ADVANCES IN CANCER RESEARCH

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FOUNDATIONS IN CANCER RESEARCH

CHROMOSOMES AND CANCER: THE EVOLUTION OF AN IDEA

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I. Introduction

This review is an attempt to summarize, from a relatively narrow personal viewpoint, developments in our thinking about the relationship between chromosome abnormalities and cancer over the last century and the ways in which chromosome studies have helped us to understand the fundamental nature of the neoplastic process. There is no attempt to present a complete history of all the important contributions and contributors but rather to offer the impressions and insights gained by one participant in the field through more than 40 years of cancer research, much of it concerned with the cytogenetics of tumors. For some of the information on the earliest workers, I am indebted to previous reviews (German, 1974; Hungerford, 1978; Hsu, 1979; Sandberg, 1980; Heim and Mitelman, 1987).

II. Boveri and Early Studies (Before 1950)

The first suggestions of a relationship between chromosome abnormalities and tumor development date to the latter part of the nineteenth century, when improvements in histopathological techniques led to the recognition of gross mitotic abnormalities in tissue sections from many different human malignancies. In 1879, the German pathologist Arnold described this phenomenon, and even included illustrations showing aberrent mitoses (Arnold, 1879). Subsequently, another German pathologist, von Hansemann, specifically suggested that such mitotic abnormalities might play a

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significant role in the initiation and further development of human tumors (von Hansemann, 1890). This was very shortly after the terms *chromosome* and *mitosis* were first introduced (Waldeyer, 1890; Flemming, 1889).

However, it was a biologist, Theodor Boveri, who first clearly enunciated a number of hypotheses concerning the role of somatic genetic alterations in the development of cancer, and a remarkable number of these have subsequently proved correct. In his studies of sea urchins, Boveri noted that mitotic abnormalities such as multipolar mitoses in doubly fertilized eggs often led to abnormal development of the organism. He extrapolated from this observation, first in 1902 (Boveri, 1902) and with more detail in 1914 (Boveri, 1914), to postulate prophetically that the development of mammalian tumors might similarly be initiated by aneuploid chromosome complements resulting from mitotic irregularities. Although he admitted to having no direct knowledge of cancer, he then extended his hypotheses to include such important ideas as the genetic instability of tumor cell populations (which he linked to spindle defects and resultant multipolar mitoses); the unicellular origin of tumors; the suggestion that defective response to external growth regulation could result from either gain or loss of critical chromosomes; and the possibility that in some cases the key genetic alterations in tumors might be submicroscopic, not involving entire chromosomes. It is truly remarkable that this spectrum of ideas, which now constitutes the basis for much of our thinking concerning the fundamental nature of human malignancy, could have been produced from the very limited experimental observations of one scientist.

Like many hypotheses that precede the development of techniques for adequate testing, Boveri's visionary concepts were largely deplored, or at least ignored, during the next 30 years. There were a few additional studies describing nuclear abnormalities and alterations in chromosome number in human tumors (Farmer *et al.*, 1906; Kemp, 1930; Levine, 1930; Ortiz Picón, 1930). Also, Bauer (1928) offered his own mutational theory of tumorigenesis, and Winge (1930) extended Boveri's suggestion of the unicellular origin of tumors to introduce the stemline concept, which would be fully defined several decades later from studies with ascites tumors. In general, however, techniques were simply not adequate for accurate counting or characterization of mammalian chromosomes, and so in a period when the human chromosome number of 48, as determined by Painter (1921), was confidently accepted, it is not surprising that other workers, such as Belling (1927), were reporting the chromosome number of human tumors as normal.

The situation did not change significantly in the 1930s and 1940s,

although many of the critical methodological developments, which ultimately came together in the 1950s, were first introduced. There were some improvements in tissue culture techniques for both solid tumors and leukemias (Kemp, 1930; Chrustchov *et al.*, 1931; Chrustchov and Berlin, 1935; Gunz, 1948), and also colchicine was first used to arrest cells in metaphase (Blakeslee and Avery, 1937; Levan, 1938). Several workers in the 1930s also discovered the value of hypotonic solutions in causing further disruption of the mitotic spindle and swelling of the cells to generate better metaphase preparations (Chrustchov *et al.*, 1931; Chrustchov and Berlin, 1935; Slifer, 1934; Lewis, 1934). Unfortunately, the potential value of these various technical improvements was not widely appreciated or utilized. This was particularly true of the techniques for culturing both normal and leukemic leukocytes, developed by several groups of Russian investigators in the 1930s, which produced usable chromosome preparations from the dividing cells (Chrustchov *et al.*, 1931; Andres and Shiwago, 1933). Regrettably, the studies of these Soviet workers were interrupted by the pseudoscientific onslaught of Lysenkoism, and their use of hypotonic solutions and other technical details had to be rediscovered, independently, decades later in other countries.

During this period (1930–1950), there were a few additional papers on the chromosome complement of both human and experimental tumors, perhaps most extensively by Koller (1947), and in general the chromosome numbers were found to be abnormal (Biesele *et al.*, 1942; Ludford, 1942; Barigozzi and Dellepiane, 1947; Gunz, 1948; Polli, 1949). Also during this period, Furth and Kahn (1937) were able to demonstrate the passage of a transplantable leukemia from one mouse to another by a single cell, adding experimental support to Boveri's hypothesis that tumors could arise from one cell. However, as in earlier decades, the quality of the chromosome preparations from neoplastic material did not permit identification of any specific karyotypic alterations, and it was only with the major technical advances of the 1950s that real evidence to support Boveri's concepts began to emerge.

III. The Stemline Concept and Modern Cytogenetics (the 1950s)

The 1950s saw two major developments that set the stage for the first adequately documented information on the karyotypic abnormalities in neoplastic cells. Interestingly, these came from two different sources: experimental tumors in animals, and normal human tissues.

The first development was the use, for chromosome studies, of long-

established transplantable rodent tumors that were being carried in the ascites form. In Japan, Makino (1957) utilized the ascites sarcoma of the rat that had been developed a few years earlier by Yoshida (1949). Similar studies were carried out by Levan and Hauschka (1952) in the United States, using mouse ascites tumors, a number of which had been established by George Klein (1951). Using "squash" techniques that had been developed in other cytogenetic studies, these workers were able to demonstrate multiple karyotypic alterations in these late-stage neoplasms. More importantly, in some cases it was possible to identify one or more characteristic chromosome abnormalities in all the cells of a given tumor, providing the first real evidence for what was then called the stemline concept and later developed into the clonal evolution model of tumor development. The presence of the same abnormal chromosome in all cells supported the original suggestion by Boveri that tumors arose from a single altered cell, and variations in the chromosome complement from cell to cell added weight to the view, enunciated earlier by Winge (1930), that the stepwise clinical and biological progression of tumors over time could result from the acquisition of additional somatic genetic changes in developing tumor populations. Later in the decade, these conclusions concerning stemlines were given added weight when similar cytogenetic findings were reported in established tissue culture lines by Chu and Giles (1958) and, more importantly, in primary radiation-induced tumors by Ford and Clarke (1963) and ultimately in human tumors by Makino *et al.* (1964).

Meanwhile, some of the same workers, as well as others, were investigating nonneoplastic human and other mammalian tissues and developing improved culture techniques that brought us finally to the era of modern cytogenetics. The use of colchicine to arrest cells in mitosis and of hypotonic solutions to disrupt the mitotic spindle and expand the cells was rediscovered by Hughes (1952), Makino and Nishimura (1952), and particularly Hsu (1952). This resulted in chromosome preparations of sufficiently good quality that the normal human chromosome number of 46 was finally accurately established 4 years later by Tjio and Levan (1956) and quickly confirmed by Ford and Hamerton (1956). These improved cytogenetic methods were promptly applied to various pathological conditions and soon allowed the identification of specific constitutional chromosome abnormalities associated with particular human disorders (e.g., Down's syndrome, Turner's syndrome) (Lejeune, 1959; Ford *et al.*, 1959). Also, a limited number of human tumors were quickly examined with these newer methods, especially leukemias, but initially no specific cytogenetic alterations were identified. It was still possible, as late as 1960, for Bayreuther, having investigated a wide spectrum of

tumors, to conclude that the chromosome complement of most human neoplasms was normal (Bayreuther, 1960).

It was at this point, serendipitously, that I entered the field of tumor cytogenetics, and so subsequent sections of this review will be even less of an objective review and more obviously represent personal involvement and perspective.

IV. Early Findings in the Pre-banding Era (the 1960s)

In 1956, I joined the faculty of the University of Pennsylvania, after 2 years of research in the Navy on radiation carcinogenesis and bone marrow transplantation. I began studying human leukemic cells *in vitro,* using a short-term culture technique developed by Dr. Edwin Osgood (Osgood and Krippaehne, 1955). The cells were grown on small slides, and I rinsed them with tap water prior to staining them with Giemsa for study. This represented one more inadvertent rediscovery of the hypotonic technique and resulted in the recognition of metaphases with countable chromosomes in my preparations. I knew nothing of cytogenetics, but found a graduate student, David Hungerford, who was attempting to obtain material for a thesis on human chromosomes.

This promptly led to a collaboration in which we, simultaneously with other laboratories around the world, began to use the modern cytogenetic techniques to look for specific chromosome abnormalities in human leukemias. In early studies of acute myelogenous leukemia, we found no consistent abnormality, but the results with chronic myelogenous leukemia (CML) were more encouraging. David Hungerford identified a characteristic small chromosome in the neoplastic cells of two patients (Nowell and Hungerford, 1960a), and then when we began, with the help of Dr. Paul Moorhead (Moorhead *et al.,* 1960), to use an improved air-drying technique developed by Rothfels and Siminovitch (1958), we reported a series of seven patients who all had the minute chromosome (Nowell and Hungerford, 1960b). The abnormality was identified as involving a chromosome in group G, the smallest of the autosomes, but with the techniques then available we could not determine whether it resulted from a deletion or from translocation. The finding was promptly confirmed by the Edinburgh group (Tough *et al.,* 1961), who were also studying human leukemias, and they designated it the Philadelphia chromosome (Ph^1), in accord with the First International Conference on Cytogenetic Nomenclature, which had suggested that abnormal chromosomes be named for the city of origin (Denver Conference, 1960).

We felt that this observation of a consistent somatic genetic change in

nearly all cases of a specific human neoplasm, and in all the cells of the tumor, strongly supported Boveri's suggestion that a tumor could arise from a critical genetic alteration in a single cell, providing a growth advantage as the progeny of that cell expanded into a recognizable neoplasm. However, our own studies in other leukemias, as well as investigations by others, in the years immediately before and after the identification of the Philadelphia chromosome, failed to find other *consistent* cytogenetic alterations (Nowell and Hungerford, 1964; DeGrouchy, 1967). For example, an abnormal chromosome noted in chronic lymphocytic leukemia in New Zealand, and designated the Christchurch chromosome, proved not to be a consistent finding (Gunz *et al.*, 1962).

Some additional information was developed on a few human solid tumors during this period by Lubs and Clark (1963), Lubs and Salmon (1965), Miles (1967), Sandberg (1966), and others, and it became apparent that in nearly all instances the chromosome pattern was abnormal. Furthermore, particularly in far-advanced tumors and malignant effusions, extensive alterations were found, with chromosome numbers in the hypotetraploid range and gross structural rearrangements producing readily recognizable marker chromosomes. There was often a definite correlation between the extent of the cytogenetic changes and the degree of clinical progression of the tumor (Atkin and Baker, 1966; DeGrouchy, 1967; Miles, 1967). As had been observed earlier in the ascites tumors, a clonal type of growth was frequently observed, with the tumor consisting of a single stemline of cells or several closely related sublines. However, this pattern was somewhat less common in the solid neoplasms than in the leukemias, and a wide distribution in the number and alterations of the chromosomes in a given tumor, as examined by the direct preparative techniques then available, was not uncommon (Lubs and Salmon, 1965; Miles, 1967). Most importantly, when stemlines were present, they typically differed from case to case, and no chromosomal change comparable in consistency to the Philadelphia chromosome was observed. There were a few types, including tumors of the ovary, testis, and meninges (Atkin *et al.*, 1967; Martineau, 1966; Zang and Singer, 1967), in which a proportion of the cases appeared to show a characteristic abnormality, but this was not sufficiently common to be totally persuasive.

Part of the difficulty was methodological. Although technical improvements continued during the 1960s, including the development of a more efficient hypotonic solution by Dr. Hungerford and co-workers and the use of phytohemagglutinin-stimulated lymphocyte cultures as an easy source of mitoses from nonneoplastic cells (Moorhead *et al.*, 1960; Nowell, 1960), it was still impossible to identify each human chro-

mosome individually, and the often poor technical quality of metaphases from tumor material, as opposed to normal cells, made even accurate counting sometimes difficult.

Despite these limitations, another area of investigation that ultimately lent further support to a general relationship between chromosome abnormalities and cancer was initiated in the 1960s. This was the recognition that certain individuals with inherited clinical syndromes (e.g., Bloom's syndrome, ataxia telangiectasia, Fanconi's anemia) showed increased spontaneous chromosome breakage in standard cytogenetic preparations made from their circulating normal lymphocytes (German *et al.*, 1965; Harnden, 1974). Since these individuals also showed an elevated incidence of leukemia and other types of malignancy, it seemed likely that this increased tendency toward the development of chromosomal abnormalities in their somatic cells was a significant tumorigenic factor (Harnden, 1974; Hecht and McCaw, 1977; German, 1983a).

Thus, by the end of the 1960s, there was general agreement that most human tumors had chromosome alterations and that these tended to be more extensive in more advanced neoplasms. However, a lack of consistent abnormalities associated with specific tumors resulted in the widespread view that these alterations (even including the Philadelphia chromosome) were probably the result rather than the cause of the neoplasm. Some investigators felt that they played a significant role in the *progression* of malignancy, but others believed that they might simply be epiphenomena with respect to tumorigenesis.

It must be remembered that this was an era in which the field theory of tumor development was still widely held. This theory speculates that neoplasms arise from many cells in a tissue made susceptible by exposure to carcinogenic agents. It was also a time when most investigators did not favor a mutational basis for tumors, whether unicellular or multicellular in origin. In part, this may have reflected the hope, more emotional than scientific, that if cancer did not arise from structural changes in the genome, reversibility of the neoplastic state might be more easily achieved. And so it was not until the 1970s that the techniques that generated more definitive evidence, at least at the level of the light microscope, for some of the mutational hypotheses originally enunciated by Boveri were finally developed.

V. Chromosome Banding and Clonal Evolution (the 1970s)

The report by Caspersson *et al.* (1970) of a successful technique for fluorescent banding of mammalian chromosomes ultimately revolu-

tionized the field of tumor cytogenetics and, by extension, general thinking about the fundamental nature of carcinogenesis. The various banding techniques developed in the early 1970s (Yunis and Sanchez, 1975) finally permitted the identification of each individual chromosome and allowed the recognition of small translocations and other rearrangements (Whang-Peng, 1977).

These methods were rapidly applied to a variety of human tumors with startling results. For example, Dr. Janet Rowley and colleagues in Chicago demonstrated that in CML the Philadelphia chromosome resulted from a translocation between chromosomes 9 and 22 (Rowley, 1973). They also identified a number of other characteristic translocations associated with particular subgroups of human acute leukemia and noted the nonrandom occurrence of isochromosome 17, deletion of the long arm of chromosome 7, and extra dosage of the long arm of chromosome 1 in various leukemias and other tumors (Rowley, 1975). In Sweden, Manolov and Manolova (1972) found a consistent alteration of chromosome 14 in Burkitt's lymphomas (14q+), which was later shown by Zech *et al.* (1976) to result from a translocation between chromosomes 8 and 14. A number of other workers in Sweden and elsewhere extended the observations on chromosome 14 to other lymphoid tumors (Mitelman, 1981) and also identified some of the first nonrandom chromosome changes in human solid tumors, such as monosomy 22 in meningiomas (Mark, 1977). In Belgium, Van den Berghe *et al.* (1974) reported a deletion of chromosome 5 (5q−) associated with a specific preleukemic refractory anemia syndrome, and a consistent interstitial deletion of chromosome 13 in retinoblastoma was reported by Wilson *et al.* (1973).

The new banding techniques were also applied to various experimental systems, and the Swedish group, for example, identified consistent cytogenetic changes in sarcomas of mice and rats, including evidence of additional karyotypic changes associated with progression to more aggressive tumors (Mitelman, 1980).

Thus, within a very few years after the introduction of banding technology, enough evidence was accumulated to indicate strongly that a high proportion of human and experimental tumors were characterized by specific cytogenetic alterations (Yunis, 1983), finally providing strong support for the Boveri hypothesis that somatic genetic changes, often visible as chromosomal abnormalities, were involved in the causation of cancer (Cairns, 1981). There remained some skeptics, however (DiPaolo, 1975), whose views were buttressed by the fact that there were still some neoplasms in which karyotypic changes were not demonstrable, as for example, approximately 50% of human acute leukemias (Whang-Peng,

1977). There were also no techniques to actually identify any of the genes involved. The frequency of karyotypically "negative" cases was subsequently reduced by the introduction of high-resolution banding methods by Yunis and Sanchez (1975) and others, but it remains clear, even today, that some neoplasms do not have genetic alterations demonstrable at the level of the light microscope.

Another result of the improved cytogenetic methods of the 1970s was further refinement of the clonal evolution view of tumor development. This was seen not only in experimental systems, as noted previously, but also in human leukemias, particularly CML (Mitelman *et al.*, 1976), as well as a few solid tumors (Orye and Delbeke, 1974). Reports of sequential acquisition of additional karyotypic changes in association with biological and clinical progression began to appear. These reports supported the stemline concept developed earlier from the studies of ascites tumors. They linked these observations to the phenomenon of tumor progression in patients—the tendency of neoplasms to become more aggressive in their behavior and more malignant in their characteristics in a stepwise fashion during their life history, a clinical process defined most clearly, in the 1950s, by Foulds (1957) and also discussed at that time by Furth (1953) and by George and Eva Klein (1957).

Furthermore, these improved cytogenetic studies of tumor material, as well as of tumor cell lines in tissue culture, provided further evidence that malignant cells were more genetically unstable than their normal counterparts, and so more likely to undergo such sequential somatic genetic alterations. As early as the 1960s, there had been some data indicating that neoplastic cells in culture were more susceptible than their normal counterparts to both spontaneous and induced mutations and chromosome breakage (reviewed by German, 1983b). This led Nichols (1963) and others to speculate that an early step in carcinogenesis might be the activation of a mutator locus comparable to those described in *Drosophila* and certain plants and perhaps related to one or more of the genetic defects in the human chromosome fragility syndromes mentioned earlier. These hypotheses were expanded in the 1970s (German, 1983b), including the possibility that genetic instability in tumor cells might result from acquired defects in DNA synthesis (Loeb *et al.*, 1975) as well as repair. Since this was a time when basic cancer research was focusing heavily on oncogenic viruses, it was also suggested that incorporation of viral genes might be a basis for instability in human tumor cells. Unfortunately, none of these suggested mechanisms were supported by adequate data from human material.

Nevertheless, when taken together, all these various lines of investigation led to more detailed expression of the view that tumors arise from a

single mutated cell; that biological and clinical progression results from subsequent *additional* genetic alterations, often visible cytogenetically, giving rise to more aggressive cell populations within the original neoplastic clone; and, morever, that the likelihood of such sequential changes in tumors are enhanced by increased genetic instability in these cells, acquired as part of the neoplastic process. Although, as noted, there remained some resistance to the basic idea that tumors result from somatic genetic change, the initial reaction to this more extensive clonal evolution concept, as published in 1976 (Nowell, 1976), was generally favorable despite its rather pessimistic implications for simple answers to cancer causation or therapy.

Another important result of the development of the chromosomal banding techniques in the 1970s was the emergence of useful clinical applications of such studies in human tumors, and particularly for leukemias. There had been a few such diagnostic and prognostic efforts in the 1960s, even including some pre-neoplastic disorders (Lubs and Clark, 1963; Spriggs *et al.,* 1962; Nowell, 1965; Enterline and Arvan, 1967), but without the ability to identify specific abnormalities (except perhaps the Philadelphia chromosome), these had little practical value. When the banding methods allowed the identification of specific alterations associated with many subgroups of leukemia, large clinical investigations on the prognostic significance of particular karyotypic abnormalities, particularly in the childhood acute leukemias (Third International Workshop on Chromosomes in Leukemia, 1981; Fourth International Workshop on Chromosomes in Leukemia, 1984), began to be undertaken. These techniques, including related molecular approaches, have now been extended as techniques have improved and as optional therapies have become more widely utilized. This aspect will receive further comment in the final section.

VI. Molecular Cytogenetics (the 1980s)

As chromosome banding techniques continued to improve through the 1970s, recombinant DNA technology was also being developed. This finally made it possible, in the 1980s, to use the clues provided by specific nonrandom cytogenetic alterations in human tumor cells to help look for the growth regulatory genes involved and the mechanisms by which the function of these genes was altered. As a result, molecular dissection of various translocations, deletions, chromosomal additions, and microscopically visible gene amplification units has been actively pursued for the last decade. Several of the oncogenes previously identified in experimental systems, such as *myc* and *abl,* were promptly identified as being

involved in the chromosome abnormalities of certain human tumors. As work progressed, it became clear that many other genes that are oncogenic in experimental tumors do not play a significant role in human lesions, leading some molecular geneticists to hope for relatively simple answers to human cancer (Weinberg, 1982; Bishop, 1983). Unfortunately, however, the molecular investigation of nonrandom karyotypic alterations has now led to the recognition that there is a very large number of other, previously unknown, oncogenes and tumor suppressor genes that *are* important in the pathogenesis of many different human malignancies, introducing degrees of complexity previously unimagined.

My involvement in these studies resulted from a collaboration with Dr. Carlo Croce in which we used a combination of cytogenetic, molecular genetic, and somatic genetic techniques to explore chromosome translocations in lymphoid tumors. Initially, we investigated the t(8;14) translocation of the Burkitt's lymphoma and, along with the contributions from a number of other laboratories (Erikson *et al.*, 1983; Leder *et al.*, 1983), helped to demonstrate that the c-*myc* gene from chromosome 8 is brought into juxtaposition with a transcriptionally active immunoglobulin heavy chain locus on chromosome 14, resulting in deregulation of the oncogene. Similar studies, by us and others, were pursued throughout the 1980s in a wide variety of B cell and T cell tumors, providing more information on the molecular basis of these neoplasms than on any other group of human malignancies (reviewed by Nowell, 1992). Nearly two dozen translocations have now been investigated in lymphoid tumors, with cloning of the chromosomal breakpoints and at least partial characterization of the genes involved, with some surprising findings. The only previously known oncogene in these many translocations is c-*myc*, which has been shown to be involved in a subset of aggressive T cell leukemias as well as in the pathogenesis of the Burkitt's lymphoma. In addition, some eight or ten previously unknown genes have now been isolated and characterized from different translocations, each associated with a subset of B cell or T cell tumors. Perhaps the best characterized is BCL-2, a gene normally located on chromosome 18, which is deregulated in a t(14;18) translocation that characterizes a high proportion of low-grade follicular lymphomas (Tsujimoto *et al.*, 1984; Cleary and Sklar, 1985; Bakhshi *et al.*, 1985). The BCL-2 gene product appears to be localized to mitochondrial membranes but, to the extent that they have been characterized, most of the other new oncogenes in these lymphoid tumor translocations seem to be DNA-binding proteins, like *myc*. More remarkable, unlike *myc*, all these new oncogenes appear to be highly lineage specific, restricted to either the B cell or the T cell in

their tumorigenic role, and also to have different effects in determining the degree of malignancy (Klein, 1991).

As these interesting findings with lymphoid tumors were compiled, similar attempts to combine cytogenetics with molecular genetics were applied to certain myeloid leukemias and nonhematopoietic tumors. Groffen *et al.*, (1984) and Shtivelman *et al.* (1985) demonstrated that the critical rearrangement in the Philadelphia chromosome was an association between the ABL protooncogene (*abl*) from chromosome 9 and a previously unknown gene on chromosome 22, designated BCR for breakpoint cluster region. Despite greater technical difficulty, several additional previously unknown genes have now been identified in translocations associated with acute myeloid leukemias (Solomon *et al.*, 1991; Erikson *et al.*, 1992), and limited results have also begun to emerge from studies of common epithelial malignancies.

Interestingly, in these latter tumors, chromosome translocations are less common than karyotypic alterations that reflect changes in gene dosage, specifically amplification units and deletions. In the 1980s it was shown that the homogeneous staining regions (HSRs) and double minutes (DMs) that had been observed earlier in metaphases from cell lines and direct preparations of neuroblastomas and other solid tumors (Biedler and Spengler, 1976) represented amplification of both known (e.g., *myc*) and previously unknown oncogenes (e.g., n-*myc*, l-*myc*) (Seeger *et al.*, 1985). At the same time, the frequency of nonrandom chromosomal *deletions* in solid tumors provided a major impetus for a detailed search for genes involved in human tumors that normally inhibit growth rather than stimulate it, the so-called tumor suppressor genes.

A genetic basis for tumor suppression was suggested in 1969 by the demonstration that certain nonmalignant cell lines could suppress the capacity to form tumors when fused with a number of different highly malignant cell types (Harris *et al.*, 1969), and this suppressive effect was later associated with individual chromosomes (Codish and Paul, 1974). At the same time, a hypothetical concept was suggested by DeMars (1970) and formulated in detail by Knudson (1971) from combined family studies and cytogenetic studies of several inherited pediatric tumors. Investigation of the familial patterns of retinoblastoma, as well as Wilms' tumors, were supplemented by cytogenetic studies using improved high-resolution techniques (Yunis and Sanchez, 1975) that demonstrated chromosomal deletions, in chromosomes 13 and 11, respectively, associated with these neoplasms. This led to the two-hit concept, which suggested that individuals could be born with a defect in one growth inhibitory gene and that, when random loss of function of the other allele occurred through nondisjunction or other mechanisms in a

somatic cell, growth inhibition would be lost and a tumor could develop from the mutated cell (reviewed by Knudson, 1985). In individuals without an inherited defect, random loss could occur in both alleles of a cell, but the low probability of this occurrence would typically result in only rare sporadic tumors, arising later in life.

After some delay, the recognition of chromosomal deletion as a clue to the presence of such suppressor genes has stimulated a search for other candidates in many of the common human malignancies, and some progress has been made despite major technical difficulties in isolating and identifying an unknown gene that normally inhibits growth. The most extensive findings have appeared in just the last few years in studies of colon cancer, where Vogelstein and his colleagues (Fearon and Vogelstein, 1990), as well as other laboratories (Groden *et al.*, 1991), have utilized chromosomal clues to help identify three or four tumor suppressor genes involved in the complex sequence of events leading to a fully developed colonic malignancy. These genes include two on the long arm of chromosome 5 (*apc, mcc*), at least one of which may be inherited in a defective state and contribute to the familial syndrome of colonic polyps and carcinomas; the *p53* gene on chromosome 17, which appears to be involved in the development of a wide variety of human neoplasms; and the *dcc* gene on chromosome 18, which codes for a cell surface protein, perhaps associated with tumor cell invasion as well as aberrant growth (Fearon and Vogelstein, 1990; Groden *et al.*, 1991). Similar clues provided by deletions at other chromosomal sites are stimulating efforts to find additional suppressor genes involved in CNS tumors (James *et al.*, 1988) and in common cancers such as breast and lung cancer (Birrer and Minna, 1988).

The delineation of a relatively reproducible sequence of cytogenetic and molecular genetic events in the pathogenesis of colon cancer has provided definitive support for the clonal evolution model of tumor development and the earlier theoretical formulations on tumor progression by Foulds (1957). However, despite continuing speculation through the 1980s concerning the underlying mechanisms, there still remains very little evidence on what specific factors may be contributing to the genetic instability of tumor cells that leads to this evolutionary process. It now appears that just as a large number of different growth regulatory genes are involved in different human tumors, so also may a variety of mutator mechanisms contribute to the genetic instability of neoplastic cells under different circumstances ranging from gross defects in the mitotic spindle (as envisioned by Boveri) to specific loci associated with DNA repair, DNA synthesis, regulation of cell cycle progression, and even gene amplification (Loeb, 1991; Livingstone *et al.*, 1992).

As the 1980s drew to a close, cytogenetics, in combination with derived molecular probes, was finding increasing clinical application in the diagnosis and management of patients with hematopoietic tumors, particularly as alternative therapies such as bone marrow transplantation and interferon were more widely utilized for these disorders. At the same time, information on the cytogenetics of solid tumors, including early benign lesions, was expanding rapidly, aided by improved techniques of tissue culture and *in situ* hybridization. This was resulting in additional, although still limited, clinical applications in the evaluation of certain difficult pediatric solid malignancies and a few carcinomas and sarcomas in adults (Nowell, 1991).

VII. Conclusions

Thus, after 50 years of speculation and an almost equal period of technical advances and data collection, it now appears well established that most tumors arise from a single cell through sequential alterations in a series of critical genes, with many of these alterations recognizable as karyotypic abnormalities. As with many advances in biomedical science in this century, the development of our understanding of the relationship between chromosomes and cancer has involved profoundly insightful scientists whose hypotheses were too early to be tested; necessary technical advances that were found, lost, and then found again, often serendipitously; and finally, the application of modern molecular methods, originally developed in other systems, to permit us to begin to unravel the specific ways in which particular cytogenetic alterations contribute to the development of individual tumors. As we move to new levels of complexity in trying to understand and control human cancer, it may be worth remembering how slow and sometimes indirect have been the multiple efforts that have brought us this far.

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PATHWAYS OF RAS FUNCTION: CONNECTIONS TO THE ACTIN CYTOSKELETON

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I. Introduction

Ras proteins belong to a large superfamily of membrane-bound guanine nucleotide-binding proteins that function in receptor-mediated processes, including growth-regulated signal transduction, regulation of cytoskeletal actin, and membrane trafficking. Initially identified in mutant form as the protein encoded by the Harvey and Kirsten tumorigenic retroviruses and by human tumor genes, Ras has been demonstrated to play a central role in the cellular responses to many factors that control proliferation and differentiation. Ras signaling capability is activated by

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GTP binding and inactivated by GTP hydrolysis. Mutant forms of Ras, which have reduced GTPase activity and are constitutively bound to GTP, have been observed in ~25% of human tumors (Bos, 1990).

The connection between mutant Ras and cancer has driven wide-ranging endeavors to understand Ras function. However, despite much effort, the molecular features of regulated guanine nucleotide cycling between GTP- and GDP-bound forms of Ras *in vivo* are still emerging, as are the connections to upstream receptors and downstream effectors in the cell. Important clues to Ras function have been revealed by the study of signal transduction processes in many systems, especially those in which genetic analysis is possible. The nomenclature from each of these systems is different, but the pathways and factors that are involved have functional and structural similarities (as discussed later). For reference, a summary of the nomenclature for molecules identified in signal transduction pathways upstream and downstream of Ras in several systems is presented in Table I. Mammalian cell components are depicted in a graphical model for signal transduction that is shown in Fig. 1.

We review here the most recent advances in the biological and biochemical mechanisms of signal transduction involving Ras proteins. We focus first on guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). Next, we summarize evidence connecting Ras to two intracellular signal transduction pathways that control transcription and cytoskeletal actin organization. Finally, we consider perspectives on Ras function from studies on the phenotypic reversion of *ras*-transformed cells and speculate on the meaning of recent observations to our understanding of Ras function.

The reader is directed to other reviews for a general evaluation of G protein structure and function (Bourne *et al.*, 1991; Kaziro *et al.*, 1991; M. I. Simon *et al.*, 1991), Ras functions in lower eukaryotes (Broach, 1991; Rubin, 1991; Sternberg and Horvitz, 1991; Firtel, 1991), Ras regulation and effector functions (Bollag and McCormick, 1991b; Lowy *et al.*, 1991; Downward, 1992c), and Ras farnesylation and other post-translational modifications (Gibbs, 1992; Khosravi-Far *et al.*, 1992). Other reviews on signal transduction (Bar-Sagi, 1992; Hall, 1992b; Polakis and McCormick, 1992; Satoh *et al.*, 1992) and Ras-related proteins (Hall, 1992a) have also appeared recently.

II. Ras Guanine Nucleotide Exchange Factors

The intrinsic rates of Ras guanine nucleotide exchange and GTPase activity are slow. In cells, three classes of activities that control GTP binding and hydrolysis by Ras superfamily proteins have been described.

TABLE I

NOMENCLATURE OF RAS SIGNAL TRANSDUCTION PATHWAY MOLECULES

Organism Ras biological function	*S. cerevisiae*[a] cAMP/nutritional response	*S. pombe*[b] mating response	*C. elegans*[c] vulval cell fate	*Drosophila*[d] R7 eye cell fate	*Xenopus*[e] oocyte meiosis	Mammals/ mitosis/ differentiation
Receptor	?	Pheromone receptor	let-23	Sevenless	?	Y kinase receptor (e.g., PDGF-R)
SH2/SH3 domain-containing adaptor proteins	?	?	*sem-5*	drk	?	Shc GRB2
Ras guanine nucleotide exchange factors	CDC25, SDC25	*ste6*	?	Son of seven-less (SOS)	?	Ras^{GRF}, mSOS1,2, *smg*GDS
Ras	RAS1, RAS2	*ras1/ste5*	let-60	Ras1	(Ras)	H-Ras, K-Ras, N-Ras
Ras GTPase-activating proteins	IRA1, IRA2	*sar-1*	?	Gap1	GAP	p120^{GAP}, p100^{GAP}, NF1
Candidate effector proteins	CYR1 / CAP	?	?	?	?	JC99 (?), JC265 (?), *rsp-1* (?)
Raf-like kinases	—	?	?	l(1)polehole	(Raf)	Raf1
MAPKKK	*STE11*	*byr2/ste8*	?	?	?	?
MAPKK	*STE7*	*byr1/ste1*	?	Dsor1	MAPKK	MAPKK / MEK
MAPK	KSS1?, FUS3?	?	?	DmERK-A (?)	MAPK	MAPK / ERK

[a] Gibbs and Marshall, 1989; Broach, 1991; Marsh *et al.*, 1991; Pelech and Sanghera, 1992; Thomas, 1992.

[b] Gibbs and Marshall, 1989; Wang *et al.*, 1991a,b; Crews and Erikson, 1992; Downward, 1992a.

[c] Sternberg and Horvitz, 1991; Pawson and Gish, 1992.

[d] Rubin, 1991; Biggs and Zipursky, 1992; Dickson *et al.*, 1992; Tsuda *et al.*, 1993; Olivier *et al.*, 1993; J. Schlessinger, personal communication.

[e] Bollag and McCormick, 1991b; Pelech and Sanghera, 1992.

[f] Bollag and McCormick, 1991b; Colicelli *et al.*, Cutler *et al.*, 1992; Downward, 1992a; Pawson and Gish, 1992; Roberts, 1992.

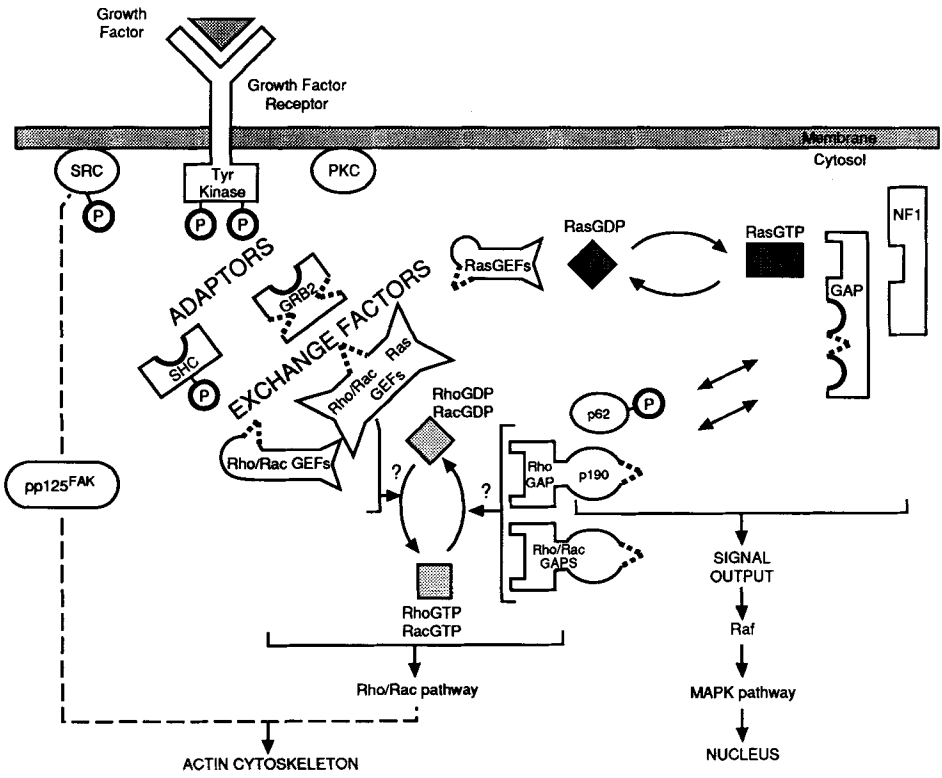

FIG. 1. Model for signal transduction pathways upstream and downstream of Ras. The model hypothesizes the sequential formation of multiprotein complexes at or near the plasma membrane whose signaling capabilities ultimately lead to changes in nuclear processes and cytoskeletal actin organization. During different phases of signal transduction, the complexes could contain tyrosine kinases, SH2/SH3 domain-containing adaptor proteins, GEFs, PKC, Ras, RasGAPs (GAP, p120^{GAP}, and NF1), GAP-associated proteins (p62 and p190), and Rho/Rac GEFs and GAPs. Information is channeled to Ras from activated growth factor receptors or Src family kinases through intermediary SH2/SH3 domain-containing adaptor proteins (Shc and GRB2) and GEFs. This information transfer may involve more than one adaptor protein. Stimulation of GEF activity is hypothesized to result by an unknown mechanism from the formation of GEF–adaptor protein complexes. The interactions between Ras, RasGEFs, and Rho/Rac GEFs and between Ras and RasGAPs lead to the transduction of a bifurcated signal that activates the downstream MAPK and Rho/Rac pathways. Other signals emanate from Src family kinases through pp125^{FAK}, affecting cytoskeletal architecture in a manner that is independent of Ras.

These regulatory factors include GTPase activators (GAPs), and two classes of guanine nucleotide exchange factors (GEFs), guanine nucleotide dissociation stimulators (GDSs), and guanine nucleotide dissociation inhibitors (GDIs). RasGAP activities, encoded by $p120^{GAP}$ and neurofibromin (NF1), have been characterized in considerable detail (reviewed by Bollag and McCormick, 1991b; Lowy *et al.*, 1991; Downward, 1992c; Hall, 1992b; see following discussion), but until recently information on mammalian GDSs and GDIs has been fragmentary.

A. Pathways Leading to Exchange Factors

Under normal cell resting state conditions, Ras is bound to GDP, and the rate of GDP dissociation is limiting in the nucleotide cycle (Satoh *et al.*, 1992). Therefore, the physiological rates of guanine nucleotide exchange that occur on Ras following cell stimulation by growth factors or upstream-acting tyrosine kinases must involve GEFs. The importance of GEFs also follows from the expectation that they are part of complexes that act as direct intermediaries between Ras and upstream activators of Ras that transduce signals from tyrosine kinase receptors.

Several recent studies have implicated GEF activities in regulating steady-state RasGTP levels. Since the normal Ras GTPase cycle is limited by the rate of GDP dissociation, one approach has utilized a Ras mutant (RasH116) that is sensitive to $p120^{GAP}$ but has a rapid intrinsic rate of nucleotide exchange that is not dependent on exchange factors. Serum and platelet-derived growth factor (PDGF) increased the proportion of RasGTP in NIH3T3 cells containing normal Ras but not in cells containing the mutant RasH116 protein (Zhang *et al.*, 1992). In another example, enhanced exchange of insulin- and epidermal growth factor (EGF) stimulated guanine nucleotide on Ras in NIH3T3 could be blocked by a RasN17 (Medema *et al.*, 1993), a dominant inhibitory mutant that appears to compete for binding of GEFs (Schweighoffer *et al.*, 1993). Finally, in PC12 pheochromocytoma cells, nerve growth factor (NGF) rapidly stimulates GEF activity in a manner sensitive to tyrosine phosphorylation (Li *et al.*, 1992). This result implies that GEF activity might be directly or indirectly controlled by tyrosine kinase activity.

The linkage(s) between GEFs and upstream receptors is not yet clear but probably involves SH2/SH3 domain-containing proteins that serve as adaptor molecules between receptors and the molecular systems that regulate Ras guanine nucleotide metabolism (see Fig. 1) (Pawson and Gish, 1992). One provocative connection that has been presented involves an SH2/SH3 domain-containing protein (GRB2) that was isolated on the basis of its ability to bind the EGF receptor C-terminus (Lowenstein *et al.*, 1992). Coinjection of quiescent rodent fibroblasts with GRB2

and normal Ras induced DNA synthesis, whereas neither protein alone had any effect. This result indicated that GRB2 activated Ras signaling function, possibly by inducing GTP exchange onto Ras (Lowenstein *et al.*, 1992). The GRB2 protein is ~60% similar to and can complement (Lowenstein *et al.*, 1992) the *C. elegans sem-5* gene, which encodes an SH2/SH3 domain-containing protein (Clark *et al.*, 1992). The *sem-5* gene has been implicated in a Ras-containing pathway that regulates vulval development in *C. elegans* (Sternberg and Horvitz, 1991). The results in this system suggest that Sem-5/GRB2 acts upstream of Ras, perhaps by influencing RasGEF activity. Recently, it has been demonstrated (Rozakis-Adcock *et al.*, 1992) that GRB2 associates with another SH2 protein (Shc) that can interact with Src family kinases and the EGF receptor (see Fig. 1) (Pawson and Gish, 1992). Like tyrosine kinases, Shc becomes phosphorylated following growth factor stimulation (McGlade *et al.*, 1992). This result links Ras and the EGF receptor through a cascade of proteins that include Shc and GRB2. Along with rapid progress in the area of exchange factors (see the following discussion), the connections between receptor tyrosine kinases and Ras will be an area of intense research in the near future and should be outlined shortly.

B. Exchange Factors in Lower Eukaroyotes

In yeast and fruit flies, genetic approaches have identified a set of related proteins that regulate guanine nucleotide exchange on Ras (see Table I). In *Saccharomyces cerevisiae*, CDC25 and SDC25 were mapped genetically upstream of RAS and were then subsequently identified as guanine nucleotide exchange factors for Ras proteins (Bollag and McCormick, 1991b; Broach, 1991). As predicted for an exchange factor, in a genetic analysis CDC25 appeared to favor interaction with Ras-GDP by several-fold compared to Ras-GTP (which CDC25 will still bind, although less avidly) (Munder and Fürst, 1992). The CDC25 homolog in *Schizosaccharomyces pombe* (*ste6*) also functions upstream of Ras (Hughes *et al.*, 1990). It was recently determined that expression of the C-terminal domain of SDC25 in animal cells can overcome the inhibitory action of the RasN17 dominant negative mutant, corroborating the unproven although widely accepted notion that RasN17 acts by competing for upstream Ras exchange factors (Schweighoffer *et al.*, 1993). Work from the same group has demonstrated that expression of the SDC25 C-terminal domain can transform NIH3T3 cells (Schweighoffer *et al.*, 1993), suggesting that truncations of mammalian Ras GEFs could play an activating role during animal cell transformation.

In *Drosophila, ras* and a gene termed *Son of sevenless* (*SOS*) that has

similarity to CDC25 have been identified as components in eye development mediated by Sevenless, a tyrosine kinase receptor (see Table I) (M. A. Simon *et al.*, 1991; Fortini *et al.*, 1992). In the genetic screen for suppressors of Sevenless that identified *ras* and *SOS*, there are five other genes that are likely to encode other components of the signaling machinery upstream and/or downstream of *ras* and *SOS* (Rubin, 1991). One of these components, originally termed E(sev)2B but renamed *drk* (downstream of receptor kinases), was recently found to encode an SH2/SH3 domain-containing protein that is capable of directly interacting with the Sevenless receptor and *SOS* (Simon *et al.*, 1993). A second group of workers cloned *drk* by an independent route, through its homology to the *C. elegans sem-5* and mammalian GRB2 genes and also showed that the *drk* protein could bind to *SOS* (Olivier *et al.*, 1993). These findings reinforce the notion that the functions of the four other genes that were identified in the screen for Sevenless suppressors will be components of pathways that involve Ras.

The molecular mechanisms by which SDC25 stimulates dissociation of GDP from Ras have been explored biochemically (Mistou *et al.*, 1992; Verrotti *et al.*, 1992). Analysis of the response of a set of Ras mutants to SDC25 stimulation suggests that the regions of Ras that are required for interaction with $p120^{GAP}$ (Mistou *et al.*, 1992) and the yeast effector adenylyl cyclase (Verrotti *et al.*, 1992) do not overlap with those required for SDC25. Mutation of amino acids 80-82 in yeast RAS [located in the distal part of the "switch II" segment defined previously as an important region for effector functions (Bourne *et al.*, 1990, 1991; Kaziro *et al.*, 1991)] severely impaired the SDC25-stimulated conversion of RAS from the GTP- to GDP-bound form (Verrotti *et al.*, 1992). The data indicate that regulation of guanine nucleotide exchange and stimulation of GTP hydrolysis may involve nonoverlapping domains of Ras. This observation is interesting because it suggests that GEFs and GAPs could be bound simultaneously, perhaps forming a ternary signaling complex. The mechanism by which CDC25 is regulated may involve phosphorylation, because it has been reported that CDC25 is a phosphoprotein that becomes hyperphosphorylated by cAMP-dependent protein kinase very rapidly following stimulation of yeast cells with glucose (Gross *et al.*, 1992).

C. Biochemical Identification of Mammalian GEFs

Biochemical activities in cell extracts that control the exchange of GDP for GTP on H-Ras were described in 1990 (Downward *et al.*, 1990;

Huang *et al.*, 1990; West *et al.*, 1990; Wolfman and Macara, 1990). These workers reported activities derived from proteins with various molecular weights and specificities for substrates (reviewed by Bollag and McCormick, 1991b), but further efforts did not lead to any structural information.

In a more fertile line of work, Takai's group has characterized *smg*-GDS, a ~60-kDa exchange factor for the Ras-related protein *smg*p21/Rap1A/Krev-1 (Kaibuchi *et al.*, 1991; Yamamoto *et al.*, 1991) that also stimulates GTP/GDP exchange on K-Ras and the Rho family protein, RhoA, but not on H-Ras (Mizuno *et al.*, 1991). The interaction of *smg*GDS with its substrates requires either farnesylation or geranylgeranylation of the substrate C-termini (Mizuno *et al.*, 1991; Ando *et al.*, 1992). Furthermore, prenylated C-terminal peptides can compete directly with fully processed substrate for *smg*GDS binding (Shirataki *et al.*, 1991). The domain of *smg*GDS required for interaction with its prenylated substrates appears to be located in the *smg*GDS C-terminus (Hiroyoshi *et al.*, 1991; Hori *et al.*, 1991; Kotani *et al.*, 1992). Overexpression of normal K-Ras and *smg*GDS in 3T3 cells induced transformation and transcriptional activation of the c-*fos* promoter, whereas either gene itself only weakly induced both phenomena (Fujioka *et al.*, 1992). Therefore, aberration of GEF function could contribute to tumorigenesis in the absence of Ras mutation, for example, in breast tumors containing activated *erbB* or *erbB2* oncogenes. It will be interesting to examine the expression of GEFs in tumors as cloned probes become available (see the following discussion).

If some or all Ras GEFs, like *smg*GDS, interact with C-terminal domains, then perhaps the C-terminal amino acid variations in Ras [the only region where Ras proteins differ significantly from one another (Valencia *et al.*, 1991)] provide the structural basis for the differential functions of various GEF-Ras complexes. Notably for *smg*GDS, specificity is dictated by C-terminal substrates that contain polybasic sequences known to be important for efficient localization of K-Ras (Hancock *et al.*, 1990, 1991) and the RhoA and RhoC (Adamson *et al.*, 1992) proteins. The likelihood that C-terminal domains direct Ras proteins to unique loci is also suggested by the observation that Rho and Rab proteins contain in their C-termini a targeting signal for different intracellular vesicles (Chavrier *et al.*, 1991; Adamson *et al.*, 1992).

A second class of molecules that affect guanine nucleotide exchange, termed guanine nucleotide dissociation inhibitors (GDIs), have also been described. The GDIs may play a role in the binding of Rho and Rab family proteins to the plasma membrane (for references see Bollag and

McCormick, 1991b). The action of a *rho*GDI under study in Takai's group overrides the stimulatory action of *smg*GDS or *rho*GDS if both types of factors are present, and *rho*GDI is reported to be more abundant than either GDS in cells (Kikuchi *et al.*, 1992). A GDI specific for Ras has not yet been described. Should one exist, it may regulate Ras biological function(s), since it has been observed that *rho*GDI can inhibit the effects of Rho on cell morphology when they are coinjected into cells (Miura *et al.*, 1993; Takaishi *et al.*, 1993).

D. Genetic Identification of Mammalian GEFs

Two approaches to identify homologs of the yeast exchange factor CDC25 in mammalian cells have recently led to the cloning of genes encoding Ras GEF activity. Two groups used degenerate oligonucleotides that encoded regions highly conserved in yeast GEFs to clone by a polymerase chain reaction three mammalian cDNAs, termed Ras guanine nucleotide-release factor (RasGRF), $Cdc25^{M}$, and $Cdc25^{H}$ (Shou *et al.*, 1992; Wei *et al.*, 1992). The full-length RasGRF sequence predicted a protein of ~140 kDa that was ~30% similar to CDC25 over a ~300 amino acid C-terminal domain (Shou *et al.*, 1992). Expression of the C-terminal domain in *Escherichia coli* yielded GEF activity capable of recognizing N-Ras and H-Ras but not the more distantly related Ral protein (Shou *et al.*, 1992). The second group identified partial mouse ($Cdc25^{M}$) and human ($Cdc25^{H}$) cDNAs that were similar to the C-terminal exchange domain of CDC25. As a chimeric molecule replacing the yeast domain, the mammalian cDNAs were able to complement functionally a CDC25 defect in a mutant yeast strain (Wei *et al.*, 1992). The proteins from both groups are highly related (probably mammalian homologs), and they exhibit similar expression in brain but not other tissues (Shou *et al.*, 1992; Wei *et al.*, 1992).

$CDC25^{Mm}$, by functional complementation of a CDC25 defect in yeast (Martegani *et al.*, 1992). Using this cDNA as a probe, these workers have gone on to identify a set of six related cDNAs that contain the same predicted C-terminal polypeptide but a variety of N-terminal extensions (Cen *et al.*, 1992). Polyclonal antibodies to these cDNAs expressed in bacteria recognize ~75- and ~95-kDa proteins in PC12 and NIH3T3 cells in different abundance, suggesting that $CDC25^{Mm}$ is a complex gene regulated in a tissue-specific fashion (Cen *et al.*, 1992). The $CDC25^{Mm}$ gene may encode the same gene as those for RasGRF, Cdc^{M}, and Cdc^{H}, though this family of sequences shows only weak similarity to *smg*GDS (Downward, 1992a). The reason for the differences in polypeptides

recognized by anti-RasGRF and anti-CDC25^{Mm} antisera is not clear but may be due to variations in mRNA coding potential generated by differential RNA splicing.

Recently, a third group that set out to clone mammalian homologs of the *Drosophila SOS* gene identified two cDNAs, termed mSOS1 and mSOS2, that appear to be part of a different structural class of exchange proteins than those mentioned previously and that interact with the GRB2 protein *in vitro* (J. Schlessinger, personal communication, 1993). Thus it appears that exchange factors that recognize Ras will encompass at least three structural classes, exemplified by *smg*GDS, CDC25^{Mm}/RasGRF/Cdc^{H}/Cdc^{M}, and mSOS1/2. As the identification and characterization of Ras guanine nucleotide exchange factors continues, one would anticipate that the connections that exist between GEFs and upstream regulators (such as the SH2/SH3 domain-containing adaptor proteins) will be defined soon.

E. Potential for Multiple Guanine Nucleotide Regulatory Functions on a Single Exchange Factor

Several lines of work suggest that RasGEFs may communicate with Rho and Rac family proteins through other molecules that control the GTP/GDP cycle of these factors. In addition to the expected congruity with CDC25 and SOS (a CDC25-related molecule from *Drosophila*), an unexpected similarity in the N-terminus of RasGRF and CDC25^{Mm} was found with the *dbl* oncogene product. This relationship is intriguing because *dbl* has been shown to have GEF activity on the Ras-related protein CDC42Hs, that in yeast is involved with budding of the daughter cell during the cell cycle and that is a member of the Rho/Rac family of proteins implicated in the control of the actin cytoskeleton (Hall, 1992a; see the following discussion). Similar *dbl* sequence motifs have also been observed in the Bcr (breakpoint cluster region) protein and the *vav* oncogene product (Downward, 1992b). The Bcr protein contains a domain separate from the *dbl* homology that contains RacGAP activity (Diekmann *et al.*, 1991), whereas Vav harbors distal SH2/SH3 domains presumed to interact with tyrosine-phosphorylated substrates (Pawson and Gish, 1992). Finally, the p120^{GAP}-associated protein p190 encodes RhoGAP activity (Settleman *et al.*, 1992a). Thus, through both GEFs and GAPs, Ras proteins may communicate with the Rho/Rac proteins to control both cytoskeletal organization and growth factor-regulated signals.

An additional level of complexity to the interactions in the cell be-

tween GEFs, GDIs, and GAPs is suggested by the report that a GDI for CDC42Hs also inhibits GAP activity for that protein (Hart *et al.*, 1992). Thus, the same GDI may regulate the GTP–GDP state by modulating not only the rate of nucleotide exchange but also the rate of hydrolysis. It has been suggested that such a factor could be useful as a shuttle molecule that directs the movement of a Ras-related protein between a site at a membrane or cytoskeletal location (where guanine nucleotide exchange would occur) and a GAP target site (where GTP hydrolysis would occur) (Hart *et al.*, 1992). Similar models have been suggested for Rac function (Hall, 1992a).

III. GAP and GAP-Associated Proteins

The early observation that Ras has a low intrinsic GTPase activity *in vitro* prompted a search for cellular activities that stimulate the rate of GTP hydrolysis. This led to the discovery of the widely expressed GTPase-activating protein, $p120^{GAP}$. $p120^{GAP}$ binds to Ras in a region that overlaps with the genetically defined effector region on Ras. In addition to the catalytic domain, $p120^{GAP}$ also contains SH2/SH3 domains, which mediate its interaction with the PDGF, EGF, and CSF-1 growth factor receptors, the Src tyrosine kinase, and the cellular proteins p62 and p190. A second GAP, NF1, was originally identified by genetic means and predicted to have GAP activity on the basis of a region of similarity to $p120^{GAP}$. NF1 is a ~320-kDa product of the neurofibromatosis type 1 gene that is defective in patients that have neurofibromatosis. Although less characterized, NF1 activity can be distinguished from $p120^{GAP}$ by using certain lipids and detergents (Bollag and McCormick, 1991a). The GAPs $p120^{GAP}$ and NF1 have garnered attention as both negative regulators and effectors of Ras signaling (see Fig. 1; for reviews see Bollag and McCormick, 1991b; Lowy *et al.*, 1991; Bar-Sagi, 1992; Downward, 1992c; Hall, 1992b; Polakis and McCormick, 1992). The emerging picture is that, like other G-protein systems (Bourne and Stryer, 1992), stimulation of GTP hydrolysis on Ras is coupled to the transduction of a signal that involves the action of a GAP. Three new lines of work support an effector role for $p120^{GAP}$ in Ras signal transduction, each involving factors that interact with it.

A. Effector Functions of $p120^{GAP}$

There is evidence that $p120^{GAP}$ can down-regulate Ras function but can also transmit Ras signals (Bollag and McCormick, 1991b; Gibbs, 1991; Clark *et al.*, 1993; Huang *et al.*, 1993). Because $p120^{GAP}$ action

stimulates the formation of the inactivated GDP-bound form of Ras, p120^{GAP} would act as a negative regulator of Ras in the simplest signal transduction models. In yeast (see Table I), the downstream effector of RAS activity is CYR1, which encodes adenylyl cyclase (Broach, 1991). Disruption of the two yeast IRA genes, which share structural and functional similarity to p120^{GAP} and NF1, increased intracellular cAMP levels and suppressed CDC25 mutations (Bollag and McCormick, 1991b). Genetic experiments have demonstrated that yeast RAS is downregulated by IRA function, and null mutations in IRA genes can be complemented by both p120^{GAP} and NF1 (Bollag and McCormick, 1991b). Similar results have been observed in other eukaryotic systems. For example, in *S. pombe,* disruption of the p120^{GAP} homolog *sar1* led to mating phenotypes identical to those observed with *ras1*^{Val17}, a mutant with impaired GTPase activity (Wang *et al.,* 1991a). In *Drosophila,* inactivation of the p120^{GAP} homolog Gap1 mimicked constitutive activation of the Sevenless receptor tyrosine kinase, which requires Ras in its signaling pathway (Gaul *et al.,* 1992). Thus, genetic evidence from *S. cerevisiae, S. pombe,* and *Drosophila* support a scenario in which Ras GAP(s) would act as a negative regulator of Ras signaling by increasing the steady-state level of inactive RasGDP.

