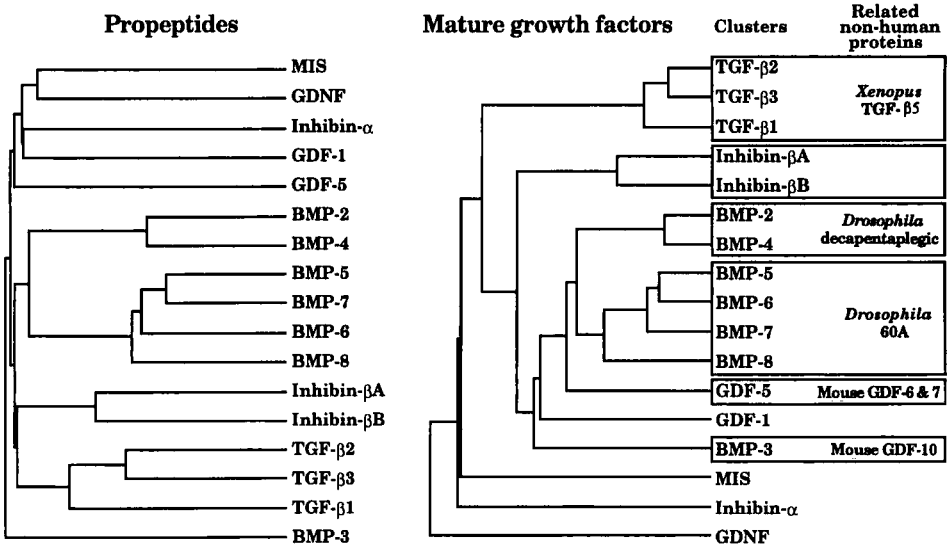


Fig. 2 TGF-β superfamily. The TGF-β superfamily comprises distinct factors that can be arranged into clusters (boxes) of related isoforms. The multiple sequence alignments of human sequences belonging to the TGF-β superfamily were generated using PILEUP (Higgins and Sharp, 1989). BMP-3, -6, -7, -8 are also called osteogenin, Vgr1, osteogenic protein-1, and osteogenic protein-2, respectively. Left: Comparison of propeptides; Right: Comparison of mature growth factors. Clusters are those suggested by Massagué *et al.* (1994). Some nonhuman proteins belonging to a defined cluster are also included on the right.

to primary sequence homology to the members of the TGF-β superfamily, TGF-β2 has remarkable similarities in three-dimensional structure to two other growth factors, namely platelet-derived growth factor-B and nerve growth factor (Swindells *et al.*, 1992; Murray-Rust *et al.*, 1993). Although no notable homology is detectable in the primary structure, all these growth factors are dimeric, fold mainly to β-sheets, and have a knot structure of intrachain disulfide bridges. All these factors also associate with the pocket domains of α_2-macroglobulin (see Crookston *et al.*, 1994). These features define a new structural superfamily of growth factors.

III. EXPRESSION OF TGF-βs AND LATENT TGF-β BINDING PROTEINS

Major sources of TGF-βs are platelets (Wakefield *et al.*, 1988; Miyazono *et al.*, 1988) and bone (Seyedin *et al.*, 1985). Latent TGF-β is found in serum at a concentration of ~10 ng/ml (O'Connor-McCourt and Wakefield, 1987), and small latent TGF-β has a plasma half-life of >100 min in the rat (Wakefield *et al.*, 1990). Blood does not, however, contain significant levels of active TGF-β1, and exogenous active TGF-β1 is cleared from rat blood with a half-life of 2.2 min (Coffey *et al.*, 1987).

A. Localization of TGF-β Transcripts and Proteins *in Vivo*

By *in situ* hybridization and immunostaining it has been found that TGF-β isoforms are expressed by a wide variety of murine embryonic tissues. TGF-β1 RNA is present in cells of endothelial and hematopoietic lineage, such as T cells in thymus and bone marrow megakaryocytes. Connective tissue cells such as osteocytes and fibroblasts and cells of the bronchial mesoderm also express this isoform (Wilcox and Derynck, 1988; Lehnert and Akhurst, 1988; Millan *et al.*, 1991; Schmid *et al.*, 1991). TGF-β2 RNA is expressed by epithelial cells, such as epithelium of the salivary gland, lens, and retina, squamous bronchial epithelium, and suprabasal keratinocytes. TGF-β2 is also the dominant isoform expressed in neural tissue, such as ventral spinal cord and forebrain. TGF-β3 is expressed in medial edge epithelium during palate formation, in cuboidal bronchial epithelium, in muscle, in capsule of the liver, and in mesenchymally derived tissues of the trachea and esophagus. All three TGF-β isoforms are expressed in some tissues, such as skeleton and the developing heart (Pelton *et al.*, 1990a; Schmid *et al.*, 1991; Millan *et al.*, 1991). The expression pattern of TGF-β isoforms in human

embryos is very close to that observed in the murine system (Gatherer *et al.*, 1990).

The expression of TGF-β1 and -β3 in epithelium is largely restricted to structures actively involved in the process of morphogenesis, whereas TGF-β2 is expressed by mature epithelium and epithelial cells of established structures undergoing differentiation (Millan *et al.*, 1991; Pelton *et al.*, 1990a; Heine *et al.*, 1987, 1990; Silberstein *et al.*, 1992).

By immunohistochemistry, TGF-β1 has been localized to perichondrium of cartilage, bone osteocytes, and stomach and intestinal epithelium. TGF-β2 is localized next to epithelial cell types in basement membranes of multiple tissues, such as kidney and gut, to dermis of skin and meninges, and to glia of the central nervous system. TGF-β3 is associated with smooth, cardiac, and skeletal muscle, and liver capsule. Epidermis, lung bronchi, and bone osteocytes contain all three isoforms of TGF-β (Flanders *et al.*, 1989; Heine *et al.*, 1987, 1990; Pelton *et al.*, 1991; Thompson *et al.*, 1989; Silberstein *et al.*, 1992).

TGF-βs 1 and 3 often colocalize with interstitial ECM components, whereas TGF-β2 is typically found associated with basement membranes. In contrast to the uniformly distributed staining of matrix found with antibodies to fibronectin, the matrix is positive for TGF-β1 immunostain only in the proximity of cells actively producing latent TGF-β1 (Flanders *et al.*, 1989). Soluble latent or active TGF-β is commonly absent from the supernatant fluids of homogenized tissues (Waltenberger *et al.*, 1993a), unless extracted by acidic ethanol or denaturants (Roberts *et al.*, 1980; Seyedin *et al.*, 1985).

Less is known about the tissue distribution of latent TGF-β binding proteins. In whole tissue mRNA blots, LTBP-1 and -2 appear to be quite ubiquitously expressed. Highest levels of LTBP-2 mRNA are found in the lung, whereas LTBP-1 is mainly expressed in the heart, placenta, lung, spleen, kidney, and stomach (Tsuji *et al.*, 1990; Morén *et al.*, 1994). LTBP-1 expression pattern at tissue level is similar to that of TGF-β1, except in thymus, where TGF-β1, but not LTBP-1, is expressed (Tsuji *et al.*, 1990). Possible expression of other LTBPs in the thymus is, however, not known.

Immunoelectron microscopic analysis of human fibroblast cultures indicates that LTBP-1 protein associates with 50-nm pericellular, fibronectin-rich fibers, and with extracellular 10- to 12-nm microfibrils. Because the microfibrils are devoid of type VI collagen, they are most likely related to fibrillin microfibrils (Taipale *et al.*, 1996). LTBP-2 was localized in developing elastic fibers in bovine aorta and nuchal ligament (Gibson *et al.*, 1995), supporting the association of LTBPs with fibrillin microfibrils. However, biochemical evidence for the association of LTBPs with fibrillins or other constituents of fibrillin fibrils has not been reported.

In gastrointestinal tissues LTBP-1 localizes to the extracellular matrix, often paralleling collagen fiber bundles (Mizoi *et al.*, 1993). The expression of

ECM-associated LTBP is also elevated in several fibrotic conditions, such as allograft arteriosclerosis, and in tuberculous pleurisy (Maeda *et al.*, 1993; Waltenberger *et al.*, 1993a,b). In tuberculous pleurisy, the staining intensity for LTBP-1 is highest in fibroblasts and mesothelia of immature fibrotic areas, but granulomas containing infiltrated T cells and macrophages are no longer positive for LTBP-1 immunostain (Maeda *et al.*, 1993). In the skin, LTBP-1 immunoreactivity is codistributed with elastin fibers and is increased in areas of solar damage (Karonen *et al.*, 1997).

B. Molecular Basis of Differential Expression of TGF-β Isoforms

In cell culture, most cells types express TGF-β1 and TGF-β3, whereas TGF-β2 is less widely produced (ten Dijke *et al.*, 1988; Derynck *et al.*, 1988). The promoter regions of TGF-βs 1–3 have been characterized (Kim *et al.*, 1989a; Geiser *et al.*, 1991; Noma *et al.*, 1991; Lafyatis *et al.*, 1990, 1991), and they show considerable differences. The promoter regions of LTBPs have not been cloned.

The promoter of TGF-β1 does not contain TATA or CAAT box types of elements, but has binding sites for AP-1 and Egr-1 transcription factors (Kim *et al.*, 1990a, 1994a,b; Scotto *et al.*, 1990). The expression of TGF-β1 is up-regulated by transformation with several oncogenes, including c-Ha-*Ras*, *src*, *jun*, *fos*, *abl*, and *ras*, and the viral transactivating proteins, HTLV-I Tax and HBV X (Kim *et al.*, 1990a,b, 1994b; Birchenall-Roberts *et al.*, 1990; Geiser *et al.*, 1991; Yoo *et al.*, 1996). The AP-1 site mediates up-regulation of TGF-β1 transcription in response to TGF-β1 or phorbol ester (Van Obberghen-Schilling *et al.*, 1988; Kim *et al.*, 1989b, 1991a), whereas the induction of the TGF-β1 gene by NGF seems to involve the Egr-1 site (Kim *et al.*, 1994a). The expression of the TGF-β1 gene can also be negatively controlled by the Wilms' tumor suppressor gene product WT1, whereas the retinoblastoma gene product can both up-regulate or down-regulate TGF-β expression, depending on the cell type (Dey *et al.*, 1994; Kim *et al.*, 1991b).

The promoter for TGF-β2 (Noma *et al.*, 1991) contains AP-1 and AP-2 sites, a cAMP regulatory element, and a TATAA consensus start site. The TGF-β2 promoter is induced by retinoic acid (Glick *et al.*, 1989). Retinoblastoma protein increases TGF-β2 transcription through the transcription factor ATF-2 (O'Reilly *et al.*, 1992; Kim *et al.*, 1992a).

The promoter of TGF-β3 contains a TATA box, a cAMP response element, a 3×TCCC motif, and a single AP-2 site. The cAMP response element mediates the induction of TGF-β3 by forskolin; the 3×TCCC motif has been suggested to have a role in tissue-specific gene expression. The AP-2 site does not appear to be functional (Lafyatis *et al.*, 1990, 1991).

It has been suggested that the genes for TGF-β1 and TGF-β3 are controlled also at the translational level, because the 5′ untranslated region of these genes suppresses the translation of heterologous mRNAs (Kim *et al.*, 1992b; Arrick *et al.*, 1991).

IV. MECHANISMS OF ACTIVATION OF LATENT TGF-β

Latent forms of TGF-β can be activated *in vitro* by acidic or basic pH (Lawrence *et al.*, 1984, 1985; Miyazono *et al.*, 1988; Lyons *et al.*, 1988), heat treatment (Brown *et al.*, 1990), and chaotropic agents or sodium dodecyl sulfate (SDS) (Miyazono *et al.*, 1988). A fraction of latent TGF-β can be activated by deglycosylation of LAP by Endo F or sialidase (Miyazono and Heldin, 1989), or by limited proteolysis of LAP by plasmin (Lyons *et al.*, 1988, 1990). The complexity of the isoforms and latent forms suggests that multiple pathways exist for activation of TGF-βs *in vivo* (Table I). However, no divergent means of activation have been found between the different isoforms to date.

Only few primary cells and established cell lines secrete significant amounts of active TGF-β into their culture medium. Active TGF-β2 is secreted by BSC-1 African green monkey kidney cells (Holley *et al.*, 1978; Tucker *et al.*, 1984a,b) and certain lines of human glioblastoma cells (De Martin *et al.*, 1987; Olofsson *et al.*, 1992). Activation of TGF-β can be induced in cell culture by treatment of keratinocytes by retinoids (Glick *et al.*, 1989) or vitamin D_3 analogs (Koli and Keski-Oja, 1993), and by treatment of cancer cells or normal fibroblasts with antiestrogens (Knabbe *et al.*, 1987; Coletta *et al.*, 1990). Active TGF-β is also induced when bone osteoblasts or MG-63 osteosarcoma cells are treated with corticosteroids (Boulanger *et al.*, 1995; Oursler *et al.*, 1993).

After it was found that cultured cells secrete TGF-β in a latent form (Lawrence *et al.*, 1984), it was thought that the activity of TGF-β in tissues would be regulated by a single biochemical activation step, whereby the mature TGF-β is released from LAP. It is now clear that at least three specific proteolytic cleavages are likely to regulate TGF-β1 activity in tissues, namely (1) the release of TGF-β1 from the matrix (Fig. 3) (Taipale *et al.*, 1992), (2) the activation of TGF-β (Lyons *et al.*, 1988, 1990; Sato and Rifkin, 1989; Sato *et al.*, 1993), and (3) negative regulation by the shedding of TGF-β binding protein betaglycan from the cell surface (López-Casillas *et al.*, 1994; Lamarre *et al.*, 1994). The complexity of the regulation is illustrated by the fact that a single proteinase, plasmin, can function in all three steps (Taipale *et al.*, 1992; Lyons *et al.*, 1988; Lamarre *et al.*, 1994). Therefore, an understanding of the extracellular regulation of TGF-β bioactivity requires the

Table I Activation of Latent Forms of TGF-β

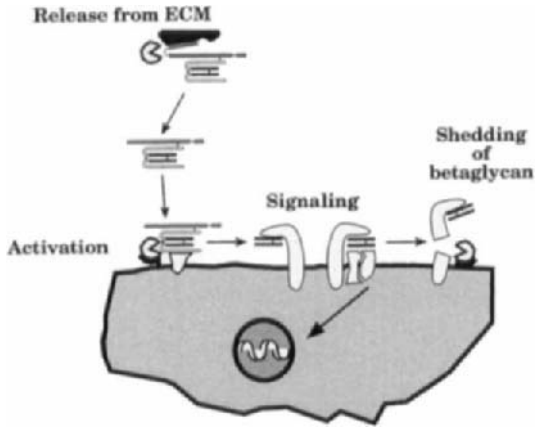
Activation	Ref.
Biochemical	
Extremes of pH	Brown *et al.* (1990)
Proteases (plasmin, cathepsin G)	Lyons *et al.* (1988, 1990)
Cell cocultivation (plasmin dependent)	Sato and Rifkin (1989)
Glycosidases	Miyazono and Heldin (1989)
Acidic cellular microenvironment	Jullien *et al.* (1989)
Thrombospondin mediated	Schultz-Cherry and Murphy-Ullrich (1993)
Treatment (*in vivo*)[a]	
Gamma radiation	Barcellos-Hoff (1993); Erhart *et al.* (1997)
Drug induced (in cell culture)	
Antiestrogens	Knabbe *et al.* (1987)
Retinoids	Glick *et al.* (1989)
Vitamin D	Koli and Keski-Oja (1993)
Glucocorticoids	Oursler *et al.* (1993) Boulanger *et al.* (1995)

[a] Induces activation of TGF-β in cell culture or *in vivo* (by an unknown mechanism).

identification and precise localization of the proteinase(s) and other components involved in all three steps.

Latent TGF-β is activated in cocultures of endothelial cells and smooth muscle cells or pericytes (Antonelli-Orlidge *et al.*, 1989; Sato and Rifkin, 1989, Sato *et al.*, 1990). When individually cultured, both cell types produce latent TGF-β, but coculturing induces the production of active TGF-β. Activation requires the binding of latent TGF-β to cell surfaces via the cation-independent mannose 6-phosphate receptor (Dennis and Rifkin, 1991; Kovacina *et al.*, 1989) or via a yet uncharacterized binding site (Sato *et al.*, 1993). The activation of TGF-β in cocultures of endothelial cells and smooth muscle cells requires close proximity between the cell types (Sato and Rifkin, 1989; Antonelli-Orlidge *et al.*, 1989; Sato *et al.*, 1990). This does not, however, indicate that activation would involve cell–cell contacts. Because latent TGF-β associates with the extracellular matrix (Taipale *et al.*, 1992), the interactions required for the activation might involve contact between one cell type with the extracellular matrix of the other (see Saxén *et al.*, 1976). The importance of proper matrix assembly to TGF-β activation is underscored by a study showing that inhibitors of the matrix cross-linking enzyme transglutaminase block activation of TGF-β in the endothelial/smooth muscle coculture model (Kojima *et al.*, 1993; Nunes *et al.*, 1997).

If endothelial cells and smooth muscle cells are cocultured in the presence of plasmin inhibitors or antibodies to LTBP-1, the production of active TGF-β is suppressed (Sato *et al.*, 1990; Flaumenhaft *et al.*, 1993). It is not clear,



Flg. 3 A model of extracellular regulation of TGF-β signaling. TGF-β is released from the extracellular matrix (ECM) by members of the serine proteinase family. Released soluble large latent TGF-β associates with the cell surface, where it is activated by proteolysis. Subsequently, activated TGF-β binds to betaglycan, which presents the ligand to its cell surface receptors, and signal is transduced to the interior of the cell. Response of the cells to TGF-β can be down-regulated by shedding betaglycan from the cell surface. Adapted from Taipale *et al.* (1992, 1994a, 1995), Lyons *et al.* (1988), Wrana *et al.* (1994a), López-Casillas *et al.* (1994), and Lamarre *et al.* (1994).

however, whether plasmin is required in this system to release TGF-β from the matrix, or to directly activate TGF-β. Similarly, antibodies to LTBP-1 could act by blocking the assembly of LTBP-1 to the matrix or by inhibiting the activation reaction at a later stage.

It is not known whether the release of large latent TGF-β from the matrix regulates local TGF-β activity in a positive or negative manner. This depends on whether the substrate for activation is the soluble or matrix-bound form of latent TGF-β. If the soluble large latent form of TGF-β were the substrate for activation, as suggested by Sato *et al.* (1993), the release from the matrix would increase the local availability of soluble large latent TGF-β, positively regulating extracellular TGF-β activity. If, however, the matrix-bound form of latent TGF-β is directly a substrate for activation, the release of TGF-β from the matrix would act as a negative regulator of TGF-β activity, by decreasing the concentration of matrix-bound latent TGF-β. Cultured cells do not normally activate significant proportions of soluble large or small latent TGF-β. Soluble large latent TGF-β1 has less than 1% of the activity of active TGF-β1 in inducing Mv1Lu growth arrest (Taipale and Keski-Oja, 1996). In addition, we failed to detect activation of TGF-β when Mv1Lu epithelial cells were cultured in TGF-β-containing fibroblast extracellular matrices (Taipale and Keski-Oja, 1996). It therefore appears that neither a pos-

itive nor negative regulatory role for the release of TGF-β from the matrix can be ruled out.

Hepatocyte growth factor, in contrast to TGF-β, is efficiently activated by cultured cells, and little difference is observed in the biological activities of active and latent forms of HGF in the presence of serum (Naldini *et al.*, 1992; Miyazawa *et al.*, 1994). However, cell culture represents an artificial model, wherein cells continuously migrate and divide in the presence of proteins normally found in damaged tissues (serum). In a sense, cell culture resembles a continuously healing wound. It is thus possible that two proteolytic states of cells exist: "TGF-β-activating" quiescent state (stable epithelium) (see Sun *et al.*, 1994) and "HGF-activating" proliferative or invasive state (cultured cells, invading cells, cancer). Therefore, the inability of cultured cells to activate TGF-β could simply reflect the fact that TGF-β is not activated by migrating and dividing cells. It is also possible that serum (as opposed to blood) lacks components that are required for TGF-β activation. The apparent lack of active TGF-β in cell culture supernatants may also be caused by the relative insolubility of active TGF-β and the high affinity of the TGF-β receptors (below).

Proteolysis, acidic cellular microenvironments (Jullien *et al.*, 1989), and the extracellular matrix molecule thrombospondin (Schultz-Cherry *et al.*, 1993) have been proposed to mediate TGF-β activation under physiological conditions. Proteolysis of LAP is the most likely mechanism of TGF-β activation *in vivo*, because proteinase inhibitors block the activation of TGF-β in several cell culture models (Antonelli-Orlidge *et al.*, 1989; Sato and Rifkin, 1989; Sato *et al.*, 1990; Huber *et al.*, 1992; Khalil *et al.*, 1996). Plasminogen-depleted medium also fails to support TGF-β activation (Sato *et al.*, 1990; Brauer and Yee, 1993). Genetic evidence suggests, however, that alternative pathways exist for the activation of TGF-β1. Mice defective in plasminogen, or in both plasminogen activators u-PA and t-PA, do not suffer from inflammatory lesions characteristic of the TGF-β1 knockout mice, indicating that these factors are not required for the expression of TGF-β1 activity (Shull *et al.*, 1992; Carmeliet *et al.*, 1994; Bugge *et al.*, 1995).

Activation of TGF-β-Related Growth Factors

Proteases have also been implicated in the activation of other members of the TGF-β superfamily. The TGF-β homologs bone morphogenetic proteins-2, -3, and -4 copurify from demineralized bone with a proteinase, BMP-1 (Wang *et al.*, 1988; Wozney *et al.*, 1988). In *Drosophila*, genetic evidence links a BMP-1 homologous proteinase, the product of the *tolloid* gene, to the signaling pathway of *decapentaplegic* (*dpp*), a BMP-2/BMP-4-related gene involved in dorsal patterning of the *Drosophila* embryo (Ferguson and An-

derson, 1992a; Hecht and Anderson, 1992; Wharton *et al.*, 1993). Both Tolloid and BMP-1 are members of the astacin family of metalloproteinases (Dumermuth *et al.*, 1991).

Recombinant activin, BMP-2, and BMP-4 appear to be secreted from CHO cells as active dimers (Israel *et al.*, 1992; Suzuki *et al.*, 1992). However, BMP-7 (OP-1) is secreted as a complex with its propeptide, and the biological activity of this complex is unknown at present (Jones *et al.*, 1994). The two cysteines involved in LAP dimer formation that are necessary for the latency of TGF-βs (Cys-233 and Cys-235 of TGF-β1) (Brunner *et al.*, 1989) are not conserved in the TGF-β superfamily. Several members of the superfamily, including GDF-8 (McPherron *et al.*, 1997), contain analogous cysteine residues.

In vivo, the activity of BMPs is controlled by specific binding proteins, such as chordin, which binds to and inactivates BMP-4 (Piccolo *et al.*, 1997). Recently it was found that proteolytic processing of chordin by BMP-1 metalloprotease leads to activation of BMP-4 (Piccolo *et al.*, 1997). This hypothesis is supported by the observation that the requirement for *tolloid* in dorsal development can be bypassed by raising the amount of *dpp* RNA in the *Drosophila* embryo (Ferguson and Anderson, 1992b; Hecht and Anderson, 1992; Wharton *et al.*, 1993).

A significant fraction of secreted recombinant TGF-β1 is defectively processed when overexpressed in CHO cells (Gentry *et al.*, 1988; Miyazono *et al.*, 1991, 1992; Saharinen *et al.*, 1996). Similarly, CHO cell-expressed BMP-7 has a long secretion half-life, suggesting defects in folding or processing (Jones *et al.*, 1994). In *Xenopus,* overexpressed Vg1 is not processed and remains inside cells in monomeric form (Thomsen and Melton, 1993; Dale *et al.*, 1993). High expression levels and unphysiologic producer cells may thus cause the defective processing of BMPs and activins.

V. SIGNAL TRANSDUCTION BY TGF-β

A. TGF-β Binding Proteins and Receptors

The majority of normal and tumor cells express between 10 and 4000 high-affinity binding sites for TGF-β1 (Wakefield *et al.*, 1987). Affinity labeling reveals two polypeptide species that bind TGF-β1 with high apparent affinity, namely type I and type II receptors. Recombinant type II receptor extracellular domain binds to TGF-β1 and -β3 with high affinity (K_D = 25–100 p*M*). TGF-β2 is not bound by this receptor (K_D > 10 n*M*). Type I receptor does not bind TGF-β in the absence of type II receptor (Laiho *et al.*, 1990a; Wrana *et al.*, 1994a).

Most cell types express also up to 10^5 medium-affinity TGF-β binding sites. These sites represent a TGF-β binding protein known as betaglycan (formerly type III receptor; Massagué, 1990; Miyazono *et al.*, 1993; Miyazono, 1997). Betaglycan binds to all TGF-β isoforms with medium affinity (apparent $K_D \sim 30$–300 p*M*). Betaglycan presents TGF-β isoforms to type II receptor and induces high-affinity binding of TGF-β2 to the type II TGF-β receptor. Endothelial and myeloid cells also express another TGF-β binding protein, endoglin, that binds TGF-β1 and TGF-β3 with high affinity ($K_D \sim 50$ p*M*), and TGF-β2 with significantly weaker affinity (Cheifetz *et al.*, 1992; Bellón *et al.*, 1993).

Several other cell surface TGF-β binding proteins have also been characterized (see, for example, Cheifetz and Massagué, 1991; Segarini *et al.*, 1992; Bützow *et al.*, 1993). A receptor function for one of these has been suggested (O'Grady *et al.*, 1992). Of soluble plasma proteins, TGF-β1 binds to the plasma protease inhibitor α_2-macroglobulin (O'Connor-McCourt and Wakefield, 1987; Philip and O'Connor-McCourt, 1991), to which a clearance function has been assigned.

Several extracellular matrix proteins have also been suggested to bind TGF-β1. These include fibronectin (Fava and McLure, 1987), thrombospondin (Murphy-Ullrich *et al.*, 1992), and collagen IV (Paralkar *et al.*, 1992). The core proteins of extracellular matrix proteoglycans decorin and biglycan have also been reported to bind to TGF-β and neutralize its activity (Yamaguchi *et al.*, 1990). However, cross-linking or stoichiometric saturable binding to TGF-β has not been demonstrated to any of the above proteins. One extracellular matrix protein, ficolin, has been cloned on the basis of its affinity for TGF-β. Ficolin is a multimeric 40-kDa protein that can be cross-linked to TGF-β1 in cell culture, but purified recombinant ficolin apparently does not bind TGF-β (Ichijo *et al.*, 1993).

Cell surface and extracellular matrix proteoglycans have been suggested to bind TGF-β1 via a 60-kDa protein (Bützow *et al.*, 1993). Direct binding of TGF-β1 to heparin has also been reported (McCaffrey *et al.*, 1992; see also Keski-Oja *et al.*, 1987).

B. Receptor Signaling

The type I and type II TGF-β receptors have been cloned and identified as transmembrane serine/threonine protein kinases (Lin *et al.*, 1992; Franzén *et al.*, 1993; ten Dijke *et al.*, 1994a). In addition, multiple serine/threonine kinases resembling the type I and type II receptors for TGF-β have been cloned, including receptors for activin and BMP-2 (Mathews and Vale, 1991; Estevez *et al.*, 1993; Ebner *et al.*, 1993; Matsuzaki *et al.*, 1993; Franzén *et al.*, 1993; ten Dijke *et al.*, 1993, 1994a,b; Wrana *et al.*, 1994b). The nucleotide se-

quences of the TGF-β binding proteins, betaglycan (Wang *et al.*, 1991; López-Casillas *et al.*, 1991), and endoglin (Bellón *et al.*, 1993), have also been determined.

Studies using a panel of TGF-β receptor mutant cell lines have shown that type I receptor is not expressed in cells defective of type II receptor. Expression of both receptor subtypes is, however, necessary for TGF-β signal transduction (Laiho *et al.*, 1990a, 1991a; Wrana *et al.*, 1992, 1994a). The loss of both receptor types in type II receptor-deficient cells is probably due to the fact that type I TGF-β receptor alone does not bind TGF-β1 (Wrana *et al.*, 1994a).

When ligand is not present, TGF-β receptor type II and betaglycan form homooligomers (Henis *et al.*, 1994). In the presence of ligand, heteromeric complexes between type II receptor and betaglycan, endoglin, or type I receptor are detected (Wrana *et al.*, 1992; López-Casillas *et al.*, 1993; Moustakas *et al.*, 1993; Miyazono *et al.*, 1994). The formation of a type I/type II receptor heteromer is required for the transduction of TGF-β signal (Wrana *et al.*, 1992, 1994a).

C. Intracellular Signaling

Genetic evidence from *Drosophila* suggested that Mothers against dpp (MAD) protein is involved in intracellular signaling of decapentaplegic (Sekelsky *et al.*, 1995; Raftery *et al.*, 1995). Subsequently, MAD was found to be a prototype of a large family of proteins that are involved in the signal transduction of TGF-β superfamily members (reviewed in Massagué, 1996). Three MAD-related proteins that mediate TGF-β signals have been identified to date, Smad2, Smad3, and Smad4 (Eppert *et al.*, 1996; Zhang *et al.*, 1996; Lagna *et al.*, 1996; Macías-Silva *et al.*, 1996). In unstimulated cells, Smad proteins are found dominantly in the cytoplasm as homomeric complexes (Wu *et al.*, 1997; Lagna *et al.*, 1996; Newfeld *et al.*, 1996) Ligand stimulation induces transient association of Smad proteins with heterodimeric TGF-β superfamily receptors (Zhang *et al.*, 1996; Macías-Silva *et al.*, 1996; Nakao *et al.*, 1997). Type I TGF-β receptor phosphorylates the C-terminal domains of Smad2 and Smad3 proteins (Macías-Silva *et al.*, 1996). Phosphorylation induces dissociation of Smad2 and Smad3 proteins from the receptor, and their heteromerization with a common subunit, Smad4. Activated Smad2/3–Smad4 complexes are translocated to the nucleus (Yingling *et al.*, 1996; Macías-Silva *et al.*, 1996; Zhang *et al.*, 1996; Nakao *et al.*, 1997), where they act as transcriptional activators (Liu *et al.*, 1996). It is likely that multiple Smad proteins are involved in signaling by one TGF-β superfamily member (Savage *et al.*, 1996).

Two other potential substrates for type I TGF-β receptor have been found

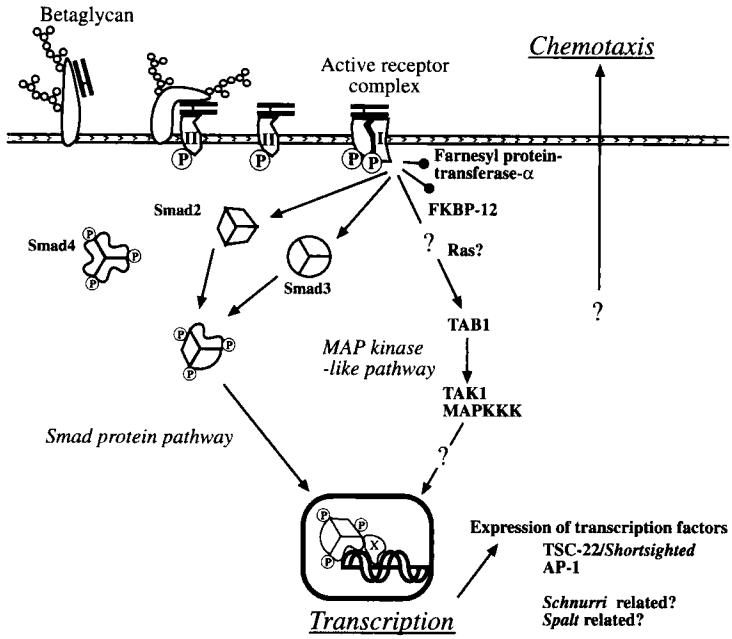


Fig. 4 Intracellular signaling by TGF-β. TGF-β binds to betaglycan (formerly type III receptor), which presents TGF-β to the type II receptor. Type I receptor binds to the complex of type II receptor and TGF-β and is phosphorylated by the constitutively active TGF-β type II receptor kinase. Type I receptor kinase is activated and propagates the signal by phosphorylating Smad2 and Smad3 (arrows). These Smad proteins heteromerize with Smad4 and are translocated to the nucleus, where they act as transcriptional activators (whose cofactors are unknown (X)). The type I TGF-β receptor also associates with two other cytoplasmic proteins, protein farnesyltransferase-α and FKBP-12 (●), whose functions in TGF-β signaling are unknown at present. A MAP-kinase-like pathway has also been suggested to be involved in TGF-β signaling. Adapted from Lopez-Casillas *et al.* (1993), Wrana *et al.* (1994a), Massagué (1996), Shibuya *et al.* (1996), Shi *et al.* (1997), and Miyazono (1997).

using yeast two-hybrid screens, protein farnesyltransferase-α (Kawabata *et al.*, 1995) and FKBP-12 (Fig. 4). Protein farnesyltransferase-α is a subunit of farnesyltransferase that catalyzes the addition of farnesyl moieties to N termini of cytoplasmic proteins, including $p21^{ras}$ family GTPases. In the absence of TGF-β, protein farnesyltransferase-α associates with the type I TGF-β receptor in an inactive form. Phosphorylation of the farnesyltransferase-α by ligand-bound type II TGF-β receptor induces its dissociation from the receptor and activation. FKBP-12 is a protein that interacts with the immunosuppressive drug FK-506, and was found to interact with the type I TGF-β receptor (Wang *et al.*, 1994a). FKBP-12 was subsequently found to function as a universal inhibitor of type I receptors of the TGF-β superfamily (Wang

et al., 1996). Because TGF-βs are extremely immunosuppressive (Massagué, 1990), this raises the possibility that FK-506 exerts part of its effects through interfering with TGF-β signaling.

TRIP-1 (Chen *et al.*, 1995) is a protein that binds to and is phosphorylated by the type II TGF-β receptor. Ligand-dependent changes in its phosphorylation or activity have not been found, and the role of this protein in TGF-β signaling remains unknown.

At present, it is not clear whether the Smad proteins are involved in all aspects of TGF-β signaling, including regulation of extracellular matrix synthesis, cell adhesion, cell growth, extracellular proteolytic balance, cell migration, and chemotaxis. Some aspects of the signaling by TGF-β could be mediated by a MAP kinase-type pathway. In this model, TGF-β activates TAB1, which binds to and activates MAPKKK TAK1 (Shibuya *et al.*, 1996; Yamaguchi *et al.*, 1995). TAK1 activation, in turn, induces transcription from a PAI-1 promoter by an unknown mechanism. One possibility is that this MAP kinase pathway is involved in the regulation of Smad protein phosphorylation.

The chemotactic responses to TGF-β (Postlethwaite *et al.*, 1987) could also involve the regulation of Ras (Mulder and Morris, 1992) and Rho (Atfi *et al.*, 1997) GTPases. Some reports have also suggested that TGF-β signaling is in part mediated through pertussis toxin-sensitive G-proteins (Howe *et al.*, 1990a; Kataoka *et al.*, 1993). TGF-β has also been reported to inhibit the coupling of $p21^{ras}$ to the activation of phospholipase C-mediated hydrolysis of phosphatidylcholine (Diaz-Meco *et al.*, 1992), and to down-regulate the activity of Src family protein tyrosine kinases (Atfi *et al.*, 1994). TGF-β stimulation also activates signaling molecules that have traditionally been thought to be involved in growth stimulation, such as ras GTPase and $p44^{map}$ kinase (Yan *et al.*, 1994; Hartsough and Mulder, 1995). TGF-β activates $p44^{map}$ kinase in proliferating, but not in quiescent, epithelial cells within 5–10 min of stimulation (Hartsough and Mulder, 1995). In fact, one report even suggests that *ras* GTPase is activated only in epithelial cells that respond to TGF-β by growth inhibition, but not in transformed epithelial cells that are growth stimulated by TGF-β (Yan *et al.*, 1994).

VI. CONTROL OF GENE EXPRESSION BY TGF-β

Stimulation of cells by TGF-β directly modulates the function of multiple transcription factors. Two factors have been identified that are regulated by TGF-β in the absence of protein synthesis. An element that confers a 50-fold inducibility of heterologous promoter by TGF-β has been mapped to region −707 to −726 in the human PAI-1 gene (Riccio *et al.*, 1992; Westerhausen

et al., 1991; Keeton *et al.*, 1991; Wrana *et al.*, 1992; Sandler *et al.*, 1994). TGF-β induces the binding of a 100-kDa protein to this element within 5 min of stimulation. TGF-β also induces the phosphorylation of CRE binding protein and increases its binding to its target sequence (Kramer *et al.*, 1991).

TGF-β modulates gene expression by regulating the synthesis of multiple transcription factors. TGF-β modulates the AP-1 transcription factor complex by up-regulating the synthesis of c-*jun* and *junB* mRNA and protein (Pertovaara *et al.*, 1989; Li *et al.*, 1990). In addition, one putative transcription factor gene, TSC-22/*Shortsighted*, has been found to be induced by TGF-β (Shibanuma *et al.*, 1992; Treisman *et al.*, 1995). TGF-β also down-regulates the expression of c-myc and B-myb transcription factors and modulates the activity of E2F1 (Pietenpol *et al.*, 1990a,b; Satterwhite *et al.*, 1994; Satterwhite and Moses, 1994; Laiho *et al.*, 1990b; Schwarz *et al.*, 1995). Genetic evidence from *Drosophila* indicates that in addition to TSC-22/*Shortsighted*, two other transcription factors, *Schnurri* (Arora *et al.*, 1995) and *Spalt* (de Celis *et al.*, 1996), are required for dpp signaling. Homologs of these factors might thus also be involved in mediating TGF-β responses.

Other TGF-β-inducible elements include Sp-1-like binding sites in human α2(I) collagen, *p15* and *p21* genes (Inagaki *et al.*, 1994; Datto *et al.*, 1995a,b; Li *et al.*, 1995a), and NF-1-like binding site (TAE) in α1(I) collagen (Ritzenthaler *et al.*, 1993).

The expression of stromelysin is inhibited by TGF-β through binding of a fos-containing protein complex to a 10-bp cis-acting element (Kerr *et al.*, 1990), denoted transforming growth factor inhibitory element (TIE). TIE is an AP-1-like sequence that lacks a *bona fide* AP-1 site. TIE is present in the promoters of multiple metalloproteinase genes and in the u-PA gene (Kerr *et al.*, 1990; Cannio *et al.*, 1993).

TGF-β has also a role in the regulation of mRNA stability—it increases the half-life of mRNAs of multiple extracellular matrix proteins (Penttinen *et al.*, 1988; Raghow *et al.*, 1987). In addition, TGF-β induces a mRNA stabilizing factor that specifically binds to the 3′ untranslated region of ribonucleotide reductase mRNA (Amara *et al.*, 1995).

VII. BIOLOGICAL EFFECTS OF TGF-β

The three major biological effects of TGF-β are inhibition of the growth of epithelial, endothelial, and hematopoietic cells, stimulation of extracellular matrix formation, and immunosuppression. Only a limited number of differences in the effects of different TGF-β isoforms have been found. TGF-β1 is a more potent growth inhibitor of several murine hematopoietic pro-

Table II Phenotypes of TGF-β Null Mice

Null allele	Phenotype	Ref.
TGF-β1	Multifocal lymphocyte-mediated inflammatory disease	Shull *et al.* (1992), Kulkarni *et al.* (1993), Diebold *et al.* (1995)
	Yolk sack hematopoiesis and vasculogenesis defect	Dickson *et al.* (1995)
	Cardiac abnormalities	Letterio *et al.* (1994)
TGF-β2	Wide range of developmental defects, including cardiac, lung craniofacial and urogenital defects	Sanford *et al.* (1997)
TGF-β3	Cleft palate	Proetzel *et al.* (1995)
	Cleft palate, abnormal lung development	Kaartinen *et al.* (1995)
Type II receptor	Yolk sac hematopoiesis and vasculogenesis defect	Oshima *et al.* (1996)
Type I receptor	Not characterized	
Betaglycan	Not characterized	
Endoglin	Not characterized; human mutations cause hereditary hemorrhagic telangectasia type I	McAllister *et al.* (1994)

genitor cell lines and human endothelial cells compared to TGF-β2 (Ohta *et al.*, 1987; Jennings *et al.*, 1988). This is probably due to the fact that cells of endothelial and hematopoietic lineage express endoglin, and often lack betaglycan, leading to decreased binding of the TGF-β2 isoform (López-Casillas *et al.*, 1993).

An important delineation of TGF-β function *in vivo* comes from studies of transgenic mice expressing TGF-β under tissue-specific promoters, and analysis of null alleles of TGF-βs and their receptors. The phenotypes of these mice are summarized in Tables II and III. Null alleles of TGF-βs and their receptors cause defects in cell proliferation, adhesion, migration, and differentiation (Table II). Overexpression of TGF-β1 in epithelium, in turn, results typically in decreased epithelial proliferation and fibrosis (Table III).

TGF-β knockout is modulated by genetic background (Shull *et al.*, 1992; Bonyadi *et al.*, 1997). Under NIH/O1a, C57BL/6J/O1a, and F1 backgrounds, approximately 50% of embryos die *in utero*, due to defects in yolk sac vasculogenesis and hematopoiesis. The modifying gene has been mapped to mouse chromosome 5 (Bonyadi *et al.*, 1997). Under all immunoproficient mouse backgrounds tested, mice that are born appear relatively healthy, but they die shortly after weaning due to tissue damage caused by invasion of inflammatory cells in multiple organs. This lethal phenotype of TGF-β1 knockout mice can be reversed by cross-breeding to an immunodeficient

Table III Phenotypes of TGF-β Overexpressing Mice

Target organ	Promotor	Phenotype	Ref.
TGF-β1			
Liver	Albumin	Fibrosis of liver and kidney, arteritis, myocarditis, atrophic changes in pancreas and testis	Sanderson *et al.* (1995)
	Albumin	Progressive fibrotic kidney disease	Kopp *et al.* (1996)
Lung	SP-C	Arrest in lung epithelial cell differentiation	Zhou *et al.* (1996)
Mammary gland	WAP	Secretory mammary epithelium defect	Kordon *et al.* (1995)
	MMTV	Ductal hypoplasia	Pierce *et al.* (1995)
Pancreas	Insulin	Fibrosis, macrophage, and neutrophil infiltration	Lee *et al.* (1995)
		Fibrosis, pancreatititis	Sanvito *et al.* (1995)
Central nervous system	GFAP	Hydrocephalus, acceleration of Alzheimer's-like disease	Galbreath *et al.* (1995), Wyss-Coray *et al.* (1997)
Skin	Keratin 1	Compact orthohyperkeratosis, reduced number of hair follicles	Sellheyer *et al.* (1993)
TGF-β2			
Bone osteoblast	Osteocalcin	Osteoporosis-like phenotype	Erlebacher and Derynck (1996)

(SCID) background, indicating that death of these mice is caused by hyperactivity of the immune system (Diebold *et al.*, 1995). These observations support the notion that TGF-β1 plays a major role in limiting inflammatory responses and invasive phenotype of cells. The study of the TGF-β1 knockout mouse may also be complicated by the fact that maternal TGF-β1 protein is transferred to fetus, and immunostaining for TGF-β1 is unchanged in first generation TGF-$\beta 1^{-/-}$ mice (Letterio *et al.*, 1994).

Knockout mice for TGF-βs 2 and 3 have also been constructed. TGF-β2 knockout mice have multiple developmental defects that in some cases simulate neural crest deficiencies (Sanford *et al.*, 1997). The TGF-β3 null mice, in turn, suffer from cleft palate and lung defects, and die soon after birth (Kaartinen *et al.*, 1994; Proetzel *et al.*, 1995). Immunohistochemical and *in situ* hybridization data also suggest that TGF-β3 plays an important role in murine palate formation (Pelton *et al.*, 1990b).

Type II TGF-β receptor null mice die *in utero* of a yolk sac hematopoiesis and vasculogenesis defect that is indistinguishable from the phenotype of the TGF-β1 null mice (Oshima *et al.*, 1996).

Interestingly, under some genetic backgrounds, TGF-β1 knockout mice have no major developmental defects, and the pathology of the TGF-β3 knockout is also relatively mild. The results differ from what would be anticipated from the expression patterns of the isoforms in mouse embryos (above). The lack of effects is probably due to the fact that multiple TGF-β superfamily members show an overlapping expression pattern. The functions of TGF-β1 could thus be carried out by TGF-β-2 and -3, or other members of the superfamily.