However, there also exists a large body of data from mammalian systems showing that p120^{GAP} has an effector function(s) for Ras signaling (Bollag and McCormick, 1991b; Gibbs, 1991; Clark *et al.,* 1993; Huang *et al.,* 1993). The first direct evidence was the demonstration that Ras and p120^{GAP} could collaboratively inhibit the coupling of a muscarinic receptor with a potassium ion channel, in guinea pig atrial membranes (Yatani *et al.,* 1990). Oncogenic Ras was more effective at inhibiting the coupling, and anti-GAP antibodies blocked the effect. The N-terminal SH2/SH3 domains of p120^{GAP} appear to be responsible for this phenomena: deletion of the Ras-binding domain relieves the requirement for Ras and suggests that Ras might regulate a conformational change in p120^{GAP} that allows it to interact with its effector substrate(s) (Martin *et al.,* 1992). Even though the physiological meaning of this phenomenon is unclear, the data argue that Ras controls the access of the p120^{GAP} SH2/SH3 domain to downstream signaling elements.

Consistent with this likelihood, it has been demonstrated that the N-terminal region of p120^{GAP} is sufficient to induce downstream signal transduction. Expression of an N-terminal domain containing only the SH2/SH3 region of p120^{GAP} (GAP37; also termed GAP-N in a later discussion) was, similar to Ras, sufficient to stimulate transcription from the c-Fos promoter (Medema *et al.,* 1992). Others have also argued that p120^{GAP} functions as a Ras effector from demonstrations that p120^{GAP}

can reverse the inhibitory effects of GAP-C (which contains only the GTPase-stimulatory catalytic domain) on oncogenic Ras-stimulated transcription from the polyoma enhancer/promoter (Schweighoffer *et al.*, 1992). Since oncogenic Ras binds but does not respond to p120^{GAP}, GAP-C overexpression would be expected to prevent Ras from interacting with an effector. This experiment suggests that p120^{GAP} will itself act as an effector since it can eliminate the GAP-C inhibitory effect.

What parts of this N-terminal region of p120^{GAP} are required for its signaling capability? In a seminal report, it has recently been demonstrated by Tocqué and colleagues that the SH3 domain is necessary for p120^{GAP} signal transduction (Duchesne *et al.*, 1993). An anti-p120^{GAP} monoclonal antibody that blocked oncogenic Ras-induced maturation of *Xenopus* oocytes recognized a region containing the SH3 domain on mammalian p120^{GAP}. The antibody did not affect the stimulation of the Ras GTPase by p120^{GAP}, implying that it must be blocking the signaling ability of its N-terminus. Microinjection of peptides containing sequences from the p120^{GAP} SH3 domain blocked oocyte maturation when it was induced by oncogenic Ras or insulin, which is Ras dependent, but not by progesterone, which is Ras independent. Significantly, peptides containing sequences from the SH3 domain of phospholipase C-γ were not active, demonstrating specificity for the p120^{GAP} SH3 domain. The p120^{GAP} SH3 peptide could not compete for the interaction of recombinant p120^{GAP} with mammalian cell extracts containing the activated EGFR, which requires the more N-terminal of the two SH2 domains of p120^{GAP} (Duchesne *et al.*, 1993). These observations provided strong evidence that p120^{GAP} is an effector for Ras and suggest that the SH3 domain is the key link to downstream signal transduction.

Perhaps not surprisingly, the effector mechanism involving p120^{GAP} is complex. In the transcriptional activation experiments employing the *c-fos* promoter, stimulation by the GAP-N protein was dependent on endogenous Ras function, since co-transfection of a dominant negative Ras mutant (RasN17) suppressed it (Medema *et al.*, 1992). Furthermore, stimulation of the *c-fos* promoter by GAP-N was augmented by co-transfection of oncogenic Ras (Medema *et al.*, 1992). The synergistic effect of oncogenic Ras and GAP-N is not inhibited by RasN17, indicating that the SH2/SH3 domains of p120^{GAP} do not activate endogenous Ras but cooperate with another signal coming from oncogenic Ras.

What might mediate this presumptive second signal? One clue may come from analyses of a set of novel Ras mutants that separate Ras biological functions from GAP binding and responsiveness. The mutations destroy the biological signaling capability of Ras but do not affect the ability of Ras to bind and respond to p120^{GAP} (DeClue *et al.*, 1991;

Fujita-Yoshigaki *et al.*, 1991; Marshall *et al.*, 1991) and NF1 (DeClue *et al.*, 1991). The mutations map to the C-terminal half of the Ras effector region, opposite the N-terminal half, which is involved in biochemical interaction with $p120^{GAP}$ (Schaber *et al.*, 1989). The character of these mutants suggests that there may be an additional effector contact for Ras in addition to $p120^{GAP}$ that is necessary for biological activity. Possible candidates for other effectors might be found among a set of cellular polypeptides of 150, 120, 105, and 50 kDa, which have been reported to associate with Ras in human A431 cells (Kaplan and Bar-Sagi, 1991), or might be identified by an ongoing somatic genetic approach to identify cellular mutants that have an altered target specificity for Ras mutants with abnormal effector domains (Stone and Blanchard, 1991).

B. p190 AND p62

The evidence that $p120^{GAP}$ delivers Ras-dependent signals into the cell provokes one to determine the pathways that lead from it. $p120^{GAP}$ associates with a variety of proteins that contain phosphotyrosine, including the platelet-derived growth factor receptor, epidermal growth factor receptor, insulin receptor, $pp60^{c\text{-}src}$, and two phosphoproteins termed p62 and p190 (see Fig. 1) (Bollag and McCormick, 1991b). The N-terminal SH2/SH3 region of $p120^{GAP}$ is necessary and sufficient for the association of $p120^{GAP}$ with these proteins (Bollag and McCormick, 1991b). Peptides encoding the region on the platelet-derived growth factor receptor that interacts with $p120^{GAP}$ impede p62 binding to it $p120^{GAP}$, implying that the platelet-derived growth factor receptor and p62 interact with the same SH2 domain on $p120^{GAP}$ (Fantl *et al.*, 1992). Since the growth factor receptor associations probably mediate upstream regulation of $p120^{GAP}$, interest in candidate effectors for downstream signaling have focussed on p62 and p190. Before its identification as a $p120^{GAP}$-associated protein, p62 was discovered as a major substrate for phosphorylation in tyrosine kinase-transformed cells (see Moran *et al.*, 1990; Bouton *et al.*, 1991). The association of p190 with $p120^{GAP}$ is increased in growth factor-treated cells and cells transformed by tyrosine kinases, and the interaction is reported to reduce the GTPase-activating activity of $p120^{GAP}$ on Ras (Moran *et al.*, 1991). These characteristics are consistent with a signaling role for p62 and p190, which recently have been purified and cloned (Settleman *et al.*, 1992b; Wong *et al.*, 1992).

The structures of the p190 and p62 cDNAs reveal provocative sequence similarities to other proteins, providing a rich source of material for further experimentation and model building. A tantalizing aspect of

their cloning was predicted sequences that pointed to a direct role in nuclear events. p62 revealed significant similarity in an ~100 residue N-terminal region to a putative hnRNP protein termed GRP33 (Wong *et al.*, 1992). Consistent with the possibility that p62 may function as GRP33, recombinant p62 was able to associate nonspecifically with RNA and DNA and was modified by dimethylation on multiple arginine residues, similar to other mRNA processing factors. Moreover, anti-p62 recognized a subpopulation of p62 in the nuclear lysate of fractionated cells. The p62 C-terminus was identified as the region where $p120^{GAP}$ binds; a tyrosine-rich segment is found in this region, and its phosphorylation appears to be necessary for p62–$p120^{GAP}$ interaction. Since this segment is separated from the GRP33 similarity, phosphorylation may regulate $p120^{GAP}$ binding rather than the potential RNA/DNA binding (Wong *et al.*, 1992).

The structure of p190 also revealed similarity to a putative nuclear factor, in this case glucocorticoid receptor gene binding factor-1 (GRF-1), a human factor reported to be a transcriptional repressor for the glucocorticoid receptor gene (LeClerc *et al.*, 1991; Settleman *et al.*, 1992b). The near identity of rat p190 and human GRF (~95% over 778 amino acids) strongly suggests that they are homologs, and in fact frameshifts in the repressor sequence can extend the similarity (Settleman *et al.*, 1992b). The likelihood that p190 will interact with DNA is currently problematic because there has been difficulty in reproducing the DNA binding activity that was originally reported for GRF-1 and because the identification of this protein as a transcriptional repressor is in question (P. Sicinsky, J. Settleman, and R. A. Weinberg, personal communication, 1993). However, in support of a nuclear function for p190, anti-p190 antisera identified a subfraction of p190 in nuclear extracts from fractionated cells (Settleman *et al.*, 1992b).

p190 also contains two other motifs that flank either side of the putative transcriptional repressor similarity. The first, located in the N-terminus, is related to signal-transducing GTPases of the Gα family, small GTP-binding proteins, and several elongation and initiation factors. It is not yet clear that this region has GTPase function; preliminary experiments to test GTP binding to p190 immunopurified from cell extracts are reported to have failed (Settleman *et al.*, 1992b). The second motif in p190, located in the C-terminus, is related to the Bcr and N-chimaerin proteins, which have been shown to harbor intrinsic GAP activity specific for the Ras-related protein Rac1 (Diekmann *et al.*, 1991). In this case, a direct test with recombinant p190 demonstrated that it can act as a GAP specific for Rho family proteins, including RhoA, RhoB, Rac1, Rac2, and CDC42Hs (Settleman *et al.*, 1992a). Thus, the complex formed between

p120^{GAP} and p190 may allow coupling of growth factor-stimulated signals to both Ras and Rho GTPases, the latter of which are involved in regulating the spatial arrangement of cytoskeletal actin (Hall, 1992a; see below). This association would provide a mechanism for coordinating cell cycle progression with cytoskeletal reorganization and could be important in cells transformed by Ras and tyrosine kinases, which exhibit radical changes in actin-containing cytoskeletal structures such as focal adhesions and stress fibers (Small, 1988; Bretscher, 1991).

It will be important to determine the effects that interaction of p120^{GAP} with p62 and p190 has on the functions of each protein. When bound to RasGTP, the p120^{GAP} SH2/SH3 domains could interact with p62 and/or p190 in a manner that either inhibits or activates their function. The p62 and/or p190 proteins could also act by preventing the interaction between p120^{GAP} and growth factor receptors, blocking the potential signaling consequences of the complexes (Settleman *et al.*, 1992b; Wong *et al.*, 1992). The possibility that p62- and p190-mediated signaling pathways operate between p120^{GAP} and the nucleus independently of Ras has also been suggested (Settleman *et al.*, 1992b). In light of the recent biological results demonstrating an essential role of the p120^{GAP} SH2/SH3 region for Ras-regulated signal transduction (Medema *et al.*, 1992; Duchesne *et al.*, 1993), p62 and p190 are very likely to be critical transducers of Ras signals. We expect that these issues will be resolved soon.

C. Possible Connections between SH3 Domain Proteins, GAPs, and GEFs

An important clue to the regulation of Ras-regulated signal transduction has recently emerged, suggesting a role for SH3 domain-containing proteins in regulating both Ras GEFs and GAPs. Perhaps the most direct support for these notions has come from Baltimore's group, which has identified a protein termed 3BP-1 that binds the c-Abl SH3 domain (Cicchetti *et al.*, 1992). Intriguingly, 3BP-1 was noted to contain a region related to proteins that encode GAP activity for the Ras-related Rho proteins. This result is germane because the p120^{GAP}-associated protein p190 also has Rho–GAP activity and shows sequence similarities with 3BP-1 (Duchesne *et al.*, 1993). If, like 3BP-1, p190 is capable of interacting with SH3 domain-containing proteins, then its interaction with p120^{GAP} may be mediated via the p120^{GAP} SH3 domain. In light of recent evidence that this domain is necessary for the signal-transducing capability of p120^{GAP}, p190 may be a key effector for Ras signal transduction (Duchesne *et al.*, 1993). The work on 3BP-1 has also reinforced

speculation from studies in yeast that SH3 domain-containing proteins may interact with and regulate GEF activities (Pawson and Gish, 1992). More direct evidence for this notion may come from the observation (mentioned previously) that mSOS1 and mSOS2, mammalian homologs of the putative *Drosophila* RasGEF, can interact directly with the SH3 domain-containing adaptor protein GRB2 (J. Schlessinger, personal communication, 1993).

The SH3 domains appear in several classes of proteins. They were initially identified in the Src family of nonreceptor tyrosine kinases (Pawson and Gish, 1992). Deletion of SH3 in Src results in activation of its transformation capability, suggesting that it has a negative regulatory function (for references see Cicchetti *et al.*, 1992). The SH3 domains are observed in other signaling molecules that act downstream of tyrosine kinases, including $p120^{GAP}$, phospholipase C-γ1, and the p85 subunit of phosphoinositide-3′kinase. Their function in these molecules has not yet been elucidated. A third class of proteins containing SH3 motifs are the adaptor proteins, such as GRB2, that contain little else except SH2/SH3 motifs (Pawson and Gish, 1992). These proteins can interact via their SH2 motif with tyrosyl-phosphate on activated growth factor receptors. Finally, SH3 domains have also been observed in structural proteins such as myosin1, spectrin, and ABP-1, an actin-binding protein from yeast, where SH3 has been suggested to mediate protein–protein contacts and allow interactions of proteins with the actin cytoskeleton (Drubin *et al.*, 1990).

The results suggesting an interaction between SH3-containing proteins and GAPs or GEFs are provocative because this information provides a missing link between signaling factors that modulate Ras activity (Pawson and Gish, 1992). Proteins with SH3 domains might control Ras through GEF functions but may also interact through GAPs with the family of Rho/Rac proteins that affect actin cytoskeletal structure (see Fig. 1 and the following discussion). In any case, although speculative at this time, the picture that is emerging points to an important role for SH3 domain-containing proteins both upstream (e.g., Shc and GRB2 connections to tyrosine kinases) and downstream (e.g., $p120^{GAP}$ connections to the RhoGAP activity on p190) of Ras.

IV. Signal Transduction Pathways Downstream of Ras

There is a large body of evidence indicating that Ras proteins are necessary components of the receptor-mediated signal transduction

pathways that control the proliferation and differentiation of normal cells. A significant portion of the signals transduced through Ras converge to stimulate immediate early gene expression (Schönthal *et al.*, 1988), which is obligatory for cell growth and differentiation to occur. An additional and potentially important feature of oncogenic Ras relative to the normal protein is its ability to alter cellular morphology rapidly.

Recent work has indicated that Ras may regulate at least two downstream signal transduction pathways, one that regulates translation, transcription, and the cell cycle, and another that controls cytoskeletal organization. The first pathway referred to later as the mitogen-activated protein kinase (MAPK) pathway (see Fig. 2), includes a set of cytoplasmic growth factor-regulated serine/threonine kinases that act on both ribosomal and nuclear factors. The second pathway, referred to later as the Rho/Rac pathway (see Fig. 3), includes a set of Ras-related

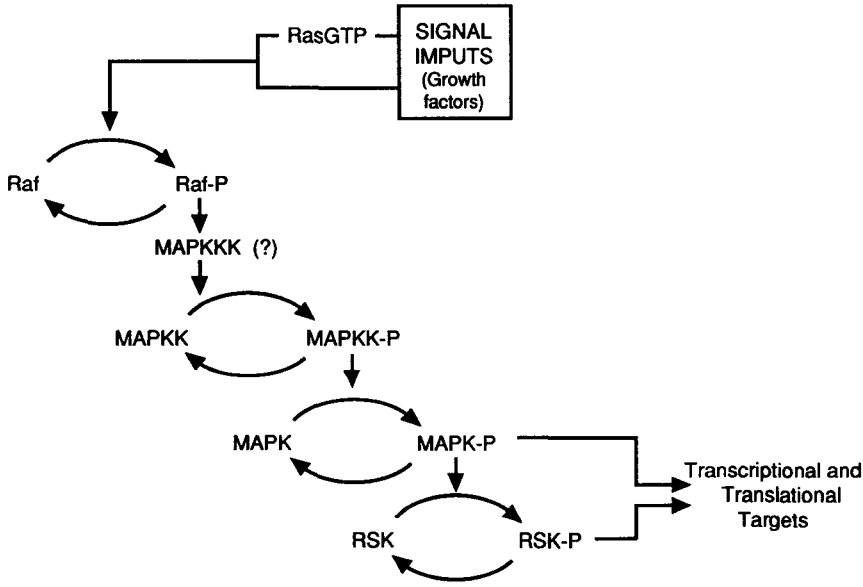

FIG. 2. MAPK pathway. Induction of Raf kinase activity is stimulated by at least two upstream signal inputs, one of which is dependent on Ras signaling (Roberts, 1992). Activated Raf stimulates phosphorylation of MAPKK, either directly or through an intermediate protein kinase(s), hypothesized to be a MAPKKK. Signal transduction through the MAPK pathway leads to activation of various transcriptional and translational targets.

GTP-binding proteins that control the spatial pattern of polymerized actin in the cell.

A. Ras and the MAPK Pathway

A significant part of Ras-regulated signal transduction involves components of the MAPK pathway, including mitogen-activated protein kinases [MAPKs, or extracellular–signal regulated kinases (ERKs)], mitogen-activated protein kinase kinase [MAPKK, which is also referred to as MAPK/ERK kinase (MEK)], Raf kinase, and the ribosomal protein S6 family of ribosomal protein kinases (RSKs). We briefly describe the significance and current understanding of the MAPK pathway and then review recent work indicating that Ras can activate it. A simple summary of the MAPK pathway is graphically depicted in Fig. 2. For a more extensive consideration of the MAPK pathway, the reader is directed to other reviews (Heidecker *et al.*, 1992; Pelech and Sanghera, 1992; Roberts, 1992; Sturgill and Wu, 1992; Thomas, 1992).

1. Significance of the MAPK Pathway

The MAPKs were originally identified as a group of 40 to 45 kDa serine-threonine kinases activated by insulin, phorbol esters, and other growth factors. Among the first known substrates for MAPK activity was Microtubule-Associated Protein-2, which accounts for the original nomenclature sometimes still used. A broad range of cell stimuli have been found to activate MAP kinases and they have been suggested by many lines of work to transduce signals during the G0/G1 cell cycle transition and in meiosis. In fact, cloning of some MAPKs (ERKs) has revealed both genetic complexity and a structural relationship to the cdc2 family of kinases that are involved in cell cycle regulation. Downstream effects of MAPK pathway activation may include, through the RSKs, regulation of translation via S6 ribosomal protein phosphorylation.

Recently, several reports have suggested that MAPK may also regulate transcription factor activities. First, MAPK stimulated ternary complex formation of the transcription factors $p62^{TCF}$ and serum response factor (SRF) with the *c-fos* promoter through phosphorylation of $p62^{TCF}$ (Gille *et al.*, 1992). Second, MAPK-induced (Seth *et al.*, 1992) and serum-induced (Alvarez *et al.*, 1991) phosphorylation of a MAPK recognition site in the c-Myc protein N-terminus increased its transcriptional transactivation activity (Seth *et al.*, 1991, 1992). The c-Jun protein may also be regulated by MAPK phosphorylation (Pulverer *et al.*, 1991; Smeal *et al.*, 1991; Smeal, 1992). Consistent with a nuclear role for MAPK pathway

kinases, MAPKs and RSKs have been observed in the cell nucleus (Chen *et al.*, 1992; Seth *et al.*, 1992), and nuclear lamins also have been identified as substrates for MAPK (Peter *et al.*, 1992).

2. MAPKK, RSKs and Raf Are Part of the MAPK Pathway

In stimulated cells, MAPKs are part of a complex phosphorylation cascade that includes MAPKK and RSKs. A large body of data suggests that MAPKK activates MAPKs through both tyrosine and threonine phosphorylation, and MAPKs activate RSKs through serine/threonine phosphorylation (reviewed by Heidecker *et al.*, 1992; Pelech and Sanghera, 1992; Sturgill and Wu, 1992). An upstream activator of MAPK, MAPKK, has been isolated as a ~45-kDa polypeptide(s) from several species (Ahn *et al.*, 1992; Crews and Erikson, 1992; Matsuda *et al.*, 1992; Nakielny *et al.*, 1992; Seger *et al.*, 1992). This polypeptide is reported to be similar to the M phase MAP kinase activator that has been isolated from *Xenopus* oocytes (Shirakabe *et al.*, 1992). A unique feature of MAPKK is its specificity for both threonine and tyrosine on MAPK, suggesting a novel mechanism for its activation (Nakielny *et al.*, 1992; Rossomando *et al.*, 1992). Sequence analysis of mammalian MAPKK cDNAs revealed that the encoded proteins are members of a protein kinase subclass that contains the products of the *S. pombe byr1* and *S. cerevisiae STE7* genes (see Table I), which are involved in phermone-dependent signal transduction in yeast (Ashworth *et al.*, 1992; Wu *et al.*, 1993; see the discussion that follows).

Recently, the Raf kinase (reviewed by Rapp, 1991) has been demonstrated to regulate the MAPKK–MAPK–RSK phosphorylation cascade. In NIH3T3 cells, which are transformed by the *v-raf* oncogene, MAPKK and MAPK activities are constitutively induced (Dent *et al.*, 1992; Howe *et al.*, 1992; Kyriakis *et al.*, 1992). The MAPKK may be a direct substrate for partially purified (Howe *et al.*, 1992; Kyriakis *et al.*, 1992) or recombinant baculovirus-synthesized (Dent *et al.*, 1992) Raf kinase *in vitro* and is activated following phosphorylation by Raf. Thus, the product of the *raf* protooncogene may directly control the MAPKK–MAPK–RSK signal transduction pathway(s). The connection between Raf and MAPKK is currently tenuous since none of the reported experiments used purified MAPKK, and thus possible intermediary kinases cannot be ruled out. Also, genetic clues from yeast suggest that upstream kinase(s) other than Raf may be the physiological activators of MAPKK (Crews and Erikson, 1992; Howe *et al.*, 1992; see the following discussion). However, the ability of Raf to activate the MAPK pathway provides a potential mechanism for explaining how it activates *c-fos* transcription, demonstrated

previously by several groups (reviewed by Heidecker *et al.*, 1992), since an activating transcription factor for the *c-fos* promoter, $p62^{TCF}$, has been identified as a MAPK substrate (Gille *et al.*, 1992).

3. Ras Is an Upstream Activator of the MAPK Pathway

In an important advance, Ras has been shown to be necessary and sufficient for upstream activation of the MAPK pathway in several cell systems. Several groups have used a dominant inhibitory Ras mutant (RasN17) (Feig and Cooper, 1988) to demonstrate that NGF-induced activation of the MAPK pathway in PC12 pheochromocytoma cells requires an endogenous Ras function (Robbins *et al.*, 1992; Thomas *et al.*, 1992; Wood *et al.*, 1992). RasN17 also blocked the stimulation of MAPK activity in rodent fibroblasts by insulin and platelet-derived growth factor (de Vries-Smits *et al.*, 1992). Conversely, when activated forms of Ras are expressed in these cells in the absence of growth factors, MAPK activity is rapidly stimulated (Leevers and Marshall, 1992; Thomas *et al.*, 1992; Wood *et al.*, 1992). In *Xenopus* oocytes, where similar results have been obtained by microinjecting p21Ras, it has been demonstrated that effector mutations, which compromise transformation activity, eliminate the ability of Ras to activate MAPK (Pomerance *et al.*, 1992).

These data are largely consistent with other results indicating that Raf acts downstream of Ras and upstream tyrosine kinases (Rapp, 1991). It has been shown that expression of dominant inhibitory Raf mutants and antisense Raf RNA can block both *ras* transformation (Kolch *et al.*, 1991; Kizaka-Kohdoh *et al.*, 1992) and *ras*-stimulated transcriptional activation events (Bruder *et al.*, 1992). Others have demonstrated by examining the phosphorylation state of Raf that Ras action is required for full autophosphorylation activity of recombinant Raf in baculovirus-infected insect cells (Williams *et al.*, 1992). Furthermore, Ras has been shown to function upstream of Raf in the Sevenless signal transduction pathway regulating R7 cell fate during *Drosophila* eye development (Dickson *et al.*, 1992).

4. Complexity of the Interactions between Ras and the MAPK Pathway

What connections lie between Ras and components of the MAPK pathway? In an exciting development, it has been reported recently that it is possible to reconstitute the Ras activation of MAPK in broken cell extracts of *Xenopus* oocytes (Hattori *et al.*, 1992; Shibuya *et al.*, 1992). The activation was shown to depend upon RasGTP (Hattori *et al.*, 1992) and Ras C-terminal farnesylation (Shibuya *et al.*, 1992), which is required for positive signaling activity. Using this system, it should be possible to

identify biochemically the long-sought Ras effector functions that lead to MAPK activation.

One potential set of intermediaries between Ras and Raf might be the conventional isotypes of protein kinase C (PKC α, β, γ), based on their ability to phosphorylate the Raf kinase both *in vitro* and *in vivo*, in recombinant baculovirus-infected insect cells (Sözeri *et al.*, 1992). Other experiments that would place PKC between Ras and Raf in the MAPK pathway include the observations that (1) injection of Ras oncoprotein into quiescent 3T3 cells rapidly induces PKC activity (Morris *et al.*, 1989) and (2) phorbol ester stimulation of MAPK activity in Rat1 cells is insensitive to RasN17 (de Vries-Smits *et al.*, 1992). However, other observations argue that PKC acts upstream of Ras. For example, Ras is required for phorbol ester-induced DNA synthesis in fibroblasts (Yu *et al.*, 1988). Moreover, RasN17 can block phorbol ester-stimulated MAPK activity in PC12 cells (Thomas *et al.*, 1992; Wood *et al.*, 1992), and overexpression of $p120^{GAP}$ blocks phorbol ester-induced MAPK activity in 3T3 cells (Nori *et al.*, 1992). Finally, phorbol ester induces the formation of RasGTP in T cells (Satoh *et al.*, 1992). Although this apparent conflict may be due to cell-type differences, it might be resolved if a synergism exists between signals from PKC and Ras, which are not yet understood, possibly involving GEFs and phorbol ester inhibition of GAP activity (Howe *et al.*, 1992; Satoh *et al.*, 1992).

There are additional data arguing against a simple linear connection between Ras and MAPK activation. First, RasN17 does not inhibit all receptor ligands from activating MAPK (Robbins *et al.*, 1992), so there must be routes from tyrosine kinase receptors that bypass Ras. Second, even though v-*ras* induces MAPK in NIH3T3 cells, it does not do so in Rat1a fibroblasts (Gallego *et al.*, 1992). Thus, signals transduced by Ras may act independently of MAPK. Finally, v-*ras* does not induce MAPK in all cells (Wood *et al.*, 1992), so there may be a bifurcation downstream of Raf, possibly leading to Raf-dependent nuclear events such as *c-fos* transcription (Heidecker *et al.*, 1992; Roberts, 1992). One is led to the conclusion that (1) cells arrange their signal transduction pathways in different ways, (2) there exists a complex branched network of signaling routes whose operation depends on the comparative importance of the experimental inputs (Roberts, 1992; Williams *et al.*, 1992), or (3) the linear models for signal transduction must be replaced by models in which upstream and downstream molecules can influence each other in a nonlinear manner. If this latter conclusion is true, then discussions of signal transduction must be conducted in terms of epistasis rather than strict biochemical linearity. One would anticipate that this situation will be

clarified by the emerging biochemical characterization of signaling complexes that contain Ras.

5. Ras and the MAPK Pathway in Yeast

Genetic analysis of *ras* in the fission yeast *S. pombe* supports the connection between Ras signaling and the MAPK pathway and provides another route to identify additional members of the cascade. In *S. pombe,* the single *ras1* gene is nonessential for cell growth but necessary for sexual differentiation functions, conjugation, and sporulation (Fukui *et al.,* 1986; Nadin-Davis *et al.,* 1986). Partial phenotypic suppression of *ras1* mutations can be achieved by overexpression of the *byr1* (*ste1*) (Nadin-Davis and Nasim, 1988) or *byr2* (*ste8*) (Wang *et al.,* 1991b) genes, which encode serine/threonine kinases (see Table I). Epistatic analyses support a scenario in which the action of *byr2* is situated between the actions of *ras1* (upstream) and *byr1* (downstream) (Wang *et al.,* 1991b).

A compelling similarity has recently been described between *byr1* and several peptide sequences generated from a biochemically purified murine polypeptide (MEK) with MAPKK activity (Crews and Erikson, 1992; Nakielny *et al.,* 1992). The relationship gleaned from the limited sequence information available suggests that MEK and *byr1* might be members of a gene family. The evidence that MAPKK activation is downstream of Ras, and the similarity between MEK and *byr1* implies that a mammalian homolog of *byr2* exists that mediates Ras activation of MAPKK (MEK) activity. However, this hypothesized intermediate protein kinase may not be Raf, since there is considerably less extensive similarity between *byr2* and Raf (~20%) than between MAPKK and *byr1* (~50%) (Howe *et al.,* 1992). One would anticipate that, if Raf is not the mammalian homolog of *byr2,* another more closely related kinase(s) (e.g., MAPKKK) that activates MAPKK remains to be identified in animal cells. If this is the case, Raf could act in parallel or upstream of MAPKKK to activate MAPKK activity.

B. Ras and the Rho/Rac Pathway

One of the characteristics of cellular stimulation by growth factors is a rapid and extensive remodeling of cytoskeletal architecture, much of which is due to the reorganization of microfilaments that contain filamentous actin (F-actin) (Small, 1988; Bretscher, 1991). In contrast to growth factor-treated normal cells, transformed cells exhibit a permanent change in cytoskeletal organization. It has been known for some time that expression of oncogenic Ras rapidly induces the reorganiza-

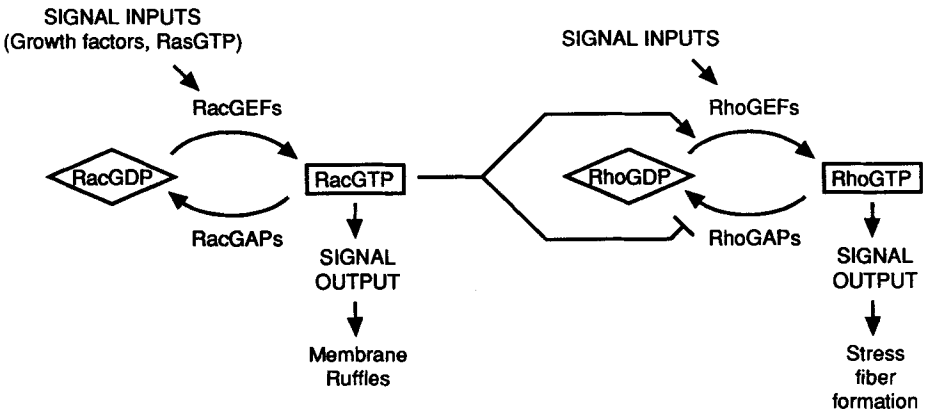

FIG. 3. Rho/Rac pathway. Rac and Rho guanine nucleotide exchange can be stimulated by two signal inputs, one of which acts upstream of Rac and the other that acts upstream of Rho but is independent of Rac (Downward, 1992b).

tion of actin (Bar-Sagi and Feramisco, 1986). But until recently the mechanism of this process was obscure.

In an important advance, studies of Ras-related GTP-binding proteins now indicate that activated forms of Ras induce changes in cytoskeletal actin through members of the Rho family, which have been implicated in the control of the cell architecture (for reviews see Downward, 1992b; Hall, 1992a). A simple model that summarizes the current data is shown in Fig. 3. The connection between activated Ras and the Rho/Rac pathway (as we have called it) is vitally important because it may represent a critical feature that differentiates oncogenic from normal Ras functions. We briefly review the function of Rho family members in regulating cytoskeleton actin and then discuss work demonstrating that activated Ras can modulate cytoskeletal actin via the Rho/Rac pathway.

1. Rho and Rac Affect Cytoskeletal Actin Organization

In mammals, the Rho family is comprised of nine GTP-binding proteins with ~50% similarity to each other and ~30% similarity to the Ras proteins. On the basis of computer similarity assessments (see, e.g., Vincent *et al.*, 1992), the family can be subdivided into two groups containing (1) the three closely related RhoA, RhoB, and RhoC proteins (Rho proteins), and (2) the two Rac1 and Rac2 proteins, TC10, two CDC42Hs proteins, and RhoG (the Rac proteins). As suggested by their relatedness to Ras, all function similarly to Ras in that their GTPase cycle is controlled by specific GAPs and GEFs (see Fig. 3). Furthermore, mutations

that stimulate (Garrett *et al.*, 1991) or impede (Ridley *et al.*, 1992) GTPase activity in Ras function operate on Rho family proteins in a similar manner to the analogous mutations in Ras. For example, RhoV14 has an impaired GTPase activity, constitutively induces changes in actin cytoskeleton (Ridley *et al.*, 1992), and functions in an activated manner similar to the analogous RasV12 mutation.

The actin cytoskeleton in fibroblasts is composed of (1) cell surface membrane ruffles, (2) the cortical network, located beneath the plasma membrane, (3) and stress fibers, which extend into the cell from cell-surface focal adhesions where integrins make contacts with the extracellular matrix (Small, 1988; Bretscher, 1991; Downward, 1992b). Early evidence that Rho proteins could regulate actin organization came from observations that introduction of recombinant Rho or C3 transferase (a bacterial ADP-ribosyltransferase from *Clostriduim botulinum* C3 for which Rho is a substrate) could affect the number of actin stress fibers at the cell membrane. Rho increased stress fiber assembly, whereas C3 transferase reduced it by ADP-ribosylation and inactivation of endogenous Rho function (reviewed by Hall, 1992a). This form of regulation may be physiologically relevant in mammalian cells, since the epidermal cell differentiation inhibitor (EDIN), an inhibitor of keratinocyte differentiation, is reported to exhibit high sequence similarity to C3 transferase and acts by ADP-ribosylating Rho family proteins (Sugai *et al.*, 1992).

In two illuminating reports, it has recently been demonstrated that in growth factor-stimulated fibroblasts, RhoA is required to regulate the assembly of actin stress fibers and focal adhesions (Ridley and Hall, 1992), whereas Rac1 is necessary for the formation of membrane ruffles (Ridley *et al.*, 1992) (see Fig. 3). Assembly of stress fibers and focal adhesions was blocked by injection of C3 transferase, which efficiently and selectively inhibits endogenous Rho function. Membrane ruffling could still be observed in C3 transferase-injected cells in response to serum (Ridley and Hall, 1992). However, injection of dominant inhibitory Rac proteins blocked both growth factor-induced membrane ruffling and stress fiber/focal adhesion formation (Ridley *et al.*, 1992). This result indicated that Rac function is required for both processes, suggesting that in some cases Rac may function upstream of Rho in a cascade of Rho family GTPases (Downward, 1992b).

2. Ras Acts Upstream of the Rho/Rac Pathway

Microinjection of activated Ras into living cells has been shown to stimulate membrane ruffling rapidly (Bar-Sagi and Feramisco, 1986). It has now been demonstrated that this effect requires the action of Rac protein(s), since co-injection of a dominant inhibitory Rac protein with

activated Ras blocked the Ras-induced ruffling response (Ridley *et al.*, 1992). This result suggests that Ras acts upstream of Rac to stimulate ruffling, consistent with the observation that ruffling induced by Rac is more rapid than that induced by Ras (Bar-Sagi and Feramisco, 1986; Ridley *et al.*, 1992).

Significantly, normal endogenous Ras function does not appear to be required for growth factor-induced ruffling: neither anti-Ras antibodies nor dominant inhibitory Ras proteins, when they were microinjected into cells, suppressed membrane ruffles induced by serum or several growth factors (Ridley *et al.*, 1992). This important result suggests that a critical feature of transformation by oncogenic Ras may be its ability to activate an intracellular Rho/Rac pathway in a manner not targeted by normal Ras. One possible mechanism for this phenomenon might be that signaling by normal Ras requires a RasGEF function, which on structural grounds may also encode an associated Rho/Rac GEF function(s), whereas oncogenic Ras is independent of exchange factors. It will be important to determine if transformation by activated Ras can be blocked by inhibiting a Rho family function(s). That RhoA overexpression results in a weakly transformed phenotype in NIH3T3 cells (Avraham and Weinberg, 1989) lends credence to the idea that Rho family proteins will be important for transformation. Moreover, RhoG (Vincent *et al.*, 1992) and RhoB (Jahner and Hunter, 1991a,b) mRNAs are rapidly induced in rodent fibroblasts stimulated by serum (RhoG) or EGF, PDGF, and the v-*src* and v-*fps* oncogenes (RhoB).

There is also biochemical evidence indicating a direct linkage between Ras and Rho family function that derives from the characterization of Ras GEFs and RasGAP-associated proteins. These data support the notion that both normal as well as activated Ras interact with particular Rho/Rac pathways. p190 contains a C-terminal domain related to Bcr and *n*-chimerin (Settleman *et al.*, 1992b) that has RhoGAP activity (Settleman *et al.*, 1992a). Mammalian RasGEFs exhibit an N-terminal relationship to Bcr and the *dbl* oncogene product (Cen *et al.*, 1992; Shou *et al.*, 1992), the latter of which has GEF activity on Rho family members (Diekmann *et al.*, 1991; Hart *et al.*, 1991). These associations strongly connote that signals involving RasGEFs and RasGAPs influence the state of guanine nucleotide on Rac and Rho proteins. Interactions between Ras and Rho/Rac GEFs and GAPs would be anticipated to provide the mechanisms for the observed biological interactions between Ras and Rho/Rac (Ridley and Hall, 1992; Ridley *et al.*, 1992) (see Fig. 1). Interestingly, constitutive expression of the N-terminal domain of GAP (GAP-N) in Rat2 fibroblasts has been reported to disrupt the actin cyto-

skeleton and cell adhesion to fibronectin substratum (McGlade *et al.*, 1993).

The induction of the Rho/Rac pathway by Ras suggests that actin organization at focal adhesions may play a key role in Ras transformation. Actin microfilaments terminate at the plasma membrane in focal adhesions, where they are tipped by factors such as talin, viniculin, tensin, and integrins, which are involved in regulating cell-substratum adhesion (Burridge *et al.*, 1988; Turner and Burridge, 1991). The importance of focal adhesions in transformation and cell growth is suggested by the localization there of $pp60^{v\text{-}src}$ (Liebl and Martin, 1992) and $p125^{FAK}$, a substrate of $pp60^{v\text{-}src}$ and a tyrosine kinase in its own right that may lie at a signaling intersection between integrins and oncogenes (Burridge *et al.*, 1992; Lipfert *et al.*, 1992; Zachary and Rozengurt, 1992) (see Fig. 1). If Ras induces changes in cytoskeletal actin through the Rho/Rac pathway, it may do so either downstream or in parallel to where $p125^{FAK}$ performs, because *ras*-transformed cells do not exhibit the increased $p125^{FAK}$ tyrosine phosphorylation (Guan and Shalloway, 1992) characteristic of cells transformed with the oncogenes v-*src*, v-*yes*, or v-*crk* (Zachary and Rozengurt, 1992), all of which act upstream of Ras.

Another emerging connection between Ras and cytoskeletal actin that should be mentioned here involves profilin and β4 thymosin, which both directly bind monomeric actin (G-actin) (for a more thorough review see Goldschmidt-Clermont *et al.*, 1992a). G-actin is itself an ATP/ADP-binding protein analogous to G-proteins in the sense that the "active" form is bound to ATP, which polymerizes more readily *in vitro* than the ADP-bound form to produce (F-actin) (Pollard and Cooper, 1986). This analogy between G-actin and G-proteins can be conceptually expanded by the recent finding that profilin and β4 thymosin regulate adenine nucleotide exchange on G-actin, with profilin serving as an actin adenine nucleotide dissociation stimulator (actinADS) and β4 thymosin serving as an actin adenine nucleotide dissociation inhibitor (actinADI) (Goldschmidt-Clermont *et al.*, 1992a). Like guanine nucleotide exchange factors, profilin can stimulate exchange of both ATP and ADP, but the net effect of its action would tend to be ATP loading since cells contain a relative excess of ATP.

The connection of these proteins to Ras comes from studies in *S. cerevisiae* that have defined cyclase-associated protein (CAP), a polypeptide that is associated with Ras and the Ras effector in yeast, adenylyl cyclase (see Table I). Expression of C-terminal CAP deletion mutants in yeast cells produces abnormal morphological phenotypes, which, intriguingly, can be rescued by profilin overexpression (Vojtek *et al.*, 1992).

The mechanism of the rescue is obscure, but based on results with profilin mutants, it appears to require the ability of profilin to bind actin and membrane-bound polyphosphoinositides, which interact with profilin. This latter feature reveals another analogy between profilin and RasGEFs, because the predicted sequence of a RasGEF contains a putative polyphosphoinositide-binding motif (Goldschmidt-Clermont *et al.*, 1992b). Taken together, these observations prompt the speculation that Ras could regulate G-actin states through an effector complex that has an associated profilin-like (actinADS) activity. Ras effector signaling would lead to the downstream activation of actin polymerization by inducing ATP loading of G-actin through profilin (or another actinADS). Thus, current findings point to two means that Ras might regulate cytoskeletal actin, one involving F-actin via a Rho/Rac pathway and another involving G-actin that might involve actin adenine nucleotide exchange factors (actinAEFs) like profilin.

Work on the cytoskeleton and cell function is proceeding rapidly, and we expect that it will lead to a greater appreciation for the signaling functions of the cell that involve direct contacts with cytoskeletal architecture. In contrast to the abundance of information connecting Ras to the MAPK pathway, there is little understanding and appreciation of the importance of the signals that feed through the Rho/Rac pathway. A central problem for understanding Ras-regulated signal transduction in the future must include an identification of the elements downstream of the Rho/Rac proteins, and an understanding of the consequences of actin reorganization on short-term and long-term cell physiology (especially in disease states such as cancer).

V. Perspectives on Ras Function

A. Reversion of Ras-Transformed Cells

Many laboratories have studied phenotypic revertants of *ras*-transformed cells with the goal of identifying the biologically critical elements required for Ras signal transduction. Some efforts have focused on isolating direct downstream effectors required for Ras function, employing reversion as phenotype for genetic approaches. However, these approaches also define key cellular functions and pathways that Ras must affect indirectly in order for it to transform cells. These clues would be important since they provide access to necessary steps that might be difficult to identify biochemically and that may provide insights into the vital aspects of Ras transformation.

1. Genetic Reversion

Early efforts in this area resulted in the isolation of K-rev1/Rap1A/*smg* p21, a Ras-related GTP-binding protein that suppresses transformation by K-Ras (Kitayama *et al.,* 1989). The mechanism of K-rev1 may be through competition with Ras for $p120^{GAP}$, as it binds $p120^{GAP}$ approximately 100-fold more tightly than do Ras proteins (Bollag and McCormick, 1991b). This biochemical activity of K-rev1 indicates that it may act by sequestering $p120^{GAP}$ from Ras, supporting a role for $p120^{GAP}$ in Ras effector function [however, K-rev1 may also stimulate separate pathways since it is largely localized in a separate cellular compartment from Ras (Bollag and McCormick, 1991b)].

Other genes encoding factors that may compete for downstream Ras effector functions are *rsp-1,* which was isolated in a screen for suppressors of v-*ras* transformation (Cutler *et al.,* 1992), and three cDNAs (JC99, JC265, JC310) isolated in a expression screen for proteins that interfere with RAS function in yeast (Colicelli *et al.,* 1991) (see Table I). The *rsp-1* gene does not have extensive similarity to known proteins, but it does harbor a leucine-based repeat sequence that is found in the regulatory region of yeast adenylyl cyclase. This homology is potentially interesting because adenylyl cyclase is the downstream target effector of yeast RAS (Broach, 1991), and there is genetic evidence that the repeat motif is required for cyclase interaction with RAS (Colicelli *et al.,* 1990). The JC99 and JC265 cDNAs are related genes that showed weak similarity to a region found in GAP-like genes, including IRAs (yeast GAPs) and NF1, whereas JC310 exhibited a small box of similarity to yeast GEFs (Colicelli *et al.,* 1991). The meaning of these relationships is quite tenuous given the limited sequence similarity but presents the possibility that, like *rsp-1,* these genes may act by competing for effector functions (perhaps fortuitously) or may in their normal role encode effector functions themselves.

In another screen for candidate Ras effectors, Lengyel's group has isolated two revertant cell lines from NIH3T3 that were transformed with c-H-*ras* (Yamada *et al.,* 1990). Fusion of the revertants with normal NIH3T3 cells and with each other yields hybrids with transformed morphology, indicating that revertants are recessive and represent two complementation groups. In both c-H-*ras*-transformed and revertant cells, there is no change in the phosphorylation state or *in vitro* kinase activity of the Raf kinase, suggesting that transformation induced by c-H-*ras* does not proceed through the MAPK pathway (Yamada *et al.,* 1991). Moreover, v-*raf* (Yamada *et al.,* 1991) and a variety of oncogenes acting

upstream of Raf (Omata-Yamada *et al.,* 1991) will retransform the mutants. However, examination of the transcription patterns of the revertants indicates that, similar to normal NIH3T3 cells, they lack some transcriptional activating signal present in the transformed cells (Yamada *et al.,* 1991). These data are consistent with the possibility that in the revertants there are defects in signaling components that (1) are required for c-H-*ras* transformation, (2) are not part of the MAPK pathway and (3) may be effectors of c-H-*ras*.

2. *Pharmacological Reversion*

In an interesting series of reports, Nishimura's group has described the action of an antibiotic compound, azatyrosine, that permanently reverts *ras*- or *raf*-transformed NIH3T3 cells to a flattened phenotype following treatment for several days in culture (Shindo-Okada *et al.,* 1989). Normal cells or those transformed by the *hst* or *ret* oncogenes are apparently unaffected, except for a reduction of ~30% in the growth rate during the course of the drug treatment. Azatyrosine has also been shown to inhibit p21Ras-induced maturation of *Xenopus* oocytes following microinjection (Chung *et al.,* 1991) and chemical carcinogenesis involving *ras* activation *in vivo* following treatment of mouse skin with benzanthracene (Izawa *et al.,* 1992). Azatyrosine will inhibit the differentiation of PC12 cells induced by mutant Ras, but not that induced by NGF (Fujita-Yoshigaki *et al.,* 1992), which requires normal Ras function (Hagag *et al.,* 1986). This observation suggests that azatyrosine may distinguish a pathway utilized by mutant but not normal Ras.

Azatyrosine-induced morphological flattening of *ras*-transformed cells correlates with induction of several genes (Krzyzosiak *et al.,* 1992), including *rrg* (*ras* recision gene), whose expression is reported to revert *ras*-transformed cells (Contente *et al.,* 1990), and *rhoB*, a member of the Rho family that is rapidly induced by v-*src* and v-*fps* and is part of the immediate early genetic response to EGF in 3T3 cells (Jahner and Hunter, 1991a,b). The *rrg* gene is closely related or identical to lysyl oxidase (Kenyon *et al.,* 1991), an extracellular matrix enzyme that modifies elastin and collagen (for references see Trackman *et al.,* 1990), and *rhoB* encodes a member of the Rho family proteins that play a role in the organization of intracellular actin (as discussed previously). Azatyrosine-treated cells also exhibit an increase in collagen III and fibronectin expression (Krzyzosiak *et al.,* 1992).

These results are of interest because they reinforce by another route the association between Ras transformation activity and cell surface or extracellular entities that control cell shape, adhesive properties, and cell–cell communication. There is growing evidence that molecules in-

volved in cell adhesion may regulate, rather than simply respond to, normal and abnormal cell growth signals (Zachary and Rozengurt, 1992). The connection of mutant Ras to actin organization in focal adhesions has already been mentioned. Integrins, which bind extracellular matrix elements like fibronectin, are found in focal adhesions and also play a role in regulating cell shape and adhesion (Zachary and Rozengurt, 1992). One additional extracellular aspect of transformation by *ras* and other oncogenes that may be important is modulation of gap junctions (see, e.g., Brissette *et al.*, 1991; de Feijter *et al.*, 1992); and references therein). Loss of cell–cell communication through gap junctions has been argued to be important for tumorigenic progression (Yamasaki, 1988), although in some coculturing experiments where normal cells have been observed to revert transformed cells, paracrine factors rather than changes in gap junction-mediated exchange may actually mediate the reversion phenomenon (Martin *et al.*, 1991).

The potential connections between Ras and systems that are involved with extracellular processes are still quite new. For all the genetic or pharmacological agents that revert *ras*-transformed cells, it will be important to determine whether they induce functions that override *ras* action or inhibit functions that are necessary. The genes and pharmacophores that can revert *ras*-transformed cells, will, in any case, prove to be important probes that can broaden our understanding of the biological and biochemical functions of Ras.

B. Models for Ras Function in Growth Factor-Mediated Signal Transduction

1. Two Pathways Emanate from Ras

Elucidation of Ras function has been a very difficult task in part because the physiology of Ras differs among organisms and cell types. Examples vary from second messenger regulation (*S. cerevisiae*) and cell fate (*C. elegans* and *Drosophila*) to mitosis (cell division) and differentiation (cell arrest) in mammalian cells. Nevertheless, in mammalian cells the factors that regulate Ras and the paths epistatic to Ras continue to be defined. In this review, we have outlined that the stream of information leading to Ras typically includes a tyrosine kinase, an SH2 domain-containing adaptor protein, and a nucleotide exchange factor(s). This information results in Ras activation and emission of a Ras-regulated signal through effector functions that are not yet completely clear. At present the data do not support a model in which Ras interaction with a specific protein leads to a specific signal response, although the recent

biological results with $p120^{GAP}$ indicate that it is at least one component that is required for Ras-induced cellular transformation. However, the information disseminating from Ras as it pertains to cellular transformation can now be divided into at least two paths, mitogenesis and morphology changes, and we have discussed the rapid definition of the components of these paths (MAPK and Rac/Rho, respectively). A graphical view of a model for growth factor-regulated signal transduction involving Ras is shown in Fig. 1.

2. Rho/Rac Pathway, Cell Morphology, and Myc

The idea that Ras regulates at least two functions by different pathways was predicted by the elegant work of Chris Marshall, Alan Hall, and their colleagues, who in 1989 were able to dissociate Ras-induced mitogenic and morphologic phenotypes based upon dependence on PKC (Lloyd *et al.*, 1989). Mitogenesis (DNA synthesis) was PKC dependent, whereas morphology changes were PKC independent. Curiously, Ras-induced *myc* mRNA expression was also PKC independent implying that *myc* lies within the morphology pathway. This situation is consistent with results from Marshall Sklar, Edward Prochownik, and colleagues, who have reported that antisense *myc* RNA will morphologically revert *ras*-transformed cells (Sklar *et al.*, 1991). Based on these observations, one would predict that the *ras* morphology path may regulate *myc* expression and the subsequent downstream effects through the action of Myc protein [believed to encode a transcription factor (reviewed by Prendergast and Ziff, 1992)]. In this context, it is interesting to note that several Myc-regulated genes encode extracellular matrix proteins (e.g., plasminogen activator inhibitor-1; Prendergast *et al.*, 1989) or integrins (e.g., LFA-α; Inghirami *et al.*, 1990). Thus, not only is the morphology pathway likely to be a very important component of the transforming Ras phenotype, but it may also lead to novel insights into the biological functions of Myc. The relationship between Ras and Myc can now be further evaluated in the context of the Rac/Rho regulatory system.

3. Does Oncogenic Ras Signal Differently Than Normal Ras?

Another reason for the difficulty in defining Ras function may come from our dogmatic models that postulate that Ras must have parallels with the G-protein signal transduction system, where there is a linear relationship among protein components that respond to stimuli originating at the plasma membrane. A similar relationship may be true to some extent for normal Ras, which is sensitive to stimulation by tyrosine kinases in a common compartment, the plasma membrane. Since on-

cogenic Ras is also plasma membrane associated, it has been assumed that it interacts with the same effector systems and elicits the same functions as normal Ras. Could this assumption be incorrect? Work with dominant negative forms of Ras and $p120^{GAP}$ could be interpreted to suggest that there are differences between normal and oncogenic Ras functions. The RasN17 mutant, which preferentially binds GDP rather than GTP, and the Ras-CVLL mutant, which contains a signal for C-terminal geranylgeranylation rather than farnesylation, both preferentially inhibit normal Ras function but have little effect upon oncogenic Ras function (Feig and Cooper, 1988; Cox *et al.*, 1992). Conversely, two different dominant negative proteins (prenylation-defective forms of oncogenic RasL61 and an N-terminal segment of $p120^{GAP}$ containing SH2/SH3 domains) preferentially inhibit oncogenic Ras function but have a considerably weaker effect against normal Ras function (Stacey *et al.*, 1991; Clark *et al.*, 1993).

There are additional paradoxes regarding oncogenic Ras function. For example, K-rev1/Rap1A appears to be localized in the Golgi apparatus, but upon overexpression it antagonizes oncogenic Ras function thought to act at the plasma membrane (Bollag and McCormick, 1991b). In another case, it has been shown that a geranylgeranylated form of Ras is transforming, but it is localized in a membrane fraction different than plasma membrane (Hancock *et al.*, 1991; Cox and Der, 1992; Cox *et al.*, 1992). Furthermore, the observation that the function of farnesylated oncogenic Ras can be blocked by soluble Ras implies that there is a soluble protein or process not at the plasma membrane that is required for Ras function (Gibbs *et al.*, 1989; Michaeli *et al.*, 1989). In this regard it is interesting to note that some of the enzymes that posttranslationally modify Ras, the endoprotease, methyltransferase, and palmitoyl transferase are associated with microsomal membranes rather than the plasma. It is intriguing to speculate that Ras is modified and localized at an intracellular membrane site and then transported to the plasma membrane. Such transport (i.e., localization of a small subpopulation in a nonplasma membrane compartment) may be particularly relevant with respect to oncogenic Ras function, which apparently is not dependent upon the plasma membrane-associated factors (tyrosine kinase, GEF) to initiate its effector functions.

One indication that mitogenic signaling may involve events at internal, vesicular membrane sites has come from the observation that p110, the catalytic subunit of phosphoinositide 3′-kinase (PI3′-K), shares sequence similarity with Vps34, a *S. cerevisiae* protein involved with protein sorting (Hiles *et al.*, 1992). The PI3′-K activity is stimulated upon its association with activated tyrosine kinases such as the platelet-derived

growth factor receptor. Thus, one can propose a simple model that has both receptor and enzyme co-localized at the plasma membrane. If the sequence similarity between Vps34 and PI3′-K indicates functional similarities, then the action of PI3′-K may involve steps in nonplasma membrane compartments. If this were true, Ras may also affect these events, as speculated previously.

Bourne has suggested that members of the Ras superfamily may be involved with mechano-coupling processes (somewhat analogous to EF-Tu) rather than classic signal transduction found with G-proteins (Bourne, 1988). For Ras-related proteins that direct vesicle fusion in secretory processes, this scenario appears to be the case and involves complexes among the Ras-related protein, membrane-bound docking proteins, and regulatory proteins (GDIs). For Ras itself, we do not know whether this model applies, although others have argued for this possibility (Chardin, 1991). However, the recent identification of Ras regulatory proteins with multiple functional domains raises the possibility that Ras indeed may also function within multi-protein complexes. In contrast to G-proteins, Ras could be present in complexes that act as signaling devices that are composed of GAP(s), GEF(s), and possibly other proteins. In such a model, even upstream factors such as GEFs might have Ras effector function. The formation of a multi-protein signaling device could also explain how Ras could affect a number of physiological responses because the exact protein components and therefore complexes could vary among cell types. This scenario provides one means by which Ras could be central to so many aspects of cell physiology and why elucidating its function(s) has proved so challenging.

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NOTE ADDED IN PROOF: Two significant advances in the regulation of Ras by exchange factors and Ras effector function have been reported. First, several groups have isolated murine and human forms of the Ras exchange factor, SOS, and demonstrated that a proline-rich region in it mediates interaction with the SH3 domain of the adaptor protein GRB2 [for a summary and list of references, see L. Feig, *Science* **260,** 767–768 (1993) and F. McCormick, *Nature* (*London*) **363,** 15–16 (1993)]. Second, a direct biochemical interaction between Ras-GTP, Raf-1 kinase, and MAPKK was implied by work that identified a complex containing these factors [see S. A. Moodie *et al., Science* **260,** 1658–1661 (1993)]. This observation argues that there is a direct linkage between Ras and Raf-1 in signal transduction, and identifies the Raf-1 kinase as a critical Ras effector molecule.

THE ROLE OF THE ADENOMATOUS POLYPOSIS COLI (*APC*) GENE IN HUMAN CANCERS

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I. Identification of the *APC* Gene

Familial adenomatous polyposis (FAP) is an autosomal-dominant disease that affects 1 in 7000–15,000 people worldwide (Utsunomiya, 1990). The FAP is characterized by the development of hundreds to thousands of adenomatous polyps in the colon and rectum, one or more of which will progress to cancer if left without surgical treatment. Tumors are not necessarily limited to the colon and rectum; patients with FAP may sustain a variety of neoplasms in other tissues (e.g. desmoids, osteomas, or fibromas) in a syndrome described by, and named for, Eldon Gardner (Gardner and Richards, 1953). Cytogenetic observation in one FAP patient (Herrera *et al.*, 1986) and linkage studies in large FAP kindreds (Bodmer *et al.*, 1987; Leppert *et al.*, 1987; Nakamura *et al.*, 1988) localized the gene responsible for FAP to chromosome 5q21-22, a region that frequently shows allelic deletion in sporadic colorectal ade-

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nomas and carcinomas (Solomon *et al.*, 1987; Vogelstein *et al.*, 1988, 1989; Ashton-Rickardt *et al.*, 1989, 1991; Miyaki *et al.*, 1990; Okamoto *et al.*, 1990; Miki *et al.*, 1991). Hence, it was considered likely that germline or somatic mutations in one or more genes within chromosome 5q21 would lead to development of familial and sporadic forms of colorectal adenoma.