A biological function for extracellular matrix-associated TGF-β1 has been suggested in the inhibition of ductal budding in murine mammary gland (Silberstein *et al.*, 1992), and in the regulation of branching morphogenesis in the lung (Heine *et al.*, 1990). Interestingly, antibodies to LTBP-1 inhibit epithelial–mesenchymal transition in cell culture model of endocardial cushion tissue formation, and the inhibition can be reversed by the addition of active TGF-β (Nakajima *et al.*, 1997). In accordance, Ghosh and Brauer (1996) have found that at least a latent form of TGF-β3 is present in the extracellular matrix of embryonic heart.

A. Regulation of Cell Proliferation by TGF-β

TGF-β reversibly inhibits the growth of most cell types. Particularly sensitive to TGF-β1 are pure cultures of epithelial and endothelial cells (Moses *et al.*, 1985). The effects of TGF-β on the growth of mesenchymal cells are bimodal. Under high mitogen concentrations TGF-β is growth inhibitory, whereas under low mitogen concentration TGF-β stimulates the growth of fibroblasts and smooth muscle cells by inducing an autocrine platelet-derived growth factor (PDGF) loop (Shipley *et al.*, 1985; Leof *et al.*, 1986; Battegay *et al.*, 1990). TGF-β induces both PDGF A and B chains and PDGF receptor (Leof *et al.*, 1986; Mäkelä *et al.*, 1987; Battegay *et al.*, 1990). TGF-β-induced indirect mitogenesis and stromal connective tissue formation can also be mediated by the induction of fibroblast growth factor-2 (FGF-2) (Pertovaara *et al.*, 1993), vascular endothelial-cell growth factor (VEGF) (Pertovaara *et al.*, 1994), and a PDGF-related protein, connective tissue growth factor (CTGF) (Igarashi *et al.*, 1993).

TGF-β1 inhibits the proliferation of epithelial cells *in vivo*. Slow-release polymers impregnated with TGF-β1 implanted in mouse mammary glands inhibit branching morphogenesis by stopping the invasion of ductal end buds (Silberstein and Daniel, 1987; Silberstein *et al.*, 1992). Growth stimulation of mesenchymal cells by TGF-β1 can also be demonstrated *in vivo*. Porcine arteries transfected with a construct encoding active TGF-β1 show intimal and medial smooth muscle cell hyperplasia (Nabel *et al.*, 1993).

MECHANISM OF GROWTH REGULATION BY TGF-β

TGF-β acts by arresting the cell cycle in middle to late G_1 (Moses *et al.*, 1985; Moses, 1992; Laiho *et al.*, 1990b). Several mechanisms for TGF-β-mediated cell cycle arrest have been postulated on the basis of correlative evidence. These include down-regulation of c-*myc* protooncogene expression (Pietenpol *et al.*, 1990a,b), suppression of the activity of CDK tyrosine phosphatase Cdc25A (Iavarone and Massagué, 1997), inhibition of retinoblastoma-protein phosphorylation (Laiho *et al.*, 1990b), suppression of the activity of cyclin-dependent kinases Cdc2 (Howe *et al.*, 1991), Cdk2 (Koff *et al.*, 1993), and Cdk4 (Ewen *et al.*, 1993), and induction of cyclin-dependent kinase inhibitors $p15^{INK4B}$ (Hannon and Beach, 1994), $p21^{Cip1}$ (Li *et al.*, 1995a; Datto *et al.*, 1995a,b), and $p27^{Kip1}$ (Polyak *et al.*, 1994). A satisfactory cause–effect relationship for any of these mechanisms has not been demonstrated, and most are associated with cell cycle arrest in general.

Few definitive experiments delineate mechanisms of TGF-β-mediated cell cycle arrest. Cell hybrid studies indicate that the resistance to growth inhibition by TGF-β is a recessive trait (Geiser *et al.*, 1992; Reiss *et al.*, 1993). Expression of SV40 large T antigen makes cells refractory to growth inhibition by TGF-β, but does not affect other aspects of TGF-β signaling (Laiho *et al.*, 1991b). SV40 large T is known to associate with multiple proteins controlling the cell cycle, such as p53, the retinoblastoma gene product (Rb, p105), and the Rb-related protein p107 (Jay *et al.*, 1981; DeCaprio *et al.*, 1989; Ewen *et al.*, 1989). Cells expressing adenovirus-transforming protein E1A are also refractory to TGF-β growth inhibition (Pietenpol *et al.*, 1990b; Missero *et al.*, 1991). E1A binds at least to four proteins, p300, p107, p105 (Rb), and p60 in mouse keratinocyte-derived cell line Pam212. Pam212 cells expressing a mutant E1A that associates only with p107 display 15% resistance to TGF-β, whereas E1A mutant binding to Rb, p107, and p60 and E1A mutant binding to p300 confer 30% and 60–70% resistance, respectively. The two mutant E1A proteins, one binding to Rb, p107, and p60 and the other to p300, complement each other in trans, conferring complete TGF-β resistance (Missero *et al.*, 1991). It thus appears that the interaction of E1A with multiple cell cycle regulatory proteins is required for the complete loss of TGF-β growth inhibition.

Mv1Lu cells overexpressing Cdk4 (Ewen *et al.*, 1993), viral-transforming proteins with pRb-binding domains, or the transcription factor E2F1 (Schwarz *et al.*, 1995) are refractory to TGF-β1-induced growth arrest. Hypophosphorylated Rb binds to E2F1 and suppresses its activity (Chellappan *et al.*, 1991; Hiebert *et al.*, 1992), and Cdk4 is capable of phosphorylating Rb (Kato *et al.*, 1993). These data indicate that TGF-β growth inhibition is likely to be mediated by the retinoblastoma protein and/or related proteins p107 and p130 (Laiho *et al.*, 1990b). Based on all evidence to date, it is like-

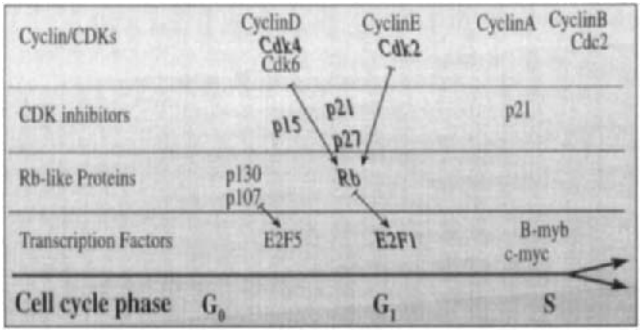


Fig. 5 TGF-β effects on cell cycle progression. In resting cells (G_0), expression of the cyclin Ds is low. Growth factor stimulation induces the expression of cyclins D, which associate in complexes with Cdk4 and Cdk6. These complexes phosphorylate target proteins required for the progression of the cell cycle in gap 1 phase (G_1). Subsequently, cyclin E is induced, and complexes of cyclin E and Cdk2 are formed. The cyclin E/Cdk2 complex promotes progression of the cell cycle through a restriction point, after which the cell is determined to enter the DNA synthesis phase (S). The activity of cyclin-dependent kinases is regulated by "cdk inhibitors," such as p15, p16, p21, and p27. Both p15 and p16 inhibit only Cdk4 complexes with cyclin D, whereas p27 and p21 inhibit Cdk4/cyclin D and Cdk2/cyclin E; p21 has multiple functions—it can suppress the elongation of DNA synthesis at individual replication forks (Yan and Newport, 1995). The factors that are modulated by TGF-β to brake the cell cycle (see text) are indicated by shadowing and reverse italic notation. Because the expression of cyclin A, Cdc2, and B-*myb* occur at late G_1 to S, the suppression of expression/activity of these proteins by TGF-β is likely to be a consequence rather than the cause of the growth arrest. Adapted from Hannon and Beach (1994), Doreé and Galas (1994), Sardet *et al.* (1995), Xu *et al.* (1995), Massagué and Polyak (1995), and Sherr (1996).

ly that multiple TGF-β signaling mechanisms converge to cause growth arrest (see Fig. 5).

In some cells, TGF-β can positively regulate $G_1 \rightarrow S$ transit. Loss of substratum connection blocks the cell cycle of anchorage-dependent cell lines at the G_1–S boundary (Shin *et al.*, 1975; O'Neill *et al.*, 1986; Singhvi *et al.*, 1994). In mouse AKR-2B fibroblasts and rat NRK-49F cells, TGF-β is able to bypass the requirement of adhesion, inducing anchorage-independent growth (Roberts *et al.*, 1981; Moses *et al.*, 1985; Han *et al.*, 1993). TGF-β and cell adhesion appear to control $G_1 \rightarrow S$ transit independently of growth factor-mediated mitogenic events (Han *et al.*, 1993).

B. Cell Motility

TGF-β is chemotactic to fibroblasts (Postlethwaite *et al.*, 1987), neutrophils (Parekh *et al.*, 1994), and monocytes (Wahl *et al.*, 1987). The loco-

motion of endothelial cells is, however, suppressed by TGF-β (Sato and Rifkin, 1989; Heimark *et al.*, 1986). Chemotaxis of neutrophils requires adhesion of cells to fibronectin (Parekh *et al.*, 1994), whereas chemotaxis of fibroblasts and fibrosarcoma cells utilizes the hyaluronan receptor RHAMM and hyaluronan (Samuel *et al.*, 1993).

C. Effects of TGF-β on Synthesis and Degradation of the Extracellular Matrix

TGF-β has a major role in the regulation of extracellular matrix synthesis, degradation, and remodeling (Fig. 6). TGF-β stimulates the synthesis of multiple extracellular matrix components, including collagens, fibronectin, vitronectin, tenascin, thrombospondin, and proteoglycans (Ignotz and Massagué 1987; Raghow *et al.*, 1987; Koli *et al.*, 1991; Pearson *et al.*, 1988; Penttinen *et al.*, 1988; Bassols and Massagué, 1988). TGF-β has also more subtle effects on the extracellular matrix. TGF-β affects the alternate splicing pattern of fibronectin (Borsi *et al.*, 1990) and the glycosylation pattern

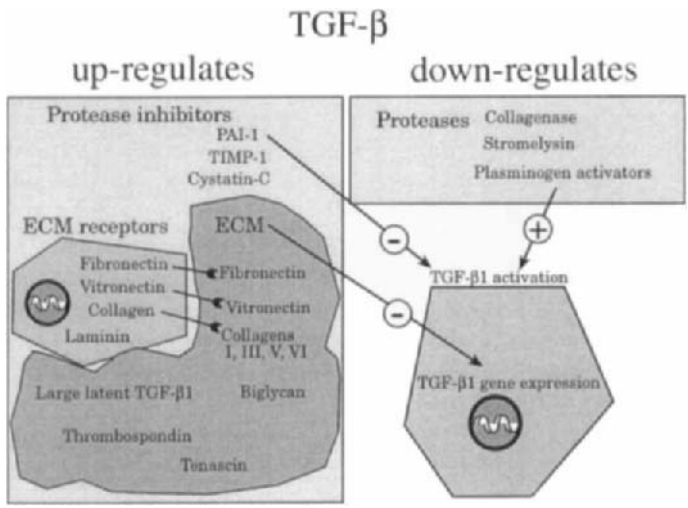


Fig. 6 TGF-β and the extracellular matrix. TGF-β stimulates the synthesis of multiple extracellular matrix (ECM) components (including large latent TGF-β1) and induces the expression of ECM receptors. Concomitantly, TGF-β down-regulates the expression of ECM-degrading proteases and up-regulates the expression of proteinase inhibitors. This results in net accumulation of the extracellular matrix, where large latent TGF-β complexes are integrated. The formed extracellular matrix, in turn, down-regulates the expression of TGF-β1 gene. Down-regulation of proteolytic activity also suppresses activation of latent TGF-β. Adapted from Lyons *et al.* (1988), Massagué (1990), Streuli *et al.* (1993), and Taipale *et al.* (1994a).

of proteoglycans (Bassols and Massagué, 1988). Typically, TGF-β stimulates the secretion of "embryonic" or early matrix proteins, such as tenascin, thrombospondin, and fibronectin, whereas its effects on the expression levels of the proteins present in mature connective tissue, such as elastin and collagen I, are more modest. TGF-β also modulates the expression of extracellular matrix receptors (Ignotz *et al.*, 1987; Heino and Massagué, 1989; Heino *et al.*, 1989).

TGF-β suppresses matrix degradation by down-regulating the expression of proteinases, such as plasminogen activators (Laiho *et al.*, 1986), collagenase (Edwards *et al.*, 1987), and stromelysin (Kerr *et al.*, 1990), and by inducing proteinase inhibitors, such as plasminogen activator inhibitor-1 (PAI-1) (Laiho *et al.*, 1986, 1987), tissue inhibitor of metalloproteinase-1 (TIMP-1) (Edwards *et al.*, 1987), and cystatin C (Solem *et al.*, 1990).

At the tissue level TGF-β1 promotes fibrotic and inflammatory reactions (Roberts *et al.*, 1980; Sanderson *et al.*, 1995). This probably represents the combined effect of stimulation of fibroblast and inflammatory cell chemotaxis, inhibition of epithelial regeneration, and induction of extracellular matrix synthesis. Abnormal metabolism of TGF-β has been linked to several fibrotic disorders, such as glomerulonephritis (Border and Ruoslahti, 1992), Alzheimer's disease (Flanders *et al.*, 1995), lung fibrosis (Broekelmann *et al.*, 1991; Anscher *et al.*, 1993), liver cirrhosis (Czaja *et al.*, 1989; Castilla *et al.*, 1991), and fibrosis of transplanted tissues during allograft rejection (Waltenberger *et al.*, 1993a). Increased levels of TGF-β have also been implicated in fibrosis of coronary arteries after myocardial infarction (Thompson *et al.*, 1988) and after coronary angioplasty (Majesky *et al.*, 1991; Nikol *et al.*, 1992), in carcinoid heart disease (Waltenberger *et al.*, 1993b) and in arteriosclerosis (Nabel *et al.*, 1993).

Interestingly, latent TGF-β1 is a component of the extracellular matrix and can be released by matrix-degrading proteinases (Taipale *et al.*, 1992, 1995; Benezra *et al.*, 1993; Falcone *et al.*, 1993). Furthermore, extracellular matrix has been found to down-regulate the expression of the TGF-β1 gene (Streuli *et al.*, 1993). TGF-β may thus act as a feedback regulator of the extracellular matrix both during its formation and its degradation.

VIII. TGF-β IN ONCOGENESIS

Because TGF-β is growth inhibitory to hematopoietic, epithelial, and endothelial cells, the loss of sensitivity to TGF-β might promote tumorigenesis of these cell types. This is, indeed, the case. Some particular cancer types, such as squamous cell carcinomas (Shipley *et al.*, 1986; Reiss *et al.*, 1993) and retinoblastoma cells (Kimchi *et al.*, 1988), are refractory to TGF-β

growth inhibition. The growth of many other cancer cell lines is inhibited by TGF-β (Moses *et al.*, 1985; Moses, 1992). However, cancer cells are typically less susceptible to growth inhibition by TGF-β than are their normal counterparts (reviewed in Fynan and Reiss, 1993). Many primary cultures of hematopoietic, epithelial, and endothelial cells respond to TGF-β by growth arrest. The majority of cancer cell lines are not growth arrested in response to TGF-β (Fynan and Reiss, 1993). Paracrine TGF-β that would signal normal cells to arrest growth may thus only reduce the growth rate of tumor cells, resulting in malignant growth.

It is important to note that cancer cells that appear to be sensitive to TGF-β growth inhibition *in vitro* may not be inhibited by it under more physiological conditions. This may be due to the fact that the *in vitro* cultures lack proper extracellular matrix structures or inflammatory and stromal cell-derived growth factors. For example, extracellular matrix and growth factors influence the response of a rat prostate cancer cell line to TGF-β (Morton and Barrack, 1995).

A. Escape from TGF-β Growth Regulation

Multiple mechanisms can account for the loss or attenuation of TGF-β growth inhibitory responses in cancer cells. These include a low expression level of TGF-β receptors, mutation of TGF-β receptors or intracellular signaling proteins, and induction of TGF-β resistance by oncogenes, growth factors, or viral transforming proteins.

1. LOW EXPRESSION LEVEL OF TGF-β RECEPTORS

The lack of TGF-β growth regulation may also be a consequence of low-level TGF-β receptor expression. This appears to be the case in myeloid leukemia cells (Falk *et al.*, 1991). Myeloid leukemia cell lines express low levels of type II TGF-β receptor mRNA (Taipale *et al.*, 1994b). Induction of cell differentiation by growth factors, retinoic acid, or phorbol esters induces type II TGF-β receptor, which increases the response of the cells to TGF-β (Taipale *et al.*, 1994b).

2. MUTATIONS IN TGF-β RECEPTORS AND MAD PROTEINS IN CANCER

Mutations in the type II TGF-β receptor have been found in human T cell malignancies (Kadin *et al.*, 1994). Similarly, Markowitz *et al.* (1995) discovered that type II TGF-β receptor was mutated and inactivated in ~90% of colon carcinomas with microsatellite instability. The mutation is localized

to a stretch of 10 adenines in the coding region of type II TGF-β receptor, and it is common in colon and gastric cancers (Myeroff *et al.*, 1995), but rare in endometrial cancers with microsatellite instability. The mutation of TGF-β receptors may be selected for in the colon, because TGF-β1 is an autocrine negative growth regulator of colon carcinoma cells (Wu *et al.*, 1992; Levine and Lewis, 1993). A study of sporadic cancers of the pancreas, liver, and breast failed to identify this microsatellite mutation (Vincent *et al.*, 1996). However, the mutation may be widespread, because we identified an identical mutation from the HT-1080 human fibrosarcoma cell line (Taipale *et al.*, 1994b).

Mutations in the Smad genes involved in the signal transduction of TGF-β superfamily members have also been linked to cancer. Smad2 is regulated in response to TGF-β1, and mutations inactivating Smad2 are present in ~6% of colon carcinomas (Eppert *et al.*, 1996; Riggins *et al.*, 1996). Smad4, which is required for signaling by BMP-4, activin, and TGF-β, was originally isolated as DPC4, a tumor suppressor of pancreatic cancer, and it is deleted or mutated in >30% of pancreatic cancers (Hahn *et al.*, 1996).

3. ONCOGENES AND GROWTH FACTORS CONFERRING TGF-β RESISTANCE

Transformation of cells with oncogenes may render the cells insensitive to TGF-β growth inhibition. Expression of the Ha-*ras* oncogene induces TGF-β resistance in epithelial cell lines derived from mink lung (Kerr *et al.*, 1991; Longstreet *et al.*, 1992), rat intestine (Filmus *et al.*, 1992), and rat liver (Houck *et al.*, 1989). Transformation of Mv1Lu mink lung epithelial cells by the Myc oncogene results in reduced sensitivity to TGF-β growth inhibition (Kerr *et al.*, 1991). It has also been reported that Mv1Lu cell lines expressing mutant p53 are refractory to TGF-β growth arrest (Ewen *et al.*, 1995).

Hepatocyte growth factor (HGF) can release epithelial and endothelial cells from TGF-β1-induced growth arrest (Taipale *et al.*, 1996). This mechanism may be relevant to the progression of breast cancer. TGF-β has been suggested to act as a tumor suppressor in the mammary gland, because transgenic mice expressing TGF-β1 from the MMTV-LTR promoter are less susceptible to chemically induced mammary tumorigenesis than are wild-type mice (Pierce *et al.*, 1995). Importantly, mice that express low levels of an oncogenic form of the HGF receptor (TRP-MET) driven by a relatively non-tissue-specific promoter (metallothionein) develop mammary tumors (Liang *et al.*, 1996). The mice have also an increased incidence of lymphoma. No tumors were detected in liver, spleen, kidney, or colon, where the transgene is also expressed. One mechanism that explains this finding is that the tumor-suppressing function of endogenous TGF-β present in the mammary gland extracellular matrix (Silberstein *et al.*, 1992) could be blocked by the

TRP-MET expression. In accordance, increased expression of HGF and MET correlates with decreased survival in breast cancer (Wang *et al.*, 1994b; Yamashita *et al.*, 1995; Nagy *et al.*, 1995).

4. ONCOGENIC VIRUSES INDUCE TGF-β RESISTANCE

Cells transformed by certain tumor viruses, such as SV40, adenovirus, Epstein–Barr virus (EBV) (Kumar *et al.*, 1991), and human papillomavirus (HPV), lose sensitivity to TGF-β-mediated growth inhibition. The effect is mediated by viral transforming proteins, such as SV40 large T antigen (Laiho *et al.*, 1990a; Pietenpol *et al.*, 1990a), adenovirus E1A (Missero *et al.*, 1991), and HPV E6 (Pietenpol *et al.*, 1990a). The loss of TGF-β growth regulation following virus infection may thus contribute to B cell lymphomas (EBV) and cervical carcinomas (HPV).

B. Loss of Sensitivity to TGF-β Growth Inhibition Is Linked to Malignant Progression

Loss of sensitivity to TGF-β growth arrest has been linked to tumor progression in two models, cutaneous T cell lymphoma (Kadin *et al.*, 1994; Knaus *et al.*, 1996) and colon adenoma–carcinoma (reviewed in Hague *et al.*, 1993).

Mac-1 cells have been isolated from the blood of a patient with an indolent form of cutaneous T cell lymphoma (CTCL). Two other cell lines, Mac-2A and Mac-2B, were isolated from skin tumor nodules of the same patient later, after the disease had progressed to a clinically aggressive form. These cells had lost their sensitivity to TGF-β (Kadin *et al.*, 1994). The resistance of the Mac-2 cells to TGF-β was caused by a mutation of a single allele of the type II TGF-β receptor acting in dominant-negative fashion (Knaus *et al.*, 1996).

Mulder *et al.* (1988) found that cultured colon adenoma cells become TGF-β resistant at high frequency. Subsequent studies found that the loss of response to TGF-β occurs concomitantly with tumor progression (Manning *et al.*, 1991; Wu *et al.*, 1992; Hsu *et al.*, 1994). An *in vitro* model of colon adenoma progression was described by Markowitz *et al.* (1994). At early passages, a benign cultured colon adenoma, VACO-235, is nontumorigenic. The cell line progresses to malignancy spontaneously during *in vitro* culture, and the tumorigenic variants have lost sensitivity to TGF-β growth inhibition.

C. TGF-β in Cancer Invasion, Angiogenesis, and Metastasis

Most adult tissues are characterized by a low number of mitoses, slow turnover of the extracellular matrix, and virtually nonexistent angiogenesis.

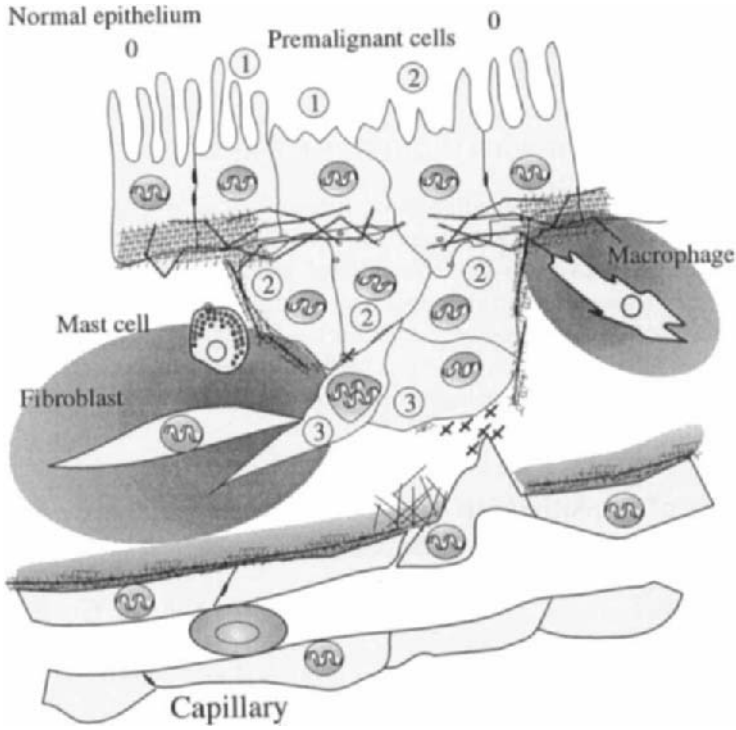


Fig. 7 Role of matrix-associated TGF-β in epithelial tumor progression. Epithelial tumorigenesis is initiated by mutation (1), which allows an epithelial cell to grow into an avascular tumor, where the cells have already lost the capability to regulate properly the growth of clonally identical cells. At this point, the cells are still sensitive to growth regulation by TGF-β derived from neighboring epithelial cell basement membranes and stromal cell extracellular matrix (TGF-β represented by shading). Subsequent mutations (2) make the cells refractory to TGF-β growth inhibition and allow invasion through the basement membrane and interstitial matrix. Further mutagenesis (3) makes the tumor cells angiogenic and metastatic. These later progression steps may involve increased secretion of active TGF-β.

Cancer frequently begins when a cell acquires a mutation that allows it to proliferate to a small, nonvascular, benign growth that is surrounded by normal tissue and a basement-membrane-like capsule (Fig. 7). The cells are sensitive to growth regulation by factors derived from neighboring normal cells and extracellular matrix, but may already have lost the capability to restrict the growth of clonally identical cells (Fig. 7). Malignant conversion is initiated by subsequent mutations, which allow the cells to ignore the growth- and invasion-suppressive signals from surrounding tissue. Progression to a highly malignant phenotype is induced by mutations that promote tumor angiogenesis and by metastasis of tumor cells to distant organs.

Latent TGF-β is present in the basement membranes and interstitial extra-

cellular matrices of multiple tissues (Taipale *et al.*, 1995). TGF-β decreases proteolytic activity of cells and inhibits epithelial and endothelial cell migration (Laiho *et al.*, 1986, 1987; Sato and Rifkin, 1989). TGF-β secreted by stromal cells and TGF-β present in the basement-membrane-like matrix surrounding benign epithelial lesions thus probably have a role in limiting the growth and invasion of the abnormal cells. This model could explain why the acquisition of TGF-β resistance in colon carcinoma progression is associated with conversion from adenoma to carcinoma (see above).

Regulation of cell invasion and proteolytic activity by TGF-β can be altered in cancer cells. For example, expression of TGF-β2 correlates with the depth of tumor invasion in melanoma (Reed *et al.*, 1994). In the lung adenocarcinoma cell line A549, TGF-β enhances the expression of urokinase-type plasminogen activator (Keski-Oja *et al.*, 1988) and its receptor (Lund *et al.*, 1991), leading to a net increase in plasminogen activation. Induction of a proteolytically active phenotype in tumor cells by TGF-β could create a positive-feedback loop by increasing the release and activation of TGF-βs from the matrix during invasion. TGF-β may also induce an epithelial–mesenchymal transdifferentiation of tumor cells to highly invasive and malignant spindle cells (Miettinen *et al.*, 1994; Caulin *et al.*, 1995; Cui *et al.*, 1994, 1996). Loss of growth arrest response to TGF-β without changes in other aspects of TGF-β signalling—as induced by viral proteins, oncogenes, and HGF—may thus result in a highly invasive and malignant phenotype.

TGF-β secreted by tumor cells, or macrophages or fibroblasts of tumor stroma, can also induce angiogenesis (Roberts *et al.*, 1986). Angiogenesis induced by increased expression of TGF-β isoforms, coupled with loss of sensitivity of tumor cells to TGF-β growth arrest, could be important in pancreatic cancer (Böttinger *et al.*, 1997; Hahn *et al.*, 1996). The mechanism of angiogenesis induction by TGF-β is not clear, but may involve up-regulation of FGF-2 and VEGF (Pertovaara *et al.*, 1993, 1994).

D. Role of TGF-β Secretion in Cancer

A large number of model systems have been used in attempts to clarify the role of TGF-β1 secretion in cancer. The studies have yielded apparently conflicting results, because TGF-β1 expression has been found both to suppress and to promote tumorigenesis. In general, the expression of TGF-β1 in epithelia suppresses tumorigenesis (Glick *et al.*, 1994; Cui *et al.*, 1994; Pierce *et al.*, 1995), and expression of the TGF-β1 transgene by TGF-β-sensitive carcinoma cells suppresses tumor growth (Wu *et al.*, 1992). Sarcoma cells, and carcinoma cells that have lost the growth inhibitory responses to TGF-β, become more invasive and tumorigenic after TGF-β1 transgene expression (Samuel *et al.*, 1992; Chang *et al.*, 1993; Cui *et al.*, 1996).

In epithelia, the expression of TGF-β1 may thus have an early tumor-suppressing function (Pierce *et al.*, 1995; Cui *et al.*, 1996). After carcinoma cells lose their sensitivity to TGF-β growth inhibition, the secretion of active TGF-β may contribute to tumor progression by affecting tumor invasion or angiogenesis (above) and the synthesis of growth factors by stromal cells (Urashima *et al.*, 1996), and by inhibiting immune responses (Torre-Amione *et al.*, 1990; Arteaga *et al.*, 1993).

E. Extracellular Control of TGF-β Activity

The effects of exogenous TGF-β can also be modulated by the deposition of TGF-β to the matrix and activation. Cui *et al.* (1994) found that loss of TGF-β1 expression is prognostic for a high risk of malignant conversion in benign skin tumors. TGF-β1-null keratinocytes initiated with ras oncogene progress to squamous cell carcinoma, whereas control keratinocytes form well-differentiated papillomas (Glick *et al.*, 1994). Control TGF-β1-null keratinocytes deposit exogenous TGF-β1 to the epidermis, but the ras-transformed keratinocytes fail to do so. Thus, in the ras-transformed TGF-β1-null keratinocytes, both autocrine and paracrine growth control by TGF-β is lost, because the cells fail to synthesize TGF-β (null phenotype) and to deposit exogenous TGF-β (ras transformation). In the normal ras-initiated keratinocytes, autocrine TGF-β1 is apparently sufficient to suppress tumorigenesis.

The failure of paracrine growth control may be related to the expression and matrix deposition of LTBP-1. LTBP-1 and TGF-β1 are associated with fibrillar structures in normal fibroblast matrix (Taipale and Keski-Oja, 1996). In contrast, SV40-transformed fibroblasts completely lack LTBP-1 fibers (Taipale and Keski-Oja, 1996). In prostatic tumors, tumor cells at various stages of differentiation, as well as the stromal cells, stain positively for TGF-β1. Cystectomized and benign prostatic tumors also stain positively for LTBP-1. However, staining by LTBP-1 is lost in malignant prostatic cells (Eklöv *et al.*, 1993). Similarly, in gastrointestinal carcinomas, TGF-β1 is found in both tumor and stromal cells, whereas LTBP-1 is found only in stromal cells and in the stromal extracellular matrix (Mizoi *et al.*, 1993). The failure of tumor cells to secrete or retain an LTBP-1-containing matrix may contribute to loss of growth control by decreasing the concentration of TGF-β1 in the vicinity of the tumor cells.

F. TGF-β Can Induce Stromal Cells to Synthesize Tumor Growth Factors

Multiple myeloma cells are resistant to growth inhibition by TGF-β1, but secrete high levels of the factor (Urashima *et al.*, 1996). TGF-β induces the

secretion of a myeloma growth factor, IL-6, by the myeloma cells and bone marrow stromal cells. Loss of sensitivity to a negative growth factor can thus promote tumorigenesis by affecting the homeostasis of the cytokine network.

G. Immunosuppression

TGF-βs are highly immunosuppressive and they inhibit the proliferation and effector functions of T cells, B cells, NK cells, and macrophages. Induction of active TGF-β production by retinoids, vitamin D_3, and corticosteroids is probably a central effect in the antiinflammatory and immunosuppressive mechanisms of these drugs (Koli and Keski-Oja, 1993, 1996; Oursler *et al.*, 1993; Glick *et al.*, 1989). TGF-β2 was purified as glioblastoma-derived T cell suppression factor and contributes to immunosuppression in glioblastomas (de Martin *et al.*, 1987) and breast cancer (Arteaga *et al.*, 1993). Importantly, reintroduction of glioma cells expressing TGF-β1 in an antisense orientation can cause remission of the primary tumor by induction of an immune response (Fakhrai *et al.*, 1996).

H. Secondary Pathogenesis Mediated by TGF-β

TGF-β has also a role in the secondary pathogenesis caused by certain tumor types and cancer therapy. These include immunosuppression in glioblastomas (Wrann *et al.*, 1987), carcinoid heart disease (Waltenberger *et al.*, 1993b), bleomycin-induced lung fibrosis (Raghow *et al.*, 1989; Khalil *et al.*, 1996), fibrosis of lung and liver induced by chemotherapy, and bone marrow transplantation in breast cancer (Anscher *et al.*, 1993).

I. Intervention

The loss of function and dominant-negative mutations in the TGF-β receptors and signal transducers may prove to be difficult targets for cancer therapy, e.g., requiring gene transfer in all tumor cells. Interference in the generation of active TGF-β by cancer cells may be more susceptible to therapeutic intervention. When the precise mechanisms of accumulation of TGF-β in the extracellular matrix and its subsequent activation are elucidated, it will be possible to design small-molecule inhibitors of active TGF-β production. Such molecules might prevent cancer drug-induced fibrosis, limit invasiveness of cancer cells, or even induce immune response against the tumor (Fakhrai *et al.*, 1996). A similar approach has been shown to be feasible in an another model. Protease inhibitors that inhibit the cleavage of membrane-bound TNF-α protect mice from lethal endotoxin shock (Mohler *et*

al., 1994). Chemicals affecting extracellular events also have the pharmacological advantage that they do not have to cross the cell membrane.

IX. PERSPECTIVE

The malignant phenotype is characterized by the loss of negative growth control. Increasing evidence suggests that altered signaling by transforming growth factor-βs contributes to multiple cancer types. Growth inhibition of cells by TGF-β requires the expression of the TGF-β gene, secretion of latent TGF-β, association of the latent complex with the matrix, release and activation of the latent complex, binding of the active polypeptide to TGF-β receptors, and intracellular transduction of the growth-suppressive signal. Cell growth is also modulated by cellular integration of signals both inhibitory and stimulating to growth. Depending on the localization of the tumor, different mechanisms may explain tumor cell resistance to TGF-β growth inhibition. In the colon, mutation of type II TGF-β receptor or Smad2 is probably the dominant mechanism. In lymphoma and cervical cancer, oncogenic viruses such as EBV and HPV may disrupt intracellular signaling by TGF-β. In breast and lymphoid cancer, oncogene activation or altered regulation of HGF may be important. Mechanisms involved in the secretion, association with the extracellular matrix, and activation of TGF-β still remain to be elucidated. It is well established that tumor cells display increased proteolytic activity and deposit a disorganized form of extracellular matrix. Therefore, the association of TGF-βs with the matrix and their subsequent proteolytic activation are also likely to be dysregulated in cancer.

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Differentiation and Cancer in the Mammary Gland: Shedding Light on an Old Dichotomy

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In this brief review, the development of breast cancer is discussed from the vantage of phenotypic differentiation, similar to what has been considered over the years for leukemias and melanomas, both of which express easily visible differentiation markers (Hart and Easty, 1991; Clarke *et al.*, 1995; Lynch, 1995; Sachs, 1996; Sledge, 1996). The review is divided into a theoretical background for human breast differentiation and a discussion of recent experimental results in our laboratories with differentiation of breast epithelial cells.

In the theoretical background, *in situ* markers of differentiation of normal breast and carcinomas are discussed with emphasis on their possible implications for tumor therapy. So far, most of the emphasis regarding differentiation therapy of tumors has been focused on the possible action of soluble factors, such as colony-stimulating factors in leukemias and retinoic acids in solid tumors (Lotan, 1996; Sachs, 1996). However, an emerging and promising new avenue in this area appears to point to additional factors, such as the cellular form and extracellular matrix (ECM) (Bissell *et al.*, 1982; Bissell and

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Barcellos-Hoff, 1987; Ingber, 1992). The recent interest in these parameters has evolved along with an increasing understanding of the molecular composition of the ECM, and of the molecular basis of the classical findings that normal cells—in contrast to tumor cells—are anchorage dependent for survival and growth (Folkman and Moscona, 1978; Hannigan *et al.*, 1996). We now know that this is the case for epithelial as well as fibroblastic cells, and that interaction with ECM is crucial for such regulation. Indeed, ECM and integrins are emerging as the central regulators of differentiation, apoptosis, and cancer (Boudreau *et al.*, 1995; Boudreau and Bissell, 1996; Werb *et al.*, 1996; Bissell, 1997; Weaver, *et al.*, 1997).

In the experimental part, we elaborate on our own recent experiments with functional culture models of the human breast, with particular emphasis on how "normal" and cancer cells could be defined within a reconstituted ECM. Special attention is given to integrins, the prominent ECM receptors. We further discuss a number of recent experimental results, all of which point to the same conclusion: namely that phenotypic reversion toward a more normal state for epithelial tumors is no longer an elusive goal. Thus "therapy by differentiation" could be broadened to include not only blood-borne tumors, but also solid tumors of epithelial origin.

I. INTRODUCTION

Globally, breast cancer is the most frequent form of cancer affecting women (Wolff *et al.*, 1996). Approximately one out of eight women will be diagnosed with this disease if they live long enough, and the incidence rates have been increasing by 2% per year over the past decade (Feuer *et al.*, 1993; Wolff *et al.*, 1996). Among the identified risk factors for breast cancer development are early menarche and late menopause (Kelsey and Berkowitz, 1988; Snedeker and Diagustine, 1996). A large placenta during fetal life also correlates with higher risk of breast cancer in adult life (Ekbom *et al.*, 1995). Conversely, pregnancy after teens, before age 35, and the associated period of lactation have been identified as preventive factors (Kelsey and Berkowitz, 1988; Snedeker and Diagustine, 1996). Because the lactational state represents the ultimate level of differentiation in the human breast, it is arguable that repeated periods of tissue-specific differentiation constitute a physiological mechanism for overruling the accumulation of carcinogenic and cancer-promoting events during a lifetime (Russo *et al.*, 1982; Snedeker and Diagustine, 1996). If this were true, it would be tempting to speculate that the signals responsible for maturation, structure formation, and tissue-specific function could integrate to counteract tumor growth and progression even in the advanced stage, where tumor cells are generally considered to be beyond normal cell regulation. In other words, although tumorigenesis has been viewed as the result of accumulation of multistep genetic aberrations (Klein and Klein, 1985; Bishop, 1987; Weinburg, 1989), and mutation is the accepted paradigm of cancer research (Bishop, 1991), would it be still possible even after the tumor is fully developed to revert the phenotype through manipulation of the microenvironment toward the differentiation of normal

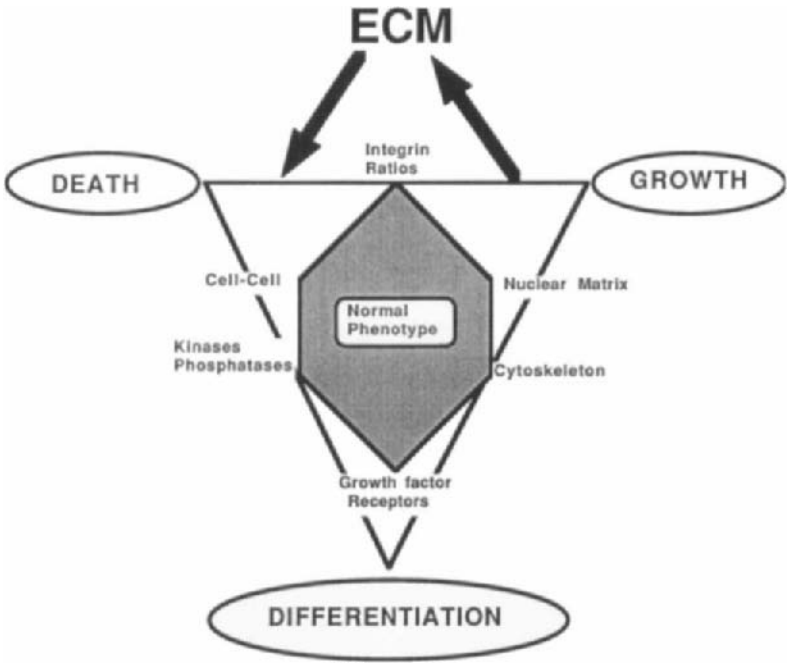

Fig. 1 Diagram illustrating the normal phenotype of a cell in a triangle between growth, death, and differentiation. The extracellular matrix (ECM) is proposed to play an important role via integrin-mediated signaling.

breast? If we consider differentiation to be synonymous with controlled behavior, growth arrest, minimal cell motility, limited life span, and structural integrity, this then is the opposite of cancer, as Pierce, Bissell, and others have postulated (Bissell, 1981; Pierce and Speers, 1988; Zutter *et al.*, 1995; Campisi, 1996). Certainly, efforts to differentiate cancer cells have been exercised at the experimental as well as at the therapeutic level as possible chemoprevention (Noda, 1993; Lotan, 1996), with reasonable success achieved for blood tumors such as leukemias, for which the maturation pathways in hematopoiesis have been studied exhaustively (Sachs, 1996, and references herein).

The groundbreaking discoveries of oncogenes and tumor suppressor genes and the new understanding of breast cancer biology in general have not translated equally well into therapy (Sledge, 1996). The very fact that breast cancer has such a diverse array of phenotypes in terms of histology and biology, ranging from most anaplastic to differentiated tubular carcinomas that are often difficult to discriminate from benign lesions, indicates that breast carcinomas have an intrinsic ability to assume considerable phenotypic maturation. Here we argue the case for the microenvironment as an ac-

tive participant not only in normal differentiation (see Fig. 1), but also during induction and progression of cancer, with a view to find novel avenues for therapeutic intervention for solid tumors (Bissell, 1981; Petersen *et al.*, 1992, 1995; Rønnov-Jessen, 1996; Rønnov-Jessen *et al.*, 1996; Weaver *et al.*, 1997).

That the differentiated state is indeed a relevant end point to pursue in terms of therapy is supported by the fact that highly differentiated tumors have a remarkably better prognosis when compared to less differentiated tumors (Tabar *et al.*, 1996). This in itself is not a novel or surprising finding. These statements have been made often over the years. Thus, what is new in this area? First, the plasticity of tumors is better documented. There is now evidence that otherwise cohesive, differentiated tumor cells become transiently more aggressive on stimulation with exogenous factors, such as hepatocyte growth factor (Birchmeier and Behrens, 1994), and that noncohesive, invasive cells, on the other hand, may be reverted by, e.g., insulin-like growth factor I, tangeretin, retinoic acid, and tamoxifen (Bracke *et al.*, 1996). In other words, invasion and metastasis are not irreversible next steps on a one-way progression ladder even for epithelial cancers. Second, adhesion molecules and extracellular matrix in general are becoming recognized as important modulators of normal and malignant phenotypes (Petersen *et al.*, 1992; Bracke *et al.*, 1996; Jones *et al.*, 1996; Varner and Cheresh, 1996; Weaver *et al.*, 1997). Third, we now know that the surrounding stromal cells are extensively recruited, as the tumor develops, to convey the formation of microenvironmental conditions resembling those of chronic wounds, the implications of which are only beginning to be understood (Clarke *et al.*, 1995; Rønnov-Jessen *et al.*, 1995, 1996, and references therein). Thus, stromal cells, which are expected to be devoid of genotypic aberrations (although this may need further scrutiny in the future), actively contribute to tumor development and progression, and consequently, peritumoral fibroblasts may prove instrumental in future attempts to regulate the behavior of the tumor.

II. MARKERS OF BREAST DIFFERENTIATION

A. Morphology

The human breast is divided longitudinally into main ducts (lactiferous ducts), of which there are 10–12, each ending in the nipple, branching interlobular ducts, and terminal duct lobular units, also referred to as alveoli (for review, see Rønnov-Jessen *et al.*, 1996). Each alveolus is the origin of further acinus formation during pregnancy and lactation (Battersby and Anderson, 1988). Thus, rather than being formed at each pregnancy from bud-

ding ducts all over again, as is the case of mice, the human acinus develops by expansion and multiplication of existing alveoli (Ferguson and Anderson, 1983). This has made the search for "stem" cells less feasible in humans compared to mice (see below). However, a transverse section through a duct or an alveolus in the human or mouse mammary gland reveals the same fundamental structures, i.e., an inner continuous layer of luminal epithelial (glandular) cells and an outer more or less continuous layer of myoepithelial cells (Rønnov-Jessen *et al.*, 1996). This basic pattern exists irrespective of the lactational status of the gland (Umemura *et al.*, 1996). The interrelationship between luminal epithelial cells and myoepithelial cells continues to be a mystery. Basically, they originate from the same developmental origin—the ectoderm. It would, however, be a misunderstanding to assume that, because of their stromallike phenotype, myoepithelial cells are derived from mesenchymal cells. These cells are always on the epithelial side of the basement membrane, as opposed to the true stromal cells, and so far there has been little published evidence for a stromal-to-epithelial conversion as an explanation for their occurrence *in vivo* (Deugner *et al.*, 1995).