By means of so-called positional cloning, we and others succeeded in identifying the gene responsible for FAP, termed the *APC* (adenomatous polyposis coli) gene, which codes 2843 amino acids (Kinzler *et al.*, 1991; Nishisho *et al.*, 1991; Groden *et al.*, 1991; Joslyn *et al.*, 1991). The *APC* gene consists of 16 exons (one noncoding and 15 coding exons); the largest, most 3′-coding exon encodes more than 2000 amino acids (Groden *et al.*, 1991; Horii *et al.*, 1993). The *APC* gene is expressed ubiquitously in various organs, but at least five forms of noncoding transcripts have been identified (Horii *et al.*, 1993). Transcriptional initiation occurs at three sites in two distinct nontranslating exons at the 5′ end of the gene, and five different forms of 5′ noncoding sequences are generated by alternative splicing. The splicing mechanism seems to be regulated in a tissue-specific fashion because one type of transcript contained a noncoding exon, which was transcribed specifically in brain (Horii *et al.*, 1993). It is uncertain as yet whether this brain-specific transcript has a unique function, because we have not examined whether or not it contains an entire *APC* coding sequence. However, the possibility exists that it may contain a differently spliced form of the *APC* coding region at the 3′ downstream and that its product may have a very specific function in the brain.

Furthermore, the alternative splicing that involves the signal recognition particle gene (*SRP19*) and exon 14 of *APC* (Kinzler *et al.*, 1991; Joslyn *et al.*, 1991) is also controlled in a tissue-specific manner, and one type of transcript is missing in some organs (Horii *et al.*, 1993).

II. Germline Mutations of the *APC* Gene in Familial Adenomatous Polyposis Patients

A. Detection of Germline Mutations

Germline mutations of the *APC* gene have been reported so far in 123 unrelated patients with FAP, including two patients with several-hundred-kilobase deletions reported (Joslyn *et al.*, 1991). Our group has characterized 103 of these mutations by means of a ribonuclease (RNase) protection analysis (Myers *et al.*, 1985a), single strand conformation polymorphism (SSCP) analysis (Orita *et al.*, 1989), or denaturing

TABLE I
TYPES OF 121 GERMLINE MUTATIONS IN THE *APC* GENE[a]

Point mutations		44
Nonsense mutation	38	
Missense mutation	6	
Frameshift mutations		77
Deletion (1–31 bp)	70	
Insertion (1 bp)	7	
Total		121

[a] 103 patients (Miyoshi *et al.*, 1992a; Nagase *et al.*, 1992a, 1992b; our unpublished observations); 8 patients (Fodde *et al.*, 1992); 5 patients (Cottrell *et al.*, 1992); 4 patients (Groden *et al.*, 1991); 1 patient (Stella *et al.*, 1992).

gradient gel electrophoresis (DGGE) (Myers *et al.*, 1985b) coupled with polymerase chain reaction (PCR) amplifications (Saiki *et al.*, 1986) of genomic DNA isolated from FAP patients (Nishisho *et al.*, 1991; Miyoshi *et al.*, 1992a; Nagase *et al.*, 1992a, 1992b; Joslyn *et al.*, 1991; Stella *et al.*, 1992; Cottrell *et al.*, 1992; Fodde *et al.*, 1992; our unpublished observations) (Table I). Of the 121 *APC* germline mutations with small changes, 77 were 1–31 base pairs (bp) deletions and seven were 1–2 bp insertions; all caused a shift of the *APC* reading frame that created new stop codons downstream. Of 44 point mutations, 38 generated stop codons and 6 led to amino acid substitutions. Thus, 115 (95%) of the 121 small mutations so far detected were predicted to result in truncation of the APC protein. Figure 1A shows the distribution of known mutant sites within the *APC* gene in FAP patients. On the basis of our results in 103 of these cases (as the other studies did not examine the entire coding region), almost all the germline mutations (97%) so far observed in the *APC* gene have occurred within the 5′ half of the coding region. In particular, more than 50% of these mutations have been found between codons 1000 and 1400 in exon 16, a region that accounts for only 14% of the entire *APC* coding region. Analysis of the predicted structure of the APC protein has revealed two potential coiled-coil structures, one of which is located in the central portion of the protein where mutations are frequently observed (Kinzler *et al.*, 1991; Joslyn *et al.*, 1991).

It is well known that deletions often occur where nucleotides are repeated, perhaps because of misalignment. Our observations, which included a C deletion from CCC (at codon 1427), an A deletion from several A's (codon 142), and an AA from AAA (codon 1250), were consistent

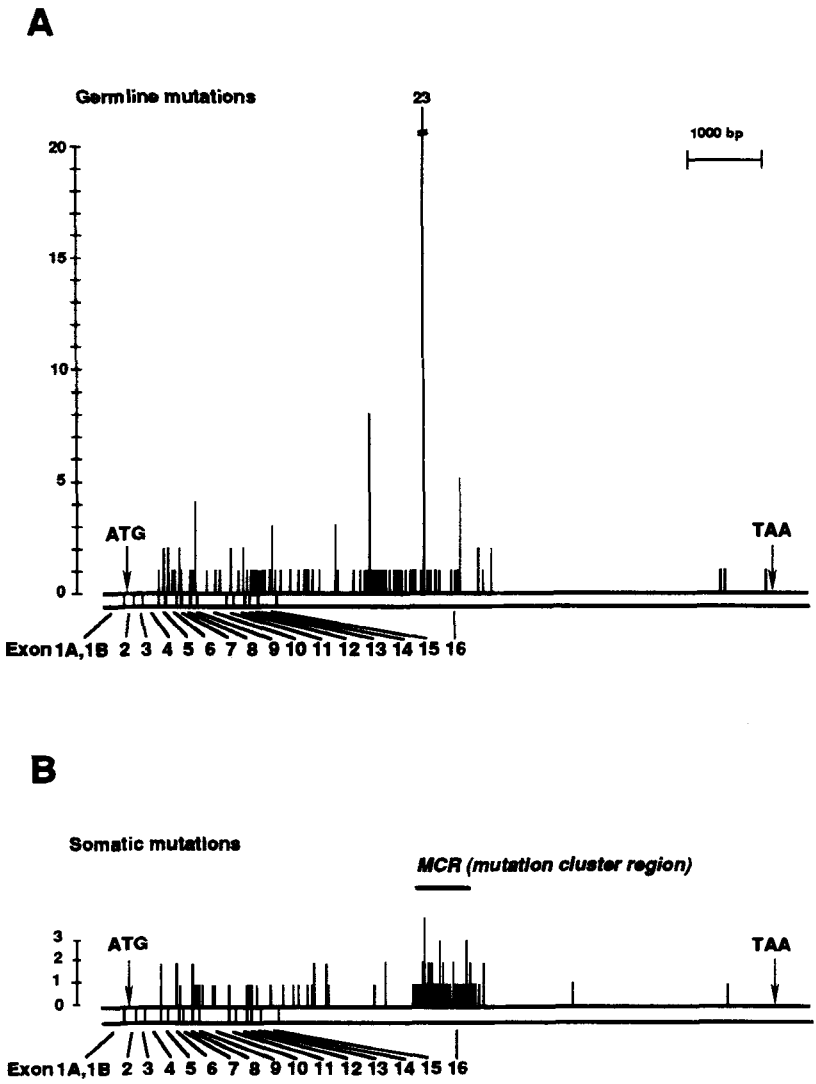

FIG. 1. Distribution of germline mutations (A) and somatic mutations (B) in the *APC* gene. The height of each bar indicates the number of FAP patients or colorectal tumors in which germline or somatic mutations have been detected at the respective positions. The locations of transnational initiation (ATG) and termination (TAA) codons are marked with arrows.

with a proposed model for generating misalignment within a stretch of common bases (Strisinger *et al.*, 1966; Kunkel, 1986). Some of the other deletions in *APC* have occurred at positions containing multiple copies of a direct repeat; for example, a 2-base deletion from a sequence containing five AG repeats (AAAGAGAGAGAGTG; codon 1465), a 5-base (AAAGA) deletion at ATAAAAGAAAAGATT (codon 1309), a 5-base (ACAAA) deletion from ATAAAACAAAGT (codon 1061), and a TGAAA deletion from TCAAATGAAAAC (codon 1546) have been detected. All these deletions may have occurred during DNA replication, probably as a result of slippage of the template strand and subsequent misalignment (Trinh and Sinder, 1991). After synthesis of the first copy of the direct repeat, the template strand could slip and misalign with the second copy of the repeat, with the result that the intervening sequences would be deleted.

Another characteristic of *APC* germline mutations is that more than 80% of known base substitutions are from cytosine to other nucleotides (Table II). Of these, only one-third have occurred at a CpG site, suggesting that deamination of 5-methylcytosine to thymine is not as common in the *APC* gene as it is in somatic mutations of the retinoblastoma and *p53* genes that are seen in many types of cancer (Yandell *et al.*, 1989; Nigro *et al.*, 1989). Although the cytosine seems to be a specific target, the majority of mutations involving C have occurred on the sense strand of *APC* without affecting cytosines on the antisense strand. This observation suggests that cytosines on the sense strand might be preferentially targeted by some carcinogens. However, the frequency of mutations at cytosine residues might be biased by the base specificity of the RNase protection assay (Myers *et al.*, 1985a), which may be more sensitive to cytosine mismatches than to mismatches at other nucleotides.

TABLE II
SUMMARY OF NUCLEOTIDE CHANGES DETECTED AS GERMLINE POINT MUTATIONS OF THE *APC* GENE IN 44 FAP PATIENTS

From/to[a]	C	T (CpG site)	G	A	Total
C	—	25 (13)	7	4	36
T	0	—	1	2	3
G	0	0	—	2	2
A	0	3	0	—	3
Total	0	28	8	8	44

[a] Listed in coding strand.

B. Association of the *APC* Germline Mutation with Various Clinical Phenotypes

Comparisons of germline mutations with clinical manifestations other than colorectal polyposis, in 29 FAP patients for whom detailed information was available, are summarized in Table III (Nagase *et al.*, 1992b). Although FAP is frequently accompanied by extra-colonic manifestations (Utsunomiya, 1989, 1990; Bussey, 1975; Haggitt and Reid, 1986; Spigelman *et al.*, 1989; Gardner and Richards, 1953), observations of

TABLE III
Germline Mutations of the *APC* Gene and Extra Colonic Lesions[a]

Patient	Codon	Nucleotide change	Amino acid change	Extracolonic lesion[b]
102	213	CGA–TGA	Arg–Stop	D,DP
33	232	CGA–TGA	Arg–Stop	—
93	280	TCA–TGA	Ser–Stop	O
24	302	CGA–TGA	Arg–Stop	—
34	302	CGA–TGA	Arg–Stop	D
1085	302	CGA–TGA	Arg–Stop	T,GP
21	414	CGC–TGC	Arg–Cys	O
1142	553	TGG–TAG	Trp–Stop	O
90	577	TTA–TAA	Leu–Stop	D
86	622	TAC–TAA	Tyr–Stop	D,T
38	625	CAG–TAG	Gln–Stop	—
1099	665	ATTAT–AAT	TT deletion	D
60	713	TCA–TGA	Ser–Stop	O
84	806	CATGA–CGA	AT deletion	O,E,GP,DC
124	857	GGAATTGG–GGG	GAATT deletion	—
1087	1041	CAA–TAA	Gln–Stop	GP
104	1102	TAC–TAG	Tyr–Stop	GP,DP
91	1156	GAAGAGA–GGA	AAGA deletion	T
43	1175	CAG–TAG	Gln–Stop	O,E
39	1211	ATG–ATTG	T insertion	—
47	1249	TGC–TGA	Cys–Stop	NI
51	1250	AAAGT–AGT	AA deletion	GP
46	1309	GAAAAGAT–GAT	AAAGA deletion	O
1163	1309	GAAAAGAT–GAT	AAAGA deletion	O,E,DP
1146	1330	CCTGAACCAA–CAA	CTAGAACC deletion	O,E,NI
16	1465	AGTGG–TGG	AG deletion	O,E
1141	1465	AGTGG–TGG	AG deletion	O,E,GP
1143	1465	AGTGG–TGG	AG deletion	O,GP,DP
85	1597	ACTG–ACG	T deletion	T,GP

[a] From Nagase *et al.* (1992b).

[b] O, osteoma; E, epidermoid cyst; D, desmoid; T, thyroid tumor, GP, gastric polyp; DP, duodenal polyp; DC, duodenal cancer; NI, no information about upper GI tract.

variable phenotypes of patients within single pedigrees have suggested that extra-colonic manifestations are not regulated simply by the mutation causing a large number of polyps in colon and rectum. Our results shown in Table I support the view that extra-colonic manifestations, i.e., osteomas, epidermoid cyst, desmoid tumors, thyroid tumors, and upper gastrointestinal polyps, do not correlate with the type or location of a particular germline mutation. However, it might be notable that of three unrelated patients who carried the mutation identified at codon 1465, all showed a large number of extra-colonic benign tumors.

We have looked for an association between the number of polyps developed in FAP patients and the locations of the germline mutations. Based on the number of adenomatous polyps, FAP patients are clinically divided into two categories, profuse and sparse types; a patient with the profuse type of FAP develops more than 5000 adenomatous polyps, and in the sparse type of FAP, a patient develops 1000–2000 polyps (Utsunomiya, 1989, 1990). Based on these broad categories, the number of polyps is usually consistent among patients within a pedigree (Maeda *et al.*, 1984). Furthermore, the average age of cancer onset in the profuse type (34.0 years) is younger than that (41.8 years) observed in the sparse type. These evidences imply the possibility of different types of genetic alteration in the two groups.

We compared the locations of germline *APC* mutations in 25 unrelated patients with the number of colorectal polyps developed in each case; 19 of the patients were sparse types, and 6 were profuse types. All but one of the mutations were considered to cause truncation of the gene product by frameshift, due to deletion or nonsense mutation (Nagase *et al.*, 1992a; our unpublished observations). The location of each germline mutation seemed to correlate with clinical type; mutations in the FAP patients with profuse polyps were observed between codons 1250 and 1464, whereas mutations in the FAP patients with fewer polyps had occurred in other regions of the *APC* gene. The result suggests that the number of colorectal polyps in FAP patients may be associated with a difference in the stability or the biological function of the truncated APC protein.

We can suggest three possibilities to explain this observation: (i) APC products truncated around codon 1300 may have almost no biological activity to suppress adenoma formation, although the shorter or longer products have some suppressor activities; (ii) APC products truncated around codon 1300 may be unstable compared with shorter or longer products that may still be capable of some suppressor function; (iii) the APC product may interact with itself or with other proteins, and if so, APC products truncated around codon 1300 might form stable

complexes capable of little or no suppressor activity. Analysis of the predicted amino acid sequence of the APC protein has indicated the presence of two regions suggestive of a coiled-coil structure, one in the NH_2-terminal domain and the other in the central portion of the protein (Kinzler *et al.*, 1991; Joslyn *et al.*, 1991), that are considered to be important domains for di- or oligomerization of APC with itself or with other proteins. Although the evidence tends to favor the third possibility, further investigations will be required to address the question of how an individual mutation relates to clinical phenotype. Although we cannot exclude the additional possibility that difference in phenotype may be caused by linked modifier genes, our results do suggest a possible correlation between the numbers of colorectal polyps in FAP patients and the locations of germline mutations of the *APC* gene.

C. Presymptomatic Diagnosis for Members of FAP Families by Direct Detection of the *APC* Mutation and/or by Linkage Analysis with Polymorphisms in the *APC* Gene

The 13 locations where mutation has been observed in two or more unrelated FAP patients will be useful for directly screening DNA in FAP families for presymptomatic diagnosis. Because most of these 13 mutations were observed in patients of more than one nationality, they are very unlikely to be the results of founder effects. Together, they account for nearly 50% of the 121 families whose small germline mutations have been identified, and most can be detected by gel electrophoresis of PCR products, some with and some without enzyme digestion. Deletions at 8 of the 121 sites are detectable as smaller PCR products, and nonsense mutations involving 5 recognition sites for restriction enzymes are detectable by the absence or presence of smaller PCR products following digestion with appropriate enzymes (Ando *et al.*, 1993). Nonsense mutations at codons 232 and 302 affected recognition sites for *Sna*I and *Taq*I, respectively, and the mutation in codon 625 created a recognition site for *Mae*I.

In particular, 5-base deletions at codon 1061 or 1309, found in 28 unrelated cases, account for nearly one-fourth of the germline mutations so far identified. Figure 2 shows one example of presymptomatic diagnosis using direct detection of the mutation. The mutation at codon 622 from TAC to TAA disrupted the recognition site of *Msp*I (CCGG). The 303-bp PCR products containing this mutation were not cleaved to two 163-bp and 140-bp fragments, and two children were diagnosed as affected. Although screening of a germline mutation for each FAP kindred usually entails an enormous amount of work, these kinds of direct-

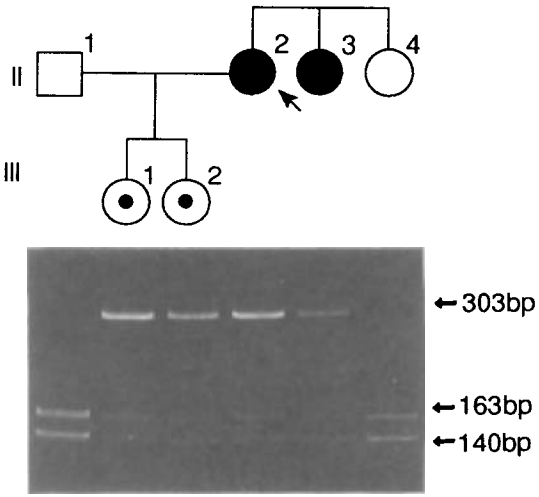

FIG. 2. Presymptomatic diagnosis by direct detection of the mutation. The 303-bp PCR products containing codon 622 of the *APC* gene were digested with *Msp*I and electrophoresed in a 10% polyacrylamide gel. The DNAs that included the mutation from TAC-CGG to TAACGG were undigested and were 303 bp, whereas DNAs derived from a normal allele were cut into 163-bp and 140-bp fragments. □, FAP patient; ●, normal individual; ○, individuals presymptomatically diagnosed as affected.

detection methods can provide presymptomatic diagnosis with a 100% accuracy in less than 24 hours after a mutation specific to each family is identified. Moreover, they do not require DNA samples from as many family members as diagnosis based on genetic linkage.

Moreover, 25 polymorphisms in the *APC* gene have been reported so far (Nagase *et al.*, 1992b; Miyoshi *et al.*, 1992a; Powell *et al.*, 1992; Groden *et al.*, 1991). All available information about these polymorphisms is summarized in Table IV. Allelic frequencies of the 5 most common polymorphisms among the 150 individuals tested (Nagase *et al.*, 1992b) or 45 individuals (Powell *et al.*, 1992) are shown; these occurred at codon 486 with or without an *Rsa*I site (0.43/0.57); at codon 545 (0.35/0.65); at codon 1493 with a *Dsa*I polymorphism (0.35/0.65); at codon 1678 with an *HgiA*I site (0.64/0.36); and at codon 1960 (0.63/0.37). Base substitutions at codons 545 and 1960 do not affect the recognition site of any restriction enzyme but can be easily distinguished by the allele-specific PCR method (Prezant and Fischel-Ghodsian, 1992). Allelic frequencies among the other 20 polymorphisms were less than 0.05. This information should be useful for presymptomatic diagnosis of FAP, as well as for studies to examine tumors for loss of heterozygosity at the *APC* locus.

TABLE IV
SUMMARY OF POLYMORPHISMS IN THE *APC* GENE

Exon	Codon	Sequence	Amino acid	Frequency[a]	Enzyme site
1	84[f]	GGA/GTA	Gly/Val	0.01[f]	
7	−24[e]	A → T		0.01[e]	
7	−23[e]	T → C		>0.01[e]	
8	+31[e]	A → G		>0.01[e]	
11	486[b,c]	TAC/TAT	Tyr/Tyr	0.43/0.57[e]	*Rsa*I
13	545[b,d]	GCA/GCG	Ala/Ala	0.34/0.66[e]	
13	548[b]	TTG/TTA	Leu/Leu	>0.01[e]	
14	+8[e]	T → C		0.97/0.03[e]	
15	741[e]	AAT/AAC	Asn/Asn	>0.01[e]	
15	870[f]	CCA/CTA	Pro/Ser	0.98/0.02[f]	
15	1055[b]	ATA/ATT	Ile/Ile	>0.01[e]	
15	1118[e]	AAT/GAT	Asn/Asp	>0.01[e]	
15	1292[e]	ACG/ATG	Thr/Met	>0.01[e]	
15	1304[b]	ATA/GTA	Ile/Val	>0.01[e]	
15	1359[e]	AAA/AAG	Lys/Lys	>0.01[e]	
15	1422[e]	GAT/GAC	Asp/Asp	>0.01[e]	
15	1493[e]	ACG/ACA	Thr/Thr	0.35/0.65[e]	*Dsa*I
15	1678[b,c]	GGA/GGG	Gly/Gly	0.64/0.36[e]	*HgiA*I
15	1750[b]	GTC/GTG	Val/Val	N.I.	
15	1756[b]	TCG/TCT	Ser/Ser	N.I.	
15	1822[f]	GAC/GTC	Asp/Val	0.90/0.10[f]	
15	1960[f]	CCA/CCG	Pro/Pro	0.63/0.37[f]	
15	2401[b]	CTA/TTA	Leu/Leu	0.01[e]	
15	2502[b]	GGT/AGT	Gly/Ser	0.01[e]	
15	2568[f]	GGA/GGG	Gly/Gly	0.96/0.04[f]	

[a] Estimated among 150 unrelated FAP patients.
[b] Miyoshi *et al.* (1992a).
[c] Groden *et al.* (1991).
[d] Fodde *et al.* (1992).
[e] Nagase *et al.* (1992b).
[f] Powell *et al.* (1992).
N.I., no information.

III. Somatic Mutations of the *APC* Gene in Colorectal Tumors

A. Detection of Somatic Mutations in Colorectal Tumors

At this writing, 85 somatic mutations in 65 colorectal tumors (19 adenomas and 46 carcinomas) have been reported (Nishisho *et al.*, 1991; Miyoshi *et al.*, 1992b; Powell *et al.*, 1992; Cottell *et al.*, 1992) (Table V).

TABLE V
TYPES OF 85 SOMATIC MUTATIONS IN THE *APC* Gene[a]

Point mutations		40
Nonsense mutation	32	
Missense mutation	3	
Splice site	5	
Frameshift mutations		45
Deletion (1–31 bp)	35	
Insertion (1 bp)	10	
Total		85

[a] 43 mutations in 37 colorectal tumors (9 adenomas and 28 carcinomas) (Miyoshi *et al.*, 1992b); 35 mutations in 10 adenomas and 15 carcinomas (Powell *et al.*, 1992); 7 mutations in 3 carcinomas and 4 cell lines (Cottrell *et al.*, 1992).

Forty of these mutations were point mutations; 32 were nonsense, and three were missense. Five occurred in introns at or very close to intron–exon junctions and would be considered to cause abnormal splicing; 45 were frameshift mutations due to 1- to 31-bp deletions or 1- to 4-bp insertions. As in the case of germline *APC* mutations, the great majority (82/85) of somatic mutations that have been identified in colorectal tumors are of a kind predicted to truncate the gene product. Although it is uncertain whether the three missense mutations (Ser to Tyr at codon 906, Glu to Gly at codon 911, and Thr to Ala at codon 1313) would significantly affect the biological function of the gene product, these mutations might point to functionally important domains of the APC protein.

Figure 1B presents the distribution of 78 known *APC* gene mutations that have occurred somatically (Miyoshi *et al.*, 1992b; Powell *et al.*, 1992) (the data from two reports were used in this figure, since both groups examined the entire coding region of the *APC* gene). Although they are scattered through the 5′ half of the *APC* gene, 46 (nearly 60%) are clustered within a very small part (less than 10%) of the coding region within the segment from codon 1286 to 1513, that has been designated MCR (mutation cluster region) (Miyoshi *et al.*, 1992b).

The ratio of point versus frame shift mutations in *APC* between germline and somatic mutations is not same; 36% point mutations versus 64% frameshift mutations for germline mutations and 47% versus 53% for somatic mutations. Point mutations seemed slightly higher for somatic mutations than for germline mutations. This tendency may or

TABLE VI
SUMMARY OF SOMATIC POINT MUTATIONS OF THE *APC* GENE IN COLORECTAL TUMORS

From/to[a]	C	T	G	A	Total
C	—	19	1	5	25
T	1	—	0	0	1
G	0	7	—	1	8
A	0	2	4	—	6
Total	1	28	5	6	40

[a] Listed in coding strand.

may not be general because the methods used and the regions examined have varied among research groups. However, data from our laboratory derived from 103 germline mutations and 43 somatic mutations have supported its generality.

Nineteen of 40 point mutations found in colorectal tumors occurred at C residues (Table VI); most of these were at *CpG* sites (14 cases), in a ratio similar to that in germline mutations (13 *CpG* sites among 44 point mutations). However, the frequency of somatic *APC* point mutations at *CpG* sites in American patients (9 in 17 cases, 53%) was much higher than that seen in Japanese patients (5 in 21 cases, 24%). Since the incidence of colorectal carcinomas is much higher in the United States than in Japan, this difference in mutation frequency at *CpG* sites might reflect differences in dietary habits.

B. Does the Formation of Adenomas Require Two Mutational Hits in the *APC* Gene?

Although some reports have suggested that loss of the normal allele at the *APC* locus causes mild or moderate adenomas to become more progressive (Bodmer *et al.*, 1987), whether the initial step of adenoma formation is caused by decreased dosage or by the complete loss of the *APC* gene product is unclear. Data from two groups (Miyoshi *et al.*, 1992b; Powell *et al.*, 1992) have indicated that both copies of the *APC* gene are inactivated in the majority of colorectal tumors (both carcinomas and adenomas) due to point mutations, insertion or deletion of several nucleotides, or loss of the chromosomal segment spanning the *APC* locus. Table VII summarizes data reported by Miyoshi *et al.* (1992b). Of 47 carcinomas, 39 (83%) bare at least one mutation in *APC* and 23 had sustained two separate mutational events; 17 of these 23 cases were

TABLE VII
MUTATIONS AT THE APC LOCUS OBSERVED IN COLORECTAL TUMORS[a]

Carcinoma Type of mutation(s) detected	Number of tumors
LOH[b] + somatic or germline mutation	15
Two mutations (somatic and/or germline)	8
LOH[b]	10
One somatic mutation	6
None	8
Total (including 4 carcinomas developed in FAP patients)	47

Adenoma Type of mutation(s) detected	Number of tumors
LOH[b] + somatic or germline mutation	4
Two mutations (somatic and/or germline)	5
LOH[b]	1
One somatic or germline mutation	4
None	2
Total (including 8 adenomas developed in FAP patients)	16

[a] From Miyoshi *et al.* (1992b).

[b] Although LOH is in fact a somatic event, LOH was scored separately from specific mutations within the *APC* gene.

confirmed to involve both alleles. Similar results were observed among 16 adenomas: 14 bare at least one inactivated *APC* allele, and in 9 of these, both copies were inactivated.

The frequency of somatic mutations reflected in Table VII is likely to be an underestimate, because the RNase protection analysis does not detect all mismatches and because we did not examine introns or the 5′ flanking region. Using the same method, we detected germline mutations in only 70% of FAP patients studied (Nagase *et al.*, 1992b). Hence, it seems likely that mutations of the *APC* gene, probably on both alleles, are involved in the great majority of colorectal tumors.

To further examine whether the dosage effect of germline mutations in patients with familial adenomatous polyposis is sufficient to cause colorectal adenomas or whether an additional somatic mutation of the normal allele is required as well (Knudson, 1985), we investigated somatic mutations of *APC* in adenomas removed from one FAP patient (Ichii *et al.*, 1992). Taking advantage of a constitutional 5-bp (AAAGA) deletion detected in the region containing a tandemly repeated AAAAGAAAAGA around codon 1309, we examined allelic loss using

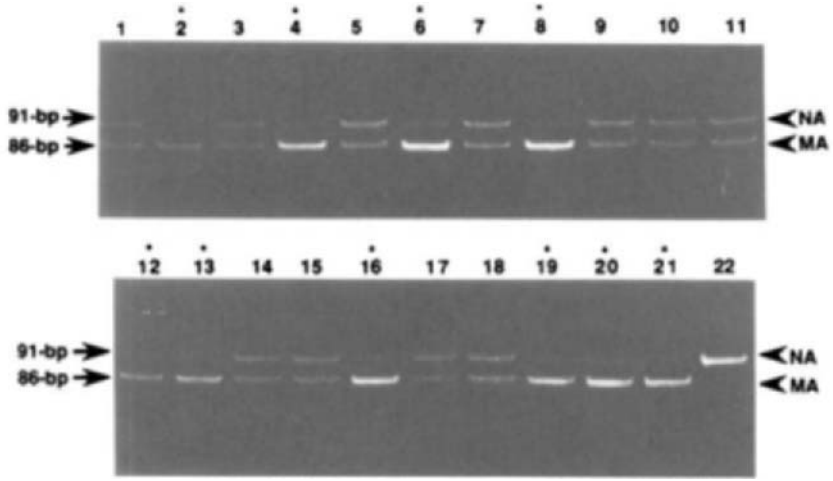

FIG. 3. Polyacrylamide gel electrophoresis of PCR products. Lanes 1, 5, 9, 14, and 18, DNA from normal mucosa of various portions of the colon and rectum; lane 11, the patient's constitutional DNA; lane 22, DNA from a normal control individual; remaining lanes, DNA from 15 adenomas. Sizes of PCR products are indicated in base pairs at the left. NA and MA denote normal and mutant allele, respectively. The LOHs detected in ten samples are marked with asterisks above the panels (Ichii *et al.*, 1992).

PCR. As shown in Fig. 3, two separate fragments, appearing as 91- and 86-bp electrophoretic bands corresponding to normal and mutated alleles, are seen in the lane containing the patient's constitutional DNA (lane 11). No 91-bp bands appear in lanes 2, 4, 6, 12, 13, 19, 20, or 21, indicating loss of the normal allele in these adenomas. The 91-bp bands in lanes 8 and 16 show significant reduction of intensity in the normal band as compared with the mutated, faster-migrating molecular spices; these faint bands probably reflect contamination of normal cells in tumor cells. According to histopathological criteria, all 15 adenomas from this patient were classified as mild (12 tumors) or moderate (3 tumors) atypia. Although the number of polyps examined was not large, these results suggested that inactivation of the *APC* gene on both alleles must be required at an initial step of generating an adenoma from normal colonic mucosa. This conclusion would agree with the two-hit hypothesis of Knudson (Knudson and Strong, 1972; Knudson, 1971, 1985) that has been verified by studies on the *RB* (Cavenee *et al.*, 1983; Hashimoto *et al.*, 1991), *p53* (Baker *et al.*, 1990), and *WT1* genes (Huff *et al.*, 1991; Brown *et al.*, 1992; Haber and Buckler, 1992).

In addition to somatic mutations in the coding region, we have noted the absence of one type of mRNA transcript in each of two primary-cultured cell lines derived from colorectal tumors. Analyses of mRNAs from these two cell lines by the reverse-transcriptase polymerase chain reaction (RT-PCR) (Noonan and Roninson, 1988) revealed that one or another of the transcriptional forms was absent in both cell lines (Horii *et al.*, 1993). This observation suggested that mutations lie in the control region or the first exon of *APC*, or that mutation(s) have affected the splicing efficiency or transcriptional initiation of the gene in the ancestral cell giving rise to these two tumors.

C. Disruption of the *APC* Gene by Somatic Insertion of a Mobile Genetic Element

Another type of *APC* alteration observed in our laboratory was disruption of the gene by somatic insertion of a long interspersed repetitive element (LINE-1 sequence : L1) into the MCR region (Miki *et al.*, 1992). This insertion, which had occurred in a colon cancer developed in a FAP patient, was composed of a 3′ portion of the L1 consensus sequence (Hattori *et al.*, 1986) and nearly 180 bp of its poly(A) tract. Since an 8-bp duplication at the target site was observed, retrotranscriptional insertion of an active L1 sequence is suspected to have caused this insertion event (Fanning and Singer, 1987). This is the only reported case where a tumor suppressor gene was disrupted by somatic insertion of a mobile genetic element. Although the biological function of L1 elements is unclear, their mobility is well documented. For example, *de novo* germline insertions of L1 sequences within the factor VIII gene resulted in Hemophilia A (Kazazian *et al.*, 1988; Woods-Samuels *et al.*, 1989). In the rat, L1 insertion into the *Igh* (immunoglobulin heavy chain) and *Mlvi-2* (Moloney leukemia virus integration 2) loci was observed (Economou-Pachnis *et al.*, 1985); others have reported somatic insertion of L1 sequence into an intron of the *myc* locus in a human breast carcinoma (Morse *et al.*, 1988) and into the 5′ flanking region of the c-*myc* gene in canine transmissible venereal tumor (Katzir *et al.*, 1985). It is interesting that the somatic insertion into the *APC* gene involved an inversion of part of the inserted L1 sequences, whereas the germline insertions into the factor VIII gene maintained the colinear organization. Although the L1 insertions into or upstream of the *myc* gene might or might not have significant effects, the *APC* insertion described previously would be expected to alter dramatically the gene product because it occurred in an exon in the middle of the gene.

IV. Somatic Mutations of the *APC* Gene in Other Human Cancers

A. Somatic Mutations of *APC* in Gastric Cancer

Since some gastric cancers are thought to originate from intestinal metaplasia (Jaervi and Lauren, 1951), it is of interest to examine the role of *APC* during development of gastric carcinoma. We have examined somatic mutations of this gene in 57 gastric cancers of four distinct cellular types (Horii *et al.*, 1992a; Nakatsuru *et al.*, 1992): very well differentiated, well or moderately differentiated, poorly differentiated, and signet-ring cell type. According to certain histopathological criteria, gastric cancers fall into three major categories: intestinal type (differentiated), diffuse type (undifferentiated), or signet-ring cell carcinoma (Lauren, 1965; Japanese Research Society for Gastric Cancer, 1981; World Health Organization, 1990). However, we divided the first category further, into very well differentiated and well or moderately differentiated types (Y. Kato, personal communication). In the very well differentiated type, nuclei, both round and ovoid, are equal in size and located near the basement membrane, with little pleomorphism. Since most specimens of this type are limited to the mucosa, they correspond to the "severe dysplasia" criterion cited by European and American pathologists. However, cancer tissue sometimes shows submucosal invasion. In our well differentiated category, tumor tissue shows high-grade atypism, both structurally and cellularly.

As we prepared DNAs from paraffin-embedded tissues with careful dissection under the microscopy, the amount of available DNA was limited. Hence, we looked for somatic mutations only between codons 742 and 1540, a segment of nearly 30% of the *APC* coding region including the MCR described previously. We identified 17 somatic mutations in 12 of the 57 gastric tumors examined; 3 were signet-ring cell carcinomas, 7 were very well differentiated adenocarcinomas, and two were well differentiated adenocarcinomas. With all nine adenocarcinomas, intestinal metaplasia was found in the surrounding tissue. No somatic mutations were detected in gastric carcinomas of the poorly differentiated, diffuse type.

Frequencies of the *APC* mutations detected in each histopathological type are summarized in Table VIII. Although materials represent only a small sample, the trend seems to suggest an increased frequency of *APC* mutations in gastric cancers with intestinal metaplasia. If borne out by larger studies, this result would imply that the inactivation of the *APC* gene may be associated with an early event during gastric carcinogenesis,

TABLE VIII
FREQUENCY OF *APC* MUTATIONS IN EACH HISTOPATHOLOGICAL TYPE[a]

	Histopathological type	Frequency
I	Very well differentiated adenocarcinoma	7/17[b] (41%)
II	Well or moderately differentiated adenocarcinoma	2/19 (11%)
III	Poorly differentiated adenocarcinoma	0/11
IV	Signet-ring cell carcinoma	3/10 (30%)

[a] From Nakatsuru *et al.* (1992).

[b] p value for the difference between frequencies of somatic mutation in types I and II was 0.035, and that between types I and III was 0.016, by Fisher's exact test.

which would correspond to the development of an adenomatous polyp in the colon or rectum. In the intestinal type but not in the diffuse type of gastric cancers, frequent loss of heterozygosity (LOH) on the long arm of chromosome 5, where *APC* is located, has been reported (Sano *et al.*, 1991). This information supports specific correlation between *APC* mutation and the intestinal type of gastric cancer.

Table IX summarizes the types of somatic mutations found in gastric cancers. Unlike germline mutations in FAP patients or somatic mutations in colorectal tumors, 9 of the 17 mutations detected were missense mutations. Furthermore, 3 separate somatic mutations were detected in each of 2 gastric carcinomas; this finding is interesting because, among the nearly 100 colorectal tumors examined to date (Miyoshi *et al.*, 1992b; Powell *et al.*, 1992), not one has been found to carry 3 mutations. In one very well differentiated gastric adenocarcinoma, 3 independent missense mutations were found in a relatively small region of *APC:* CGT to CAT (Arg to His) at codon 1171, TTC to TCC (Phe to Ser) at codon 1197, and ATA to ACA (Ile to Thr) at codon 1259, all had occurred in a

TABLE IX
TYPES OF SOMATIC MUTATIONS
OF THE *APC* GENE IN GASTRIC CANCER[a]

Point mutations		10
Nonsense mutation	1	
Missense mutation	9	
Frameshift mutations		7
Deletion (1–5 bp)	7	
Total		17

[a] From Nakatsuru *et al.* (1992).

single allele in the tumor (Nakatsuru *et al.*, 1992). This cluster of amino acid changes must have a significant influence on the structure of the protein product in the tumor. Missense mutations in gastric carcinomas may indicate that one or more of specific mutagens possibly in food participate in genetic alterations of gastric mucosal cells.

Since we have discovered that mutations in *APC* can contribute to development of very well differentiated adenocarcinomas and to signet-ring cell carcinomas, these pathologically distinct types of gastric cancer may undergo some common or similar mechanisms in their carcinogenesis, and additional genetic changes could account for the differences in pathology.

B. *APC* Mutations in Other Types of Cancer

We have also searched for somatic mutations of *APC* in primary sporadic tumors of kidney, liver, lung, esophagus, and pancreas for the following reasons: (i) frequent LOH at sites on chromosome 5q has been reported in tumors of kidney (Morita *et al.*, 1991a,b), liver (Fujimori *et al.*, 1991; Ding *et al.*, 1991), esophagus (Boynton *et al.*, 1992), and lung (Ashton-Rickardt *et al.*, 1991; D'Amico *et al.*, 1992, Tsuchiya *et al.*, 1992); (ii) tissues of these five organs express the *APC* gene (Horii *et al.*, 1992a, 1993); and (iii) cancers in these organs are relatively frequent malignancies with poor prognosis.

The results of screening these types of tumors for APC mutations are summarized in Table X. Among the tumors tested, we could detect somatic mutations in the *APC* gene only in pancreatic cancers (Horii *et al.*, 1992b), but no mutation has so far been detected in the other types

TABLE X
Somatic Mutations of the *APC* Gene Examined in Primary, Nonintestinal Carcinomas[a]

Origin of tumor[b]	Examined area (by codons)	Tumors examined	Somatic mutations detected
Kidney	279-1666	14	0
Liver	279-1666	5	0
Lung	582-1666	55	0
Pancreas	742-1666	10	4
Esophagus	742-1666	50	0

[a] From Horii *et al.* (1992b) and our unpublished observations.

[b] Kidney, renal cell carconoma; liver, hepatocellular carcinoma; lung, non-small lung cell carcinoma; pancreas, adenocarcinoma; esophagus, squamous cell carcinoma.

of tumors examined although all the 14 renal cell carcinomas, 5 of the hepatocellular carcinomas, and 7 of the 55 lung cancers had lost heterozygosity for markers at this locus on chromosome 5q (Morita *et al.*, 1991a,b; Fujimori *et al.*, 1991; Tsuchiya *et al.*, 1992). It is still possible that domains of the gene that have not yet been examined could be mutated in these types of carcinoma. We think it more likely, however, that a tumor suppressor gene or genes other than *APC,* but also located on the long arm of chromosome 5, may be responsible for these tumors.

By far, most reported genetic alterations in pancreatic tumors have been point mutations of *K-ras* (Yanagisawa *et al.*, 1991; Almoguera *et al.*, 1988; Smit *et al.*, 1988; Gruenewald *et al.*, 1989; Mariyama *et al.*, 1989; Nagata *et al.*, 1990; Stork *et al.*, 1991) or of *p53* (Barton *et al.*, 1991; Ruggeri *et al.*, 1992) or else loss of expression of *DCC* (Hoehne *et al.*, 1992). We found mutations in the *APC* gene as well. In the multistep process of carcinogenesis in the colon and rectum, an accumulation of genetic alterations in genes including *K-ras, p53,* and *DCC* is considered to be crucial (Fearon and Vogelstein, 1990). Hence, the same or similar carcinogenetic mechanism(s) may come into play in the pancreas as in the colon.

Although the biological function of the APC gene product is still unclear, mutations of *APC* have so far been observed in adenocarcinomas developed in various digestive organs. It is certain that an inherited mutation of the *APC* gene causes a great number of adenomatous polyps in colon and rectum, but polyposis in the stomach is very rare, and no clinical manifestation associated with FAP has been reported in pancreatic tissue. Reasons for these tissue-specific phenotypic differences might include: (i) APC protein plays a more significant growth-regulating role in colorectal mucosal cells than in gastric mucosal cells or pancreatic exocrine and ductal cells; or (ii) the chance of a second hit to the *APC* gene is much higher in the colon and rectum because colorectal epithelial cells are subject to very different environmental conditions from the other organs. Different spectrums of the somatic mutations observed in gastric and colorectal tumors may imply the later possibility, but further investigations are needed to address this question.

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MOLECULAR CYTOGENETICS OF RENAL CELL TUMORS

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I. Introduction

The development and progression of cancer is associated with alterations of genes that control growth and differentiation. The accumulation of genetic changes, such as inactivation of tumor suppressor genes, and the alteration of function of oncogenes, growth factors, and their receptors, as well as genes related to metastatic growth characterize the

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multistep nature of tumor development (Fearon and Vogelstein, 1990). The mutational inactivation of one allele and the loss of the wild-type allele of tumor suppressor gene is a well-characterized sequence of genetic events associated with the development of cancer (Knudson, 1987). Recent genetic studies are focused on detection of loss of chromosomal segments that may harbor loci of tumor suppressor genes. Allelotyping, i.e., using DNA polymorphism to determine the allelic status at each chromosome arm, is now a widely used method to establish specific genetic changes in cancer (Vogelstein *et al.*, 1989). However, solid tumors are characterized not only by loss of chromosomal segments but also by trisomies or partial trisomies and by translocations. A balanced translocation could not be detected by allelotyping, and a trisomy might be easily overlooked and designed as loss of heterozygosity by restriction fragment length polymorphism (RFLP) analysis. Using cytogenetic methods, one can recognize loss of chromosomal segments, trisomies, and translocations.

The aims of this review are threefold: First, I show that cytogenetic analysis, especially in combination with DNA analysis, remains a powerful technique to detect new tumor-associated genetic alterations; second, I show that molecular cytogenic methods are efficient to stratify renal cell tumors; third, I will try to emphasize the complexity of genetic alterations in subtypes of renal cell tumors and indicate that specific genetic changes are useful in the diagnosis as well as in the separation of high-risk groups for therapy.

II. Differential Genetics of Renal Cell Tumors

Renal cell carcinoma is the most common malignant tumor arising from the kidney and affects about 7 of 100,000 adults (Javadpour, 1984). Epidemiological studies indicate that renal cell carcinoma is somewhat more common in Scandinavians and North Americans and that the incidence is lower in Asians and Africans. Although a moderate association between tobacco use and the incidence of renal cell carcinomas has been described, no conclusive evidence could be established for the role of environmental effects in their development (Bennington and Labscher, 1968). The vast majority of renal cell tumors occur in sporadic form. Predisposition to tumor development was described only in rare families and individuals with von Hippel–Lindau disease (Cohen *et al.*, 1979; Kovacs *et al.*, 1989a; Lamiell *et al.*, 1989; Li *et al.*, 1982). There is no satisfactory method for an early detection of renal cell tumors, and almost 40% of the patients have a metastatic tumor at the time of diagnosis. The most effective therapy for renal cell carcinoma localized to

the kidney is the surgery, whereas a metastatic tumor is practically incurable. The overall response to biological response modifiers is low, and the treatment is only palliative. The goal of future therapy is to target tumor suppressor genes. Therefore, it is important that genetics be incorporated into the evaluation of renal cell neoplasm in an attempt to provide a foundation for future diagnosis and selective treatment.

In recent years, a large number of cytogenetic and RFLP studies were carried out to detect specific chromosomal aberrations in renal cell carcinomas. Although the molecular basis of genetic changes is not yet established, the combination of such chromosomal and DNA alterations stratify distinct subtypes of kidney tumors (Table I). We must be constantly aware that we are not dealing with a single disease, called renal cell carcinoma, but with genetically well-characterized types of tumors, each with a unique natural history. The genetic changes, which are associated with tumor progression, may help to estimate the biological behavior of renal cell carcinomas. This classification is very recent and still not widely accepted (Kovacs, 1990). However, it is important to understand that these tumors develop on the basis of separate molecular mechanism and do not have an association with each other. Nonpapillary renal cell carcinomas show a loss of chromosome 3p segments in a proportion of tumors similar to the frequency with which the Philadelphia chromosome is observed in chronic myelogeneous leukemia. Papillary renal cell tumors have a genetic marker in the form of trisomy 17, a genetic change that has not been found in nonpapillary renal cell carcinomas. None of the papillary renal cell tumors shows a rearrangement of chromosome 3p or 5q segments (Kovacs *et al.*, 1989c). Renal oncocytomas and chromophobe renal cell carcinomas have characteristic chromosomal and mitochondrial DNA alterations.

III. Genetics of Nonpapillary Renal Cell Carcinomas

A. Sporadic Renal Cell Carcinoma

Sporadic nonpapillary renal cell carcinomas account for about 80% of the renal cell tumors (Kovacs, 1993). Histologically, they display solid, trabecular, tubular, or cystic growth pattern. Although most of these tumors are made up of clear cells, large areas or the entire tumor may be composed of granular cells. The incidence of nonpapillary RCCs is about 1.5–2 times higher in males than in females. Until now, more than 400 renal cell tumors have been processed for chromosome analysis. After a critical reevaluation of the published data, fewer than 200 cases remain for the present review.

TABLE I

DIFFERENTIAL GENETICS OF RENAL CELL TUMORS

Tumor type	Alterations of chromosomal (%) and mitochondrial DNA																				
	−Y	+7	+17	+3q	+8	+12	+16	+20	−3p	+5q	−6q	−8p	−9	−14q	−1	−2	−6	−10	−13	−17	−21	mtDNA
pRCA[a]	77	100	100	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	−
pRCC	93	75	80	34	18	34	62	28	—	—	—	—	—	15	—	—	—	—	—	—	—	−
npRCC	26	18	—	—	—	10	—	—	96	70	14	22	14	41	—	—	—	—	—	—	—	−
chRCC	—	—	—	—	—	—	—	—	25	—	—	25	18	—	100	95	88	88	95	76	88	+
RO	Normal/abnormal karyotypes; translocation (11q13;?); −Y,−1.																					+

[a] pRCA, papillary renal cell adenoma; pRCC, papillary renal cell carcinoma; npRCC, nonpapillary renal cell carcinoma; chRCC, chromophobe renal cell carcinoma; RO, renal oncocytoma.

1. Loss of Chromosome 3p Segment

The deletion of chromosome 3p segment was detected in the karyotypes of about 30–50% of the renal cell carcinomas in most cytogenetic studies (Berger *et al.*, 1986; DalCin *et al.*, 1988; deJong *et al.*, 1988; Limon *et al.*, 1990; Maloney *et al.*, 1991; Miles *et al.*, 1988; Teyssier and Ferre, 1990; Walter *et al.*, 1989; Yoshida *et al.*, 1986). However, using an appropriate cell culture technique and excluding papillary and chromophobe renal cell tumors and oncocytomas from the series, more than 90% of the nonpapillary renal cell carcinomas display the loss of short arm of chromosome 3 (Carroll *et al.*, 1987; Kovacs *et al.*, 1987b; Kovacs and Frisch, 1989; Presti *et al.*, 1991). The chromosome 3p rearrangement is the solely karyotype change in about 10% of these tumors. The RFLP analysis revealed loss of constitutional heterozygosity at chromosome 3p in 53–100% of renal cell carcinomas (Anglard *et al.*, 1991; Bergenheim *et al.*, 1989; Kovacs *et al.*, 1988a; Ogawa *et al.*, 1992; van der Hout *et al.*, 1991b; Morita *et al.*, 1991; Zbar *et al.*, 1987). These data suggest that alteration of a tumor suppressor gene at chromosome 3p region is associated with the development of nonpapillary renal cell carcinomas. To test this hypothesis, single chromosomes containing 3p, 11, or X chromosomal segments were introduced into the human renal cell carcinoma cell line YCR via microcell fusion (Shimizu *et al.*, 1990). As expected, the complementation with chromosome 3p segment resulted in modulation of tumor growth, whereas introduction of other chromosomes did not change the biological behavior of the YCR cell line.

The locus of the putative suppressor gene is not yet determined. An interstitial deletion at 3p13-p21 or at 3p14-p23 segment was described in most cytogenetic studies. Other investigators described an unbalanced translocation between chromosome 3p13 and 5q22, as the most common alteration in nonpapillary renal cell carcinomas (Kovacs *et al.*, 1987b; Kovacs and Frisch, 1989; Kovacs and Kung, 1991; Presti *et al.*, 1991), which might easily be "misread" as an interstitial deletion in chromosome preparations of poor quality. Thus, high-resolution chromosome analysis of tumor cells suggests that the 3p13-pter segment is the smallest overlapping deletion in nonpapillary renal cell carcinomas. RFLP analysis of renal cell carcinomas suggests that at least two loci on chromosome 3p—one at 3p14 and one at 3p21.3—are involved in interstitial deletions (Yamakawa *et al.*, 1991). Other investigators found terminal and interstitial deletions distal to chromosomal band 3p21.2 (Anglard *et al.*, 1991; Bergenheim *et al.*, 1989) or an interstitial deletion between chromosomal bands 3p21.3 and 3p24 (van der Hout *et al.*, 1991a). Recently, candidate tumor suppressor genes were isolated from the chro-

mosome 3p21 region. Erlandsson *et al.* (1990, 1991) identified the acylpeptide hydrolase gene in the vicinity of the D3F15S2 locus, the reduced expression of which was found in renal cell carcinoma tissues. LaForgia *et al.* (1991) suggested that the protein-tyrosinase phosphatase gamma may be involved in the initiation of renal cell carcinomas and small cell lung carcinomas. The lack of expression of aminoacylase-1 gene was shown in small cell lung cancer cell lines (Miller *et al.*, 1989). Although one allele of these genes was deleted, a mutation of the remaining allele was not shown. Either these genes are linked to and deleted with the tumor suppressor gene, or simply they are only one of the genes located to the large chromosome segment deleted in cancer tissues.

In most cytogenetic studies, the breakpoint cluster of translocation was localized to the chromosome 3p14.2 band, which is the locus of the most common fragile site FRA3B (Sutherland and Hecht, 1985). Fragile sites are located within chromosomal bands, which are frequently involved in rearrangements in cancer cells (Yunis and Soreng, 1984). However, there is no direct evidence that 3p14.2 or other fragile sites are involved in the specific chromosomal rearrangements in nonpapillary renal cell carcinomas (Kovacs and Brusa, 1988). The lack of association between fragile site FRA3B and recombinational or deletional breakpoint at chromosome 3p in renal cell carcinomas is also supported by the analysis of induced chromosome fragility of tumor cells carrying a 3p deletion (Tajara *et al.*, 1988). High-resolution chromosome analysis localized the most distal breakpoint to the border of chromosome 3p13 and 3p14.1 chromosomal bands, which is also the site of breakpoint cluster in nonpapillary renal cell carcinomas (Kovacs *et al.*, 1987b, 1988a; Presti *et al.*, 1991). That this chromosomal band represents a "hot spot" in various tumors is suggested by the finding of a submicroscopic homozygous deletion, which involves the D3S3 locus in DNA of the small cell lung cancer cell line U2020 (Rabbits *et al.*, 1990). Although the deletion was localized by genetic and physical mapping to the chromosome 3p12 band (Latif *et al.*, 1992), a deletion mapping of renal cell carcinomas obtained from individuals with constitutional t(3;6) and t(3;8) has placed the D3S3 locus to the 3p13-p14.1 segment spanning the two breakpoints (unpublished observations, 1992). Thus, the homozygous deletion in the U2020 cell line and the breakpoint cluster in nonpapillary renal cell carcinomas are localized to the same chromosomal region.

2. Trisomy of Chromosome 5q Segment

Chromosome 5q22 sequences are frequently rearranged in a specific manner in renal cell carcinomas (Kovacs *et al.*, 1987b), but to date only one paper has paid attention to this genetic change (Presti *et al.*, 1991).

In a large series of karyotype analyses, 20 of 75 nonpapillary RCCs showed an unbalanced t(3;5) leading to monosomy of chromosome 3p and trisomy of chromosome 5q segments, 15 tumors had a trisomy 5, and 3 tumors had a translocation between chromosome 5q22 and chromosomal regions other than 3p (Kovacs and Frisch, 1989). The net result of these alterations is a partial trisomy of chromosome 5q22-qter segment in 50% of the cases. The duplication of one allele of chromosome 5q was confirmed by RFLP analysis of tumor tissues from patients with hereditary and sporadic cancer (Kovacs and Kung, 1991). Recently, the loss of heterozygosity at chromosome 5q21 was demonstrated in 33% of renal cell tumors (Morita *et al.*, 1991). To establish the nature of chromosome 5q alteration, we employed the RFLP technique for analysis of tumor cells, which were karyotyped for the number of chromosome 5q segments (unpublished observations, 1992). Using the same polymorphic DNA markers that Morita *et al.* (1991) used, we confirmed the duplication of one homolog of 5q22-qter sequences corresponding to prior cytogenetic findings. In addition, we found the duplication of one allele of loci at chromosome 5q22 in 7 out of 17 carcinomas, which have two normal appearing chromosome 5. We could not confirm loss of heterozygosity at chromosome 5q21-22 in any of the 75 tumors analyzed.

Taking into account the results of cytogenetic and molecular studies, the chromosome 5q22 band is affected by allelic duplication in about 70% of nonpapillary renal cell carcinomas. This chromosomal band is the site of a breakpoint cluster in mitotic recombination between chromosome 5q and other chromosomes leading to the partial trisomy of 5q22-qter. There are many growth factors and receptors located next to the trisomic chromosome 5q22-qter segment, the altered dosage and overexpression of which might provide proliferative advantage for tumors cells (Warrington *et al.*,1992). However, the duplication of only a small fragment at the chromosome 5q22 band in many tumors excludes this possibility. The *APC* and *MCC* genes are localized to this chromosomal site (Kinzler *et al.*, 1991a,b; Groden *et al.*, 1991), but they are not involved in partial trisomies in all cases. Likely, alteration of a gene or a breakpoint cluster region distal to the *APC* and *MCC* genes is the specific event associated with the development of nonpapillary renal cell carcinomas. It might be that a tumor suppressor gene is located at this region, disruption or transpositional inactivation of which is important for tumor development.

3. Monosomy of Chromosome 6q, 8p, 9, and 14q

A monosomy or deletion of chromosome 14q occurs in about 30–50% of nonpapillary renal cell carcinomas (deJong *et al.*, 1988; Kovacs *et*

al., 1987b; Kovacs and Frisch, 1989; Maloney *et al.*, 1991; Presti *et al.*, 1991; Walter *et al.*, 1989). The chromosome 14q22-qter segment represents the smallest overlapping deletion. Other nonrandom alterations such as loss of chromosome 6q23-qter and 8p11-pter segments and monosomy 9 occurs in 14, 22, and 14% of the cases, respectively. Alteration of these chromosomal regions are implicated in the genetics of various types of tumors as well.

B. Familial Renal Cell Carcinoma

Susceptibility to renal cell carcinoma was found in rare families with normal karyotype (Li *et al.*, 1982; Pathak *et al.*, 1982). The association between renal cell carcinoma development and inherited constitutional translocation 3;8 was reported (Cohen *et al.*, 1979). Each member of this family carrying a balanced translocation developed multiple and/or bilateral renal cell carcinomas at an earlier age of onset. It was suggested that one allele of a putative tumor suppressor gene is disrupted by translocation. According to Knudson's two-hits model, the second hit should affect the normal, nontranslocated chromosome 3p (Knudson, 1987). However, no tumor tissue was available to test this hypothesis. The association between constitutional translocation 3;6 and development of multiple and bilateral renal cell carcinomas was recently reported (Kovacs *et al.*, 1989a). Contrary to the predictions, the normal chromosome 3 homolog was retained and the derivative chromosome 6 carrying the translocated 3p13-pter segment was lost in tumor tissues. Very recently, cytogenetic and molecular genetic analysis of multiple tumors obtained from members of the family with t(3;8) yielded similar results (Li *et al.*, 1993).

The question raised by these findings is whether the constitutional translocation has directly been involved in the inactivation of one allele of the tumor suppressor gene. Cytogenetically, distinct chromosomal sites are affected by translocations in the two families. One of the breakpoints was localized to chromosome 3p14.2 (Wang and Perkins, 1984) and the other one to 3p13; thus, at least a subband of 3p14.1 separates the two breakpoints. Deletion mapping of the chromosome 3p region in tumors obtained from both families confirmed this finding. The loss of heterozygosity at D3S3 (PMS1-37), D3S42 (YNZ86.1), and D3S687 (CI3-528) loci was found in all tumors obtained from an individual with t(3;6), whereas tumors from individuals with t(3;8) retained the heterozygosity for these loci (unpublished observations, 1992). Therefore, it is unlikely that these translocations disrupt the same tumor suppressor gene. The loss of the translocated 3p segments in tumor cells may well be

the result of instability of derivative chromosomes. Mitotic activity in normal kidney (probably during embryonal development) may simply result in a large number of cells with loss of the translocated 3p segment carrying one allele of the putative tumor suppressor gene. A mutational inactivation of the remaining allele in some of these cells may be instrumental in the development of multiple tumors. However, the functional effect of these chromosomal rearrangements could be clarified only when genes in proximity to the breakpoints become available for analysis. Recently, DNA probes were mapped around the chromosome 3p14.2 band in an effort to clone this breakpoint (van der Hout *et al.*, 1991b; Yamakawa *et al.*, 1992).

C. Renal Cell Carcinoma Associated with von Hippel–Lindau Disease

Von Hippel–Lindau (VHL) disease is an autosomally inherited disorder with predisposition to the development of tumor-like lesions and tumors in multiple organs (Lamiell *et al.*, 1989; Maher *et al.*, 1990). The symptoms are generally manifested between the ages of 20 and 40 years. The major lesions are retineal, cerebellar, brain stem, and spinal cord haemangioblastoma, renal, pancreatic, and epididemal cyts and pheochromocytoma. Multiple bilateral cysts of the kidney have been diagnosed in more than 50% of the gene carriers (Lamiell *et al.*, 1989). Multiple nonpapillary renal cell carcinomas were found in 25–30% of these patients. There is no sex predominance for the development of renal cell carcinoma in von Hippel–Lindau patients. The gene responsible for the VHL phenotype was localized to chromosome 3p25-26 by familial linkage analysis (Seizinger *et al.*, 1988; Hosoe *et al.*, 1990).

Until now, 46 renal cell carcinomas obtained from von Hippel–Lindau patients were karyotyped (Decker *et al.*, 1988; Goodman *et al.*, 1990; Jordan *et al.*, 1989; King *et al.*, 1987; Kovacs and Kung, 1991; Kovacs *et al.*, 1991a). All tumors showed the loss of chromosome 3p segment, which was the only karyotype change in 20 of 46 tumors. The RFLP analysis of multiple tumors from von Hippel–Lindau patients showed that chromosome 3p alleles inherited from the nonaffected parents were lost in each tumor (Kovacs and Kung, 1991; Tory *et al.*, 1989). These data suggest a relationship between the loss of the wild-type allele of the *VHL* gene and the development of multiple lesions. Judging from the RFLP and chromosome analyses, loss of a large chromosomal segment of 3p13-pter inherited from nonaffected parent is the specific genetic change in renal cell carcinomas of von Hippel–Lindau patients. Because it is lost with the deleted chromosome 3p segment, it

was suggested that the *VHL* gene is the *RCC* suppressor gene (Tory *et al.*, 1989). A deletion at 3p21 or 3p23 would effectively eliminate one allele of the *VHL* gene. However, none of the hereditary or sporadic tumors showed such a distal deletion suggesting that loss of more proximal regions is necessary before a cancer arises. It is more likely that the homozygous expression of *VHL* gene is responsible for the development of renal cysts and the inactivation of both alleles of the *RCC* gene for initiation of renal cell carcinomas. The clinical observation that the vast majority of renal cysts persist during life and do not develop a tumor supports this hypothesis (Maher *et al.*, 1990).

The genetic changes affecting other chromosomes are also similar to those found in sporadic renal cell carcinomas. Trisomy of the chromosome 5q22-qter segment was recorded in 54% of the tumors, in many cases as a result of a nonhomologous mitotic recombination between chromosome 3p and 5q. Both parental alleles of the chromosome 5q segment were involved in the genetic changes in multiple tumors in patients with von Hippel–Lindau disease (Kovacs and Kung, 1991). Alteration of chromosomes 6q, 8p, and 14q was found at lower frequency than in sporadic cases (Kovacs *et al.*, 1991a). However, 24 of the 46 renal cell carcinomas were smaller than 1 cm in diameter, i.e., in an early stage of development, whereas almost all sporadic tumors were larger than 3 cm in diameter at the time of analysis.

D. Nonhomologous Mitotic Recombination

The loss of one allele of the putative *RCC* gene may occur at least three ways: nondisjunctional loss of the entire chromosome 3, deletion of a large chromosome 3p segment, and a nonhomologous mitotic recombination between chromosome 3 and other chromosomes. The translocation between chromosome 3p and 5q results in all cases in loss of one homologous chromosome 3p and partial trisomy of chromosome 5q segment. The RFLP analysis of tumor cells showed that both parental chromosomes 5 are retained and that one of them is partially duplicated (Kovacs and Kung, 1991). This result could be explained by the model of nonhomologous mitotic recombination (Fig. 1). In the late S phase or G2 phase, when two chromatids are available, a translocation between two nomhomologous chromatids may occur. After adjacent segregation, when daughter cells each receive a rearranged chromatid as well as normal chromatids, they are trisomic for one and monosomic for the other chromatid segment in exchange (Fig. 1/I). After alternate segregation, when both rearranged chromatids move into one of the daughter cells, the cell division results in a normal cell and in a cell with a balanced

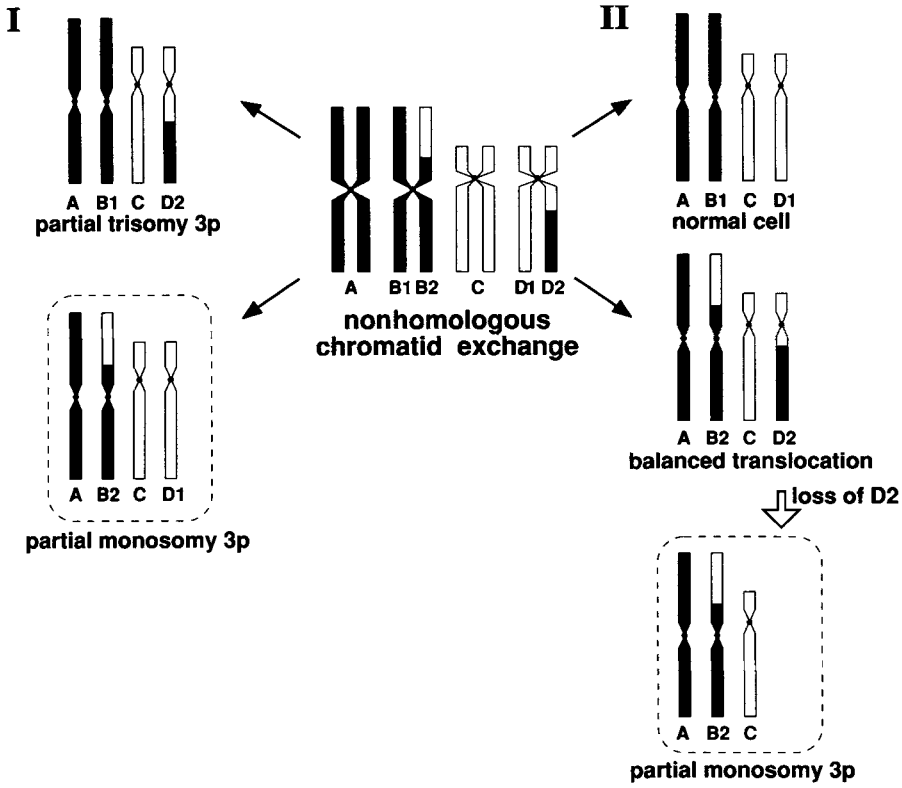

FIG. 1. Model of nonhomologous mitotic recombination in renal cell carcinomas (A and B represent chromosomes 3, whereas C and D are the partner chromosomes in exchange). Adjacent or alternate segregations after exchange between two nonhomologous chromatids (B2 and D2) lead to either unbalanced (I) or balanced (II) translocation. I, For recombination between chromosomes 3 and 5 and adjacent segregation, one daughter cells is trisomic for 3p and monosomic for 5q, whereas the other cell is monosomic for 3p and trisomic for 5q. II, For involvement of chromosomes 3 and 8 and alternate segregation, one cell receives normal chromatids, whereas the other two rearranged chromatids result in a balanced translocation 3;8. This cell requires a nondisjunctional loss of derivative chromosome D2, to eliminate the 3p segment. The chromosome patter within dash-lined boxes fits the karyotype changes found in nonpapillary renal cell carcinomas (see Fig. 2) (Kovacs and Kung, 1991).

translocation (Fig. 1/II). Using this model, we can explain unbalanced translocations leading to loss of one and duplication of other chromosomal segments as well as balanced translocations. A partial monosomy of chromosome 3p and partial trisomy of chromosome 5q segments (Fig. 2A) or partial monosomy of both chromosome 3p and 8p segments

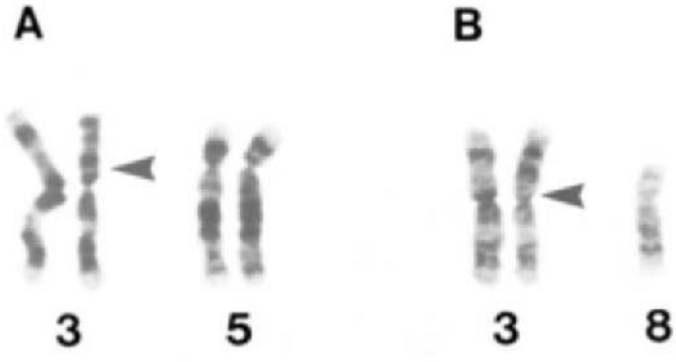

FIG. 2. Partial G-banded karyotypes showing the normal (left) and rearranged (right) chromosome 3 and chromosomes 5 and 8 involved in mitotic recombination. A, Unbalanced translocation (3;5) with two normal chromosomes 5. The breakpoint on chromosome 3 is at p13 (arrow). B, Translocation (3;8) and one copy of the normal chromosome 8. The breakpoint on chromosome 3p is marked by the arrow.

(Fig. 2B) are frequent cytogenetic findings in nonpapillary renal cell carcinomas. Of interest, karyotype changes corresponding to the other daughter with partial trisomy 3p and monosomy 5q was not yet detected in tumor cells. Likely, cells with duplication of chromosome 3p segment (three copies of the wild-type *RCC* gene) have less chance for a malignant transformation than normal cells or they are simply not viable.

In families with a predisposition to renal cell carcinomas, a balanced translocation (3;6) and (3;8) is transmitted through the germline, and all cells of the kidney have this genetic alteration identical to the few precursor cells in sporadic cases having a balanced translocation after alternate segregation (Fig. 1/II). Therefore, a nondisjunctional loss of derivative chromosome 8 or 6 results in a high number of precursor cells in familial cases, and multiple nonpapillary renal cell carcinomas will develop.

This model could be used for explanation of genetic changes occurring in cysts and tumors of von Hippel–Lindau patients. The *VHL* gene is mapped to the distal chromosomal 3p25-26 bands, whereas the *RCC* gene is located more proximally, likely somewhere around the 3p13-21 chromosomal region. A mitotic recombination, between chromosomes 3p13 and 5q22 in a patient carrying the *VHL* gene, results in cells with karyotype aberrations. One of the daughter cells has only the mutated allele of the *VHL* gene and one copy of the wild-type *RCC* gene (Fig. 1/I). This cell expresses the *VHL* phenotype, i.e., develops a renal cyst, which

increases the number of cells with one copy of the wild-type *RCC* gene. A chromatid exchange between chromosomes 3 and 8 results in a daughter cell with balanced translocation 3;8 having two copies of the wild-type *RCC* gene, one copy of the wild-type and one of the mutated *VHL* gene. Nondisjunctional loss of the derivative chromosome 8 removing the wild-type *VHL* gene (and *RCC* gene) results in a cyst. A subsequent mutation in cells of cysts may lead to the development of nonpapillary renal cell carcinoma.

Both types of segregation may occur at the same frequency by chance. The mechanism of alternate segregation requires more steps to eliminate one allele of the gene(s); therefore, it is less effective. The karyotypes of 18 tumors obtained from one patient with VHL disease support this theory. Seven tumors showed a karyotype alteration corresponding to the adjacent segregation (Fig. 1/I), whereas loss of chromosome 3p suggesting an alternate segregation (Fig. 1/II) occurred in only two tumors (Kovacs *et al.*, 1991a).

The nonhomologous mitotic chromatid exchange is not limited to the alterations of the aforesaid chromosomes. This model could be used for the explanation of any other unbalanced translocations occurring in sporadic and hereditary renal cell carcinomas and in other types of tumors as well. A somatic crossing over is well documented in mammalian cells, and its cytological evidence, referred to as quadriradial chromosome configuration, is found in mitotic cells of cultured normal human tissues (German, 1964). Such quadriradials were interpreted as a consequence of interchange between two chromosomes with a point of exchange at apparently homologous sites. A somatic recombination via homologous chromatid interchange resulting in a partial uniparental disomy of chromosome 3 was demonstrated in a subclone of lymphoblastoid cells from a patient with Bloom's syndrome, a cancer-predisposing condition with genetic instability (Groden *et al.*, 1990). Mitotic cells of individuals with Bloom's syndrome do accumulate an abnormally large number of mutations, many of which involve large chromosomal segments. All cytogenetic changes that characterize cells from individuals with chromosome instability syndromes are to be found, with much less frequency, in cells from normal individuals.

A recombination between homologous and nonhomologous chromosomes is a frequent genetic event playing an important role in the evolution, and it occurs during individual development at a much higher frequency than thought (Steinmetz *et al.*, 1987). Short tandemly repeated DNA sequences, which are present at about 1000 loci in the human genome, are implicated to be the sites of recombination (Wahls *et al.*, 1990). A "genetic accident" at recombination sites might be a major

source of chromosomal translocations resulting in mosaicism in normal tissues (Tycko and Sklar, 1990).

E. Somatic Mosaicism in Normal Kidney Tissue

It was proposed that tumor cells arise from normal diploid cells and acquire karyotype alteration due to genetic instability (Nowell, 1986). Evidence for the role of genetic instability in tumor development comes from studies of chromosomal instability syndromes (Schröder, 1982). However, the vast majority of cancer patients have no inherited susceptibility to chromosomal alterations. The instability in these cases is the result of postzygotic events such as chromosomal mutation with random numerial or structural chromosomal changes in a subpopulation of somatic cells (Holliday, 1989).

There is increasing evidence that chromosomal aberrations showing tissue-specific distribution may occur in normal human tissues (for a review see Hall, 1988). For example, only 1 of 85 lymphocytic cells of a renal cell cancer patient with constitutional translocation (3;6) showed an abnormal chromosomal set, whereas 15 of 62 kidney cells had structural and numerical karyotype alterations (Kovacs *et al.*, 1989a). Nonrandom clonal chromosomal abnormalities, especially trisomy of chromosomes 7 and 10 as well as loss of the Y chromosome, occurs also in short-term cultures of normal kidney, lung, and brain tissues of patients with cancer (Elfving *et al.*, 1990); Heim *et al.*, 1989; Lee *et al.*, 1987; Kovacs and Brusa, 1989). Structural or numerical karyotype alterations may occur up to 18% of metaphase cells of normal kidneys (Emanuel *et al.*, 1992). Of interest, 2 of 2413 normal parenchymal renal cells obtained from patients with renal cell cancer have a deletion of the chromosome 3p segment (unpublished observations, 1991). These data suggest that a large number of cells may contain gross chromosomal alterations in phenotypically normal tissues. Taking into account that karyotyping is not sensitive enough to detect all genetic changes, the frequency of somatic mosaicism in normal tissues should be higher than generally assumed.

F. Model of Development and Progression of Nonpapillary Renal Cell Carcinoma

One allele of a putative tumor suppressor gene at chromosome 3p is inactivated by gross chromosomal alterations in 96% of nonpapillary renal cell carcinomas. As predicted by the Knudson model, the other

allele should be inactivated by mutation before a tumor develops. Chromosome aberration arises during mitosis, and the cell division per se increases the risk of genetic errors of various kinds (Ames and Gold, 1990). There is a rapid, almost exponential cell proliferation during the embryonal and fetal period of life. When the kidneys have attained their normal size, the cells are replaced only to balance cell loss. Thus, the chance for gross chromosomal alterations is much higher during embryonal development than during cellular turnover and regeneration. Gene mutations occur independently from the cell cyclus, and the rate of gene mutations is time dependent. It is likely, that most chromosomal aberrations leading to somatic mosaicism arise during embryonal development, whereas most gene mutations are acquired during the postnatal period of life. Therefore, the following model for the development of sporadic and hereditary nonpapillary renal cell carcinomas is proposed.

Genetic accidents during embryonal development result in mosaicism for various gross chromosomal aberrations, such as deletion 3p, nondisjunctional loss of chromosome 3, or mitotic recombination between 3p and 5q. A mutation at the remaining allele of the suppressor gene occurs in many cells during life. The homozygous inactivation of the *RCC* gene remains phenotypically silent in nonproliferating, differentiated tubular cells. When these cells are involved in cellular turnover or regeneration, they cannot stop to divide due to lack of function of suppressor gene. In addition, most of these cells have three copies of chromosome 5q22 segment or loss of the chromosome 6q, 8p, or other segments, which become an important genetic alteration when cells begin to proliferate. The genetic noise affecting the chromosome 3p segment in kidney cells of individuals with constitutional translocations is much higher than in sporadic cases. Therefore, all individuals carrying the germline translocation will develop multiple and/or bilateral renal cell carcinomas, if they live long enough (Li *et al.*, 1993).

Renal cell carcinoma in von Hippel–Lindau disease arises from preexisting cysts (Solomon and Schwarz, 1988). This developmental sequence is suggested by some clinical and histopathological data. Renal cysts are frequently detected in the second decade of the life, but renal cell carcinomas has not been reported in patients aged less than 20 years (Maher *et al.*, 1990). The correlation between the number of cysts and tumors in kidneys from *VHL* gene carriers also support this hypothesis (unpublished observations, 1991). Both the *VHL* and *RCC* genes are located near the chromosomal region, which is deleted in nonpapillary renal cell carcinomas. The loss of this chromosomal segment during the embryonal development results in renal cyst. The growth of multiple

cysts increases the number of cells having only one homolog of the wild-type *RCC* gene, the mutational inactivation of which may result in the development of multiple renal cell carcinomas.

According to the proposed model, a gross chromosomal deletion removing one allele of the *RCC* gene is the first genetic event in the vast majority of hereditary and sporadic nonpapillary renal cell carcinomas. We do not know the number of cells affected by gross chromosomal alterations and/or mutations. Taking into account not only the genetic but also all epigenetic factors, an "optimal condition" for the development of one sporadic renal cell carcinoma is given in only 7 of 100,000 individuals.

Most tumor cells are genetically unstable and acquire additional aberrations during clonal expansion. The accumulation of multiple genetic alterations and their association with the tumor phenotype from hyperplastic growth to frankly malignant tumor is well documented in colorectal carcinoma (Fearon and Vogelstein, 1990). The development and progression of nonpapillary renal cell carcinoma does not relate to an adenoma–carcinoma sequence. None of the known tumor suppressor genes (*RB, APC, DCC, WT, p53*) are affected. However, as discussed previously, alterations of at least six chromosomes are involved in the genetic changes of nonpapillary renal cell carcinomas. To determine the sequence of genetic alterations during clonal progression, we have divided renal cell carcinomas into groups corresponding to the number of karyotype alterations (Table II). The frequency of specific aberrations was then determined for each group. This survey shows that loss of the chromosome 3p segment is the first genetic change. Trisomy of chromosome 5q22-qter region is the second genetic alteration, which arises in many tumors together with the loss of chromosome 3p. The third step is the monosomy of chromosome 14 in the majority of cases, followed by a monosomy of chromosomes 8p and 9. The order of genetic alterations is

TABLE II
GENETIC CHANGES IN NONPAPILLARY RENAL CELL CARCINOMAS

Number of alterations	Karyotype changes (%)						Number of tumors
	−3p	+5q	−6q	−8p	−9	−14q	
1	77	—	—	15	8	—	13
2	100	50	8	4	—	8	24
3	100	48	13	8	—	52	23
4–5	94	58	17	41	6	53	17
>6	100	70	24	42	50	75	24

not constant, in some exceptional cases even the monosomy 8 or 9 might be the first visible karyotype change. A trisomy of chromosome 5q or monosomy of chromosomes 6q and 14q was not found as the only karyotype change. The loss of chromosome 3p and trisomy of 5q seqments occurs at the same frequency in carcinomas with and without metastatic growth (Table III). This finding indicates that alterations of genes at chromosome 3p and 5q are associated with the development of renal cell carcinomas. A monosomy of chromosome 14q occurs in only 23% of tumors without metastatic growth, whereas it is found in 73% of tumors with metastasis. Recently, a similar association between loss of chromosome 14 and poor outcome in a subset of neuroblastoma was reported (Fong *et al.*, 1992). It is likely that a gene at chromosome 14q is responsible for the alteration of tumor phenotype leading to a more aggressive, metastatic growth.

In summary, nonpapillary renal cell carcinomas are characterized by genetic events affecting multiple chromosomal regions such as chromosome 3p, 5q, 6q, 8p, 9, and 14q. These data pinpoint a network of genes, the altered function of which is involved in the development and progression of these tumors.

IV. Genetics of Papillary Renal Cell Tumors

Papillary renal cell tumors account for approximately 10% of renal cancer. Histologically, they consist of papillary or tubulopapillary growth of small cuboidal cells with scanty cytoplasm or large columnar cells with eosinophilic or basophilic granular cytoplasm. There is a 6:1 to 8:1 preponderance of males over females. Papillary renal cell tumors have genetic changes that are distinct from those of nonpapillary carcinomas (Table I). Not only the chromosomes involved in karyotype alterations but also the genetic events are different. Even though nonpapillary renal cell carcinomas are marked by sequential losses of specific chromosomal regions, papillary renal cell tumors display trisomies such as trisomy of

TABLE III
GENETIC CHANGES ASSOCIATED WITH TUMOR AGGRESSIVENESS

Metastatic growth	Karyotype changes (%)						Number of tumors
	−3p	+5q	−6q	−8p	−9	−14q	
no	96	52	10	18	7	30	74
yes	98	50	23	30	34	73	26

chromosomes 3q, 7, 8, 12, 16, 17, and 20, with the exception of the loss of the Y chromosome.

A. Genetic Alterations Associated with Papillary Renal Cell Tumors

1. Loss of the Y Chromosome

The loss of the Y chromosome is one of the specific karyotype changes occurring in more than 80% of tumors that arise in male patients. The papillary renal cell tumor develops preferentially in males, the male:female ratio is 6:1 to 8:1. Nonpapillary renal cell carcinoma, which develops in the same organ at the same age of onset, has a missing Y chromosome in only 27% of the cases, and the male:female ratio is 1.5:1. The loss of the Y chromosome, in combination with trisomy of chromosomes 7 and 17, is the first visible karyotype change in papillary renal cell tumors (Kovacs *et al.*, 1991b). The loss of Y chromosome-specific DNA sequences was confirmed by the RFLP analysis of tumor tissues (unpublished observations, 1992).

How does the loss of the Y chromosome foster the development of papillary renal cell tumors? Gonadoblastomas occur exclusively in individuals with dysgenetic gonads having a Y chromosome (Verp and Simpson, 1987). It was postulated that a regulatory gene referred to as a gonadoblastoma locus at the Y chromosome (*GBY* gene) may act as an oncogene (Page, 1987). It is unlikely that alteration of the same gene is associated with the development of papillary renal cell tumors, because they are characterized by the loss of the Y chromosome. It is more likely that a tumor suppressor gene is localized at one of the homologous region on the X and Y chromosomes, a mutational and deletional inactivation of which is instrumental in the initiation of tumors. Peltomäki *et al.* (1991) suggested that the pseudoautosomal region is the site of specific genetic alterations in solid tumors. We are not able to confirm this finding in renal cell tumors (unpublished observations, 1992). Two of the papillary renal cell carcinomas that developed in females showed translocations involving the Xq22 band. The Xq22 and Yp11 chromosomal regions harbor homologous sequences and might be the loci of the tumor suppressor gene affected in papillary renal cell tumors.

2. Polysomy of Chromosome 7

Trisomy or tetrasomy of chromosome 7 occurs in all papillary renal cell adenomas and in 75% of papillary carcinomas. Trisomy of chromosome 7 is one of the most common karyotype alterations occurring in

various tumors and normal tissues. Trisomy 7 occurs in 18% in non-papillary renal cell tumors. We do not know the genes that are affected by this gross chromosomal change. Polysomy of chromosome 7 and alteration of the *EGFR* gene has been implicated in the genetics of malignant glial tumors (Libermann *et al.*, 1985). Overexpression of the *EGFR* gene was found in 60–93% of "renal cell carcinomas generally" (Freeman *et al.*, 1989; Ishikawa *et al.*, 1990; Weidner *et al.*, 1990). Taking into account that trisomy of chromosome 7 occurs in approximately 30% of kidney cancers (all types together), a correlation between trisomy 7 and overexpression of the *EGFR* gene in papillary renal cell tumors could be excluded. Likely, the dosage alteration of another gene is the important event. Both hepatocyte growth factor/scatter factor (HGF/SF) and its receptor (c-*MET* proto-oncogene) are mapped to chromosome 7. The HGF/SF has a mitogenic and morphogenic effect on renal tubular cells by activation of the c-*MET* protooncogene (Montesano *et al.*, 1991; Nagaike *et al.*, 1991). One can suggest that an autocrine growth stimulation involving the scatter factor and its receptor trigger the growth of cells having three copies of both genes. However, no data are available on the function of these genes in renal cell tumors.

3. Trisomy of Chromosome 17

Trisomy of chromosome 17 occurs in all papillary renal cell adenomas and in 80% of the papillary renal cell carcinomas. The specific combination of trisomy of chromosomes 7 and 17 in the vast majority of tumors, with additional trisomy of chromosome 12 in some cases, suggests that the *ERBB* gene family, mapped to these chromosomes, might be involved in the genetic changes. The low expression of the *ERBB-2/HER-2* gene was found in 80–90% of the "renal cell carcinomas" (Freeman *et al.*, 1989; Weidner *et al.*, 1990). However, as with the *EGFR* gene and trisomy of chromosome 7, a correlation between the altered expression of *ERBB-2* and trisomy of chromosome 17 could be excluded. The *p53* tumor suppressor gene at the short arm of chromosome 17 is implicated in the genetics of nearly all types of tumors (de Fromentel and Soussi, 1992). One allele of the *p53* gene is duplicated in all papillary renal cell tumors with trisomy 17 and the expression of *p53* gene is 3–6 times higher in tumor tissues than in corresponding normal kidneys (unpublished observations, 1991). Because the overexpression of the *p53* gene is characteristically (but not exclusively) associated with the presence of mutations in the coding sequences of the gene, we have analyzed 40 papillary renal cell tumors for mutation. However, PCR–SSCP analysis of the exons 2 to 11 of the *p53* gene failed to detect any mutation. The RFLP analysis revealed the duplication of the same allele in multiple

papillary renal cell tumors from the same kidney. In another case, both tumors from the left kidney showed the duplication of one allele, whereas the other allele was duplicated in both tumors from the right kidney (unpublished observations, 1991). These data suggest that a somatic mutation or imprinting and allelic dosage of genes (chromosomes) may be the initial alteration at chromosome 17 in papillary renal cell tumors.

4. Trisomies Associated with Malignant Growth

Trisomy of chromosome 16 occurs in 62% of papillary renal cell carcinomas. The long arm of chromosome 16 harbors two genes, the uvomorulin and *CAR* genes, alteration of which is implicated in the aggressive growth of tumors. One allele of the uvomorulin (E-cadherin) gene is duplicated in carcinomas having trisomy 16. All but one papillary renal cell carcinomas showed a reduced expression of the uvomorulin gene, whereas in one tumor it was overexpressed. The lack of expression of the uvomorulin gene was shown to be associated with cell dissociation and metastatic tumor growth (Behrens *et al.*, 1989). Recently, a putative cell adhesion regulator (*CAR*) gene was cloned and mapped to a chromosomal site near the uvomorulin gene locus (Pullman and Bodmer, 1992). Of interest, the loss of heterozygosity at this chromosomal region is associated with the malignant progression of various types of tumors (Carter *et al.*, 1990; Sato *et al.*, 1991; Tsuda *et al.*, 1990). The frequent loss of constitutional heterozygosity at the same chromosomal region was detected in a subset of Wilms' tumors as well (Maw *et al.*, 1992).

Trisomy of chromosome 12 occurs in 34% of papillary renal cell carcinomas. There is a frequent karyotype change (27%) in Wilms' tumors as well. Polysomy of the short arm, i.e., i(12p), and deletion of the long arm of chromosome 12 is a highly specific genetic alteration in male germ cell tumors (Atkin and Baker, 1983; Murty *et al.*, 1992). A trisomy 12 occurs in over 80% of ovarian granulosa–stromal cell tumors, in most cases as the sole karyotype change (Fletcher *et al.*, 1991). Trisomy 20 was found in 28% of papillary renal cell carcinomas. This chromosome aberration is relatively frequent in embryonal tumors such as rhabdomyosarcoma, hepatoblastoma, and Wilms' tumor. Trisomy 8 occurs in 18% of papillary renal cell carcinomas, whereas the nonpapillary renal cell carcinomas show the loss of chromosome 8 sequences in 22% of the cases.

Cytogenetic analysis of 32 papillary renal cell carcinomas revealed a trisomy of chromosome 3 in 6 tumors. Another 5 tumors showed a partial trisomy of the chromosome 3q11-qter segment due to an unbalanced translocation between chromosome 3q11 and other chromosomes. Thus, a partial trisomy of chromosome 3q11-qter sequences

occurs in 34% of papillary renal cell carcinomas. This karyotype pattern could also be explained by the genetic mechanism of nonhomologous mitotic recombination. However, the recombinational breakpoint in papillary renal cell tumors is localized not at the short arm of chromosome 3, but at chromosome 3q11, and the chromosomes in exchange are different. A duplication of the chromosome 3q sequences seems to be the important alteration, because only the daughter cell with trisomy of chromosome 3q and monosomy of the partner chromosome is seen in papillary renal cell carcinomas.

5. *Possible Functional Effect of Trisomies*

The identification of nonrandom trisomies in karyotypes of papillary renal cell tumors seems to provide evidence contrary to the known mechanism of mutational–deletional two-hits model of carcinogenesis. Trisomies, especially the combination of trisomies, are highly specific genetic changes in papillary renal cell tumors: none of the tumors showed a monosomy of such chromosomes. Trisomy of specific chromosomes is a common genetic change in experimental tumors and transformed rodent cells as well (Aldaz *et al.*, 1989; Cowell, 1980; Cram *et al.*, 1983). In such cases, preferential duplication of chromosomes carrying the mutant gene was shown (Bianchi *et al.*, 1990; Bremmer and Balmain, 1990; Wirschubsky *et al.*, 1984). There may be no contradiction between the two genetic mechanisms, i.e., the loss of chromosomal segments with the wild-type gene or duplication of chromosomes carrying the mutant gene. The effect of mutation on cell proliferation–differentiation may be dependent on gene dosage (Klein, 1981). In cells that overcome the trans-acting restrictive control of the wild-type allele, the ratio might be one to zero when the wild-type allele is lost, or two to one or three to one when the mutant allele is duplicated. Another possible explanation is that modifier genes (imprinting genes), which are sensitive to gene dosage, are located on chromosomes 7 and 17, and other chromosomes involved in genetic changes of papillary renal cell tumors (Sapienza, 1990).

B. Adenoma–Carcinoma Sequence

On the basis of karyotype alterations and histological–clinical characteristics, we are able to distinguish two groups of papillary renal cell tumors. Papillary renal cell *adenomas* are characterized by a combination of tri- or tetrasomy of chromosome 7 and trisomy of chromosome 17 in each case (Table 1). Until now, the cytogenetics of only 10 papillary renal cell adenomas have been published (for a review see Kovacs *et al.*, 1991b).

Nine of the tumors were diagnosed in males, and the loss of the Y chromosome was detected in 7 of them. The size of benign papillary tumors varied between 2 mm and 5.5 cm in diameter. The cytogenetic finding that a constant combination of alteration of three chromosomes (−Y,+7,+17) occurs in small as well as in large tumors suggests a relative stability of these genetic changes during growth. Thus, the size of papillary renal cell tumors does not correlate with their biological behavior.

Papillary renal cell *carcinomas* show genetic changes in addition to those of renal cell adenomas (Table I). Until now, karyotypes of 32 papillary renal cell carcinomas (27 tumors from males) were published (Carroll *et al.*, 1987; DalCin *et al.*, 1989; deJong *et al.*, 1988; Kovacs, 1989; Kovacs *et al.*, 1991b; Miles *et al.*, 1988; Wolman *et al.*, 1988). The size of the tumors varied between 7 mm and 19 cm in diameter. The most frequent alteration associated with the malignancy is trisomy of chromosome 16 followed by trisomy 12, partial trisomy 3q12-qter, trisomy 20, as well as trisomy 8 and monosomy 14. The association of these chromosomal alterations with malignant behavior suggests that these genetic changes are a prerequisite for an aggressive growth of papillary renal cell tumors. Renal cell adenomas may reach a large size without any sign of malignancy, but a malignant transformation accompanied by complex genetic changes may occur in small adenomas. It is not the size, but the accumulation of genetic alterations, that is associated with malignant behavior.

C. Parenchymal Lesions Associated with Papillary Renal Cell Tumors

For many years, multiple small papillary adenomas were described in kidneys of patients with renal cell carcinomas (Apitz, 1944; Cristol *et al.*, 1946). Recently, a detailed histological analysis revealed 42 microscopic parenchymal lesions on average in kidneys with papillary renal cell carcinoma (Kovacs and Kovacs, 1993) and less than one (0.4) such alteration per kidney was detected in cases with nonpapillary renal cell carcinoma. The vast majority of these tubulo-papillary structures were intermingled with normal parenchymal elements. No strict border between the lesions and normal tissues or compression of surrounding normal parenchymal was seen; therefore, they do not fulfill the morphological criteria necessary for the diagnosis of an adenoma. These lesions resemble maturing and adenomatous nephrogenic rests, which are known to be associated with the development of Wilms' tumor (Beckwith *et al.*, 1990). These findings suggest that Wilms' tumor in children and papillary renal cell

tumors in adults develop from similar precursor lesions of embryonal origin.

D. Shared Genetic Changes in Wilms' Tumor and Papillary Renal Cell Tumor

Wilms' tumor shares some genetic features with papillary renal cell carcinoma. Hyperdiploidy with nonrandom trisomies is the most common cytogenetic alteration in Wilms' tumor (Kaneko *et al.*, 1991). Hyperdiploidy with combination of specific trisomies is a characteristic of papillary renal cell tumors as well. Trisomies of chromosomes 3, 7, 8, 12, 16, 17, and 20 are, in a variable degree, common in both types of tumor. The most intriguing data from our point of view are the karyotypic findings in three Wilms' tumors published by Kaneko *et al.*, (1991). Each tumor showed a combination of trisomy 7 and 17 as well as additional karyotype changes such as trisomy 12 and 20 among others. This combination of karyotype changes is pathognomonic for papillary renal cell carcinomas of adults. Whether these tumors represent an early transformation of nephrogenic rest into less differentiated papillary renal cell carcinomas in children or they are typical Wilms' tumor is not yet known. The occurrence of persisting renal blastema and "Wilms' tumor" in adults (Babaian *et al.*, 1980; Scharfenberg and Beckman, 1984) as well as papillary adenoma in children (Stambolis, 1977) suggests that no strict age- and phenotype-defined border exists between childhood- and adult-type lesions. A genetic analysis of such exceptional cases is necessary to have more insight into the nature of these lesions.

E. From Developmental Disturbances to Papillary Renal Cell Carcinomas

Papillary renal cell tumor is an extraordinarily useful model for exploring the multistep nature of tumorigenesis from embryonal developmental disturbances to frankly malignant tumors of elderly patients. Since some basic knowledge regarding the normal nephrogenesis will be necessary for the following discussion, a quick sketch of some steps of the early stages of normal kidney development is provided here. The kidneys develop from mesodermal blastema. Their morphogenesis is triggered by inductive interactions between the epithelial "ureteric" bud and the mass of "nephrogenic mesenchyme." As a result of bilateral inductive events, the epithelial ureteric bud will produce the collecting duct system, and the nephrogenic mesenchyme will go to develop a

normal nephron. The blastemal cells undergo an aggregation, vesiculation, and segmentation to form glomerular and tubular structures, and the latter makes an anastomosis with the "inducer"-collecting tubule. The renal architecture of such elements will be built up layer by layer from the inducable nephrogenic blastema, which remain detectable at the periphery of the renal lobes until the 36th week of pregnancy. Considering the complexity of this inductive communication system, it is no wonder that even in normal conditions sometimes errors occur. Thus, incompletely differentiated cells, referred to as nephrogenic rests, are left over from the fetal developmental phase. This is a fairly common event and may be subsequent to the alteration of gene(s), which control the proliferation and differentiation of blastemal cells. The Wilms' tumor is thought to originate in cells of nephrogenic rests. Histological observations on multiple nephrogenic restlike parenchymal lesions in kidneys with papillary renal cell carcinomas suggest that the initial developmental sequence is shared by Wilms' tumor of children and most of the papillary renal cell tumors of adults (Fig. 3) (Kovacs and Kovacs, 1993).

The development of Wilms' tumor is associated with the alteration of genes at chromosome 11p13 and 11p15 chromosomal region (for a review see Haber and Housman, 1992). The Wilms' tumor gene (*WT1*) was

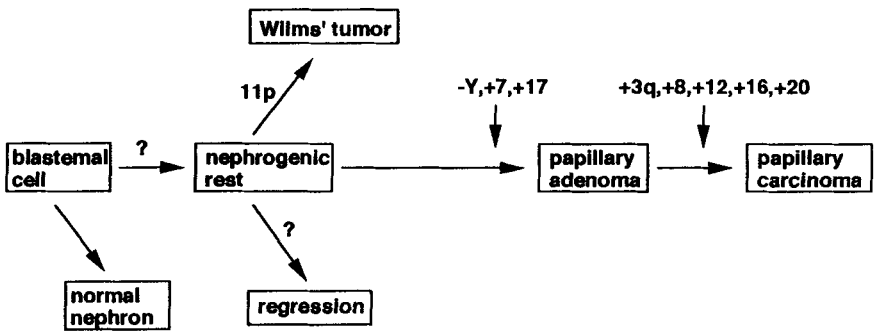

FIG. 3. Model of development of papillary renal cell tumors. How nephrogenic rests develop and regress is not yet known. The predicted developmental sequence for Wilms' tumor is the mutational inactivation of genes at chromosome 11p in nephrogenic rests (Haber and Housman, 1992). It is proposed that many of the nephrogenic rests persist during life, and some with genetic alteration of a putative tumor suppressor gene at X–Y chromosomes and with combined trisomy of chromosome 7 and 17 develop benign papillary adenoma. A papillary renal cell carcinoma develops (in most cases) in cells having additional genetic changes such as trisomy of chromosomes 3q, 8, 12, 16, and 20 (G. Kovacs *et al.*, 1992).

recently cloned from the chromosome 11p13 region (Call *et al.*, 1990; Gessler *et al.*, 1990). The involvement of *WT1* in the urogenital organ development and its mutation in Wilms' tumors was shown (Haber and Housman, 1992). We do not know whether these genetic changes are associated with the maturation block of cells in nephrogenic rests. When the mutation of genes at chromosome 11p region is responsible for the persistence of blastemal cells in kidneys, and both Wilms' tumor and papillary renal cell tumor are developed from the precursor lesions, the chromosome 11p region should be altered in papillary renal cell tumors as well. The lack of alteration at chromosome 11p region in papillary renal cell tumors, however, excludes the possibility that gene(s) in this region are involved in the development of the precursor nephrogenic rests (G. Kovacs *et al.*, 1992). The lack of linkage between familial Wilms' tumor development and chromosome 11p13 or 11p15 region support this hypothesis (Grundy *et al.*, 1988; Huff *et al.*, 1988). Recently, the loss of heterozygosity for markers at chromosome 16q13-22 was also implicated in the etiology of some Wilms' tumors (Maw *et al.*, 1992). However, a genetic linkage of familial Wilms' tumor predisposition to this chromosomal region has been ruled out (Huff *et al.*, 1992). It is more likely that a yet unidentified gene(s) is responsible for the maturation arrest and "overproduction" of embryonal blastemal cells, the inherited or somatic mutation of which may result in a faulty induction stimuli of differentiation. The continuous cell growth, after cessation of normal kidney development, results in nephrogenic rests. Presumably, most of the nephrogenic rests regress in early ages (Beckwith *et al.*, 1990). When cells of the nephrogenic rests acquire alteration of genes at chromosome 11p (Haber and Housman, 1992) and/or other chromosomal region such as 16q, 6, 12 (Kaneko *et al.*, 1991; Maw *et al.*, 1992), a Wilms' tumor develops. When cells of nephrogenic rests acquire genetic changes such as alteration of a putative tumor suppressor gene at the homologous region of the X and Y chromosomes and trisomy of chromosomes 7 and 17, they proliferate slowly and undergo some degree of differentiation. After many decades of slow proliferation, they may result in papillary renal cell adenoma and, subsequently to additional genetic changes, in papillary renal cell carcinoma.

The early stage of tumorigenesis, until nephrogenic rests develop, is thought to be shared by Wilms' tumor and papillary renal cell tumor. However, the alterations at chromosome 11p in Wilms' tumor and at the Y chromosome in papillary renal cell tumors indicate clearly that they have distinct genetics. The alteration of the Y chromosome is one of the initial genetic changes in papillary renal cell tumors, but it is a rare karyotype alteration in Wilms' tumors. There is a strong 6:1 to 8:1 male

preponderance for the development of papillary renal cell tumors, whereas Wilms' tumor develops equally in both sexes.

Some families with inherited predisposition to the development of Wilms' tumor were described, but an inherited form of papillary renal cell tumor has not yet been reported. As previously suggested, the inherited genetic alteration in families with Wilms' tumor yields a large number of maturation arrested cells, i.e., nephrogenic rests, which highly increase the risk for Wilms' tumor development. Most of these children will be cured. However, not only Wilms' tumors but also the nephrogenic rests will be "cured" by the therapy; therefore, no more precursor lesions will be left over for late papillary renal cell tumor development.

F. Papillary Renal Cell Tumors with Translocation Involving the Xp11.2 Breakpoint

Recently, a new cytogenetic subtype of papillary renal cell carcinoma having a translocation (X;1)(p11.2;q21) was described by Meloni *et al.* (1993). Of interest, one of the four cases had a trisomy of both chromosomes 7 and 17 and another one a trisomy of chromosome 17, which are the characteristic genetic changes for the main group of papillary renal cell tumors. Two additional renal cell tumors of adults with translocation between the X chromosome and chromosome 1 have been described. Both tumors were characterized by identical translocation involving the Xp11.2 and 1p34.3 chromosomal bands (Yoshida *et al.*, 1986; Kovacs *et al.*, 1987b).

Renal cell carcinoma is rare in children, comprising less than 7% of renal cell tumors in individuals under 21 years of age (Raney *et al.*, 1983). Only two cases have been analyzed cytogenetically. DeJong *et al.* (1986) karyotyped a renal cell cancer obtained from a 2.4-year-old boy. Each tumor cell has a 46,Y,t(X:1)(p11.2;q21) karyotype. The second case was reported by Tomlinson *et al.* (1991). A 17-month-old boy had multiple lymph node metastasis at the time of surgery. The tumor cells displayed a 46,Y,t(X;17)(p11.2;q25) karyotype, while the karyotype of peripheral blood lymphocytes was normal.

In addition to the common breakpoint, tumors belonging to this genetic group have two common features. First, each tumor had a trabecular/papillary growth pattern of extremely large clear cells, of which the cellular phenotype is rarely seen among papillary renal cell tumors with trisomy of chromosome 17. Second, all cases were observed in male patients. The common breakpoint at chromosome Xp11.2 in tumors suggests that this region may contain a tumor suppressor gene, the

alteration of which is associated with the development of this subtype of papillary renal cell tumors. The chromosomal band Xp11.2 is implicated also in the development of synovial sarcoma, another mesodermally derived epithelial malignancy that is characterized by a specific translocation (X;18)(p11.2;q11.2) (Turc-Carel *et al.*, 1987), and in the genetic changes of uterine leiomyomas as well (Mark *et al.*, 1990).

V. Renal Oncocytoma

Renal oncocytoma is a benign tumor of the kidney. The well-circumscribed tumor is composed of acinar-arranged, large eosinophilic cells. Electron microscopic studies showed that cells of oncocytomas are densely packed with mitochondria. Only few data on the cytogenetics of renal oncocytomas are published so far. Some of these cases represents other types of renal tumors such as "oncocytic" or chromophobe renal cell carcinomas and show loss of chromosome 3 or other karyotype changes characteristic for such tumors (Psihramis *et al.*, 1986; Dobin *et al.*, 1992). Employing RFLP techniques, no loss of heterozygosity at chromosome 3p sequences was found in renal oncocytomas (Brauch *et al.*, 1990; G. Kovacs, unpublished observations, 1993). Karyotypes of only a few renal oncocytomas are available for the present survey. From these data, however, we can outline some characteristics of the genetic alterations.

A. Chromosomal Alterations

A subset of renal oncocytomas displays a mixed population of cells with normal and abnormal karyotypes showing clonal chromosomal abnormalities (Crotty *et al.*, 1992; Dobin *et al.*, 1992; Kovacs *et al.*, 1987c, 1989b). Seven renal oncocytomas have balanced or unbalanced translocations involving different chromosomes. Translocations (7;9)(q22;q13) and (X,13)(q11;p13) were found in one case (Kovacs *et al.*, 1987c), a translocation (1;13)(q21;q34) was found in another case, and a translocation (20;?)(p13;?), in the third tumor (Kovacs *et al.*, 1989b). Dobin *et al.* (1992) described a renal oncocytoma showing a translocation (14;17)(p11;p13). Three oncocytomas shared a common breakpoint at chromosome 11q13. One of them had a 46,XY,t(9;11)(p23;q13) karyotype (Walter *et al.*, 1989). Presti *et al.* (1991) described an oncocytoma with a 46,XX,t(5;11)(q35;q13) karyotype. The third case (Kerman *et al.*, 1991) displayed a 45,XY,-1,-11,+der(11)t(1;11)(p11;q11) chromosomal pattern. Reevaluation of the published karyotype of the latter case suggests the breakpoint to be at chromosome 11q13. Thus, the chromosome

11q13 is a common breakpoint in these cases. This chromosomal band represents a "hot spot" region containing genes, the rearrangement or amplification of which is associated with the development and progression of various type of tumors (for a review see Lammie and Peters, 1991). A paracentric inversion of chromosome 11 in benign parathyroid adenomas places the *PTH* gene from 11p15 adjacent to the *PRAD 1* gene at 11q13 leading to its elevated expression. The amplification of the chromosome 11q13 region containing the *INT2, HST1, PRAD 1,* and *EMS1* genes has been found in breast cancer, transitional cell carcinoma of the urinary bladder, non-small cell lung carcinoma, and squamous cell carcinoma of the head and neck region. It is unknown whether these genes are involved in the initiation and progression of renal oncocytomas. The chromosome 11q13 band harbors two fragile sites, namely FRA11A, a rare folic acid type, and FRA11H, a common amphidicolin type fragile site, which might also be instrumental in the translocations. However, the alteration of a gene(s) at this chromosomal region is more likely than the consistent involvement of one of the many fragile sites.

Recently, Crotty *et al.* (1992) have suggested that coincident loss of the Y chromosome and chromosome 1 as the sole abnormality marks a subset of renal oncocytomas. Until now, seven oncocytomas having the 44,X,-Y,-1 karyotype have been reported (Crotty *et al.*, 1992; Jordan *et al.*, 1992; Meloni *et al.*, 1992; Miles *et al.*, 1992; Psihramis *et al.*, 1988). Crotty *et al.* (1992) also described a renal oncocytoma with a balanced translocation 15;21 and loss of the Y chromosome and another one with monosomy 22 as the sole karyotype change. Thus, renal oncocytomas comprise a cytogenetically heterogeneous group of tumors.

An end-to-end fusion or telomere-to-centromere rearrangement of chromosomes was observed in some of the renal oncocytomas. Telomeric association of chromosomes occurs in normal cells from patients with ataxia teleangiactasia (Hayashi and Schmid, 1975), in senescent human fibroblasts (Benn, 1976) and also in tumors (Kovacs *et al.*, 1987a). The role of these genetic changes in the tumor development as well as the correlation between the heterogeneous chromosomal DNA and constant mitochondrial DNA alterations, if any, is not yet established.

B. Mitochondrial DNA Alterations

The genetic mechanism underlying the accumulation of mitochondria in renal oncocytomas is not known. The high number of mitochondria may reflect some disturbances in regulation of mitochondrial division.

The D-loop region is the site where protein–DNA interaction occur directing the mitochondrial DNA replication (Zeviani *et al.*, 1989). All proteins involved in the replication are thought to be encoded by nuclear genes. Molecular analysis of mitochondrial DNA from oncocytoma tissues revealed an altered restriction pattern after HinfI digestion (Kovacs *et al.*, 1989b; Welter *et al.*, 1989), which might reflect a mutation in one of the restrictions fragment (Singh *et al.*, 1987). Whether mitochondrial DNA alteration is responsible for the continuous replication resulting in the enormously high number of mitochondria in tumor cells or the chromosomal DNA alterations at 11q13, 1, and Y, remains to be analyzed.

VI. Chromophobe Renal Cell Carcinoma

Recently, a new phenotypical variant of renal cell tumor, referred to as chromophobe renal cell carcinoma (RCC), was described (Thoenes *et al.*, 1988). Chromophobe renal cell carcinoma is characterized by cells having a pale, fine reticular cytoplasm. By electron microscopic analysis, chromophobe cells display pathognomic cytoplasmic vesicles and a variable number of mitochondria with an altered morphology.

A. Chromosomal Aberrations

Until now, the chromosome analysis of three chromophobe RCCs has been published (Kovacs *et al.*, 1988b; Kovacs and Kovacs, 1992). These tumors showed an unusually low chromosome number between 34 and 39. In two fully karyotyped cases, the common loss of chromosomes 1, 2, 6, 10, 13, 17, and 21 has been found. It was suggested that these karyotype changes reflect a clonal selection of cells with specific chromosomal losses. Telomeric association between different chromosomes as well as pulverization of multiple chromosomes were also found. Both types of alterations may lead to extreme loss of chromosomes in descendent cells. The RFLP analysis using chromosome 3p-, 5q-, 17p-, and 17q-specific DNA probes showed allelic changes that have not been seen in other types of renal cell tumor (A. Kovacs *et al.*, 1992). To establish the genetic changes of chromophobe renal cell carcinomas, we have employed the comparative genomic hybridization (CGH) technique. Hybridization of DNA extracted from 17 chromophobe renal cell carcinomas to normal metaphasis chromosomes revealed a constant loss of chromosomes 1, 2, 6, 10, 13, 17, and 21 (Table I). The loss of chromosome 1 occurred in 100% of the cases, whereas the loss of other chromosomes occurred in

76–95% of tumors. This finding is very unusual, and we do not have any interpretation for such an extreme loss of chromosomes in a constant combination.

B. Mitochondrial DNA Alterations

Chromophobe renal cell carcinomas are characterized by a variable number of normal and morphologically altered mitochondria as well as by cytoplasmic vesicles, which are thought to originate from mitochondria (Bonsib and Lager, 1990; G. Kovacs, unpublished observations, 1992). The mitochondrial DNA may be extensively rearranged in some of the chromophobe renal cell carcinomas (A. Kovacs *et al.*, 1992). Whether these changes affect the function of mitochondrial genes, and if so, what genes are involved, is not yet known. Whether the extensive alteration of chromosomal DNA in chromophobe renal cell carcinomas results in the alteration of mitochondrial DNA and in the abnormal morphology of mitochrondria also remains to be established. Further molecular biological studies will be necessary to understand the complexity of genetic events affecting the chromosomal and mitochondrial DNA in chromophobe renal cell carcinomas and renal oncocytomas as well.

VII. Conclusions

The identification of specific chromosomal and mitochondrial DNA alterations affecting sites of yet unknown genes in renal cell tumors offers a fascinating series of questions to scientists interested in diverse areas. There is a high rate of cell proliferation and complex process of cell differentiation and maturation during the fetal period of life. Mature cells of the nephron reach the stage of terminal differentiation at the 36th weeks of gestation, when the proliferation genes become suppressed. When the kidneys have attained their normal size, the cells are replaced only to balance loss. Papillary renal cell tumors arise from cells corresponding to the developmental stage before the 36th week of gestation, whereas nonpapillary renal cell carcinomas develop from differentiated tubular cells. Likely, genetic alterations at chromosome 3q, 7, 8, 12, 16, and 17 and at the X and Y chromosomes in papillary tumors pinpoint a network of genes the normal function of which is important in the embryonal and early fetal stage of kidney development. The complex genetic changes at chromosome 3p, 5q, 6q, 8p, 9, and 14q in nonpapillary renal cell carcinomas may identify loci of genes, which control the limited cell proliferation such as normal cellular turnover or

regeneration. Cloning these genes and establishing their function in the morphogenesis and regeneration of nephrons may help researchers to understand how distinct renal cell tumors develop and progress. This will be a formidable task, since there are many genes involved in the regulation of growth, morphogenesis, and differentiation of the kidney.

Future studies will establish, especially through the definition of gene alterations, the true nature of distinct types of renal cell tumors and, as a result, will determine the prognosis because it is through the use of such biological information that new approaches to treatment will be developed. As soon as this happens, the determination of the genetic status of renal cell tumors will be an essential component of the oncological–pathological service.

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REVERSE TRANSFORMATION, GENOME EXPOSURE, AND CANCER

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I. Introduction

The basic molecular lesions underlying cancer are still far from clear. Cancer involves the loss of specific differentiation properties and the assumption of an unregulated growth pattern. However, despite identification of many different oncogenes, tumor suppressor genes, and transcription factors, the conceptual structure of genome regulation in normal mammalian cells has not been elucidated, and therefore the nature of the defect resulting in cancer has been obscure. The principal unsolved conceptual questions relating cancer to the developmental biology of the cell appear to be the following:

1. What is the genome regulatory mechanism underlying normal differentiation in mammalian cells?
2. How is this process altered in cancer?
3. What nuclear and cytoplasmic structures are involved?
4. What is the molecular nature of carcinogenesis?

On the basis of studies on reverse transformation and the phenomenon of genome exposure, a theory was proposed describing two separate levels of regulation of genome activity in mammalian cells (Puck *et al.*,

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1990). The first of these involves an exposure step that causes a differentiation-specific set of genes to become sensitive to interaction with control molecules present in the medium. Thereafter, particular exposed genes are activated or inactivated as required by the needs of the tissue in which the cell resides. Cancer is viewed as a failure of the first or gene exposure step. Earlier versions of the theory describing roles of the cytoskeleton, the cell membrane, and the nuclear elements in genome exposure have been described elsewhere (Puck *et al.*, 1990; Puck and Krystosek, 1992).

The present review cannot include all the important recent literature describing interrelationships between the cytoskeleton, the cell membrane, the nucleus, and cancer. Instead, newer developments in the previously described conceptual picture of reverse transformation (redifferentiation) are reviewed, and the entire scheme is presented as a theoretical formulation of the role of genome exposure in cancer, the consequences of which can now be tested experimentally.

II. Review of Reverse Transformation (Redifferentiation)

The process whereby a cancer cell is induced to resume a normal phenotype by treatment with a specific chemical agent, usually a normal metabolite, was first described by two separate laboratories in 1971 (Hsie and Puck, 1971; Johnson *et al.*, 1971). The name *reverse transformation* was applied to this phenomenon (Puck *et al.*, 1972), and further developments were described in subsequent papers (Porter *et al.*, 1974; Puck *et al.*, 1972; Hsie *et al.*, 1971; Puck, 1977a; Puck, 1978; Puck *et al.*, 1981: Rumsby and Puck, 1982; Gabrielson *et al.*, 1982). This reaction was important in several ways. First, it demonstrated that malignant properties like loss of growth control could be replaced by their normal counterparts in particular cells by supplying an appropriate metabolite. Second, it provided a system in which the structure and function of cells with and without malignant properties could be accurately compared. When a cancer cell is compared with its presumed normal precursor, there is often uncertainty as to which cell type of the tissue of origin is the actual progenitor of the cancer. However, in reverse transformation of the Chinese hamster ovary (CHO) cell by the addition of cyclic AMP derivatives, the reaction is readily reversed on removal of the drug. Therefore, the genome of the cell is constant, and the change in properties must be due to some modification of its regulatory mechanism. Finally, this phenomenon suggested a new principle for cancer treat-

ment, different from the application of cytotoxic agents that can kill normal as well as cancer cells.

On the addition of dibutyryl, 8-brom-, or various other derivatives of cyclic AMP to a culture of CHO cells, a sequence of changes in cell properties is initiated. The entire process requires 72–96 hours for completion. (a) The condensed, pleomorphic malignant cells whose membrane is studded with knoblike vesicles is converted into a typical spindle-shaped fibroblast with a smooth membrane (Fig. 1). (b) The cells lose the ability to multiply in agar suspension but grow readily on glass and plastic surfaces. The surface growth is monolayered instead of being three dimensional like the original transformed culture. (c) Major changes occur in the membrane including replacement of the oscillating knobbed structures by a smooth plasma membrane surface and the deposition of fibronectin in the membrane (Nielson and Puck, 1980). New patterns of protein synthesis and protein phosphorylation occur, and a profound change in the cytoskeleton, which involves all three elements—the microtubules, the microfilaments, and intermediate filaments—takes place (Gabrielson *et al.*, 1982; Chan *et al.*, 1989; Miranti and Puck, 1990; Rumsby and Puck, 1982). Klebe *et al.*, (1991) have further shown that reverse transformed CHO-K1 cells reassume normal morphogenetic patterns of cell–cell association and have emphasized the roles of the extracellular matrix and transmembrane signaling in the new social behavior.

Englesberg and co-workers have shown that in defined medium, insulin strongly controls the growth of CHO-K1 cells and determines whether the cells assume the normal fibroblastic or transformed phenotype (Mamounas *et al.*, 1989, 1991; Mendiaz *et al.*, 1986). This action appears to involve specific membrane transport mechanisms for amino acids. It may also be associated with other components of the information transmission system between the cell membrane and specific nuclear elements (Mendiaz *et al.*, 1986; Mamounas *et al.*, 1989, 1991; Qian *et al.*, 1991).