We would like to propose that the precise structural composition of the mammary gland as a double-layered tube has to be considered the ultimate level of organization. So far, it has not been possible to model this structure outside an organism, and thus we do not know how crucial this is for maintenance of function or lack thereof during cancer development. There is some evidence to suggest that the myoepithelial cells may have two opposite functions during tissue remodeling and resting conditions. In the former, they serve to pave the way for emerging ductules in the stroma, consistent with the fact that they express a number of proteases (Niranjan *et al.*, 1995). Under resting conditions, myoepithelial cells or their products may contribute to maintain the differentiation of luminal cells or the luminal-derived carcinoma cells, and thus, may elicit a "tumor-suppressive" function (Bani *et al.*, 1994; Liu *et al.*, 1996).

Clearly, myoepithelial cells are altogether missing or are less differentiated in the full-blown invasive breast cancer in most species (Rudland *et al.*, 1995). Here the basement membrane is lost, and cancer cells express markers for luminal epithelial cells rather than myoepithelial cells (for review, see Rønnov-Jessen *et al.*, 1996). Is it meaningful then, even theoretically, to believe that a cancer cell and its progeny can be reverted all the way back to a double layered tube, if the cancer—monoclonal as it may be—originates from a predetermined luminal epithelial cell?

Understanding the evolution of lineage has been considered important in the search for the cellular origin of cancer in any tissue. The problem, as mentioned above, is that, because we know little about the precise interrelationship between luminal epithelial and myoepithelial cells, it is difficult to search for a "predetermined" luminal epithelial cell, assuming this exists (Rønnov-

Jessen *et al.*, 1996). In the mouse mammary gland, it is believed that the end buds, the epithelial cords budding from the ducts, contain the stem cells (Williams and Daniel, 1983). The end buds comprise two epithelial cell types: an outer layer of cap cells and an inner solid mass of body cells. Looking at the dynamics in the growing end bud by time-lapse video microscopy and thymidine labeling, it has been reported that the basal cap cells give rise to myoepithelial cells, by maturation along the subtending duct, as well as to body cells, which comprise the later luminal epithelial cells by migration into the central cord of epithelial cells (Williams and Daniel, 1983; Dulbecco *et al.*, 1982). Thus, cap cells have been ascribed stem cell-like properties, which fits well with their low level of differentiation. Therefore, in the mouse, fully developed myoepithelial cells of the duct presumably derive from cap cells in the end buds, although definitive proof is lacking even here. The transitional phenotypes are located in the interlobular duct and are referred to as basal cells with intermediate levels of differentiation (Sonnenberg *et al.*, 1986). Numerous attempts have been made to extrapolate these results to the human breast, but there is no solid evidence for the presence of end buds or cap cells in the human mammary gland. The presumed cellular equivalent, the so-called clear cells, have been found throughout the glandular tree (for review, see Rønnov-Jessen *et al.*, 1996). Novel data that human breast cancers in fact widely do express the most obligate myoepithelial marker, the oxytocin receptor, are an indication that cancer cells may possess the ability to differentiate more along this lineage than previously believed (Bussolati *et al.*, 1996). That breast carcinoma cells, in addition, respond to oxytocin by differentiation and growth inhibition suggests that the myoepithelial phenotype should also be considered in assays of breast cancer differentiation (Cassoni *et al.*, 1996).

To clarify the discussion concerning the morphology and the existence of putative stem cells in the human breast, measures to purify the different lineages and to regenerate the double-layered duct/alveolus would be extremely useful.

B. Estrogen Receptors

Another trait that needs to be addressed if one is to understand the origin of carcinoma cells and how they relate to any normal counterpart, is estrogen receptor (ER) status. It has been quite a task to extract sensible information about ER function under normal and malignant conditions based on the standard tissue culture assays (Bissell and Werb, 1995). The fact is, that if measured biochemically, the estrogen receptor is expressed in about 60% of breast carcinomas but is hardly measurable in normal breast tissue (Ricketts *et al.*, 1991; Khan, 1995). By this definition then, the ER-negative car-

cinomas should be the most highly differentiated tumors in that, in this respect, they would resemble normal tissue the most! This of course is not the case. Rather, all evidence from histopathological and clinical trials point to the opposite scenario: moderate ER expression correlates with morphologically differentiated tumors and better survival (Tavassoli and Man, 1995; Joslyn *et al.*, 1996). Therefore, the ER-positive breast carcinoma cell appears to be a relatively differentiated cell. Furthermore, if one accepts that normal breast tissue is essentially devoid of ERs, then ER-positive carcinomas would have to arise from a multipotent stem cell similar to those of teratocarcinomas (Sell and Pierce, 1994). However, once immunocytochemical assays became available, it was discovered that the sensitivity of the original biochemical ER analysis was too low to measure the level of ER expression in normal breast tissue (Petersen *et al.*, 1987; Khan, 1995). Using the assay, the level of expression in normal breast tissue from reduction mammoplasties was found to be an average of 7% of the luminal epithelial cells, and the stroma was devoid of receptor expression (Petersen *et al.*, 1987; Khan, 1995). Based on studies in the mouse, it had been hypothesized previously that the ERs in the stroma signal to the epithelium. However, these studies were conducted with labeled ligands that gave too high a background (Underwood, 1983). More recent data on mice agree with those of human studies in that it is now known that stroma is indeed ER negative or weakly ER positive (Haslam and Shyamala, 1981; Haslam and Nummy, 1992; Sapino *et al.*, 1993).

If breast cancer were to occur in a stochastic manner within the epithelium with an initial ER level of approximately 7%, then it is puzzling that as many as 60% of the tumors are ER-positive. One possibility is that at least 60% of breast epithelial cells could turn on the ER if "activated" by an as yet unknown mechanism that may be operative in the early stages of malignancy. It is also likely that the 7% is an average value for all breast epithelium (Petersen *et al.*, 1987). Thus, Ricketts *et al.* (1991) reported that the total cellular pool may be divided into classes of nonexpressors or low and high expressors, respectively. Therefore, another possibility could be that even though the high expressors represent only a small percent of the total normal cellular population (16%), they may be at a significantly higher risk of developing breast cancer and thereby account for the 60% prevalence of ER-positive tumors. In this respect, it is interesting that ER positivity is recorded in up to 80% of *in situ* carcinomas and that ER expression is highest in lesions that are associated with invasive carcinoma. Once the ER-positive tumor is developed, however, ER expression is gradually lost with progression as a result of selection of "less differentiated" ER-negative cells (Robertson, 1996).

The appreciation of ER expression as a differentiated trait has been somewhat confounded by the large body of information gathered from experimental cancers in nude mice. Here, estrogen acts analogous to a tumor promotor, because ER-positive tumor cells will not develop into tumors unless

estrogen is provided either from the mouse or exogenously through the diet (Soule and McGrath, 1980). This, and the fact that estradiol increases the incidence of mammary tumors in mice and rats (Snedeker and Diagustine, 1996), raise an interesting question, namely whether estrogen acts as a carcinogen for normal cells (Khan, 1995). This question has been difficult to answer in a physiological setting because normal breast epithelial cells—as mentioned above—should definitely not be considered a homogeneous population in terms of ER expression. The picture has remained confusing because thus far no one has been successful in maintaining the ER expression under experimental conditions (Rønnov-Jessen *et al.*, 1996). Thus, to understand how to maintain ER in "normal" cells in culture remains a major future challenge.

One approach to overcome the difficulties of maintaining ER-positive normal cells in short-term culture has been to transfect the ER into established normal breast epithelial cell lines (Zajchowski *et al.*, 1993). Surprisingly, in these cell lines, the response to estrogen is the direct opposite of what is observed with tumor cells and normal cells in short-term culture; they are in fact growth inhibited (Zajchowski *et al.*, 1993). One of our laboratories has transfected a spontaneously immortalized, intrinsically ER-negative breast epithelial cell line with an ER construct and obtained the same experimental outcome, i.e., the cells were indeed growth inhibited (Lundholt *et al.*, 1996).

From these experiments, we are left with the question of whether the paradoxical response in normal, transfected cells is due to the fact that these cells are intrinsically different from ER-positive tumor cells, or whether the transfected receptor lacks additional sequences that usually give the growth signal. Unfortunately, it appears that the latter may be the correct answer, because if overtly malignant, ER-negative breast carcinoma cells are transfected in a similar manner, they, too, behave like normal transfected cells—that is, they are growth inhibited by estrogen (Levenson and Jordan, 1994; Petrangeli *et al.*, 1994). It therefore seems reasonable to conclude that transfection of an apparently functional ER, eliciting all the downstream events that can be measured biochemically (Lundholt *et al.*, 1996), does not evoke the same fundamental growth response as in nontransfected cell lines. Thus, much more needs to be learned about normal ER function and lineage evolution during tumor development. This means that we must also learn about how tissue-specific gene expression is maintained in the normal breast, i.e., we must dissect the elements of microenvironmental control (Bissell and Barcellos-Hoff, 1987).

C. Integrins

The organization of epithelial cells into branching ducts and alveoli depends on the action of various soluble factors, cell–cell, and cell–ECM in-

teractions, and the establishment of epithelial polarity. The epithelial cell membrane is segregated into specialized apical and basolateral domains separated by tight junctions, and the cytoskeleton is organized into fibers terminating at junctional complexes and transmembrane integrin heterodimers facing the basement membrane (Taylor-Papadimitriou *et al.*, 1983; Eaton and Simons, 1995). The largest class of ECM receptors, the integrins, are now known to be the cellular antennas that sense the subtleties of the ECM and convert the chemistry of the microenvironment into subcellular signaling, which is then translated into form and function. Normal human breast epithelial cells express at least two β-integrins (β1 and β4) and four α-integrins (α1, α2, α3, α6) (Bergstraesser and Weitzman, 1994; Glukhova *et al.*, 1995). The expression of integrins in breast cancer is usually altered quantitatively and/or qualitatively (Zutter *et al.*, 1990; Mechtersheimer *et al.*, 1993). Based on their pairing and assumed function under normal and malignant conditions, the integrins will be described as (1) α6β4 in the normal breast, (2) α6β4 in breast cancer, and (3) β1-integrins in the normal breast and in breast cancer.

1. α6β4 IN NORMAL BREAST

Expression of the α6β4-integrin as a dimer in the human mammary gland has not been analyzed in great detail until recently because of lack of relevant antibodies. However, when studied individually in the normal breast, both β4 and α6 are expressed primarily by myoepithelial cells (Berdichevsky *et al.*, 1994). This should not be surprising, because α6β4 is expressed generally by basal cells such as those in the skin or the bronchial tree (Carter *et al.*, 1990; Sheppard, 1996). With regard to luminal epithelial cells, it is generally assumed that β4-integrin is expressed at the basolateral surface of the cell membrane *in vivo*. However, at the light microscopic level, it is difficult to determine whether the observed β4-integrin staining derives from myoepithelial or luminal cells (Koukoulis *et al.*, 1991; Natali *et al.*, 1992). With regard to α6-integrin, there is no clear consensus on the luminal epithelial expression pattern. It has been described either as strictly limited to the basal cell surface (Natali *et al.*, 1992), variably expressed also at the lateral membranes (Mechtersheimer *et al.*, 1993), or homogeneously expressed on the entire cell surface (Friedrichs *et al.*, 1995). The apparent wider subcellular distribution of α6-integrin should be seen in light of the fact that, in addition to β4-integrin, α6 also pairs with β1-integrin (Sonnenberg *et al.*, 1990). It is important to realize, however, that α6-integrin will preferentially pair with β4-integrin if the latter is present (Cress *et al.*, 1995; Schoenenberger *et al.*, 1994). The α6β4 pairing leads to formation of stable anchoring contacts

or hemidesmosomes to the basement membrane, typical of polarized, resting cells with little or no motile behavior (Carter *et al.*, 1990; Cress *et al.*, 1995). The quiescent state of the cells is most likely signaled via β4-mediated induction of p21/WAF1/CIP1, an inhibitor of the cyclin-dependent kinases (Clarke *et al.*, 1995). This fits nicely with the fact that myoepithelial cells under normal conditions are virtually noncycling (Rønnov-Jessen *et al.*, 1996). As expected from these studies, p21/WAF1/CIP1 under normal conditions is expressed at least occasionally in some myoepithelial cells (Barbareschi *et al.*, 1996).

2. α6β4 IN BREAST CANCER

At first glance, the reports on α6β4-integrin expression in cancer seems quite confusing. Clearly, whereas it is up-regulated in some forms of cancers, including squamous cell carcinomas of the skin (Giancotti and Mainiero, 1994), astrocytomas (Previtali *et al.*, 1996), and thyroid carcinomas (Serini *et al.*, 1996), other cancers, such as those of breast and prostate, are almost completely devoid of α6β4 as determined by β4 staining (Cress *et al.*, 1995; Koukoulis *et al.*, 1991). It is noteworthy, however, that the up-regulation of α6β4 in some cancers, e.g., in squamous carcinomas, may be a product of the inability of the tumor cells to differentiate and to become α6β4 negative as they would during the process of normal skin differentiation (Giancotti and Mainiero, 1994). However, if α6β4 is indeed up-regulated specifically, it may be instrumental in the malignant phenotype in a manner different from that governing the normal conditions as outlined above. Thus, some of the reported oncogenic activities of α6β4 include tyrosine phosphorylation of Shr/Grb2 and induction of the *ras* pathway (Mainiero *et al.*, 1995), phosphorylation of $p185^{HER2}$ (Campiglio *et al.*, 1994), EGF-induced deterioration of adhesive structures (Mainiero *et al.*, 1996), and escape from cell death mechanisms (Dowling *et al.*, 1996).

Nevertheless, *in vivo* β4-integrin is down-regulated in invasive breast cancer as compared to normal breast tissue (Koukoulis *et al.*, 1991). In comparing the different reports in the literature concerning α6-integrin in breast cancer, it is clear that this integrin is not down-regulated to the same extent as β4, because it is expressed in at least 50–70% of breast carcinomas compared to reports of β4 expression in the range of 0–22% of all carcinomas [compare Friedrichs *et al.* (1995) with Koukoulis *et al.* (1991) and Taylor-Papadimitriou and Alford 1995)]. The significance of this difference again should be viewed in light of the level of β1-integrin expression in the normal breast and breast cancers (see below) and the fact that β1-integrin is the other integrin that α6 may pair with, as mentioned above (Sonnenberg *et al.*, 1990).

3. β1-INTEGRINS IN NORMAL BREAST AND BREAST CANCER

The hypothesis that ECM, and hence its receptors, have information and direct tissue-specific form and function was set forth a decade and a half ago (Bissell *et al.*, 1982). That this is indeed the case has been amply demonstrated for many tissues but especially the mammary gland of rodents and more recently humans (for reviews, see Adams and Watt, 1993; Lin and Bissell, 1993; Hay, 1995; Roskelley and Bissell, 1995; Rønnov-Jessen *et al.*, 1996; Bissell, 1997). β1-Integrin has been shown to be involved in the regulation of milk protein gene expression (Streuli *et al.*, 1991) as well as apoptosis (Howlett *et al.*, 1995; Boudreau *et al.*, 1995). Knockouts of β1-integrin are embryonically lethal (Fassler and Meyer, 1995; Stephens *et al.*, 1995). In 1994, Matlin and collaborators (Schoenenberger *et al.*, 1994) put forth an imaginative hypothesis that stated that if phenotype, *in casu,* morphogenesis should depend only qualitatively on the expression of integrins at the cell surface, then minor changes would not be expected to be important. On the other hand, if a correct phenotype depends on the exact balance of various integrins at the plasma membrane, then a change in the relative amounts of individual integrins would be quite significant (Schoenenberger *et al.*, 1994). Because α6-integrin may pair with β4 as well as with β1, and the ratio between α6 and β4 appears to increase with cancer development, it becomes essential to know what happens to the level and location of β1 in cancer cells (Weaver *et al.*, 1997). The equation needs to also include α2 and α3, the other two integrin subunits compatible with β1.

In the race to be the β1 partner on the cell surface, it has been reported that α2 wins over α3, which in turn wins over α6, but that there is usually an excess of β1 on the cell surface of nontransformed cells (Schoenenberger *et al.*, 1994). In the normal human breast, α2-integrin is expressed equally well on the basolateral cell surface of both luminal and myoepithelial cells, whereas α3 is slightly weaker in luminal epithelial cells (Koukoulis *et al.*, 1991; Glukhova *et al.*, 1995; Taylor-Papadimitriou and Alford, 1995). Looking at mRNA expression (which is not as informative) and in at least one case of protein staining, α2 shows the same tendency as α3 in that there is a little higher expression in myoepithelial cells compared to luminal epithelial cells (Koukoulis *et al.*, 1991; Zutter *et al.*, 1993). The expression of α2 and α3 at the cell–cell junctions as well as at the cell–ECM junctions reflects their assumed function in directing morphogenesis and maintaining cell survival (Berdichevsky *et al.*, 1994; Howlett *et al.*, 1995).

In breast carcinomas, α2-integrin has been reported to be absent or close to absent in about 50% of the cases (Mechtersheimer *et al.*, 1993; Taylor-Papadimitriou and Alford, 1995). Also, around 40% of breast carcinomas show decreased levels or absence of α3 (Mechtersheimer *et al.*, 1993). For

comparison, β1-integrin is not down-regulated at the protein level to the same extent and in one report no decrease was found in the majority of carcinomas (Mechtersheimer *et al.*, 1993; Zutter *et al.*, 1993). The equation may thus be concluded as follows: with little or no β4, significant reductions in α2 and α3, and available β1, there is room for pairing of α6 with β1 in breast cancer as opposed to what is seen in normal cells. This fits nicely with a reported reciprocal expression of α2β1 with α6β1 in around 50% of breast cancers (Oda *et al.*, 1994). The α6β1 phenotype is particularly characteristic of one histological variant of breast cancer, that of the invasive lobular cancer, which shows little morphological differentiation (Koukoulis *et al.*, 1993). The conclusion of the equation is compatible with observations on prostatic carcinomas whereby the α6β4 pathway is essentially bypassed in favor of the α6β1 pathway (Cress *et al.*, 1995). The biological significance of this observation is supposed to involve the ability of cells to migrate and metastasize. Whereas α6β4 is associated with stable anchoring contacts, α6β1 colocalizes with focal contacts at the cellular protrusions (Cress *et al.*, 1995; Koukoulis *et al.*, 1991, 1993). In this respect, it is also interesting that the nonmalignant HBL-100 breast epithelial cells, although clearly not normal, almost exclusively express α6β4, whereas the highly malignant breast carcinoma cell line, MDA-MB435, shows the opposite constitution. The invasive phenotype of the latter may in fact be dramatically reduced by dominant negative knockout of α6β1 [compare Sonnenberg *et al.* (1990) with Shaw *et al.* (1996)]. A related phenomenon has been reported for a colonic carcinoma cell line in which progression from a poorly tumorigenic variant to a more aggressive variant was associated primarily with an increase in β1 and a parallel decrease in β4 integrin expression (Lopez-Conejo *et al.*, 1996). It is important, however, to realize that a shift in β1 relative to β4 integrin in itself may not be sufficient to trigger an aberrant phenotype. In this respect it is quite intriguing that a tumorlike shift in the ratio between β1 and β4 integrins is already grossly apparent at the level of noninvolved breast tissue from the majority of cancer patients (Jones *et al.*, 1992). The importance of the relative ratios of integrins at the cell surface in determining the phenotype of normal and malignant cells was tested most recently in our laboratories and will be discussed in more detail below (Weaver *et al.*, 1997).

III. CULTURE MODELS OF MAMMARY GLAND DIFFERENTIATION

All cancer biology, indeed all biology, relies on well-characterized model systems. When dealing with human cells, the only option is to explant the relevant cells and develop culture assays aimed at answering specific ques-

tions. Classical cancer research has benefited from monolayer culture assays of immortality, transformation, and progression (Nettesheim and Barrett, 1985; Harris, 1987). However, for epithelial cells, these assays do not measure differentiation beyond the level of squamous metaplasia (Bissell, 1981; Masui *et al.*, 1986). Morphological differentiation appears to require more elaborate model systems.

A. Basement Membrane (EHS) Assay

We have searched for a set of criteria to define safely "normal" or "near-normal" for cultured human breast epithelial cells, without incorporating the complexity of the myoepithelial cells until such time that we know more about them. Analogous to mouse mammary epithelial cells (Barcellos-Hoff *et al.*, 1989; Aggeler *et al.*, 1991), normal luminal epithelial cells, when confronted with a physiologically relevant reconstituted basement membrane (EHS; Matrigel, Collaborative Research), were shown to recapitulate a number of normal features, independent of the presence of a basal layer of myoepithelial cells. First, they rapidly (within 7–10 days) organized a near-perfect acinus-like sphere (Petersen *et al.*, 1992). Some of these structures may well have been clonal because they were preceded by an initial burst of growth that declined to almost zero once the final size (<50 μm) was reached. The growth arrest was not the result of suboptimal culture conditions, because the same medium readily supported growth of similar cells in monolayer culture or tumor cells in EHS (Petersen *et al.*, 1992). Second, when sectioned, these structures contained true acini with a central lumen formed by apical cell membranes and cells with basally located nuclei. The growth arrest was synchronized with acinus formation. We determined the mean number of cells in an equatorial section of the spheres to be around 8 cells, and thus similar to that seen in acini *in vivo*. Because an important measure of differentiation in the mammary gland is the ability to polarize the cellular axis in a correct manner, we stained the cells for an integral, apical-specific protein, sialomucin, as well as the basement membrane component, type IV collagen. The localization of both proteins was found to be comparable to what is found in acini *in vivo*, i.e. apical deposition of sialomucin and basal deposition of type IV collagen at the cell–ECM junction (Petersen *et al.*, 1992). It is interesting to note that both the final size of spheres (50 μm), the number of cells in aggregates (~8), the initial growth burst, the time before growth arrest (7 days), and the apical lumen formation and basolateral type IV collagen deposition have all been found previously for Madin–Darby canine kidney (MDCK) cells in collagen cultures, implying that these are fundamental epithelial and glandular behavior in the presence of relevant three-dimensional structures (Wang *et al.*, 1990). The fact that ER-negative

epithelial cells were able to form almost normal acini on cultivation in a basement membrane matrix indicates that neither ER expression nor myoepithelial cells are strictly required for this phenomenon to occur in culture and that the EHS matrix and the characteristics of cultured luminal breast cells are compensating for the other cell types that exist *in vivo* (Petersen *et al.*, 1992; Rønnov-Jessen *et al.*, 1996).

Analogous to the rodent model (Medina *et al.*, 1987; Schmidhauser *et al.*, 1990; Lin and Bissell, 1993), the effect of the ECM on human breast epithelial differentiation was not restricted to freshly explanted primary cultures with a finite life span. Rather, two nonmalignant, but immortal, cell lines (MCF-10A and HMT-3522) from benign breast lesions responded essentially in the same manner, that is with acinus-like formation and growth arrest. Although our assay was performed in a serum-free medium to obtain normal behavior, others have reported similar data using MCF-10A, even with serum in the medium (Basolo *et al.*, 1996). A related assay, also based on three-dimensional culturing, but in type I collagen instead of a reconstituted basement membrane, has been used for human breast epithelial cells (Berdichevsky and Taylor-Papadimitriou, 1991; Shearer *et al.*, 1992). In contrast to what has been reported for MDCK cells, however, this assay does not contain sufficient cues to polarize mammary epithelial cells correctly nor for them to deposit an endogenous basement membrane (Howlett *et al.*, 1995; Lu *et al.*, 1995). Thus the basement membrane (EHS) assay appears to be the first assay that allows recapitulation of critical aspects of normal breast in culture.

With the establishment of the EHS assay for human breast cells, the way was paved for the rapid identification of even subtle deviations from a differentiated behavior. Thus, within the same period of 7–12 days, during which "normal" cells organized an acinus, HBL-100 cells formed small, dispersed colonies, and tumor cells from either biopsies or established cell lines formed large irregular clusters of cells that did not growth arrest, deposit a basement membrane, or polarize correctly in response to the EHS (Petersen *et al.*, 1992, 1995; Howlett *et al.*, 1994, 1995). Thus as predicted, the tumor cells failed to sense the ECM properly, which was consistent with our hypothesis that the ability to respond to ECM correctly is a function of a new class of "suppressor genes" that are lost or altered when cells become malignant (Petersen *et al.*, 1992). The reason for this dramatic difference in response to the ECM could lie in defects anywhere from the ECM to integrins to cytoskeleton to nuclear matrix and to the genes themselves (Bissell *et al.*, 1982) and need not depend on the same genetic defects in different tumor cells. Our initial observations on the different behavior of "normal" and "malignant" human breast cancer cells inside EHS were confirmed by others (Shearer *et al.*, 1992; Bergstraesser and Weitzman, 1993). Since, then, it has been observed that the morphogenic effect of EHS on normal and can-

cer cells makes the use of EHS a more sensitive assay than the soft agar assay for predicting whether cells are tumorigenic in nude mice (Basolo *et al.*, 1996). Moreover, not only does the EHS assay serve to reveal normal behavior, it also supports growth of carcinoma cells better than the soft agar assay (Oridate *et al.*,1996).

To test the hypothesis that the assay could shed light on possible tumor suppressor genes, we tested the behavior of the highly malignant MDA-MB435 breast carcinoma cells that had been stably transfected with the tumor suppressor gene NM-23H1/NDP-kinase (Leone *et al.*, 1993). Whereas the neotransfected control cells behaved analogous to other breast cancer cells in the EHS assay, the NM-23-transfected cells showed an altered morphology and growth and polarized deposition of a basement membrane (Howlett *et al.*, 1994). In particular, they reached a final size of about 50 μm, as opposed to the neotransfected cells that kept on growing. It would be too naive to attribute these findings to a specific function of this particular tumor suppressor gene, because similar data in the EHS assay were obtained with the same cell line transfected with quite another gene, a novel H-cadherin (Lee, 1996), which may thus also be considered a tumor suppressor by our definition. Rather these data collectively point to the fact that as long as aspects of the phenotype can be compensated for, the exact error leading to the initial loss need not be corrected. The data also indicate that structural reorganization, if successfully imposed on the cells, is a very strong regulator of phenotype that can lead to reestablishment of a basement membrane and polarity, even in the absence of critical epithelial markers such as keratins and cadherins, which are permanently lost in MDA-MB435 cells (Pierceall *et al.*, 1995).

B. HMT-3522 Epithelial Cell Line of Breast Cancer Development

For a number of years, all our understanding of the early malignant lesions has had to rely on experimentally generated malignant transformation as obtained by SV-40 transfection, or benzo[*a*]pyrene treatment of cultured cells from normal individuals (Chang *et al.*, 1982; Stampfer and Bartley, 1985) (for reviews, see Rønnov-Jessen *et al.*, 1996; Weaver *et al.*, 1996). In general, human cells do not undergo the spontaneous transformation in culture often seen with rodent cells. The artificially generated malignant cells are quite aggressive to begin with and cannot therefore be taken to represent the early stages of cancer. Attempts to overcome the problem of spontaneous transformation of normal human cells have included the possible generation of established cell lines from benign lesions assumed to have initiated and immortalized prior to explantation (Pauley *et al.*, 1993). Success in estab-

lishing such "spontaneously" immortal cell lines from the human breast has been experienced in two cases. Originally, one of our laboratories generated the HMT-3522 S1 cell line under chemically defined culture conditions (Briand *et al.*, 1987) from a person with fibrocystic disease. Later, a different isolate was made from a similar source but with serum in the medium (the MCF-10A) (Soule *et al.*, 1990). The two cell lines share similar characteristics of being nonmalignant up to a very high passage number and showing very few chromosomal aberrations in the early passages. Clearly, they form an excellent basis for generating model systems for malignant progression. We have concentrated on the HMT-3522 cell line. The first question to address was whether they would progress spontaneously to a malignant state. The cells were kept under very defined culture conditions for more than 10 years and > 500 passages. It is relevant here to mention that most SV-40-immortalized cells become malignant around passage 100 (Rønnov-Jessen *et al.*, 1996). However, no signs of tumorigenicity were recorded in these non-xenobiotic-exposed cells. Interestingly, the cells have drifted substantially with regard to their karyotype such that later passages are grossly aneuploid. Still, however, they do not become tumorigenic (Vang Nielsen *et al.*, 1994). This correlates with the recent findings that aneuploidy has been found in breast tumors long before they are overtly malignant (Leal *et al.*, 1995; Teixeira *et al.*, 1995). It is also interesting to note that the other spontaneously immortal human breast epithelial cell line, MCF-10A, resembles HMT-3522 in its lack of tumorigenicity even in high passages.

It is a widely held notion that growth autonomy is a well-defined premalignant step in the multistep process of cancer development. In the absence of spontaneous transformation within a reasonable time frame, the HMT-3522 cells were forced to become autonomous with respect to EGF/amphiregulin by omitting this cytokine from the chemically defined medium in passage 120 (Madsen *et al.*, 1992). With this treatment, the cells remained relatively unaffected on the culture flask, but refrained from further growth for a few weeks (Briand *et al.*, 1996). After this latency period, growth commenced in a collective fashion from a slow to a more usual rate within one or two passages. Apart from being EGF autonomous, there were no discernible signs of further transformation of these lines (referred to as S2) grown on tissue culture plastic, because they remained nontumorigenic in nude mice. However, after exactly 118 passages in EGF-free medium, the cells suddenly gave rise to tumors, and the cells explanted from one of these tumors was named HMT-3522 T4-2. This spontaneous shift in phenotype coincided with the acquisition of an extra short arm of chromosome 7 (trisomy 7p) as the only obvious detectable karyotypic change. Other karyotypic changes, however, have more recently been identified using comparative genomic hybridization (collaborative studies with the Joe Gray and Dan Pinkel laboratories; Weaver *et al.*, 1998). The transformation was repro-

ducible in passage 238 as revealed by thawing frozen stocks of earlier passages and closely following them through the critical passages by inoculations in nude mice (Briand *et al.*, 1996). In other words, we have now succeeded in making a controlled progression series within the same cell line and without imposing any additional destabilizing exogenous transformation steps. Other attempts to make a progression series within a single cell line have included at least one additional measure to facilitate transformation (Pauley *et al.*, 1993). The HMT-3522 series has given us a unique opportunity to focus on the transformation-associated phenotype in the absence of the usual "noise" arising from tremendous dissimilarities among the normal and malignant cell lines.

An initial question that needed immediate clarification was whether the series from S1, EGF dependent, through S2, EGF independent, to tumorigenic T4-2 could be sufficiently resolved in our differentiation assay inside the reconstituted basement membrane. Indeed, all S1 passages behaved normally as described above by forming small acinus-like structures (Petersen *et al.*, 1992; Weaver *et al.*, 1998). More interestingly, however, not only T4-2 but also S2 diverged from such behavior and exhibited their own characteristic abnormal phenotype. As seen in Fig. 2, at least one population within S2 formed very large balls of cells many times larger than those formed by S1 cells. T4-2, on the other hand, formed intermediate-sized clusters with irregular boundaries and dissemination into the matrix (Fig. 2). Also, on sectioning of the gels and staining for endogenous basement membrane, the S1 cells stained as expected with a continuous line at the cell–ECM junction, S2 cells were almost negative, and T4-2 clusters in general were either not polarized or inversely polarized, but stained for type IV collagen (Fig. 3).

The fact that S2 cells apparently do not form their own basement membrane but proliferate continuously is compatible with their classification as benign hyperplasia. Thus, S2 cells resemble keratin K19-negative benign hyperplastic epithelium (Rønnov-Jessen *et al.*, 1996). That T4-2 cells apparently reexpress type IV collagen, albeit in an uncoordinated manner, has precedent for breast carcinomas *in vivo,* as mentioned previously. Thus, some invasive carcinomas in fact do stain for basement membrane (BM) components either in the cytoplasm or as an abortive attempt to form a BM. Staining for BM in breast cancer is considered a differentiated trait and correlates with better prognosis (Albrechtsen *et al.*, 1981; Natali *et al.*, 1992). Also, BM staining is a consistent feature of early *in situ* cancers and it is therefore not unreasonable to classify the derived T4-2 cell line as either an early cancer cell line or a differentiated cancer. Accordingly, in contrast to the existing established breast carcinoma cell lines, T4-2 is likely to be more subject to regulatory microenvironmental cues, and as such an excellent model for further exploration of possible differentiation therapy or reversion studies.

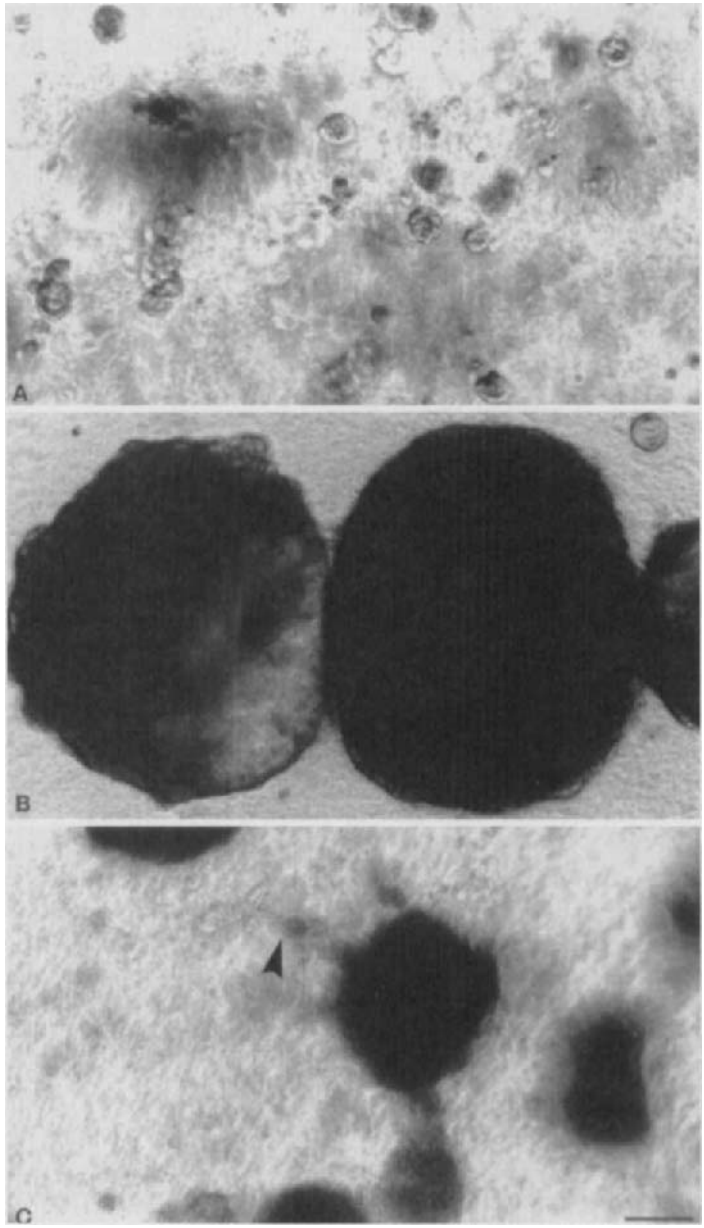

Fig. 2 Morphological characterization of the HMT-3522 series in the EHS assay. Phase-contrast micrographs of S1 cells (A), S2 cells (B), and T4-2 cells (C) plated inside EHS and cultured for 12 days in serum-free conditions. Whereas the "normal" S1 cells form typical acinus-like spheres, a subpopulation of the "benign" S2 cells form megaclusters reminiscent of hyperplasia, and the T4-2 cells form smaller irregular colonies with some penetration of cells into the EHS, similar to invasion (arrowhead in C). Magnification, ×90. Bar = 100 μm.

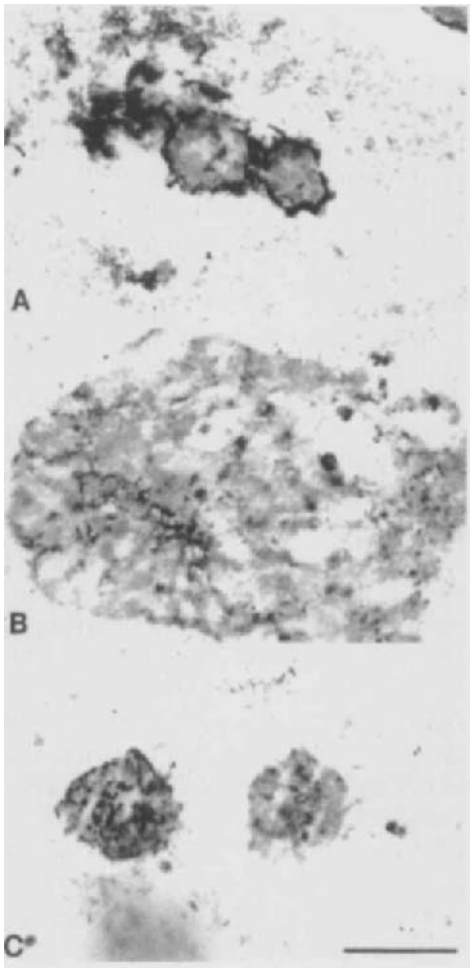

Fig. 3 Characterization of basement membrane deposition of the HMT-3522 series. Light micrographs of cryostat sections of S1 cells (A), S2 cells (B), and T4-2 cells (C) immunoperoxidase stained for human type IV collagen and counterstained with hematoxylin. Note that although the S1 cells deposit an almost continuous basement membrane, this is lost in the S2 cells. The T4-2 cells deposited a disorganized and apolar basement membrane. Magnification, ×150. Bar = 100 μm.

C. Reversion of Malignant HMT-3522-T4 Phenotype

One remarkable feature that clearly discriminated the T4-2 cell line from our previously tested established breast cancer cell lines was the distinct expression of β1-integrin on the cell surface of T4-2 cells [compare Howlett *et al.* (1995) and Weaver *et al.* (1997)]. We therefore used this new cell line in

the previously described assay aimed at establishing the role of β1-integrin in morphogenesis of normal versus tumor cells. This could not be tested in the tumor cell lines that were tested previously because they did not contain much β1 (Howlett *et al.*, 1995). The T4-2 line responded to a β1-integrin-inhibitory antibody in a surprising manner by forming regular spheres with an occasional central lumen (Weaver *et al.*, 1997): They exhibited growth arrest within the experimental period, similar to what we had recorded with the nonmalignant S1 line without the antibody (Petersen *et al.*, 1992; Howlett *et al.*, 1995; Weaver, *et al.*, 1997). A quantification of the number of acini showed that almost all cells were in fact reverted by the treatment, leaving essentially no irregular clusters of cells after 10–12 days of cultivation. The growth arrest was also genuine as evidenced by thymidine incorporation and cyclin D1 expression. Moreover, sectioning and staining for endogenous type IV collagen showed a distinct organized and polarized BM at the cell–ECM junction similar to our observation in the S1 cells. The morphological, proliferative, and polarized reversion was also associated with a reorganization of the cytoskeleton. Thus, whereas the untreated or mock-treated T4-2 cells showed a diffuse staining for actin, E-cadherin, and β-catenin, the β1-integrin antibody-treated cells polarized these molecules to the cell–cell junction (Fig. 4; see color plate) (Weaver *et al.*, 1997). That the S1 cells and the β1-integrin-treated T4-2 cells were in fact different was evident only from the fact that the latter stained for β-catenin and E-cadherin at the cell–ECM junction in addition to the staining at the cell–cell junction (Fig. 4). In addition, in contrast to S1 cells, very few lumina were observed at the apical surface. Finally, we established a link between our culture observations and traditional tumorigenicity in nude mice in that β1-integrin-treated T4-2 cells gave rise to significantly fewer and smaller tumors than did the mock-treated control cells (Weaver *et al.*, 1997).

Because the behavior of T4-2 cells could be almost completely corrected by an inhibitory antibody to β1-integrin, it was tempting to assign conversion to the malignant phenotype in this system to an overexpression of β1-integrin at the cell surface as compared to S1 cells. The expression level of β1-integrin was therefore measured on immunoblots of total proteins and of biotin-labeled surface proteins. In both cases, the level of β1-integrin was higher in T4-2 cells versus S1 cells. Thus, if the action of the β1-integrin antibody could be truly attributed to specific blocking of the signaling pathway and cells were not selected for, then we had conclusive evidence for the role of this integrin in the dramatic reversion.

The specificity of the function blockade was further tested by use of other inhibitory antibodies against β1-integrin and one β1-integrin stimulatory antibody. The latter did not inhibit. Also, Fab fragments were generated to distinguish between receptor clustering and inhibition of signaling. Fab fragments inhibited as well as the intact antibody. Finally, we could exclude se-

lection of possible contaminating S1 cells because repeated cycles of reexplantation into monolayer and reembedding into EHS with anti-β1-integrin-treated and mock-treated cells showed that the "malignant" and "normal" phenotypes were reversible (Weaver *et al.*, 1997).

Neutralizing β1-integrin was previously shown to lead to a less malignant behavior in terms of invasion and tumorigenicity *in vivo* (Fujita *et al.*, 1992; Schiller and Bittner, 1995). However, the mechanism(s) for this lowered tumorigenicity was neither explored nor understood. Thus, the dramatic differentiating effect of down-regulating β1-integrin in tumor cells is not only a novel finding for β1-integrin action on tumor cells, but it also reveals a very important principle for glandular function. In an effort to explain the finding with the integrin balance theory in mind (see Section II, C), we speculated that the corrected phenotype was not only the result of a diminished β1-integrin signaling per se but also of restoring the signaling of one or more of the other integrins. In other words, the malignant behavior was conveyed by an imbalance of the array of available integrins at the cell surface. Cell surface labeling showed that there was less β4-integrin on T4-2 cells compared to the nonmalignant S1 cells. The ratios between β1 and β4 were 1.85 and 5.18 in S1 and T4-2 cells, respectively. In further support of the imbalance hypothesis were the facts that the level of cell surface β4-integrin in the T4-2 cells was essentially normalized on treatment with the β1-integrin antibody, and that, conversely, treatment of S1 cells with either β4- or α6-integrin function-altering antibodies resulted in a disorganized phenotype somewhat reminiscent of that seen in malignant cells (Weaver *et al.*, 1997).

As discussed in the introduction, the concept that signaling from the microenvironment may revert an otherwise overtly malignant phenotype is not unprecedented, although a mechanism has not been put forward. For example, it has been reported that the information imparted by pure thrombospondin may revert malignant hemangiomas to differentiated nontumorigenic capillary-cord-like cells, and that the expression of thrombospondin inversely correlates with malignant behavior of melanoma, lung, and breast carcinoma cell lines (Zabrenetsky *et al.*, 1994; Sheibani and Frazier, 1995).

What is equally significant about the collective observations we have made with this breast epithelial series inside a three-dimensional reconstituted basement membrane assay is a new integrated view of how normal tissues function *in vivo*. Our results may point to a hitherto unknown switch that may be pulled, the net effect of which in a three-dimensional assay is to override several genotypic abnormalities associated with malignant behavior. Our data should also be viewed within the context of integrin signaling whereby the apparent redundancy of integrins has been a puzzle to solve. Further understanding may be gained from the concept that multiplicity does not indicate redundancy but serves to maintain an equilibrium of signaling

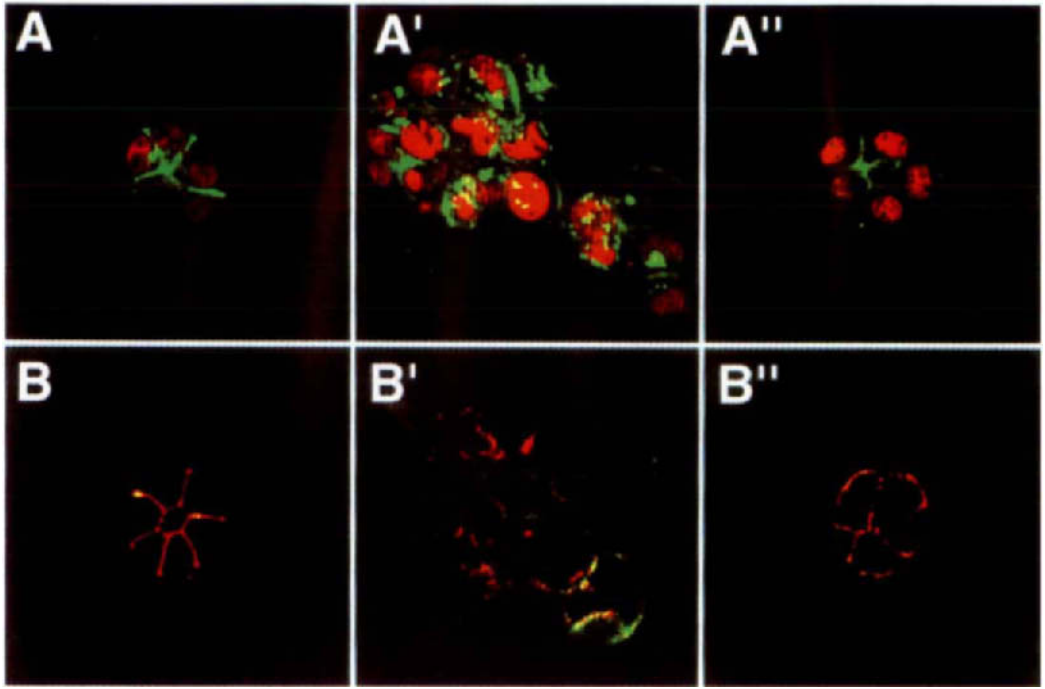

Fig. 4 β1-Inhibitory antibody treatment of tumor cells leads to the formation of reverted acini. (A–A″) Confocal fluoresence microscopy images of F-actin. Both the S1 (A) and T4-β1 reverted acini (A″) showed basally localized nuclei (propidium iodide) and organized filamentous F-actin (FITC), whereas T4-2 mock-treated colonies (T4-2 IgG) had disorganized, hatched bundles of actin and pleiomorphic nuclei (A′). (B–B″) Confocal immunofluorescence microscopy images of E–cadherin (FITC) and β-catenin (Texas red): In S1 (B) and T4-β1 reverted acini (B″), E-cadherin and β-catenins were colocalized and superimposed at the cell–cell junctions. Reproduced from Weaver *et al.* (1997). *J. Cell Biol.* **137**, 231–245, by permission of The Rockefeller University Press.

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from different pathways responding to a microenvironment in such a way as to maintain adequate homeostasis and growth when necessary.

IV. CONCLUSION

The recent spectacular ability to identify and clone the susceptibility genes for many forms of cancers has led to much excitement and also much expectation for a rapid understanding and cure for breast and other epithelial cancers.

The central thesis of this short review, focused mainly on our current work, is that we will not understand breast cancer unless we understand normal epithelial biology. We argue that a simple genetic model of breast cancer, even if multistep, cannot, and does not, explain much of the literature and our current data. The fact that malignant behavior can be reverted by manipulation of a single ECM signaling pathway (even if so far in a single model system) argues that many of the changes that were responsible for this behavior (unregulated growth, aberrant morphogenesis, lack of a basement membrane, loss of cell–cell interaction, levels of cyclins, etc.) were lost not by mutation, but by changes in their levels or localization as a result of a global disorganization of tissue structures. Because we can shuttle these cells back and forth in dramatic changes in phenotype, and retain the same malignant genotype, it is clear that the tissue phenotype is dominant over the cellular genotype.

Thus, we argue that breast cancer is the result of not just genetic change, developmental regulation, or loss of growth regulation, but an interweaving of all of these factors. This is an insight that should help us to unravel the molecular mechanisms that allow the genes and the microenvironment to come together to create tissues and specificity. To understand how these specialized cues are lost as cells become malignant may require collaboration of oncologists, molecular and cell biologists, but also pathologists, physiologists, computation biologists, and bioengineers.

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Inherited Carcinomas of the Kidney

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Studies of families with inherited carcinomas have provided powerful tools to identify the genes involved in the pathogenesis of human cancers. In this review, we summarize the clinical, pathological, and genetic characteristics of the inherited carcinomas of the kidney. We emphasize the observation that different genes predispose to histologically different types of renal carcinoma. Hereditary papillary renal carcinoma, a recently described inherited disorder, is discussed in detail along with the predisposing gene, the MET protooncogene. The data support a classification of renal carcinomas based on molecular genetics.

I. INTRODUCTION

Until recently, adenocarcinomas of the kidney were regarded as a single entity. Recent evidence from pathologic, cytogenetic, and family studies have changed this view (Kovacs, 1993; Storkel and van den Berg, 1995; Weiss *et al.*, 1995; this review). Now, it seems appropriate to regard adenocarcinomas of the kidney as a group of malignancies caused by mutations in different genes. This review focuses on the data that have led to these conclusions, with particular emphasis on the results of family studies. By studying families with inherited cancers it is possible to identify the genes responsible for the inherited neoplasms, and to characterize cancers initiated by mutation of single genes. Emphasis will be placed on hereditary papillary renal carcinoma, a newly recognized, inherited malignancy (Zbar *et al.*, 1994, 1995; Schmidt *et al.*, 1997). Information on the genes known to predispose to kidney cancer will be outlined. Information will also be presented on an inherited carcinoma of the kidney in the rat (Eker and Mossige, 1961).

II. INHERITED CARCINOMAS OF THE KIDNEY: HISTOLOGY

The inherited carcinomas of the kidney may be classified according to histologic appearance of the neoplasms, or by clinical syndromes. Members of kidney cancer families inherit a tendency to develop a particular histologic type of renal neoplasia (Table I). Members of families with von Hippel–Lindau disease develop clear cell renal carcinomas; there is no predisposition to develop papillary renal carcinomas (Lubensky *et al.*, 1996) or any other histologic type of renal neoplasia. Conversely, members of hereditary papillary renal carcinoma families develop papillary renal carcinomas; there is no predisposition to develop clear cell renal carcinomas (Zbar *et al.*, 1994, 1995; Schmidt *et al.*, 1997) or any other histologic type of renal neoplasia. Indi-

Table I Classification of Inherited Tumors of the Kidney

Histology/laterality/other tumors	Clinical syndrome	Predisposing event	Ref.
Clear cell			
Bilateral/with other tumors	von Hippel–Lindau disease	Germ-line mutation in VHL gene	Latif *et al.* (1993a)
Bilateral/without other tumors	3;8 and 3;6 translocation	Germ-line balanced translocation	Cohen *et al.* (1979), Kovacs *et al.* (1989a)
Bilateral/with other tumors	Tuberous sclerosis	Germ-line mutation in TSC2	Bjornsson *et al.* (1996)
Unilateral/without other tumors	Non-VHL, nonpapillary renal carcinoma, associated with supernumerary nipples	Not known Not known	Teh *et al.* (1997a) Mehes (1995)
Papillary			
Bilateral/without other tumors	Hereditary papillary renal carcinoma	Germ-line mutations in the MET protooncogene	Schmidt *et al.* (1997)
Unilateral/without other tumors	Not defined	Not known	This review
Oncocytoma			
Bilateral/without other tumors	Hereditary renal oncocytoma	Not known	This review
Transitional cell carcinoma of the renal pelvis and ureter/with other tumors	HNPCC	Mutation mismatch repair genes	Lynch *et al.* (1990, 1997)
Adult nephroblastoma	HPT-JT	HRPT2 mutation	Teh *et al.* (1997b)
Nephroblastoma	Wilms' tumor	Germ-line mutation WT-1	Beckwith (1997)
Angiomyolipoma/with other tumors	Tuberous sclerosis	Germ-line mutation TSC1, Germ-line mutation TSC2	

viduals with inherited kidney cancer develop multiple, independent tumors in each kidney; all kidney tumors from a patient with an inherited carcinoma of the kidney have a similar histologic appearance. These observations indicate that the genes that predispose to kidney carcinoma cause specific histologic types of renal carcinoma. The fact that morphologically distinct renal epithelial tumors are produced by germ-line mutations in different genes is a distinguishing feature of the inherited carcinomas of the kidney, and separates these disorders from the inherited carcinomas of the breast and colon.

III. INHERITED CARCINOMAS OF THE KIDNEY: CLINICAL SYNDROMES

The hallmark of the inherited carcinomas of the kidney is the presence of multiple, bilateral renal neoplasms (Fig. 1, see color plate). The lesions vary in size from dysplastic lesions that can be seen only with the microscope, to lesions 10–15 cm in diameter; the tumors vary in number from 1–2 per kidney to as many as 50 tumors per kidney. The characteristics that serve to distinguish the various types of inherited kidney carcinoma are the histologic appearance of the renal tumors and the presence of other independent neoplasms (Table I). The inherited carcinomas of the kidney are all inherited as autosomal dominant disorders. The penetrance (the proportion of gene carriers who develop disease) is dependent on the disease gene and the particular mutation in the disease gene.

A. Clear Cell Renal Carcinomas

von Hippel–Lindau (VHL) disease is a multisystem neoplastic inherited disorder characterized by a predisposition to develop clear cell renal carcinomas, as well as retinal angiomas, central nervous system hemangioblastomas, pheochromocytomas, pancreatic cysts, epididymal cystadenomas, and endolymphatic sac tumors (Melman and Rosen, 1964). Families with VHL differ in the types of tumors they develop (Chen *et al.*, 1995b; Glenn *et al.*, 1991; Neumann and Wiestler, 1991; Zbar, 1995; Zbar *et al.*, 1996). The most common form of VHL is characterized by a predisposition to develop retinal angiomas, central nervous system hemangioblastomas, and clear cell renal carcinomas (VHL type 1). The next most common form of VHL (VHL type 2B) is characterized by a predisposition to develop pheochromocytomas in addition to the manifestations of VHL type 1. The rarest form of VHL (VHL type 2A) is characterized by a predisposition to

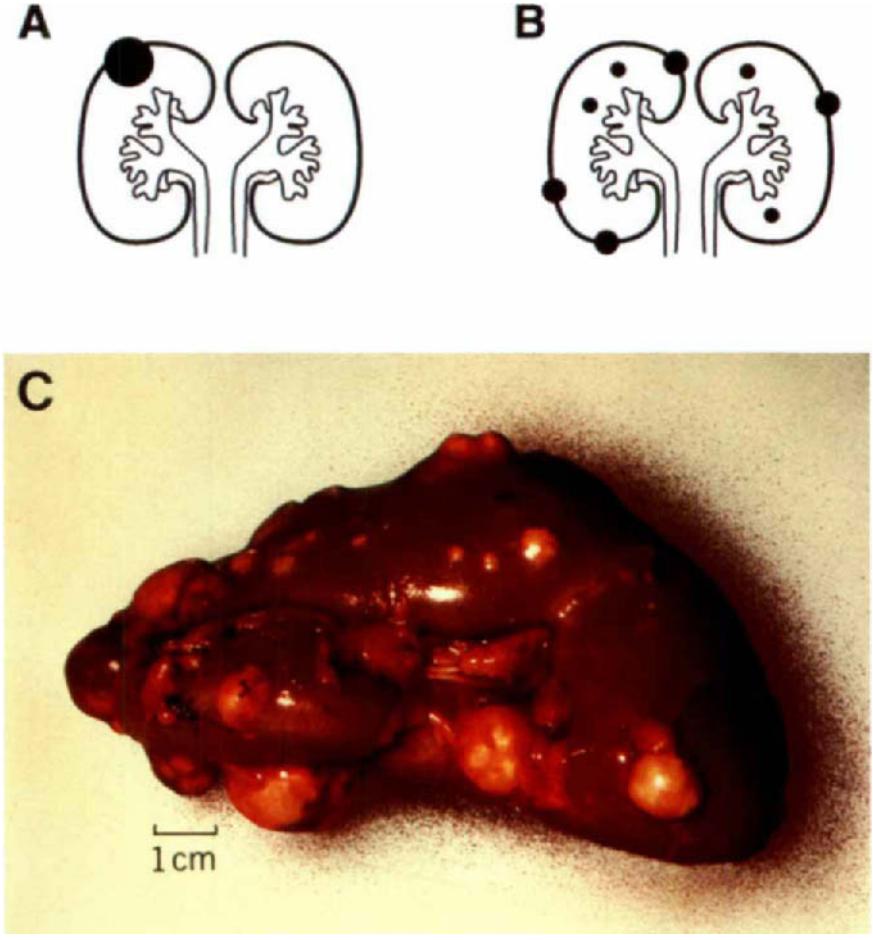

Fig. 1 Schematic illustration of sporadic and inherited renal cell carcinoma. (A) Patients with sporadic renal carcinoma have a single tumor in one kidney. (B) Patients with inherited renal carcinoma have multiple tumors in both kidneys. (C) Photograph of kidney from a patient with inherited (papillary) renal carcinoma (Zbar *et al.*, 1994). Note that the numerous tumor nodules vary in size. Tumors were also present in the patient's other kidney. All renal tumors had a papillary histologic appearance.

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develop pheochromocytoma with little or no tendency to develop renal carcinoma.

Families with a constitutional, balanced translocation between chromosomes 3 and 8, or between chromosomes 3 and 6, also have a predisposition to develop clear cell renal carcinomas (Cohen *et al.*, 1979; Kovacs *et al.*, 1989a; Li *et al.*, 1982, 1993; Li, 1988). Renal tumors are usually detected after age 40, somewhat later than in VHL. Thyroid carcinomas developed in two family members who were chromosome 3;8 translocation carriers (Li, 1988; Li *et al.*, 1993). The number of affected individuals is small, so it is uncertain whether the thyroid carcinomas that have occurred in translocation carriers were related to the chromosome translocation. In contrast to VHL, the inheritance of a constitutional 3;8 translocation is not consistently accompanied by a predisposition to develop other neoplasms.

Clear cell renal carcinomas have also developed in patients affected with tuberous sclerosis (TSC) (Aoyama *et al.*, 1996; Bjornsson *et al.*, 1996; Sampson *et al.*, 1995), and in patients with supernumerary nipples (Mehes, 1995; Urbani and Betti, 1996). The clinical information available on the renal carcinomas associated with these disorders is limited. Available data suggest that supernumerary nipples are, in general, associated with single clear cell renal neoplasms. In a review of 16 TSC patients with renal carcinomas, 43% of the tumors were bilateral (Waschecka and Hanna, 1991).

In addition to these well-recognized forms of inherited clear cell renal carcinoma, there are families with two or more members affected with renal carcinomas that do not fit the above categories (Teh *et al.*, 1997a). These families have received various designations, reflecting the difficulties in classification. The history is usually that of a parent and child affected with single clear cell renal carcinomas. Occasionally, the parent has had a single clear cell renal carcinoma and the child had bilateral clear cell renal carcinomas. There are other pedigrees with stronger evidence for an inherited predisposition to develop clear cell renal carcinoma. In one family, a husband with cancer had three children affected with renal cancer by two different wives (B. Zbar and W. M. Linehan, unpublished data). Teh *et al.* (1997a) have identified one family in which single clear cell renal carcinomas were present in members of three generations, and another family in which there were five patients affected with single clear cell renal carcinoma in two generations.

Chance may explain the finding of families with two members affected with clear cell renal carcinoma. In an international epidemiologic study of 1774 patients with renal cancer, there were 56 patients with one first-degree relative with kidney cancer, and 7 patients with two first-degree relatives with kidney cancer; in the 2359 matched controls (without renal cancer) there were 50 controls that had one first-degree relative with kidney cancer and no controls with two first-degree relatives with kidney cancer (Schlehofer *et al.*, 1996). These results suggest that most nuclear families

with two members affected with single clear cell renal carcinomas arise by chance.

Biologic characteristics of selected families with clear cell renal carcinomas suggest a genetic basis other than germ-line mutations in the VHL gene. The three-generation family with clear cell renal carcinoma without evidence of VHL, and the detection of renal cancer in the offspring of two different spouses with a common affected husband (in the absence of evidence for germ-line VHL mutations), suggest the existence of genes other than VHL that predispose to clear cell renal carcinomas.

B. Papillary Renal Carcinomas

Hereditary papillary renal carcinoma (HPRC) is characterized by a predisposition to develop multiple, bilateral renal tumors with a papillary histologic appearance (Zbar *et al.*, 1994, 1995; Schmidt *et al.*, 1997). Although other malignancies have been observed in patients with HPRC (see below), or in their first-degree relatives, because the number of patients affected with HPRC is small, it is uncertain whether these maligancies are related to mutations in the HPRC gene. In contrast to VHL, the major neoplastic manifestation of HPRC appears to be confined to the kidney. The youngest individual found to have a HPRC tumor was 24 years of age. One estimate of the median age for renal tumor detection in carriers of the HPRC gene is 40 years. The time from tumor detection to metastases is not known.

Two families studied at the National Institutes of Health had multiple members affected with papillary renal carcinoma that did not fit into the clinical pattern observed for typical HPRC families (B. Zbar and W. M. Linehan, unpublished). In one family (family 166), affected family members had single papillary renal carcinomas rather than the bilateral, multiple papillary renal carcinomas characteristic of HPRC. In the other family (family 154), affected family members had bilateral, multiple papillary renal neoplasms. However, in contrast to the well-differentiated features that are typical of HPRC, the histologic appearance of the papillary renal carcinomas in this family was poorly differentiated, with variation in cell morphology in different parts of the neoplams. It is uncertain whether the biologic differences observed between families 154 and 166 and typical HPRC families reflect allelic variation or genetic heterogeneity.

C. Transitional Cell Renal Carcinomas

Transitional cell carcinomas of the renal pelvis and ureter may occur in patients with hereditary nonpolyposis colon carcinomas (HNPCC) (Lynch *et*

al., 1990, 1997). Hereditary nonpolyposis colon carcinoma is an inherited predisposition to develop single carcinomas of the colon; there is an associated predisposition to develop carcinomas of other organs, notably endometrial carcinomas. In HNPCC the renal neoplasms that develop presumably reflect the error in DNA repair that is the fundamental characteristic of this disorder.

IV. PAPILLARY RENAL CARCINOMA: PATHOLOGY AND GENETICS

Papillary renal carcinomas comprise about 10% of sporadic renal carcinomas (Storkel and van den Berg, 1995; Thoenes *et al.*, 1986, 1990). Papillary renal carcinomas are defined by the presence of a characteristic pattern of tumor cell growth (Amin *et al.*, 1997a; Delahunt and Eble, 1997; Kovacs, 1989; Kovacs *et al.*, 1997; Mancilla-Jimenez *et al.*, 1976). Tumor cells are arrayed on fingerlike projections attached to a basement membrane that contains a central fibrovascular core. Tumors are classified as either eosinophilic or basophilic. Recognizing that renal carcinomas vary in the percentage of tumor occupied by a papillary architecture, different authors have proposed requiring either 50 or 75% of the tumor to have a papillary architecture to be classified as papillary renal carcinoma (Kovacs, 1989; Mancilla-Jimenez *et al.*, 1976). However, there are no indications that the 50 or 75% threshold reflects an underlying biologic phenomenon, and it can be difficult to quantitate the percentage of a renal tumor that is papillary.

Delahunt and Eble (1997) suggested that papillary renal carcinomas be divided into two subtypes based on distinct morphological and immunochemical characteristics. Type 1 papillary renal carcinoma is characterized by the presence of papilla covered with a single or double layer of small epithelial cells with scant clear or pale cytoplasm. Type 2 papillary renal carcinoma is characterized by papilla covered with epithelial cells with abundant eosinophilic cytoplasm arranged in a pseudostratified or irregularly stratified manner. Type 1 papillary renal carcinomas were accompanied by multiple microscopic papillary tumors in 26/62 (41%) of cases; type 2 papillary renal carcinomas were accompanied by microscopic papillary tumors in 4/38 (10%) of cases.

Kovacs recognized that papillary renal carcinomas were genetically distinct from other (nonpapillary) forms of renal carcinoma (Kovacs, 1989, 1993). Kovacs found that papillary renal adenomas were characterized by polysomy of chromosomes 7 and 17. Malignant papillary renal carcinomas were characterized by polysomy of chromosomes 7, 12, 16, and 17, and in men, loss of the Y chromosome. Although trisomy and tetrasomy of chro-

mosome 7 may be found in clear cell renal carcinomas (Zhao *et al.*, 1995), the combination of trisomy of chromosomes 7 and 17 was found to be a characteristic of papillary renal carcinomas. This observation has been confirmed (Corless *et al.*, 1996; Dijkhuizen *et al.*, 1996).

Sporadic papillary renal tumors are often multiple, even in patients without a family history of papillary renal carcinoma (Kovacs and Kovacs, 1993). There may be a single sporadic papillary renal carcinoma and multiple microscopic papillary lesions, or there may be bilateral, multiple lesions greater then 1 cm in diameter. It is not known whether the patients with sporadic papillary renal carcinomas with multiple microscopic tumors and the patients with bilateral, multiple, clinically detectable papillary tumors represent parts of the same disease continuum or distinct entities. There appears to be a disagreement between the results of Kovacs and Kovacs (1993), and the results of Delahunt and Eble (1997). Kovacs and Kovacs made serial sections of the normal kidney from patients with sporadic papillary renal carcinomas and found that virtually all sporadic papillary renal carcinomas were accompanied by multiple microscopic papillary renal neoplasms. Delahunt and Eble found that the frequency of detection of microscopic papillary renal neoplasms in patients with sporadic papillary renal carcinomas depended on the morphology of the primary tumor. Multiple microscopic papillary neoplasms were more frequent in type 1 papillary renal carcinomas. These discrepancies may reflect sampling problems, or perhaps a fundamental biologic difference between subtypes of papillary renal carcinoma.

In end-stage renal disease with acquired renal cystic disease, the incidence of renal carcinomas is increased and the renal carcinomas that develop are frequently papillary (Ishikawa and Kovacs, 1993).

A. Families with Papillary Renal Carcinoma

Several reports appeared in the last 20 years describing families with members affected with renal cell carcinoma (Li *et al.*, 1982). In a few families, all affected family members had renal tumors with a papillary growth pattern (Bernades *et al.*, 1972; Bernues *et al.*, 1995; Franksson *et al.*, 1972, Pearson, 1969). Because papillary renal carcinomas were detected in members of a single generation, it was uncertain whether this condition was an inherited disorder. Zbar *et al.* identified several families with multiple members affected with papillary renal carcinoma in two or three generations (Zbar *et al.*, 1994, 1995; Schmidt *et al.*, 1997). Affected family members developed bilateral, multiple renal tumors with a papillary growth pattern. The disorder was designated hereditary papillary renal carcinoma. The pattern of inheritance of HPRC was consistent with autosomal dominant transmission with reduced penetrance. The predisposition to develop papillary renal car-

cinoma was not linked to loci on chromosome 3p (the site of the VHL gene); papillary renal carcinoma families did not show germ-line mutations of the VHL gene, and there were no VHL mutations in papillary renal carcinomas. The cytogenetic, linkage, and mutational analyses of papillary renal carcinoma families supported the concept that there was a distinct gene, HPRC, that predisposed to papillary renal carcinoma.

1. LINKAGE ANALYSIS IN HPRC FAMILIES

Schmidt *et al.* (1997) determined that the MET protooncogene was the gene responsible for HPRC. The HPRC gene was located at chromosome 7q31–34 by linkage analysis in five HPRC families. The maximal lod score (lod = 6.75 at $\theta = 0.0$) (two-point linkage analysis) was obtained with probe D7S1801. The most likely location of the HPRC gene was in a 27 centimorgan interval bounded by D7S496 and D7S1837. There was no suggestion of genetic heterogeneity in the families examined. One family (160), by itself, was of sufficient size to give a lod score greater than 3 (a lod score of 3.0 is considered significant evidence of linkage).

Cytogenetic studies of papillary renal tumors supported the results of linkage analysis. The most consistent cytogenetic finding in sporadic and inherited papillary renal carcinomas was trisomy of chromosome 7, suggesting that a gene(s) located on chromosome 7 was involved in the pathogenesis of papillary renal carcinomas (Kovacs, 1993). The location of the region on chromosome 7 containing the putative papillary renal carcinoma gene was refined by studies of renal carcinomas from one patient with HPRC. The sole alteration in one of this patient's renal tumors was the duplication of the distal portion of chromosome 7q21–q35 (Bernues *et al.*, 1995). This result suggested that the 7q21–35 region harbored a gene(s) of critical importance in the pathogenesis of papillary renal carcinoma. Of particular importance, the results of the linkage analysis placed the HPRC gene at 7q31.1–7q34, in the center of the region defined as critical by the cytogenetic studies. Cytogenetic studies of papillary renal carcinoma, like cytogenetic studies of clear cell renal carcinoma, pointed to the location of a gene important in the pathogenesis of the neoplasm.

2. RADIOLOGIC DIAGNOSIS OF HEREDITARY PAPILLARY RENAL CARCINOMA

A critical element in the linkage analysis was the identification of renal tumors in asymptomatic members of HPRC families (Choyke *et al.*, 1997). These tumors were detected in asymptomatic members of HPRC families by computerized axial tomography scans with contrast enhancement (Press *et al.*, 1984). The renal tumors were hypovascular and could be mistaken for

cysts. When region of density measurements were obtained, these lesions demonstrated a 12- to 63-unit increase in Hounsfield density after intravenous contrast. Ultrasound analysis was less sensitive than computerized tomography in detecting renal tumors in HPRC families.

3. GERM-LINE AND SOMATIC MUTATIONS OF THE MET PROTOONCOGENE IN PAPILLARY RENAL CARCINOMAS

The MET protooncogene was shown to be the HPRC gene by mutation analysis (Schmidt *et al.*, 1997). Although the MET protooncogene had been identified in 1984 (Cooper *et al.*, 1984), the genomic structure had not been determined. As a first step in the analysis, Duh *et al.* determined the genomic structure of the MET protooncogene (Duh *et al.*, 1997). Exon 2 and exons 9–21 of the MET gene were examined by single-strand conformational polymorphism in affected members of HPRC families, and in a panel of sporadic papillary renal carcinomas. Schmidt *et al.* (1997) found five different germ-line mutations in the MET protooncogene in HPRC families and four somatic mutations in sporadic papillary renal carcinomas. The nucleotide changes in the MET gene identified in these families were not found in a panel of normal chromosomes, and therefore were unlikely to be polymorphisms.

All mutations were missense mutations located in the tyrosine kinase domain of the MET gene; 6/9 mutations involved nonconservative changes in amino acids. Germ-line mutations in the MET protooncogene were detected in HPRC families from the United States, Canada, Germany, Spain, The Netherlands, and Sweden. Somatic mutations in the MET gene were detected in 3/60 sporadic papillary renal carcinomas.

In contrast to VHL, wherein many of the germ-line and somatic mutation were predicted to inactivate the protein product, in HPRC all mutations in the MET gene were of the missense type; no mutations predicted to inactivate the MET protein were identified. Also, in contrast to the VHL and other tumor suppressor genes, the mutations identified were localized to a small region of the gene (Fig. 2). The observations suggested that missense mutations in the tyrosine kinase domain of the MET protooncogene produced a gain of function.

4. GERM-LINE MUTATIONS IN MET IN PATIENTS WITHOUT A FAMILY HISTORY OF PAPILLARY RENAL CARCINOMA

Germ-line mutations in the MET protooncogene were detected in three patients without a clear family history of papillary renal carcinoma (Fig. 3). In one patient (family 164) without a family history of papillary renal carcinoma, bilateral papillary renal carcinoma, stomach carcinoma, and rectal car-

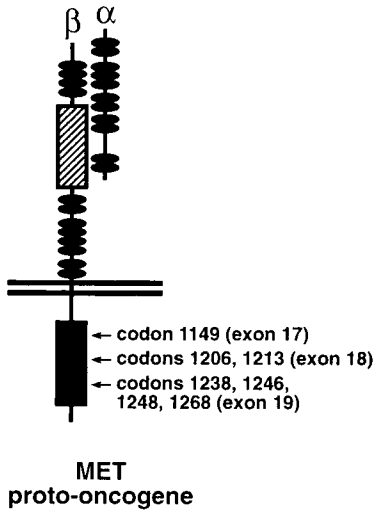

Fig. 2 Illustration of the MET transmembrane receptor tyrosine kinase with location of germ-line and somatic mutations in the MET protooncogene indicated. Note that all mutations were confined to the tyrosine kinase domain. All mutations were missense.

cinoma were present. The germ-line mutation in MET in this patient was Y1248C. A second patient (family 158) was referred because of the finding of bilateral papillary renal carcinoma; although the patient's parents had no history of renal neoplasia, an aunt had a history of a nephrectomy. A germ-line mutation in the MET gene (V1206L) was found in the patient and his deceased aunt. In a third case, a 27-year-old male (family 165) was referred because of a history of bilateral papillary renal carcinoma. The patient's mother had had a bilateral nephrectomy for "clear cell renal carcinoma." A germ-line mutation was found in this patient (D1246N). Clearly, germ-line mutations in the MET protooncogene may be found in patients without an extended history of renal neoplasia. It may be that large families with papillary renal carcinoma are uncommon, and that the more common form of hereditary papillary renal carcinoma is small families with one or two affected individuals. The MET protooncogene may be a low-penetrance cancer-causing gene with onset of detectable renal neoplasia at late ages.

B. Manifestations of Germ-Line Mutations in the MET Gene

Knowledge of the clinical manifestations of germ-line MET mutations will facilitate recognition of patients with these mutations. There are four dis-

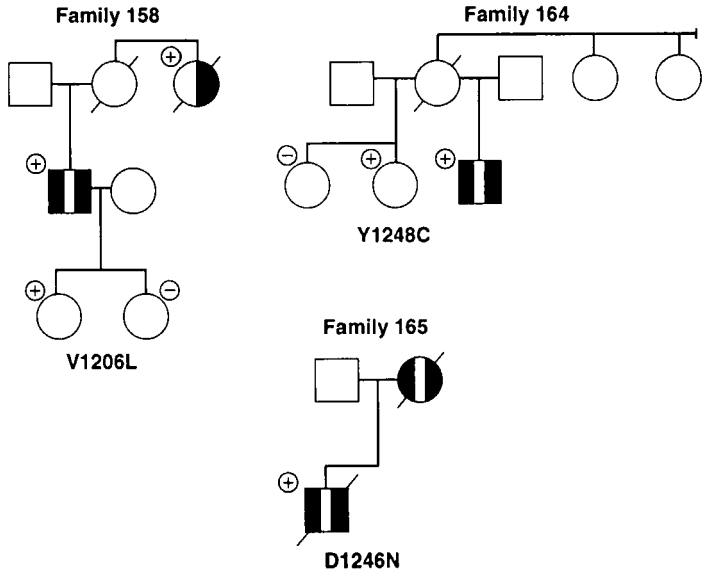

Fig. 3 Germ-line mutations in the MET protooncogene in individuals without a clear family history of renal neoplasia. +, The presence of a germ-line mutation in the MET protooncogene; –, the absence of germ-line mutation in the MET protooncogene. The germline mutation is indicated beneath the pedigree.

tinct currently recognized presentations of germ-line mutations in the MET gene: (1) families with multiple members affected with bilateral, multiple papillary renal carcinoma; (2) bilateral, multiple papillary renal carcinoma in patients with one relative with a history of renal neoplasia; (3) bilateral, multiple papillary renal carcinoma in patients without a family history of renal neoplasia; and (4) a single solid renal tumor in an asymptomatic individual with a family history of HPRC.

1. FREQUENCY OF MET MUTATIONS IN SPORADIC PAPILLARY RENAL CARCINOMAS

The general principle that has emerged from studies of the inherited cancer syndromes is that there is consistent mutation of the tumor suppressor genes in the sporadic form of the disease. This is the case for the VHL gene in sporadic clear cell renal carcinomas, and for the APC gene in sporadic colon carcinomas. Multiple endocrine neoplasia type 2 is a form of inherited human cancer caused by the inheritance of a mutant protooncogene (RET) (Eng, 1996; Ponder and Smith, 1996). This disorder is characterized by a predisposition to develop pheochromocytomas, medullary carcinoma

of the thyroid gland, and hyperplasia of the parathyroid gland. About 40% of sporadic medullary carcinomas of the thyroid show somatic RET mutations identical to the MEN 2B mutation (M918T) (Eng *et al.*, 1994; Eng, 1996; Hofstra *et al.*, 1994).

Mutations in the MET protooncogene have been found, so far, in only a small proportion of sporadic papillary renal carcinomas. The frequency of detection of somatic MET mutations in sporadic papillary renal carcinomas is 3/60 (5%) compared to a frequency of detection of germ-line MET mutations of 6/7 (86%) (Schmidt *et al.*, 1997, L. Schmidt *et al.*, unpublished). There are several possible explanations for the low frequency of MET protooncogene mutations in sporadic papillary renal carcinomas. (1) There may be mutations in the MET protooncogene in sporadic papillary renal carcinomas that have not been identified yet. (2) Somatic mutations in MET may occur in only one of the morphological subtypes of papillary renal carcinoma. (3) There may be a single mutation in the MET gene, analogous to the M918T mutation in RET, that is responsible for a substantial fraction of sporadic papillary renal carcinoma; such a mutation has not been found. Alternatively, mutations in the MET protooncogene may play a role in the pathogenesis of only a small proportion of sporadic papillary renal carcinomas.

In papillary renal carcinomas, there may be somatic mutations in other genes on chromosome 7, perhaps hepatocyte growth factor/scatter factor (HGF/SF), the ligand for the MET protooncogene, or another receptor, tyrosine kinase, located on chromosome 7. Alternatively, increased expression of the MET protein and its ligand without mutation might occur in some sporadic papillary renal carcinomas, and thus contribute to tumorigenesis according to the scenario depicted in Fig. 4A.

2. SIMILARITIES BETWEEN MUTATIONS IN MET, RET, AND C-KIT IN HUMAN NEOPLASIA

There are striking homologies in the location of the mutations observed in the MET, RET, and c-KIT protooncogenes (Schmidt *et al.*, 1997). c-KIT is a receptor tyrosine kinase that is expressed on stem cells in the bone marrow; c-KIT is the receptor for STEEL factor (Tsujimura, 1996). RET, also a receptor tyrosine kinase, expressed in thyroid cells, is the receptor for glial-derived growth factor (Ponder and Smith, 1996). All three transmembrane tyrosine kinase receptors have mutations in the tyrosine kinase domains (Bolino *et al.*, 1995; Hofstra *et al.*, 1994; Nagata *et al.*, 1995, Piao and Bernstein, 1996). In three instances, the mutations in MET were located at codons homologous to mutations in RET or c-KIT (Schmidt *et al.*, 1997). The MET M1268T mutation found in two sporadic papillary renal carcinomas is homologous to the MEN2B mutation M918T. The MET D1246H mutation, found in a sporadic papillary renal carcinoma, and the MET D1246N germ-

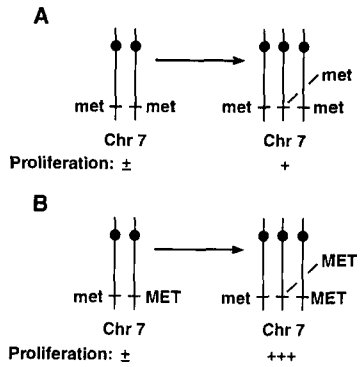

Fig. 4 Polysomy of chromosome 7, cell proliferation, and hereditary papillary renal carcinoma. (A) Polysomy of chromosome 7 without MET mutations affords a slight proliferative advantage; (B) polysomy of chromosome 7 with duplication of a mutant MET protooncogene provides a major proliferative stimulus.

line mutation are homologous to the human c-KIT mutation D816V and the mouse c-KIT mutation D814V. The RET M918T mutation produces medullary carcinoma of the thyroid, pheochromocytomas, neuromas, and parathyroid gland hyperplasia (Hofstra *et al.*, 1994). The c-KIT D816V mutation produces mastocytosis with an associated hematologic disorder (Nagata *et al.*, 1995).

So far, seven residues in the tyrosine kinase domain of MET have been identified that, in mutant form, lead to disease. Three residues were identified in the tyrosine kinase domain of RET that, in mutant form, lead to disease. How these mutations alter protein function is unknown. Subtle conformational changes may lead to activation of the enzymatic function of the proteins.

C. Possible Mechanisms of Disease Production

The function of the mutations in RET and c-KIT have been studied by transfection (or infection) of mutant constructs into suitable indicator cells (Piao and Bernstein, 1996; Piao *et al.*, 1996; Santoro *et al.*, 1995). Numerous foci formed when the RET MEN2A and 2B mutations were introduced into NIH 3T3 cells, indicating that the RET mutant constructs had transforming ability. Transfected NIH 3T3 cells formed tumors in nude mice. Biochemical studies indicated that the RET mutant constructs produced autophosphorylation and constitutively active proteins. When the c-KIT mutant was introduced into interleukin-3-dependent indicator cells, Kitaya-

ma *et al.* (1995) found IL-3-independent proliferation of cells *in vitro*, and growth of cells in nude mice.

Based on the experiments with RET and c-KIT with missense mutations in the tyrosine kinase domains, we predict that transfection of recombinant mutant MET genes would lead to constitutive phosphorylation of the MET protein and transformation of NIH 3T3 cells. A schematic model of the relationship between chromosome 7 trisomy, MET mutations, and cellular proliferation is given in Fig. 4. Duplication of one chromosome 7 (trisomy 7) with a wild-type MET protooncogene is associated with growth stimulation (Ermis *et al.*, 1995). Duplication of one chromosome 7 (trisomy) with a mutant MET protooncogene is associated with a greater growth stimulatory effect.

The identification of missense mutations in the tyrosine kinase domain of MET fits into a broader picture of alteration of cellular growth by the mutation of receptor tyrosine kinases. Hunter summarized the wide variety of cell surface receptors involved in disease including neu, cytokine receptors, RET, and c-KIT (Hunter, 1997).

1. WHICH CHROMOSOME 7 ALLELE IS DUPLICATED IN TUMORS OF PATIENTS WITH HPRC?

Dijkhuizen (1997) studied multiple renal tumors from a patient with HPRC and found that the same chromosome 7 allele was duplicated in all five tumors. Preliminary results suggest that the mutant MET allele is duplicated in papillary renal tumors from patients with HPRC (Z. Zhuang, unpublished data). Trisomy 7 may increase the dosage of the mutant MET allele (Fig. 4B). Trisomy involving chromosomes bearing oncogenes has been observed with murine dimethylbenz[*a*]anthracene (DMBA)-induced skin papillomas/carcinomas and murine T cell lymphomas (Aldaz *et al.*, 1989; Bianci *et al.*, 1990; Bremmer and Balmain, 1990; Burns *et al.*, 1991; Wirchubsky *et al.*, 1984).

2. SIGNIFICANCE OF THE TRISOMY OF CHROMOSOMES 12, 16, 17, AND 18 IN PAPILLARY RENAL CARCINOMA

Kovacs showed that trisomy of chromosomes 7, 12, 16, 17, and 18 were characteristic of papillary renal carcinomas (Kovacs, 1993). The studies of renal tumors from members of HPRC families suggest that chromosome 7 trisomy may be a marker for the presence of a mutated (activated) receptor tyrosine kinase. The consistent constellation of trisomy 7, 12, 16, 17, and 18 in papillary renal carcinoma may represent the outline of a growth control pathway, with the trisomic chromosomes harboring mutated receptor tyro-

sine kinases that are the members of the pathway. Dijkhuizen (1997) found that the same chromosome 17 allele was duplicated in two cases of sporadic multiple papillary renal carcinoma, suggesting that a gene(s) located on chromosome 17 was involved in the pathogenesis of these neoplasms.

D. Evaluation of Papillary Tumors for Clonality

Few studies have been performed to evaluate the clonality of the multiple papillary renal carcinomas in patients with HPRC or in patients with sporadic papillary renal carcinoma with multiple microscopic neoplastic foci. Chromosome X inactivation analysis of multiple papillary carcinomas from one patient with HPRC demonstrated that in this patient the tumors arose independently (Z. Zhuang, and I. Lubensky, unpublished observations).

E. Significance of Second Primary Tumors in Patients with HPRC

Of the 67 individuals affected with HPRC, there were 2 patients with carcinoma of the pancreas (in the same family), 2 patients with carcinoma of the breast, 1 patient with carcinomas of both the stomach and rectum, 1 patient with carcinoma of the common bile duct, 1 patient with malignant melanoma, 1 patient with fibrosarcoma, and 1 patient with carcinoma of the lung (Zbar *et al.*, 1994, 1995; Schmidt *et al.*, 1997). Further study is necessary to determine whether the second tumors found in some patients with hereditary papillary renal carcinoma represent a coincidence, or an uncommon consequence of germ-line mutations in the MET protooncogene.

F. Somatic (X;1)(p11;q21) Translocation

The somatic chromosome translocation (X;1)(p11;q21) is a recurrent chromosomal abnormality in a subset of papillary renal cell carcinoma, sometimes as the sole cytogenetic abnormality (Meloni *et al.*, 1993; Shipley *et al.*, 1995). van Kessel's and Cooper's groups reported that the translocation results in a fusion of the transcription factor TFE3 on the X chromosome to a novel gene, designated PPRC on chromosome 1 (Beckmann and Kadesch, 1990; Sidhar *et al.*, 1996; Weterman *et al.*, 1996a,b). Through this fusion the reciprocal translocation products that are formed are both expressed in papillary renal carcinomas. This change appears to produce gain-of-function of a protein. There is evidence that the translocation is associated with papillary renal carcinomas with distinct clear cell features.

G. Causes of Bilateral Papillary Renal Carcinomas

As indicated above, bilateral papillary renal carcinoma occurs both in a hereditary and a nonhereditary setting. Germ-line mutations in the MET protooncogene occur in patients with bilateral papillary renal carcinoma with or without a family history of renal neoplasia. There may be patients with bilateral papillary renal carcinoma that is not caused by germ-line mutations in the MET protooncogene. In these patients, several other causes need to be considered, including viral infection, somatic mutation in a growth regulatory gene early in development [perhaps similar to the McCune–Albright syndrome (Ringel *et al.*, 1996; Shenker *et al.*, 1993)], growth stimulation in end-stage renal disease (ligand-driven renal carcinoma?), and mutation of the TFE3 and PRCC genes.

If the bilateral renal neoplasms in some patients are caused by somatic mutational events that occur early during kidney development and differentiation, there should be identical mutations in each papillary renal neoplasm, but not in the normal tissues of the patient. Somatic mutations during renal development and differentiation might underly several types of unexplained bilateral renal carcinomas, renal oncocytomatosis, as well as papillary renal carcinomas. Cytogenetic studies of bilateral renal tumors have been performed (Dal Cin *et al.*, 1996; Henn *et al.*, 1993).

H. Genetic Heterogeneity of Papillary Renal Carcinomas

Genetic heterogeneity exists in the causation of papillary renal carcinoma. There are three genes that can predispose to papillary renal carcinomas: TFE3, PPRC, and MET. The MET protooncogene is involved in inherited predisposition to papillary renal carcinoma, and also in sporadic papillary renal carcinoma. The TFE3 and PPRC genes are implicated in the pathogenesis of sporadic papillary renal carcinoma. It will be necessary to study a large panel of papillary renal carcinomas to determine the frequency of mutation of MET, TFE3, and PPRC in sporadic papillary renal carcinomas.

V. CLEAR CELL RENAL CARCINOMAS: PATHOLOGY AND GENETICS

Clear cell renal carcinomas comprise about 80% of sporadic renal carcinomas (Thoenes *et al.*, 1986, 1990). This type of renal carcinoma is defined by a characteristic appearance of the cells that comprise the neoplasm (Ko-

vacs *et al.*, 1997). When viewed in hematoxylin- and eosin-stained sections, the cytoplasm of the tumor cells appears empty, unstained, and therefore clear. This appearance is a result of extraction, and removal of lipid in the cytoplasm of these cells by the solvents used in the paraffin embedding procedure. Variants of clear cell renal carcinomas exist with different histologic appearances (Fleming, 1993; Kovacs *et al.*, 1997; Weiss *et al.*, 1995).

A. Germ-Line VHL Mutations and Phenotypes

Germ-line mutations in the VHL gene have been reviewed and summarized (Chen *et al.*, 1995b; Zbar, 1995; Zbar *et al.*, 1996). Mutant VHL alleles produced three distinct phenotypes: VHL type 1, with renal carcinoma, hemangioblastoma of the central nervous system, and retinal angiomas without pheochromocytoma; VHL type 2A, with pheochromocytoma without renal carcinoma, and infrequent hemangioblastomas of the central nervous system and retinal angiomas; and VHL type 2B, with renal carcinoma, hemangioblastomas of the central nervous system, and retinal angiomas with pheochromocytoma. Germ-line mutations responsible for each type of VHL have been identified and catalogued (see http://www.ncifcrf.gov/kidney; Zbar *et al.*, 1996). Mutations predicted to truncate the VHL protein produce VHL type 1; mutations (missense) that lead to full-length VHL proteins lead to VHL type 2. A few specific missense mutations in the VHL gene lead to VHL type 2A.