Perhaps the most surprising change of all occurs in the structure and function of the nucleus, involving the process we have called *genome exposure* and which we have postulated to underlie the changes in properties related to malignancy.

III. The Genome Exposure Defect in Cancer

Addition of cyclic AMP derivatives to CHO-K1 cells so as to bring about reverse transformation (redifferentiation) produces important changes in gene expression. To understand how these changes might be

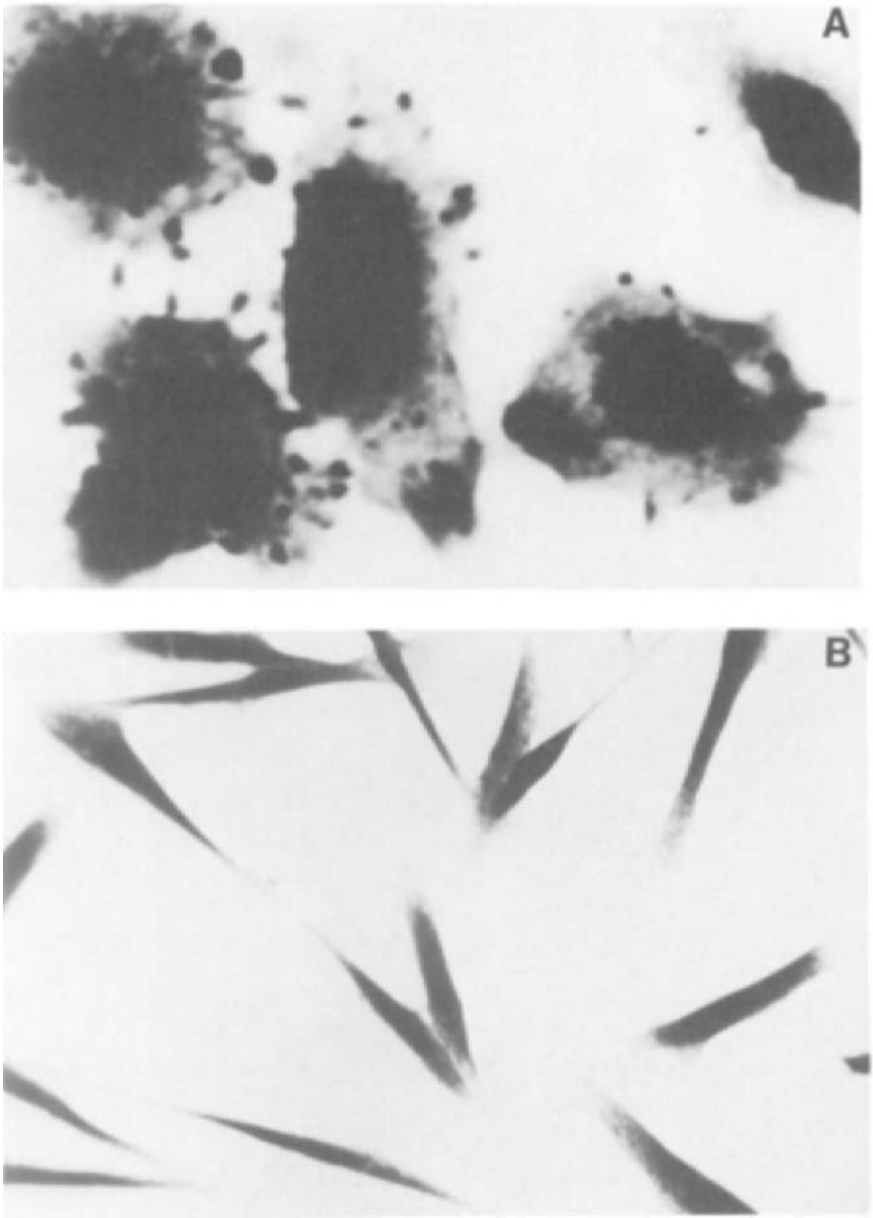

FIG. 1. Demonstration of the change in morphology accompanying the reverse transformation of CHO cells. A, Cells in normal medium; B, cells in normal medium to which was added dibutyryl cyclic AMP (10^{-3} *M*) plus testosterone (5 μg/ml).

orchestrated at the genome level, chromatin organization was investigated. Cell nuclei from malignant, reverse transformed, and normal Chinese hamster ovary fibroblasts were treated with DNase I under conditions where only active or potentially active genes are hydrolyzed by the enzyme (Schonberg *et al.,* 1983). Such experiments demonstrated that both the normal and the reverse transformed cells exhibit much more DNase I-sensitive DNA than did the malignant cell. It was postulated, therefore, that the malignant cell has less active or potentially active DNA than the normal cell and has presumably lost activity of specific differentiation genes, which include those that limit cell reproduction. (We use the term *active* or *potentially active* DNA to include activity in transcription or in other kinds of function, such as regulatory activity.)

It was also demonstrated that an intact cytoskeleton is essential for the reverse transformation reaction including restoration of genome exposure (Hsie and Puck, 1971; Ashall and Puck, 1984). Therefore, the cytoskeleton was postulated to control activation of at least some of the DNA responsible for differentiation, whose activity has been lost in the cancer cell. Those genes that are active in the normal but not the cancer cell and that are detected by their sensitivity to DNase I were named *exposed* genes, whereas the enzymatically resistant genes were termed *sequestered.*

The reverse transformed cells were shown to possess roughly the same amount of exposed DNA (approximately 10–30% of the total genome) as the normal cells, whereas cancer cells have much less exposed DNA in their nuclei under the same defined digestion conditions (Ashall and Puck, 1984). It was demonstrated that this change in DNase I sensitivity on passage from the malignant to the normal phenotype is highly gene specific (Ashall *et al.,* 1988). By this time a large number of different kinds of studies from various laboratories have produced further support for our proposal that cell cytoskeleton action is involved in the difference in genome expression in normal and cancer cells. A sample of such findings is listed (Antecol *et al.,* 1986; McPherson and Ramachandran, 1980; Leung *et al.,* 1992; Otto, 1982; Hamada *et al.,* 1981; Ben-Ze'ev, 1991; Klebe *et al.,* 1991; Rumsby and Puck, 1982).

Studies were undertaken (Krystosek and Puck, 1990) to determine the location of exposed and sequestered DNA, respectively, in the nuclei of cells with normal and cancerous phenotypes by means of a nick translation reaction adapted from that originally described by Hutchison and Weintraub (1985). In this reaction cells are fixed so as to permeablize the membrane. A solution containing DNase I is added in a concentration just sufficient to introduce an appreciable number of nicks in the exposed

DNA. The solution also contains an optimal amount of *E. coli* DNA polymerase enzyme plus labeled bases whose incorporation into DNA at the site of the nicks can be visualized by an appropriate detection reaction. Such experiments reveal that the normal cell has a large deposit of exposed DNA distributed around the periphery of the nucleus, a result that had also been reported by Hutchison and Weintraub for the cells that they had studied. However, the surprising new result was that the malignant CHO cells had almost completely lost this region of exposed DNA. Use of the analytical *in situ* technique allows DNase I concentrations as low as 0.2 ng/ml to be used, and the procedure is more suitable than the gel methodology for screening cell lines and biopsies. By now, studies on 16 different cancer cell lines have revealed that all have suffered severe diminution of this nuclear peripheral exposed DNA (Puck and Krystosek, 1992; Krystosek and Puck, 1993) (Fig. 2). That there is a common nuclear defect in a large number of different cancers raises the intriguing possibility that most cancers share a common nuclear defect, even though there may be several different pathways to the development of the ultimate pathology.

These experiments reveal another unexpected result. A second region of exposed chromatin is evident in association with the nuclear nucleoli. Unlike exposure in the nuclear periphery, however, this labeled site occurs both in normal and cancer cells. We have proposed that the nuclear periphery contains differentiation genes whose function has been lost by the cancer cell while the nucleolar gene exposure represents genes essential in both cancer and normal cells (Puck *et al.*, 1991). Evidence is now forthcoming that the nucleolar region is rich in spliceosomes (Krystosek and Puck, 1993). Recent work has demonstrated that human chromosomes can span large stretches of interphase nuclear space and be simultaneously engaged at the perinucleolar area and the lamina (Puck *et al.*, 1992). It should now be possible to identify the genes situated in the different nuclear locales.

These considerations would account for the most visually striking deposits of DNase I-sensitive chromatin in normal cells and the departure from this pattern in malignant cells. Many uncertainties remain. Are there other less obvious sites of accessible chromatin and do they correspond to active genes? What is the location in the nucleus of the genes that a cell is precluded from using? To what extent do sequestered DNA sequences also occur in the regions of exposed DNA? How is genome exposure involved in action of regulatory as well as transcriptional DNA sequences? What is the physical and chemical nature of the interactions of the cytoskeleton with the genome DNA? How is the transcription machinery related to the genome exposure process?

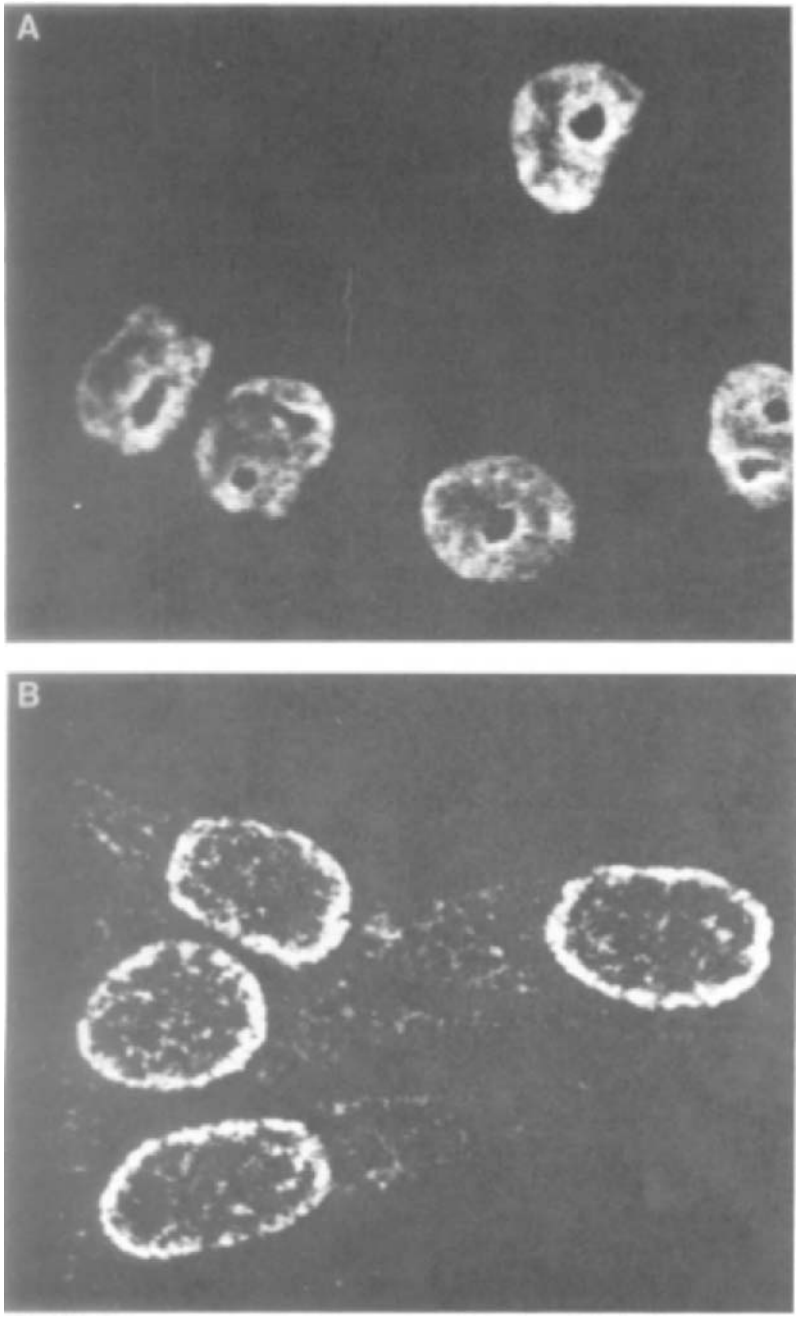

FIG. 2. A, Malignant CHO-K1 cell nuclei grown in normal medium after the nick translation reaction for genome exposure. Definite exposure rings are evident around the nucleoli but few if any can be seen at the nuclear periphery. B, Nuclei of reverse transformed CHO-K1 cells after the nick translation reaction for genome exposure. Exposure is evident around both the nuclear periphery and the nucleoli, but that of the latter is obscured by the brightness of the former.

IV. Restoration of Genome Exposure in Transformed Cells

It seems reasonable to attribute the principle change in phenotype of the reverse transformed cell to the change observed in genome exposure. Reappearance of exposed DNA at the nuclear periphery requires 72 hours or more for its completion, suggesting a slow process that may require one or more cell divisions. Such a relationship is also consistent with the need for elaboration of new cytoskeletal arrangements. The nuclear digestion technique can be adapted to the use of cDNA probes to identify the specific sequences whose sensitivity to digestion by DNase I is changed in malignancy. Such specificity has been demonstrated. The change in nuclease accessibility was shown to occur at specific gene regions rather than as a general shift in the digestion of bulk chromatin. Thus, ribosomal RNA gene probes are highly sensitive to DNase I both before and after cAMP treatment of CHO-K1 cells, whereas the gene for HMG-CoA reductase, which is activated by treatment with cAMP was resistant to the enzyme before cAMP treatment and sensitive afterwards (Ashall *et al.*, 1988). Work with fluorescence *in situ* hybridization utilizing a large variety of probes to map their locations in the interphase nucleus is now in progress.

Reverse transformed cells are more spread than untreated CHO-K1 but somewhat less so than normal fibroblasts in culture. A brief incubation with cytochalasin B or colcemid causes a rapid loss of the sensitivity of chromatin to DNase I. We interpret this result to indicate that there is interplay between cytoskeletal structure and chromatin organization in the nucleus that can be altered rapidly upon collapse of the cytoskeleton. This relationship appears to be even more stable in the normal human fibroblast than in the reverse transformed cell. Normal Chinese hamster fibroblasts appear to be more susceptible to colcemid than normal human fibroblasts (Ashall and Puck, 1984).

Studies with the *src*-transformed vole fibroblast clone, 1T, indicated that the tyrosine kinase inhibitor herbimycin A, reported by others to revert morphologically cells transformed by tyrosine kinase-type oncogenes (Uehara *et al.*, 1988), could also restore stretched cell morphology and normal exposed chromatin structure in this cell line showing that there are multiple ways of achieving reverse transformation. An increased chromatin exposure is produced regardless of the inducer. The relationship between the region of peripheral genome exposure and gene activity has received support from studies of Barbara Hamkalo and co-workers (Clark *et al.*, 1991) who demonstrated that transcriptionally active RNA polymerase molecules are preferentially located in

the nuclear periphery confirming this region as one of active gene transcription.

The genome exposure reaction can be influenced by a variety of parameters including concentration of DNase I, time of exposure to reverse transforming agents, cell density, and other as yet not precisely defined factors possibly including cell cycle distribution.

V. Differentiation Induction in Malignant Cells as Reverse Transformation

The earliest studies on reverse transformation were performed with spontaneously and virally transformed fibroblast cell lines (Hsie and Puck, 1971; Johnson *et al.*, 1971). Cell transfection of immortalized fibroblasts with oncogenes provides additional transformed cell lines that can be reverse transformed by various treatments (Samid *et al.*, 1984; Tagliaferri *et al.*, 1988; Sakai *et al.*, 1989). Restoration of normal fibroblast morphology and growth control have been the hallmarks of the normalized phenotype. Expression of differentiation properties in such fibroblastic lines has been studied much less frequently. New protein species are induced during reverse transformation (Miranti and Puck, 1990) and treated cells increase their commitment to already expressed proteins such as collagen (Hsie *et al.*, 1971) and/or are able to elaborate already expressed molecules such as fibronectin into their proper organizational domains (Nielson and Puck, 1980).

Although the range of regulators initiating reverse transformation and inducible differentiation in other malignant cell lines clearly overlap (cyclic AMP, okadaic acid, and retinoic acid being examples), these two programs of cellular change have usually been treated as if they were totally different phenomena. Reverse transformation has usually been studied with experimentally transformed rodent fibroblast, whereas induced differentiation has been applied to study how physiological modulators can normalize cell lines derived from malignancies, often of human origin. Differentiation inducers are often specific for a given lineage. In optimal cases induction of differentiation results in cessation of proliferation as cells commit to a terminal differentiation process. Reverse transformation by contrast is often reversible when the inducing condition is withdrawn (Hsie and Puck, 1971), although in specific cases genetic variants, i.e., flat revertants, can be selected. Unlike cells of hematopoietic lineages, which have definite life spans, transformed fibroblasts are immortalized, and reverse transformation will yield "normalized" cells, which grow in regulated fashion. However, even in CHO cells,

under appropriate conditions, cAMP addition can produce apoptosis (Gurley *et al.*, 1992).

New transcription both from early response genes and for differentiation-specific proteins occurs during differentiation induction which, aside from insights into the reversible nature of cancer, provides model systems for molecular biological exploration of gene regulation. The diversity of tumor cell types and their inducing molecules has focused attention on differences between various cell lines rather than on similarities in the way the cell phenotype is changeable. Our work indicates a clear dichotomy between chromatin structure in normal cells and tumor cell lines. The ability to interconvert these forms by reverse transformation made possible the test of the simple hypothesis that cellular normalization in inducible differentiation of malignant cells would likewise lead to the reappearance of exposed DNA at the nuclear periphery. The results of such tests indicate that without exception this is true in all cases examined (Krystosek and Puck, 1993). These included retinoic acid-induced differentiation of human neuroblastoma, HL60 leukemia, U937 histiocytic lymphoma, and mouse F9 teratocarcinoma cells; nerve growth factor-induced differentiation of PC12; and cyclic AMP analog-induced differentiation of C6 glioma and NG108-15 neural tumor cells. As in reverse transformation, the chromatin changes appear during a time period of 24–96 hours following inducer addition to cells in culture. Some of these cell lines such as HL60 and F9 synthesized new lamin isoforms (Lebel *et al.*, 1987; Paulin-Levasseur *et al.*, 1989) during this time period, suggesting that extensive remodeling of the nucleus results from inducer treatment. The C6 rat glioma cell, which represents a model of the redifferentiation action, furnishes an especially clear example of this action (Haag *et al.*, 1993). As in the case of reverse transformation of CHO-K1, newly established chromatin structures are rapidly reverted upon treatment with colcemid. We conclude that the terms *reverse transformation* and *redifferentiation* describe essentially the same phenomenon.

Further support of this conclusion arises from a series of studies by Cho-Chung and colleagues examining the molecular consequences of protein kinase A activation in both oncogene transfectants and human cancer cell lines susceptible to differentiation induction (Cho-Chung, 1989; Katsaros *et al.*, 1987; Tagliaferri *et al.*, 1988; Tortora *et al.*, 1991). Growth inhibition in both cases in response to 8-Chloro cyclic AMP involves an alteration of the ratio of two specific isoforms of protein kinase A regulatory subunits. The RI_{α} form characteristic of malignant cells is down-regulated, whereas the RII_{β} is up-regulated in the process of cell normalization. In describing the response of cells to 8-Chloro cyclic AMP, Cho-Chung also refers to phenotypic normalization of

oncogene-transfected fibroblasts as reverse transformation. The term *growth inhibition* is used by this group to describe cases where no specific differentiation markers were measured and where the morphological response was less obvious than the fibroblast paradigm (HT1080, MCF-7). The term *differentiation induction* was used for cases such as HL60 where specific differentiation markers could be measured. We have shown that examples from all three cases—reverse transformation (CHO-K1; *src*-transformed vole cells), differentiation induction (HL60), and growth inhibition (HT1080, HeLa)—show increased genome exposure upon treatment with 8-Chloro cyclic AMP. Moreover, the newly regained accessible chromatin is localized at the nuclear periphery.

The *in situ* assay revealing the state of exposure in nuclear chromatin points to previously unrecognized similarities in initiating phenotypic change in transformed cells. Induced differentiating cells almost universally show a spectrum of new properties such as an early calcium ion flux, phosphorylation changes, cytoskeletal reorganization, synthesis of new cytoskeletal proteins, and adoption of new cellular and nuclear morphologies that also occur in reverse transformation. These are precisely the events that seem to produce the nuclear chromatin changes we have described. Thus, these observations support our thesis that differentiation induction in malignant cells is essentially the same as reverse transformation. In both cases, the more exposed chromatin allows cells to activate new patterns of gene expression. Inducible differentiation often demonstrates more clearly the appearance of new and often abundant gene products as well as morphologies typical of normal cells in a given lineage.

Numerous cases of malignant cells being treated with compounds that are "growth-inhibitory" now abound in the literature. We predict that experiments testing cytoskeletal organization, genome exposure, and gene expression will show a number of these paradigms actually to be instances of reverse transformation, i.e., differentiation induction.

VI. Relationship to Other Work on DNase I Sensitivity

Structure of DNA within nuclear chromatin has been widely studied by determining accessibility to the action of macromolecules such as DNase I, using diverse methodologies. The underlying assumption is that DNase I sensitivity measures a special chromatin configuration and that accessibility to a probe such as an endonuclease is indicative of the way in which endogenous regulatory molecules such as transcription factors can approach or be excluded from the same loci. Early work by

Weintraub and Groudine (1976) and by Axel and co-workers (Garel *et al.*, 1977) showed that genes expressed in a given cell type are more sensitive to digestion when DNase I is applied to isolated nuclei than is the rest of the chromatin or the same genes in tissues where they are not active. As reviewed by Weisbrod (1982), DNase I sensitivity reflects a potential for a gene to be expressed in a given cell and not necessarily its transcriptional activity at the moment, since in some cases DNase sensitivity remains after transcription ceases.

The area susceptible to digestion around the globin gene in nucleated red blood cells extends 8 kb to the 3′ side and 6–7 kb at the 5′ suggesting that there is a large-scale change in conformation at the transcribed loci (Stalder *et al.*, 1980). More recent evidence suggests that for other genes the DNase I-sensitive domain can be as large as 47.5 kb and represents a sequence length constrained between chromosomal anchorage loops (Levy-Wilson and Fortier, 1989). Studies in which digestions are performed with micrococcal nuclease show the release of nucleosomes from active gene regions suggesting that the traditional histone core structure still dominates the DNA packaging. However, studies with S1 nuclease and chemicals modifying DNA were interpreted to indicate a potentially open or single-stranded nature to the DNA helix of transcribed genes (Larsen and Weintraub, 1982; Kohwi-Shigematsu *et al.*, 1983).

Refinements in digestion procedures have led to the insight that still lower concentrations of DNase I produce nicks at select regions (especially promoters), which were termed *hypersensitive sites* (reviewed by Elgin, 1988). In contrast to the total gene structure, nucleosomal organization may be altered or lacking over small sequence stretches. Some such hypersensitive sites may extend as far as 54 kb upstream of a coding sequence (Curtin *et al.*, 1989). These improved methods also allow the nicked DNA to be recovered, rather than destroyed, allowing mapping of the hypersensitive sites at the single nucleotide level.

New studies lead to replacement of the old distinction between nuclease sensitivity and resistance of a gene by the proposal of a hierarchy of control points in defined stretches of chromatin. Not only are differences in chromatin accessibility demonstrated between tissues expressing and not expressing a given gene, but the stepwise unveiling of the hypersensitive sites is shown to occur during differentiation. Furthermore, some of these sites represent commitment to a differentiation program rather than a strict indication of the start of transcription from that locus (Yu and Smith, 1985; Wotton *et al.*, 1989; Lubbert *et al.*, 1991). Groudine and Weintraub (1982) further proposed that the ability to propagate DNase I hypersensitive sites across cell division could be a mechanism for determination of cell fate. Actual transcriptional activa-

tion can lead to still further generation of hypersensitive sites, described as a spreading pattern in the case of the *fos* gene (Feng and Villeponteau, 1990).

Even though the mapping of hypersensitive sites has proceeded to the single nucleotide level, the protein interactions with DNA remain sketchy. Weisbrod and Weintraub (1979) suggested that specific nonhistone chromosomal proteins (HMG14 and 17) could confer DNase sensitivity to the globin gene. Jump and Oppenheimer (1980) showed that thyroid hormone receptor is released by DNase I digestion of hepatic nuclei, presumably indicating its involvement at the sensitive sequences. The action of the topoisomerase inhibitor, novobiocin, in reversing DNase sensitivity suggests that this enzyme may play a role in setting the superhelical tension necessary for the accessible chromatin configuration (Villeponteau *et al.*, 1984). Binding of glucocorticoid receptor to target DNA sequences can change both general DNase sensitivity of a gene region as well as generate discrete hypersensitive sites (Zaret and Yamamoto, 1984). The latter action may represent a shift in the position of nucleosomes at the receptor binding site (Carr and Richard-Foy, 1990). Evidence previously cited that the DNase-sensitive regions have a relationship to matrix anchoring regions, as well as the demonstration that sequence-specific DNA-binding proteins are associated with the nuclear matrix (Dworetzky *et al.*, 1992) and that the matrix itself contains cell type-specific components (Fey and Penman, 1988) suggests that our knowledge of chromatin organization must be greatly expanded in relationship to its protein components.

We have found that treatment of transformed cells with a much larger amount of DNase I in the nick translation reaction for genome exposure will elicit the appearance of a nuclear peripheral region of exposed DNA resembling that in normal cells. Presumably the failure of the transformed cells to yield the reaction under standard conditions means that DNA present in the peripheral region is sequestered so that a higher concentration of DNase I is required to compete with the masking agents. Elucidation of the specific DNA and protein components residing in the nuclear periphery is required.

Evidence for histone participation in the genome exposure reaction in the CHO-K1 cell is provided by the action of Na butyrate. The Na butyrate hyperacylates histones and relaxes condensed chromatin structures so as to permit increased accessibility of DNA (Allfrey *et al.*, 1964; Yoshida *et al.*, 1990). Addition of butyrate to CHO cells mimics some of the actions of cAMP (Wright, 1973; Storrie *et al.*, 1978) and is known to affect the differentiation state of particular cells (Leder and Leder, 1975). Genome exposure studies in our laboratory have demonstrated

that 1.0 m*M* Na butyrate is a powerful inducer of genome exposure in CHO-K1 cells. The extent to which the DNA sequences exposed in the same cells by different agents are the same or different remains to be determined.

Work in this field has greatly benefitted from the development of the technique of *in situ* nick translation, which substitutes a labeling method for degradation or extraction of sensitive chromatin. This synthetic approach offers opportunities to study DNase I-sensitive chromatin within the preserved structure of fixed cells. Hutchison and Weintraub (1985) originally used the technique with interphase cells to demonstrate that the same nick translation procedure that labels active globin genes as determined with DNA probes, if adapted as an *in situ* procedure, labels discrete areas of the cell nucleus. For some mammalian cell lines, they demonstrated a preferential labeling at the periphery of the nucleus. Their work followed the studies by Cedar and colleagues, who demonstrated that the *in situ* technique labeled Giemsa light bands on metaphase chromosomes (Kerem *et al.*, 1984) and could distinguish between active and inactive metaphase X chromosomes (Kerem *et al.*, 1983). More recently, Santos and Yunis (1992) returned to metaphase chromosomes to approach active gene status at higher resolution. They directly scored chromosomal breaks resulting from DNase I introduced into lymphocytes followed by a collection of metaphases. They found that recurring chromosomal breaks correlate with band positions of known lymphoid-expressed genes. There is, in fact, a distinction between T and B cells in the pattern of their expressed genes and induced breaks.

Our work extends the use of these various techniques to a comparison of chromatin structure in normal and malignant cells. In contrast to the study of single genes, our assay measures a composite of gene expression in the cell and reflects the role of cytoskeletal control in the process. The role of DNA binding proteins in changing DNA conformation, the organization of sensitive regions into large domains, and the possible role of DNA methylation are now ready for detailed analysis.

Paradoxically, even though we have emphasized enhanced sequestration of chromatin in tumor cells, certain specific genes in specific tumors may have an abnormally enhanced sensitivity to DNase I. This occurs, for example, in the case of the c-*myc* gene, which, because of chromosomal translocation, is repositioned within 7 kb of an immunoglobulin enhancer. The translocated allele is overexpressed and has a unique pattern of DNase I hypersensitivity in promoter sites (Siebenlist *et al.*, 1984). Inappropriate gene expression may be due to the activated configuration of this gene region so as to prevent normal repressing interactions. Retroviral integration loci also map near DNase I hypersensitive

sites (Rohdewohld *et al.*, 1987), suggesting that the accessibility status of chromatin may have evolutionary consequences as regards to the degree to which a host can exchange DNA with other vectors. Thus, DNase I sensitivity may be an indirect sensor of a range of DNA protein interactions regulating gene expression and repression. The lamins A, B, and C, which are parts of the intermediate filament system of the cytoskeleton, and topoisomerase II exhibit nuclear distributions that include a heavy concentration in the region of the exposed nuclear peripheral DNA. It is, therefore, readily conceivable that these play a role in the genome exposure reaction.

DNase I sensitivity of different chromatin regions needs to be viewed in terms of other physical properties of DNA that can be changed by physiological or chemical intervention. Particularly interesting in this regard is the dynamic pattern of methylation of deoxycytidine residues in DNA. Methylation changes the physical properties of DNA (Diekmann, 1987). Active gene regions tend to be undermethylated, and sites of DNase I sensitivity can correspond to demethylated regions (Wotton *et al.*, 1989; Lubbert *et al.*, 1991). Implications of these facts for cancer have been reviewed by Jones (1986).

The exposed structure of active genes renders them especially sensitive to damage both by γ-radiation and to cancer chemotherapeutic agents (Chiu *et al.*, 1989). Increased knowledge of the dynamics of active chromatin should further illuminate these relationships.

In summary, control of exposure of large domains of DNA proceeds through molecular mechanisms that include involvement of the cytoskeleton, and probably other organelles, and many other processes like phosphorylation and dephosphorylation, nucleosome dynamics, DNA methylation, effects of nuclear hormone receptors, transcription factors, and Ca flux (Bunn *et al.*, 1990). These processes probably include commitment to defined programs of differentiation as well as minute-to-minute expression status of given genes.

VII. Theoretical Formulation about Signal Transduction Mechanisms Governing Genome Exposure

Disruption of normal nuclear regulation is the key event in the process of malignant transformation but has been largely covert since, except for the case of temperature-sensitive viral oncogenes, the successive stages of cellular change have not been available for study on an hour-to-hour basis. Events antecedent to the nuclear changes and the consequent changes in gene regulation and growth control that follow and lock in

place the continued expression of the malignant phenotype need to be delineated at the molecular level. The ability to reverse transform cells now makes such investigation possible (Puck, 1977c).

We have presumed the genome exposure reaction to be a mechanism operating in normal development to position appropriate genes for transcriptional activation at appropriate times in a lineage-dependent manner. Restoration of normal chromatin accessibility in malignant cells will presumably employ the same components as are operative in setting up the changing patterns of normal differentiation. The cellular signaling systems mediating mitogenesis, differentiation, and transformation are known to be redundant and to involve "cross-talk" between intracellular metabolic pathways. Multiple genetic mutations affect the progressive development of human cancer. There should, therefore, be multiple signaling pathways capable of restoring normal growth control even in the absence of the ability to correct an abnormal oncogene. Restoration of a normal cytoskeleton is presumed to be a key event in the normalization of order throughout cellular compartments and reflects the way in which cytoskeletal and nuclear interactions control normal developmental processes (Puck, 1977a,b,c; Puck and Krystosek, 1992).

In the classic case of reverse transformation of CHO initiated by cyclic AMP analogs, the major signaling pathway to be activated appears to be the phosphorylation by protein kinase A of serine and threonine residues of numerous cellular proteins. The early calcium ion flux seen after cyclic AMP addition (Bunn *et al.*, 1990) could provide the opportunity for further involvement of cellular protein kinases activated by calcium and/or phospholipid metabolites, as well as new cytoskeletal interactions (Dienhart *et al.*, 1990; Puck *et al.*, 1990). Increased spreading of cells on the substrata may also provide new patterns of integrin–extracellular matrix molecule interactions that activate ionic fluxes and affect membrane-associated kinases themselves. The reverse transformation manifested by attachment to fibronectin of CHO cells transfected with integrin genes (Giancotti and Ruoslahti, 1990) indicates the importance of these auxiliary pathways of stimulation.

New patterns of phosphorylation have been identified by two-dimensional gel analysis (Gabrielson *et al.*, 1982). Phosphorylation events are rapid, and, in particular, phosphorylation of vimentin and the appearance of a new isoform of actin and cytoskeleton reorganization are early events in reverse transformation occurring well in advance of other protein changes (Miranti and Puck, 1990). The most important features of the new fibroblastic morphology appear to be the restoration of a newly organized cytoskeleton, which renews communication between the cellular fiber systems and the nucleus leading to structural remodel-

ing and activation of the normal configurational state of chromatin. Indeed, Parodi *et al.* (1979) showed that nuclear shape and chromatin dispersion varies in the course of reverse transformation as a function of the spread fibroblastic shape of the entire cell. Using *ras*-transformed fibroblasts, Pienta and Coffey (1992) have shown that the disrupted actin filament system of transformed cells is of major importance in preventing the cell nucleus from achieving the flattened shape during spreading of the cell on the substratum. This key deficit is apparently corrected by reorganization of the cytoskeleton in reverse transformation.

The molecular basis of the nuclear remodeling remains to be delineated. We have postulated large-scale redeployment of chromatin positions to new, multiple, physical loci mediating the required global rearrangement. In our original formulation (Puck, 1977a,b,c; Puck *et al.*, 1990), it was suggested that the physical loci correspond to multiple individual cellular fibers that attach to discrete chromatin regions and determine the exact composition of exposed and sequestered genomic regions. We postulated as a working hypothesis either the ability of cytoskeletal fibers to penetrate the nuclear envelope or to engage other nuclear components, thus achieving a solid-state continuity as a transcompartmental multicomponent fiber system. Other models are possible. Although nuclear microfilaments and microtubules have occasionally been seen in electron microscopy, and their constituent proteins occur as minor (contaminating?) components in biochemical analysis of isolated nuclear preparations, their significance remains in question. On the other hand, there is substantial new evidence for the continuity of the intermediate filament–nuclear lamina system of fibers, and there is a growing literature on the binding phenomena among various nuclear proteins (Georgatos and Blobel, 1987; Georgatos *et al.*, 1988; Yuan *et al.*, 1991; Foisner *et al.*, 1991). The recent demonstration (Foisner *et al.*, 1991) that interaction of plectin with lamin B and vimentin is regulated by phosphorylations with either protein kinase A or C is entirely consistent with our view of kinase activity involvement in the communication between cytoskeletal and nuclear compartments. Suggestions about the ability of the nuclear lamina (and envelope) to engage chromosomal regions have also been offered by others (Blobel, 1985; Nigg, 1989). Changing patterns of lamin polypeptide expression during development are of pertinent interest (Nigg, 1989). Traub (1985) suggested that intermediate filaments are a reservoir of protein subunits that could interact with DNA and histones and whose function is modulated by the calcium signaling systems.

A redundant and recognizable DNA motif would also seem to be required to provide a binding site by which chromatin could be modulated.

We postulated that the families of repetitive DNA sequences throughout the genome serve such a function. Thus chromatin conformation and positioning in interphase are mediated essentially by cytoskeletal attachment to specific repetitive DNA sequences. The situation is similar to that occurring in mitosis, where microtubules engage the repetitive sequences of the centromeres. In both cases, intermediary proteins like those of the kinetochore may be involved (Law *et al.*, 1989; Puck, 1977c). Current work on transcription factors (reviewed by He and Rosenfeld, 1991; Curran and Franza, 1988) elucidates their ability to oligomerize, to bring together noncontiguous DNA domains, to bind at "response element" sequences, which are essentially gene-associated short repetitive sequences, and to associate with transcriptional complexes at the nuclear matrix. Topoisomerase II is another nuclear protein whose anchoring functions (i.e., involvement at the base of chromosomal loops) would suggest its repetitive and discontinuous deployment along chromatin. There is, therefore, a protein-based array of repetitive conformational motifs in nuclear chromatin that could serve as targets for interaction with cytoskeletal-modulated nuclear communication. Superimposed on this, the extensive phosphorylation potential of nuclear matrix and nuclear envelope proteins provides yet another modulating mode of conformationally changing the nuclear environment. Thus would be created motifs differing between normal and transformed cells or indeed normal cells in different states of differentiation and activity.

The net effect of the altered nuclear-cytoskeletal interactions is conformationally to change the accessibility pattern of specific chromatin regions with respect to transcriptional activation. Previously sequestered chromatin becomes transcribable, and new patterns of gene expression serve to institute growth control and allow cells to progress along differentiation pathways. As already mentioned, signaling pathways are redundant, and activation of kinase systems will produce new patterns of phosphorylated transcription factors (He and Rosenfeld, 1991) and the induction of early response genes (Curran and Franza, 1988). Transcriptional activation may therefore occur as a result of the convergence of newly activated and newly synthesized DNA-binding proteins with newly exposed chromatin.

Where inducing agents other than cAMP, such as retinoic acid, other vitamins or protein/differentiation factors, and cytokines, are employed, patterns essentially similar to those previously outlined presumably take place. Activation of kinases would be prominent among these events. Even in the absence of kinase A activation protein kinase C, and calcium-dependent kinases often share substrate specificity with protein kinase A. Inhibition of tyrosine kinase-type oncogenes by agents such as her-

bimycin A is effective in reverse transformation, probably through rapid modulation of focal adhesion structures as a unique entry point for reorganization of the cytoskeleton. Thus, reverse transformation, differentiation inducers, and oncogene inhibition may all utilize the same basic mechanism.

Other workers have also provided experimental support for the idea of a continuum of signal transduction from the extracellular matrix through the cytoskeletal fibers and into the nuclear chromatin domains (Ben-Ze'ev, 1991; Pienta *et al.*, 1989; Ingber, 1991) and that there are structural mechanisms for gene positioning in the interphase nucleus (De Boni, 1988; Lawrence *et al.*, 1988; Manuelidis and Borden, 1988; Huang and Spector, 1991). These ideas will increasingly guide experimental designs to understand gene regulation in normal development and its alterations in cancer and other diseases.

VIII. Further Unsolved Problems and Some Experimental Predictions of the Model

Knowledge of the molecular intermediates in the pathway of reverse transformation and differentiation induction is clearly incomplete at this time. The general outline, however, indicates areas that can be probed directly. What is the function of the expression of early response transcription factors? Can any of these gene products produce large-scale conformational changes in chromatin beyond their effects at response elements? How are the chromatin structural changes related to the gross morphological changes in nuclei, e.g., those reported during reverse transformation of CHO (Parodi *et al.*, 1979) or early HL60 differentiation induction by retinoic acid (Yen *et al.*, 1985)? Are exposed and sequestered DNA subsets indicative of different chromatin protein compositions? Will such proteins show major distributional discontinuities within the nucleus as does exposed DNA itself? Even though genome exposure seems like a large-scale phenomenon when compared to individual DNase I hypersensitive sites in genes, is it in turn dependent on events such as positioning of whole chromosomes within the interphase space (Puck *et al.*, 1992)? Does DNA move within the interphase nuclear space during the restoration of genome exposure? Are mechanico-chemical enzymes such as dynein and kinesin involved in the normalization of cytoskeletal (Gyoeva and Gelfand, 1991) and nuclear structures (Zhang *et al.*, 1990).

Despite the common appearance of exposed chromatin in all normal cells examined so far, we would predict differences according to cell type in the specific genes involved in the accessible chromatin. We are

currently testing this idea using *in situ* hybridization with YAC and cosmid probes to achieve gene localization. Can the overall amount of exposed DNA within normal cells change due to physiological stimulation? Might this be expected in cases of cell activation (macrophages, etc.), intense stimulation (neurons), or re-entry of quiescent cells into the proliferative cycle? We are experimenting with three-dimensional reconstruction of the nuclear labeling pattern using confocal microscopy and image analysis. There may be other cases where the amount of accessible DNA is inappropriately decreased but not as drastically as in malignancies. Severe genetic abnormalities preventing normal development and toxicological insults may well cause defects in genome exposure. Shoeman *et al.* (1991) have suggested that various tissue pathologies in HIV infection, including the emergence of neoplasms, could be due to the disruption of cytoskeletal systems by the HIV-encoded protease cleaving vimentin (Honer *et al.*, 1991).

It is essential to determine as many different conditions as possible that will cause a malignant cell to adopt a more normal nuclear structure. The *in situ* nick translation assay can serve as a rapid screening tool for this purpose. Restoration of normal genome exposure may occur by such diverse means as transfection of tumor suppressor genes, wild-type copies of genes deleted or mutated in cancers, or genes encoding protein components mediating cell–cell and cell–substratum interactions. Other processes to be studied involve inactivation of oncogenes by addition of antisense oligonucleotides or inhibitory antibodies inactivating autocrine stimulatory loops or growth of cells in extracellular matrix environments that allow a transmembrane reorganization of the cytoskeleton. Multiagent differentiation protocols are often needed for maximal yield of responding cells (Waxman *et al.*, 1986; Hemmi and Breitman, 1987; Ponzoni *et al.*, 1992). The resulting understanding will be important not only in cancer therapy, but in the elucidation of differentiation and development as well.

Our view of signal transduction has special implications for the nature of protooncogenes, i.e., genes whose protein products are required for maintenance of normal differentiation and whose function can be perturbed by mutation resulting in disordered cellular growth. Association of some oncogene and protooncogene products with focal adhesions, cytoskeleton, and nuclear matrix (Eisenman *et al.*, 1985; Klempnauer, 1988; White and Cipriani, 1989; Greenfield *et al.*, 1991; Zhou *et al.*, 1991) and the possession by others of protein kinase activity, are entirely consistent with this view. Nuclear, cytoplasmic, focal adhesion, and plasma membrane oncogenes presumably all affect the same

set of signaling pathways maintaining nuclear structure and chromatin organization, but act on on different steps of the common pathways.

IX. Therapeutic and Preventative Significance

A long-standing hope in this field has been that the principles of reverse transformation will allow new forms of human cancer treatment using more physiologic regulators to induce growth control as an alternative to chemotherapeutic agents that are destructive to normal cells as well. Moreover, terminally differentiated cells lose the capacity for self-renewal or are subject to apoptotic cell death as their end-phase fate. The goals of chemotherapy may thus be met in an alternative fashion since it has now been demonstrated that reverse transformation (re-differentiation) can proceed to the apoptosis stage (Martin *et al.*, 1990; Gurley *et al.*, 1992). Recently, retinoic acid has been employed successfully in clinical trials for treatment of leukemias (Parkinson and Smith, 1992). The potent cyclic AMP analog 8-Chloro cyclic AMP is being evaluated in clinical trials (Cho-Chung, 1989).

The dynamics of reverse transformation are much different from those of cytotoxic drugs conventionally used in cancer treatment, and therefore different regimens for these two kinds of agents may be required. Cytotoxic drugs are designed to kill cancer cells by irreversible distortion of basic structures and metabolic processes. Reverse transformation agents first produce a reversible change in phenotype, which may then lead to end-point differentiation. They may, therefore, require continuous administration until all malignant cells have reached the point of irreversible, end-point differentiation.

Reestablishment of normalization in some of the refractory human cancers characterized by multiple genetic defects may seem unlikely (Fearon and Vogelstein, 1990). However, Goyette *et al.* (1992) reported that although progression of colorectal cancer displays multiple tumor suppressor gene defects, inhibition of tumorigenicity can be accomplished by correction of any single defect by use of chromosome transfer. This principle need not be limited to large genomic regions; antisense 40-mer oligonucleotides can specifically target a gene for inactivation. In tissue culture studies a combination of differentiation-inducing or reverse transformation agents can act synergistically (Waxman *et al.*, 1986). These considerations may also be applicable in clinical therapy. A further advantage of synergistic action is that the needed doses can be reduced to tolerated levels. Reverse transformation agents such as retinoic acid and other dietary substances are also potential

chemopreventive agents capable of inhibiting chemical carcinogenesis as tested with the tracheal epithelial cell transformation bioassays (Steele *et al.*, 1990).

The ideal reverse transformation agent will correct all pathologic manifestations of the cancer cell. In some cases where this cannot be achieved, sensitivity of the cancer cell to other kinds of therapeutic agents may be augmented. Thus, antisense-mediated inactivation of overexpressed c-*raf*-1 oncogene in human squamous carcinoma sensitizes the cells to radiation (Kasid *et al.*, 1989). Interruption of the autocrine stimulatory pathway by monoclonal antibody to epidermal growth factor receptor not only inhibits growth but also sensitizes human prostatic carcinoma cells to the cytotoxic effects of the tumor necrosis factor (Fong *et al.*, 1992). Reverse transformation agents such as retinoic acid can turn off c-*myc* synthesis; overexpression of *myc* is known to lead to down-regulation of HLA expression (Gross *et al.*, 1990) and development of resistance to interferon (Einat and Kimchi, 1988). Ability to down-regulate the oncogene may be expected to improve the immune response to tumor cells. It is likely that many of these effects follow from the ability of tumor cells treated with reverse transformation agents to express appropriate genes previously sequestered in inactive chromatin.

The ability to trace key events in the signal transduction pathways maintaining tumor cells in proliferative growth allows interventions to be targeted at intermediate steps. These might not be obvious from the lineage of the cells or their specific oncogene and chromosomal defects. Tortora and colleagues showed that antisense oligonucleotides specific for the RI_{α} protein kinase A regulatory subunit applied in culture for HL60 cells block proliferative growth, up-regulate the RII_{β} gene, and allow differentiation to proceed (Tortora *et al.*, 1991). A priori, this would seem an unlikely strategy in a cell line known to have amplified and overexpressed c-*myc* as well as having lost *p53*. The primary oncogene defect, therefore, need not be targeted directly.

A further consideration arises from the unique roles of the cytoskeleton in transformation and cell signaling in normal cells. Pienta and Coffey (1991) have suggested that cytoskeleton-based cell adhesion and motility phenomena offer potential targets for anti-cancer drug development. Extracellular or intracellular events could presumably be manipulated so as to prevent movement and transmembrane signaling of growth responses. Ingber's and Folkman's (1989) work on angiogenesis suggests ways of interfering with the extracellular matrix–cytoskeletal connection to extinguish the growth potential of endothelial cells—a necessary component of solid tumor growth.

X. Summary

The reverse transformation reaction whereby malignant cells are restored to a more normal phenotype has been reviewed. The primary causative action is ascribed to the genome exposure reaction in which a peripheral nuclear DNA region is restored to high sensitivity to DNase I, like that in normal cells. Various aspects of genome exposure around the nucleoli and the nuclear periphery are considered. The special role of the cytoskeleton in regulating exposure resulting in normal differentiation on the one hand and malignant transformation on the other is discussed. The action of the two-level system for regulation of the mammalian genome previously proposed is reviewed in relation to normal differentiation and malignancy with brief indication of roles played by various metabolites, transcription factors, protooncogenes, cell organelles, and processes like specific phosphorylation and dephosphorylation. Possible implications for cancer therapy and prevention and for the fields of genetic disease and toxicology are indicated.

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PEPTIDE-BINDING HEAT SHOCK PROTEINS IN THE ENDOPLASMIC RETICULUM: ROLE IN IMMUNE RESPONSE TO CANCER AND IN ANTIGEN PRESENTATION

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I. The Curious Paradox of Heat Shock Proteins as Tumor-Specific Antigens

The observations that inbred mice and rats can be immunized against their own tumors or against tumors of the same genetic background were convincingly made between 1943 and 1962 (Gross, 1943; Foley, 1953; Baldwin, 1955; Prehn and Main, 1957; Klein *et al.*, 1960; Old *et al.*, 1962; for a review see Srivastava and Old, 1988). They provided the underpinnings for the idea of immunogenicity of cancers and, by deduction, of the existence of tumor-specific antigens. In essence, these studies showed that mice vaccinated with inactivated cancer cells are immune to subsequent challenges of live cancer cells. The phenomenon was shown to be individually tumor specific, in that mice were immune specifically to the tumors that were used to immunize them and not to other tumors (Basombrió, 1970; Globerson and Feldman, 1964), hence the nomenclature *individually distinct* tumor rejection antigens. The demonstration of immunogenicity of cancer cells led to a search for the cancer-derived molecules, which elicit resistance to tumor challenges. The general structure of these experiments was to fractionate cancer-derived proteins and test them individually for their ability to immunize mice against the cancers from which the fractions were prepared (for other approaches to identification of tumor-specific antigens, see Srivastava and Old, 1988; Old, 1981; Boon, 1991). A number of proteins have been identified by this method (Table I) and a surprisingly large proportion of them are related to a class of proteins known as heat shock proteins (HSPs) or stress-induced proteins (Lindquist and Craig, 1988). And there lies a curious paradox. The HSPs are among the most highly conserved and abundant proteins in living systems; they are found across the phylogenetic ladder from archaebacteria to primates and differ but modestly among different species, let alone within an inbred strain. All in all, they are the most unlikely candidates for tumor-specific antigens.

The observations that have helped define and substantiate this paradox are described here, as are the recent results that have now begun to explain it. The basis of tumor-specific immunogenicity of HSPs lies at the intersection of a number of diverse areas including antigen presentation, protein folding and assembly, and priming of cellular immune response to infection and sheds light on them. these studies also suggest some radically novel opportunities for human cancer immunotherapy.

TABLE I
NONVIRAL ANTIGENS THAT ELICIT PROTECTIVE IMMUNE RESPONSE TO TUMOR CHALLENGES

Source	HSPs	Others	Reference
Rat hepatoma[a]	gp96		Srivastava and Das (1984)
MCA-sarcomas	gp96		Srivastava *et al.* (1986); Palladino *et al.* (1987); Udono and Srivastava (1993b); Feldweg and Srivastava (1993)
UV-sarcoma	gp96		Blachere and Srivastava (1993)
MCA-sarcomas	hsp90		Ullrich *et al.* (1986); Udono and Srivastava (1993a)
MCA-sarcomas	hsp70		Udono and Srivastava (1993a,b)
B16 melanoma	Albumin-like[b]		Hearing *et al.* (1986)
Rat histiocytoma	Albumin-like[b]		Deshpande and Khar (1993)
UV-sarcoma		gp76	Ransom *et al.* (1981)
Colon carcinoma		gp30	Sato *et al.* (1987)
MCA-sarcomas		p75/82	DuBois *et al.* (1982)
MuLV-leukemia		gp175	Rogers *et al.* (1984)
ras-transformed cells		p50	Torigoe *et al.* (1991)

[a] gp100 was shown to react with anti-gp96 antiserum on a Western blot (unpublished observations, 1989).

[b] Albumin is heat shock inducible in fetal liver, although it is not a classical HSP (Srinivas *et al.*, 1987).

A. THE MAJOR TUMOR-IMMUNOGENIC HSPS—GP96, HSP90, AND HSP70

Immunogenicity of HSPs to tumors has been most extensively demonstrated in case of methylcholanthrene (MCA)-induced sarcomas of BALB/c mice, although evidence is increasingly accumulating in other systems as well (see Table I and the data that follow). The MCA-induced tumors such as Meth A, CMS5, and CMS13 elicit individually specific immunogenicity in mice immunized with them. A search for the tumor-derived molecules that elicit such specific immunity led to the identification of glycoproteins of 96,000 Da size (gp96) as the active principle in immunization by a number of tumors (Srivastava *et al.*, 1986; Palladino *et al.*, 1987; Feldweg and Srivastava, 1993). Independently and simultaneously, Ullrich *et al.*, (1986) identified the active immunizing principle from one of the same tumors as 84,000 and 86,000 Da (p84/86) isoforms of one protein. The fortuitous reasons that led to the identification of different proteins from the same tumor are discussed at length

elsewhere (Srivastava and Maki, 1991). However, sequence characterization of the two molecules revealed that gp96 and p84/86 shared considerable homology (Srivastava *et al.*, 1987, 1988; Maki *et al.*, 1990) and were members of the same HSP family: p84/86 is the cytosolic hsp90 and gp96 is its counterpart in the endoplasmic reticulum (ER), in the same manner as the Grp78 (BiP) is the ER-resident counterpart of the cytosolic hsp70. The gp96 transcripts were subsequently shown to be inducible by heat shock and other stresses (Maki, 1991), and heat shock elements were identified in 5′ flank of the gp96 gene (Maki *et al.*, 1993). More recently, immunization of C3H mice with gp96 isolated from the ultraviolet-(UV) induced C3H fibrosarcoma 6138 (Ward *et al.*, 1990) has been observed to elicit tumor-specific cytotoxic T lymphocytes (CTLs) against the 6138 sarcoma (Blachere and Srivastava, 1993; see also Section III,C).

In order to test the generality of tumor-immunogenicity of HSPs, Udono and Srivastava, (1993a,b) recently immunized BALB/c mice with hsp70 preparations from the Meth A sarcoma and found them to be specifically immunogenic against the Meth A sarcoma (see Section III,A). Mice are currently being immunized with other HSPs—hsp65 and hsp28. Konno *et al.*, (1989) had earlier observed a correlation between tumor immunogenicity and the expression of an hsp70-like protein in *ras*-transformed tumor cells.

Among these three HSPs (gp96, hsp90, and hsp70), the gp96 has been studied most extensively with respect to the molecular basis of its specific immunogenicity and the cellular mechanism of this activity. The present discussion therefore focuses on gp96, although evidence from other HSPs is discussed where corresponding information is available.

B. Specificity of Immunogenicity of HSPs Derived from Tumors and Lack of Immunogenicity of HSPs Derived from Normal Tissues

Immunity elicited by immunization with tumors is tumor specific: a mouse immunized against a given tumor is resistant to that tumor but not to other tumors (Prehn and Main, 1957; Old *et al.*, 1962). This immunity is so exquisitely specific that tumors of the same histological type and induced by the same carcinogen in an inbred colony of mice, or even two tumors induced in the same animal by the same carcinogen, are antigenically distinct (Basombrió, 1970; Globerson and Feldman, 1964). This phenomenon is not restricted to MCA-induced tumors but is ob-

served in tumors induced by a wide spectrum of agents including UV-irradiation as well as in spontaneous tumors tested (for a review see Srivastava and Old, 1988). The molecular basis of this specificity and diversity has been a continuing enigma in cancer immunology; however, recent discoveries have begun to shed some light on its possible origin (see Section V,A).

In consonance with the specific immunogenicity of tumors, immunization with HSPs derived from tumors also elicits resistance specific to the tumor from which it is derived, but not to other antigenically distinct tumors. Thus, gp96 isolated from Meth A sarcoma elicits immunity against Meth A but not CMS5. Conversely, CMS5 gp96 immunizes against CMS5 but not Meth A (Fig. 1; see also Srivastava *et al.*, 1986). This specificity has also been observed in immunization with gp96 derived from a rat hepatoma (Srivastava and Das, 1984) and hsp90 derived from the Meth A sarcoma (Ullrich *et al.*, 1986) as well as hsp70 obtained from the Meth A sarcoma (Udono and Srivastava, 1993a,b).

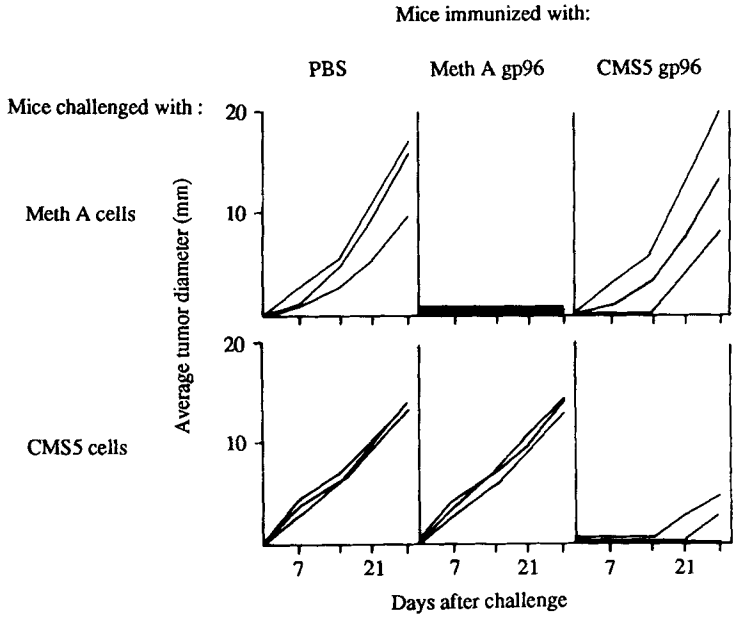

FIG. 1. Growth of Meth A and CMS5 in BALB/c mice after immunization with Meth A gp96 or CMS5 gp96. Lines represent tumor growth in mice challenged with 125,000 Meth A cells or 75,000 CMS5 cells.

An interesting demonstration of the specificity of immunogenicity of gp96 was made by Palladino *et al.*, (1987) in the case of three BALB/c sarcomas—Meth A, CMS5, and CMS13. The Meth A and CMS5 sarcomas are antigenically distinct as are the CMS13 and CMS5 sarcomas. However, Meth A and CMS13 sarcomas show partial cross-reactivity in tumor transplantation experiments. The gp96 preparations derived from the three tumors elicit an identical pattern of reactivity: administration of Meth A gp96 protects mice completely against Meth A, partially against CMS13, but not against CMS5 challenges. Administration of CMS5 gp96 protects mice against CMS5 but not against Meth A or CMS13 challenges, and injection of CMS13 gp96 protects mice significantly against CMS13, partially against Meth A, but not against CMS5 challenges (Table II).

Like other HSPs, gp96, hsp90, and hsp70 are expressed to varying degrees of abundance in normal tissues as well. However, immunization of mice with gp96 or hsp70 derived from liver or spleen of BALB/c mice does not elicit resistance to Meth A sarcoma (Fig. 2) Udono and Srivastava, 1993a,b).

TABLE II
IMMUNOGENICITY OF GP96 PREPARATIONS FROM CROSS-REACTIVE AND ANTIGENICALLY DISTINCT BALB/C SARCOMAS METH A, CMS5, AND CMS13

Antigen preparation[a]	Tumor challenge (% of inhibition of tumor growth)[b]		
	Meth A	CMS13	CMS5
Meth A gp96	>90 (0/3)[c]	59 (2/3)	<10 (3/3)
CMS13 Con A	65 (3/3)	>90 (0/3)	<10 (3/3)
CMS5 Con A	<10 (3/3)	<10 (3/3)	80 (2/3)

[a] Antigen preparations used were Con A-Sepharose, DEAE-Sephadex-fractionated Meth A gp96, and Con A-Sepharose-chromatographed cytosol protein fractions of CMS5 and CMS13 sarcomas. Preparations were made according to the procedures described by Srivastava *et al.* (1986). Groups of three BALB/c mice each were immunized twice at 7-day intervals with an amount of antigen equivalent to that obtained from 5×10^7 cells and challenged 7 days later with an intradermal injection of 1×10^5 Meth A, 8×10^4 CMS5, or 2.2×10^5 CMS13 sarcoma.

[b] Percentage of inhibition of tumor growth based on average tumor diameter 14 days after tumor challenge.

[c] Numbers in parentheses, number of tumor-bearing mice over number of mice.

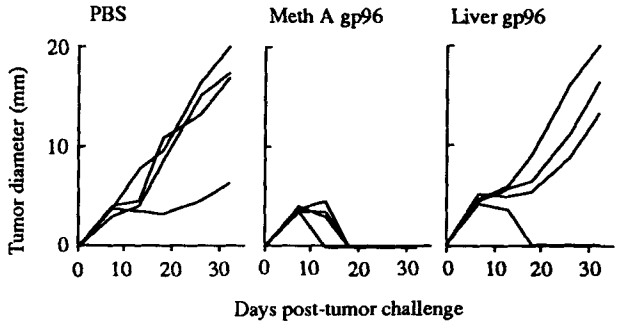

FIG. 2. Lack of tumor immunogenicity of gp96 derived from normal mouse liver. Mice were immunzed twice at weekly intervals with PBS, or 2 units gp96 derived from Meth A cells or BALB/c liver. Mice were challenged with 80,000 Meth A cells. Each line represents the kinetics of tumor growth in one mouse.

C. Lack of Tumor-Specific Polymorphisms in HSP Genes and Evidence That Carbohydrates Are Not Responsible for Immunogenicity

The specificity of immunogenicity of HSPs derived from tumor cells and the lack of tumor immunogenicity of HSP preparations derived from normal tissues suggested that HSPs might be hot spots for mutations during malignant transformation such that the HSP genes will show variation between normal tissues and tumors and among tumors. However, sequencing of gp96 cDNAs from BALB/c spleen, Meth A and CMS5 did not reveal any tumor-specific, individually distinct polymorphisms (unpublished observations, 1987). Moore *et al.*, (1990) looked for and similarly failed to identify tumor-specific DNA sequence polymorphisms in hsp90 genes. This directed our attention to the role of N-linked sugars of gp96 (there are no O-linked sugars in gp96). However, the following observations ruled out their role in tumor-specific immunogenicity of gp96, (i) Meth A cells cultured in the presence of tunicamycin can be used to immunize BALB/c mice specifically against Meth A. (ii) The gp96 protein derived from Meth A cells grown in the presence of tunicamycin did not bind to Con A-Sepharose and successfully immunized mice specifically against Meth A (unpublished observations, 1990). In the case of hsp90 and hsp70, the question of a role for sugars in the specific immunogenicity does not arise, as they are not glycosylated to begin with. Although it is conceivable that specificity might reside in other post-translational modifications, it is unlikely in

view of the fact that immunity to tumors is T cell mediated and that there is not much precedent for cellular immunity to non-peptide determinants. Thus, there appears little reason to believe that specific immunogenicity of HSPs lies in HSPs *per se*.

II. Hypothesis That HSPs Chaperone Antigenic Peptides

The lack of diversity in HSPs led us to consider molecules associated with them. Because the gp96 used to immunize mice is homogeneous by all criteria —(a) there is a single band on overloaded silver-stained gels; (b) there is a single amino terminus during Edman degradation; (c) antipeptide antibody to gp96 depletes a preparation of its immunogenic activity—our attention was focused on small moieties. A number of HSPs bind to a wide array of molecules, including peptides (Flynn *et al.*, 1989, 1991), and we proposed that gp96 molecules may not be immunogenic *per se* but may act as carriers of antigenic peptides (Srivastava and Maki, 1991; Srivastava and Heike, 1991). In view of the predominant localization of gp96 in the endoplasmic reticulum (Booth and Koch, 1989), we further suggested that gp96 acts as peptide-acceptor for peptides transported to ER and may be accessory to loading of MHC class I molecules (Fig. 3).

III. Evidence in Support of the Hypothesis

A. Antigenic Peptides Can Be Eluted Off gp96 and hsp70 Molecules

In order to test directly whether peptides were associated with gp96, experiments similar to those performed with MHC class I molecules have been carried out. Approximately 3-mg purified gp96 was stripped at pH 2, and the low molecular weight eluted material was analyzed by HPLC on a C18 reverse chromatography column. A number of peaks were obtained and were analyzed by mass spectroscopy (unpublished observations, 1992). We have tested the stripped high molecular material for its integrity by SDS-PAGE and have found it to be still intact. Restripping of this material under identical conditions did not result in further recovery of peptide peaks. Mass spectroscopic analysis of the eluted peptides has shown that the peptides vary in size from 400 to 2000 Da. One of the peptides has been sequenced and, as expected, is unrelated to gp96. We are awaiting mass and sequence characterization of other gp96-associated peptides.

We have suggested a general role for gp96 as a peptide-acceptor

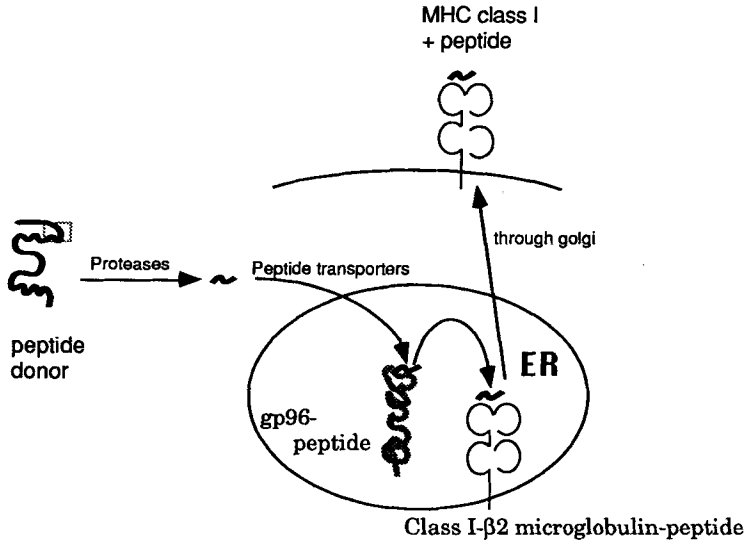

FIG. 3. Schematic representation of the proposed role of gp96 HSP in antigen presentation by MHC class I molecules.

(chaperone) in the ER lumen. If gp96 indeed acts in this capacity, it should bind peptides derived from any cellular proteins and not merely immunogenic tumor antigens. In addition to the structural approach described in the preceding paragraph, the question of peptide binding by gp96 has also been approached immunologically in an influenza viral system. The following question has been posed: Can peptides eluted from a gp96 preparation from influenza-virus infected cells sensitize non-influenza infected cells for lysis by influenza-specific CTLs? It is observed that gp96-eluted peptides can indeed sensitize non-influenza-infected cells for lysis by NP-specific CTLs (Blachere and Srivastava, 1993).

The hsp70 molecules have also been demonstrated to bind tumor-antigenic peptides. The hsp70–peptide interaction has been shown to be ATP-sensitive, and addition of ATP releases the peptides (Flynn *et al.*, 1989, 1991). Administration of hsp70 preparations derived from Meth A sarcoma has been shown to confer Meth A-specific immunity (see Section I,A); however, if the hsp70 preparation is applied to an ATP-agarose column and eluted by ATP, it loses its immunogenicity (Udono and Srivastava, 1993b). The peptides eluted from hsp70 preparations by addition of ATP have been resolved on a C18 column, and the individual fractions are being characterized by mass spectroscopy. These

observations demonstrate the validity of one of the central tenets of our hypothesis, i.e., HSPs chaperone antigenic peptides.

B. gp96 Binds ATP and Is an ATPase

Should gp96 be involved in peptide sequestering or assembly, it should be able to bind and perhaps hydrolyze ATP, as a source of energy for these activities. This has been found to be true for another peptide-binding ER luminal HSP, the grp78 or BiP (Flynn *et al.*, 1989, 1991). In accordance with this proposed role, gp96 sequence was observed to contain A and B type consensus sequences characteristic of ATP-binding proteins (Walker *et al.*, 1982; Chin *et al.*, 1988). Further, gp96 has been shown to be able to bind ATP *in vitro* and gp96 preparations obtained from metabolically iP-labeled cells are found to contain ATP and ADP bound to them (Li and Srivastava, 1993a).

Highly purified, apparently homogeneous gp96 preparations have been assayed for and determined to possess ATPase activity (Li and Srivastava, 1993a). This activity can be depleted from a gp96 preparation by passing through an anti-gp96 monoclonal antibody affinity column. The ATPase activity of gp96 is optimal at 42°C when assayed at pH 7.2 and is dependent on exogenous supplied Mg^{2+}, but not Ca^{2+}. The ATPase activity of another ER-resident HSP, Grp78 or BiP has been observed to be stimulated by addition of peptides (Flynn *et al.*, 1989, 1991), and ATP hydrolysis is believed to result in release of BiP-associated peptides. In contrast, the ATPase activity of gp96 was not stimulated by addition of a number of peptides. In order to gain a clue with respect to other factors that may modulate the ATPase activity of gp96, other protein substrates were examined, and dephosphorylated casein was found to stimulate the ATPase activity of gp96. It would appear that ATP hydrolysis by gp96 is stimulated by gp96–protein interactions rather than protein–peptide interactions. Implications of this possibility are discussed in Section VI.

C. Immunization with gp96 Molecules Elicits Cytotoxic T Cells against Tumors and Virus-Infected/Transformed Cells

The immunological consequence of vaccination with gp96 has so far been measured by protection from a tumor challenge. Because this protection is mediated by CD8+ T lymphocytes (Udono and Srivastava, 1993c), we tested directly the ability of gp96 to elicit a CTL response. This has been carried out in several antigenic systems. The CTLs have been generated against the sarcoma 6138 by immunizing C3H HeN

mice with gp96 derived from the 6138 sarcoma (Blachere and Srivastava, 1993). Mice were immunized with 5 μg of gp96 in PBS and mixed lymphocyte-tumor cultures (MLTC) (with splenocytes of 6138—gp96 immunized mice and 6138 cells) tested. (There is no *in vitro* priming in this system, i.e., spleen cells from naive unprimed mice cultured with 6138 cells do not show any cytotoxicity). Class I-restricted CTL activity was detected against the 6138 cells but not against the antigenically distinct 6139 cells against which an NK-like (not blockable by anti-Class I antibody) activity is observed. The Class I restricted cytotoxicity against the 6138 cells becomes significantly more pronounced on day 12 after a second round of *in vitro* stimulation. This did not happen on the NK-like activity on 6139 target cells.

Because the peptide-chaperoning role hypothesized for gp96 is a general one (i.e., valid for any cellular protein and not only for tumor antigens), we have tested our hypothesis in non-tumor systems as well. The gp96 preparations were obtained from flu(PR8)-infected BALB/c fibroblasts and used to immunize BALB/c mice. T cells generated from these mice and stimulated *in vitro* with flu-infected cells showed significant antigen-specific, MHC class I-restricted CTL activity (Blachere and Srivastava, 1993). (No *in vitro* priming is seen, i.e., T cell cultures generated from unimmunized mice an stimulated *in vitro* with flu-infected cells show no Class I restricted cytotoxicity.)

Similar observations have been made with SV40-transformed cells (Blachere and Srivastava, 1993). Mice were immunized with gp96 derived from SV40-transformed PSC3H cells or with non-SV40-transformed C3H fibroblasts. The MLTCs generated from these mice and stimulated with PSC3H cells were tested on PSC3H and non-SV40-transformed fibroblasts. Class I-restricted cytotoxicity against PSC3H was seen in MLTCs generated from mice immunized with PSC3H gp96 but not the fibroblast-derived gp96.

Generally, exogenously added antigens elicit a CD4+, MHC class II-restricted response, whereas endogenously synthesized antigens are presented in the context of MHC class I molecules and elicit a CD8+ response (Townsend and Bodner, 1989). In light of this paradigm, the mechanism by which immunization of mice with gp96–peptide complexes elicits a CD8+ CTL response is not clear. This is discussed in Section IV.

D. Gp96 Genes Are Interferon Inducible

An analysis of the 5′ flanking region of the *gp96* gene showed the presence of an imperfect interferon-responsive element at -519 (Levy

et al., 1988). This led us (in collaboration with Dr. Berish Rubin, Fordham University, Bronx, New York) to investigate the interferon inducibility of *gp96* transcripts. It was observed that *gp96* transcripts are indeed up-regulated by exposure of He La cells to α- and γ-interferon, but not by β-interferon. While the interferon inducibility of *gp96* may not be of direct relevance to us, it is of some interest because all the elements involved in antigen presentation by MHC class I molecules—the proteosome subunit genes, the transporter genes, and the Class I itself are interferon responsive. The interferon responsivity of gp96 is consistent, in this aspect, with a role for gp96 in antigen presentation by MHC class I.

E. gp96 Facilitates Folding/Assembly of MHC Class I Molecules

The assembly of MHC class I–β2 microglobulin–peptide has been shown to be ATP dependent (Levy *et al.*, 1991; Luescher *et al.*, 1992). In view of the peptide and ATP-binding property of gp96 and its ATPase activity, we tested directly whether gp96 can assist in assembly of MHC class I complex. A number of antibodies against MHC class I molecules are conformation dependent, and Townsend and colleagues have used these antibodies and the RMA-S cell line to demonstrate the role of peptides in folding of Class I (Townsend *et al.*, 1989). Extracts of metabolically labeled (3 hours) RMA-S cells were precipitated with the B22.249 antibody, which preferentially recognizes the fully folded D^b molecule. As shown by Elliott *et al.* (1991), a relatively small amount of Class I heavy chain is precipitated, because it is not fully assembled. However, addition of a presentable peptide to the lysate allows a significantly higher recovery of Class I heavy *and* light chains. When increasing quantities of purified gp96 (in the absence of exogenously added peptide) was added to the RMA-S lysate, the same result was obtained as obtained by addition of peptide, i.e., a significantly higher quantity of heavy and light chains were precipitated (Li and Srivastava, 1993b). Thus, gp96 can substitute for peptide in assembly in Class I–β2 microglobulin complex. These experiments are presently being carried out in a number of conditions,including use of other peptides, precipitation with antibodies to the folded K^b molecules, depletion of ATP, and addition of ATPase inhibitors. Our observations have at least two interpretations: (i) the Class I–β2 microglobulin complex is being stabilized by gp96 by transfer of peptides associated with gp96 or (ii) gp96 by itself is somehow stabilizing the Class I–β2 microglobulin complex even in the absence of peptide.

F. Evidence Not Yet Obtained

Although the evidence cited in support of the "HSPs chaperone antigenic peptides hypothesis" is compelling, it is not yet final and conclusive. Such evidence would consist of (i) isolation and structural characterization of a known CTL epitope of a viral antigen (or a tumor) from the peptides eluted from gp96 or hsp70 preparations from a cell expressing that viral antigen (or that tumor) ; (ii) reconstitution of an "empty" gp96 or hsp70 molecule derived from another source with the identified peptide, and (iii) immunization of naive mice with such reconstituted gp96 or hsp70 molecule so as to elicit a CTL response restricted to that particular epitope. Several variations on this general theme can be constructed. Attempts to satisfy these criteria are underway in our laboratory.

G. Conceptual Requirement for a gp96-like Molecule for Peptide Charging of MHC Class I Molecules

There is clearly a mechanistic requirement for a molecule in the lumen of the ER, which will facilitate charging of MHC class I molecules with peptides. The gp96 molecules appear to be ideal candidates for such a role. Alexander *et al.* (1989, 1990) have postulated the existence of molecules that participate in loading "empty" class I MHC molecules with peptides, before the MHC-peptide complex is released for transport to cell surface. These hypothetical molecules would be similar to BiP, which binds to Ig heavy chain, before it is assembled with the light chain (Lindquist and Craig, 1988). Townsend *et al.* (1990) have recently demonstrated assembly of class I MHC molecules *in vitro* and hint at the interaction of another molecule in the assembly process. This is a 105-kDa molecule that coprecipitates with D^b heavy chains after assembly is promoted in lysates with β2 microglobulin alone. The coprecipitation is prevented if β2 microglobulin is denatured; it is also inhibited by a peptide that binds D^b but not K^b. Townsend *et al.* consider it "conceivable that a catalyst of class I assembly would occupy the peptide-binding site and be displaced by the peptide. If it were resident in the ER, it could serve both to catalyze assembly and retain peptide-free class I molecules." Similarly, Levy *et al.* (1991) suggested that "luminal proteins may exist that bind peptides very efficiently, thereby concentrating peptides in the ER. As soon as a peptide enters the ER lumen, it is trapped by these proteins and is further processed into the assembly pathway." In view of the ability of gp96 to bind peptides and ATP, its ATPase activity,

and its localization in the ER lumen (where it is the major component), it is reasonable to suggest this role for gp96.

An ER chaperonin p88/p90 has recently been identified (Degen and Williams, 1991; Hochstenbach *et al.*, 1992): it is an ER membrane (as opposed to luminal) protein, is related to the calnexin molecule (Galvin *et al.*, 1992), and is distinct from gp96. This protein is associated with partial complexes of membrane immunoglobulins, T cell receptors, and MHC class I, but it is not present in fully assembled complexes. Hochstenbach *et al.* (1992) suggest that p88/p90/calnexin might participate in assembly of multisubunit complexes. The peptide-transfer role suggested by us for gp96 is distinct from this role.

H. Mechanisms by Which Nonendoplasmic Reticulum HSPs Such as hsp90 and hsp70 Associate with Antigenic Peptides

The gp96 protein is localized in the ER, and this location is ideal for encountering antigenic peptides. It is not clear however as to where the non-ER HSPs such as hsp90 and hsp70 bind antigenic peptides. The HSPs are generally believed to be present in the cytosol, where the proteins are synthesized on the ribosomal machinery and are presumably degraded by proteosomes or other proteolytic machinery into short peptides. The peptides are then translocated into the lumen of the ER, where we suggest they are accepted by gp96 for transfer to MHC class I. It is conceivable that hsp90 and hsp70 bind antigenic peptides upstream to the ER lumen, i.e., in the cytosol, soon after the proteins are degraded into peptides. However, other subcellular locales where this might happen should also be considered. The view that HSPs are cytosolic proteins is based largely on the biochemist's operational definition of the cytosol (as the 100,000-g supernatant) and is too simplistic. The HSPs occupy a variety of cellular sites, including the cell surface, endosomes, ER, and nucleus (Srivastava *et al.*, 1986; Altmeyer *et al.*, 1993; Mazzarella and Green, 1987; Lakey *et al.*, 1987; VanBuskirk *et al.*, 1989; Welch *et al.*, 1991) and may encounter peptides at any of these sites. It is of interest in this regard that a comparison of the relative tumor-specific immunogenicities of gp96, hsp90, and hsp70 from the Meth A sarcoma shows that gp96 and hsp70 are far more immunogenic than hsp90 (Udono and Srivastava, 1993b). Among other possibilities, this observation may reflect the fact that some intracellular sites may allow a better opportunity for HSP–peptide interactions than others.

IV. Mechanism by Which HSPs Elicit Specific Immunity

Immunization of mice with gp96 leads to tumor-specific protection, which is dependent on CD4+ and CD8+ T lymphocytes (Udono and Srivastava, 1993c). Similarly, antigen-specific, MHC class I-restricted CD8+ CTLs can be generated by immunization with gp96 in a number of systems (Blachere and Srivastava, 1993). The mechanism by which immunization with HSP–peptide complexes elicits a CD8+ response is unclear. A Class I-restricted response usually requires presentation of antigens through the endogenous pathway, and it would appear that the HSP–peptide complexes, even though provided exogenously, are able to channel the peptides into the Class I presentation pathway. A clue into this mechanism may emerge from the observation that depletion–functional inactivation of macrophage completely abrogates the tumor-specific immunogenicity of gp96; in contrast, immunization with whole tumor cells is not sensitive to depletion of macrophage (Udono and Srivastava, 1993c). It is conceivable that macrophage possesses receptors for gp96 and other HSPs, allowing them to bind HSP–peptide complexes that are then directed to the endogenous presentation pathway through a compartment distinct from the lysosomal compartment. Such receptors are presently being sought. The role of macrophage in channeling exogenous antigens into the endogenous presentation pathway has also recently been suggested in two other systems (Debrick *et al.*, 1991; Hosmalin *et al.*, 1992).

A number of studies have recently reported that it is possible to elicit MHC class I-restricted CTL response by immunization with soluble peptides. The peptides are usually given in incomplete Freund's adjuvant (Aichele *et al.*, 1990; Schulz *et al.*, 1991) or are modified by a lipophilic tail (Deres *et al.*, 1989). The precise mechanisms that allow peptides access to the class I presentation pathway in such immunizations are not yet known. It is possible that the HSPs transfer the chaperoned peptides into the class I presentation pathway by similar means.

V. Implications for Immunity to Cancer

A. What Are Tumor Antigens?

If our suggestion as to the mechanism of specific immunogenicity of gp96 is correct, the question of structural basis of specificity of immunogenicity of gp96 HSPs can be rephrased: What is the source of HSP-associated immunogenic peptides and their tumor specificity? The

source of the immunogenic peptides clearly lies in altered (mutated) cellular proteins, and their specificity lies perhaps in randomness of mutations. It is now clear that any alterations in a coding gene (productive mutations) are potentially presentable by MHC class I antigens and recognizable by the cellular immune system (Townsend and Bodner, 1989). These mutations can be caused by application of MCA or other carcinogens, or they may occur spontaneously. Because mutations are random events, each tumor will possess a specific repertoire of mutations, which will be stably inherited; the number of different types of mutational repertoires will be practically unlimited in this scenario (Fig. 4). The antigenicity created by randomness of mutations thus explains the extraordinary diversity and specificity or immunogenic tumor antigens. Also inherent in this proposal is the concept of hierarchy of tumor antigens (Herin *et al.*, 1987; Degiovanni *et al.*, 1988; Knuth *et al.*, 1989; Wolfel *et al.*, 1989; Van den Eynde *et al.*, 1989). If a number of peptides are antigenic, it is reasonable to assume that if one selects tumor cells

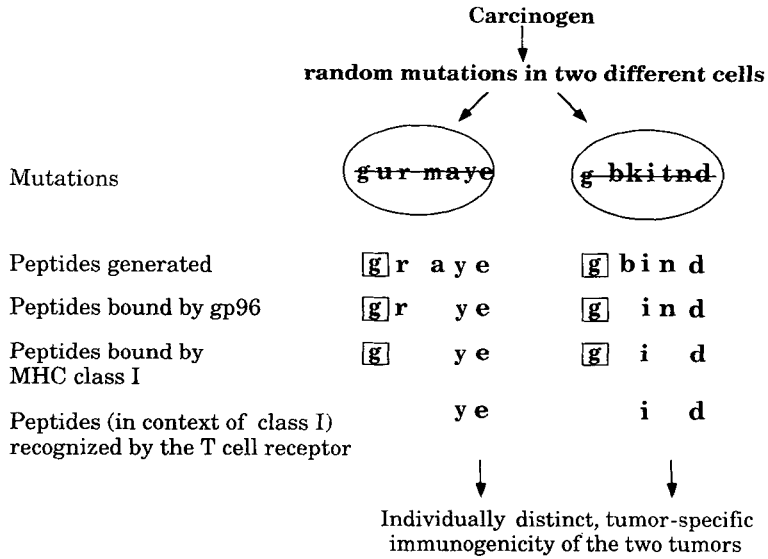

FIG. 4. Specificity of immunogenicity of tumors results from different random mutations in each cell. Subsets of altered peptides generated by mutations are presented by gp96 to MHC class I and a smaller subset of these are recognized by the immune system. An altered peptide such as g, which is a hot spot (i.e., is altered in both cells and represents mutations such as those in *ras* or *p53*) is not recognized by the immune system. As the peptides recognized as immunogenic for each tumor are distinct (y e in case of one tumor and i d in the other), each tumor elicits an individually distinct immune response.

that do not express a particular epitope, other epitopes will be exposed and become immunologically detectable. "It was an early belief that tumor-specific antigens are a special set of proteins which are induced as a result of malignant transformation and elicit tumor immunity. About two decades and several hundred publications later, that belief turns out to be substantially incorrect. It now appears that there are no tumor-specific molecules, but only tumor-specific epitopes of common molecules" (Srivastava, 1991).

It is now possible to revisit another long-standing question in tumor immunology: Is there a relationship between the immunogenic tumor antigens and the transforming event(s) (see Hopkins *et al.*, 1981)? Considering that a cell may acquire any number of random mutations, only a small proportion of which will be transforming (i.e., mutations in oncogenes), the answer is clearly in the negative. The events responsible for development of the transformed cell are independent of the events that elicit its recognition by the immune system. The question arises, however, whether the mutational alterations responsible for malignant transformation may also be recognized by the immune system, in addition to other random mutations. Mutations in *p53, ras,* and other oncogenes or tumor suppressor genes are among the most common genetic alterations in human cancer. It would make perfect sense for these alterations to be immunogenic, and if cancer were to have evolved to be therapeutically amenable, altered oncogene products would serve as excellent cancer vaccines. However, although there are occasional and notable examples of immune response to altered *ras* proteins (Jung and Schluesener, 1991; Peace *et al.*, 1991), and there is an extensive ongoing effort in a number of laboratories to uncover the cellular immune response to altered *p53*, there is little evidence that altered oncogene products may be generally immunogenic. Indeed, in case of immune response to the spontaneous breast cancers of v-Ha-*ras* transgenic mice (Sinn *et al.*, 1987), it has been demonstrated that although the tumors are highly antigenic and anti-tumor CTLs can be generated, the CTLs do not recognize the activated *ras* products (Heike *et al.*, 1993). This makes perfect sense in terms of the evolution of cancers: if activated oncogenes were detected by the immune system and the cells harboring them were destroyed, malignant cells would not survive. Tolerance of immune system for the oncogene products is yet another means of immunological suppression caused by tumors. The tolerance of an animal for an oncogene product has been demonstrated in case of the *neu* oncogene, whose activation–amplification is associated with a large proportion of human breast cancers (Slamon *et al.*, 1987). When a vaccinia virus construct expressing the *neu* oncogene of rats is expressed in mouse tumor

cells, the rat-*neu* expressing mouse tumors elicit potent immunity in *mice*. However, the same construct expressed in syngeneic rat tumor cells, does not elicit immunity in rat (Bernards *et al.*, 1987). Clearly, the rat is tolerant to its *neu* product, and the mouse anti-*neu* is directed against a foreign protein. These observations dampen the early hopes of using oncogene products as targets of cancer immunotherapy. Immunotherapy is to be directed to the incidental repertoire of mutations that a malignant cell acquires.

B. HSPs and Other Tumor Antigens: A Unified View

Immunogenic antigens of experimental tumors have been identified by two approaches: (i) the transplantation approach whereby antigens are detected by their ability to protect against tumor challenges (Table I) and (ii) the T cell approach whereby antigens defined by tumor-specific CTLs have been sought to be characterized. A number of different molecules have been identified as immunogenic tumor antigens using these approaches. In this section, an attempt will be made to understand why different antigens have been isolated using these two approaches and sometimes using the same approach and what the relationship between these various antigens is.

The transplantation approach can, in principle, detect two types of antigens—molecules that elicit a T helper response (because the antigens are exogenously provided when mice are immunized) and molecules like HSPs, which "chaperone" the CTL epitopes. It would be reasonable to predict that the non-HSP antigens described in Table I will turn out to be help-eliciting antigens. In contrast to the transplantation approach, the CTL approach, pursued by Boon (1992) detects directly the antigenic epitopes recognized by CTLs—these would be different from the helper antigens and the chaperonins detected by the transplantation approach. It is thus immediately evident that the antigens detected by the two approaches are bound to be different.

It is possible to conceive of a situation where these types of approaches would converge. If the peptides eluted from the gp96 or hsp70 molecules isolated from a given tumor were characterized by mass spectroscopy and the antigenic peptides identified, one or more of the antigenic peptides should be identical to the CTL epitopes detected by the Boon approach. Regarding the dichotomy between the helper and CTL-epitopes detected by the transplantation approach, it is not clear why some fractions derived from some tumors elicit help more effectively than fractions derived from other tumors. This must have to do with the

intrinsic characteristics of the antigenic epitopes. Ideally, these experiments should be carried out in a tumor system, in which a non-HSP molecule has been identified as eliciting protective tumor immunity and in which tumor-specific CTLs can be generated. Such a system will permit a convergence of all the roads to tumor immunity.

C. Human Cancer Immunotherapy

Identification of HSPs as chaperones of antigenic peptides has a direct bearing on immunotherapy of human cancer. One of the major conceptual difficulties in cancer immunotherapy has been the possibility that human cancers, like cancers of experimental animals, are antigenically distinct. Clearly, there is some recent evidence for the existence of common human tumor antigens (Kawakami *et al.*, 1992); Darrow *et al.*, 1989), and this augurs well for prospects of cancer immunotherapy. Nonetheless, in light of the overwhelming evidence from experimental and human systems, it is reasonable to assume that, at the very least, human tumors would show tremendous antigenic diversity and heterogeneity.

The prospect of identification of the immunogenic antigens of individual tumors from cancer patients (or even of only several different types of immunogenic antigens in case the antigens are shared) is daunting to the extent of being impractical. The possibility that HSPs chaperone antigenic peptides of the cells from which they are derived circumvents this hurdle. Because of the reasons and evidence described in Section III, an HSP preparation is expected to chaperone the entire repertoire of antigenic peptides of a given cell. Immunization with HSPs thus offers a number of profound and unique advantages over other methods of immunization against cancer.

1. The HSPs "carry" a variety of immunogenic peptides, derived from the cells from which they are isolated. Immunization with them thus obviates the necessity for isolation and characterization of the antigenic molecules.

2. Immunization with soluble antigens usually leads to a CD4+ helper T cell response because of the preferential presentation of exogenous antigens by the MHC class II antigens. Thus, even if a tumor antigen were to be identified, immunization of patients with it will yield a predominantly helper response, which may not be necessarily protective. In contrast, immunization with gp96 HSPs is able, by an as yet undetermined mechanism, to elicit a CD4+ and a CD8+ T cell response (Udono and Srivastava, 1993c).

3. Immunization with biochemically undefined tumor extracts inevitably carries the risk of inoculating the mice or patients with potentially transforming or immunosuppressive agents such as transforming DNA or TGFβ. Immunization with purified HSP preparations eliminates these risks.

4. Immunization with HSPs elicits significant tumor immunity and CTLs without the use of adjuvants. Even though adjuvants that may further potentiate the immunity elicited by HSPs may be sought, their availability is not a precondition for a significant protective response.

These advantages make autologous cancer-derived HSPs uniquely useful and radically novel immunogens against human cancer. It is advisable, however, to introduce a note of caution here: It was demonstrated that gp96 elicits tumor immunity in a dose-restricted manner and that immunization with very high doses may result in toxicity (Srivastava *et al.*, 1986). The basis of this dose-restricted activity and high-dose toxicity is not yet clear. It is conceivable that gp96 (and other HSPs) may elicit a measure of autoimmunity due to the fact that they chaperone a broad spectrum of antigenic peptides. Although the host would normally be tolerant to the self-peptides, administration of large quantities of HSP–peptide complexes may break this tolerance. This idea has not yet been experimentally substantiated, and it would be useful to do so, before human vaccination with tumor-derived HSP preparations is considered.

VI. Implications for Antigen Presentation

A. Promiscuity of Peptide-Binding by HSPs

In contract to MHC antigens, HSPs are not polymorphic: individuals of a given species do not show genetic variation in the HSP genes. If gp96 molecules are accessory to charging MHC class I with peptides, the same gp96 molecule should be able to transfer peptides to different Class I molecules. However, Class I molecules of different haplotypes bind different sets of peptides. It can be deduced from this that gp96 must be able to bind a broader spectrum of peptides than any of the Class I molecules. Indeed, the ability of a peptide to bind gp96 must be the first selection step in the presentability of a peptide. Furthermore, because peptide binding to gp96 is a step in the eventual transfer of the peptide to Class I, the binding must be reversible. There is no such requirement in case of Class I–peptide binding, which may explain the observation that immunization of mice with Class I–peptide complexes does *not* lead to tumor immunity (deduced from Srivastava *et al.*, 1986;

unpublished observations, 1985). The suggested reversibility of gp96–peptide binding suggests that there must be physiological mechanisms for this reversal. Our studies on modulation of ATPase activity of gp96 (which is expected to result in release of peptides, as in the case of BiP; see Flynn *et al.*, 1989, 1991) by interaction with other proteins (see Section III,B) suggest one such possible mechanism (Li and Srivastava, 1993a).

Lack of polymorphism in gp96 should have an interesting consequence on immunization of allogeneic animals with gp96. The gp96 molecules isolated from cells of any haplotype infected with the same virus should contain an identical set of antigenic peptides and, therefore, can be used to immunize mice of any haplotype successfully. It has been observed earlier that mice immunized with allogeneic cells expressing a particular foreign antigen are able to generate CTLs against the foreign antigen in context of the haplotype of the immunized mouse, regardless of the haplotype of the immunizing cell (Gooding and Edwards, 1980). These observations have led to suggestions that there must exist some mechanism(s) for *de novo* charging of preexisting Class I molecules in the immunized mice. It is suggested here that the HSP–peptide complexes of the immunizing allogeneic cells prime the immunized mice for generating a CTL response restricted by the haplotype of the immunized mice (see Section IV). Experiments to confirm this suggestion are presently in progress (also see Section VI,B).

B. HSPs as Agents of "Priming" of CTL Responses *in Vivo*

It is clear that HSPs may chaperone antigenic peptides as accessories to antigen presentation by MHC class I molecules (as in case of gp96) or as a part of their general scavenging purpose (as possibly in case of hsp90 and hsp70). It remains to be seen however whether the ability of the HSP–peptide complexes to prime CTL responses is merely an artifact, albeit a possibly useful one, or it has a physiological significance. It is submitted here that it does indeed have a wider physiological significance in immune response to cancer and infections.

It is assumed that priming of CTL precursors *in vivo* occurs by recognition of an MHC class I–peptide complex on an infected cell or a tumor by a suitable T cell receptor borne on the CTL precursor. This clearly has to be so, at least in part. However, if the mechanism for priming of CTLs were to be limited to this, the priming process would stop once the target cells were lysed by the CTLs. Another difficulty arises in instances where the infected or tumor cells are lysed by antibodies or

non-specific effector mechanisms before recognition by CTLs can occur. It is suggested that the ability of HSPs to chaperone antigenic peptides and to prime CTLs (i) allows for a prolongation of the priming process even after the target cells are lysed; (ii) permits priming of CTL precursors even if the target cells are lysed by antibodies or other mechanisms before CTLs can be primed; (iii) permits priming of CTLs even in instances where infected cells or tumor cells do not express sufficient cell surface MHC class I antigens, as an escape mechanism, as in the case of adenovirus-infected cells (Gooding and Wold, 1989) and a proportion of tumors (Tanaka *et al.*, 1985). In the latter instance, priming byHSP–peptide complexes may be the only mechanism for priming of CTL precursors.

C. Relationship between the HSPs and the MHC Molecules

"Nothing in biology makes sense except in the light of evolution" (Theodosius Dobzhansky, cited in Ayala, 1977), and the suggestion that HSPs prime CTL precursors *in vivo* meets this criterion. We have proposed earlier that, lack of sequence homology notwithstanding, HSPs represent functional forerunners of MHC molecules—both groups of molecules are highly conserved and ubiquitously expressed, involved in defense, and most significantly, bind peptides (Srivastava and Heike, 1991). The HSPs are older molecules and evolved in an evolutionary era of limited interindividual diversity and hence the lack of polymorphism in HSP genes; the MHC on the other hand are more recent and arose in an era of considerably increased genetic diversity among individuals. However, the two molecules perhaps performed similar functions in different periods of our evolutionary history, and their roles have converged in antigen presentation as we understand it today. The demonstration by Flajnik *et al.* (1991a,b) of a sequence homology between the peptide-binding domain of MHC class I and the corresponding putative region in molecules of the hsp70 family is consistent with this suggestion.

I extend this train of thought here to suggest that HSP–peptide complexes prime CTL precursors *in vivo* in the same manner that MHC–peptide complexes can. The roles of the two molecules are not competing, but rather complementary: the MHC–peptide complexes can prime only when expressed on a cell surface and not in solution (unpublished observations), whereas the HSP–peptide complexes can prime only when released from cells. The exquisite synergism of this complementarity may be an essential component of host defense.

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THE ASSOCIATION OF EPSTEIN–BARR VIRUS (EBV) WITH T CELL LYMPHOPROLIFERATIONS AND HODGKIN'S DISEASE: TWO NEW DEVELOPMENTS IN THE EBV FIELD

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I. Introduction

The Epstein–Barr virus (EBV) is a gamma-herpes virus, endemic in human populations throughout the world. Infection is normally asymptomatic, typically first occurring during early childhood. Delayed primary EBV infection frequently results in the development of a transient lymphoproliferative disorder, clinically manifest as infectious mononucleosis (Niederman *et al.*, 1968). In addition to benign lymphoproliferation, EBV is associated with a range of lymphoid malignancies. The EBV was first discovered in cell lines derived from endemic (African) Burkitt's lymphoma (BL) tissues (Epstein *et al.*, 1964), and there is strong evidence that the virus contributes to the development of such tumors, although its precise pathogenetic role remains unclear (Klein, 1983; Lenoir and Bornkamm, 1987; Magrath, 1990). The EBV is also associated with the development of high-grade B cell non-Hodgkin's lymphomas (NHLs) in patients with both congenital and acquired immunodeficiency states. Such tumors have been most extensively studied in allograft recipients with iatrogenic immunodeficiency (Hanto *et al.*, 1983; Ho *et al.*, 1985; Locker and Nalesnik, 1989; Young *et al.*, 1989) and in human immunodeficiency virus (HIV)-seropositive individuals (Subar *et al.*, 1988; Hamilton-Dutoit *et al.*, 1991a, 1993a,b), but they also occur in inherited immunodeficiency conditions such as the X-linked lymphoproliferative syndrome (Purtilo *et al.*, 1982).

The realization that EBV was linked to the development of certain lymphomas, albeit rare tumors occurring under unusual conditions, had important implications because it suggested that the virus might also play a role in the pathogenesis of more common lymphoproliferative lesions. Little evidence has, however, been found to link EBV to the development of the majority of B cell malignancies; most studies detect markers of EBV infection in less than about 5% of high-grade B cell lymphomas arising in individuals without overt immunodeficiency (Pagano *et al.*, 1973; Ziegler *et al.*, 1976; Bornkamm *et al.*, 1976; Andiman *et al.*, 1983; Wutzler *et al.*, 1986; Weiss *et al.*, 1987; Anagnostopoulos *et al.*, 1989; Staal *et al.*, 1989; Ohshima *et al.*, 1990; Hamilton-Dutoit and Pallesen, 1992a; Geddes *et al.*, 1992).

Although efforts to implicate EBV as an important factor in sporadic B cell lymphomagenesis have been largely unsuccessful, it has become apparent that EBV infection may occur in a much wider range of lymphoproliferative lesions than was previously realized. Thus, recent studies have shown that EBV nucleic acids and antigens are present in tumor cells in about one-half of Hodgkin's disease (HD) cases, and in a substantial number of peripheral T cell lymphomas (PTLs) and related lesions.

In this review, we will look at the evidence for EBV infection in these tumors and discuss the significance of these unexpected findings.

II. Biology of EBV

A. General

The EBV genome is a linear double-stranded DNA molecule some 172 kb in length encoding approximately 100 genes (Farrell, 1989). It is separated into short and long unique sections by a variable number of tandemly reiterated 3.1-kb internal repeats and is flanked at either end by multiple tandem 0.5-kb terminal repeats. In common with other herpes viruses, EBV is able to establish both replicative (productive/lytic) and latent (persistent) infections. During replicative infection, there is extensive transcription of the linear viral genome, broad expression of viral proteins, and production of mature virions accompanied by host cell lysis and death. In contrast, viral gene expression is restricted during latent infection, and new virions are not produced, the virus establishing a persistent infection of the host cell.

The EBV shows tropism for both lymphocytes and epithelial cells. Primary infection takes place by the oropharyngeal route. Following recovery, healthy virus carriers continue to shed EBV intermittently into their saliva indicating the presence of productive viral infection in the normal oropharynx and/or salivary glands (Morgan *et al.*, 1979). The nature of the cell type supporting this infection remains unclear. The normal site of EBV latency is also controversial, although recent studies indicate that this probably lies within the hematopoietic system (Gratama *et al.*, 1988; Rickinson, 1988; Klein, 1989; Yao *et al.*, 1989).

The EBV infects B lymphocytes via a 140-kDa glycoprotein receptor, designated CD21 or CR2, which also functions as receptor for the C3d complement component (for a review see Nemerow *et al.*, 1990). Binding to CR2 is mediated by the viral membrane glycoprotein gp350/220. Following entry into the host cell, the viral genome circularizes by fusion of the terminal repeats and is then maintained as a multicopy episomal plasmid (Kieff and Liebowitz, 1990). Replication of episomes is restricted, occurring in step with host cell proliferation to maintain stable genome copy numbers.

The B lymphocytes infected *in vitro* characteristically enter the cell cycle and continue to proliferate indefinitely (Pattengale *et al.*, 1973). Similarly, peripheral blood B lymphocytes from EBV carriers will spontaneously give rise to permanent clones of transformed cells, lymphoblastoid cell lines (LCLs), when placed in culture. This process of

immortalization is thought to be the primary biological activity of EBV underlying its pathogenetic role in lymphoproliferative lesions.

The EBV-transformed B lymphocytes are controlled *in vivo* by an HLA-restricted cytotoxic T cell response (Rickinson, 1986; R. J. Murray *et al.*, 1992). Impairment of this control is thought to account for the development of EBV-associated lymphomas in immunosuppressed individuals (Rickinson, 1992). The target antigens for virus-specific T cell surveillance are not well defined. In a recent study, R. J. Murray *et al.* (1992) showed that cytotoxic T cells in healthy virus carriers have multiple reactivities against different viral antigens. Even though target antigens varied according to the host HLA class I type, the dominant targets were provided by Epstein–Barr virus nuclear antigens (EBNAs) 3A, 3B, and 3C.

The EBV strains can be subdivided into two types, A and B (or 1 and 2), according to the organization of the genes encoding EBNAs 2, 3A, 3B, 3C (Dambaugh *et al.*, 1984; Adldinger *et al.*, 1985; Rowe *et al.*, 1989). These differ biologically, the transforming capacity of EBV type B being reduced compared with EBV type A (Rickinson *et al.*, 1987). Initially, it was thought that type B virus showed geographical restriction to equatorial Africa and New Guinea (Zimber *et al.*, 1986; Young *et al.*, 1987), but subsequently type B virus has been found in throat washings from both normal and HIV-seropositive individuals (Sixbey *et al.*, 1989).

B. Gene Expression during Latent Infection

Integration of EBV into the host cell genome has been described in *in vitro* infected BL cell lines (Hurley *et al.*, 1991a) but is rare following infection of resting B lymphocytes (Hurley *et al.*, 1991b). Thus, expression of episomal viral genes during latency appears to be sufficient for establishing lymphocyte transformation and immortalization. Much interest has centered, therefore, on defining the gene products expressed during latent infection in order to identify those responsible for the immortalizing properties of EBV.

Three distinct forms of EBV latent gene expression have been described *in vitro* in B cells, designated *latency I*, *latency II*, and *latency III* (Kerr *et al.*, 1992; Rowe *et al.*, 1992).

The best studied of these, latency III, is exemplified by latently infected LCLs (for a review see Kieff and Liebowitz, 1990), which constitutively express six nuclear antigens (EBNAs 1, 2, 3A, 3B, 3C, leader protein), three latent membrane proteins (LMPs 1, 2A, 2B), and two highly abundant ($\approx 10^5$–10^7 copies per cell), EBV-encoded, nonpolyadenylated, nontranslated, small RNAs (EBER-1 and EBER-2).

These cells express high levels of B cell activation and adhesion molecules. The EBNA mRNAs expressed in latency III are produced by alternative splicing of long primary transcripts initiated from either a *Bam*HI C (*Cp*) or a downstream *Bam*HI W (*Wp*) promoter (Speck and Strominger, 1989). The LCLs are largely nonpermissive for viral replication.

The EBV positive BL cell lines show two forms of latency on serial passage (Rowe *et al.*, 1987a). Some (group I lines) retain the phenotype of the original tumor (latency I) and express only EBERs 1 and 2 together with EBNA-1, EBNA mRNA transcription being driven by a *Bam*HI F (*Fp*) promoter (Sample *et al.*, 1991). Others (group III lines) progressively acquire a latency III LCL-like phenotype with broad expression of viral latent genes (including *Cp*/*Wp* driven EBNAs).

In addition to these well-characterized forms of latency, studies of EBV-associated malignancies have indicated that other latent infection patterns exist *in vivo* (Young *et al.*, 1988; Pallesen *et al.*, 1991a; Deacon *et al.*, 1993). Subsequently, a third latency form (latency II) was also identified *in vitro* in somatic cell hybrids (Kerr *et al.*, 1992) and in BL cells following surface immunoglobulin (Ig) ligation (Rowe *et al.*, 1992). Latency II is transcriptionally distinct, being characterized by expression of EBERs 1 and 2, LMPs 1, 2A, and 2B, and *Fp* driven EBNA-1 (in the absence of other EBNAs).

C. Function of Latent Gene Products

The EBNA-1 expression is consistently found in all types of EBV latency, suggesting that it is essential for the successful establishment of EBV latent infection. The EBNA-1 transactivates the EBV-latent origin of plasmid replication (*oriP*) (Yates *et al.*, 1985) and is vital for the stable maintenance of viral episomes. The functions of EBERs 1 and 2 are unknown but do not appear to be required for establishment of latent infection or B cell transformation (Swaminathan *et al.*, 1991). The transforming activity of EBV is associated with expression of EBNA-2 and LMP-1. The EBNA-2 antigen is the only EBV gene known to be required for immortalization of B cells. The EBV strains deleted for EBNA-2 are unable to transform lymphocytes. This defect can be overcome by either complementation or recombination of the nontransforming EBV with vectors encoding EBNA-2 (Cohen *et al.*, 1989; Hammerschmidt and Sugden, 1989). The EBV type A and type B viruses, which vary in the organization of their EBNA-2 genes, show differences in transforming efficiency (Rickinson *et al.*, 1987). Expression of EBNA-2 following gene transfer lowers the serum requirement for

cell growth in cultured rodent fibroblasts (Dambaugh *et al.*, 1986). The EBNA-2 antigen has been shown to induce the B cell activation antigen CD23 and to transactivate LMP-1 (Wang *et al.*, 1990), suggesting that EBNA-2 may act by regulating expression of cellular or viral genes.

The LMP-1 protein functions as an oncogene in rodent cell lines. Transfection and expression of LMP-1 in rodent fibroblasts results in reduced contact inhibition and serum dependency, anchorage-independent growth, and tumorigenicity in nude mice (Wang *et al.*, 1985, 1988; Baichwal and Sugden, 1988). In transfection studies, LMP-1 causes phenotypic changes in B cells including up-regulation of the *bcl*-2 cellular oncogene, with consequent protection of the cells from apoptotic death (Henderson *et al.*, 1991), and induction of CD23 and the cell adhesion molecules LFA-1, LFA-3, and ICAM-1 (Wang *et al.*, 1990).

The results of these *in vitro* studies implicate EBNA-2 and LMP-1 as central effector molecules in the pathogenesis of EBV-induced disease. Little is known about the function of the remaining latent gene products.

D. Gene Expression during Lytic Infection

Control of latency in EBV-infected cells is strict, although the mechanisms by which this is maintained are poorly understood. One key element in this system has been identified as the immediate early BZLF1 gene, which is down-regulated in the standard EBV genome. Expression of the BZLF1 gene product (BZLF1 protein or ZEBRA) is both necessary and sufficient for disrupting latency, apparently transactivating other viral genes and initiating the viral lytic cycle (for a review see Miller, 1990).

The subsequent viral replicative cycle is associated with expression of large numbers of early and late lytic genes with production of the early antigen (EA), viral capsid antigen (VCA), and membrane antigen (MA) systems (for a review see Kieff and Liebowitz, 1990; Miller, 1990).

III. Detection of EBV Markers in Tissues

A. Detection of Viral Antigens

The *in situ* demonstration of EBV-encoded antigens in lymphoid cells provides convincing evidence of viral infection. The EBNA-1 antigen is at first sight an ideal marker for such a purpose since expression of this antigen is consistently found in all known forms of EBV infection. Indeed, detection of EBNA-1 using the anti-complement immunofluores-

cence test with selected human sera is of proven value for demonstrating EBV infection in cells (Reedman and Klein, 1973). However, even though this test gives good results when applied to cytological preparations, it is of limited value when used on tissue sections as the sera frequently give nonspecific background staining. It has also proved difficult to raise monoclonal antibodies to EBNA-1 of sufficient specificity and sensitivity for use in standard immunocytochemical assays, although specific EBNA-1 staining has been reported in epithelium (Sandvej *et al.*, 1992).

Recently, monoclonal antibodies to EBNA-2 (PE2) and to LMP-1 (S12 and CS.1-4) have been described (Mann *et al.*, 1985; Rowe *et al.*, 1987b; Young *et al.*, 1989) and are well suited for use with standard immunohistological methods in order to demonstrate latent EBV infection of lymphoid cells *in situ* (Young *et al.*, 1989; Pallesen *et al.*, 1991a, 1992; Hamilton-Dutoit and Pallesen, 1992a). We have tested these monoclonal antibodies on both paraffin and frozen sections from a wide range of benign and malignant tissues. Cross-reactions are not seen with PE2. Monoclonal antibodies CS.1-4 show weak reactivity with smooth muscle cells and with a fraction of plasma cells, but this does not interfere with recognition of specific labeling. In contrast, the S12 antibody cross-reacts with an antigen regularly present in epithelial cells, thereby limiting its application. Both CS.1-4 and S12 generally give reliable staining in formalin-fixed paraffin-embedded sections. Sometimes PE2 can be used on paraffin sections, but this usually requires microwave treatment of the section.

Monoclonal antibodies to lytic cycle antigens (EA, VCA, MA) are available for immunohistological use, although most lymphomas contain few cells positive for these markers (Pallesen *et al.*, 1992; Hamilton-Dutoit and Pallesen, 1992a; Sandvej *et al.*, 1992). The BZLF1 gene expression can be detected immunohistologically using monoclonal antibody BZ.1 to the BZLF1 protein (Young *et al.*, 1991) allowing *in situ* detection of lytic cycle induction (Pallesen *et al.*, 1991b, 1992; Hamilton-Dutoit and Pallesen, 1992a). This is usually only possible in frozen sections, but BZ.1 may sometimes give staining in formalin-fixed paraffin-embedded sections, particularly following microwave treatment.

B. Detection of Viral Nucleic Acids

1. Filter-Based Hybridization

The EBV DNA and EBV RNA can be detected in purified tissue extracts by dot-blot and Southern blot analyses and by Northern blotting,

respectively, using standard procedures. In general, fresh or snap-frozen tissues are required for optimal results using these techniques. Southern blot analysis of the terminal regions of the EBV genome can be used to determine the size and number of episomal bands (and the possible presence of linear genomes), providing information on the clonality of the virus and whether a replicative or latent infection is present in a lesion (Raab-Traub and Flynn, 1986; Katz *et al.*, 1989).

2. Polymerase Chain Reaction Analysis

The polymerase chain reaction (PCR) can be used to detect EBV DNA in both fresh and paraffin-embedded archive tissue (Herbst *et al.*, 1990). However, depending on the amplification conditions chosen, a variable percentage of reactive lymph nodes will also give a positive signal for EBV indicating that the technique is sufficiently sensitive to detect EBV in latently infected nonneoplastic lymphocytes (Brocksmith *et al.*, 1991). Thus, a positive PCR signal in a lymphoma must be interpreted with care, since this does not prove that the virus is present in the tumor cell population.

Teramoto *et al.* (1992) have shown that this problem may be overcome by using PCR to detect EBV DNA in single cells, removed individually from tissue sections with a micromanipulator. These workers amplified EBV DNA from single Hodgkin and Reed–Sternberg (HRS) cells and nonneoplastic lymphocytes (in one case) picked out of HD tissue sections known to be EBV positive by traditional PCR. However, this technique is difficult and time consuming and is likely to give misleading results if insufficient numbers of cells are analyzed. Therefore, an *in situ* technique (see Sections III,A and III,B,3) is more appropriate for routinely identifying the cellular source of a positive EBV PCR signal.

By using the technique of reverse transcription PCR, it is possible to amplify EBV RNA transcripts, thus providing a sensitive method for the analysis of patterns of EBV gene expression in tissues (Brooks *et al.*, 1992; Deacon *et al.*, 1993). In theory, reverse transcription PCR could also be used to analyze EBV gene expression in individual virus-infected cells removed from lymphomas by micromanipulation. Although we are not aware of any reports of the use of such a technique, it has the potential for providing very valuable information not obtainable by other methods.

3. In Situ Hybridization

Hybridization techniques based upon analysis of tissue extracts provide little or no information concerning the cell type infected in an EBV-positive specimen. Identification of the cellular source of viral nucleic

acids requires, therefore, the use of *in situ* hybridization (ISH). The EBV DNA can be localized in paraffin sections using ISH with either isotopic- or nonisotopic-labeled probes (for a review see Hamilton-Dutoit and Pallesen, 1992b). Sensitivity is increased by using *Bam*HI W probes specific for the viral internal repeats. The sensitivity of isotopic paraffin section ISH for detecting EBV DNA in AIDS-related lymphomas is similar to that possible with Southern blot analysis of frozen tissue (Hamilton-Dutoit *et al.*, 1991b).

Recently, the sensitivity of paraffin section EBV ISH has been markedly improved. This has followed the realization that an *in situ* method for detection of EBER-1 and EBER-2 transcripts, originally described by Howe and Steitz (1986), could be adapted and applied to formalin-fixed, paraffin-embedded tissues (Wu *et al.*, 1990; Weiss *et al.*, 1991; Niedobitek *et al.*, 1992; Hamilton-Dutoit *et al.*, 1993a). The EBER transcripts are so abundant during latent infection that enough transcripts remain, even following paraffin embedding, for single-figure EBV genome copies to be detected in routine tissue sections using nonisotopic-labeled probes. The sensitivity of EBER ISH is such that single-virus-positive nonneoplastic lymphocytes can be detected in lymphoid tissue (Hamilton-Dutoit *et al.*, 1993a). This technique combines the advantages of ISH (good morphology and maintenance of tissue integrity) with a sensitivity comparable to that of PCR. It is now the method of choice for detection of latent EBV infection in tissue sections.

IV. EBV and T Cell Lymphoproliferations

A. EBV In T Cell Lines and Nonneoplastic T Cells

1. T Cell Lines

Early studies showed that EBV receptors were found on B cells, but apparently not on T cells or T cell lines (Jondal and Klein, 1973). One exception was the Molt 4 T cell leukemia line, which was initially found to carry complement receptors (Jondal and Klein, 1973) and later shown to express EBV receptors (Jondal *et al.*, 1976; Menezes *et al.*, 1977). For some years, the expression of EBV receptors on Molt 4 cells was regarded as an anomaly associated with the transformed phenotype of the leukemic cells. Indeed, although electron microscopy confirmed that Molt 4 cells could bind EBV, the virus did not appear to penetrate the cells, and no viral antigen production was detected by immunofluorescence (Menezes *et al.*, 1977). The EBV infection and antigen expression could be induced in Molt 4 cells by implanting them with EBV receptor

positive Raji cell membranes, suggesting that the EBV receptor in normal Molt 4 cells was not functional for viral penetration (Shapiro *et al.*, 1982). Binding of EBV to Molt 4 cells was shown to be uninhibited by incubation with the CR2/CD21 specific monoclonal antibody OKB7, providing further evidence that the EBV receptor on these cells differed from that found on B cells (Stocco *et al.*, 1988).

Several studies have identified additional T cell leukemia lines that appear to have EBV receptors. Tatsumi *et al.* (1985) screened cell lines for EBV binding using FITC-conjugated EBV and found this in two T cell lines, JM and HPB-ALL. Fingeroth *et al.* (1988) used the CR2/CD21 specific monoclonal antibodies HB5 and B2 to identify apparent EBV receptors on the T cell leukemia lines HPB-MLT (HPB-ALL), Jurkat 5 (JM), and MOLT 3 (a line from the same patient as MOLT 4). All lines were $CD1^+$, $CD4^+$, and $CD8^+$. These cells were not investigated for reactivity with OKB7, the monoclonal antibody most closely associated with EBV receptor function in B lymphocytes. Nor was it reported whether the virus was internalized after binding.