B. VHL Mutations in Sporadic Clear Cell Renal Carcinomas

Clear cell renal carcinomas are characterized by mutations of the VHL gene (Bailly *et al.*, 1995; Foster *et al.*, 1994; Gnarra *et al.*, 1994; Kenck *et al.*, 1996; Latif *et al.*, 1993a; Shuin *et al.*, 1994; Whaley *et al.*, 1994). Studies of sporadic clear cell renal carcinomas from patients in the United States, France, Great Britain, Germany, and Japan show a frequency of somatic VHL mutations of about 50%. Inactivation of the VHL gene by hypermethylation of the promoter has been demonstrated in approximately 20% of sporadic clear cell renal carcinomas (Herman *et al.*, 1994; J. G. Herman unpublished; H. Brauch unpublished). Therefore, about 70% of sporadic clear cell renal carcinoma samples show inactivation of the VHL gene by somatic mutation or hypermethylation. Because no panels of sporadic clear cell renal carcinomas have been studied both for structural changes in the VHL gene and for hypermethylation, the figure of 70% alterations of the VHL gene in clear cell renal carcinomas must be regarded as a rough estimate. The

percentage of clear cell renal carcinoma with somatic mutations of the VHL gene or hypermethylation is important because percentages appreciably lower than 100% suggest the existence of genes other than VHL that can lead to clear cell renal carcinoma.

The VHL mutations found in sporadic renal carcinomas were predicted to inactivate the VHL gene product (Bailly *et al.*, 1995; Foster *et al.*, 1994; Gnarra *et al.*, 1994; Latif *et al.*, 1993a; Shuin *et al.*, 1994; Whaley *et al.*, 1994; Zbar *et al.*, 1996). What is remarkable is the diversity of somatic VHL gene mutations. Virtually all the VHL somatic mutations detected to date are different from one another. Of germ-line VHL mutations, 70% were predicted to compromise elongin binding (Zbar *et al.*, 1996) (see below). There have been no correlations between VHL somatic mutations and prognosis. Wilhelm *et al.* found no correlation between the presence of VHL mutations in clear cell renal carcinomas and factors that predict the biologic behavior of the neoplasm (Wilhelm *et al.*, 1995b). Clear cell renal carcinomas with VHL mutations were just as likely to be associated with aggressive characteristics as clear cell renal carcinomas without VHL mutations.

Environmental carcinogens have been suspected as a cause of clear cell renal carcinoma because of characteristic patterns of somatic VHL mutations (Gnarra *et al.*, 1994). Bruning *et al.* (1997) and Brauch *et al.* (1997) provided the first possible link between a specific carcinogen, somatic mutations in the VHL gene, and renal cell carcinoma. They studied workers exposed to high concentrations of trichloroethene, and found that 23/23 individuals with kidney cancer had evidence suggesting a somatic mutation in the VHL gene. Of tumors with a band shift, 4/23 have been sequenced so far and these tumors showed somatic mutations in the VHL gene. The frequency of change detected by SSCP was considerally higher than found in individuals with renal carcinoma not exposed to trichloroethene.

Occupational risk for renal cell carcinoma has been suspected in other types of workers, including firefighters and painters (Delahunt *et al.*, 1995). It would be of interest to study the patterns of somatic VHL mutations to determine whether there is evidence for a carcinogen footprint in clear cell renal carcinomas associated with specific environmental exposures.

C. Cytogenetic Changes in Clear Cell Renal Carcinomas

The most characteristic cytogenetic change in clear cell renal carcinomas is a loss the of the distal part of chromosome 3p (Kovacs *et al.*, 1988). This observation has been confirmed repeatedly by loss-of-heterozygosity studies (Kovacs *et al.*, 1988; Presti *et al.*, 1993; Thrash-Bingham *et al.*, 1995; van der Hout *et al.*, 1993; van den Berg *et al.*, 1996a; Zbar *et al.*, 1987). Addi-

tional cytogenetic changes in clear cell renal carcinomas include duplication of chromosome band 5q22 and deletion of chromosome arms 6q, 8p, 9p, and 14q (Cairns *et al.*, 1995; Kovacs, 1993; Morita *et al.*, 1991; Moch *et al.*, 1996). There have been reports describing associations between loss of chromosome 9p and 14q segments and prognosis (Moch *et al.*, 1996; Wu *et al.*, 1996). These areas of chromosome loss may indicate the location of genes that may be important in the progression of clear cell renal carcinoma. These characteristic cytogenetic changes have been used as the basis of a genetic classification of renal tumors (see below) (Bugert and Kovacs, 1996; Bugert *et al.*, 1997).

D. Pathogenesis of Clear Cell Renal Carcinomas Associated with the Chromosome 3;8 Translocation

The family with the chromosome 3;8 translocation associated with a predisposition to develop clear cell renal carcinoma focused attention on the possible role of genes located on chromosome 3p in the pathogenesis of renal carcinoma (Cohen *et al.*, 1979). The identification of the VHL gene on chromosome 3p supported the suggestions raised by studies of the 3;8 translocation family. Because the translocation breakpoint (3p14.2) was located far from the VHL gene (3p25), it was unclear whether the VHL gene played a role in the pathogenesis of 3;8 translocation-associated clear cell renal carcinoma. Further studies have clarified the role of the VHL gene in the pathogenesis of chromosome 3;8 translocation renal carcinoma.

The 3;8 translocation appears to increase the susceptibility of the derivative 8 chromosome to chromosomal loss. This idea is supported by the results of molecular genetic and cytogenetic studies. Clear cell renal carcinomas found in chromosome 3;8 translocation patients had lost the derivative 8 chromosome (Li *et al.*, 1993); the derivative 8 chromosome contains the distal portion of chromosome 3p. Consequently, loss of the derivative 8 chromosome produced loss of one copy of the VHL gene. The remaining copy of the VHL gene was mutated in some (two out of four) renal tumors (Gnarra *et al.*, 1994; Knudson, 1995; Schmidt *et al.*, 1995). The results suggest that clear cell renal carcinomas develop in members of chromosome 3;8 translocation families by a series of at least three steps: (1) inheritance of the chromosome 3;8 translocation, (2) loss of the chromosome 8 bearing the distal portion of chromosome 3p (a somatic event leading to loss of one copy of the VHL gene), and (3) somatic mutation of the VHL gene located on the normal chromosome 3. These three events occur within a single renal epithelial cell, and initiate malignant transformation.

The process of formation of clear cell renal carcinomas in patients with the chromosome 3;8 translocation appears to be a varation on the events lead-

ing to sporadic clear cell renal carcinomas. In sporadic clear cell renal carcinomas, a somatic point mutation or hypermethylation inactivates one copy of the VHL gene; the other copy of the VHL gene is lost as a consequence of chromosomal mutation (somatic loss of 3p). In clear cell renal carcinoma associated with the chromosome 3;8 translocation, the loss of 3p by chromosomal mutation is accelerated by the instability of the derivative 8 chromosome.

The chromosome 3;8 translocation suggested that there was a gene located at the translocation breakpoint that was important in the pathogenesis of clear cell renal carcinomas. The translocation breakpoint gene (FHIT) has been cloned (Ohta *et al.*, 1996), and to date, no alterations have been found in FHIT in clear cell renal carcinomas (Kondo *et al.*, 1997; van den Berg *et al.*, 1996b).

E. Interstitial Deletions on Chromosome 3p

Several groups have reported detecting small interstitial deletions on chromosome 3p in sporadic renal carcinomas (van den Berg *et al.*, 1996a; van den Berg and Buys, 1997; Yamakawa *et al.*, 1991). If correct, these interstitial deletions on 3p would pinpoint the location of tumor suppressor genes on 3p important in the pathogenesis of renal cell carcinoma. Wilhelm *et al.* and Bugert *et al.* were unable to confirm the reports of interstitial deletions on 3p in sporadic renal cell carcinomas (Wilhelm *et al.*, 1995a; Bugert *et al.*, 1996). Their work showed a terminal deletion of chromosome 3p with a breakpoint clustering between 3p11.2 and 3p14.1. About one-third of the 3p breakpoints were located in a small region at 3p11.2. There has been one report of a homozygous deletion at chromosome 3p in a renal carcinoma cell line (Lisitsyn *et al.*, 1995).

F. Knudson's Model

The Knudson model applies to clear cell renal carcinomas (Knudson, 1995). The initiating event in clear cell renal carcinomas appears to be the inactivation of both copies of the VHL gene. One copy of the VHL gene is lost as a consequence of the loss of the short arm of chromosome 3 by chromosomal mutation; the other copy of the VHL gene is inactivated by intragenic mutations. Hypermethylation is an alternative mechanism for inactivation of the VHL gene. The VHL gene has been referred to as a "gatekeeper" of renal cell growth, analogous to the APC gene in colonic epithelial cell growth (Kinzler and Vogelstein, 1996).

These events are particularly amenable to analysis in the clear cell renal car-

cinomas associated with von Hippel–Lindau disease. Loss of the wild-type allele of the VHL gene appears to be an early event in the pathogenesis of clear cell renal carcinomas associated with von Hippel–Lindau disease. Of 26 clear cell renal lesions, ranging from cysts with a single cell lining to microscopic renal cell carcinomas, 25 showed *in situ* loss of the wild-type allele and retention of the inherited mutated VHL allele (Lubensky *et al.*, 1996).

G. Genetic Heterogeneity of Clear Cell Renal Carcinomas

The frequency of detection of somatic mutations in the VHL gene in clear cell renal carcinomas has been remarkably constant from series to series. All reports indicate about 50% of clear cell renal carcinomas show structural rearrangements in the VHL gene (Bailly *et al.*, 1995; Foster *et al.*, 1994; Gnarra *et al.*, 1994; Shuin *et al.*, 1994). Possibly, there remain a subset of VHL mutations in sporadic clear cell renal carcinomas that have not been identified. Against this idea is the observation that, although with improvements in technology the proportion of germ-line VHL mutations detected has increased to 90% (C. Stolle and H. Kazazian, unpublished), the proportion of sporadic VHL mutations detected has remained unchanged.

Current studies suggest that the VHL gene is inactivated in about 70% of sporadic clear cell renal carcinomas. The results raise the possibility that there is another gene(s) responsible for initiating clear cell renal carcinoma in the 30% of clear cell renal carcinomas that lack inactivation of the VHL gene by mutation or hypermethylation. This idea is supported by the existence of small nuclear families with clear cell renal carcinoma that do not appear to be associated with VHL (Teh *et al.*, 1997a).

VI. OTHER RENAL CARCINOMAS

A. Chromophobe Renal Carcinomas: Pathology and Genetics

Chromophobe renal carcinomas are characterized by finely reticulated cytoplasm that stains lightly (chromophobic) with routine hematoxylin and eosin stains (Crotty *et al.*, 1995; Kovacs *et al.*, 1997). These tumors are characterized by a positive reaction with Hale's colloidal iron technique. On electron microscopic examination, there are numerous intracytoplasmic membrane-bound vesicles. On cytogenetic examination, chromophobe renal carcinomas are characterized by widespread chromosome loss. Loss of heterozygosity has been found for DNA markers on chromosomes 1, 2, 6, 10,

13, 17, and 21 (Bugert *et al.*, 1997; Schwerdtle *et al.*, 1996). Shuin *et al.*, 1996). Kovacs *et al.* (1992) reported mitochondrial alterations in chromophobe renal carcinomas. van den Berg (1997b) suggested that a subset of oncocytomas may be precursors for chromophobe renal carcinomas.

No families have been reported with members affected with chromophobe renal carcinomas; there have been no case reports of patients with bilateral, multiple chromophobe renal carcinomas.

B. Oncocytomas: Pathology and Genetics

Renal oncocytomas are characterized by large cells with granular eosinophilic cytoplasm (Amin *et al.*, 1997b; Kovacs *et al.*, 1989b, 1997). Under electron microscopic examination, densely packed mitochondria are seen. Cytogenetic studies show two types of renal oncocytomas: one type is characterized by loss of chromosome 1p (Thrash-Bingham *et al.*, 1996); the other type is characterized by a somatic chromosome translocation, with 11q13 as the common breakpoint (van den Berg, *et al.*, 1995; Neuhaus *et al.*, 1997; Dijkhuizen, 1997). The chromosome 11q13 breakpoint has been finely mapped (Sinke *et al.*, 1997). One important distinction between renal oncocytomas and clear cell renal carcinoma is that renal oncocytomas do not show loss of chromosome 3p. Kovacs studied two renal oncocytomas and reported that the mitochondrial DNA from the tumors had a new, somatic restriction endonuclease (*Hinf* I site) site (Kovacs *et al.*, 1989b). This observation has not been confirmed.

There have been at least eight case reports of patients with bilateral, multiple renal oncocytomas (Fairchild *et al.*, 1983; Hara *et al.*, 1982; Israeli *et al.*, 1995; Kadewsky and Fulgham, 1993; Mead *et al.*, 1990; Zhang *et al.*, 1985). A entity termed "renal oncocytomatosis" has been described characterized by the presence of hundreds of small oncocytomas in the kidney (Warfel and Eble, 1982). Two families have been identified with three members affected with bilateral renal oncocytomas (W. M. Linehan, and B. Zbar, unpublished). These families appear to define a distinct clinical entity, "hereditary renal oncocytoma," analogous to hereditary papillary renal carcinoma. Teh *et al.* (1997c) described a patient with bilateral renal oncocytomas associated with a reciprocal, balanced translocation between chromosomes 9q and 8q, and a constitutional VHL mutation.

C. Medullary Renal Carcinoma with Sickle Cell Trait

Medullary carcinoma of the kidney associated with sickle cell trait is a recently recognized distinct entity that is a rare cause of renal tumors in young

African Americans (Davis *et al.*, 1995). These renal tumors are believed to arise from the collecting ducts of the renal medulla, and are characterized by an inflamed desmoplastic stroma.

D. Collecting Duct Carcinomas of the Kidney

Collecting duct carcinomas are characterized by irregular channels lined by a atypical epithelial cells (Flemming and Loewi, 1986; Rumpelt *et al.*, 1991). Loss of heterozygosity studies showed loss on chromosomes 1q, 6p, 8p, and 21q (Polascik *et al.*, 1996). No families with collecting duct carcinomas of the kidney have been identified.

E. Renal Carcinoma in the Eker Rat: Pathology and Genetics

Currently, there is one animal model available to study inherited carcinomas of the kidney. This inherited renal carcinoma developed spontaneously in a Wistar rat. The Eker renal carcinoma is an inherited renal malignancy in the rat caused by an insertion of an intracisternal A particle in the rat homolog of the tuberous sclerosis 2 (TSC2) gene (Eker and Mossige, 1961; Kobayashi *et al.*, 1995; Yeung *et al.*, 1994). The mutation is predicted to lead to an inactive protein. The histologic appearance of the Eker tumors is compatible with human chromophobe cell renal tumor according to the Bannasch nomenclature (Hino *et al.*, 1996). The pathogenesis of renal tumors have been studied in this rat model. Loss of the wild-type allele of the TSC2 rat homolog has been detected in preneoplastic lesions estimated to contain fewer than 100 cells (Kubo *et al.*, 1995). These observations supports the two-mutation model for the origin of renal tumors in the Eker rats, and strongly emphasize the loss of the wild-type allele being of pathogenetic importance in the origin of the preneoplastic cell islands.

Everitt *et al.* (1992) and Walker *et al.* (1992) treated Eker rats with a chemical carcinogen that targeted both renal epithelial and mesenchymal cells. The carcinogen caused an increase in tumors of epithelial origin in susceptible animals; the number of carcinogen-induced mesenchymal tumors was unaffected by the presence of the mutation at the susceptibility locus.

F. Renal Carcinomas in Patients with Tuberous Sclerosis

Tuberous sclerosis (TSC) is an autosomal dominant disorder characterized by seizures, mental retardation, and tumors in the brain, heart, skin, and kid-

ney. Most tumors are benign with the exception of those in the kidney, where malignant tumors may occur. The demonstration of a mutation in the TSC2 gene in the Eker rat kidney tumor has prompted efforts to determine whether the TSC1 and/or TSC2 genes are involved in the pathogenesis of human renal carcinoma. There are two approaches being used: testing for mutations in the TSC2 gene in sporadic renal carcinomas with particular emphasis on chromophobe renal carcinomas, the human tumor that most closely resembles the Eker rat tumor, and study of the renal tumors that develop in tuberous sclerosis patients.

Angiomyolipomas, which are benign tumors with vascular, smooth muscle and fat elements, are the most common TSC renal tumors. Rarely, renal carcinomas occur in TSC patients. In an unselected series of 256 TSC patients, Sampson *et al.* found two cases of renal carcinoma (Sampson *et al.*, 1995). When renal carcinomas occur in TSC patients, they may be single or bilateral (Washecka and Hanna, 1991). Although clear cell renal carcinomas are the most common type of renal carcinoma, other histologic types have been observed.

A loss of heterozygosity analysis of TSC-associated renal carcinomas has been reported (Bjornsson *et al.*, 1996). Of interest is the unusual immunochemical staining for a melanocyte-associated marker, HMB-45, in four of six TSC-associated renal carcinomas, a marker that is positive with angiomyolipomas.

G. Additional Inherited Tumors of the Kidney

There are a number of other kidney tumors that occur on an inherited basis. These tumors include angiomyolipomas (tuberous sclerosis), Wilms' tumor (Beckwith, 1997), and mixed mesenchymal tumors of adults (jaw–kidney association) (Szabo *et al.*, 1995; Teh *et al.*, 1997b).

VII. GENES THAT PREDISPOSE TO CARCINOMAS OF THE KIDNEY

A. VHL Disease Tumor Suppressor Gene

The von Hippel–Lindau disease tumor suppressor locus was placed by linkage analysis in a small interval on 3p25 (Hosoe *et al.*, 1990; Maher *et al.*, 1991; Yao *et al.*, 1993) and subsequently cloned by positional cloning technology (Latif *et al.*, 1993a). The cloned gene was identified as a tumor suppressor gene by demonstrating inactivating mutations in the germ line of VHL patients and sporadic clear cell renal carcinomas (Crossey *et al.*, 1994;

Gnarra *et al.*, 1994; Latif *et al.*, 1993a) and growth suppression by VHL transgenes (Chen *et al.*, 1995a; Iliopoulos *et al.*, 1995). The VHL gene resides in distal 3p25, 100 kb telomeric to the plasma membrane Ca^{2+}-transporting ATPase isoform 2 (PMCA-2) gene (Kuzmin *et al.*, 1994; Latif *et al.*, 1993b) located between D3S1597 and D3S1263 in the 6-cM interval on the genetic map (National Center for Biotechnology Information, 1997). The gene lies within a single invariant 22-kb *Eco*RI genomic fragment and spans 13 kb of DNA. It contains three exons [exon 1 consists of 340 bp of coding sequence plus 70 bp of the 5′ UTR, exon 2 has 123 bp of coding sequence, and exon 3 comprises 179 bp of coding sequence, including the termination codon followed by a 4-kb 3′ UTR (Renbaum *et al.*, 1996)] and two introns of 3.5 and 4.5 kb (Renbaum *et al.*, 1996). (GenBank accession numbers: L15409, g7 cDNA; U68055, intron 1; U68176, intron 2; and U49746, 3′ UTR.)

The minimal promoter is only 106 bp long, TATAless, and overlaps with the first exon that contains a CpG island around a single Not1 site (Kuzmin *et al.*, 1995; accession number U19763). Several transcription factor binding sites were detected in the promoter sequence, notably a 12-bp site for the NRF1 factor that coordinately controls expression of growth-related genes. The VHL gene is universally and abundantly expressed in adult and fetal tissue and cell lines as a 4.8- to 5.5-kb transcript. Two noncanonical polyadenylation signals (ATTAAA) separated by a 500-bp sequence and alternative splicing of exon 2 may account for size differences observed on blots. During development the VHL gene is expressed in a temporal and spatial fashion, especially in brain and kidney (Kessler *et al.*, 1995; Richards *et al.*, 1996).

Analysis of the deduced peptide of 24 kDa encoded by the longer splice form did not reveal any homologies to other proteins except for the acidic pentamer (GXEEX) repeated eight times after the first methionine also found in a trypanosome glycan membrane protein. The presence of the second methionine immediately after the acidic repeat element suggests that the mRNA could be translated into two proteins, one with an acidic N terminus. Two proteins might be produced from the second splice form, indicating that potentially four distinct VHL proteins might be generated, providing a molecular basis for functional diversity.

Further analysis of the pVHL sequence revealed two separate conserved motifs implicated in protein binding at amino acid residues 88–139 (motif 1) and residues 156–195 (motif 2) (E. Koonin, personal comunication). Motif 1 is encoded partly (50%) by exon 2, suggesting that the pVHL form without this exon would probably not have a functional motif 1. These two conserved motifs were found in a true *Caenorhabditis elegans* homolog of VHL and in a fungal open reading frame (ORF) (gi769816), but not in yeast or bacteria. The second motif was found in elongin A and protochlorophillide reductase subunit (CHLN_MARPO). The function of motif 1 is unknown at present, but motif 2 is implicated in binding elongin subunits B and C.

Protein binding studies *in vitro* and *in vivo* have also identified these two

separate protein binding sites within the pVHL sequence (Duan *et al.*, 1995; Kishida *et al.*, 1995; Kibel *et al.*, 1995) and demonstrated that mutations of the second site (motif 2) prevented binding to elongin subunits. Because the elongin ABC complex relieves RNA pol II pausing during RNA elongation, it was speculated that pVHL may negatively regulate expression of growth-related genes, including protooncogenes. If this speculation proves correct it would provide a simple explanation for the tumor suppressor function served by the VHL gene. However, it is clear that the product(s) of the VHL gene is a multifunctional protein(s) that might be involved in several important regulatory pathways, as suggested by the presense of two highly conserved protein binding motifs (E. Kunin, personal communication). Another function of pVHL is its negative effect on the expression of the VEGF gene, the ultimate endothelial cell mitogen and motogen, by controlling the stability of its mRNA (Gnarra *et al.*, 1996; Iliopoulos *et al.*, 1996; Semeister *et al.*, 1996). Pause *et al.* (1997) found that VHL–elongin B/C complex bound to Hs-Cul-2, a protein with a similarity to cdc53. The exact function of Hs-Cul-2 is unknown; it may regulate the degradation of proteins involved in the cell cycle.

The intracellular location of pVHL remains uncertain. Conflicting data suggest that pVHL either resides in the cytoplasm (Iliopoulos *et al.*, 1995) or shuttles between nucleus and cytoplasm, depending on cell density (Lee *et al.*, 1996). Kuzmin *et al.* (unpublished) showed that pVHL is located in the nuclei in a speckled pattern, but translocates to the cytoplasm during mitosis.

In summary, VHL is a unique, small, highly conserved gene encoding a multifunctional protein(s) involved in a number of basic regulatory pathways. The disruption of the ABC elongin complex is an established function of pVHL.

B. Papillary Renal Carcinoma Genes

The MET oncogene was discovered in a classical transformation assay using DNA from a chemically transformed osteosarcoma-derived human cell line (Cooper *et al.*, 1984). The cloned gene was a hybrid involving 5′ sequences of the TPR gene from chromosome 1 and sequences belonging to a novel gene mapped to chromosome 7q31 (Cooper, 1992). Subsequently, this new gene was identified as a transmembrane tyrosine kinase receptor, the receptor for the hepatocyte growth factor (HGF) (National Center for Biotechnology Information, 1997; Cooper, 1992). The gene was classified as a protooncogene by virtue of its amplification and/or overexpression in a large number of epithelial tumors and by demonstrating the oncogenic potential of the truncated intracellular forms of the receptor (Cooper, 1992; Rubin *et al.*, 1993). The MET protooncogene belongs to the large family of receptor protein tyrosine kinases that are of pivotal importance for the transmission

of developmental cues during development, cell proliferation, and tissue morphogenesis. The MET subfamily of receptor tyrosine kinases includes also protooncogenes RON and SEA. They share high homology in the cytoplasmic kinase domain and around the tyrosine residues involved in the multifunctional docking site. In the extracellular part they share similar cysteine-rich domains and a canonical consensus sequence for protease cleavage.

MET is located on chromosome 7q31.2–q31.3 in the genetic interval 125–126 cM between markers D7S486 and D7S2487. The coding sequence of 1390 aa (accession number 125484) consists of 21 exons (Duh *et al.*, 1997) spread over a genomic region of approximately 110 kb and contains a typical signal sequence of 25 aa (residues 1–25), an extracellular domain (residues 25–932), and a transmembrane domain (residues 933–955), followed by the cytoplasmic domain (956–1390). This precursor of 170 kDa is heavily glycosylated and proteolytically cut into two subunits at residue 307; the smaller 50-kDa subunit remains extracellular and bound to the larger subunit by disulfide bonds. The kinase domain (residues 1078–1345) contains two specific tyrosine phosphorylation sites at Y1234 and Y1235 that activate the receptor. Two downstream tyrosines, Y1349 and Y1356, are involved in the multifunctional docking site, which after phosphorylation mediates the interactions with multiple SH_2-containing signal transducers connecting to different signal transduction pathways. Ligand (hepatocyte growth factor) binding to MET induces dimerization and activation of tyrosine kinase activity, leading to autophosphorylation of the catalytic kinase and the docking domains. The gene is expressed as a single mRNA in tissues of epithelial origin, most highly in liver and intestine, and also in microglia, endothelial cells, monocytes, and hematopoietic precursors. The minimal promoter is GC rich and contains binding sites for transcriptional factors AP2 and PEA3, which are regulated by PKC.

MET is oncogenic either by virtue of amplification or constitutive activation of the kinase activity by mutations (see above) or truncation of the extracellular domain. Trisomy or tetrasomy 7 was observed in a variety of human tumors as well as the truncated fusion protein tpr-met. The discovery of germ-line distinct activating mutations of MET in HPRC (Schmidt *et al.*, 1997) has demonstrated that MET is the cancer-causing gene for HPRC, a subset of sporadic papillary renal carcinomas, and may be also involved in the development of a wide spectrum of carcinomas. It is tempting to suggest that tumors with tetrasomy or trisomy 7 contain an activated mutated MET or overexpress simultaneously the ligand along with the receptor (Olivero *et al.*, 1996).

Specific chromosomal translocations are frequent in human tumors. In hematopoietic malignancies they often involve members of the bHLH transcription factor gene family, resulting in deregulation or ectopic expression of the particular gene, converting it into a powerful oncogene. In contrast, in soft tissue sarcomas the translocations result in fusion of two different

transcription factors, one donating a transcriptional activation domain and the other a DNA binding domain. Translocations involving chromosome Xp11.2 band were found in sporadic papillary renal carcinomas of both sexes. Recently the genes involved in the t(X;1)(p11.2;q21.2) translocation were identified by molecular cloning (Sidhar *et al.*, 1996; Weterman *et al.*, 1996a,b). The chromosome Xp11.2 gene, TFE3, is a member of the bHLH family of transcription factors and became fused with the N-terminal region of a novel gene on 1q21.2, termed PRCC, that supplies a proline-rich domain. This fusion probably follows the classical pattern because proline-rich regions were found in many transcriptional activators (Seipel *et al.*, 1992). Another interesting feature of these translocations is the removal of the normal TFE3 transcript, leaving the dominantly acting fusion protein alone. This is important because in kidney cells an isoform of the TFE3 gene is expressed that has a dominant negative effect on its activity.

In summary, MET and TFE3 activated by mutations or fusion comprise a pair of oncogenes that are involved in the pathogenesis of papillary renal carcinomas.

C. Tuberous Sclerosis Gene

Tuberous sclerosis is an autosomal dominant multisystem disorder characterized by the widespread development of tumors classified as hamartomas. The disease loci TSC1 and TSC2 were assigned to chromosomes 9q and 16p, respectively, by linkage analysis and the 16p gene was cloned (European Chromosome 16 Tuberous Sclerosis Consortium, 1993). No phenotypic differences have been observed between TSC linked to TSC1 and TSC linked to TSC2. Knudson and co-workers have identified the rat homolog of TSC2 as the cancer-causing gene for the hereditary renal carcinoma in the Eker rat (Yeung *et al.*, 1994). The TSC2 was shown to be a tumor suppressor gene by virtue of inactivating mutations in germ line and tumors and by suppression of tumorigenicity by the wild-type gene transfected into Eker renal carcinoma cells (Jin *et al.*, 1996). The TSC2 gene is located in 16p13.3 very close to the PKD1 gene between D16S309 and D16S291. The 5.5-kb TSC2 transcript covers a genomic region of about 45 kb (European Consortium, 1993) and is widely expressed (Wienecke *et al.*, 1997).

Multiple splice variants of TSC2 that are differentially expressed in a tissue-specific manner were described and provide a possible explanation of the organ-specific alterations caused by TSC2 mutations. The 198-kDa protein product, tuberin, was sublocalized to the perinuclear compartment consistent with the Golgi apparatus localization (Wienecke *et al.*, 1996). TSC2 showed a region of homology to the GTPase-activating protein rap1GAP (GAP3). Tuberin itself showed GAP activity toward the RAS-related RAP1 protein consistent with a tumor suppressor function by analogy with other

tumor suppressor genes (Xiao *et al.*, 1997). In summary, TSC2 is a tumor suppressor gene causing the chromophobe renal carcinomas in Eker rats.

VIII. RECOGNITION OF FAMILIES WITH INHERITED KIDNEY CANCER

Detecting individuals with inherited carcinomas of the kidney is important for the affected individuals, for members of the affected individual's family, and for future progress in the genetic basis of human renal cancer. Detecting individuals with inherited carcinomas of the kidney is difficult because of the historic tendency to lump all histologic types of renal carcinoma together under the label "adenocarcinoma of the kidney," the lack of a widely accepted system of pathologic classification of renal tumors, the rarity of these disorders, the low penetrance of some of the responsible genes, and the neglect of the family history. The development of a new pathologic system for renal tumor classification (Kovacs *et al.*, 1997) and the recognition that many types of kidney cancer may be a manifestation of an inherited illness may lead to an increased awareness of these entities.

The presence of bilateral renal neoplasms, particularly bilateral multiple renal neoplasms, should alert the physician to the possibility of an inherited susceptibility. Although multiple tumors within one kidney may be a manifestation of intrarenal metastasis, and one tumor in each kidney may reflect a primary tumor and distant metastases, the presence of multiple tumors in both kidneys is a strong indication of an inherited susceptibility to renal malignancy. A family history of renal neoplasia should also alert the physician to the possibility of an inherited susceptibility.

Usually patients are unaware of the histologic appearance of their renal malignancy. Even when pathology reports are consulted, the histologic type of renal malignancy may not be stated clearly—again, because of the historic tendency to lump all histologic types of renal carcinoma together. To pursue the diagnosis of an inherited renal malignancy, it is necessary for the physician to review the histologic slides obtained from the renal tumors of affected family members in consultation with a specialist in renal tumor pathology.

IX. TOWARD A GENETIC CLASSIFICATION OF RENAL TUMORS

With the realization that adenocarcinomas of the kidney are a group of malignancies caused by mutation in different genes, it seems appropriate to

use genetic techniques to supplement traditional pathologic methods of classication. The genetic techniques are of two types: methods that detect mutations in specific genes that predispose to renal malignancy, and methods that detect gross chromosomal changes (chromosomal gains or losses) that accompany renal neoplasia. With currently available technology, detection of mutations in the VHL and MET genes is time consuming and expensive. However, these methods may prove useful in patients suspected of having an inherited susceptibility for renal malignancy. Testing for germ-line mutations in the VHL gene is appropriate for patients with bilateral, multiple clear cell renal carcinomas. Testing for germ-line mutations in the MET protooncogene is appropriate for patients with bilateral, multiple papillary renal carcinomas.

Simpler to perform than mutation assays are assays that measure gross chromosomal alterations. Such methods include loss-of-heterozygosity assays, comparative genomic hybridization, and fluorescence *in situ* hybridization. These methods may prove most suitable for the analysis of sporadic renal malignancies. Bugert and co-workers (Bugert and Kovacs, 1996; Bugert *et al.,* 1997) developed a panel of polymorphic DNA markers and a sequential analytic procedure for rapid detection of chromosomal changes in renal carcinomas. This panel was devised to permit investigators without a background in cytogenetics to identify rapidly chromosomal changes that occur in renal carcinomas. This panel of polymorphic DNA markers permits investigators to classify renal carcinomas in terms of the gross chromosomal changes that accompany the various types of renal neoplasia (Steiner and Sidransky, 1996). It will be important to correlate the results obtained using the methods developed by Bugert and Kovacs with the biologic behavior of renal neoplasms, particularly clinical stage and prognosis.

X. CONCLUSIONS

Family studies afford extremely powerful tools to understand the genetic basis of human renal neoplasia. Two genes that predispose to renal carcinoma, the VHL tumor suppressor gene and the MET protooncogene, have been identified by family studies. Mutations in the VHL gene predispose to clear cell renal carcinomas. Mutations in the MET protooncogene predispose to papillary renal carcinomas. The TFE3 and PPRC genes, identified as somatic changes in papillary renal carcinomas, are the subject of intensive investigation. Of particular interest is the frequency of mutation of these genes in sporadic papillary renal carcinoma, and whether mutation of these genes is specific for a morphologic subtype of papillary renal carcinoma. The TSC2 gene plays a role in the pathogenesis of renal carcinoma in the Eker rat; its

role in the pathogenesis of human renal carcinoma is the subject of active investigation.

The genetic analysis of renal tumors may disclose heretofore unrecognized levels of genetic heterogeneity. The genetic studies of papillary (chromophilic) renal carcinoma are an example of the complexity of the genetic etiology of human renal carcinomas. Somatic mutations of either the MET protooncogene or the TFE3/PPRC genes can lead to sporadic papillary renal carcinomas. Until this genetic etiology of renal tumors is completely understood, it will be difficult to devise rational treatments and to estimate prognosis.

The existence of families with non-VHL, nonpapillary renal carcinoma, and the families with hereditary renal oncocytomas, suggests that there are other genes in addition to VHL and the MET protooncogene that predispose to renal neoplasia; these remain to be identified. Ten years ago (Zbar *et al.*, 1987) the suggestion that there was a renal carcinoma gene was a speculation. Today, it seems possible, that there may be at least one predisposing gene for each histologically distinct form of renal carcinoma. The consistent cytogenetic changes found in clear cell renal carcinomas and papillary renal carcinomas suggest that there are multiple genes involved in the progression of these neoplasms that remain to be discovered. Genetic research on the basis of human renal carcinomas is a fertile area of investigation that continues to benefit from the systematic identification and study of families with renal neoplasia.

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The Labyrinthine Ways of Cancer Immunotherapy—T Cell, Tumor Cell Encounter: "How Do I Lose Thee? Let Me Count the Ways"

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XVII. CODA-2: Sample Flow Chart of an Arborizing Clinical Trial
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I. INTRODUCTION

The single mutation ($V_3 \rightarrow S_3$ in the word "love") makes the metaphrase of Elizabeth Barrett Browning's opening line of her 43rd Sonnet from the Portuguese relevant and allows us to compare some of the restlessness of her poetry with the insecurities that confront us, in current cancer immunology. These arise from the burgeoning number of ways, now being recognized, by which metastatic tumor cells may escape immune killing. Examples of some of these ways were raised at the Tumour Immunology Workshop, held recently, as a satellite of the 15th International Papilloma Virus Workshop, on Daydream Island in the beautiful Whitsunday Passage of Queensland's Great Barrier Reef, Australia.

At least three categories of very different major mechanisms of immune escape can be identified: (1) loss or absence of targetable surface epitopes, (2) synthesis by the tumor cell of surface, shed, or secreted molecules capable of inactivating a cytotoxic response, and (3) defective capillary neovasculature of malignant tissue that fails to facilitate cytotoxic cell egress into the cellular substance of a metastasis. Examples from any or all categories could conceivably appear in a single patient during the cellular evolution that occurs with progression of malignancies and will contribute to the heterogeneous behavior of metastases. The randomness of the mutational processes involved in the evolution of a malignancy will ensure heterogeneity both within a patient's metastases and among patients' tumors of the same lineage. An immune response may cause the majority of metastatic deposits to regress, but the response will be recorded as only partial because of progression of clonal variants that evade the immune reaction (rogue metastases). This situation is more usefully viewed as an anticipatable problem requiring special adjunct therapy, rather than as a failure of the particular immunogenic strategy. This broad and necessarily cursory review is intended as an airing of the difficulties posed by highly evolved malignant disease, which mandates a many-faceted approach to immunotherapy if we are earnest in our attempts to cure later stage cancer.

The importance of defining the ways tumor cells escape from the effector arm of the immune response is twofold. Through an understanding of the mechanisms, appropriate adjunct procedures may be designed to overcome the particular ploy or ploys in use by a particular patient's disease, or rogue metastases. Second, a different, more complex approach to conducting and evaluating clinical trials of immunotherapeutic strategies will become necessary to accommodate diagnosis of the escape route occurring in a few rogue

metastases or even of nonresponsive total disease and thereby open the array of available, retaliatory, adjunct procedures.

A redirected approach to clinical trials should help address what Osband and Ross (1990) have identified as the anecdotal nature of clinical studies of immunotherapy. The interplay of a large number of hereditary variables, of randomly acquired tumor cell abnormalities, and of myriad multicellular responses in the immune system to differing, immunogenic stimuli may require jigsaw-like puzzles to be assembled for each patient to complete their immunotherapeutic program and outcomes.

The issues posed by the problem, in perhaps the majority of patients, of the heterogeneity of vulnerability of metastatic deposits to a particular immune response evoked by a vaccine of restricted antigenic possibilities, seem clear. Not only do significant amounts of bulk disease need an extensive series of applications of a particular vaccine, but recurrent or rogue metastases may require a new vaccine to accommodate antigenic variation occurring during tumor progression. A pragmatic view for modification of clinical trial form will be offered after the problems have been reviewed.

To reduce the complexity of the argument it is assumed that the immune rejection of tumor is a cell-mediated reaction involving recognition of T cell epitopes, and that the form of the tumor-associated antigens (TAAs) acquired by the professional antigen-presenting cells (APCs)—from epitopic peptide to native protein—does not make a qualitative difference to the antitumor response. The dosage of antigen, the type of adjuvant, the frequency and site of vaccination, etc., can all influence the nature and persistence of the immune response, but these are not the focus of this review.

II. CANCER AS A MOVING TARGET

> "Always moving as the restless spheres" (Christopher Marlowe, *Conquests of Tamburlaine*)

The process of selection and expansion of mutant clones that occurs during progression of human malignant disease leads to a wide range of random mutational, recombinational, deletional, and regulatory gene changes. The positive view of these variations is that neoantigens may be produced, in two ways:

1. The up-regulation of lineage-specific markers to immunologically "visible" levels (e.g., differentiation antigens) (Rosenberg, 1996; Van den Eynde and Brichard, 1995; Boon and van der Bruggen, 1996).
2. The occurrence of nonlethal mutations in widely expressed ("housekeeping") proteins (reviewed in Van den Eynde and Brichard, 1995; Boon and van der Bruggen, 1996).

Both of these events may provide new TAAs for new or different T cell clones to recognize and engage, after appropriate activation. Together they make a strong case for the use of autologous tumor cell vaccines (rather than defined antigenic peptides) to ensure the recognition of unknown, but potentially targetable, TAAs generated by random mutational processes (e.g., Sahin *et al.*, 1995).

The negative side of this genetic plasticity relates to three complex aspects:

1. Tumor epitope loss
   a. The tumor cells may down-regulate the synthesis of useful TAA epitopes and escape immune surveillance or
   b. they may down-regulate HLA antigens (especially HLA-B) or
   c. down-regulate β_2-microglobulin, thereby inactivating HLA class I antigen presentation, or
   d. down-regulate specific TAA epitope transport via TAP-1, -2 proteins and thus fail to exhibit potentially targetable epitopes on the tumor cell surface.
2. Immunosuppressive tumor products
   a. With more challenging consequences for therapy, tumor cell variants may up-regulate the expression of T cell immunosuppressive cytokines (such as IL-10 and TGF-β), which may paralyze either the activation or effector limbs of the antitumor response, or
   b. up-regulate the expression of antiapoptotic agents such as FLIP to protect the tumor cells from cell-mediated cytolysis.
   c. They may express surface molecules such as TNF-α or FasL and kill activated tumor-reactive cytotoxic T lymphocytes (trCTLs).
   d. The high levels of trCTL precursors (trCTLp), augmented in peripheral blood after each vaccination with modified autologous tumor cells, are maintained for only 3–4 weeks postvaccination. It has not been determined whether this decline in trCTLp is due to
   e. "Karoushi" (death by overwork) (Glickstein and Huber, 1995) or
   f. presently poorly understood CTL regulatory mechanisms but including FLIP modulation or
   g. the induction of anergy during ingress of tumor-infiltrating lymphocytes (TILs) or
   h. T cell apoptosis induced by tumor products.
3. Defective tumor capillaries
   The capillary endothelium of the intratumoral neovasculature is frequently defective, particularly in presenting the homing signals that facilitate the massive trCTL diapedesis into tumor tissue, which is presumed to be necessary for T cell killing of the malignant cells.

It must be emphasized that our current understanding of the apparently serial changes observed during tumor progression, typified by the orderly

steps best codified for adenocarcinoma of the colon (Vogelstein *et al.*, 1988), are randomly acquired genetic changes that become dominant as a tumor characteristic only if they offer a selective growth or survival advantage over the rest of the normal or altered cell population (*vide infra*).

Directionality of phenotypic expression of the randomly acquired genotypic changes is seen to result from the need for a particular change to be manifest by its dependence on the presence of some other change(s). Thus, autonomous growth due to an autocrine growth factor is possible only when its specific receptor has been expressed. This may occur previous or subsequent to another change, such as the activation of the protease necessary for release of the growth factor if, like TGF-α, it is present as a membrane bound pre-pro form. Similarly, autonomous cell replication must be present before local invasion of surrounding tissue can take place as a result of tissue matrix protease activity. The pathological description will read as adenomatous clonal growth → extracellular matrix lysis → invasion of adjacent tissue, even though the principal mediators may have been expressed in the reverse order.

III. GENOMIC INSTABILITY AND THE MUTATOR PHENOTYPE

> "How Mutability in them doth play/Her cruel sports to many men's decay" (Edmund Spenser, *The Faerie Queene*)

The notion that the development of tumors represents a form of somatic evolution has a century-old tradition, which has been exaggerated to the extent of Huxley's proposal that every tumor could be regarded as a new species (Huxley, 1958). A central view of the mechanism for the evolution of a tumor from a normal cell is that random mutational changes in cells may very rarely result in a cell acquiring a new characteristic, which allows it to multiply with a competitive advantage over its lineage cohorts and expand into an abnormal clone (Nowell, 1976). Other cellular functions may similarly undergo random change in a direction toward any of the many phenotypic characteristics that delineate the full-blown malignant cell. Some of these aberrations will lead to further clonal expansion into new tissue areas due to loss of the normal responses to the constraints of its original milieu (Loeb, 1991). The random order of the acquisition of mutations ensures, among patients, variability of the speed with which the initial clones of abnormally accumulating cells progressively gain morphological and functional markers associated with progression toward malignancy.

It is thought that a major selection mechanism to prevent the development of lethal tumors is immunological surveillance of all cells for the presence of

neoantigens (potential TAAs), which can become the targets for effector cell-mediated killing (Doherty *et al.*, 1984; Ioachim, 1990; Kripke, 1988). On the other hand there are some observations that have been marshalled to question this anticancer role proposed for immune surveillance (Prehn, 1994). TAAs may be mutational in origin or result from overexpression of lineage-specific proteins (*vide supra*) or from rekindling of the expression of embryonic proteins such as CEA (Tsang *et al.*, 1995), BAGE, GAGE (Boel *et al.*, 1995), and MAGE-1 and -3 (Gaugler *et al.*, 1994). There is as yet no inventory of the proportion of each type of mechanism whereby these changes have occurred, even in the best studied tumors, but a brief discussion of those that occupy the current vision of research in this area follows.

There is currently controversy as to whether the driving force in tumor progression is an increase in the rate at which mutations can occur in preneoplastic or tumor cells via the mutator phenotype of the tumor cells (Loeb, 1991, 1994; Parshad *et al.*, 1996), or is mainly attributable to selection and clonal expansion, or, irenically, variable dominance of one mechanism in the presence of the other. Melanoma has a long record of documented genetic deletions as an example of the consequences of frequent mutations (e.g., Dracopoli *et al.*, 1985). Tomlinson *et al.* (1996) using various computer simulations of tumor progression—based on a model from colorectal cancer—argue "that selection without increased mutation rates is sufficient to explain the evolution of tumours," a view previously entertained by Loeb (1991) but later considered less likely by him (Loeb, 1994) [e.g., up to 15% of all sporadic colorectal cancers have been found to be due to inactivation of mismatch repair (Liu *et al.*, 1995, Kinzler and Vogelstein, 1996)]. Tomlinson *et al.* (1996) concede that some tumors do acquire a mutator phenotype, but maintain that their modeling shows that this is not a sine qua non of carcinogenesis. The argument only exists until the data on the frequency of mutator phenotypes for individual tumors have been determined. The importance of the existence of mutator phenotypes in the context of this review lies in the frequency with which tumor cells may down-regulate, delete, or otherwise change TAA expression so that they become invisible to the specific effector cells of the immune system. Rapid changes in dominant antigens is a well-known strategy of parasite evasion of immune protection mechanisms (Cross, 1990), and is frequently effected by specific mutational and recombinational events [akin to the generation of T cell receptor (TCR) or immunoglobulin rearrangements] in a surface or shed/secreted, immunodominant protein, to provide a smoke screen for the parasite as a "moving target."

Until recently the hypothesis that the mutator phenotype was the significant early change in cancer cells had few data to support it (Loeb, 1991, 1994). The discovery of ubiquitous mutations in repeat sequences (microsatellites) in colonic cancers in hereditary, nonpolyposis, colorectal cancer (HNPCC) (reviewed in Kinzler and Vogelstein, 1996) has been shown to

be a manifestation of defective mismatch repair genes causing accelerated accumulation of mutations and progression to malignancy in the few sporadic adenomata that occur in these patients. This makes a sharp contrast to the slower progression in the myriad adenomata of familial adenomatous polyposis (FAP) patients, which have normal mismatch repair (Lynch *et al.*, 1996; Reitmair *et al.*, 1996). Mutations attributable to defective DNA mismatch repair have been found in a variety of replication-error-positive gastrointestinal tumors in coding regions of the IGFIIR and TGF-β1RII genes (Souza *et al.*, 1996). They have also been found in noncoding nucleotide repeats in some 30% of breast cancers. This microsatellite instability correlates with poor disease prognosis (Paulson *et al.*, 1996). Microsatellite instability has been proposed as a marker of a mutator phenotype in cancer (Loeb, 1994), and has been associated with defects of DNA mismatch repair (Peltomaki *et al.*, 1993; Kinzler and Vogelstein, 1996).

In addition to genetic, mutation-based changes resulting in tumor cell evolution, it is now being recognized that epigenetic phenomena may contribute to the genetic instability/plasticity that is seen to underly tumor progression (e.g., Klein and Costa, 1997a,b; Zingg and Jones, 1997; Usmani 1993). In particular, the occurrence of hypo- and hypermethylation of cytosine in CpG dinucleotides in tumor DNA is a focus of much contemporary interest and research to explain, and perhaps offer intervention strategies for alteration of epigenetic gene activation or silencing, respectively (Klein and Costa, 1997a). Aberrant cytosine methylation can have both mutagenic as well as epigenetic effects on the expression of normal genes and is, therefore, especially relevant to lineage-specific protein expression (and thus an important set of potential targets for immunotherapeutic strategies) as well as to the regulation of genes of more general purpose concerned with cell replication, cell mobility, cell adhesion, and intercellular communication, etc., which contribute to tumor progression and escape from immune surveillance (Zingg and Jones, 1997). A set of papers in a special issue of *Mutation Research* (Klein and Costa, 1997a,b) offers an excellent overview of the current status of DNA methylation and its aberrations in both somatic and heritable changes in gene expression in cancer and other diseases.

The formation of 5-methylcytosine by methylation of the C-5 position in cytosines, only within CpG dinucleotides, is effected by DNA (cytosine-5) methyl-transferase (Mtase) using 5-adenosyl methionine as methyl donor. This is a unique epigenetic modification of DNA in vertebrates and is essential for normal embryonic development (Gonzalgo and Jones, 1997; Turker and Bestor, 1997). CpG dinucleotides are underrepresented in the mammalian genome except in areas constituting about 1%, thereof, which are termed CpG islands, ~0.5–1 kb long, which tend to be associated with the *5′* end of housekeeping genes. Hypermethylation of CpG islands is associated with silencing gene transcription and occurs only in normal cells on inactive genes in the X chromosome and in parentally imprinted genes, where-

as hypomethylation predisposes to gene activity. Abnormally high DNA methylation of the murine major urinary protein (MUP) family genes accompanying reduction in the transcriptional and phenotypic expression of the group 1 MUP proteins in livers of adult animals, which were generated as nuclear–cytoplasmic hybrids (created by *in vitro* nuclear transplantation into eggs of different genotype), testifies to the stability, through a series of mitoses, of the epigenetic changes in gene transcription (Reik *et al.*, 1993). Systematic breeding experiments showed that more than 50% of the first, backcross generation male progeny also showed increased *Mup* methylation and reduced hepatic MUP levels—the data being interpreted as the first demonstration of epigenetic inheritance of specific alterations of gene expression (Roemer *et al.*, 1997)—testifying to the stability of the epigenetic (DNA methylation) changes through meiosis and embryogenesis. During early development gamete-specific DNA methylation patterns are converted to somatic cell-specific methylation patterns via poorly understood waves of demethylation and *de novo* methylation (Turker and Bestor, 1997). The mechanism of the establishment, at the time of implantation, of tissue-specific methylation patterns with subsequent developmental modifications is obscure at present, but is clearly of immense importance to our understanding of the variation in gene methylation and expression that is seen in cancer (Bestor and Tycko, 1996).

Mutations in the tumor suppressor protein p53 are found in approximately one-half of solid tumors. One-quarter of these point mutations are C → T transitions at CpG dinucleotides (Greenblatt *et al.*, 1994) and all of the mutational hot-spots in the p53 gene occurring at CpGs are hypermethylated, suggesting involvement of 5-mCyt as an endogenous mutagen (Gonzalgo and Jones, 1997). Mtase may facilitate cytosine deamination and thus contribute to high rates of mutation. Limiting supplies of the methyl donor may also contribute to these mutations (e.g., Laird and Jaenisch, 1994). Recognition of mutant epitopes in p53 by CTLs has been mooted in cancer patients possibly to carry a positive benefit of the methylation/mutation process in some cancers toward an antitumor immune response (Melief and Kast, 1995). The frequency of endogenous 5-mCyt deamination of CpG (yielding C → T transitions) is estimated to be highest in cancers of the bladder, breast, and colon, whereas lung cancers have a high proportion of transversions (pyrimidine ⇌ purine), anticipated from the role of exogenous carcinogens in the latter (Greenblatt *et al.*, 1994). Variable DNA methylation occurring in cancer has been correlated with colon cancer in the context of mismatch repair within microsatellites (Aaltonen *et al.*, 1993) and is at the seat of the reduction of precancerous polyps of the colon by treatment of Mtase heterozygotic mice versus APC mutant mice with 5-aza-2′-deoxycytidine (5-aza-CdR) (Laird *et al.*, 1995). The nucleotide analog 5-aza-CdR, a covalent inhibitor of Mtase (Riggs and Jones, 1983), can cause hypometh-

ylation and gene activation in genes with hypermethylated sites. Clearly a much greater understanding of how *de novo* methylation activity is directed at any given gene promoter to down-regulate tumor suppressors, or of recognized epitope-carrying proteins, or how failure of maintenance methylation to allow up-regulation of genes whose products may further tumor progression or inhibit an immune response, will be needed before intervention strategies to counter the abnormal DNA methylation events can be devised. Different malignancies appear to have different changes in global and specific methylation densities, some of which were reviewed by Laird and Jaenisch (1994). Much work and exciting data lie ahead to allow the bottom lines in this area to be written.

There is thus a strong and extending case that, in many cancers, faulty DNA repair mechanisms, perhaps due to mutations in one or a few of the 40–50 proteins involved in DNA repair, contribute to a higher than normal mutation rate. Furthermore, with the increasing duration of a tumor with its successive foci of selection and clonal expansion, the likelihood of mutational disturbance of enzymes and proteins concerned with DNA repair becomes increasingly probable. DNA mismatch repair proteins are usually thought to operate in strand-specific transcriptionally mediated DNA repair during S phase. However, randomly templated mismatch repair can occur in nonreplicating mammalian cells, but its actual contribution to mutational progression in quiescent cells remains to be evaluated (MacPhee, 1995, 1996). The actual proportions of each cancer type that are mutators will undoubtedly be established in the near future and such information will be most important in analyzing the basis for the resistance of various subpopulations of cancers to individual immunotherapeutic strategies.

IV. INDUCTION OF AN ANTITUMOR IMMUNE RESPONSE IS NOT THE MAJOR PROBLEM

There is an abundance of experimental and clinical evidence that a variety of immunization procedures are capable of inducing a specific antitumor cellular immune response (Berd *et al.*, 1991; Barth *et al.*, 1994; Herr *et al.*, 1994; Ellem *et al.*, 1997; Boon *et al.*, 1994; Dranoff *et al.*, 1993; Schmidt *et al.*, 1996). This response is characterized by immunity to challenge with a normally lethal injection of the experimental tumor in mice, or, in patients, development of a delayed-type hypersensitivity (DTH) reaction using an irradiated tumor cell suspension as antigen, augmentation of trCTL or mixed lymphocyte tumor cell reactivity in cells from a patient's peripheral blood, specific cytokine release by tumor-reactive lymphocytes, and ultimately, tumor regression. In murine models using histocompatible, transplantable tu-

mors, nonimmunogenic tumors can be rendered immunogenic by gene transfer to force cytokine secretion by irradiated, transduced cells of the tumor, with GM-CSF performing best of some 30 cytokine or cytokine-related molecules (Dranoff *et al.*, 1993; G. Dranoff, personal communication). Other cytokines may be as effective when control of their concentration produced at the vaccination site is carefully regulated, e.g., IL-2 has only a restricted range for successful immunization against specific tumor challenge (Schmidt *et al.*, 1996), the reasons for which are not understood at present.

The concept that the mechanism of GM-CSF-driven immunity is reliant on its requirement for the activation of the most effective antigen-presenting cell (APC)—the dendritic cell (Guery and Adorini, 1995)—has led to vaccination with activated dendritic cells preloaded with tumor antigens (e.g., Grabbe *et al.*, 1995; Mayordomo *et al.*, 1995; Young and Inaba, 1996). An industry has developed to identify new antigens for each type of malignancy in the hope of discovering useful, lineage specific, tumor antigens. These could contribute to the base of rapidly produced, cheaper vaccines, in spite of the difficulty posed by the individual requirement for T cell epitope mapping in the context of the heterogeneity of HLA types in outbred human populations. In human trials vaccination with chemically mutated, autologous tumor cells alone produced a wave of increase in trCTLp and of antitumor bulk peripheral blood lymphocyte (PBL) killing in peripheral blood (Herr *et al.*, 1994; Ellem *et al.*, 1997), which is, however, short-lived whether in the presence (Ellem *et al.*, 1997) or absence (Herr *et al.*, 1994) of a significant tumor burden. A combination of allogeneic melanoma cell lines, or their shed proteins, which express a range of melanoma antigens, together with an adjuvant such as BCG (Morton *et al.*, 1992; Bystryn, 1995) or alum (Bystryn, 1995), induce DTH and mixed lymphocyte reactions to melanoma antigens in the majority of patients so treated (Barth *et al.*, 1994), and a significant number of patients show at least a heterogeneous response in their metastatic disease (Morton *et al.*, 1992; Bystryn, 1995).

V. IMMUNE BASIS FOR THE "SECOND-ORDER BYSTANDER EFFECT"

Further evidence for the ubiquity of mechanisms for stimulating an immune response against autologous tumor has been the recent recognition that an immune bystander effect accompanies several strategies that kill a subpopulation of the tumor cells in a metastasis. Thus, partial transduction of tumor cell populations with the herpes simplex thymidine kinase gene not only enables the phosphorylation of the drug gancyclovir to kill the trans-

duced cells, but it is found that surrounding, nontransduced cells also perish by cell–cell transfer of the toxic metabolites both *in vitro* (Kuriyama *et al.*, 1995) and *in vivo* (Freeman *et al.*, 1993; Caruso *et al.*, 1993)—the first-order bystander effect. Further analysis of this system, *in vivo*, has shown that this "suicide gene" treatment of brain tumors and metastatic melanoma, in mice, is followed by specific systemic immunity to challenge with cells of the targeted tumor (Vile *et al.*, 1994; Barba *et al.*, 1994), as occurred following the use of cytosine deaminase as a "suicide gene" prior to the administration of 5-fluorocytosine (Mullen *et al.*, 1994). This immune component contributes to bystander killing with the development of the systemic immune response and is thus a second-order bystander effect. Induction of the immune response is attributed to the release of cytokines at the site of cell necrosis or apoptosis following drug-mediated suicide, which leads to hemorrhagic necrosis and enhanced inflammatory cell traffic, including APCs, with subsequent stimulation of an antitumor immune response (Freeman *et al.*, 1997). Similar bystander effects have been shown to accompany local induction of a strong hypersensitivity response at the site of melanoma vaccination by using hapten-coupled, autologous melanoma cells in patients who had been presensitized to that hapten (Berd *et al.*, 1991). Distant metastases also became inflamed, some showing regression, even though the haptenized epitopes were not present in them.

Yet another approach has been to initiate an analog of allograft rejection of metastases by injecting into them a preparation of a DNA plasmid containing a gene encoding a foreign HLA antigen (HLA-B7). The resultant gene transfer into some of the metastatic cells provoked a cytotoxic T cell reaction that led to the killing not only of the transduced cells, but also of the other cells in the injected metastasis in one patient (of 10), and inhibition of growth in another. Tumor-infiltrating lymphocytes (TILs) from the regressing metastasis of the former patient, after expansion *in vitro*, caused complete remission of distant metastatic disease when infused in an adjunct adoptive transfer strategy (Nabel *et al.*, 1996). Clearly a second-order bystander effect from a specific CTL reaction against a "foreign antigen" was produced through neighborhood engagement in a vigorous immune disturbance. Again, by exploring the ramifications of antitumor immunity following viral infection or transfection with a viral gene, Schmidt *et al.*, (1996) found that just loading tumor cells with a viral peptide (from influenza haemagglutinin), containing an MHC class I-compatible motif, was capable of inducing immunity to challenge with the same tumor (vaccination) and of preventing small loads of preexisting tumor from growing (theraccination). The peptide required a special vehicle for effective cell loading ("transloading") and the antitumor efficacy was dose dependent. Because the antigenic peptide was foreign but the immunity was tested with unmodified tumor, this

is clearly a "second-order bystander effect" type of immunity, the establishment of which was shown to be $CD4^+$ and $CD8^+$ T cell dependent (Schmidt *et al.*, 1996).

It is of interest to recall that the bystander effect was probably responsible for some older observations of induced tumor regression. The sporadic occurrence of local regressions following intralesional injections of a variety of immunological adjuvants has yet to match the effectiveness noted in the first reports, of what may now be interpreted as "bystander effects," more than a century ago. Coley, in 1891, made his astute observations on the regression of local and, in some cases, distant tumors of a spectrum of carcinomata and sarcomata, in a high proportion of patients who experienced cellulitis in the form of erysipelas involving the tumor (Coley, 1891, 1893). More recently, complete elimination of murine tumors has been found to follow ischemic necrosis of tumors induced by ligation of their blood supply (Foley, 1953; Baldwin, 1955) or by the vessel thrombosis following the administration of endotoxin or TNF-α, despite a remnant shell of viable tumor cells. This is due to an immune reaction stimulated by the inflammatory response in the necrotic tumor tissue, causing the elimination of the residual malignant cells (Havell *et al.*, 1988; North and Havell, 1988), and is another example of secondary bystander effects.

VI. THE VANISHING TARGET

> "Vanished quite slowly, beginning with the end of the tail, and ending with the grin" (the Cheshire cat, Charles Lutwidge Dodgson, *Alice in Wonderland*)

The vanishing target in the case of malignancies avoiding immune surveillance is a well-documented and probably common route for escape from immune destruction. Because specific recognition by activated T lymphocytes is seen as the initial step in alerting an effector response to tumor cells, presentation of an epitope derived from a TAA, bound firmly in the groove of an appropriate member of the HLA class I complex, is the necessary trigger for the release of perforin/granzyme B or for FasL barrage together with release of the cytokines (IL-2, IFN-γ, TNF, GM-CSF) for recruitment of the troops to eliminate the tumor cells.

Review of the data available in 1993 (Garrido *et al.*, 1993) on HLA class I antigen loss from tumors indicates that benign and premalignant lesions in the epithelia of breast, colon, cervix uteri, and larynx were HLA class I^+, as were virtually all *in situ* carcinomata and severely dysplastic colon adenomata. Depending on the number of antibody reagents available to define individual HLA-A or -B locus products and individual HLA alleles, frequencies of loss were 16 to 29% in laryngeal, colorectal, and gastric carcinomata,

with 29 and 38% being recorded for lung cancer and basal cell carcinoma (BCC), all being considered to be underestimates. A minimum of 40% loss of some component of HLA class I was estimated in these malignancies. The dependence of HLA class I surface expression on its interaction with β_2-microglobulin [β_2M] has led more recently to the recognition that 38.5% of 13 newly established melanoma cell lines lack β_2M and thus functional HLA class I expression, which was confirmed by histochemical staining of tumor biopsies (Restifo *et al.*, 1996). Furthermore, archival pretreatment tumor sections were available for three of five patients receiving immunotherapy and these showed β_2M staining, indicating that progression of the rogue metastases used to establish the tissue cultures after immunotherapy (by adoptive transfer of TILs) occurred through selection of β_2M^- variants. Restoration of class I antigens followed transfection of negative cells with the wild-type β_2M gene (Restifo *et al.*, 1996).

Selection of melanoma antigen-negative variants in two HLA-A2$^+$ patients receiving MelanA/MART-1 and tyrosinase peptide vaccination occurred in rogue metastases that progressed, while others expressing TAAs regressed after specific CTLs were generated (Jäger *et al.*, 1996); no mention of HLA status of regressing versus progressing metastases was made. In the other patients there was an inverse correlation between TAA expression and the presence of CD8$^+$ cytotoxic T cells in peripheral blood lymphocytes (Jäger *et al.*, 1996). Other studies of HLA class I down-regulation in melanoma using mAbs to monomorphic class I determinants found that only 16% of primary but 58% of metastatic lesions were not reactive (e.g., Ferrone and Marincola, 1995). Interestingly, HLA-B antigens are more frequently down-regulated in melanoma (Marincola *et al.*, 1994), although, together with lack of class I expression, these cells become more susceptible to NK cell killing (Versteeg *et al.*, 1989; Maio *et al.*, 1991). The algebraic sum of these disparate influences leads to prognostically unpredictable outcomes (Ferrone and Marincola, 1995). A detailed catalog of altered HLA class I phenotypes in human tumors has been published (Garrido *et al.*, 1997).

Loss of epitope presentation by class I HLA on the surface of a tumor cell can arise from functional deficiency or down-regulated production of the variety of molecules involved: (1) Mutational disruption of the disulfide bridge of the C2 domain of class I antigens prevents their transport from the endoplasmic reticulum to the plasma membrane (Miyazaki *et al.*, 1986). (2) A critical role is played by the transport associated with antigen-processing molecules (TAP-1, TAP-2) in the ATP-dependent translocation from cytoplasm to endoplasmic reticulum (ER) of the short peptides resulting from proteasome activity, which may bind to suitable HLA class I molecules for surface presentation as a potential T cell epitope (Germain, 1994). Provision of peptides by the TAP system is necessary for correct folding of ER-sited class I molecules, bound to the chaperone-like protein calnexin, to form a

stable complex with peptide and β_2M, thus to allow migration to the cell surface to occur (Germain, 1994). Mutation of the TAP-1 gene in humans leads to lymphocyte expression of only 1% of the normal amount of surface HLA class I, and is accompanied by deficient antibacterial defenses (de la Salle *et al.*, 1994). Mice with disrupted TAP-1 genes have very low class I expression and gross deficiency of $CD8^+$ lymphocytes (van Kaer *et al.*, 1992). It can thus be expected that disturbances to TAP-1/2-guided peptide delivery will result in HLA class I down-regulation. A well-documented case of down-regulation of TAP-1 and loss of the immunodominant T cell antigen was found in metastatic melanoma during 6 years of progression. Transduction of the down-regulated melanoma cell line with cDNA of both the TAA (MART-1/MelanA) and the TAP-1 restored their expression and the sensitivity of the cell line to MART-1/MelanA-specific CTL killing (Maeurer *et al.*, 1996).

Interference with TAP activity has been found to occur in 38% of class I-defective cervical carcinomata (Cromme *et al.*, 1994b), and has subsequently been found to be more frequent in the metastases than in the primary tumor (Cromme *et al.*, 1994a). Of the 44.8% non-small-cell and squamous carcinomata of the lung that had lost class I molecules, 60.9% had also lost TAP-1 expression (Korkolopoulou *et al.*, 1996). It is clear that TAP down-regulation is a significant contributor to class I molecule loss and to the immunological anonymity of the many tumors, leading to the cute title "Tap off–tumors on" of a recent summary (Seliger *et al.*, 1997). Importantly, however, HLA class I, β_2M, LMP2 and 7 (proteasome components), and TAP expression can be up-regulated by all types of IFNs (David-Watine *et al.*, 1990; Girdlestone, 1995), with some differences being noted in *in vitro* and *in vivo* systems (Massa *et al.*, 1993). We have found that cell lines derived from melanomata of four patients tested in our GM-CSF-transduced autologous melanoma cell vaccination trial have diminution in the surface density of some HLA antigens (compared to autologous lymphoblastoid cells) and that this is reversible *in vitro* with either IFN-α or IFN-γ, with the latter being significantly more effective at lower concentrations (see Fig. 1).

Fading antigen stimulation following down-regulation of HLA class I molecules or loss of TAAs at the vaccination site, together with the consequent decline in local cytokine production, would be expected to lead to cytokine deprivation of the trCTLs. The consequences of cytokine withdrawal—especially IL-2—on the activated CTLs are reduction of expression of the antiapoptotic proteins Bcl-2 and Bcl-x and increased apoptosis (Akbar and Salmon, 1997; Akbar *et al.*, 1996; Broome *et al.*, 1995; Borthwick *et al.*, 1996). Interestingly, potential cytotoxic tumor-recognizing lymphocytes, which come to reside in tumor metastases (tumor infiltrating lymphocytes) and which are considered anergic, may represent activated trCTLs that have been induced into a quiescent state by salvation rather than damnation.

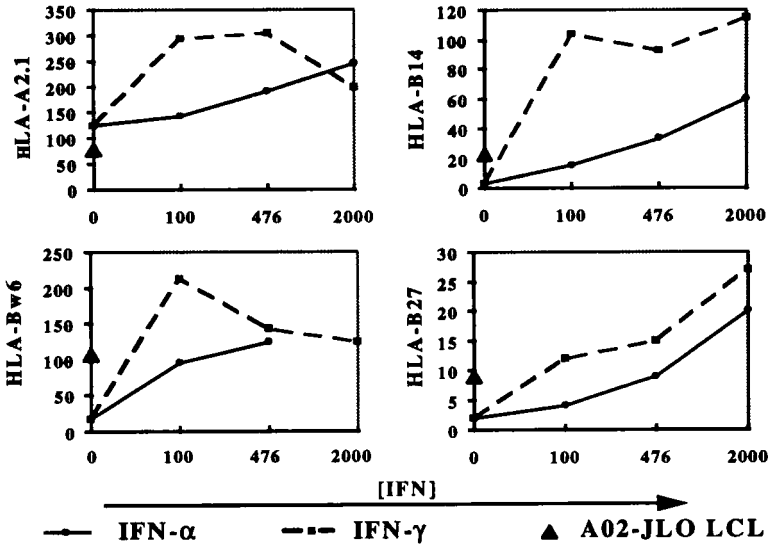

Fig. 1 Response of HLA expression (mean fluorescence intensity, vertical axis) on melanoma cell line from patient A02 to treatment with IFN-α 2b (●) or IFN-γ (■) at the specified doses (units/ml horizontal axis). Cells were treated with IFN for 4 days, harvested and immunofluorescently stained for surface expression of the markers shown using monoclonal antibodies recognizing HLA-A2.1, HLA-B14, HLA-B27, and HLA-Bw6. As a positive control, the expression on the autologous Epstein-Barr virus transformed lymphoblastoid cell line is shown (▲).

Doomed CTLs may be saved from the apoptosis of cytokine deprivation by the stromal factors produced by the normal connective tissue scaffolding (Akbar *et al.*, 1993; Gombert *et al.*, 1996) of an organized tumor mass, resulting in a nonreplicating, quiescent, tumor-reactive, primed T lymphocyte population that is reactivable by IL-2 (Akbar and Salmon, 1997; Gombert *et al.*, 1996), thus fulfilling the criteria of TILs. This represents an alternative mechanism for the induction of CTL anergy to the proposal that IL-10 or other inhibitory cytokine production by the tumor deposit is responsible for the CTL deactivation.

VII. DEFEAT OF THE NK CELL DEFAULT

> "For those whom thou think'st thou dost overthrow/die not, poor death, nor yet can'st thou kill me" (John Donne, *Holy Sonnets*)

Down-regulation of HLA class I antigens by tumor cells is not a risk-free maneuver, however, due to the existence of lymphocytes capable of killing

some types of malignant cells, by nonantigen-dependent means—natural killer (NK) cells (Herberman *et al.*, 1975; Kiessling *et al.*, 1975). In contrast to the MHC-restricted, antigen-specific, effector CTL killing, the large, granular subset of $CD3^-$, TCR^-, $CD16^+$, $CD56^+$ lymphocytes are inhibited from killing by expression of the full complement of syngeneic MHC class I antigens on their potential targets (Kärre *et al.*, 1986) but are actively cytotoxic against cells lacking exhibition of class I. This was codified in Kärre's (1985) "missing self" hypothesis. NK cells are thought to survey body tissues (other than brain) (e.g., Yamasaki *et al.*, 1996) for cells lacking self markers and may thus kill tumor cells and virus-infected cells in which MHC class I down-regulation has occurred. Although much has yet to be learned about the process of NK surveillance, numerous receptors that have been discovered on NK cells have either a positive or negative effect on their activity, so that NK cell regulation appears to involve the fine adjustment of the algebraic sum of these opposite signals. Lanier and Phillips (1996), Lanier (1997), Moretta *et al.* (1996), and Lanier *et al.* (1997) have reviewed the rapidly moving field of NK cell receptors for polymorphic MHC class I molecules. Many of the genes for these receptors are clustered in an NK complex on syngeneic regions of chromosome 6 in the mouse (including the Ly49 family of inhibitors and the NKR-P1 family of activators), chromosome 4 in the rat, and chromosome 12p12.3–13.1 in humans [so far including the single NKR-P1 (CD161) receptor with an unknown ligand but having either inhibitory activating effects when engaged by monoclonal antibody as well as the NKG2 family of inhibitors]. The NKG2 set may be the human equivalent of the murine Ly49 family, but they form heterodimers with another membrane protein, CD94, in humans rather than being homodimeric, as in Ly49 members. A separate family of inhibitory NK cell receptors (KIR or NKIR, with genes on human chromosome 19q13.4, of type 1 glycoproteins of the immunoglobulin superfamily) exists and has been reviewed by Lanier *et al.* (1997). Importantly, although HLA class I specificity of the various NKIR and CD94–NKG2A receptors is still being detailed, these receptors occur on overlapping subsets of NK cells, thereby refining detection to a single MHC class I allele loss (Lanier, 1997). The expression of several NKIR and CD94–NKG2 receptors on single human NK clones increases the difficulty of analyzing the ontogeny of NK cell specificity for self class I molecules (Lanier, 1997).

However, the NK cell activity for eliminating HLA down-regulated tumor cells escaping from antigen-specific CTL effector function may also be abrogated. HLA-G, a nonclassical MHC class I molecule, has been proposed to protect fetal trophoblast cells lacking classical HLA class I molecules from maternal NK cell attack (King *et al.*, 1989). Pazmany *et al.* (1996) have shown that the B lymphoma LCL 721.221, which is susceptible to NK killing, is protected therefrom by expression of HLA-G operating through

the NK inhibitory receptors NKIR-1 and -2. Alternative splicing of HLA-G transcripts produces at least five different mRNAs encoding HLA-G1–4 membrane-bound isoforms and a fifth soluble form. Rouas-Freiss *et al.* (1997) have shown that expression of HLA-G1 and HLA-G2 (both associated with β_2-microglobulin in the cell membrane) protects the standard human NK target, the K562 erythroleukemic cell line, from being killed by the childhood T cell leukemic NK-mediating YT2C2 clone, lacking the NKIR-1 and -2 receptors (and thus presumably mediated by an unknown KIR). Importantly, they found that NK cell cytotoxicity toward K562 cells of peripheral blood mononuclear cells, or of polyclonal NK cells isolated from them, all obtained from 20 different healthy donors, was inhibited by HLA-G1 and HLA-G2 expression in the K562 targets. Rouas-Freiss *et al.* (1997) proposed that HLA-G1 may be a public ligand for NK cell inhibitory responses, because it shows limited polymorphism and appears to stimulate inhibitory receptors on NK cells from donors representing a wide variety of HLA types. We can anticipate that a search among rogue metastases of a variety of malignancies, progressing in the presence of a tumor-reactive CTL immune response and showing down-regulation of classical HLA class I antigens, may well reveal HLA-G1 or HLA-G2 expression protecting the cells in these metastases from backup, NK cell cytolysis.

The special case of virally induced malignancies exemplifies other strategies for avoiding the natural, immune default response of NK cells. Several viruses encode proteins that result in the down-regulation of MHC class I molecules, thereby facilitating their escape from CTL destruction of infected cells. Cytomegalovirus (CMV) in humans encodes four proteins (US2, US3, US6, US11) that effectively eliminate HLA class I expression; to counter an anticipated NK cell cytolytic response to CMV-infected cells, CMV also encodes a homolog of MHC class I that contributes to the immune evasion of the virus by interfering with NK cell activation (Farrell *et al.*, 1997; Reyburn *et al.*, 1997). Reyburn *et al.* (1997) have shown this homolog to be a CMV protein (UL-18) that binds to CD94, the other member of the heterodimeric complex with NKG2 forming an inhibitory receptor on NK cells, thereby providing a negative signal to the NK cells. This may prove to be a paradigm for some tumor viruses.

In the context of malignant escape Stewart *et al.* (1996) have found that leukemic cell lines derived from patients with adult T cell leukemia (ATL) will grow (as will normal human xenografts) in severe combined immunodeficiency (SCID) mice (lacking functional T and B lymphocytes, but displaying active natural immunity, in particular NK cell activity). The ATL lines do not express viral genes although they do contain proviral DNA sequences of HTLV-1, the causative lentivirus. Reinfection of the ATL lines with HTLV-1 induces viral gene product formation and results in the rejection of these RV-ATL cells by the SCID mice via NK cell killing, the sensi-

tivity and resistance of the two ATL lines to NK cell cytolysis being also demonstrable *in vitro*. They showed that the virally directed macromolecular synthesis in some way induced NK cell sensitivity, but that the cells resulting from the primary infection *in vivo* were presumably selected by the immune system for suppression of viral transcription by NK cell activity. How general this mechanism may be, and the nature of the stimulus of viral metabolism to NK cell effector surveillance, remain to be elucidated, particularly in relation to whether the same conceptual framework can embrace the phenomena of murine, rat, and human NK studies—particularly in heterotransplantation experiments (Glas *et al.*, 1995). NK cell proliferation in viral infections appears to be mediated by both T cell-independent and T cell-dependent phenomena (e.g., Warren, 1996).

Although the emphasis of study of the determinants involved in NK cell cytotoxic function has been on MHC class I antigen expression on target cells, some other sparse evidence suggests that MHC class II may also play a role. The Burkitt's lymphoma cell line Daudi does not produce β_2-microglobulin and thus lacks functional surface HLA class I antigen, but is class II positive but susceptible to NK cell cytolysis. The Daudi cell NK susceptibility is enhanced three-fold by incubating the cells with anti-HLA-DR (class II) antibody. Similar observations on other NK-resistant lymphoma cells were made (Lobo and Spencer, 1989; Lobo and Patel, 1994). Finer analysis of HLA-class II requirements, using K562 (HLA-class I^- class II^-) cells transduced with either HLA-DR1β (class II not expressed) or HLA-DR1$\alpha\beta$ (class II^+), showed the necessity for at least a single HLA class II antigen to be expressed for resistance to NK cytolysis of HLA class I^- targets. Both the β and $\alpha\beta$ transductants were susceptible to LAK cell killing so that the NK resistance to the $\alpha\beta$ class II^+ cells was not due to some general mechanism, such as antibody-dependent cell cytotoxicity (Jiang *et al.*, 1996) or expression of antiapoptotic proteins such as FLIP (*vide infra*).

Clearly, loss of HLA class II expression can be an important factor in tumor cell resistance to NK cell killing of HLA class I down-regulated tumor, so that loss of HLA class I and class II antigens would be anticipated to confer a tumor phenotype free from specific or natural immune constraints.

VIII. T LYMPHOCYTE INADEQUACIES

"Their ineffectual feuds and feeble hates" (Matthew Arnold, *Balder Dead*)

The immunosuppression of T cell responses that often accompanies a progressing, large tumor burden has been variously attributed to suppressor T cell or macrophage function or to release of T cell-suppressive cytokines by the tumor (Aoe *et al.*, 1995; Zier *et al.*, 1996). Although still poorly defined, the signal transduction pathways in T lymphocytes, which are responsible

for their various states of activity, are believed to be dysfunctional in some cancer patients and in mice carrying certain tumors. This malfunction has been proposed to be at the heart of failing T cell responsiveness. Zier *et al.* (1996) argue that the spectrum of degrees of activation of T cells represents the function of different signaling pathways. Although decreased levels of the ζ chain of the TCR/CD3 complex and lower levels of the $p56^{lck}$ and $p59^{fyn}$ phosphotyrosine kinases are more common changes in the signaling pathways of T cell populations from mice bearing Renca (a renal cell carcinoma), and some evidence in mice suggests that splenic Mac 1^+ macrophages may have a role in these changes (Aoe *et al.*, 1995), the data at the single T cell level may be more useful in understanding the cellular mechanisms (Kelso, 1995; Bucy *et al.*, 1994). However, the reversibility of the defects that have been found in NF-κ and c-Rel transcriptional proteins, and the low levels of IFN-γ mRNA found in splenic T cells of tumor-bearing mice following treatment of the cells with flavone-8-acetic acid and IL-2 (Ghosh *et al.*, 1994), may make therapeutic intervention possible.

At the single-cell level, the patterns of cytokine expression are highly diverse and vary quantitatively from cell to cell in a continuum of rates of production (Kelso, 1995; Bucy *et al.*, 1994). At the population level, during an adaptive immune response the cytokine profiles of $CD4^+$ cells may be skewed toward what has been codified as the TH_1 and TH_2 types, with yet other profiles characterizing certain inflammatory responses (Mosmann and Sad, 1996; Abbas *et al.*, 1996). Evidence suggests that the population profile is determined by the dominant cytokines present during T cell activation, normally orchestrated by the "natural or innate immune response"—toward the TH_1 profile by NK cells producing IFN-γ or macrophages and dendritic cells (DCs) producing IL-12, or toward the TH_2 profile by mast cell, basophil, or T cell production of IL-4 (Romagnani, 1992; Fearon and Locksley, 1996). The factors involved in determining the "first cause" innate immune response are still being sought in the particular stimuli to which NK cells, macrophages, mast cells, basophils, and other "nonspecific" inflammatory cells respond to shape the character of the subsequent specific immune reactions. Given the development of a randomly heterogeneous population of T lymphocytes (Kelso, 1995; Bucy *et al.*, 1994), the question arises as to the mechanism for managing the composition of this population so that an appropriate balance of cytokine production (toward a TH_1 or TH_2 or other response) and CTL effectors is produced—is it selection or instruction or a combination of both? The DC has recently been proposed to be the key sentinel in this process (Ibrahim *et al.*, 1995). Evidence for an instructional mechanism has come from the finding that many naive T cells are multipotential for cytokine production (Kelso and Groves, 1997), but subsequent selection by cross regulatory cytokines (IFN-γ, IL-4, IL-10) may amplify/reinforce the effect of earlier instructional signals (Abbas *et al.*, 1996).

Antigen dosage may have complex switching outcomes of preference for $TH_1 \leftrightarrows TH_2$ or serial switching effects; production and release of cytokine receptors by the tumor (Mattei *et al.*, 1994) in a fashion analogous to the pox virus modulation of IFN inhibition, by encoding a soluble, type I IFN receptor (Symons *et al.*, 1995), may also favor one helper response rather than the other by removing cytokines produced by the natural immune response cells or by the TH_1 or TH_2 reinforcing cytokines (Mosmann and Sad, 1996; Abbas *et al.*, 1996). The presence of the costimulatory molecule B7.1 or B7.2 on the antigen-presenting cells provides an added complexity to the understanding of TH_1 or TH_2 determination, because the evidence points toward B7.1 encouraging a TH_1 response and B7.2 a T_2H response, although antigen dosage can modulate the outcomes (Parish, 1972; Hosken *et al.*, 1995). Among tumors, melanomata have been found to express many cytokines, which not unexpectedly include IL-10 and TGF-β, ensuring an anti-CTL pro-B cell outcome as one means of avoiding the CTL–tumor cell confrontation (Mattei *et al.*, 1994; Chen *et al.*, 1994; Musiani *et al.*, 1997). Clearly, cytokine abnormalities as a result of production by the tumor of significant amounts of immunomodulatory molecules, which counter the generation of tumor-specific $CD8^+$ T cell cytotoxicity, may well be involved in steering T cells into or selecting populations with inappropriate signal transduction activity.

IX. STROMAL SHIELDING?

"Union gives strength" (Aesop's Fable, *The Bundle of Sticks*)

Clinical trials of antitumor vaccination strategies are based mainly on the data from murine models in which a state of antitumor immunity is usually tested by challenge injection with a tumor cell suspension after an immune response has been allowed to develop. Only rarely does the vaccination follow the challenge (theraccination) to simulate the clinical situation, usually because the tumor growth is so rapid that it "outraces" the establishment of an effective immune response. However, the issues are not as simple as this, as the foregoing discussions suggest.

The most straightforward variable that may possibly determine the outcome of immune rejection of challenge tumor might seem to be the effector:target cell ratio. It was established by Winn (1960) that there is an inverse, *in vivo* relationship between the number of immune lymphoid cells admixed and coinjected with a given number of tumor cells and the amount of tumor present after a defined period; diminished tumor was attributable to the proportion of tumor cells killed by their early cytotoxic interaction after injection (Winn, 1960). More recent data show that the effectiveness of

antitumor immunity is limited by the size of the challenge inoculum when it is given to immunized mice, or when it is given just 3 days before vaccination (Dranoff *et al.*, 1993). Tumor growth, using a slower growing sarcoma, for increasing intervals (up to 28 days) before vaccination, had a diminishing outcome of therapeutic response (Mayordomo *et al.*, 1995). Tests in experimental systems that are thought to be closer to the late-stage cancer [e.g., melanoma stage IV (AJCC)] clinical situation involving patients with significant tumor burdens, however, invoke many of the other possibilities for tumor escape featured in this review.

A particular phenomenon that may be most relevant to the acquisition of tumor shielding is the growth of blood vessels and stroma into the inoculum of dispersed challenge cells. The possible screening of the tumor cells from potentially lethal trCTLs by an abnormally unresponsive neovasculature introducing a blood/tumor barrier is dealt with below. However, a case has been made that the stroma, which invades the growing tumor along with the capillaries, also introduces inhibitory influences (Singh *et al.*, 1992). It had been noted that when immune mice were challenged with the targeted tumor, the quantity of tumor cells rejected could be up to 33-fold greater if they were given as a cell suspension rather than as a solid tumor fragment, even though minimal and equivalent quantities of cells in the two forms produced tumors in nude mice. Furthermore, suspended tumor cells were more capable of producing tumor if they were injected into polyurethane (nonantigenic) sponge implants rather than directly into subcutaneous tissue. Introduction of tumor cells that grow progressively in allogeneic mice led to tumor rejection when fragments were transplanted into hosts for which the stroma was allogeneic but not in hosts for which the stroma was isogeneic. They concluded that just the antigenicity of stroma could lead to rejection of immunogenic cancer cells within it. They pointed out the allogeneic normal keratinocytes growing on stroma depleted of antigen-presenting cells are not rejected by the host (Ramrakha *et al.*, 1989). Further dissection of the mechanism of the stromal shielding and sensitizing effects needs consideration of the various other mechanisms discussed herein, particularly the modulation of surface expression of antigens and protective molecules when the cells are at close quarters together with blood vessel shielding.

X. THE trCTLp

"Lease hath all too short a date" (Shakespeare, *18th Sonnet)*

The T cells generated by vaccination of patients with GM-CSF-transduced autologous melanoma cells are capable of high activity for tumor-specific cell killing *in vitro,* which is class I restricted and CD8 mediated. The rise in pe-

ripheral blood trCTLp response to some vaccines seems to be of limited duration—even in the absence of residual tumour (Herr *et al.*, 1994), when assessed either by limiting dilution assays (Herr *et al.*, 1994; Ellem *et al.*, 1997) or by specific tumor cell lysis by bulk cultures of PBLs ((Ellem *et al*, 1997). Ethical considerations are responsible for the limited data in human malignant disease due to restriction of frequent access only to a patient's blood (which contains only 2% of the total lymphocyte population) for a quantitative estimate of tumor-specific cytotoxic T cells, rather than multiple biopsy of the tissues (tumour, spleen, or lymphoid), which may be reservoirs of the other 98% of a patient's lymphocytes (Westermann and Pabst, 1990). However, the data on the kinetics of CTL responses in the more facile murine experimental systems show a similar pulsed nature. Thus, in response to a primary viral infection, the T cell activation, replication, and accumulation lasts about 1 week; death of about 95% of these cells occurs between 1 and 4 weeks after infection, but a minority of memory cells can persist for many years (Ahmed and Gray, 1996). These data may be a paradigm for the human trCTL response, because Herr *et al.* (1994) have measured PB trCTLp responses following autologous melanoma cell vaccination in a patient with no residual disease, over a 13-year span, each of which responses had resumed baseline levels after 3 weeks, thus being consistent with the cytotoxic T cell kinetics of primary viral infection. Whether there was an anamnestic response attributable to memory cells shown in the 30 successive 1–2 monthly vaccinations is not clear, and the absence of tumor left open the question of whether there was, or was not, a correlation between blood CTLp levels and antitumor activity in the tissue deposits.

Another view of abbreviated immunity derived from observations that a murine melanoma line, B7-K1735, transduced with the B7.1 costimulatory ligand (CD80), while inducing an effective primary immune response, had a greatly reduced protection of immunized animals against tumor challenge at 90 days, with the fall in resistance being gradual, from 30 to 40 days after vaccination (Townsend *et al.*, 1994). However, this decline in immunity did not occur when the B7-EL4 thymoma murine system was used. The limited window of effectiveness in the melanoma system was attributed either to the lack of production of memory-type cells or to a requirement for costimulatory signals for the reactivation of long-term surviving T cells, but no adequate explanation for the observed differences with the thymoma model were offered. Further analysis of these systems is needed concerning (1) the quantitative relationships between size of tumor challenge and growth rate of tumor in relation to the rate of reinduction of these secondary responses, particularly (2) the dependence of rejection of other tumor systems on the challenge cells being presented as a suspension rather than a solid tumor or fragment thereof (e.g., Singh *et al.*, 1992) and (3) the unexpected cross-re-

activity between the tumors used—for example, B7-K1735 protects not only against autologous challenge, but also confers immunity against another melanoma, CM19 (also UV induced), the fibrosarcoma UV-5498-4, as well as an SV40-transformed renal carcinoma, as does the B7-EL4 thymoma against EL4 and the syngeneic thymoma C6VLB (Townsend *et al.*, 1994), possibly due to shared TAAs in each case. The number of recognized and unrecognized variables not shared by solid tumor versus suspended cell vaccination and the puzzling unique ability of ionizing radiation to eliminate the immunogenicity of the solid tumor (unlike most tumor vaccines) make uncertain any conclusions concerning mechanisms of abbreviated immunity in this model. In addition are the many findings that emphasize the difficulty in reconciling all the properties of the murine experimental models with their clinical counterparts (Colombo and Rudolfo, 1995).