A T cell with apparently functional EBV receptors has been described by Koizuma *et al.* (1992). The MT-2 cell is an HTLV-1-positive T cell line, isolated from cord blood lymphocytes by co-cultivation with HTLV-1-positive leukemic cells. Koizuma *et al.* (1992) found that MT-2 cells expressed CR2/CD21 (as shown by reactivity with monoclonal antibody OKB7) at a density comparable to Raji cells. The cells were able to bind FITC-labeled EBV and became infected with the virus, expressing EBNA-1 and LMP-1 as shown by both immunofluorescence and Western blotting. Expression of EBNA-1 was blocked by preincubating the cells with the OKB7 antibody, indicating that the CR2/CD21 molecule was required for virus internalization.

A recent study has added to the complexity surrounding the nature of the T cell EBV receptor. Hedrick *et al.*, (1992) reported a T cell line (HSB-2) that bound biotinylated EBV but showed no reactivity with anti-CR2 monoclonal antibodies (HB5, OKB7, B2, and 6F7). The absence of CR2 on HSB-2 was confirmed by the lack of expression of CR2-specific mRNA, as shown by Northern blotting and PCR. The EBV binding in these cells was specific and could be blocked by preincubation of cells with nonlabeled virus, aggregated C3, and a monoclonal antibody (72A1) against gp350/220, the viral glycoprotein that mediates EBV binding to B cells. Interestingly, preincubation with the anti-gp350/220 monoclonal antibody 2L10, together with rabbit anti-mouse Ig, caused partial inhibition of EBV binding to HSB-2 cells but not to B cell controls. This suggests that additional epitopes of the gp350/220 glycoprotein may be involved in viral binding to the HSB-2 receptor compared

with those present on B cells (Hedrick *et al.,* 1992). Following binding of EBV to HSB-2 cells, the virus was internalized (as shown by electron microscopy), EBV DNA could be demonstrated in cultured cells by Southern blotting, and EBNA-1 transcripts could be detected by PCR. Although HSB-2 did not express CR2, Hedrick *et al.* (1992) detected transcripts with some homology (under low stringency conditions) to a CR2 cDNA probe, which covers the N-terminal half of CR2, including the EBV binding epitopes. The size of the HSB-2 message was distinct from that of the B cell CR2 message. Thus, HSB-2 cells appear to carry a functional EBV receptor, which is phenotypically distinct from CR2/CD21 on B cells.

In addition to the T cell lines described previously, an EBV positive natural killer (NK) cell line (phenotype: $CD2^-$, $CD3^-$, $CD4^-$, $CD8^-$, $CD56^+$, $HLA\text{-}DR^+$; genotype: germline T cell receptor (TCR) alpha/beta and gamma/delta chains) has recently been described (Yoneda *et al.,* 1992). The cells were reported to be negative for CR2/CD21 using HB5. Previous studies have also reported occasional cases of EBV infection in abnormal lymphoproliferations of large granular lymphocytes showing an NK phenotype and genotype (Kawa-Ha *et al.,* 1989; Hart *et al.,* 1992). Little is known concerning the route of viral infection in NK cells.

2. Nonneoplastic T Cells

Initially, EBV was not thought to be able to bind or infect benign T lymphocytes. The first study to suggest otherwise was published by Tsoukas and Lambris (1988). They analyzed the phenotype of thymocytes and found that a subpopulation of immature thymic T cells (predominantly $CD1^+$, $CD2^+$; "dull" for CD3) expressed CR2/CD21. Reactivity was most pronounced with monoclonal antibody HB5 but was also seen with OKB7 and B2. These antibodies were able to immunoprecipitate a protein from thymocyte lysates of similar size (145 kDa) to CR2 found on B cells. Subsequently, the same group confirmed the presence of functional EBV receptors on these immature thymocytes (Watry *et al.,* 1991). They showed specific binding of biotinylated EBV to the T cells, which could be inhibited by preincubation with unconjugated virus, aggregated C3, anti-CR2 monoclonal antibodies HB5 (together with anti-mouse Ig) and OKB7 (alone), and anti-gp350/220 monoclonal antibody 72A1. Following binding, the virus was internalized, and episomal EBV DNA could be demonstrated in cell cultures and the EBNA-1 protein, detected by Western blotting.

The EBV has also been shown to bind to normal peripheral blood T cells. Sauvageau *et al.* (1990) used a double-labeling technique with specific monoclonal antibodies and FITC-labeled EBV to demonstrate binding of

the virus to about 50% of peripheral blood suppressor/cytotoxic ($CD8^+$) T cells from normal donors. No significant binding was reported in the helper cell ($CD4^+$) subpopulation. Binding to $CD8^+$ cells appeared to be specific as it could be blocked by preincubation with anti-gp350/220 monoclonal antibody 72A1. However, no EBV antigen production could be detected by immunofluorescence suggesting that internalization of the virus did not occur. The receptor involved appeared to be present at a much lower density than the EBV receptor found on B cells. The $CD8^+$ cells that bound EBV did not react with anti-CR2 monoclonal antibody OKB7; the reactivity with other anti-CR2/CD21 monoclonal antibodies was not reported. Furthermore, viral binding could not be blocked using the OKB7 antibody, suggesting that the viral receptor on $CD8^+$ cells differed from that found on B cells (Sauvageau *et al.*, 1990).

In contrast, Fischer *et al.* (1991) have reported CR2/CD21 expression on approximately one-third of normal peripheral blood T cells (from both $CD4^+$ and $CD8^+$ subpopulations). The CR2/CD21 was detected at lower density than on B cells, using the monoclonal antibodies HB5, BL13, and BD6, which react with different CR2 epitopes. Reactivity of the cells with monoclonal antibody OKB7 was not reported, possibly accounting for the apparent discrepancy between this report and that of Sauvageau *et al.* (1990). Whether the CR2/CD21 molecule reported by Fischer *et al.* (1991) can act as a functional EBV receptor remains to be investigated.

In summary, there is accumulating evidence that EBV can bind to and sometimes infect both normal T cells and neoplastic T cell lines. In some cases, the receptor is clearly different from the CR2/CD21 EBV receptor on B cells. However, the published data concerning EBV infection of T cells are complex, and some of the data are contradictory. Much work is still required to characterize the EBV receptor(s) on T cells before their possible biological role can be established. Interestingly, however, transfection experiments have shown that introduction of EBV DNA into T cells results in their immortalization (Stevenson *et al.*, 1986), suggesting that if the virus can gain access to a T cell *in vivo* it may be able to cause cellular transformation.

B. Atypical T Cell Proliferation Associated with Acute EBV Infection

Yoneda *et al.* (1990) described a transient, benign proliferation of polyclonal peripheral blood T lymphocytes ($CD3^+$, $CD8^+$) occurring in a 21-year-old man, which were shown to contain EBV DNA and to

express EBNA-1. The patient was presented with an acute infectious mononucleosislike illness; serology suggested either primary EBV infection or a secondary alteration of normal seropositive EBV immunity.

The EBV DNA and EBNA expression has also been reported in peripheral blood T cells (phenotype: $CD2^+$, $CD3^+$, $CD4^+$, $CD8^-$) from a child with chronic EBV infection, T cell lymphoproliferation, and clinicopathological features of Kawasaki disease (Kikuta *et al.*, 1988). Whether EBV positive T cells are a feature of other cases of Kawasaki's disease has not been reported.

Several reports have documented fatal lymphoproliferative lesions associated with primary EBV infection, variously interpreted as virus-associated hemophagocytic syndrome or fatal infectious mononucleosis, in which clonal EBV positive T cell proliferations have been demonstrated. Craig *et al.* (1992) reported the case of a 20-month-old girl with serological evidence of recent EBV infection, who developed a rapidly progressive, fatal, widespread proliferation of T cells ($CD2^+$, $CD3^+$). Southern blotting revealed monoclonal TCR beta chain rearrangement, germline Ig genes, and evidence of EBV DNA with a single (clonal) band on terminal analysis. Gaillard *et al.* (1992) and Mori *et al.* (1992) have described similar cases of "fatal infectious mononucleosis" arising in a 16-year-old girl and a 2-year-old boy, respectively. Both patients developed disseminated lymphoproliferative disease following primary EBV infection in which the predominant cell population was composed of EBV DNA^+, $CD8^+$ T lymphocytes. Gaillard *et al.* (1992) found clonal rearrangements of the TCR beta and gamma chain genes in their case, whereas Mori *et al.* (1992) showed a clonal pattern on terminal analysis of the EBV genome in their patient's tumor.

The relationship between these cases and other examples of fatal infectious mononucleosis is unclear. However, relatively few cases of fatal infectious mononucleosis have been subjected to detailed immunophenotypical and genotypical examination, and it remains possible that clonal T cell proliferations may be more common in these lesions than is at present recognized.

Similarly, it is not clear whether these cases of T cell proliferation arising in association with primary EBV infection are related to the T cell lymphomas that have been described in patients with chronic EBV infection (Jones *et al.*, 1988; Ishihara *et al.*, 1989).

C. EBV and T Cell Lymphomas

The EBV was first shown to be present in the tumor cells of PTLs by Jones *et al.* (1988), who used Southern blotting and ISH to demonstrate

EBV DNA in three such cases. Subsequently, EBV genomes and gene products have been found in a substantial proportion of human post-thymic T cell malignancies and related lymphoproliferative disorders (Ohshima *et al.*, 1990; Su *et al.*, 1991; Hamilton-Dutoit and Pallesen, 1992a; Ott *et al.*, 1992; Anagnostopoulos *et al.*, 1992). EBV has not yet been reported in association with T lymphoblastic (thymic or bone marrow-derived lymphoblastic) lymphomas or leukemias (Su *et al.*, 1991; Ott *et al.*, 1992; Zhou *et al.*, 1993).

In view of the well-established B cell tropism of EBV, it is surprising that among patients without overt immunodeficiency the virus appears to be more frequently associated with NHLs of T cell rather than of B cell origin. This has been shown to apply in both Asian (Ohshima *et al.*, 1990; Zhou *et al.*, 1993) and Western populations (Hamilton-Dutoit and Pallesen, 1992a; Ott *et al.*, 1992).

In the following sections, we will summarize the current knowledge about the association of EBV with various distinct histological and/or clinical types of PTL, based on the current literature and our own recent data. It should be emphasized that morphological subtyping of PTLs is not an exact science. The interobserver reproducibility of modern T cell lymphoma classifications is poor (Hastrup *et al.*, 1991), suggesting that common categories of PTL, such as the angioimmunoblastic lymphadenopathy (AILD)-type and various pleomorphic T cell lymphomas, may be classified differently by different workers. The results of studies correlating EBV with morphological lymphoma types should be viewed in this light. Clearly, the problem of observer reproducibility also makes comparison of studies difficult and, possibly, of limited value. Problems of classification become even more apparent when dealing with lesions variously designated as lymphomatoid granulomatosis, lethal midline granuloma, polymorphic reticulosis, and angiocentric immunoproliferative lesion. The nature of these is uncertain; however, they most probably are composed of a spectrum of conditions ranging from abnormal lymphoproliferative reactions, to prelymphomatous lesions, to overt T cell malignancies of variable morphologic type (for a review see Chan *et al.*, 1987; Chott *et al.*, 1988).

A further obstacle to defining the association between EBV and PTL precisely is the problem of distinguishing certain types of pleomorphic T cell lymphomas containing HRS-like cells ("Hodgkin-like T cell lymphoma") and variant cases of HD (sometimes called "T cell Hodgkin's disease"). Since the nature of HRS cells remains poorly defined, the distinction of these "borderline" lesions is perhaps largely a conceptual or semantic problem. The HRS cells have usually been reported to lack

B and T cell-specific immune markers. However, in a proportion of histologically typical HD cases (for example of nodular sclerosis type, for which pathologists show a high degree of diagnostic consensus), a heterogeneous profile of B or T cell-associated immune markers has been reported (Angel *et al.*, 1987; Falini *et al.*, 1987; Casey *et al.*, 1989; Agnarsson and Kadin, 1989; Dallenbach and Stein, 1989; Drexler *et al.*, 1989; Schmid *et al.*, 1991; Zukerberg *et al.*, 1991).

The problems of differentiating PTL from HD persist at the genotypic level. Although a germline configuration of the TCR and Ig genes is usually found in HD, rearrangements of these various genes may also occur (see, e.g., Weiss *et al.*, 1986; Knowles *et al.*, 1986; Sundeen *et al.*, 1987; Griesser *et al.*, 1987; Drexler *et al.*, 1989; Knecht *et al.*, 1991).

The most likely explanation for this diversity in immunophenotype and genotype is, of course, that HD (as defined by morphological criteria) is not a uniform disease entity. Rather, it is a heterogeneous group of conditions that may also include genuine PTLs. Alternatively, HRS cells may be the result of fusions between B and T cells and/or antigen-presenting cells, thus explaining their highly variable phenotype.

Because neither immunophenotypic nor genotypic analysis provide reliable criteria for distinguishing between PTL and HD, we adhere to the classical definition of HD and its subtypes, based on strict morphological criteria (Lukes and Butler, 1966). In the differential diagnosis between HD and PTLs containing HRS-like cells, we have strongly emphasized the importance of the presence in the latter of a mixture of small, medium-sized, and large atypical lymphoid cells, which we presume to represent the neoplastic cell population. In HD, in which HRS cells comprise a minor cell population, the accompanying lymphocytes should not be "atypical." Unfortunately, this feature is defined by rather subjective criteria. Moreover, various degrees of reactive lymphocyte transformation and activation are to be expected in both HD and PTLs as a result of the neoplastic process itself or in response to other immunogenic factors, the morphology of both presumably partly reflecting the sum of cytokine production in the involved tissues. Thus, even if morphological distinction between typical and atypical lymphoid cells is possible, it is unlikely that these categories correspond directly to benign and malignant cell populations.

Further problems arise in the interpretation of tumor immunophenotype in frozen tissue sections because of the suboptimal morphology usually obtained. Thus, it may be difficult, if not impossible, to differentiate small and intermediate-sized neoplastic T cells from reactive T cells, especially if the latter are numerous. Therefore, our immunohistological

results are primarily based on assessment of the atypical, transformed (large) cells, which usually show recognizable nuclear abnormalities.

D. The Clinico-Pathological Spectrum of EBV-Associated Peripheral T Cell Lymphomas

1. Types of PTL Reported in Association with EBV

The EBV has been reported to be able to infect a range of immunophenotypically, and therefore probably also functionally, distinct subpopulations of neoplastic T cells including those with $CD4^+/CD8^-$, $CD4^-/CD8^+$, $CD4^-/CD8^-$, $CD3^{+/-}$, TCR-alpha/$beta^{+/-}$, TCR-gamma/$delta^{+/-}$, and various aberrant (Pallesen, 1988; Hastrup *et al.*, 1989) phenotypes (Jones *et al.*, 1988; Ishihara *et al.*, 1989; Bonagura *et al.*, 1990; Su *et al.*, 1990, 1991; Richel *et al.*, 1990; Miyashita *et al.*, 1991; Gaillard *et al.*, 1992; Anagnostopoulos *et al.*, 1992; Su and Hsieh, 1992; Ott *et al.*, 1992).

The EBV has also been demonstrated in cases of the highly activated, $CD30^+$ PTL with anaplastic large cell (ALC) lymphoma morphology (Herbst *et al.*, 1991a; Pallesen *et al.*, 1992; Ott *et al.*, 1992; Borisch *et al.*, 1992) (Tables III and V).

A rather poorly defined group of EBV-positive lesions, mostly arising in the upper aerodigestive tract and often showing pronounced angiocentricity, has recently been described, designated variously as sinonasal PTL, lethal midline granuloma, lymphomatoid granulomatosis, or angiocentric lymphoproliferative lesion. These lesions are more common in Asian patients (Chan *et al.*, 1987; Aozasa *et al.*, 1989), and their close association with EBV (in both Asian and Western cases) has only recently been recognized. Thus, Harabuchi *et al.* (1990) found EBV in the tumor cells of five sinonasal PTLs in Japanese patients. Katzenstein and Peiper (1990) demonstrated EBV in 21 out of 29 cases (72%) of lymphomatoid granulomatosis using paraffin section PCR. Ho *et al.* (1990) described the presence of EBV genomes in 9/9 sinonasal PTLs in Chinese patients from Hong Kong, and Gaulard *et al.* (1992) recently reported finding EBV in 7/7 European nasal PTL cases. Medeiros *et al.* (1992) demonstrated EBER-1 in lymphoma cells of 5/12 cases of angiocentric immunoproliferative lesions, most of which were localized to the lung, nasal region, or skin. These lymphomas show a range of phenotypes (frequently $CD2^+$, $CD3^{+/-}$, $CD4^{+/-}$, $CD7^+$, $CD8^{+/-}$, $CD56^+$) and genotypes (sometimes with TCR alpha/beta and gamma/delta in germline configuration), suggesting that at least some may be derived from NK-like cells (Chan *et al.*, 1987; Weiss *et al.*, 1988, 1992a; Chott *et al.*, 1988; Aozasa *et*

al., 1989; Harabuchi *et al.*, 1990; Ho *et al.*, 1990; Su *et al.*, 1991; Medeiros *et al.*, 1991; Gaulard *et al.*, 1992; Su and Hsieh, 1992).

A number of studies have found a strong association between EBV and AILD, and AILD-like lymphomas, the reported frequency ranging from 23 to 96% of cases (Ohshima *et al.*, 1990; Knecht *et al.*, 1990; Su *et al.*, 1991; Anagnostopoulos *et al.*, 1992; Ott *et al.*, 1992; Weiss *et al.*, 1992b). These cases will be discussed in more detail later (see Section IV,F,2).

The EBV was not detected in either of the two cases of HML-1$^+$ mucosa-associated T cell lymphoma of the gastrointestinal tract so far reported (Ott *et al.*, 1992) (Table I).

2. Clonality of Episomal EBV in PTL

In most of the EBV-positive cases described previously in which terminal analysis of the viral genome has been reported, monoclonal or, less frequently, oligoclonal EBV episomes were found, indicating that in most cases infection occurred before clonal expansion of the tumor cells (Jones *et al.*, 1988; Kawa-Ha *et al.*, 1989; Ishihara *et al.*, 1989; Ohshima *et al.*, 1990; Ho *et al.*, 1990; Bonagura *et al.*, 1990; Su *et al.*, 1991; Miyashita *et al.*, 1991; Hart *et al.*, 1992; Medeiros *et al.*, 1992; Ott *et al.*, 1992). This finding suggests a pathogenetic role for the virus in the development of at least some PTLs.

3. EBV-Positive PTL in Immunodeficiency States

By analogy with EBV-associated high-grade B cell NHLs, it might be speculated that an underlying immune defect predisposes to the development of EBV-positive PTL. Indeed, a case of EBV-positive T-ALC has been reported in an HIV-seropositive individual (Fig. 1a) (Pallesen *et al.*, 1992). However, PTLs are in general rare in both AIDS patients and in organ transplant recipients. Thus, if immunodeficiency is involved in the development of EBV-positive PTL, it is likely to be in the form of a rather specific defect of EBV immunity. Circumstantial evidence of such as yet undefined immune defects comes from reports of EBV positive T cell proliferations developing in patients with a history of abnormal or chronic EBV infection (Jones *et al.*, 1988; Kikuta *et al.*, 1988; Ishihara *et al.*, 1989; Bonagura *et al.*, 1990; Richel *et al.*, 1990; Su and Hsieh, 1992). A specific defect of EBV immunity was demonstrated in the case reported by Bonagura *et al.* (1990) and was thought to have been predisposed to the development of the patient's EBV-positive PTL.

The AILD was originally defined as a nonneoplastic lesion seen in

patients with severe immune dysfunction. More recently, it has been recognized that a large proportion of such lesions already show evidence of lymphoma (of either T or B cell type) at diagnosis or go on to develop malignancy with time. The strong association of AILD and AILD-like lymphoma with EBV raises the possibility that the virus might play a direct, initiating role in the development of these lesions. Alternatively, the presence of EBV in AILD might be a marker of viral reactivation in the setting of immune dysfunction, rather than a primary pathogenetic event (Weiss *et al.*, 1992b). This could explain the particular tendency for the virus to infect only a minor population of cells in most such lesions. This will be discussed in more detail later.

4. EBV-Associated PTL in Relation to HTLV-1 Infection

In most cases of EBV-positive T cell proliferations studied for the presence of HTLV-1, no evidence of dual viral infection has been found (Jones *et al.*, 1988; Kikuta *et al.*, 1988; Kawa-Ha *et al.*, 1989; Ishihara *et al.*, 1989; Su *et al.*, 1990, 1991; Bonagura *et al.*, 1990; Borisch *et al.*, 1992). In the survey performed by Su *et al.* (1991), five HTLV-1-positive PTLs from Taiwan were EBV negative. However, Ohshima *et al.* (1990) demonstrated simultaneous EBV and HTLV-1 infection by electron microscopy in tumor cells in 1 of 17 cases of Japanese adult T cell lymphomas that contained HTLV-1 proviral DNA.

5. EBV Receptors in PTLs

Expression of the B cell EBV receptor (CD21 antigen) has been demonstrated in T cells in some cases of PTL, for example in two of the three lymphomas described by Jones *et al.* (1988) and in the case reported by Bonagura *et al.* (1990). However, in general, CD21 antigen is absent (at least in detectable amounts) from lymphoma cells in EBV-positive PTL (Kawa-Ha *et al.*, 1989; Su *et al.*, 1990, 1991; Herbst *et al.*, 1991a; Hart *et al.*, 1992; Gaillard *et al.*, 1992; Medeiros *et al.*, 1992; Ott *et al.*, 1992). It is, therefore, not clear how EBV enters the tumor cells in PTLs. One possibility is that virus infection occurs in a CD21-positive precursor cell after which receptor expression is down-regulated. Alternatively, other routes of cell entry may exist, for example, via a different EBV receptor such as those described in T cell lines *in vitro* (see Section IV,A). It should be noted that the general lack of CD21 expression on EBV-infected lymphoma cells in PTL is reminiscent of the situation found *in vivo* in other EBV-associated lesions. For example, in both EBV-positive HD (Uccini *et al.*, 1990; Section V,A,5) and high-grade B cell AIDS-related lymphoma (Hamilton-Dutoit *et al.*, 1993b), CD21 antigen expression is lacking in the majority of tumors. Thus, EBV-infected tumor cells in these (and

indeed in EBV-associated epithelial lesions) may be frequently CD21 negative, suggesting that care should be taken in drawing conclusions concerning the route of viral infection on the basis of cellular expression of this antigen.

6. EBV Types A and B in PTLs

Little information is available concerning the type of EBV-infecting tumor cells in PTLs. Miyashita *et al.* (1991) described the first case of EBV-type B-associated PTL in a survey comprising nine EBV-associated malignant lymphomas. The remaining eight tumors contained EBV type A, but no information about the lymphoma phenotype was provided for these cases. Hennig *et al.* (1992) reported both EBV types A and B in Western nasal PTL of midline granuloma type. This group has now studied six such lymphomas, finding EBV type A in three cases and EBV type B in the other three cases (B. Borisch, personal communication). It is not clear which factors determine the EBV type associated with different tumor entities. Type A virus predominates in European sporadic HD and in Chinese nasopharyngeal carcinoma (Gledhill *et al.*, 1991; Chen *et al.*, 1992). A relatively increased frequency of type B virus is found in immunodeficiency-associated lymphomas developing in HIV-infected individuals (Boyle *et al.*, 1991). This and other published evidence suggests that immunosuppression may favor selection of EBV type B.

E. EBV in Peripheral T Cell Lymphomas in Asian Populations

1. The Frequency of PTLs in Asians and Caucasians

Comparative studies have shown marked differences in the age-adjusted incidences of the major histological types of malignant lymphomas in Japan and the United States. The overall incidence rate of malignant lymphoma in Japan is less than one-half of that in the United States (Kadin *et al.*, 1983). In particular, follicular lymphomas and HD are relatively uncommon in Asians, whereas diffuse NHLs have an almost equal incidence in the two regions. Similar incidence rates are found for extranodal lymphomas, whereas nodal lymphomas are less frequent in Asians.

Many immunological lymphoma surveys have shown that PTLs are proportionately more common in Asian than in Caucasian populations. For example, T cell lymphoma constituted: 18–70% of NHLs in Japan (Kadin *et al.*, 1983; Mitsui *et al.*, 1983; Tajima *et al.*, 1990); 23% of NHLs in Singapore, of which only two cases (7%) were HTLV-1 positive (Sng *et*

al., 1992); 29% of NHLs in Hong Kong (Ng *et al.*, 1986); 39% of NHLs in Taiwan, of which 10 cases (19%) were HTLV-1 positive (Su *et al.*, 1985, 1988); and at least 18% of NHLs in mainland China (Xu *et al.*, 1984). The latter study may not, however, be truly representative since nearly one-third of cases were immunologically unclassifiable.

In contrast, corresponding figures from Western countries reveal a relative incidence of PTLs of about 10–20% of all NHLs (see, e.g., Habeshaw *et al.*, 1979; Gajl-Peczalska *et al.*, 1979; Kadin *et al.*, 1983; Pallesen *et al.*, 1983; Tubbs *et al.*, 1981; Weiss *et al.*, 1985; Lennert and Feller, 1992). However, although PTLs are proportionately more common in Asians, the absolute age-adjusted incidences of PTLs in Chinese and in Japanese individuals resident outside HTLV-1 endemic areas are similar to, or at most only slightly greater than, those in Caucasians (Kadin *et al.*, 1983; Ng *et al.*, 1986). Sinonasal PTLs of lethal midline granuloma type appear to be relatively common in China and Japan as compared with Western countries (Chan *et al.*, 1987; Aozasa *et al.*, 1989).

2. EBV-Associated PTL in Asian Populations

It is generally held that EBV-associated PTL is more common among Oriental than Western patients. This belief was borne of the early reports from groups in Japan, Hong Kong, and Taiwan of EBV in PTLs in Asian patients, as documented in case reports (Ishihara *et al.*, 1989; Su *et al.*, 1990; Miyashita *et al.*, 1991), small series (Kawa-Ha *et al.*, 1989; Harabuchi *et al.*, 1990; Ho *et al.*, 1990), and larger surveys (Ohshima *et al.*, 1990; Su *et al.*, 1991). The studies performed by Ohshima *et al.* (1990) and by Su *et al.* (1991) used both Southern blotting and DNA ISH to detect EBV in 11% of Japanese and 20% of Taiwanese T cell lymphomas, respectively.

3. EBV in PTL in Chinese Patients

There is no published data concerning the association of EBV with PTLs occurring in mainland Chinese patients. The high sensitivity of EBER ISH and its applicability to routine paraffin-embedded tissue sections have allowed us to study such cases in detail for their association with EBV (Zhou *et al.*, 1993). In particular, we have correlated the presence of EBV and the number of EBER-positive lymphoma cells in individual cases of Chinese PTL with their morphology and anatomic site. These results have been compared with those from a large series of Western (Danish) PTLs.

The Chinese lymphomas were drawn from the archives of several hospitals in Beijing. Neither frozen tissue nor EBV serological data were available for these patients. The cases were classified morphologically

according to the up-dated Kiel classification (Stansfeld *et al.*, 1988), with due regard to the limitations concerning the reproducibility of PTL classification noted previously (Hastrup *et al.*, 1991). Immune phenotyping was performed with B and T cell-specific (L26/CD20, polyclonal anti-CD3) and -associated (LN1/CDw75, Ki-B5, MT-1/CD43, UCHL1/CD45R0) antibodies that can be applied to paraffin sections using standard immunocytochemical procedures. The sensitivity of the reactions was often enhanced following microwave treatment of the paraffin sections. The EBV was shown by EBER-1 ISH and by immunodetection of LMP-1 using the CS.1-4 monoclonal antibodies.

Using these methods, we identified three T lymphoblastic lymphomas and 39 PTLs (including two cases of T-ALC). The former were negative for EBV products as were the two cases of T-ALC. The results of the remaining 37 PTLs are given in Table I.

In Chinese PTL, EBV is more frequently associated with extranodal (12/14; 86%) than with nodal (12/23; 52%) lymphoma (Table I) (Fisher's exact test, two tailed: $2p = 0.08$). The largest group of extranodal PTLs were from the upper aerodigestive tract (midline granuloma type), and these were strongly associated with EBV (Table I). Two of the remaining four EBV-positive extranodal Chinese PTLs showed angiocentricity and angioinvasion. In nodal Chinese PTLs, EBV was found in lymphomas of AILD, Lennert's, and pleomorphic, medium and large cell types (Table II).

The LMP-1-positive tumor cells were always much less frequent than

TABLE I

EXPRESSION OF EBER-1 IN DANISH AND CHINESE PERIPHERAL T CELL LYMPHOMAS (EXCLUDING ANAPLASTIC LARGE CELL LYMPHOMAS) IN RELATION TO SITE

Site	Number of EBER-1 positive cases (%)		
	Danish	Chinese	
Lymph node	16/50 (32)	12/23 (52)	NS ($2p > 0.3$)[a]
Extranodal, total	2/17 (12)	12/14 (86)	
Upper aerodigestive tract	1/2[b]	8/9 (89)	
Skin	1/13 (8)	1/2	
Other sites	0/2[c]	3/3[c]	
Total	18/67 (27)	24/37 (65)	

[a] χ^2 test (two tailed).

[b] Both cases localized in tonsils.

[c] Other sites composed of spleen (TCR-gamma/delta and CD56 positive phenotype) and gastric mucosa (HML-1 positive) in the two Danish cases, and caecum, the paratesticular region, and an undetermined site in the three Chinese cases.

NS, not significant.

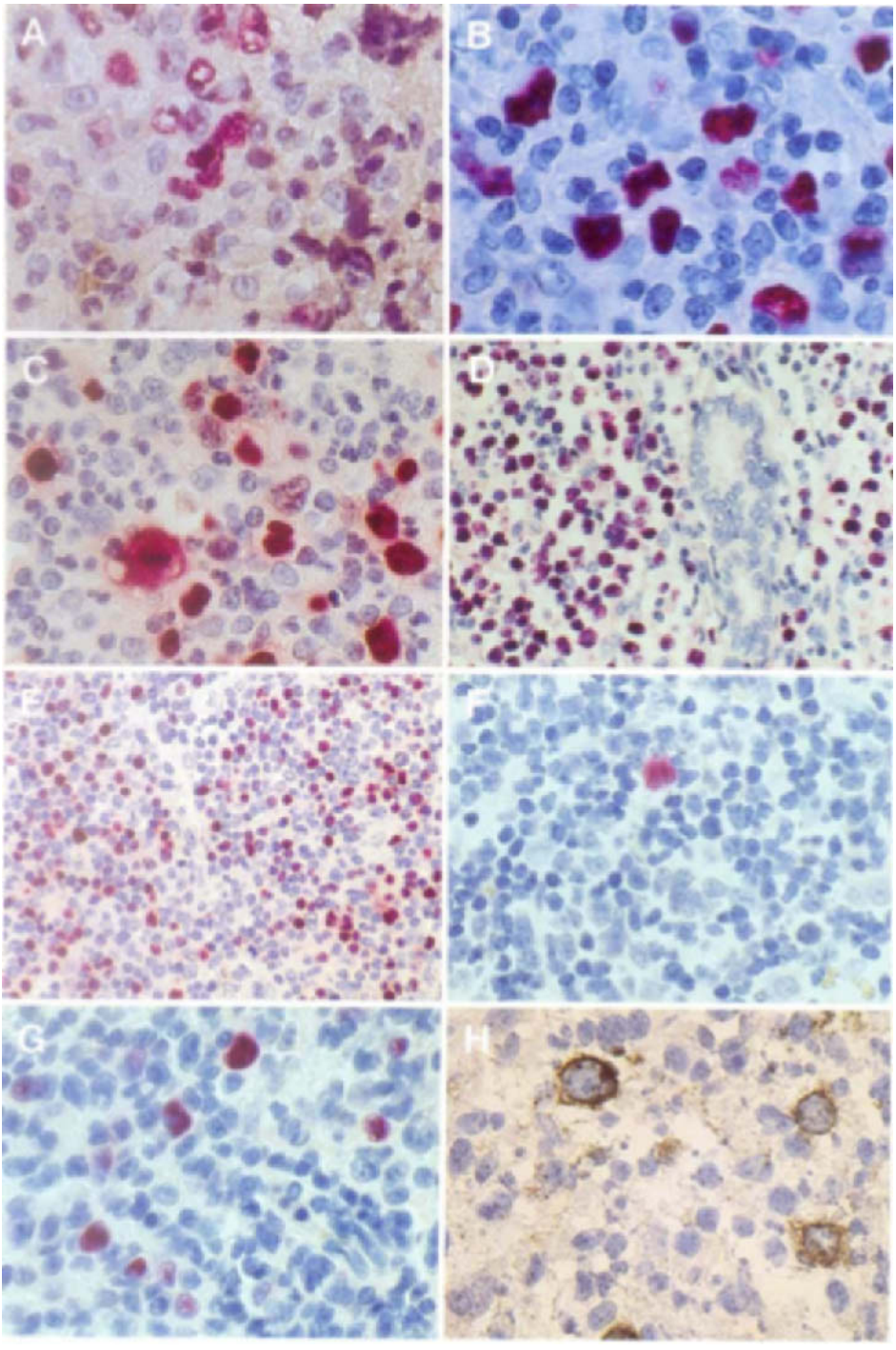
TABLE II
EXPRESSION OF EBER-1 AND LMP-1 IN CHINESE NODAL PERIPHERAL T CELL LYMPHOMAS IN RELATION TO HISTOLOGIC TYPE

Lymphoma type	EBER-1[a] (%)	LMP-1[a] (%)
AILD	4/6 (67)	3/6 (50)
Lennert's	2/2	2/2
T-zone	0/2	0/2
Pleomorphic, medium/large	6/12 (50)	4/11 (36)
ALC	0/1	0/1
Total	12/23 (52)	9/22 (41)

[a] Number of positive cases in relation to all cases studied. All analyses were performed on formalin-fixed, paraffin-embedded material.

AILD, angioimmunoblastic lymphadenopathy-like T cell lymphoma; ALC, anaplastic large cell lymphoma.

FIG. 1. The Epstein–Barr virus in peripheral T cell lymphoma. (a) EBER-1 expression in nodal anaplastic large cell lymphoma of T cell type from a HIV-seropositive individual. Some, but not all, of the bizarre tumor cells show hybridization signals in their nuclei. ×120. (b) Partial expression of EBER-1 in the tumor cells of a Chinese node-based PTL. Large cells are labeled. In contrast, most of the predominant intermediate-sized cells are EBER-1 negative, although these also belong to the neoplastic cell population by morphological criteria. A few small EBV-negative lymphocytes and a granulocyte are shown, enabling a comparison of cell sizes. ×188. (c) Expression of EBER-1 in a fraction of lymphoma cells only, in a nodal PTL. EBV RNA is predominantly found in the large tumor cells. A cell in mitosis shows EBER-1 localized to the cytoplasm. ×120. (d) EBER-1 in a Chinese PTL of the midline granuloma-type from the upper aerodigestive tract (oral mucosa). EBV RNA is expressed in the majority (perhaps all) of the lymphoma cells, whereas the glandular epithelial cells are negative. ×98. (e) Partial expression of EBER-1 in an AILD-type lesion of a lymph node. It is not possible by morphological criteria to distinguish between neoplastic and non-neoplastic positive lymphocytes. Many of the positive cells show transformation but may nevertheless belong to a non-neoplastic cell population. ×75. (f) EBER-1 expression in a lymph node removed in 1986 from a 69-year-old male with a pleomorphic T cell lymphoma. Only very rare lymphoma cells are labeled. ×120. (g) A second biopsy from the case shown in Fig. 1f removed in 1991 (5 years later) following lymph node relapse. In this node, EBER-1 is focally expressed in up to 15% of the lymphoma cells, and the histological features have progressed to a more aggressive (large cell) pattern (case no. 9 in Table V). ×120. (a–g) Paraffin section *in situ* hybridization, digoxigenins labeled EBER-1 antisense riboprobes. (h) Same case as in Fig. 1g showing LMP-1 immunohistological staining of a frozen section with monoclonal antibodies CS.1-4. LMP-1 is strongly expressed, predominantly in the cytoplasm of the large lymphoma cells. Immunoperoxidase, ×120.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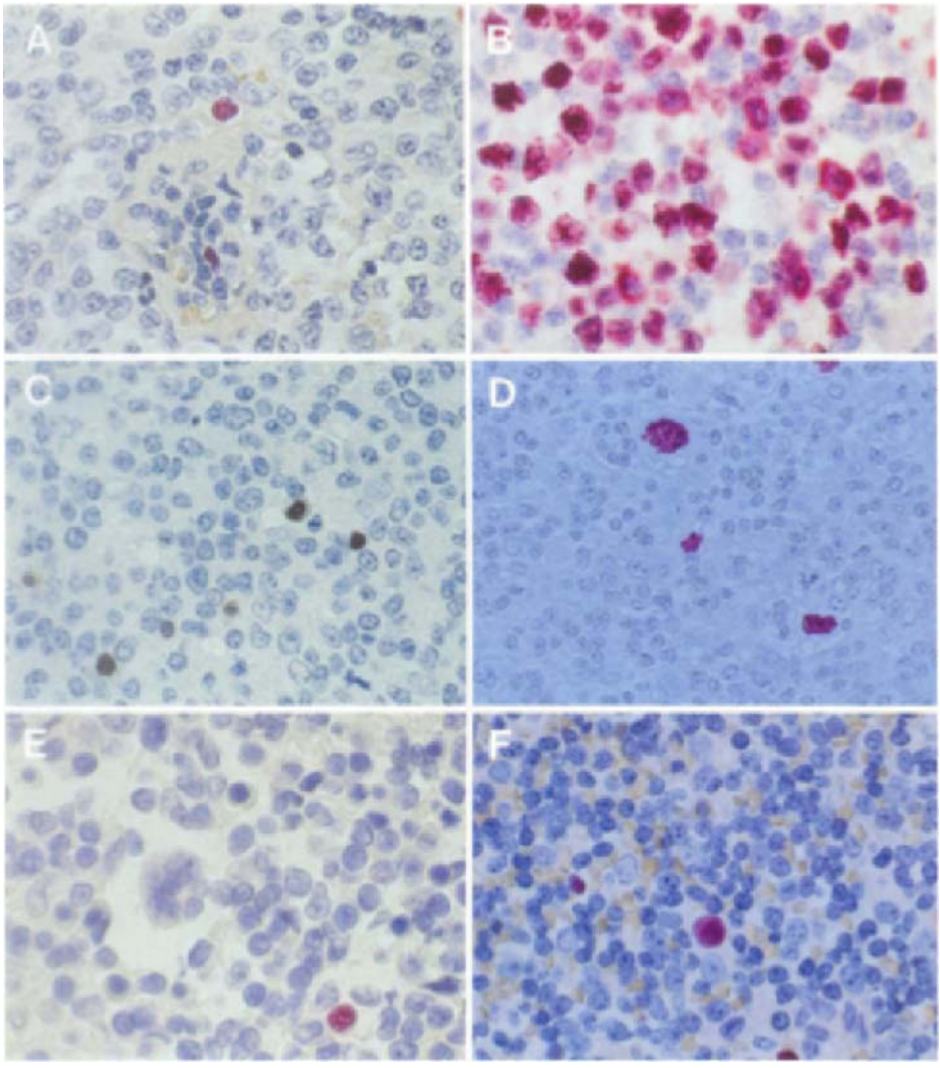
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EBER-positive cells. We found a considerable variation in the number of EBV-infected cells between individual cases of nodal PTL. The EBER-1-positive cells ranged from less than 5 to 70% of the total cell population in nodal PTL (Fig. 1b,c); however, nine of the 12 cases contained less than 10% EBER-positive cells.

In contrast, 9 of 11 extranodal EBV-positive cases (82%) showed EBER-1 expression in more than 50% of the cells, and in 6 of these almost all tumor cells were positive. In particular, the PTLs of the upper aerodigestive tract (midline granuloma-type) showed nearly 100% EBER expression in the lymphoma cells (Fig. 1d).

In some of the PTLs exhibiting partial EBER expression, both EBER-positive and -negative cells could be seen within the morphologically recognized tumor cell population (Fig. 1b,c). In other cases, in particular of AILD-type, it was difficult or impossible to distinguish morphologically between tumor and nonneoplastic T cells. Thus, in these lymphomas, EBER-positive cells might represent either a minor fraction of the tumor cells or reactive nonneoplastic lymphocytes or a combination of both (Fig. 1e). A similar observation that only a proportion of the tumor cells in EBV-positive PTLs contained viral DNA was made by Ohshima *et al.* (1990) using DNA ISH. The possible significance of a partial tumor cell infection with EBV will be discussed in more detail later (see Section IV,1,2).

FIG. 2. The Epstein–Barr virus in peripheral T cell lymphoma and Hodgkin's disease. (a) EBER-1 expression in a lymph node removed in 1983 from a 58-year-old man with AILD. Scattered positive cells (<0.1%) are seen, some of which show transformation. Immunohistological examination revealed considerable activation of both B and T cell compartments. ×120. (b) Second lymph node biopsy from the patient illustrated in Fig. 2a, removed in 1992 following nodal relapse (after 9 years). EBER-1 ISH of the second biopsy shows the majority of the blastic lymphoma cells to be positive. Histology was of typical B immunoblastic lymphoma with plasmablastic differentiation. ×120. (c) Paraffin section of a lymph node with AILD (same case as in Fig. 1e) stained with monoclonal antibody BZ.1 to the BZLF1 immediate early antigen following microwave pretreatment of the section. A fraction of cells show strong nuclear and sometimes weak cytoplasmic labeling which indicate a cellular switch form a latent to a replicative virus phase. Immunoperoxidase staining. ×120. (d) EBER-1 expression in a lymph node from a case of HD, mixed cellularity. All recognizable HRS cells show a positive nuclear signal. ×98. (e) EBER-1 expression in a lymph node from a case of HD, nodular sclerosis. HRS cells are uniformly negative, but occasional positive small lymphocytes may be found. ×150. (f) EBER-1 expression in a lymph node from a case of HD, nodular sclerosis. HRS cells are negative (not shown), whereas accompanying positive lymphocytes are increased in number and show transformation. The three positive cells are composed of a small lymphocyte, an immunoblast, and an intermediate-sized lymphoid cell. ×150. (a–b) and (d–f) Paraffin section ISH, digoxigenin-labeled EBER-1 antisense riboprobes. (c) Immunoperoxidase staining.

Thus, applying EBER ISH to Chinese PTLs, we found a much higher percentage of EBV-associated cases than has been reported in previous surveys of lymphomas from Asian patients (Ohshima *et al.*, 1990; Su *et al.*, 1991). Moreover, our study confirms the previously reported strong association between EBV and PTL of the upper aerodigestive tract, in particular affecting the nasal region. This association is as strong as that reported between EBV and nasopharyngeal carcinoma, EBV and endemic BL, and (more recently) EBV and AIDS-related B immunoblast-rich primary central nervous system lymphoma (MacMahon *et al.*, 1991; Hamilton-Dutoit *et al.*, 1993a).

F. EBV in Peripheral T Cell Lymphomas in Western Populations

1. Association of EBV with Danish PTLs

In a previous survey performed on frozen tissues from 201 sporadic NHLs, we identified EBV gene products in 8/82 cases of PTL (10%). In contrast, only 4% of high-grade B cell NHLs were EBV associated (Hamilton-Dutoit and Pallesen, 1992a). In this study, EBV was first identified by demonstrating EBV gene products (LMP-1, EBNA-2) *in situ* using immunohistochemistry on cryostat sections, the presence of EBV DNA being confirmed subsequently using paraffin section ISH.

Because some EBV-positive lymphomas may not express either LMP-1 or EBNA-2, the results of this study may have underestimated the true number of EBV-associated PTLs. Therefore, we have extended our studies on Danish PTLs using EBER ISH.

The PTLs were selected for this study from the archives of our laboratory according to the availability of frozen tissue. Immune phenotyping was performed using large panels of monoclonal antibodies on cryostat sections according to previously published principles (Pallesen, 1988; Hastrup *et al.*, 1989). In many cases, the diagnosis of T cell neoplasia was substantiated by the demonstration of clonal TCR-beta gene rearrangements (O'Connor *et al.*, 1985) or was supported by the demonstration of aberrant tumor cell phenotypes (Weiss *et al.*, 1985; Hastrup *et al.*, 1989). In addition to EBERs, EBV gene products (LMP-1, EBNA-2, BZLF1 protein, and the lytic cycle antigens) were demonstrated by immunocytochemistry using monoclonal antibodies as previously described in detail (Pallesen *et al.*, 1991a, 1992; Hamilton-Dutoit and Pallesen, 1992a; Sandvej *et al.*, 1992).

The results of our revised survey of Danish PTL for the presence of EBV are given in Table I (related to anatomical site), in Table III (related to the histological type of nodal PTLs), and in Table IV (related to the

TABLE III
EXPRESSION OF EBER-1, LMP-1, AND EBNA-2 IN DANISH NODAL PERIPHERAL T CELL LYMPHOMAS IN RELATION TO LYMPHOMA TYPE[a]

Lymphoma type	EBER-1[b] (%)	LMP-1[b] (%)	EBNA-2[b] (%)	BZLF1[b] (%)
AILD	5/13 (38)	4/13 (31)	0/13	1/13
T-zone	0/2	0/2	0/2	0/2
Pleomorphic, small	0/1	0/1	0/1	0/1
Pleomorphic, medium/large	11/30 (37)	11/30 (37)	2/30	6/30
IB	0/2	0/2	0/2	0/2
ALC	1/12	1/12	0/12	0/12
Unclassifiable	0/1	0/1	0/1	0/1
Total	17/61 (28)	15/61 (25)	2/61 (3)	7/61 (11)

[a] Updated Kiel classification.

[b] Number of positive cases in relation to all cases studied. EBER-1 *in situ* hybridization performed on paraffin sections. LMP-1, EBNA-2, and BZLF1 expression shown by immunohistochemistry using CS.1-4, PE2, and BZ.1 monoclonal antibodies, respectively, on frozen sections.

AILD, angioimmunoblastic lymphadenopathy-like T cell lymphoma; ALC, anaplastic large cell lymphoma; IB, immunoblastic lymphoma.

TABLE IV
EXPRESSION OF EBER-1, LMP-1, AND EBNA-2 IN DANISH EXTRANODAL PERIPHERAL T CELL LYMPHOMAS IN RELATION TO LYMPHOMA TYPE AND SITE

Lymphoma type	Site	EBER-1[a] (%)	LMP-1[a] (%)	EBNA-2[a] (%)
Pleomorphic, small	Skin	0/2	0/2	0/2
Pleomorphic medium/large	Skin	1/9	0/9	0/9
Pleomorphic medium/large	Tonsil	1/2	0/2	0/2
Pleomorphic medium/large	Stomach	0/1[b]	0/1	0/1
ALC	Skin	0/6	0/6	0/6
ALC	Stomach	0/1	0/1	0/1
ALC	Small intestine	0/1[b]	0/1	0/1
ALC	Axilla	0/1	0/1	0/1
Unclassified	Spleen	0/1[c]	0/1	0/1
Total		2/24[d]	0/24	0/24

[a] Number of positive cases in relation to all cases studied. EBER-1 *in situ* hybridization performed on paraffin sections. LMP-1 and EBNA-2 expression shown by immunohistochemistry using CS.1-4 and PE2 monoclonal antibodies on frozen sections.

[b] HML-1 positive.

[c] TCR-gamma/delta and CD56 positive phenotype.

[d] The two EBER-1 positive cases were BZLF1 negative.

ALC, anaplastic large cell lymphoma (T cell type).

histological type of extranodal PTLs). Details of EBER-1 expression (number of infected cells present in individual positive cases) in relation to histology and site are presented in Table V.

The EBV, as defined by EBER-1 expression in lymphoma cells, was found in 19/85 Danish PTLs (22%), including 17/61 nodal (28%) and 2/24 extranodal (8%) cases (Table III and IV). Unexpectedly, T-ALC (nodal as well as extranodal) was only rarely associated with EBV since only 1 (nodal) of 21 cases (5%) contained EBV. Thus, if T-ALC cases are excluded, about one-third of Danish nodal PTLs are EBV associated.

Comparing Danish and Chinese PTLs (excluding T-ALC), there was a tendency for EBV to be more frequently associated with Chinese (52%) than with Danish (32%) nodal PTLs, although this difference was not significant (Table I).

Extranodal lymphoma site was frequently associated with EBV in Chinese PTLs (12/14 cases; 86%) but was only rarely associated with EBV in Danish PTLs (2/17 cases; 12%). However, this difference was

TABLE V

DEGREE OF EBER-1 EXPRESSION IN EBV POSITIVE CASES OF DANISH PERIPHERAL T CELL LYMPHOMA IN RELATION TO MORPHOLOGY AND SITE

Case no.	Lymphoma type	Site	Percentage of EBER-1 positive cells
1	AILD	LN	1–5
2	AILD	LN	10
3	AILD	LN	15
4	AILD	LN	25
5	AILD	LN	80
6	Pleomorphic, medium/large	LN	1
7	Pleomorphic, medium/large	LN	1–5
8	Pleomorphic, medium/large	Tonsil	10–20
9	Pleomorphic, medium/large	LN	10–20
10	Pleomorphic, medium/large	LN	30
11	Pleomorphic, medium/large	LN	30–40
12	Pleomorphic, medium/large	LN	40
13	Pleomorphic, medium/large	LN	50
14	Pleomorphic, medium/large	LN	50
15	Pleomorphic, medium/large	LN	70
16	Pleomorphic, medium/large	LN	70
17	Pleomorphic, medium/large	LN	90
18	Pleomorphic, medium/large	Skin[a]	100
19	ALC	LN	30

[a] Angiocentricity or angioinvasion not prominent.

AILD, angioimmunoblastic lymphadenopathy-like T-cell lymphoma; ALC, anaplastic large cell type; LN, lymph node.

attributable to the high number of upper aerodigestive (in particular sinonasal) PTLs (which show a strong association with EBV) in the Chinese material, and a high number of cutaneous PTLs (that were rarely associated with EBV) among the Danish extranodal lymphomas, making comparison of the two series impossible.

Our finding that EBV is associated with certain T cell lymphomas such as AILD, pleomorphic medium and large cell type, and Lennert's lymphoma is in accordance with previous studies (Bornkamm *et al.*, 1976; White *et al.*, 1989; Ohshima *et al.*, 1990; Knecht *et al.*, 1990; Su *et al.*, 1991; Hamilton-Dutoit and Pallesen, 1992a; Ott *et al.*, 1992; Anagnostopoulos *et al.*, 1992).

2. *Comparison of the Danish Survey with Other Studies on EBV in PTL*

Our figures concerning the relative frequency of EBV-associated nodal Danish and Chinese T cell lymphomas of different histologic types are very similar to the results reported by Ott *et al.* (1992) for German PTLs using different methods of EBV nucleic acid detection.

One exception is the surprisingly rare occurrence of EBV in T-ALC (CD30 antigen positive) in our material, only 1 of 21 cases being EBV positive (not including the case illustrated in Fig. 1a). A much higher infection rate of T-ALC lymphoma cells might be expected (Hamilton-Dutoit and Pallesen, 1992a), particularly in view of the similarities between this cell type and HRS cells (in which EBV infection is frequent). In other studies, however, EBV has been detected more frequently in T-ALC. Thus, Ott *et al.* (1992) found 3/8 T-ALC cases positive for EBV with Southern blotting and DNA ISH. Herbst *et al.* (1991a) reported 5/18 cases (28%) of T-ALC to be EBV positive as defined by PCR. Virus infection of tumor cells was confirmed in two of these five cases by LMP-1 immunohistology and in at least three of the cases by EBER ISH. Pallesen *et al.* (1992) reported one LMP-1-positive case of T-ALC in a series of AIDS-related lymphomas (Fig. 1a, case not included in the present series), and Borisch *et al.* (1992) described one case of recurring cutaneous T-ALC that was positive for EBV using PCR and DNA ISH. We have no explanation for the discordant results concerning the EBV association of T-ALC. However, we believe that the results showing a low association of EBV with both nodal and extranodal T-ALC in our material are genuine, since our EBER ISH technique is sensitive enough to detect occasional EBER-positive small lymphocytes in some of the cases where residual lymphoid tissue was present in the sample.

A further difference between our study and that of Ott *et al.* (1992) is the higher proportion of EBV-positive extranodal PTLs found in the

latter. Both studies were performed on European patients, and this apparent discrepancy probably reflects incidental variations in case selection, such as differences in the numbers of skin and gut lymphomas in the two series.

A considerable variation has been found in different studies (Asian as well as Western) in the proportion of EBV-positive PTLs, which are of the AILD type. The EBV positivity was found in AILD in 23% of cases by Ohshima *et al.* (1990), in 38% of Danish cases (this study), in 40% by Su *et al.* (1991), in 57% by Ott *et al.* (1992), in 63% by Knecht *et al.* (1990), in 67% of Chinese cases (this study), in 68% by Anagnostopoulos *et al.* (1992), and in 96% by Weiss *et al.* (1992b). It is not clear whether this variation is genuine or whether it reflects either the use of different histologic criteria for the diagnosis of this lesion (see Section IV,C) or the use of different methods for the detection of EBV.

3. Immunophenotypes of Danish EBV Positive PTLs

Immunophenotyping of the 19 EBV-positive Danish PTLs revealed that 10 cases were of mainly helper cell phenotype ($CD4^+/CD8^-$), although several cases showed loss of one or more pan-T cell markers. None had a cytotoxic/suppressor phenotype ($CD4^-/CD8^+$). The remaining 9 cases exhibited other phenotypes ($CD4^-/CD8^-$; $CD4^+/CD8^+$). Sixteen cases expressed CD3 antigen in at least a proportion of the lymphoma cells. Thus, as previously mentioned, a broad range of T cell subpopulations may become infected by EBV.

G. Latent and Replicative EBV Gene Products in Neoplastic T Cells

Other EBV-associated diseases, including various types of B cell lymphoma, HD, and nasopharyngeal carcinoma, show variation both in latent gene expression and in the presence or absence of replication in a proportion of tumor cells. This prompts the question as to whether EBV-infected neoplastic T cells may express latent or replicative genes other than EBNA-1 and the EBERs (which are thought to be expressed in all latently infected cells). In particular, it is of interest to determine whether antigens, which are potentially both oncogenic and immunogenic (such as EBNA-2 and LMP-1), may be expressed in PTLs.

1. Patterns of Latent Gene Expression in PTLs

The pattern of latent gene expression has been reported in detail in relatively few cases of PTL. In a previous series, we showed that of eight

LMP-1-positive PTLs, only two also expressed EBNA-2 (Hamilton-Dutoit and Pallesen, 1992a). That is, six cases had a latency II-like pattern and two cases a latency III-like pattern of gene expression.

Previously, Harabuchi *et al.* (1990) described a latency III-like pattern in 3/3 cases of Japanese nasal PTL of the lethal midline granuloma type. Gaulard *et al.* (1992) reported LMP-1 expression in 7/7 similar tumors from European patients but did not comment on EBNA-2 expression.

Hart *et al.* (1992) described a latency I-like phenotype in their case of acute NK cell leukemia/lymphoma.

Finally, Anagnostopoulos *et al.* (1992) described the expression of LMP-1 in 17/32 cases of AILD-type PTL, but information about EBNA-2 expression was not provided.

In the present study, in which all LMP-1-negative PTLs were studied in addition for EBER-1 expression, the following distribution of latency types was found among EBV-positive Danish PTLs: Latency I-like ($EBER^+$, $LMP\text{-}1^-$, $EBNA\text{-}2^-$) in 2/16 cases (13%) (nos. 9 and 18 in Table V); latency II-like ($EBER^+$, $LMP\text{-}1^+$, $EBNA\text{-}2^-$) in 12/16 cases (75%); and latency III-like ($EBER^+$, $LMP\text{-}1^+$, $EBNA\text{-}2^+$) in 2/16 cases (13%) (nos. 11 and 15 in Table V).

In positive cases, the number of lymphoma cells expressing LMP-1 and, in particular, EBNA-2 was always smaller than the number expressing EBER-1. In the absence of LMP-1, EBNA-2 was not demonstrated. These results are broadly similar to our findings in EBV-positive AIDS-related B cell lymphomas (Pallesen *et al.*, 1992; Hamilton-Dutoit *et al.*, 1993b). They suggest that LMP-1 is probably expressed only at certain stages of the tumor cell cycle. In most infected lymphoma cells, LMP-1 expression is down-regulated, presumably in response to viral and/or host factors.

In the Chinese PTLs (which could not be studied for EBNA-2 because only paraffin sections were available), we found LMP-1 expression in a fraction of EBER-1 positive lymphoma cells in 9/10 nodal lymphomas (the one negative case being of pleomorphic, medium and large cell type) and in 9/12 extranodal PTLs. Among the eight EBER-1 positive PTLs of the upper aerodigestive tract, only one was negative for LMP-1.

The present study has shown that different patterns of EBV gene expression occur in Danish PTL. It remains to be investigated, however, whether $LMP\text{-}1^+$ and $EBNA\text{-}2^-$ PTLs show transcriptionally distinct EBNA-1 expression from the *Fp* rather than *Cp* or *Wp* promoters, as has been demonstrated in other EBV-positive tumors and cell lines with

latency II-like gene expression (Deacon *et al.*, 1993)—see Sections II,B and V,C,5.

2. EBV Replication Is Induced in Some PTLs

Few data are available concerning the capacity of EBV to escape latency and enter the replicative cycle in PTLs. This phenomenon may be detected by demonstrating linear EBV DNA in Southern blots or virus particles by electron microscopy; alternatively, tumor cell expression of the immediate early BZLF1 antigen (which controls the switch between latent and replicative EBV infection), early productive cycle antigens, late structural antigens (such as VCA or the gp350/250 protein), or MA can be shown by immunohistochemistry with appropriate monoclonal antibodies.