The short duration of the peripheral blood level of trCTLp following cytokine adjuvanted activation of CTLs, alluded to above, may well be an indication of the termination of the antitumor activity sponsored by the vaccination, but this remains to be tested. Although it is tempting to view the termination of the PB trCTLp wave as a manifestation of "Karoushi" (Glickstein and Huber, 1995), this is thought to imply antigenic overstimulation of peripheral T cells. Intimations of the similarity of the "pulse shape" of the accrual and disappearance of PB trCTLp among patients with a heavy tumor burden and those with no residual disease suggests that antigen overstimulation from tumor is an unlikely terminator. However, stimulation by cross-reactive but lower affinity self-epitopes may substitute for TAA to induce apoptotic elimination of the trCTL due to chronic stimulation (reviewed in Goodnow, 1996) or anergization by weakly binding antigens (Evavold *et al.*, 1993). It is probable that the wave of trCTLp observed in peripheral blood from 1 to 3 weeks after tumor cell vaccination is contributed by cells that have been stimulated to proliferate, express Fas/APO-1/CD95, and are thus susceptible (Renno *et al.*, 1995, Salmon *et al.*, 1994) to Fas receptor/Fas ligand (Fas/FasL)-mediated apoptotic death following subsequent TCR ligation (Renno *et al.*, 1995; Salmon *et al.*, 1994; Lenardo, 1991) or NK, Mac-1, or $CD4^+$ T cell regulatory activity (Piazza *et al.*, 1997). In the absence of these mortal stimuli other means of disposal (e.g., via TNFR, DR3) (Chinnaiyan *et al.*, 1996) of accumulating CTLs may operate as part of normal immunoregulatory processes (Chinnaiyan *et al.*, 1996). The ligand for DR3 has yet to be published.

An additional factor has just been recognized as a probable mediator of resistance and sensitivity to FasL for regulation of the limited life span of the bulk of activated T cells. Irmler *et al.*, (1997) described an antiapoptotic protein, variously termed FLIP/Casper/CASH/FLAME/I-FLICE (e.g., Wallach, 1997), which has been shown to be present for the first day of T lymphocyte

activation by phytohemagglutinin. The presence of FLIP at generous levels coincides with the resistance of the activated T lymphocytes to FasL killing, despite their exhibition of plenty of Fas (CD95). After 6 days of the mitogenic stimulation the levels of FLIP in the T cells had dropped to undetectable levels, coinciding with their acquisition of sensitivity to FasL killing. Irmler *et al.* (1997) propose that the induction and disposal of FLIP may constitute an important mechanism for ensuring homeostasis in the cellular effectors of cell-mediated immunity.

The occurrence of lymphoproliferative disease in *lpr/lpr* or *gld/gld* mice with concomitant autoimmune pathology has led to the realization of the importance of ridding the immune system of excess CTLs. The *lpr* and *gld* mutations occur in Fas and FasL, respectively, effectively eliminating the Fas/FasL trigger for apoptosis in activated T cells (Takahashi *et al.*, 1994). Activation-induced cell death (AICD) is differentially operative in $CD4^+$ helper T cells of the TH_1 rather than TH_2 lineage owing to the much higher levels of FasL expression by TH_1 cells after activation. However, in mixed cultures of TH_1 and TH_2 cells, activation is accompanied by "bystander killing" of TH_2 by the FasL-rich TH_2 cells (Ramsdell *et al.*, 1994). It has been suggested that within the set of $CD8^+$ T lymphocytes the T1 subset (secreting IFN-γ) is eliminated by Fas/FasL apoptosis but the T2 subset (secreting IL-5) is not (Carter and Dutton, 1995), so that the requirement for a peripheral mechanism to ensure that the organism does not overload with activated T lymphocytes is concentrated on the principal effectors of HLA-restricted cytotoxic and helper activity. These use high- and low-level FasL expression, respectively, as one limb of their cytotoxic armamentarium (Nagata and Golstein, 1995; Lenardo, 1996). The related TNF/TNFR1 system can modulate Fas/FasL activity and vice versa, so that a multivariate system of regulation is created by the addition of glucocorticoid effects on the production of cytokines and their receptors on the T lymphocytes (Paliogianni *et al.*, 1993; Moreno *et al.*, 1996). Differential sensitivity of Fas/FasL apoptosis, typically IL-2 driven (Lenardo, 1991), and that induced by glucocorticoids (Moreno *et al.*, 1996) allows further flexibility in CTL regulation based on local versus systemic factors.

The balance between tolerance and immunity is complex in its regulation and will need much deeper understanding of the interplay between the effector molecules and the molecular circuitry involved in the retention, competition, elimination, or expansion of T cell clones (Goodnow, 1996) that contribute to the stability of the pool size of T cells (Rocha *et al.*, 1989). Whatever the mechanisms responsible for regulating CTL levels following tumor vaccination, the "danger" of prolonged or excessive presence of trCTLs in the self–nonself context (Langeman and Cohn, 1996) appears to override their potential therapeutic benefits with regard to dismissive or permissive choices for their continued prevalence.

XI. OPPORTUNISTIC EXPRESSION OF THE FasL WEAPON BY TUMOR CELLS

The induction of Fas (CD95) on T lymphocytes following their activation provides their Achilles heel for elimination, not only to contain clonal expansion but also to prevent T cell-, NK-, or neutrophil-initiated inflammation in the body's sites of "immune privilege" (Griffith *et al.*, 1995; Suda *et al.*, 1995; Iwai *et al.*, 1994). These privileged sites, including eye, brain, testis, etc., are protected by the constitutive expression of FasL by the cells therein, rendering them capable of killing potentially destructive, activated Fas^+ T cells that may trespass into their antigenically provocative territory (Caspi *et al.*, 1986). However, the genetic plasticity of tumor cells allows virtually random expression of genes (*vide supra*), and clones of tumor cells decorated with the products of such vicarious transcription will assume a state of immune privilege if FasL is one of the genes so activated. This has in fact now been demonstrated to be a potential means of retaliation against T cell-mediated immune responses in cell lines of colon carcinoma (O'Connell *et al.*, 1996), melanoma (Hahne *et al.*, 1996), and hepatocellular carcinoma (Strand *et al.*, 1996). The latter two lineages of lines had further lowered their sensitivity to FasL suicide, fratricide, or killing by T cells, NK cells, and neutrophils, by down-regulation of Fas (receptor) expression. FasL expression was clearly protective for *in vivo* tumors in immunized mice (Hahne *et al.*, 1996). Furthermore, FasL is known to be cleaved from its membrane seating by a metalloprotease (Kayagaki *et al.*, 1995) as a 26-kDa soluble protein (Tanaka *et al.*, 1995) (sFasL). Serum samples from 18 of 35 patients with melanoma had elevated levels of sFasL (Hahne *et al.*, 1996), which is capable of activating the Fas/FasL apoptotic pathway in Fas^+ cells, and sFasL has been shown to have high toxicity to a Fas^+ murine T cell lymphoma line but not to the parental Fas^-, nontransfected line (Kayagaki *et al.*, 1995; Tanaka *et al.*, 1995). It would be anticipated that high levels of circulating sFasL could contribute to a generalized depression of the effector T cell arm of the immune system (Mariani *et al.*, 1995) such as has been observed in terminal cancer of many types (Aoe *et al.*, 1995).

In addition to Fas (CD95) down-regulation by tumor cells, another mechanism of escape from immunologically mediated FasL apoptosis has been described by several groups. Irmler *et al.* (1997) identified a protein they designated FLIP (FLICE-inhibitory protein), which in normal tissues is expressed predominantly in muscle and lymphoid tissue, is structurally related to the viral inhibitors of apoptosis induced via the TNF receptor family, but has an additional inactive caspaselike domain in the long form (55-kDa $FLIP_L$) and potently inhibits the induction of apoptosis through the known human death receptors. $FLIP_L$ was found to be present at high levels in malig-

nant melanoma tissue (in biopsies of skin metastases of five of five patients) and in the cell lines derived from malignant melanomata originating in a variety of patients. These latter were shown to be Fas^+ but were resistant to soluble FasL killing. Normal skin melanocytes did not contain detectable levels of FLIP. This suggests that FLIP expression is induced in many malignancies of cells of the melanocytic lineage and may be responsible for their evolution to FasL resistance and immune escape (Irmler *et al.*, 1997). FLIP expression may thus be an extra protective element for tumor cells in their mortal combat with T cells and, in some as yet ill-understood fashion, help to avoid the recruitment of neutrophils to the field of battle, an invasion that has been identified as the crucial factor in the rejection of FasL-expressing allogeneic pancreatic β islet cells (Kang *et al.*, 1997). Allogeneic β cells not engineered to express FasL are, by contrast, not rejected when cotransplanted with muscle cells (syngeneic with the host) that do express FasL (Lau *et al.*, 1996). This latter is presumed to destroy potentially β cell-reactive effector CTLs. Unlike the islet cells the muscle cells are shielded against apoptotic death from the FasL swords of inflammatory cells by lack of Fas and via their high expression of FLIP (Irmler *et al.*, 1997). Wallach (1997), surveying, prospectively, the reports from five groups of the presence and effects of FLIP—variously termed FLIP/Casper/CASH/FLAME/I-FLICE—indicated the pressing need for clarification of the activities of the various splice variants of FLIP in different cell lines before FLIP regulation of the apoptotic pathway is fully understood.

A parallel to the Fas/FasL attenuation of CTL antitumor activity has just been recognized from an investigation of the notoriously inefficient immune response to the many antigens on human breast adenocarcinoma cells to which there are CTL responses (Gimmi *et al.*, 1996). The readily shed, omnipresent, highly glycosylated mucin antigen, DF3/MUC-1, has been shown to be immunosuppressive to activated T cells by inducing apoptosis therein. This was shown by using either supernatant from cultures of a variety of breast cancer lines, or purified DF3, to abrogate CTL toxicity against DF3/MUC-1-transfected NIH 3T3 cells, *in vitro*, as well as by lethal challenge with the B16 murine melanoma transduced with DF3 in mice immunized against the parental B16 melanoma (Gimmi *et al.*, 1996). The actual pathway for DF3/activated T cell apoptosis has not yet been discovered.

XII. T CELLS AS OFFICERS RATHER THAN TROOPS

Although *in vitro* cytotoxicity assays of $CD8^+$ lymphocytes are commonly used to estimate efficacy and tumor specificity of an effector response of

T lymphocytes, there is a body of evidence indicating that the cytokines released by T cells on contact with tumor cells are a better predictor of *in vivo* antitumor activity (Goedegebuure *et al.*, 1994; Barth *et al.*, 1991; Aruga *et al.*, 1995). Thus a series of noncytolytic $CD8^+$ TIL lines, expanded from a murine sarcoma by stimulation *in vitro* and adoptively transferred into mice bearing the same sarcoma, were more or less effective in their antitumor activity in relation to their production of GM-CSF and IFN-γ, *in vitro,* in response to exposure specifically to cells of that poorly immunogenic sarcoma (Barth *et al.*, 1991). Others have reported that release of GM-CSF and IFN-γ during *in vitro* culture, without tumor antigen stimulation, also correlated with antitumor activity in the same murine sarcoma model (Aruga *et al.*, 1995), and that GM-CSF production *in vitro* by TILs exposed to autologous melanoma cells was an independent predictor of clinical response to adoptive transfer of these expanded TILs (Schwartzentruber *et al.*, 1994). In the murine sarcoma model it has just been shown (Aruga *et al.*, 1997) that tumor regression was mediated by $CD8^+$ T cells alone from tumor-draining lymph nodes after positive selection *in vitro* by tumor antigen stimulation, but not by similarly prepared $CD4^+$ T cells. The $CD8^+$ T cells released both GM-CSF and IFN-γ when stimulated by specific tumor in an IL-2 dose-dependent fashion, whereas the ineffective $CD4^+$ subset released GM-CSF but not IFN-γ on specific tumor cell exposure. Curiously, the $CD4^+$ cells released greater amounts of IFN-γ and GM-CSF than the $CD8^+$ subset, nonspecifically on exposure to a different sarcoma. As expected, the $CD4^+$ subset released more than 10 times the amount of IL-2 specifically by contact with the immunizing tumor (Argua *et al.*, 1997). Because antibody neutralization of either, or both, IFN-γ and GM-CSF decreased but did not eliminate the antitumor efficacy of the combined T cell population, direct effects of these cytokines in metastatic tumor suppression are worth exploring. A case has been made for the involvement of radioresistant host monocyte/macrophages in mediating the effects of IFN-γ and GM-CSF, possibly by upregulation of antigen presentation by tumor cells (Aruga *et al.*, 1997). Clearly cytokine profiling of lymphocytic populations during immunotherapeutic experiments should be added to the standard cytotoxicity tests in order to allow correlations with antitumor effects to be made.

A role analogous to that proposed for GM-CSF/IFN-γ-secreting T cells as officers rather than troops in the battle against tumor cells arises in autoimmune disease (Parmiani, 1993; Naftzger *et al.*, 1996) and is expounded via Steinman's "tale of smart bombs and the infantry" (Steinman, 1996). Analysis of the inflammatory infiltrates in the lesions of experimental autoimmune encephalomyelitis, multiple sclerosis, and other organ-specific autoimmune diseases has led to the realization (Brocke *et al.*, 1996) that there are multiple antigenic targets that attract an active inflammatory infiltrate containing

> an extremely diverse collection of T cells, B cells and macrophages . . . Autoreactive T cells are only a small component . . . [but these] rare cells in the . . . ensemble regulate the behaviour of a vast population of non-specific cells. *In vivo* administration of native self peptides or altered self peptide ligands for these rare self-reactive cells can lead to the disappearance of an entire autoimmune infiltrate from a target organ over a few hours.

We are reminded of the induction of tolerance and loss of resistance, in tumor-immune mice, to challenge with an adenovirus-induced tumor of C57BL/6 mouse origin following injection, in incomplete Freund's adjuvant, of low levels of a synthetic peptide cognate with the dominant Ad5E1B-CTL epitope responsible for tumor immunity (Toes *et al.*, 1996). Injection of this peptide epitope, whether simultaneously with or 3 days or 3 weeks before tumor challenge of mice previously immunized with the irradiated Ad5E1 tumor, effectively abrogated the preexisting tumour immunity. The Ad5E1B-CTLs, which were responsible for tumor immunity after tumor cell vaccination, were no longer detectable after injection of the Ad5E1B-encoded CTL epitope, thus explaining the state of tolerance induced by peptide injection. An understanding of this tolerogenic peptide maneuver may be as important for immunotherapeutic strategies to treat autoimmune disease as it is to avoid its occurrence in cancer immunotherapy (Toes *et al.*, 1996; Aichele *et al.*, 1995).

The expectations of a response, which includes multifunctional, heterogeneous, interacting leukocytes and networking of multiple cytokines, are that such complexity must reduce to an outcome that represents fine-tuning of the organization of disparate troops if they are to seek and destroy a well-camouflaged enemy—the tumor cell. Whether this matrix of cells and bioactive proteins always results in an inflammatory legion that is properly armed for an unmodified target will require meticulous but imaginative analysis. Given the mutability of the target and the possible variation in the genetic biases of the immune responses among patients, the final solution may take some time. Even in the apparently simpler situations of the stable or the progressing lesions of leprosy and leishmaniasis, in which IFN-γ plays a key role in differentiating these two types of response (tuberculoid versus lepromatous and localized cutaneous versus diffuse cutaneous, respectively), there remains the outstanding question of what determines an overall healing response or parasite victory and particularly what determines the spontaneous reversal reactions from lepromatous to tubercloid lesions that occur not infrequently (Modlin *et al.*, 1983; Yamamura *et al.*, 1992). Is the occurrence of a spectrum of lesions from the extremes of localized cutaneous and diffuse cutaneous through the intermediate lesions of mucocutaneous leishmaniasis (Pirmez *et al.*, 1993; Caceres-Dittmar *et al.*, 1993) an example of "indecision"—failure of fine-tuning for an efficacious outcome—within the immune system, and is this paradigmatic for inappropriate immune responsiveness in many cancer patients?

XIII. TUMOR REJECTION IS A COMBINED ACTION

There are many reports in the literature that show, in mice vaccinated against a specific tumor, that the cellular infiltrates that accompany regression of tumor, or rejection of tumor challenge, are complex and change during the process of tumor cell removal. In particular, the sequence of neutrophils, macrophages, and finally T lymphocytes has been seen in the immune rejection of tumor cells secreting various cytokines when they are used to immunize mice (Colombo *et al.*, 1992; Stoppacciaro *et al.*, 1993; Cavallo *et al.*, 1992). Although the polymorphonuclear leucocytes contribute to tumor cell destruction (Midorikawa *et al.*, 1990; Lloyd and Oppenheim, 1992), the acquisition of specific antitumor immunity is dependent on the involvement of $CD4^+$ and $CD8^+$ T cells (Cavallo *et al.*, 1992; Dranoff *et al.*, 1993). IFN-γ (Tuttle *et al.*, 1993) and TNF-α have been assigned a role in tumor cell killing by noncytolytic $CD8^+$ CTLs (Barth *et al.*, 1991), and networking via cytokines between granulocytes and T lymphocytes has been thought to be the basis of the complex rejection reaction (Colombo *et al.*, 1992; Stoppacciaro *et al.*, 1993; Cavallo *et al.*, 1992; Golumbek *et al.*, 1991). Among granulocytes, eosinophils have been the dominant type at regression sites of tumor challenge in mice immunized with GM-CSF-transduced tumor cells (Dranoff *et al.*, 1993), and at the analogous site (the DTH site) in melanoma patients in a clinical trial of this vaccination strategy in melanoma patients (Ellem *et al.*, 1997). The analogy between a local and systemic eosinophil presence during tumor regression and allograft rejection has been emphasized previously (Ellem *et al.*, 1997), but their role is still a matter for conjecture. Although tumor cells transduced with IL-5, when used as vaccine, call to the site a spectacular eosinophil infiltrate, this results in neither immunity nor rejection of the tumor cells—so that the eosinophils alone are not enough (Krüger-Krasagakes *et al.*, 1993).

XIV. THE BLOOD–TUMOR BARRIER

There are several indicators that the microvasculature of tumors may be barren of CTL homing signals, in addition to the structural and functional abnormalities that are presently being discovered (e.g., Hamberg *et al.*, 1994).

Leukocyte targeting to, and traffic through, sites of inflammation and secondary lymphoid organs are governed by several members of the three major families of cell adhesion molecules (CAMs)—the integrins, the Ig super family, and the selectins. The former two categories bind to proteins whereas the selectins recognize specific sialylated carbohydrate moieties on pro-

teins. An extensive series of chemoattractants adds another set of markers, allowing further differentiation of leukocytes by the nature of the cytokine receptors they express on activation.Thus a rich matrix of addressin, integrin, and chemokine receptor molecules exists for establishing a complex set of specific homing signals for the different subclasses of cells that will contribute to the immune/inflammatory response in tissues (e.g., Springer, 1994).

As yet, much of the detail defining specific invitations for diapedesis is still in the process of being codified. Separate streams of lymphocyte recirculation have been recognized for skin, gut, lung, and their associated lymphoid tissues (Mackay *et al.*, 1992a,b). Importantly, naive and memory/activated lymphocytes prefer different pathways of recirculation based on the expression of adhesion receptors (Mackay *et al.*, 1992a). For peripheral tissues and lymph nodes, memory/activated lymphocytes (L-selectin^{lo}) emigrate through flat "skin" capillary endothelium, whereas naive (L-selectin^{hi}) lymphocytes migrate through high endothelial venules (HEVs) of lymph nodes. However, L-selectin^{hi} neutrophils, although capable of binding directly to lymph node HEVs (e.g., in a Stamper–Woodruff assay), do not home to peripheral nodes, indicating the requirement for other factors to differentiate their responses from those of naive lymphocytes (Springer, 1994). This is relevant to the possibility that elimination of tumor deposits requires the combined action of specialist, specific, and nonspecific types of inflammatory cells. (Steinman, 1996; Stoppacciaro *et al.*, 1993; Cavallo *et al.*, 1992; Midorikawa *et al.*, 1990; Lloyd and Oppenheim, 1992). Concerning the precision, exclusiveness, and specificity of T cell homing, it would seem prudent to be ready to adapt the received wisdom of the above model of T lymphocyte trafficking to new data, in the light of the caveats raised recently (Westermann and Pabst, 1996). However, the basic requirement for cytokine activation of an appropriate set of endothelial luminal signals to attract a particular set of infiltrating cells is well established.

The major issue posed by tumors is whether restricted access of the demonstrably high levels of trCTLs in peripheral blood explains why they do not in many cases eliminate their tumor cell targets *in vivo*. Do they have access to the tumor cells veiled by a microvasculature whose endothelial lining does not present appropriate signals for the T cell attachment, rolling, activation, arrest, flattening, tighter adhesion, and transendothelial migration, which are the recognized steps of diapedesis (Mackay *et al.*, 1992a,b)? Certainly, metastatic melanoma tissue rarely contains TILs in significant numbers (Elder and Murphy, 1991), although there are enough present to establish frequent cultures, thereof, with appropriate IL-2 stimulation. These TILs were perhaps sequestered in the tumor, via a tumor-reactive T cell receptor, in an anergic state, caught during the random, undirected tissue wandering of a

small percentage of all lymphocytes (in contrast to the massive diapedesis of homing), which may be expected to occur as "noise" in the system but which must be an important element in immune surveillance. Thus the most exclusive blood–organ barrier, the blood–brain capillary endothelium, allows intravenously injected, activated rat, ovalbumin-specific, $CD4^+$ T lymphocytes access to the CNS of normal rats to the same extent as autoreactive, myelin basic protein-specific T cells (with frequencies of 1:5000 and 1:10000 of the total spinal cord lymphocyte population, respectively), whether injected separately or together (Tabi *et al.*, 1995).

The microvasculature of tumors is newly found in response to the release of angiogenesis factors by tumor cells (Fidler and Ellis, 1994), and tumor growth (beyond 1–2 mm in diameter) is dependent on the development of this neovasculature. The platelet–endothelial cell adhesion molecule PECAM-1 defines an elaborate plexus of blood vessel endothelium throughout all tumor nodules (Berger *et al.*, 1993), but expression of other endothelial markers, such as von Willebrand Factor and the α_6 laminin receptor, which are normally expressed strongly by dermal vessel endothelium, was variably deficient. Furthermore, whereas normal blood vessels are closely associated with $chymase^+$ mast cells, these are markedly deficient in tumor microvasculature (Berger *et al.*, 1993). The degranulation of these mast cells releases TNF-α, which is responsible for the induction of endothelial leukocyte adhesion molecule 1 (ELAM-1/E-selectin) in local, postcapillary venule endothelial cells (Walsh *et al.*, 1991), thereby encouraging T cell extravasation. The perivascular mast cells, as the major source for releasing TNF-α are "ideally poised to serve as gatekeepers of the dermal microvasculature." Through this cytokine, expression of ICAM-1, VCAM-1, and class I and II major histocompatibility antigens is induced in addition to ELAM-1, all contributing to local accumulation of inflammatory cells (Walsh *et al.*, 1991). VCAM-1, the immunoglobulin superfamily protein involved in both primary and secondary adhesion steps of memory or activated lymphocyte homing to extraintestinal sites of inflammation (Butcher and Picker, 1996), is usually expressed in lung capillaries, but is absent in the neocapillaries of pulmonary metastases of murine melanoma B16/129-10 (Piali *et al.*, 1995). Coculture of these murine melanoma cells with an endothelioma cell line (bEnd 3) (but separated from them by a 0.4-μm Nucleopore filter membrane) abolished the up-regulation of VCAM-1 normally seen in response to TNF-α, IL-1α, or lipopolysaccharide (LPS) (Piali *et al.*, 1995).

A hapten-sensitization vaccine developed by Berd's group induced hypersensitivity-type inflammation in melanoma metastases, but failed to induce E-selectin in tumor endothelia, whereas ICAM-1 and HLA-DR expression was variable (Berger *et al.*, 1993). Thus, despite the observed T cell infiltration, the character of the infiltrate may have been inappropriate for elimi-

nation of the tumor cells. The authors concluded that tumor endothelium may be refractory to stimulation by proinflammatory signals that normally promote angiocentric inflammation. Leukocyte–endothelium interaction has been observed to be much reduced in tumor microvascular during direct observation, compared with normal microvessels of the same size. Even the provocative inflammatory stimuli for leukocyte adherence, via up-regulated E-selectin (ELAM-1) and L-selectin by TNF-α and LPS failed to increase cell wall sticking in the tumor vasculature compared with normal capillaries (Wu *et al.*, 1992). During extensive studies of blood flow, permeability, and intratumoral pressure, it was observed incidentally that fluorescently labeled leukocytes did not stick to the intratumoral capillary endothelium (Yuan *et al.*, 1994), as they did to normal tissue capillary endothelium.

That the neovasculature of tumors has a distorted architecture has been recognized for 30 years (see review by Peterson, 1979). The consequences of more distance between capillaries, frequent cul-de-sacs and blood channels, or sinusoids with poor flow, greater permeability, but inferior nutrient delivery, have been the bane of the chemotherapist's attempts to gain high concentrations of drug in neoplastic tissue. The blood flow to tumors has been noted to be very heterogeneous—even within a tumour—and the inefficiency of delivery of even oxygen has been seen to be responsible for radiation resistance (Thomlinson and Gray, 1955). It is thus possible that systemically delivered, bioactive molecules such as IFN may not achieve the concentrations within the tumor masses for adequate up-regulation of HLA to occur, because this is a dose-dependent response (e.g., Fig. 1), as are IFN toxic effects. Larger molecules, such as antitumor antibodies, are very poorly distributed to tumor tissue, amounts less than 0.001% of the injected dose localizing per gram of tumor tissue being commonly found in clinical studies (Sands, 1988; Epenetos *et al.*, 1991). IFN binding by receptors on the tumor cells closest to capillaries could contribute to inequalities of intratumoral distribution (Kennel *et al.*, 1991; Sung *et al.*, 1990)—the "binding site barrier" (van Osdol *et al.*, 1991).

It is also possible that soluble forms of the selectins, integrins, and immunoglobulin superfamily proteins may circulate in blood at elevated levels in cancer patients. Thus a twofold increase of both VCAM-1 in the blood of patients with a variety of tumors and E-selectin in gastrointestinal and breast cancer, and a three- to fivefold increase of serum ICAM-1 in metastatic cancer (Gearing and Newman, 1993), may interfere with leukocyte–endothelial cell attachment and thus contribute to the minimal tumor infiltration by inflammatory cells. Investigations of such a systemic depression of diapedesis could contribute to understanding the terminal depression of immunocompetence that accompanies some cases of widespread metastatic disease, but the use of well standardized methodology to evaluate this hypothesis has yet to occur (Gearing and Newman, 1993).

XV. CODA-1: PROPOSED SOLUTIONS

"For when a flye offendeth him or byteth/He with his tayl awey the flye smytheth/Al esily" (Geoffrey Chaucer, *Legend of Good Women*)

The depressingly large number of ways by which metastatic tumors may escape cytotoxic T lymphocyte responses poses a challenge not only to the therapy of late-stage malignancies but also to single-strategy, randomized control trials to determine the possible contribution of immunotherapeutic measures for these diseases. In the latter context the occurrence of heterogeneity, in the somatic genotype of metastases in the same patient and in the germ line among patients, creates the anticipated dilemma of subject variation, which calls for patient–patient variation in treatment. This may be practicable for those phenotypes (e.g., HLA down-regulation, Fas-L expression, CTL inhibitory cytokine production, lack of capillary endothelial signaling, etc.) for which another additional method of intervention is currently recognized. It is perhaps simplest to deal first with some possible strategies for handling the recognition and treatment of rogue (progressive) metastases occurring in tumor-vaccinated patients showing partially responding stage IV disease as an example of a branch on the arborizing structure for immunotherapeutic trials.

This discussion of the first part herein will be confined to examples of suggested theoretical interventions to control progressing rogue metastases in the presence of a demonstrable CTL response to autologous tumor cells. Subsequently, a revised interventional form of clinical trial will be outlined. An immune response to vaccination may be measured by significant DTH reaction, elevated trCTLp frequency in PBLs, efficacy of bulk PBMC culture killing of autologous tumor, or IFN-γ, GM-CSF-producing trCTLs from the same source. In those patients who have circulating tumor cells in their blood an additional test may prove rapid for recognizing a useful immune response. Because one tumor cell can be detected in 2–5 ml of blood by using the reverse transcription polymerase chain reaction (RT-PCR) for a tumor with a nonhematogenous lineage marker (e.g., tyrosinase–melanoma, serum albumin–liver) (Smith *et al.*, 1991; Stevens *et al.*, 1996), it would be expected that vaccination should clear the patients' blood of targetable tumor cells. However, the reproducibility of RT-PCR data on blood samples from cancer patients by many groups has proven difficult and interpretations are inconsistent at this time (Buzaid and Balch, 1996) The utility of the RT-PCR assessment of disease needs verification as does the use of soluble TAAs (prostatic serum antigen, carcinoembryonic antigen, etc.) as monitors for disease response.

Although the above tests will indicate whether a T cell response has occurred, it is not clear which tests correlate best with *in vivo* regression of tu-

mor. Although the indications are that cytokine production (GM-CSF and IFN-γ) may be a better predictor of tumor response than is the level of CTL activity *in vitro,* and that this favors the "trCTL as officers rather than troops" paradigm arising from Steinman's studies of autoimmune infiltrates (Steinman, 1996), there is a clear need for more data with a broader spectrum of tumors and patients to confirm this or discover better measures of disease response, or even whether this is the correct question to be asking of the initial response to a single vaccine trial.

The all-important decision of which tests to use for assessment of the first endpoint in an immunotherapeutic strategy depends on knowing which immune response is appropriate for disposing of tumors *that have not evolved highly deviant immune escape mechanisms.* In turn, this raises the issue of what is an appropriate target cell in these tests, because, although autologous tumor may express unique TAAs that can be used in some immunotherapeutic strategies, they may also have evolved mechanisms for avoiding the consequences of effector cell activity. At this stage we need to perform as many tests as possible to select, for later routine use, those which will provide the most robust indicators that a basic, potentially effective immune response has occurred. There is a need to ensure that the target cells are expressing all the patient's HLA antigens on the target cell surface, but are not expressing FasL, TNF-α, IL-10, or antiapoptotic proteins such as FLIP, sentrin, Bcl-x_L etc. (*vide infra*).

Thus the following suggestions:

1. Biopsy sections of the tumor tissue that will be used for cell preparations for *ex vivo* manipulation (for vaccine and for target cells) and cell samples from the manipulated populations should be subject to cytoimmune techniques (fluorescence or enzyme-linked immunocytochemistry, FACs analysis) to determine their expression of HLA, FasL, and CD95 (Fas), TNF-α, TGB-β, and IL-10.

2a. Cell culture of biopsied cells should be attempted, which, for melanoma at least, can be expected to be very successful (>90% of metastases yielding permanent cultures (M. Down *et al.,* unpublished; Jaffee *et al.,* 1993), and the profile for the same five groups of proteins should be established, to compare with directly *in vivo* sourced cells to check for any modifications of the *in vivo* profile during tissue culture. Such profiles will indicate whether any of the commoner mechanisms of effector escape are present in those samples. Cultured biopsy cells to be used as targets for CTL activity should have a "control" subset pretreated with IFN-γ to ensure up-regulation of tumor cell HLA and thus TAA epitope presentation.

2b. TILs should also be cultured to establish their antitumor cytotoxicity as well as their profiles of cytokine production, to enable clarification of the preferred indicator of outcomes.

3. After three vaccinations and four DTHs (8 weeks) the patient's tumors are reevaluated. Accessible regressing metastases are biopsied to monitor the cellular composition of the inflammatory infiltrate (double immunostaining for CD markers) accompanied by antibody and *in situ* hybridization probes to establish their cytokine secretion profiles (IL-2, -4, -10, -12, GM-CSF, IFN-γ, TGF-β, TNF-α) (e.g., Rogers *et al.*, 1997), to correlate tumor cell destruction with particular patterns.

4. Progressive rogue metastases and accessible metastases in patients with unresponsive disease should be biopsied and examined as in (1) and (2) above. This should lead to recognition of the particular escape route taken, thereby suggesting appropriate action, and, most importantly, to identify the minimum number of tests needed in the long run for patient management. Parsimony in the use of expensive tests and complex therapies in patients with advanced malignancy raises the ever more recurring problems of medical economics involving decisions concerning what is potentially doable against what is affordable. This debate—a politician's nightmare—is not addressed in this review.

XVI. SOME OBVIOUS STRATAGEMS

A. The Vanishing Target

Failure of adequate expression of stable HLA on the tumor cell surface renders the tumor cell invisible to the effector cells of specific immunity, often without the indemnifying benefits of NK recognition and killing, which might have been expected from the "missing-self" paradigm (Ljunggren and Kärre, 1990). As detailed above, the interferons cause up-regulation of HLA, β_2-microgobulin, TAP-1, and TAP-2 as well as the proteasome components LMP-2 and LMP-7, at least *in vitro*, and, importantly, differences have been noted *in vivo* (Massa *et al.*, 1993). The problem of blood-borne delivery of bioactive molecules, chemotherapeutic drugs, as well as immune cells has been alluded to above in the context of a blood–tumor barrier. Systemic IFN-α may up-regulate tumor HLA, thereby increasing the immune visibility of rogue metastases. However, much higher intralesional concentrations of IFN-α may be needed than can be comfortably achieved systematically, owing to the concentration dependence of the HLA response, and such levels could be reached by infusion into wayward metastases or as an intralesional depot in microspheres, without systemic toxicity. The more effective but toxic IFN-γ could also be used intralesionally, to reduce its systemic effects while maximizing its modulation of tumour HLA.

B. Bioactive Proteins

Bioactive proteins produced by tumor cells may interfere with the effector cell activity in at least three ways; (1) T cell death by apoptosis or (2) T cell inactivation by anergization or (3) supportive cytokine sequestration by release of cytokine-binding proteins.

The basic strategy to protect activated (Fas^+) T cells from FasL-induced apoptosis by tumor cell expression of FasL, or by ligation of their Fas receptors by the cells (?NK,?T cell, ?macrophage) that normally regulate CTL expansion in immune responses, could involve gene transfer into the trCTL population of one of several candidate genes whose products are known to inhibit the FasL/Fas pathway of apoptosis. This strategy will first be modeled in mice, using the FasL-expressing murine melanoma B16/F10 (Hahne *et al.*, 1996) engrafted on the histocompatible C57BL/6 mouse. Only after completion of the murine studies can the decision to proceed with "proof of hypothesis" studies be considered in the clinical context. So that the protected trCTL population can be eliminated from the mouse or patient, to avoid the potential of lymphoproliferative or autoimmune reactions developing from unlimited clonal expansion, the herpes simplex thymidine kinase (HStk) gene will be cotransferred, so that when the appropriate tumor responses have occurred, or manifestations of autoimmune danger develop, the trCTL can be eliminated by treating the mouse or patient with gancyclovir.

The protective genes to be used for transfer into activated T lymphocytes could include either sentrin (Okura *et al.*, 1996), FAP-1 (Sato *et al.*, 1995), IAPs (Liston *et al.*, 1996), Bcl-2 (Oltvai and Korsmeyer, 1994) Bcl-x_L (Boise and Thompson, 1997), or the ICE inhibitors CrmA and P35 (Clem and Miller, 1994), all of which, when overexpressed intracellularly, are capable of inhibiting both FasL/Fas and TNF/TNFR1-induced apoptosis. Sentrin is a small protein whose cDNA was recently cloned and sequenced (Okura *et al.*, 1996). It has homology with yeast Smt3, ubiquitin, Nedd8, and the ubiquitin domain of BAG-1. Sentrin has a very specific pattern of binding to the death domains of Fas and TNFR1 but not to the death domains of CD40 or FADD/MORT1. It does not itself contain a death domain, but blocks Fas or TNFR1 signaling for apoptosis by a mechanism that still remains to be elucidated. The other proteins protecting cells from apoptosis have a longer history.

C. Blood–Tumor Barrier

Histochemistry of biopsied metastases will indicate deficient homing signal expression of the endothelium of the tumor neovasculature, thereby ex-

cluding potentially tumor-destructive cells. Chisari's group (Ando *et al.*, 1994) have addressed the issue of the barrier between blood and virus antigen-expressing cells in mice transgenic for hepatitis B surface antigen (HBsAg) and, "treated" by adoptive transfer of anti-HBsAg $CD8^+$ CTLs. Liver parenchymal cells expressing HBsAg were directly accessible to the CTLs, undergoing cytotoxic changes, whereas vicarious expression of HBsAg in brain and kidney cells was not detected through their blood–organ barriers. However, direct (extravascular) injection of the CTLs (intracerebral or subcapsular for brain or kidney, respectively) led to cytotoxic deletion of $HBsAg^+$ cells in those organs. Thus, a trCTL-enriched, expanded lymphocyte population from either peripheral blood, or harvested by leukapheresis, will be injected into rogue metastases directly. Confirmation of the blood–tumor barrier will be made by immunocytochemical study of the presence of endothelial addressins, Ig superfamily members, and the sulfated glycoproteins involved in lymphocyte diapedesis on the capillary endothelium of the tumor neovasculature. This will involve the selectins P (for a general marker) (Berger *et al.*, 1993) and E (as the lymphocyte addressin) (Shimizu *et al.*, 1992), the sulphated glycoproteins Sgp 50, 90–100, and 200 for the selectin binding (Hemmerich *et al.*, 1994), and the various members of the immunoglobulin superfamily (ICAM-1 and -2, VCAM-1, MAdCAM-1, CD31) for interaction with leukocyte integrins LFA-1, VLA-4, $\alpha 4\beta 7$, and CD31, respectively. Leukocyte markers in addition to the above will also be sought within and without the capillaries (e.g., CD44, CD2, CD45R). Comparison between the endothelial markers expressed in regressing versus progressing tumor may allow a correlation between the ability of endothelium to be induced to express the leukocyte rolling, adhesion, and diapedesis sequence allowing CTL contact with and destruction of tumor cells. Further validation of the defect in the presence of $chymase^+$ mast cells will also be sought (Berger *et al.*, 1993). Evidence for alternative endothelial cell activation will also be evident by staining for the CD40–CD40L pair on endothelium and local $CD4^+$ cells, respectively (Yellin *et al.*, 1995). The mechanism of release of the chemokines IP-10 and Mig, which are ligands for the CXC chemokine receptor (CXCR3) expressed on activated T cells (Loetscher *et al.*, 1996) and what modulations in the activity of the IP-10/Mig-CXCR3 system occur in the many tumors that preferentially accumulate effector cells (Liao *et al.*, 1995) will probably provide an understanding of yet another escape route for tumors to avoid effector cell action, but, hopefully, also a maneuver for therapeutic intervention.

To potentiate the delivery of bioactive molecules such as IFN-α to rogue metastases when they are too numerous or in inaccessible sites, the observations that the radiation-sensitizing drug pentoxifylline will increase intratumor blood flow and red cell flux, increase oxygenation, but decrease the interstitial pressure of the tumor tissue (Lee *et al.*, 1994) suggest that the drug

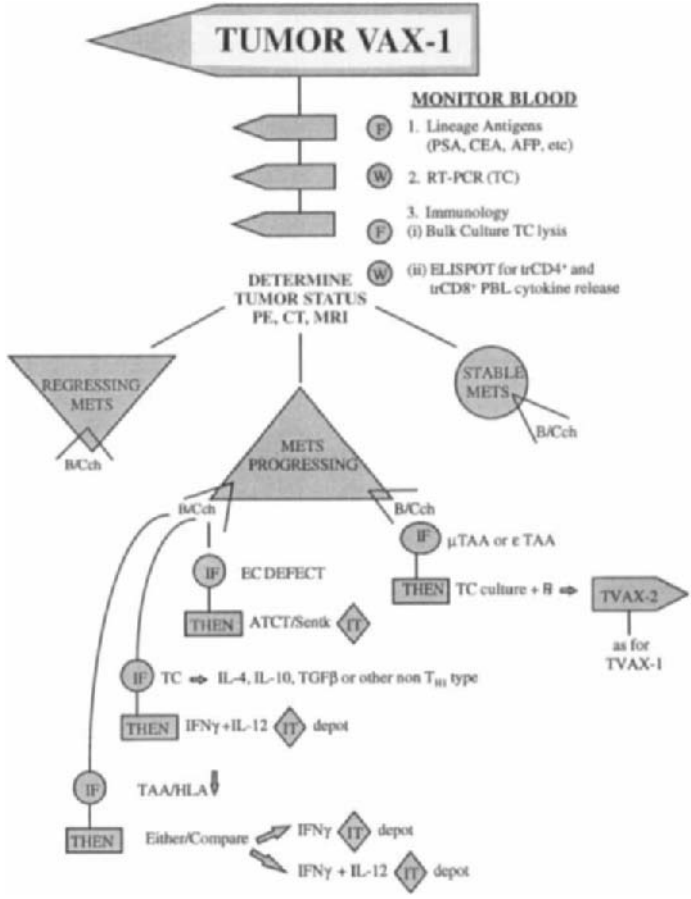

Fig. 2 Example of flow chart for arborizing clinical trial of immunotherapy. Abbreviations: ATCT/Sentk, adoptively transferred trCTLs transduced with the sentrin and herpes simplex thymidine kinase genes; B/Cch, biopsy/cytochemistry; CEA, carcinoembryonic antigen; CT, computed tomography; EC, capillary endothelium; ELISPOT, quantitative assay for cytokine-producing hematopoietic cells; F, fortnightly; HLA, human leukocyte antigen class I; METS, metastases; MRI, magnetic resonance imaging; PE, physical examination; PSA, prostate serum antigen; TAA, tumor-associated antigen; εTAA, grossly diminished or absent TAA in TC; μTAA, mutated TAA; TC, tumor cell; trCTL, tumor-reactive cytotoxic T lymphocyte; TVAX, a TH_1 type, tumor vaccine; W, weekly; ℜ, manipulation of TCs to render them potent immunogens, e.g., *ex vivo* cytokine gene transfer (Dranoff *et al.*, 1993) or *in vivo* HLA B7 gene intratumor tissue delivery (Nabel *et al.*, 1996); IT, intratumor tissue delivery; IT depot, intratumor tissue injection in a depot form.

may be of value, with or without nicotinamide (Lee *et al.*, 1993), for facilitating cytokine delivery to metastases. However, the observation that pentoxifylline inhibits T lymphocyte activation (Bemelmans *et al.*, 1994; Wang

et al., 1997) and endothelial cell, integrin-mediated cell adhesion (Kovach *et al.*, 1994; Tozawa *et al.*, 1995) will require careful monitoring of the duration of possible IFN-induced HLA up-regulation in relation to the length of effective T cell activity and diapedesis during and after a pulse of this drug.

These obvious experimental approaches to instances from the three major categories of immune escape mechanisms may well contribute to cancer management. They are offered as a salve for the depressing messages brought together in this review with the hope that they will stimulate new approaches to solving these complex problems of metastatic cancer.

At our present stage of understanding and competence in managing metastatic cancer, immunotherapy has its most likely successful application in stage II or stage III disease from which macroscopic residual disease has been removed. An evaluation of the frequency of the various ways tumor evolution leads to the avoidance of immune constraint will point to the most urgent developments for retaliatory measures. Protective manipulation of trCTLs against tumor cell weaponry only to yield to gancyclovir suicide with the quietus of both combatants, converts the ambiguities of the last line of that sonnet from the Portuguese to a *mot juste:* "I shall but love thee better after death."

XVII. CODA-2: SAMPLE FLOW CHART OF AN ARBORIZING CLINICAL TRIAL

"Lend fresh interest to a twice told tale" (George Gordon Byron, *Hints from Horace*)

Because a variety of vaccine formulations known to induce $CD4^+$, $CD8^+$, and trCTLp responses may prove to be equally effective, the flow diagram given in Fig. 2 suggesting the skeleton of the prototypic arborizing trial dispenses with preliminaries and begins with the tumor vaccine (TVAX).

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CD95(APO-1/Fas)-Mediated Apoptosis in Normal and Malignant Liver, Colon, and Hematopoietic Cells

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I. THE CD95 SYSTEM

A. The CD95 Receptor and Its Ligand

In an attempt to generate monoclonal antibodies that react with cell surface molecules and down-regulate tumor cell growth, we discovered the anti-APO-1 monoclonal antibody (mAb). Anti-APO-1 mAb reacts with a glycosylated cell surface molecule of approximately 45 to 52 kDa, which we termed APO-1 (for apoptosis 1). Binding of anti-APO-1 (IgG_3, κ) to cell surface APO-1 induces apoptosis in apoptosis-sensitive cells. Another antibody, called anti-Fas, was described that showed similar functional activity on binding to the Fas antigen. Cloning of the Fas and APO-1 cDNAs showed that their sequence was identical, and the molecule is now named CD95. We found that CD95 is a type I transmembrane receptor but can also occur in soluble form. CD95 belongs to the NGF/TNF receptor superfamily characterized by cysteine-rich extracellular domains. The human CD95 gene, *APT,* was localized to chromosome 10q23 and the mouse gene to chromosome 19. Expression of the CD95 gene and cell surface protein are enhanced by IFN-γ and TNF and by activation of lymphocytes.

Apoptosis via CD95 can be triggered by agonistic antibodies such as anti-APO-1 and by the natural ligand of the receptor, CD95L. CD95L was cloned from the cDNA of a killer cell (PC60-d10S) and shown to be a TNF-related type II transmembrane molecule. The mouse and human CD95L genes were mapped to chromosome 1. Killer cells expressing the ligand were shown to kill target cells in a Ca^{2+}-independent fashion via CD95–CD95L interaction. In addition, human CD95L overexpressed in COS cells was found in the supernatant and induced apoptosis in a soluble form. Soluble CD95L is generated from the transmembrane form by the activity of a metalloprotease (Krammer *et al.,* 1994a; Nagata and Golstein, 1995; Peter *et al.,* 1998).

B. The CD95 Signal

The cytoplasmic domain of CD95 does not contain any known consensus motifs for enzymatic activities such as kinases or phosphatases. However, the signaling pathway by which the apoptosis signal is propagated has been partially elucidated. Despite a considerable homology of the intracellular part of CD95 with the intracellular part of TNF receptor I in the so-called death domain, signaling via the two receptors seems to follow at least partially distinct routes. Dimerization of CD95, e.g., by $F(ab')_2$ anti-APO-1, was found to be insufficient to induce an apoptotic signal. Multimerization of CD95,

however, induced apoptosis. This finding suggests that in analogy to TNF, which triggers cytotoxicity as a trimer via TNF receptor I, CD95L may also act as a trimer to induce apoptosis via CD95.

In a search to identify intracellular signaling molecules coupling to oligomerized CD95, several cytotoxicity-dependent CD95-associated proteins (CAPs) were coimmunoprecipitated with CD95 from several cell lines and from normal cells. CAP1–3 (27–29 kDa) and CAP4 (55 kDa), instantly detectable after cross-linking of CD95, were associated only with aggregated (the signaling form of CD95) and not with monomeric CD95. CAP1 and CAP2 were identified as two different forms of serine-phosphorylated MORT1/FADD. The association of CAP1–4 with CD95 was not observed with C-terminally truncated nonsignaling CD95. In addition, CAP1 and CAP2 did not associate with a CD95 cytoplasmic tail carrying the *lpr*^{cg} amino acid replacement. Our data indicate that *in vivo* CAP1–4 are the CD95 apoptosis-transducing molecules. Using nanoelectrospray tandem mass spectroscopy, we sequenced CAP4 and CAP3. CAP 3 was found to have N-terminal homology to CAP4 and probably represents a minor CAP4 splice form. CAP4 is a "chimeric" molecule with two death effector domains (DEDs), homologous to the DED of FADD/MORT1/CAP1,2, and an ICE-like protease domain (therefore we called CAP4 FLICE). FLICE occurs in two isoforms, which are now called caspase 8a and 8b. On CD95 triggering, the death domain (DD) of the receptor attracts FADD via the DD of FADD. FADD then attracts FLICE via its DED and recruits it into the DISC. In fact, both caspase 8 isoforms are recruited into the DISC. Caspase 8 may then autocatalytically be cleaved to generate an active caspase 8 to cleave other downstream caspases and initiate a signaling cascade of further proteolytic cleavage. Inhibition of caspase activity by protease inhibitors, as well as by transient expression of the pox virus-derived serpin inhibitor CrmA, substantially suppressed CD95-triggered cell death. Thus, activation of caspases is a critical event in CD95-mediated cell death. Taken together, CD95-mediated apoptosis requires CD95 cell surface expression at sufficient density, multimerization by soluble or membrane-bound CD95L, and an apoptosis-sensitive cell in which antiapoptotic programs have been switched off. Only if these requirements are met, cells are susceptible to induction of CD95-mediated apoptosis. CD95 expression alone is not sufficient. This statement is supported by the following finding. CD95 is expressed on cells of many lineages, including lymphoid and nonlymphoid tissue; e.g., human colon epithelium expresses a high density and human liver cells a low density of CD95. Expression is similar in mice. Mice, however, that have a defect in the CD95/CD95L system show overt gross pathology primarily in the lymphoid organs. This suggests that CD95-mediated apoptosis plays a major role in the immune system and that this system is most sensitive to CD95-mediated apoptosis (Krammer *et al.*, 1994a,b; Peter *et al.*, 1998).

C. Defects in the CD95/CD95L System

Several mouse mutations have been identified that cause similar, complex disorders of the immune system, manifested as lymphadenopathy and autoimmunity. One is the *lpr* (lymphoproliferation) mutation, a recessive mutation causing lymphadenopathy and autoimmune disease similar to the human disease systemic lupus erythematosus (SLE). The mutations lpr^{cg} [allelic to *lpr*) and *gld* (generalized lymphoproliferative disease)] cause very similar diseases. In all three cases aberrant T cells accumulate; they express the Thy-1 antigen that is characteristic of T cells but lack both CD4 and CD8 cell surface molecules ("double-negative" cells). They also express polyclonal α and β T cell receptors and a number of cell surface molecules usually found on B cells, such as the B220 antigen.

The CD95 gene has been identified as the target of the *lpr* and lpr^{cg} mutations. In *lpr* mice the insertion of a retroviral early transposable element (ETn) into intron 2 of the CD95 gene causes a splicing defect, premature termination, and a greatly reduced expression of the receptor mRNA. In lpr^{cg} mice a point mutation (isoleucine to asparagine) in the intracellular "death domain" abolishes transmission of the apoptotic signal by the receptor. In *gld* mice a point mutation in the C terminus of CD95L impairs its ability to interact successfully with the receptor and to cause apoptosis. Thus, a failure of apoptosis accounts for the complex immune disorder in *lpr* and *gld* mutant mice.

The major features of the pathology exhibited by *lpr* and *gld* mutant mice are accumulation of the aberrant class of T cells described above, and autoantibody production by B cells. Because B cells require help from T cells to produce antibodies against T-dependent antigens, it is possible that autoantibody production by the mutant mice is a side effect of a T cell defect, leading to misdirected help for autoantibody-producing B cells. Alternatively, autoantibody production might be the direct result of a defect in the B cells. Transplantation experiments in which T cells had an equal opportunity to "help" normal or *lpr* mutant B cells led to exclusive production of *lpr* autoantibodies, implying that the *lpr* defect directly affects B cells. Breeding experiments showed that the development of the complete *lpr* pathology is strongly dependent on the genetic background of the *lpr* mutation, and in some cases autoimmunity can occur in the absence of lymphadenopathy, reinforcing the view that autoantibody production is not a consequence of excess aberrant helper T cells. Thus, autoimmunity and lymphadenopathy are distinct aspects of the *lpr* pathology, due to B cell and T cell defects, respectively (Krammer *et al.*, 1994a,b).

Insight into the molecular basis of the *lpr* and *gld* mutations has greatly influenced the discussion of the physiological role of CD95-mediated apoptosis in the immune system. In the normal lymphoid system apoptosis occurs

in primary lymphoid organs such as the bone marrow, liver, and thymus, and is used to eliminate useless precursor cells with nonrearranged or aberrantly rearranged nonfunctional antigen receptors. Furthermore, apoptosis is essential for deletion of autoreactive T cells in the thymus and is, therefore, a mechanism to guarantee central self-tolerance. In peripheral lymphoid organs, such as lymph nodes and spleen, a similar apoptotic deletion mechanism is operative in T and B cells. Peripheral deletion by apoptosis is another safeguard of the immune system to assure self-tolerance and to down-regulate an excessive immune response. Lymphocytes that escape this process might replenish the pool of cells that determine immunological memory. The mechanism of peripheral deletion was previously unknown and has now been elucidated, at least in part. The data show that the CD95 system contributes substantially to elimination of peripheral lymphocytes. This is shown for T cells, as discussed in the following section.

D. CD95 Involvement in Peripheral T Cell Depletion

In order to investigate directly in the human system whether CD95 is involved in TCR-mediated apoptosis, we used $F(ab')_2$ anti-APO-1, itself incapable of inducing apoptosis due to insufficient cross-linking of CD95, to block CD95. In addition, soluble human CD95–Fc fusion proteins were used to neutralize CD95L. Malignant Jurkat T cells were triggered via immobilized anti-CD3 in the presence of $F(ab')_2$ anti-APO-1 or CD95–Fc fusion proteins. These reagents completely prevented anti-CD3-induced apoptosis. Dexamethasone-induced apoptosis, however, was not prevented by $F(ab')_2$ anti-APO-1 and CD95–Fc. The same findings were obtained with an activated human T cell clone and activated peripheral human $CD4^+$ T cells. In these cells apoptosis induced with anti-CD3 antibodies and by stimulation with the superantigen SEB was also blocked by the above-mentioned reagents.

Taken together, these results suggest that CD95 and CD95L are specifically involved in TCR-triggered apoptosis. T cell apoptosis may occur as "fratricide" by interaction of the membrane-bound receptor with the membrane-bound ligand on neighboring T cells that kill each other. Further experiments showed that TCR-triggered CD95-mediated apoptosis is also found in single Jurkat T cells. Thus, a single TCR-activated T cell in the absence of costimulation may autonomously decide to die by apoptosis employing, at least in part, the CD95 pathway. These results suggest a minimal model in which TCR-induced death in activated T cells involves autocrine CD95/CD95L-mediated suicide. A model involving a soluble CD95L faces the difficulty to explain specificity and directionality of T cell apoptosis. In fact, we were able to induce apoptosis with supernatant transferred from ac-

tivated T cells to $CD95^+$ cells. Therefore, CD95L may have a short half-life and may act only at a short distance at a sufficiently high concentration or when properly crosslinked. In addition, soluble CD95L might only induce apoptosis in TCR-triggered T cells. Collectively, TCR-induced CD95-mediated apoptsis may occur in several forms: fratricide, paracrine death, and autocrine suicide (Dhein *et al.*, 1995).

Elucidation of the TCR/CD95 death mechanism may shed new light on peripheral T cell tolerance by deletion, on suppression of the immune response, and on the development of memory in the surviving T lymphocyte pool. Furthermore, it may open new exciting possibilities for experimental and therapeutic interventions.

We have described some of the basic features associated with the CD95 system. In the following section we discuss the CD95 system with respect to its role in the liver, the colon, and the hematopoietic system.

II. CD95 IN LIVER DISEASE

The liver belongs to the group of many nonlymphoid tissues that are CD95 positive. CD95 is expressed in the developing (French *et al.*, 1996) and the mature (Leithäuser *et al.*, 1993) liver, and primary hepatocytes from mice (Ni, 1994) and humans (Galle *et al.*, 1995) have been demonstrated to be sensitive toward CD95-mediated apoptosis *in vitro*. A physiological role of CD95 in maintaining liver homeostasis has been suggested because mice deficient in CD95 develop—in addition to other abnormalities—substantial liver hyperplasia (Adachi *et al.*, 1995). Moreover, the striking finding of acute hepatic failure in mice on CD95 triggering (Ogasawara *et al.*, 1993) has stimulated general interest in the involvement of CD95-mediated apoptosis in human liver disease.

A. Destructive Liver Diseases

The liver is highly sensitive to induction of apoptosis by the agonistic anti-Fas antibody. Mice injected intraperitoneally with 10–100 μg anti-Fas died within several hours (Ogasawara *et al.*, 1993). Biochemical, histological, and electron microscopic analyses revealed severe liver damage by apoptosis as the most likely cause of death. Subsequent studies have confirmed and extended these data. Transgenic mice expressing human Bcl-2 were protected from CD95-mediated cytotoxicity (Lacronique *et al.*, 1996), in contrast to their nontransgenic counterparts. Likewise, Bcl-2 expression under control of the α1-antitrypsin promoter prevented hepatic destruction after anti-Fas antibody treatment. However, it did not delay death of the animals, demon-

strating involvement of other organs in addition to the liver in CD95-mediated death (Rodriguez *et al.*, 1996a). Furthermore, preservation of liver structure and function has been described for linomide treatment after CD95 stimulation; linomide is a substance capable of inhibiting apoptosis induced by various stimuli (Redondo *et al.*, 1996) and also is used for treatment with caspase inhibitors (Rodriguez *et al.*, 1996b).

These findings suggested that CD95 is involved in apoptosis in the liver and pose the question whether CD95-mediated cell death plays a role in liver failure in man. We investigated a panel of livers that had been explanted in the course of liver transplantation after acute or chronic liver failure due to hepatitis A, B, or C, to alcoholic liver damage, or to Wilson's disease, a copper storage disease (Galle *et al.*, 1995). In all these cases an increased rate of hepatocyte apoptosis was detectable by deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick end-labeling (TUNEL) staining when compared to normal liver. In addition, in all groups of patients, with the exception of those with alcoholic cirrhosis, a strong increase in membranous expression of CD95 was noted by immunohistochemistry.

Further analysis of CD95L mRNA by *in situ* hybridization revealed two distinct patterns of expression related to the underlying cause of liver disease:

1. In viral liver disease CD95L expression colocalized with lymphocytic infiltrates, suggesting that activated T cells might kill hepatitis B virus (HBV) antigen-expressing hepatocytes by CD95/CD95L interaction and, thus, clear HBV from the liver. This is in line with experiments in HBV transgenic mice that pointed to an important role of activated T cells during viral hepatitis (Chisari, 1992). This role has recently been confirmed by demonstrating that interference with the CD95 system using soluble receptor decoys (Nagata, 1995) indeed prevents T cell-mediated liver destruction in HBV transgenic mice. Because HBV-infected individuals can become either chronic virus carriers or can clear the virus, individual differences may exist in induction of liver cell apoptosis by cytotoxic T cells (and natural killer cells). Furthermore, various states of hepatitis exist in which liver cell apoptosis might be dysregulated, possibly involving CD95/CD95L or the CD95 signaling pathway.

2. In contrast to lymphocytic expression of CD95L in viral hepatitis, in patients with toxic damage of the liver due to alcohol consumption (Galle *et al.*, 1995) or as result of excessive copper storage in Wilson's disease (Strand *et al.*, 1998), CD95L mRNA was found to be expressed in hepatocytes. The presence of CD95L mRNA and of CD95 in the same cell points to a new mechanism of liver cell damage. Initial investigation in rats revealed that CD95L is not expressed in liver (Suda *et al.*, 1993). Similar results were obtained by us investigating normal human liver. However, our data demonstrate that in toxic liver damage human hepatocytes can up-regulate the

CD95 system. CD95L might be expressed as a membrane-bound form and might mediate apoptosis as "fraticide" interacting with CD95 on neighboring cells. Alternatively, CD95-mediated apoptosis may occur in an autocrine or paracrine fashion via a soluble form of CD95L. Up-regulation of CD95 in toxic liver damage might involve the p53 tumor suppressor gene, possibly as a result of DNA damage. Treatment of hepatoma cells with DNA-damage agents (Müller *et al.*, 1997) but also with copper results in a transient increase in p53 wild-type expression and in transcriptional transactivation of CD95 expression.

B. Malignant Liver Diseases

In view of the finding that the CD95 and the perforin/granzyme B system mediate T cell cytotoxicity, alterations of the CD95 system could conceivably result in the escape of tumor cells from immune surveillance. Indeed, there have been observations that different tumors, including hepatocellular carcinoma, that had originated from tissues normally expressing CD95 might down-regulate CD95 expression (Leithäuser *et al.*, 1993; Higaki *et al.*, 1996). Loss of CD95 may result in rescued sensitivity of the tumor cells toward the cytotoxic action of T lymphocytes. Analysis of 22 hepatocellular carcinomas indeed revealed complete (9 out of 22) or partial (13 out of 22) loss of CD95 expression (Strand *et al.*, 1996). This suggests a reduced susceptibility to T cell cytotoxicity. Thus, reintroduction of CD95 expression in such tumors might present a tool of therapeutic intervention (Shimizu *et al.*, 1996). Moreover, we investigated the capability of tumor cells to express CD95L as a possible means to actively destroy T lymphocytes. Killing of lymphocytes has been demonstrated as a result of CD95L expression in different nonlymphocytic cells, creating immune-privileged sites (Bellgrau *et al.*, 1995; Griffith *et al.*, 1995) or a state of immune suppression (Badley *et al.*, 1996). In three out of seven hepatocellular carcinomas, CD95L mRNA and protein were present (Strand *et al.*, 1996). Furthermore, in coculture CD95L-positive hepatoma cells killed Jurkat lymphocytes. This suggests a novel mechanism of immune evasion of tumor cells.

III. CD95 IN THE COLON

A. CD95 Expression in Normal, Reactive, and Neoplastic Colonocytes

In humans, CD95 is constitutively expressed at high levels in the entire colonocytic compartment, i.e., enteroblasts, differentiating and senescent

cells, and goblet and columnar cells; the special differentiated cells such as M cells in the dome epithelium covering mucosa-associated lymph follicles also express CD95. There is only one exception: Paneth cells are CD95 negative. Under normal conditions, the Paneth cell is a rare component of crypts in the proximal part of the colon. It is, however, numerically increased in so-called Paneth cell metaplasia, e.g., in ulcerative colitis. CD95 is localized at the basolateral cell surface of enterocytes and absent at the luminal cell surface (Leithäuser *et al.,* 1993). This pattern of expression is retained in inflammatory large bowel disease and also in the great majority of colon adenomas. In colon carcinoma, however, the situation is different. A comprehensive immunohistological study revealed that polarity of expression tends to be lost. Further, in 39% of colon carcinomas CD95 expression was diminished and in 48% of carcinomas, predominantly of the nonmucinous type, CD95 was reduced beyond detection levels. Complete loss of CD95 was more frequent in carcinomas that had already metastasized. Comparative analysis revealed that the aberrant mode of CD95 expression correlated with that of abnormal MHC class I, class II, and invariant chain expression, and also with that of the secretory component (Möller *et al.,* 1994). Comparing the staining intensity of colon epithelial cells *in situ* and cytospin preparations of colon carcinoma cells, the latter have, as a rule, essentially severely down-regulated CD95. The level of CD95 surface expression, however, can be enhanced by IFN-γ and, additively, by TNF-α (Möller *et al.,* 1994). Thus, it looks as if malignant transformation of colon epithelium often leads to down-modulation/loss of CD95. Thus, carcinoma cells either lack CD95 even in the context of heavy lymphohistiocytic stromal infiltration, or CD95 is inducible by IFN-γ/TNF-α.

B. CD95L Expression in Normal and Malignant Colon Epithelia

By *in situ* hybridization, CD95L transcripts have not been found in normal colonocytes (Möller *et al.* 1996; De Maria *et al.,* 1996). The absence of CD95L in colon epithelium was recently confirmed at the protein level using monoclonal antibody (Sträter *et al.,* 1997). Again, there is one exception: Paneth cells express high copy numbers of CD95L mRNA (Möller *et al.,* 1996). Hence, the Paneth cell displays a program that is complementary to that of all other colonocytes. In ulcerative colitis, these patterns are unchanged. This implies that autocrine suicide or paracrine fratricide does not seem to operate in this cellular system. This might be different in malignancy. CD95L transcripts and functional CD95L expression have been demonstrated in colon carcinoma cell lines, e.g., SW480, SW620, and COLO 205 (O'Connell *et al.,* 1996). We were able to reproduce these data (unpublished). Whether CD95L neoexpression also occurs *in vivo* will have to be

shown. Nevertheless, these data open the possibility that common colon carcinoma cell lines are in some state of paralysis as to CD95/CD95L expression. If, however, a cell line expresses CD95L in order to grow, it must have developed a strategy to circumvent CD95L-mediated autocrine suicide. This might be the reason for the relative resistance encountered *in vitro* (see below).

C. Effects of Agonistic CD95 Antibody on Normal Colon Epithelial Cells

To investigate whether normal colonic epithelium enters apoptosis on CD95 ligation, freshly isolated colon crypts have to be examined. This approach is hampered by the fact that freshly isolated crypt cells rapidly enter an anoikis-type of spontaneous apoptosis (Sträter *et al.*, 1996). Anoikis can be inhibited, at least for the differentiated colon epithelium, by immediate embedding of such crypts in collagen gel. Following incubation of these embedded crypts with agonistic CD95 antibody, apoptosis is induced in a time- and dosage-dependent manner. Cells all along the crypt axis are affected, suggesting a similar susceptibility of colonocytes independent of their maturational position within the crypt. Interestingly, goblet cell death occurred with slower kinetics, suggesting a lower susceptibility to CD95-mediated apoptosis. Thus, normal colonocytes are constitutively susceptible to CD95 signaling, the effect of which is death irrespective of the state of maturation (Sträter *et al.*, 1996).

D. Biological Role of General Susceptibility in the Normal State

What might be the physiological role of the general susceptibility of the colon epithelium toward CD95-mediated apoptosis? Presently, there is no satisfying answer to this question. The CD95 system, however, might be relevant in the following context. We showed that intraepithelial lymphocytes in the normal mucosa and in the mucosa affected by ulcerative colitis do not express CD95L (Möller *et al.*, 1996; Sträter *et al.*, 1997). However, in ulcerative colitis, the number of interstitial CD95- and CD95L-expressing mononuclear cells was increased. Many of these cells were situated in close proximity to the epithelium, i.e., located directly beneath the basement membrane. These microareas showed increased apoptotic events in both the mononuclear and the epithelial cell populations. We argued that, by some kind of overshoot reaction among immune cells, epithelial damage may accidentally occur via soluble CD95L meeting the colonocyte by diffusion

through the basement membrane. This may be the mechanism of the development of the early aphthoid lesion typical of ulcerative colitis.

A second relevant case relates to the finding of CD95L-positive Paneth cells. Paneth cells are known to release into the crypt lumen an array of factors, including lysozyme, short antimicrobial peptides called defensins, and TNF-α (Ouelette *et al.*, 1989; Kagan *et al.*, 1994; Keshav *et al.*, 1990; Tan *et al.*, 1993). Nevertheless, soluble CD95L released from Paneth cells obviously does not inflict any mucosal damage. It is conceivable that under normal conditions due to tight junctions, sCD95L cannot reach the basolaterally located CD95 receptor. This might be different, e.g., during viral infection or whenever the polarity of the epithelial cell is lost and CD95 is exposed at the luminal surface, too. In this hypothetical case, luminal soluble CD95L would selectively kill such an abnormal CD95-expressing enterocyte. This hypothesis alludes to the well-known enigma that, compared with the incident rates in stomach and colon, epithelial tumors in the small intestine (where Paneth cells reside in each crypt!) are extremely rare.

E. Susceptibility of Colon Carcinoma Cells toward CD95 Ligation

Quite in contrast to normal colonocytes, colon carcinoma cells constitutively show relative or absolute resistance to CD95-mediated death (Owen-Schaub *et al.*, 1994; von Reyher *et al.*, 1998b). This was initially attributed to the relatively low levels of CD95 surface expression on colon carcinoma cell lines. This view seemed further supported by the fact that IFN-γ increases both CD95 surface levels and susceptibility toward CD95-triggered death (Yonehara *et al.*, 1989; Möller *et al.*, 1994; Owen-Schaub *et al.*, 1994). However, cycloheximide and actinomycin D were also shown to enhance the effectivity of the CD95 antibody (Owen-Schaub *et al.*, 1994). Thus, inhibition of protein synthesis and gene transcription, respectively, also sensitized the cells. We therefore undertook a study to address this issue systematically. Determination of surface CD95 levels and specific death rates under different conditions of pretreatment with IFN-γ, actinomycin D, cycloheximide, and Brefeldin A (inhibition of protein export) revealed that the sensitizing effect of IFN-γ is not explained by an increase of surface CD95 levels. Under pathophysiologically altered conditions, the low levels of CD95 constitutively expressed on colon carcinoma cell lines are sufficient for effective signaling. Our results confirm and extend the data on reversibility of primary resistance to CD95-mediated apoptosis by inhibition of gene transcription, protein synthesis, and export. Kinetics of action suggest that different anti-apoptotic proteins are constitutively expressed in colon carcinoma cell lines, some of which seem to have a half-life of around 10 min (von Reyher *et al.*,

1998b). However, in the colon carcinoma system these antiapoptotic, life-sustaining factors are still undefined. Members of the Bcl-2 family appear to be possible candidates.

F. A Central Role for Protein Kinase C

It has been shown previously that protein kinase C (PKC) plays a role in apoptosis (Cohen and Duke, 1992; McConkey, 1994) and, more specifically, in CD95-mediated apoptosis (Ni *et al.*, 1994). In colon carcinoma cells such as HT-29, COLO 205, and SW480, treatment with phorbol esters inhibited CD95-mediated apoptosis by counteracting IFN-γ-, actinomycin D-, and cycloheximide- but not Brefeldin A-mediated sensitization (von Reyher *et al.*, 1998b). The involvement of PKC in this context was demonstrated by antagonizing the phorbol myristate acetate effects by a specific and selective PKC inhibitor, Goe6983 (von Reyher *et al.*, 1998b). To date, 11 PKC isoenzymes (α, β_1, β_2, γ, δ, ϵ, η, θ, ζ, λ/ι, μ) are known to differ in cellular and subcellular distribution and dependence on Ca^{2+} and activation by different agents (for review see Marcks and Gschwendt, 1996). We could show that inhibition of apoptosis by tetradecanoyl-phorbol-13-acteate (TPA) was five times more efficient in COLO 205 than in HT-29 cells. This correlated with the different amounts of PKCα expressed in both cell lines, suggesting that the α isoform, but not PKCβ_2, δ, ϵ, ζ, or μ, is important in TPA-induced inhibition of apoptosis (Weiss *et al.*, 1998). PKCα has numerous putative targets, including proteins of the ras/raf/Map kinase pathway and caspases.

G. Active Disintegration: A Novel Integral Mechanism of the CD95-Mediated Apoptotic Program of Colon Carcinoma Cells

Enforced disruption of matrix contact of sessile cells was shown to induce apoptosis (Frisch and Francis, 1994). This route to death was termed "anoikis," i.e., death caused by homelessness. The molecular mechanisms of anoikis has been clarified, at least in part. It has been shown that epithelial cells crucially depend on β_1 integrin-mediated contact (Frisch and Francis, 1994; Meredith *et al.*, 1993). β_1 integrins, especially $\beta_1\alpha_5$, the fibronectin receptor, but also collagen receptors, confer proliferative and/or life-sustaining signals to the cell, provided the integrin receptor is bound to its matrix ligands (for review cf. Burridge and Chrzanowska-Wodnicka, 1996). We showed that anoikis is also operative *in vivo:* colonocytes in freshly isolated colonic crypts undergo rapid apoptosis. Anoikis could be prevented by immediate exposure of crypts to collagen. This effect was antagonized by

blocking integrin β_1 antibody (Sträter *et al.*, 1996). Inducing apoptosis via CD95 cross-linking in IFN-γ-pretreated colon carcinoma cell monolayers led to rapid detachment from the culture flask. We observed that apoptosis was initiated while detachment proceeded, and was completed in suspension. We then studied the mechanisms by which dying cells detached. Detachment from hyaluronate was observed to be due to active shedding of the CD44 hyaluronate receptor (Günthert *et al.*, 1996). Other adhesion receptors, including integrins, were also depleted from the cell surface, however, by an alternative process: integrins were removed by membrane blebs. Integrins on blebs were functionally intact. Blebs readily adhered to collagen and collagen binding of blebs was inhibited by blocking integrin β_1 antibody. Inhibition of blebbing in CD95-triggered, dying cells by cytochalasin B effectively interfered with detachment but did not prevent the ongoing apoptotic death (von Reyher *et al.*, 1998a). These data indicate that one consequence of CD95 signaling is induced loss of adhesion, a phenomenon we have termed "active disintegration" (Günthert *et al.*, 1996). By active disintegration the cells remove receptors important for survival signals of the microenvironment.

H. Hypothetical Involvement of the CD95/CD95L System in Formation of Colon Carcinomas

Presently, we cannot point to any definite role for the CD95/CD95L system in normal growth and physiological death of normal colon epithelium. However, taking the above data into consideration, it is tempting to speculate on the putative role CD95 and CD95L might play in the development and progression of colon cancer. In colon adenomas an inflammatory response is observed regularly. Membrane-bound or soluble CD95L, from infiltrated mononuclear cells, might cause apoptotic damage in adenomas similar to that in reactive epithelial cells in colitis. In fact, in adenomas, apoptotic events are highly increased in number (Sträter *et al.*, 1995). CD95-resistant subclones may evolve in progressing adenomas. Interestingly, we observed subsets of CD95 weakly positive or negative cells in a minority of adenomas (Möller *et al.*, 1994). Considering the high frequency of colon carcinomas with down-modulated or abrogated CD95 expression (see above), this acquired abnormality might create a selective growth advantage, likely to favor the survival of malignant subclones within the adenoma, which later might become dominant. Moreover, if malignant transformation may also lead to neoexpression of CD95L in the malignant clone, two major effects would have to be envisaged: (1) Neoplastic cells that have down-regulated expression of CD95 might gain selective advantage. In fact, this mechanism would contribute to the high incidence of CD95-negative colon carcinomas

observed. Likewise, negative cells in which the CD95-signaling pathway is inhibited might be favored. Inhibition of CD95 signaling and survival from apoptosis by active disintegration may contribute to immortality of the malignant cells and to their capacity to metastasize. (2) Expression of CD95L in the neoplastic clone may efficiently counterattack a T cell-driven antitumor immune response (O'Connell *et al.*, 1996).

IV. CD95 AND HEMATOPOIETIC MALIGNANCIES

CD95 was originally detected on lymphoid cell lines. Further analysis of the expression pattern in cell lines and tissues revealed a widespread distribution. Still, the critical role of the CD95 system for growth control is best demonstrated in the immune system, where genetically defined pathology that results from deficiencies in the receptor or the ligand is found (Nagata and Golstein, 1995; Rieux-Laucat *et al.*, 1995; Fisher *et al.*, 1995; Drappa *et al.*, 1996; Debatin, 1996). Consequently a number of studies have addressed the role of CD95 in lymphohematopoietic malignancies. During normal development of T and B cells expression of CD95 is differentially regulated. Although CD95 expression on hematopoietic stem cells is weak, the majority of thymocytes in mice and humans express intermediate levels of CD95 (Barcena *et al.*, 1996; Debatin, 1994; Debatin *et al.*, 1994; Stahnke *et al.*, 1997). However, no definite role for this surface receptor in negative selection of thymocytes involving active triggering of apoptosis has been demonstrated. Maturation of T cells and export to the periphery as naive mature T cells are associated with down-regulation of the receptor, which is reexpressed at high levels following activation of T cells (Klas *et al.*, 1993). No conclusive data are available concerning CD95 expression during early B cell ontogeny (Barcena *et al.*, 1996; Stahnke *et al.*, 1997; DiGiuseppe *et al.*, 1996). However, B cell precursors in human bone marrow as well as circulating naive B cells in the peripheral blood express only low levels of CD95. Similar to T cell activation, activation of mature B cells in secondary lymphoid tissues *in vivo* is associated with increased levels of CD95. In addition to regulation at the level of receptor expression or DISC formation, sensitivity or resistance to CD95 cross-linking by the natural ligand or agonistic antibodies is determined by molecules that may modulate the intracellular signal pathway, such as Bcl-x_L (Reed, 1997; Peter *et al.*, 1998). In contrast to the role of the CD95 system in lymphopoiesis, only limited data are available for hematopoietic cells (Barcena *et al.*, 1996; Stahnke *et al.*, 1997; Maciejewski *et al.*, 1995a,b; Robertson *et al.*, 1995). Several reports have described increased expression of CD95 with increasing maturation of myeloid cells and increased expression of CD95 on activated hematopoietic

progenitors. The finding of increased CD95 expression on myeloid progenitor cells in diseases such as aplastic anaemia and myelodysplasia suggests a role for CD95-mediated apoptosis in diseases of bone marrow failure (Lepelley *et al.*, 1996; Gersuk *et al.*, 1996; Maciejewski *et al.*, 1995a,b).

Corresponding to the sensitivity of activated T cells for CD95-induced apoptosis, strong expression of the CD95 cell surface receptor and CD95 sensitivity is found in leukemic cells from patients with HTLV-I-induced adult T cell leukemia (ATL) (Debatin *et al.*, 1990, 1993). Thus ATL cells exhibit the apoptosis-sensitive phenotype found in long-term activated T cells (Klas *et al.*, 1993). CD95 is also expressed in the majority of acute leukemias that represent precursor T cell phenotypes, such as in childhood acute leukemia/lymphoma (ALL) (Kondo *et al.*, 1994; Debatin and Krammer, 1995; Lücking-Famira *et al.*, 1994). However, the majority of samples derived from patients with T-ALL are resistant toward CD95-induced apoptosis, resembling the relative resistance of most human thymocytes with intermediate levels of CD95 expression. A number of studies performed in hematologic malignancies, including acute lymphoblastic leukemia of precursor B cell phenotype, acute myeloid leukemia, chronic B cell leukemia, chronic myelogenous leukemia, non-Hodgkin's lymphoma, Hodgkin's disease, and plasma cell disorders, have found variable expression in these diseases (Chauhan *et al.*, 1997; Hata *et al.*, 1995; Panayiotidis *et al.*, 1995; Owen-Schaub *et al.*, 1995; Kondo *et al.*, 1994; Selleri *et al.*, 1997; Dirks *et al.*, 1997; Robertson *et al.*, 1995; Shima *et al.*, 1995; Wang *et al.*, 1997). In general, low levels of CD95 expression are detected in many patient samples, although strong expression is only found in minor subpopulations. This is a somewhat surprising finding because most lymphoid and myeloid cell lines that have been originally derived from patients with leukemia, lymphoma, or plasmocytoma express intermediate to high levels of CD95. However, many CD95-expressing cell lines are resistant to CD95-triggered apoptosis similar to normal cells. Studies on constitutive expression of CD95 in clinical tumor samples may be misleading because several factors, including cytokines, may be able to up-regulate receptor expression. Thus IFN-α and IFN-γ as well as TNF have been demonstrated to induce increased levels of receptor expression (Fellenberg *et al*, 1997; Maciejewski *et al.*, 1995b) and cytotoxic drugs have also been implicated in the activation of the system (see below).

Failure to up-regulate CD95 expression or defects in the receptor, the ligand, or the signal molecules may result in apoptosis defects in tumor cells. Blockade of CD95-mediated apoptosis *in vivo* may be caused by the production of soluble receptor variants, by alternative splicing, that counteract the apoptosis-inducing signal, or, alternatively, may be due to mutations in the receptor. Increased levels of soluble CD95 have been found in some patients with T and B cell leukemias but not in myeloid leukemias, despite

CD95 expression on the malignant cells (Munker *et al.*, 1996; Knipping *et al.*, 1995). Mutations of the CD95 molecule in *lpr* mice and CD95L in *gld* mice constitute the first description of a pathology associated with an apoptosis gene defect (Nagata and Golstein, 1995). However, neither *lpr* nor *gld* mice develop lymphoid or hematopoietic malignancy but rather suffer from a syndrome of lymphoproliferation due to the inability to delete long-term activated T cells. The disease of *lpr* and *gld* mice is recapitulated in a group of patients with lymphoproliferation and autoimmunity in which mutations of CD95, primarily located in the death domain of the molecule, have been found (Rieux-Laucat *et al.*, 1995; Fisher *et al.*, 1995; Drappa *et al.*, 1996). Patients develop extensive lymphadenopathy and hepatosplenomegaly with autoimmune disease, such as immune thrombocytopenia. So far no detailed analysis in mutations of CD95 and CD95L in leukemias or lymphomas has been performed. However, in an analysis of 20 T-ALL samples we found only two mutations in the extracellular domain of the receptor and the promoter, but no mutations in the CD95 death domain (Beltinger and Debatin, 1998). Because most T-ALL samples from patients are resistant toward CD95-induced apoptosis, this would suggest that other factors may determine CD95 resistance in these cells.

The CD95 System and Anticancer Drugs

Since the introduction of the folic acid antagonist aminopterin (methotrexate) into leukemia therapy by the pediatric oncologist Sidney Farber (Farber *et al.*, 1948) almost 50 years ago, cancer treatment of leukemias and solid tumors has been turned from a mostly unsuccessful therapeutic attempt into an effective therapeutic approach in some malignancies. Especially in the treatment of leukemias and pediatric solid tumors the majority of patients can be cured by therapeutic protocols that use combinations of different cytotoxic drugs given over a certain period of time. In addition to sophisticated high-dose therapy protocols, adjuvant chemotherapy has become an important treatment option in cancers of colon and breast. However, the widespread use of cytotoxic drugs, the prototypes of which were mostly developed in the 1960s and 1970s, also revealed that only some tumors are chemosensitive. Especially the most prevalent malignancies have been proved to be more or less resistant to chemotherapy (and irradiation). In addition, the side effects of chemotherapy limit the development of megadose protocols for tumors resistant to conventional doses of cytotoxic drugs. Resistance to chemotherapy, however, also still is a major problem, even in cases involving chemosensitive tumors such as leukemias or sarcomas, for which the majority of relapse patients present with tumor cells that are much more resistant to chemotherapy than are the primary cells.

How does chemotherapy operate? Anticancer drugs have primarily not been designed for their cytotoxic effect but have been found in assays that are based on inhibition of proliferation and clonogenicity. Further analysis of compounds found in these screening assays then demonstrated that most of the active substances somehow interfered with metabolism, mitosis, or DNA replication. Consequently, cytotoxic drugs have been classified as DNA-damaging agents, such as cyclosphosphamide, cisplatin, and doxorubicine, as antimetabolites, such as methotrexate and 5-fluorouracil, as mitotic inhibitors, such as vincristine, as nucleotide analogs, such as 6-mercaptopurine, or as inhibitors of topoisomerases, such as etoposide. However, studies of anticancer drugs largely neglected the fact that cells in which proliferation was inhibited or metabolism was disturbed have to die to be removed. Thus, in retrospect it is not surprising that it was found that most cytotoxic drugs used in anticancer therapy today kill their target cells by induction of apoptosis (Dive *et al.*, 1992; Gorczyca *et al.*, 1993; Kaufmann *et al.*, 1993; Fisher, 1994). However, although induction of apoptosis has been shown following treatment of different tumor cells with different cytotoxic drugs and gamma-irradiation, molecular mechanisms other than toxicity have not been defined until recently. With the concept that some drugs may be cytotoxic through DNA damage, p53 as the guardian of the genome has come into play, suggesting that p53 may drive and activate the apoptosis machinery following treatment with, e.g., DNA-damaging agents (Lowe *et al.*, 1993; Miyashita and Reed, 1995). Thus, p53 has been shown to be involved in various forms of apoptosis induced by cellular stress (Levine, 1997). Although p53 may represent a cellular master switch that regulates several distinct cellular responses, evidence suggests that key downstream elements of the apoptosis machinery are directly involved in apoptosis induced by anticancer drugs. Thus, resistance toward chemotherapy in some cases has been found to be associated with increased levels of expression of antiapoptotic molecules of the Bcl-2 family, such as a Bcl-2 and Bcl-x_L (Miyashita and Reed, 1993; Dole *et al.*, 1995; Sumantran *et al.*, 1995; Minn *et al.*, 1995). Although the levels of Bcl-2 expression, e.g., in lymphoid tumors, did not clearly correlate with clinical response to chemotherapy, reduced expression of Bax was found to be associated with poor outcome in breast cancer (Hermine *et al.*, 1996; Coustan-Smith *et al.*, 1996; Krajewski *et al.*, 1995). p53 was found to be involved in transcriptional expression of the proapoptotic Bax gene following DNA damage. Thus some forms of chemotherapy-induced apoptosis mechanistically seem to involve up-regulation of Bax expression following p53 activation through DNA damage, which leads to activation of the apoptosis program (Miyashita *et al.*, 1994; Miyashita and Reed, 1995).

Recent additions to this scenario now suggest a major role for the CD95 system in the process of drug-induced apoptosis. Cytotoxic drugs common-

ly used in effective chemotherapy of, e.g., leukemias strongly induce CD95L expression in CD95-positive tumor cells following drug treatment (Hata *et al.*, 1995; Müller *et al.*, 1997). CD95L is produced, expressed in a membrane form, or secreted by the tumor cells exposed to the drug. Binding of CD95L to the receptor then triggers the apoptosis cascade in chemosensitive cells. This scenario reflects the activation-driven death in activated T cells following T cell receptor stimulation (Stahnke *et al.*, 1997). Triggering of CD95/CD95L interaction by anticancer drugs was originally discovered in leukemia cells but was also found to be involved in drug-induced apoptosis in other chemosensitive tumors, such as hepatoblastoma, neuroblastoma, and brain tumors (Hata *et al.*, 1995; Müller *et al.*, 1997; Micheau *et al.*, 1997; Fulda *et al.*, 1997). In some tumors, in addition to induction of CD95L expression, treatment of tumor cells with cytotoxic drugs also induces upregulation of CD95, which seems to depend on p53 (Munker *et al.*, 1995, 1997). So why are tumors chemosensitive? A summary of the recent discoveries would suggest that regardless of the primary target by which the cytotoxic drug initiates the cell death program in the tumor cell, activation of the key effectors for apoptosis, such as the caspase cascade, is crucial for the antitumor effect, and inhibition of caspase activation in tumor cells is associated with resistance against anticancer drugs (Los *et al.*, 1997; Kaufmann *et al.*, 1993; Bellosillo *et al.*, 1997). Although it is not entirely clear whether this concept applies to all drugs, apoptosis induced by glucocorticoids may use a different effector system (Geley *et al.*, 1997). The initial event (phase I) may target diverse cellular functions such as metabolism, DNA, or the mitotic apparatus. This cellular damage is sensed by p53 and/or leads to activation of cellular stress programs (Herr *et al.*, 1997). The consequence of this phase II events is the activation of the relatively uniform apoptosis program of the cell in which CD95L/CD95 interaction represents one of the important trigger mechanisms for the apoptosis cascade leading to protease activation (phase III). However, CD95 and CD95L may represent only a part of an amplifier machinery, because drug-induced induction of other apoptosis-inducing ligands (TNF-α and TRAIL) has also been found (Jeremias *et al.*, 1998). Alterations of the initial phase, the amplifier, or the execution phase may lead to drug resistance. Thus, in addition to established mechanisms of drug resistance, such as increased drug efflux by membrane pumps, the failure to activate apoptosis programs due to mutations or functional alterations of key molecules and pathways represents another mode of drug resistance in tumor cells (Friesen *et al.*, 1996; Knipping *et al.*, 1995). In this respect the recent study showing a favorable outcome for patients with CD95-positive acute myeloid leukemia (AML) undergoing chemotherapy as compared to CD95-negative AML seems to demonstrate that studies of CD95 expression in tumors in relationship to therapy may be clinically relevant (Min *et al.*, 1996). In addition to the importance of CD95/CD95L in-

teraction for effective chemotherapy, both molecules also participate in reciprocal interactions with cells of the immune system (Debatin, 1997). Thus CD95-positive tumor cells may become targets for killer T cells, NK cells, or LAK cells (Micheau *et al.*, 1997; Yoshihiro *et al.*, 1997). Factors that up-regulate CD95 expression in tumor cells, such as cytotoxic drugs or cytokines, may therefore be involved in induction of tumor regression by immune cells if these cell resist the attack by CD95L, which may be produced by the tumor cell.

V. CONCLUSIONS

In this review we have discussed the role of the CD95 system in homeostasis of the immune system and of the liver. So far, no obvious role of the CD95 system has been seen for the organization of the tissue of the normal colon. In addition, we have discussed the role of the CD95 system for liver disease, colon disease, and malignancy, and for malignant lymphoid disorders. Finally, the eminent role of CD95 in chemotherapy as recently elucidated has been amply discussed. Although our discussions are primarily focused on the CD95 system, it is obvious that, as other cell death systems are studied in more detail, their role in the above processes will become apparent.

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