Linear EBV DNA was found in four PTLs (out of seven successfully analyzed, composed of two AILD, one Lennert's, and one pleomorphic-type lymphomas) in the study by Ott *et al.* (1992), and in the case described by Bonagura *et al.* (1990). Virus particles were demonstrated in one case of HTLV-1- and EBV-positive PTL by Ohshima *et al.* (1990).

Neither BZLF1 protein nor MA were detected in the five EBV PCR-positive cases of T-ALC in the study of Herbst *et al.* (1991a). In our previous study (Hamilton-Dutoit and Pallesen, 1992a), we demonstrated BZLF1 protein in 4/8 EBV-positive PTLs. Early antigen was expressed in only one of the cases, whereas neither VCA nor MA was detected. Gaulard *et al.* (1992) reported BZLF1 protein expression in 7/7 EBV-positive sinonasal PTLs.

In the present study (Table III) we detected the BZLF1 protein in frozen sections of 8/17 (47%) Danish PTLs (seven of pleomorphic, medium and large cell type and one of AILD type). Sometimes, this antigen could even be demonstrated in paraffin sections following microwave treatment (Fig. 2c). One BZLF1 positive PTL was extranodal (case no. 8 in Table V); neither LMP-1, nor EBNA-2 was detected in this lymphoma. EA was detected in only a few cells of 2/8 BZLF1 positive cases. Neither VCA nor MA was demonstrated in any case.

Thus, EBV replication can take place in neoplastic T cells *in vivo*. However, there is rarely evidence of late viral replicative stages suggesting that either production of infectious virions is blocked in most cases or lytic proteins are difficult to demonstrate due to rapid *in vivo* destruction of permissively infected cells.

H. Prognostic Implications of EBV Infection in Peripheral T Cell Lymphoma

Two surveys have compared the prognosis of EBV-positive versus EBV-negative PTLs in Japanese and Taiwanese patients, respectively (Ohshima *et al.*, 1990; Su *et al.*, 1991). These results suggest that infection of PTLs with the virus is associated with a more aggressive course, although this remains to be tested in larger series. Thus, EBV-positive PTLs should probably be considered as a separate clinico-pathologic group in in which novel treatment approaches may be indicated.

No data on the influence of EBV infection on the prognosis of PTLs in Western patients are yet available.

I. Conclusion: The Role of EBV Infection in Peripheral T Cell Lymphomas—Possible Pathogenetic Models

A wide range of PTL types may be EBV positive, and these tumors show considerable variability in their pattern of infection. This heterogeneity suggests that the pathogenetic role of the virus is unlikely to be the same in all cases. Recognition that EBV may be associated with some PTLs is recent, and for most types of EBV-positive PTL there is as yet little firm evidence to indicate the precise part the virus might play in the development of the tumor. However, it is possible to propose different models that could explain some of the features seen in different EBV-infected PTLs.

1. Uniform Infection of the Tumor Cell Population in PTLs by EBV

One group of PTLs consists of lesions in which all recognizable tumor cells carry the virus. These may be analogous to EBV-positive high-grade B cell NHLs and nasopharyngeal carcinoma, in which essentially all viable tumor cells express EBERs (Hamilton-Dutoit *et al.*, 1993a; Wu *et al.*, 1991). This pattern may be seen in both nodal and extranodal PTLs, but it is in our experience best exemplified by the nasal T cell lymphomas. The consistent presence of EBV in these lesions suggests that the virus has a central pathogenetic role either in initiating or in promoting lymphomagenesis. This is supported by the finding of monoclonal virus in many cases (see Section IV,D,2). Whether virus infection is a primary event or whether EBV acts as a secondary cofactor acting on

cells that have already undergone genetic change is unknown. This uncertainty is reminiscent of that surrounding the precise role of EBV in endemic BL (Klein, 1983; Lenoir and Bornkamm, 1987).

2. Partial Infection of the Tumor Cell Population in PTLs by EBV

Various studies have made the interesting and presumably important observation that in many PTLs EBV appears to infect only a fraction of the apparent tumor cells (Ohshima *et al.*, 1990; Richel *et al.*, 1990; Anagnostopoulos *et al.*, 1992; Medeiros *et al.*, 1992) (Table V). Several models could explain this situation.

1. Some of these cases may be polyclonal atypical T cell lesions in which EBV infection has not occurred in all proliferating clones and in which the virus is a coincidental "passenger" with either a minor or nonexistent role in the development of the lesion.

2. A second possibility is that partial infection occurs following loss of EBV from some of the proliferating cells in an oligo- or monoclonal lesion. In theory, this might result from unequal distribution of viral episomes in successive host cell divisions. Alternatively, circumstances may exist in which latent EBV infection becomes unstable with consequent loss of the virus from cells. Interestingly, it has recently been reported that transfection and overexpression of the EBV BZLF-1 gene in Raji cells is associated with loss of EBNA expression and a fall in EBV genome copy number (Takada *et al.*, 1992). Whether a similar process can occur in EBV-infected cells *in vivo*, eventually resulting in elimination of the virus, is not known.

3. A third model, and one that we believe best explains the findings in most cases, involves EBV as an important but not essential cofactor in a stepwise process of malignant transformation. In this model, virus infection first occurs in T cells that have already undergone some degree of malignant change. A relatively low number of virus-infected tumor cells would be present at this stage, the virus being found in an oligo- or polyclonal form. Virus-infected T cells would be given a growth advantage (possibly similar to that conferred on B cells by EBV infection), which could contribute to clonal selection, the latter presumably being associated with the gradual accumulation of genetic change. For this to occur EBV-positive T cells would have to avoid immune surveillance, suggesting the presence of defects in EBV immunity. The eventual emergence of a transformed cell clone would be accompanied by an increase in the relative number of EBV-infected cells and a rise in the

number of large cells and in the proliferative activity being evident histologically. The EBV terminal repeat pattern analysis at this stage would show a change from an oligoclonal to a monoclonal pattern. At each stage, variable numbers of EBV-infected nonneoplastic B and T cells would also be present in the lesion, as suggested by the findings in AILD-type lymphomas (Anagnostopoulos *et al.*, 1992).

This last model allows for the finding of a variable and often surprisingly low number of EBV-infected lymphoma cells in PTLs (Table V) (Anagnostopoulos *et al.*, 1992), the occurrence of PTLs with oligoclonal and monoclonal forms of EBV (see, e.g., Ott *et al.*, 1992), and the apparent link reported between EBV infection of PTLs and decreased patient survival (Ohshima *et al.*, 1990; Su *et al.*, 1991). In some cases, the EBV-infected clone might come to dominate the tumor to the extent that the lesion became indistinguishable from those lymphomas with uniform tumor cell EBV infection described in Section IV,I,1.

Additional evidence for the last model is provided by studying the evolution of individual tumors. We previously described a case that could support the idea that lymphoma development can accelerate, at least in some cases, following EBV infection of lymphocytes that have already undergone neoplastic transformation (Hamilton-Dutoit and Pallesen, 1992a).

The patient, a 74-year-old male (case no. 11 in Hamilton-Dutoit and Pallesen, 1992a), had been treated for a nodal PTL of pleomorphic type 5 years previously. This lesion was shown to contain very few (<<0.1%) EBER-1-positive tumor cells (Fig. 1f). At relapse, the immune phenotype was identical to the original lymphoma, but the histology had changed suggesting progression to a higher grade of malignancy, and the lymphoma contained about 15% EBER-1- and LMP-1-positive tumor cells (case no. 9 in Table V; Fig. 1g,h). The clinical course was aggressive. A similar evolution was seen in the PTL case described by Richel *et al.* (1990).

We have recently observed another case that adds further complexity to the possible mechanisms by which EBV may contribute to lymphoma development but supports the idea that the virus may have a tumor-enhancing effect under certain conditions. A 58-year-old man developed lymphadenopathy and clinical symptoms compatible with AILD in 1983 (case not included in the present study). The lymph node biopsy was diagnosed as AILD both at presentation and also on later revision. The ISH showed occasional (<0.1%) EBER-1-positive lymphocytes, some showing blastic transformation (Fig. 2a). Their phenotype was not

identified. Considerable activation of both B and T cells was seen in the initial lymph node biopsy (Fig. 2a). Nine years later, the patient again developed lymphadenopathy. Histological and immunophenotypical examination of a lymph node biopsy revealed typical monoclonal B immunoblastic lymphoma with plasmablastic differentiation. The ISH revealed EBER-1 in the great majority of the tumor cells (Fig. 2b). It has not been possible in this case to compare possible rearrangements of the TCR and Ig genes in the two biopsies. However, it is tempting to suggest that AILD initial EBV infection of small lymphocytes (B and/or T cells) may eventually lead to the development of overt NHLs of either T or B cell type.

V. EBV and Hodgkin's Disease

A. Epidemiological and Serological Evidence of an Association between EBV and Hodgkin's Disease

Although the nature of the HRS cell remains unclear (see Section IV,C), there is increasing evidence that EBV plays a pathogenetic role in a substantial proportion of HD cases (Pallesen, 1992). The indirect evidence for this will be briefly reviewed and this will be followed by a detailed discussion of the more direct evidence for an association between EBV and HD that has recently been reported.

1. Epidemiological Evidence of an Association Between EBV and HD

Various epidemiological studies have suggested a possible role for EBV in HD (for a review see Mueller, 1987). In contrast to most malignancies, Hodgkin's disease has a bimodal age-incidence curve (Gutensohn and Cole, 1980). In developed countries, the first peak is seen in young adults between the ages of 15 and 34 years, whereas the second peak occurs in the elderly. In economically developing countries, the first peak is less pronounced and occurs in children between the ages of 5 and 15 years, a fall in incidence being seen during young adulthood. It has been suggested that the increased incidence of HD in young adults in economically advantaged populations may be related to the effect of delayed age at primary infection with a common virus (Gutensohn and Cole, 1977). Support for this has come from studies of childhood social environment that have shown that factors favoring late infection with common viruses, such as small sibship size, early birth order, high mater-

nal education, and low-density housing in childhood, are associated with an increased risk of HD in young adults (Gutensohn and Cole, 1980, 1981). The risk of HD in older age groups is independent of social class variables.

The incidence of the different histological subtypes of HD also shows variation with age. Thus, the nodular sclerosis (NS) subtype of HD (the predominant type among young adults) has a unimodal curve with a single peak between the ages of 15 and 34 years. In contrast, the mixed cellularity (MC) subtype of HD shows an increased incidence with age; if an infectious agent is involved in the pathogenesis of this group of HD, then it is perhaps more likely to be as a result of reactivation of infection following age-related decline in T cell immunity.

If HD is related to infection with a common virus, then EBV is a leading candidate for such an agent. The epidemiology of HD occurring in young adults is similar to that of infectious mononucleosis. Furthermore, cohort studies have shown a two to four times increased risk of developing HD following infectious mononucleosis (Rosdahl *et al.*, 1974; Munoz *et al.*, 1978). The excess risk of HD is greatest within 3 years of the diagnosis of infectious mononucleosis.

2. *Serological Evidence of an Association between EBV and HD*

Sera from patients presenting with HD show moderately elevated levels of anti-EBV antibodies to VCA, to both components (diffuse and restricted) of EA, and (less consistently) to EBNA, in a proportion of cases, compared with controls (Johansson *et al.*, 1970; Levine *et al.*, 1971; Henle and Henle, 1973; Hesse *et al.*, 1977). These findings have been confirmed in a number of subsequent studies. Johansson *et al.* (1970) found elevated anti-VCA titers in about one-half of their patients. In addition, they reported a striking correlation between the level of EBV antibodies and the histological subtype. Their cases were classified using the now outdated Jackson and Parker (1947) HD classification into paragranuloma, granuloma, and HD sarcoma. An inverse relationship was shown between the level of anti-EBV titers and the degree of lymphocyte infiltration in the tumors, high levels being seen in HD sarcoma and near normal titers in the lymphocyte-rich paragranuloma group (Johansson *et al.*, 1970). These studies could not be used to show whether the elevated EBV titers in HD indicated a primary etiological role for the virus or whether they reflected reactivation of the virus associated with the underlying disease process or its treatment. In order to address this question, Mueller *et al.* (1989) studied sera obtained from

HD patients prior to the onset of their disease. They found antibody patterns similar to those reported in presentation sera, with elevated anti-EBV titers to VCA (both IgG and IgA), to the diffuse form of EA, and to EBNA. Altered antibody patterns were most apparent in those sera taken from patients 3 years or more before HD diagnosis. Thus, enhanced EBV activity appears to predate the presentation of HD suggesting that dysregulation of the viral-host balance occurs long before the onset of overt disease. According to Mueller *et al.* (1989), the EBV viral titers in pre-diagnosis HD sera indicate a level of viral activity compatible with a relatively severe, delayed primary EBV infection, or a change in host control in older patients due to diminished immune competence. Although these findings imply a primary pathogenetic role for the virus in HD, it remains possible that elevated EBV titers in HD patients are a secondary marker of abnormal immunity (inherited or acquired) that predisposes to both viral reactivation and to the development of HD.

B. EBV Genomes in Hodgkin's Disease Tissues

1. Detection of EBV Genomes in HD Tissue Extracts

Direct evidence linking EBV with HD has only recently been reported. Epidemiological and serological studies suggesting a possible role for EBV in HD prompted a number of groups to look, unsuccessfully, for viral nucleic acids in HD tissue extracts (Lindahl *et al.*, 1974). It was only when improved filter hybridization techniques and probes became available, that the sensitivity of viral DNA detection was sufficient to allow the demonstration of EBV genomes in HD. The first successful report came from Weiss *et al.* (1987) who used Southern blotting to demonstrate EBV DNA in a small series of HD biopsies. Subsequent filter hybridization studies confirmed these findings, detecting EBV DNA in between 17 and 44% of HD cases (Weiss *et al.*, 1989; Anagnostopoulos *et al.*, 1989; Boiocchi *et al.*, 1989; Staal *et al.*, 1989; Libetta *et al.*, 1990; Armstrong *et al.*, 1992). Interestingly, the highest detection rate (44%) was reported from a study performed mainly on DNA extracted from formalin-fixed, paraffin-embedded tissue blocks (Libetta *et al.*, 1990). A particularly high frequency of EBV genomes (five of seven cases positive) was found in HD biopsies from HIV-seropositive patients (Uccini *et al.*, 1990).

Even higher EBV DNA positivity rates (up to 87%) have been reported in HD tissue extracts using PCR, the detection rate depending largely on the sensitivity of the PCR technique employed (Herbst *et al.*, 1990; Uhara *et al.*, 1990; Masih *et al.*, 1991; Knecht *et al.*, 1991; Weiss *et al.*,

1991; Armstrong *et al.*, 1992). Interpretation of these results is complicated by the ability of PCR to detect viral DNA in EBV positive nonneoplastic lymphocytes, even when these are present only in very low numbers. Thus, a positive EBV PCR indicates the presence of the virus in a tissue but provides no clear evidence as to the cellular source of the signal (Masih *et al.*, 1991; Coates *et al.*, 1991; Khan *et al.*, 1992; Armstrong *et al.*, 1992). Because HRS cells are themselves relatively infrequent in HD tissues, quantitative PCR techniques cannot be used to show whether the virus infects tumor cells in a particular case.

2. Detection of EBV DNA in HRS Cells Using in Situ Hybridization

Analysis of the viral terminal repeat region was performed in several of the first HD cases to be shown to be EBV positive by Southern blotting (Weiss *et al.*, 1987, 1989; Anagnostopoulos *et al.*, 1989). These results suggested that the virus infected a monoclonal cell population, and by implication HRS cells. However, formal proof that EBV did indeed infect HRS cells in such cases first came from DNA ISH using ^{35}S-labeled *Bam*HI W internal repeat probes (Weiss *et al.*, 1989; Anagnostopoulos *et al.*, 1989). These and subsequent studies have clearly shown that EBV DNA is localized to HRS cells (Weiss *et al.*, 1989; Anagnostopoulos *et al.*, 1989; Uhara *et al*,. 1990; Uccini *et al.*, 1990; Herbst *et al.*, 1990; Brousset *et al.*, 1991a,b; Niedobitek *et al.*, 1991), although the limits of sensitivity of ISH for detecting the virus means that not all EBV-infected cases give a positive result (unpublished observations). In most DNA ISH studies, viral genomes have not been detected in accompanying lymphocytes. Some studies have, however, reported rare EBV DNA-positive cells among the supposed nonneoplastic small cell population in a few cases of HD (Guarner *et al.*, 1990; Coates *et al.*, 1991). This finding is not unexpected, given that an ISH technique with sufficient resolution (i.e., a nonisotopic method) and sensitivity is used for EBV DNA detection.

3. Clonality of Episomal EBV in HD

Most EBV-positive HD cases successfully analyzed for the configuration of the infecting virus' terminal repeat region have been reported to contain monoclonal episomes. This implies that the virus infects a clonal cell population (i.e., HRS cells) and that infection occurred prior to clonal expansion (Weiss *et al.*, 1987, 1989; Anagnostopoulos *et al.*, 1989; Boiocchi *et al.*, 1989; Staal *et al.*, 1989; Gledhill *et al.*, 1991; Jarrett *et al.*, 1991). This technique is particularly valuable in HD since other genotypic methods for testing clonality may not be successful because of

the absence of clonal rearrangements of the *Ig* or *TCR* genes in many cases.

Occasional cases of HD have shown an oligoclonal EBV terminal repeat pattern (Weiss *et al.*, 1987; Katz *et al.*, 1989; Boiocchi *et al.*, 1989; Masih *et al.*, 1991; Jarrett *et al.*, 1991). This finding may indicate either genuine oligoclonality of the HRS cells or the detection of other EBV clones present in EBV-infected nonneoplastic lymphocytes that may occur in some HD cases (Weiss *et al.*, 1991; Herbst *et al.*, 1992; Khan *et al.*, 1992).

The demonstration of clonally expanded populations of EBV-infected cells in HD biopsies strongly supports an etiological role for the virus in a substantial proportion of cases. Masih *et al.* (1991) have, however, reported that, in addition to HD cases, clonal EBV may also be found in some control lymph nodes with benign hyperplasia. This unexpected finding implies that detection of clonal EBV does not necessarily indicate the presence of a monoclonal tumor cell population in a lesion. This finding has, however, not yet been reproduced in other laboratories.

4. EBV Types A and B in HD

Few studies have examined the type of virus present in EBV-positive HD cases. Gledhill *et al.* (1991) found EBV type A, but no type B, in each of 8 cases of HD investigated. In a follow-up report from the same group, Jarrett *et al.* (1991) found only type A virus in 30 EBV-positive HD cases. Whether this predominance of EBV type A will also be found in HD from other geographical regions and clinical groups (for example in HIV-seropositive individuals) remains to be tested.

5. Receptors for EBV on HRS Cells

Interestingly, it appears that HRS cells in the majority of HD cases do not show immunohistological expression of CR2/CD21 antigen (Uccini *et al.*, 1990). Studying our own material with monoclonal antibodies OKB7, HB5, and B2 (which recognize different epitopes of the CR2/CD21 molecule), we detected only OKB7 reactivity in 1 out of 20 cases of HD. However, some other cases did show staining of HRS cells with HB5 and B2. This immunoreactivity, which had a different pattern to that seen with OKB7, was not related to the EBV status of the HRS cells, and its significance (i.e., whether it represents a functional EBV receptor) is unclear.

In contrast to the foregoing findings, Jiwa *et al.* (1992) reported immunohistological expression of CD21 in 9 out of 12 EBV-positive (75%) compared with 1 out of 13 EBV-negative (8%) HD cases using anti-CD21 monoclonal antibodies (HB5 and an unspecified antibody from

Biogenesis, Bournemouth, United Kingdom). This study was mainly conducted on paraffin tissue sections. The association of CD21 antigen expression in HRS cells with EBV infection found in this series will need to be confirmed by other studies, particularly performed on frozen section.

C. Latent EBV Gene Products in Hodgkin's Disease Tissues

Poppema *et al.* (1985) were the first to report the immunocytological detection of a latent EBV gene product (EBNA-1) *in situ* in HRS cells in a HD-like lesion. Their patient presented with an infectious mononucleosis-like illness and developed episodic lymphadenopathy over several months. Serology was interpreted as showing chronic or reactivation EBV infection rather than primary infectious mononucleosis. Examination of the lymph nodes revealed a lesion morphologically indistinguishable from HD (MC type), and large $EBNA^+$ cells were detected in lymph node suspensions. The conclusion of this case report, that HRS cells in (apparent) HD could express EBV latent antigens, was later confirmed using two separate techniques: (i) Wu *et al.* (1990) used paraffin section ISH to demonstrate abundant EBER-1 RNA transcripts in HRS cells in six out of eight EBV genome-positive HD cases. (ii) Pallesen *et al.* (1991a) used monoclonal antibody frozen section immunohistology to identify abundant LMP-1 expression in HRS cells in about one-half of a large series of HD cases.

These studies showed that both viral transcription and translation could occur in EBV-infected HRS cells, supporting the idea that EBV was not present merely as a "silent passenger" in HD. That the transformation-associated protein LMP-1 (but not EBNA-2) was expressed in HRS cells was thought to be of particular relevance to HD oncogenesis because of its well-established oncogenic and transforming properties *in vitro* in both human and animal cells.

1. LMP-1 Expression in HRS Cells

Our finding that EBV-infected HRS cells express LMP-1 was subsequently confirmed by other groups (Herbst *et al.*, 1991b; Brousset *et al.*, 1991b; P. G. Murray *et al.*, 1992; Armstrong *et al.*, 1992). Herbst *et al.* (1991b) used a different (polyclonal) anti-LMP-1 antibody for this purpose. In all reported cases, LMP-1 was localized to HRS cells and was never seen in other tissue constituents, including small lymphocytes.

In our initial report of LMP-1 expression in HD we detected the protein in HRS cells in 40/84 cases (48%) using pooled monoclonal

antibodies CS.1-4 and frozen section immunohistochemistry. In most instances the antibodies also worked on corresponding formalin-fixed, paraffin-embedded tissue sections, although some false negative results occurred, and the number of cells showing LMP-1 reactivity in positive cases was clearly lower than in frozen sections stained in parallel. More recently, we have included microwave pretreatment of paraffin sections in the staining technique, and the sensitivity of LMP-1 immunodetection on paraffin sections using CS.1-4 monoclonal antibodies has been greatly improved. In addition, background staining and artifactual nuclear reactivity, which may occur in paraffin sections treated by proteolysis alone, are no longer seen.

Other groups have detected LMP-1 in HRS cells in HD with varying frequencies. The HRS cell LMP-1 expression was reported by Herbst *et al.* (1991b, 1992) in 18/47 (38%) and 18/46 (39%) HD cases, respectively; in 36/107 cases (34%) by Delsol *et al.* (1992); in 16/39 cases (41%) by Armstrong *et al.* (1992); and in 22/46 cases (48%) by P. G. Murray *et al.* (1992). In a recent study of HD in children by Weinreb *et al.* (1992), LMP-1 expression was seen in HRS cells in 37/74 cases (50%).

Most of these studies have been performed on paraffin sections and are therefore likely to represent the minimum figures for frequency of LMP-1 expression in HRS cells.

An analysis of LMP-1 at the mRNA level has been performed by Deacon *et al.* (1993) using reverse transcription PCR. The amplification product was identified in 11 of the 12 EBV (HRS cell) positive but in none of the 5 EBV-negative HD biopsies (the latter remained negative even after long overexposure of the autoradiographs). Transcripts with the same splice pattern originally identified in LCL cells with the 2.8-kb LMP-1 mRNA, were found in the 11 cases. However, using alternative primers, it was demonstrated that transcripts representing the longer 3.7 kb LMP-1 mRNA, initiated by a promoter upstream of the 2.8-kb mRNA start site, were present in 10 of the 12 EBV-positive HD biopsies. Thus, LMP-1 transcription in EBV-positive HD resembles that seen in nasopharyngeal carcinomas.

At the protein level, Deacon *et al.* (1993) reported expression of an appropriately sized full-length protein detected by immunoblotting for LMP-1 in extracts of HD biopsies. They found the variable size of the LMP-1 band in each EBV-positive HD biopsy to be consistent with the presence of a different virus isolate in each of the cases.

In a clinical study of the patients included in our original paper on LMP-1 expression in HD, we found no significant differences between LMP-1-positive and -negative cases with regard to progression-free survival, sex, age, systemic symptoms, clinical stage, or mediastinal involve-

ment (Vestlev *et al.*, 1992). It was not, however, possible in this retrospective study to correlate LMP-1 expression in HRS cells with an eventual degree of immune dysfunction in the corresponding patients. In order to address this and other limitations of the study, a larger, prospective survey is planned.

2. EBER Expression in HRS Cells

As noted in Section III,B,3, the sensitivity of EBER ISH for detecting EBV in HD tissues is comparable to, or possibly even higher than, that of PCR analysis. Several studies have reported that, at least when working with fixed, paraffin-embedded biopsies, PCR gives a much higher frequency of false negative results compared with EBER ISH (Weiss *et al.*, 1991; Armstrong *et al.*, 1992).

Wu *et al.* (1990) were the first to report expression of EBER-1 in HRS cells using ISH. A signal was seen in six out of eight cases of HD, all positive for EBV by Southern blotting.

Weiss *et al.* (1991) used ISH to identify EBER-1 in HRS cells in 11 out of 23 HD cases (48%) not including HD, nodular LP type. In addition, 13 cases of HD–LP (nodular) type were examined all of which were negative for EBER-1 in the HRS variant cells. In positive cases, EBER-1 was expressed in virtually all HRS cells, with the exception of one tumor that showed focal expression of EBER-1 only. The authors speculated that in this case (an HIV-positive patient) infection of HRS cells might have taken place *after* neoplastic transformation had occurred.

Using ISH, Weiss *et al.* (1991) were the first to demonstrate EBER expression in the residual benign lymphocyte population. These cells were found in HD biopsies containing both EBV-positive and -negative HRS cells, as well as in controls of normal/reactive lymphoid tissues. They demonstrated the presence of infrequent small and medium-sized lymphocytes in 20/23 of their HD cases (87%). In cases with EBER negative HRS cells, they found positive lymphocytes in 9/12 cases (75%) of HD, not including cases of LP, nodular type. The latter harbored EBER-1 positive lymphocytes in 9/13 cases (69%). Control tissues (lymph nodes, tonsils, spleen) contained EBER-positive lymphocytes in 7/10 cases (70%). Double-labeling experiments showed that these EBER-positive lymphocytes usually expressed the CD20 B cell antigen. However, a minor fraction expressed the CD43 T cell-associated antigen. Thus, both HD and reactive lymphoid tissues apparently harbor at least two EBV-positive lymphocyte populations, one of B cell and the other of possible T cell derivation. However, expression of CD43 is certainly not restricted to the T cell lineage, and therefore the issue of whether EBV

infects normal, resting T lymphocytes *in vivo* remains to be established using T cell-specific markers.

In a subsequent study, Herbst *et al.* (1992) reported expression of EBERs in HRS cells in 23/46 cases (50%) of HD, including 2/2 cases of LP, nodular type. In positive cases, EBERs were expressed in virtually all tumor cells. In addition, EBER-positive small lymphocytes were recognized in 42/46 cases (91%) including 19/23 HRS cell EBV-negative cases (83%). Double-labeling studies showed down-regulation (weak positivity or negative reaction) of the CD20 antigen and no labeling with the T cell-specific antibody beta-F1 of EBER-positive small lymphocytes. Thus, these lymphocytes were either of B cell phenotype or could not be assigned to a particular cell lineage.

Using EBER ISH, Armstrong *et al.* (1992) have obtained similar frequency rates for EBV-associated HD, 19/39 cases (49%) showing expression of EBERs in HRS cells.

In contrast, Khan *et al.* (1992) demonstrated EBERs in only 6/33 cases (18%) of HD. In six other cases (18%), EBERs were detected in the residual lymphocyte population only.

Our own studies have detected EBER-1 (see color plate, Fig. 2d) in 40/84 HD cases (48%), these figures being in close concordance with the results of our earlier immunohistological study of LMP-1 expression in HRS cells (Pallesen *et al.*, 1991a). Positive small lymphocytes were demonstrated in only 6/44 cases (14%) with EBER-1-negative HRS cells (Fig. 2e; Table VI). In contrast, in a study of 25 HD cases from a Danish clinico-pathological project (LYGRA) we found 14/25 cases (56%) to contain EBER positive lymphocytes.

TABLE VI
EBV GENE EXPRESSION IN 84 DANISH CASES OF HODGKIN'S DISEASE[a]

HD type	LMP-1$^+$ HRS cells[b] (%)	EBER$^+$ HRS cells (%)	EBER$^-$ HRS cells and lymphocytes (%)	EBER$^-$ HRS cells but EBER+ lymphocytes (%)
LP	1/10 (10)	1/10 (10)	7/10 (70)	2/10 (20)
NS	16/50 (32)	16/50 (32)	30/50 (60)	4/50 (8)
MC	23/24 (96)	23/24 (96)	1/24 (4)	0/24
Total	40/84 (48)	40/84 (48)	38/84 (45)	6/84 (7)

[a] Cases selected on the basis of the availability of frozen tissue. The results of LMP-1 and EBNA-2 expression have been published previously (Pallesen *et al.*, 1991a). The EBER ISH was performed on paraffin sections, LMP-1 immunostaining on frozen sections.

[b] LMP-1 was detected only in HRS cells and never identified in small lymphocytes.

HD, Hodgkin's disease; HRS cells, Hodgkin and Reed–Sternberg cells; LP, lymphocytic predominance type; MC, mixed cellularity type; NS, nodular sclerosis type.

Using ISH, it is apparent that EBER-1-positive lymphocytes in HD are not an entirely resting cell population. In HD cases, both with and without EBV-positive HRS cells, EBER expression can be seen in small, intermediate-sized, and large (immunoblast forms) lymphocytes (Fig. 2f). Thus, these EBV-infected, nonneoplastic lymphocytes show morphological evidence of activation, perhaps as a consequence of impaired immunity of the host. These cells do not express either LMP-1 or EBNA-2, this down-regulation of viral gene expression possibly underlying their avoidance of immune recognition by the host.

In our recent series of Chinese HD cases (Zhou *et al.*, 1993; Section V,F,2), we have identified EBER-1 expression in 17/28 cases (61%). Occasional small EBER-1-positive lymphocytes were seen in 5 out of 12 cases (42%) with EBV-negative HRS cells.

3. Comparison of LMP-1 and EBER-1 Expression in HRS Cells

We have recently reexamined the cases from our original report of LMP-1 expression in HRS cells (Pallesen *et al.*, 1991a) and have found a nearly perfect correlation between the results of LMP-1 frozen section immunohistology and those of EBER paraffin section ISH (Table VI) for the detection of EBV. We have also compared the sensitivity of the modified (microwave pretreatment) paraffin section LMP-1 staining method with that of EBER ISH for EBV detection in HD. We found almost absolute concordance between the results of the two methods when applied to a series of paraffin-embedded Chinese HD cases (Zhou *et al.*, 1993) and to a series of 25-year-old formalin-fixed, paraffin-embedded HD biopsies from the Danish LYGRA HD project. Thus, under appropriate conditions (i.e., using either frozen sections or microwave-pretreated paraffin sections) we consider CS.1-4 immunostaining to be as sensitive, and less resource consuming, as EBER ISH for detection of EBV gene expression in HRS cells.

In contrast to our findings, Herbst *et al.* (1992) found a lower EBV detection rate in HD when using LMP-1 immunostaining (18/46; 39%) compared with EBER ISH (23/46; 50%). Of 23 HD cases with EBER-positive HRS cells, these authors found 18 to contain LMP-1 positive HRS cells. In the latter, LMP-1 was expressed in only a proportion (10 to 80%) of the HRS cells, whereas EBERs were detected in virtually all HRS cells. However, most of the cases in the latter study were stained for LMP-1 on paraffin sections without the use of microwave pretreatment, possibly accounting for this discrepancy. Similarly, Armstrong *et al.* (1992) found LMP-1 positivity in 16/39 (41%) HD cases as compared

with 18/39 (46%) with EBER-positive HRS cells. The LMP-1 staining was performed on paraffin sections without microwave pretreatment.

Even though LMP-1 immunostaining is extremely well suited for the demonstration of EBV in HRS cells in HD, it may not be completely reliable as a method for screening other tumor types for EBV infection because expression of the protein may be variably down-regulated in different EBV-associated lesions.

Close concordance between LMP-1 immunostaining and DNA ISH has been reported by Delsol *et al.* (1992) using ModAMeX-processed tissues (about 35% of HD cases being EBV DNA and LMP-1 positive). These authors did not recognize small EBV DNA-positive lymphocytes in their material.

4. HRS Cells Do Not Express EBNA-2

Although the EBNA-2 gene is apparently intact in EBV genome-positive HRS cells (Gledhill *et al.*, 1991), immunohistological studies using antibodies to EBNA-2 have shown no expression of this antigen in HD (Pallesen *et al.*, 1991a; Herbst *et al*,. 1991b; Brousset *et al.*, 1991b). These findings have been supported by transcriptional analysis studies of EBV infection in HD using ISH (Niedobitek *et al.*, 1991) and reverse transcription PCR (Deacon *et al.*, 1993), which have found no evidence of EBNA-2 gene expression.

5. Expression of Other Latent Gene Products in HD Tissues

With the exception of LMP-1 and EBNA-2, studies of EBV latent protein expression in HD have been hampered by a lack of satisfactory antibodies for immunohistochemical staining and by the paucity of HRS cells in most HD biopsies, which prevents their analysis by immunoblotting. To overcome this problem, Deacon *et al.* (1993) have characterized the pattern of latent gene expression in EBV (HRS cell)-positive HD using reverse transcription PCR to detect specific viral mRNAs. Primer-probe combinations that had previously been successfully applied to the analysis of EBV transcription in biopsies of nasopharyngeal carcinomas were used.

a. EBNA-1. As mentioned previously, Poppema *et al.* (1985) demonstrated EBNA-1 in HRS cells in cytological specimens from a biopsy with a HD-like lesion using human hyperimmune antiserum. However, we have never been able to obtain satisfactory results when applying this method to frozen sections (see Section III,A). We have tested, however, a number of anti-EBNA-1 monoclonal antibodies that invariably have

shown either low affinity or insufficient specificity when used for immunohistology. Attempts to amplify the reactions of the low-affinity antibodies using various methods resulted in inconsistent immunocytochemical staining of EBV-positive cells in control cell line pellets and tissues. In HD biopsies we have not been able to obtain positive reactions for EBNA-1 in HRS cells above background staining, which is invariably high following amplification. Similar results were obtained with frozen tissues from various EBV-positive NHLs and nasopharyngeal carcinomas and from most cases of acute infectious mononucleosis (unpublished observations).

In contrast, using reverse transcription PCR, Deacon *et al.* (1993) were able to demonstrate EBNA-1 mRNA in 9/12 EBV (HRS cell)-positive HD cases but in none of the five cases with EBV-negative HRS cells. Moreover, the spliced EBNA-1 mRNA products were characteristic of *Fp* promoter-driven transcription in all nine cases (see Section II,B). In one case, *Cp/Wp*-driven EBNA-1 transcription was also detected. The presence of *Fp*-driven EBNA-1 transcription was independent of the histological HD subtype.

b. EBNA-3A, 3B, 3C, and Leader Protein. Deacon *et al.* (1993) found no evidence of *Cp/Wp* EBNA transcription using reverse transcription PCR in the great majority of HD cases tested, suggesting strongly that EBNA-3A, 3B, 3C, and leader protein expression is absent in these tumors.

c. LMP-2A/B. The LMP-2A and/or 2B transcripts were detected in 8 out of 12 cases of EBV (HRS cell)-positive HD using reverse transcription PCR (Deacon *et al.*, 1993). No signal was found in the EBV-negative HD biopsies. In 2 of the cases, only LMP-2A or 2B was detected. The remaining biopsies showed both types of transcriptions.

d. BamHI A Transcription. Rightward transcription of *Bam*HI A sequences is seen in some latent EBV infections and appears to be a consistent feature of nasopharyngeal carcinoma (Gilligan *et al.*, 1991). In the study by Deacon *et al.* (1993), *Bam*HI A transcripts were found in 11 of 12 EBV-positive HD cases, indicating that this gene is not exclusively expressed in EBV-infected epithelial cells as was previously suggested.

6. HRS Cells Show EBV Latency II Expression

In view of the results obtained by Deacon *et al.* (1993) and summarized in Section V,C,5, it may be concluded that HRS cells show latency

type II (see Section II,B), resembling that found in undifferentiated nasopharyngeal carcinoma (Young *et al.*, 1988) in several aspects: (1) selective expression of EBNA-1 from a *Bam*HI F promoter (as is seen in BL cells); (2) down-regulation of the *Bam*HI C and W promoters and their associated EBNA mRNAs; (3) expression of LMP-1 and, in most cases, LMP-2A and 2B transcripts; and (4) expression of *Bam*HI A transcripts.

A latency II-like pattern of EBV gene expression has been shown using immunohistology in some AIDS-related B cell lymphomas (Pallesen *et al.*, 1992; Hamilton-Dutoit *et al.*, 1993b) and in some PTLs (see Section IV,G,1).

D. EBV Replication in Hodgkin's Disease

We have analyzed the control of EBV latency in HRS cells by examining frozen HD tissues for expression of the BZLF1 protein. Using immunohistology with monoclonal antibody BZ.1, we were able to detect occasional BZLF-1-positive HRS cells in only 3 out of 47 LMP-1-positive HD cases (6%) (Pallesen *et al.*, 1991b). Two of the cases belonged to the MC, and one belonged to the NS subgroup of HD. None of the 3 BZLF-1 positive lymphomas expressed EA, VCA, and MA, either in HRS cells or in accompanying lymphocytes.

Small, linear EBV DNA fragments have been reported in Southern blots of DNA extracts from occasional HD cases, suggesting the presence of either whole or defective virions in these biopsies. Using this technique, however, it is impossible to establish whether virus replication occurs in HRS cells or in accompanying lymphocytes in these lesions (Anagnostopoulos *et al.*, 1989; Katz *et al.*, 1989; Masih *et al.*, 1991).

Joske *et al.* (1992) have investigated expression of the EBV late replicative gene BLLF-1 in 20 EBV DNA-positive HD cases using reverse transcription PCR. Even though they found ample LMP-1 RNA, particularly among the MC subtype, they found BLLF-1 RNA transcripts in only a single case.

These findings suggest that control of viral latency in HD is tight and that if activation of the replicative cycle does occur, then it results in abortive virus production. Factors such as host immunity and/or host cell differentiation may be important in maintaining latency of EBV (Gradoville *et al.*, 1990; Pallesen *et al.*, 1991b, 1992).

Clearly, it will be of interest to establish the frequency of viral replication in AIDS-related, compared with sporadic, HD.

E. EBV Is Associated with Histological Subtype in Hodgkin's Disease

Pallesen *et al.* (1991a) found LMP-1 expression to be related to a histological subtype in HD. Almost all MC cases expressed LMP-1, whereas the histologically less aggressive subtypes NS and LP expressed LMP-1 in only about one-third and one-tenth of cases, respectively. The association of MC subtype and EBV has been subsequently confirmed in some (Staal *et al.*, 1989; Weiss *et al.*, 1991; Delsol *et al.*, 1992; P. G. Murray *et al.*, 1992; Weinreb *et al.*, 1992) but not all (Herbst *et al.*, 1991b; Jarrett *et al.*, 1991) studies.

Because the HD subtype LP, nodular (nodular paragranuloma) is now well recognized as a B cell lymphoproliferative disorder, this type was expected to be more frequently associated with EBV than other histological types of HD. However, our original finding of a low rate of EBV association (10%) in this type (Pallesen *et al.*, 1991a) has subsequently been confirmed in other studies. Thus, EBV was demonstrated in HRS cell variants in 0/10 LP, nodular HD cases by Delsol *et al.* (1992), in 0/13 cases by Weiss *et al.* (1991), and in 1/12 cases by P. G. Murray *et al.* (1992). Somewhat higher EBV detection rates were reported in the HD-LP, nodular type by Uhara *et al.* (1990) (1/4 cases), by Weinreb *et al.* (1992) (4/13 cases), by Herbst *et al.* (1992) (2/2 cases), and by Armstrong *et al.* (1992) (2/2 cases).

F. EBV-Positive Hodgkin's Disease in Different Populations

1. EBV in Western HD

As noted previously, PCR overestimates the true frequency of HRS cell EBV infection in HD as it detects virus in nonneoplastic lymphocytes. Accurate estimates of the incidence of EBV in HD in Western patients require the use of *in situ* techniques of virus detection. The results of LMP-1 immunostaining and EBER ISH from several studies suggest that EBV infects HRS cells in approximately 50% of HD cases (Pallesen *et al.*, 1991a; Herbst *et al.*, 1992; Armstrong *et al.*, 1992; Weinreb *et al.*, 1992; P. G. Murray *et al.*, 1992).

2. EBV in Asian HD

Hodgkin's disease is uncommon in Asian compared with Caucasian populations, accounting for only 9% of all lymphomas in Hong Kong and Taiwan (Ho *et al.*, 1984; Su *et al.*, 1985) and only 6% of all lymphomas

in Japan (Kadin *et al.*, 1983). Hodgkin's disease has been reported in 18–22% of all lymphomas from mainland China (Harrington *et al.*, 1987; Yan *et al.*, 1991), but the real figure may be lower than this because of the probable tendency to include PTLs in this group in the absence of immunophenotypic analysis (unpublished observations).

In order to study the association between EBV and HD in a developing country, we examined 28 cases of HD from mainland China for expression of EBER-1 and LMP-1 using RNA ISH and immunohistology, respectively. The EBV-positive HRS cells were found in 17/28 cases (61%), with excellent agreement between the results of analysis with the two techniques. The EBV gene expression in HRS cells varied according to the histological subtype, being detected in 10/11 HD-MC (91%), 6/14 HD-NS (43%), 0/1 HD-LP, 0/1 lymphocytic depletion, and 1/1 unclassified HD. In 5 out of 12 cases with EBER and LMP-1 negative HRS cells, occasional small, medium, and large (transformed) lymphoid cells, which we believe belong to the normal reservoir of nonneoplastic EBV-containing cells, expressed EBER-1 but not LMP-1. Thus, the overall frequency as well as the subtype distribution (MC>NS) of Chinese EBV-positive HD is similar to that found in developed countries.

3. EBV-Positive HD in Immunodeficiency States

Development of HD in HIV-seropositive patients is being reported with increasing frequency (Ree *et al.*, 1991; Pelstring *et al.*, 1991). These tumors appear to be clinically more aggressive, carry a worse prognosis, and respond poorly to therapy, compared with HD in HIV-seronegative patients. Recently, EBV was shown to be more strongly associated with HD in HIV-seropositive compared with HIV-seronegative patients. Uccini *et al.* (1990) detected EBV DNA in 5/7 AIDS-associated HD cases by Southern blotting and DNA ISH. Audouin *et al.* (1992) found immunohistological expression of LMP-1 in HRS cells in each of 18 AIDS-associated HD cases.

The rarity with which HD is reported in immunosuppressed organ transplant recipients (Penn, 1984) and in patients with primary immunodeficiencies suggests that if impaired immunity is important in the pathogenesis of HD then it is likely to be highly selective. Interestingly, Mueller *et al.* (1989) have pointed out that the EBV antibody pattern seen in HD patients (in particular increased anti-EBNA and IgG/IgA anti-VCA titers in association with low levels of IgM anti-VCA antibodies) is different from that seen in immunosuppressed graft recipients developing EBV-associated B cell lymphomas (in whom there is a low titer to EBNA but a detectable IgM anti-VCA response). This implies

that different changes in host control of EBV may be associated with the development of different diseases.

Relatively few cases of HD developing in primary immunodeficiency have been investigated for an association with EBV. However, a recent survey on archival biopsy material from the Immunodeficiency Cancer Registry (Minnesota) disclosed HD in 3/3 patients with hyper IgM syndrome and in 2/5 patients with ataxia telangiectasia. All 5 cases of HD were EBER ISH positive (Ambinder *et al.*, 1992).

These data suggest that certain immune defects may be predisposed to EBV-associated HD, whereas other immunodeficiency states (e.g., post-organ transplant therapy, cancer chemotherapy, severe combined immunodeficiency, and Wiskott–Aldrich syndrome) increase the risk of EBV-associated NHL rather than HD.

Because immunodeficiency-associated HD cases are rare, multicentric studies will be required in order to establish the role (if any) of EBV in their pathogenesis. The technical advances that have made possible detection of EBV gene expression in paraffin sections now allow such studies to be performed retrospectively on archive material.

4. Association of EBV-Positive HD with Age

Jarrett *et al.* (1991) reported an excess of EBV-positive HD cases in young (less than 15 years) and older (more than 50 years) patients compared with young adults. They believed that their results supported the hypothesis that HD is a heterogeneous condition with different etiologies in different age groups. Other groups have not, however, found an association between age and the frequency of EBV-positive HD in Caucasians (Boiocchi *et al.*, 1989; Libetta *et al.*, 1990; Herbst *et al.*, 1990; Coates *et al.*, 1991; Vestlev *et al.*, 1992; Weinreb *et al.*, 1992). That the rate of EBV-positive HD in children appears to be at least equal to that in adults is by itself of interest. Since a substantial proportion of children remain EBV seronegative up to the age of 15 years in Western countries, Weinreb *et al.* (1992) have argued that the finding of similar rates of EBV-positive HD at all ages suggests that EBV infection of HRS cells is not coincidental but rather indicates a specific etiological role for the virus.

In our study of HD from mainland China, there was a tendency for EBV-positive HD to be associated with age. Thus, in those HD cases in which the age of the patient was recorded, 5/5 cases (100%) were EBV positive in patients under 20 years of age, compared with 8/15 cases (53%) in patients over 20 years of age. However, the number of cases in these groups was too small to allow statistical analysis of the significance of this trend.

5. Correlation between EBV (HRS Cell) Positive HD and Serology

Given that HD patients frequently show abnormal patterns of EBV serology (see Section V,A,2), it would be of interest to compare EBV gene expression in HRS cells with serology in individual patients.

Brousset *et al.* (1991a) found no correlation between the presence of EBV DNA in HRS cells in HD shown by ISH and patient serology.

Similarly, in preliminary studies we have found no correlation between EBV antibody titers and expression of EBV genes in a series of HD cases from Massachusetts General Hospital ($N = 18$; LMP-1 studied in archive biopsy material) (N. Mueller, N. L. Harris, and G. Pallesen, unpublished observations, 1992) or cases from the Danish LYGRA-series ($N = 25$; LMP-1 and EBER-1 examined in archive biopsy material) (G. Pallesen, P. Ebbesen, P. Levine, and A. M. Nordentoft, unpublished observations, 1992).

G. Influence of EBV Infection on the Prognosis of Hodgkin's Disease

Brousset *et al.* (1991a) found no difference in response to chemotherapy, relapse rates, or survival comparing EBV-positive (as defined by DNA ISH) and -negative HD. The median follow-up period was only 20 months.

Similarly, Vestlev *et al.* (1992) compared LMP-1 expression with clinical parameters such as sex, age, systemic symptoms, clinical stage, number of tumor sites, mediastinal involvement, plasma copper, LDH, and ESR. The LMP-1 expression was significantly associated with only the histological subtype (Vestlev *et al.*, 1992). However, the median follow-up period for EBV-positive and -negative cases (13 months and 22 months, respectively) was rather short for a tumor such as HD, and further studies will be required to resolve this issue.

Interestingly, Weinreb *et al.* (1992) found that LMP-1 expression in HRS cells was independently associated, not only with MC subtype morphology but also with clinical stage IV disease.

Fellbaum *et al.* (1992) have also shown that the presence of EBV DNA in HD cases did not influence the prognosis of the disease. However, EBV infection in these HD cases was not defined by *in situ* methods. Thus, the amplified EBV PCR signal obtained could have been derived from normal rather than neoplastic cells.

H. Conclusion: The Significance of EBV Infection in Hodgkin's Disease

It is now evident that the following conclusions can be drawn: (i) HRS cells are in a substantial fraction of HD cases infected with EBV. (ii) The infecting virus is usually monoclonal. (iii) Infected HRS cells show transcription of the EBV genome with translation of viral proteins. (iv) The infection is predominantly latent, with a distinctive (latency II) pattern of gene expression. (v) At least one of the products of EBV gene expression (LMP-1) in HRS cells is potentially oncogenic and immunogenic. (vi) EBV infection is associated with the histological subtype of HD (MC>NS>LP).

There is not yet proof that EBV plays a role in HD pathogenesis. However, the evidence detailed previously suggests strongly that this is the case and that HD should be added (albeit conditionally) to the list of EBV-associated tumors. This is a remarkable state of affairs when one considers that it is little more than 5 years since the first definite report that EBV DNA could be detected in HD tissues (Weiss *et al.*, 1987).

There is, however, much that is unclear concerning the relationship between EBV and HD. The PCR studies show that EBV is present in a large proportion of HD cases, but these results cannot be used to determine how many tumors contain the virus in HRS cells and how many have the virus confined to reactive lymphocytes. The combined results of Southern blot, DNA and RNA ISH, and single-cell PCR (Teramoto *et al.*, 1992) studies indicate that about one-half of HD cases from different epidemiological populations show HRS cell EBV infection. Two possibilities exist regarding those cases that do not appear to be associated with HRS cell EBV infection: (i) that HRS cells in these cases are truly EBV negative, and (ii) that HRS cells in these cases are also infected with EBV, but that this cannot be demonstrated using available methods of virus detection.

That the first possibility is true for at least some HD cases is strongly suggested by the fact that: (a) All PCR studies reported to date include a fraction (albeit sometimes small) of HD cases with no evidence of EBV DNA in their tissues. (b) Studies show that HD occurs in some patients who have no serological evidence of previous EBV infection (Johansson *et al.*, 1970; Mueller *et al.*, 1989).

With regard to the second possibility, present techniques of DNA ISH are not sufficiently sensitive to ensure detection of all HD cases containing EBV positive HRS cells (unpublished observations). However, Deacon *et al.* (1993) showed that HD cases without *in situ* evidence of EBV

gene expression (i.e., negative for LMP-1 and EBER-1 by immunohistology and RNA ISH, respectively) were also consistently negative for EBV gene expression when analyzed by reverse transcription PCR. Although this study was performed on a relatively small series of cases, it would appear to exclude technical artifact as an explanation for the failure of *in situ* methods to detect EBV gene expression in HRS cells in such a large proportion of HD cases.

It is conceivable that occasional HD cases might exist containing EBV genome-positive HRS cells in which there is no expression of viral genes. Indirect support for this possibility comes from the observation that in many HD cases with EBV-positive HRS cells, not all such cells are either LMP-1 or EBER positive [although Herbst *et al.* (1992) disagree with regard to EBERs], and the intensity of gene expression often varies between positive cells within a single tissue section (unpublished observations). If, as seems probable, EBV gene expression depends on both viral and host factors (for example, the cell cycle of HRS cells), then some HRS cells might not show viral transcription. However, it is difficult to imagine a situation in which all HRS cells in a given tumor should completely down-regulate viral gene expression. It is generally held that latent EBV infection is invariably associated with EBER expression, although exceptions to this rule might conceivably occur.

Thus, even though it is formally possible that HD cases could exist in which all EBV-infected HRS cells were silent for viral transcription (presumably with the exception of EBNA-1), we believe that this must be, at most, a rare occurrence. Therefore, present evidence indicates to us that EBV is genuinely absent from HRS cells in the great majority of the one-half of HD cases that are negative for HRS cell EBV gene expression. Although HD-MC cases are more likely to be EBV-associated than other subtypes, no absolute phenotypic (or clinical) features that can be used to distinguish between EBV-positive and -negative tumors have so far been reported. This is an important observation because it shows that the virus cannot be regarded as an absolute prerequisite for HD oncogenesis. Clearly, HD may comprise a range of lesions of varying etiology (some associated with EBV), all of which result in an almost similar clinical and morphological picture. As noted by Klein (1992), the situation in HD is not unlike that found in BL in which the endemic lymphoma is highly associated with EBV, whereas only a minority of the morphologically indistinguishable nonendemic lymphomas are infected. In BL a common link between EBV-positive and -negative lymphomas is the invariable presence of Ig/*myc* translocation. It remains to be seen whether an analogous unifying factor can eventually be identified in all HD cases.

The pathogenesis of HD cases not associated with EBV remains unknown. The association of EBV-positive HD with particular age groups remains unclear (see Section V,F,4). However, it is interesting to note the trend for HD in young adults to be less frequently associated with EBV, in spite of the fact that this was the group originally thought to be most likely to be caused by a virus on epidemiological grounds. This suggests the intriguing possibility that another common virus may in fact be involved in the development of HD in these patients.

The contribution made by the virus to the development of those cases associated with EBV infection is unknown. It is tempting to speculate that LMP-1 expression might confer a growth advantage on HRS cells. If this is so, then preliminary studies (albeit with short follow-up times) have found no evidence that it affects the eventual prognosis of the associated cases. It is possible that LMP-1 expression induces a cytotoxic T cell response against the host HRS cells (Murray *et al.*, 1988), which counterbalances any growth advantage provided by the protein. The high frequency of LMP-1-positive HD cases in AIDS (Audouin *et al.*, 1992) lends some support to this possibility. An important part of future studies will be to determine prospectively the prognostic influence of the presence of both EBV DNA and EBV gene expression in HRS cells and to analyze the immune status of these patients.

The pathogenesis of HD is certainly complex, and EBV is probably only one of a number of possible factors involved in the development of the disease. However, the possibility that prevention or treatment of some cases of HD might be feasible using EBV as a target for antiviral therapy or vaccination should be pursued.

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THE ROLE OF DIRECT CELLULAR COMMUNICATION DURING THE DEVELOPMENT OF A HUMORAL IMMUNE RESPONSE

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I. Introduction

The induction of B cell proliferation and differentiation requires the direct, physical interaction and communication between a B cell and an activated, T helper (Th) cell. In this manner, sequential biochemical signals are delivered in an ordered fashion to a resting B cell, promoting the expression of gene products necessary for B cell cycle progression. These two cell types communicate with one another through the action of counterreceptors. These receptors are integral membrane proteins whose ligands are receptor proteins expressed upon the surface membrane of the opposed cell. The co-ligation of these counterreceptors is transient, but delivers biochemical signals to both the B and Th cells. Typically, signals emanating from one set of counterreceptors recruit the next set of receptors into the process allowing the two cells to continue a constant dialogue with each other. This process is initiated following the appearance of antigen that both the B and Th cells recognize via antigen-specific receptors expressed upon their membranes. The occupancy of these receptors by antigen does not, by itself, provide all the

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biochemical signals necessary for eliciting cell growth and/or differentiation. However, these receptors are essential for allowing the selection of the appropriate B and Th cell clones for participation in the developing immune response. The efficient selection by antigen of lymphocytes for inclusion in this response requires the involvement of structural and functional features of secondary lymphoid organs. These anatomical sites of lymphocyte localization facilitate antigen capture by phagocytic cells and promote the ability of antigen-reactive lymphocytes to interact with each other. This review focuses upon several interrelated models that highlight the importance of secondary lymphoid tissue function and cellular interactions during the development of a thymus-dependent (TD) humoral immune response.

II. Contributions of Lymphoid Tissue Architecture

Immune responses occur in secondary lymphoid organs, such as lymph nodes and spleen. These organs act as filtration units for tissue-derived fluids (lymph nodes) and blood (spleen), as well as sites at which lymphocytes and macrophages preferentially localize. There are structural components of secondary lymphoid tissues that facilitate the trapping of pathogens required to initiate the response and help the appropriate antigen-reactive B and Th cells find one another. For the purposes of the present discussion, we will utilize a typical lymph node to illustrate relevant features of secondary lymphoid tissues essential for the successful development of a humoral immune response.

Antigens found in tissue fluids enter the lymphoid system by draining into a lymphatic vessel. These vessels eventually empty into the subcapsular sinus of a lymph node (see Fig. 1). This entrance is lined with macrophages that phagocytose most of the antigenic material coming in from the periphery. The phagocytosed antigen is subsequently degraded within the lysosomal vesicular system of the macrophage, with some of the resultant peptides transported back to the surface in association with MHC class II molecules (Rothbard and Gefter, 1991). This allows the macrophage to function as an antigen-presenting cell (APC) for Th cells (Fig. 2), resulting in the expansion and differentiation of the antigen-reactive Th cell clones into more mature effector cells (see Section III). Simultaneously, B cells expressing antigen receptors [membrane immunoglobulin (mIg)] selectively take up and process antigen, and similar to macrophages, display antigenic peptides upon their surfaces in association with MHC class II molecules (Watts *et al.*, 1991). These antigen-presenting B cells can now physically associate with appropriate effector Th cells and receive the necessary signals to enter the

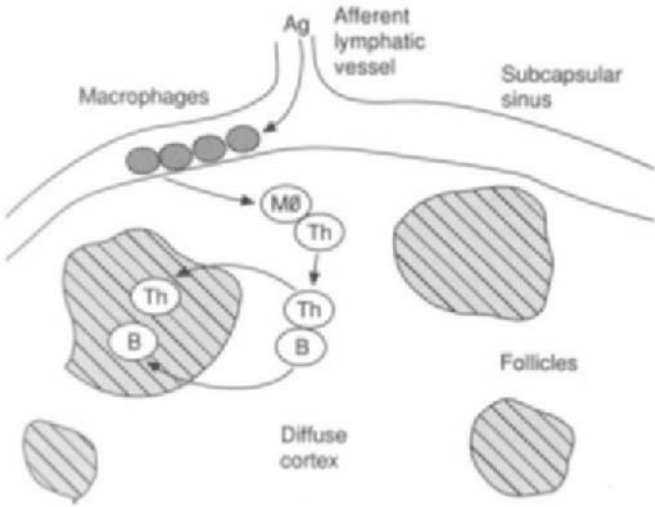

FIG. 1. Schematic of a lymph node. Antigen enters the lymph node via the afferent lymphatic vessel and immediately encounters macrophages in the subcapsular sinus. Some of the antigen-laden macrophages function as antigen-presenting cells for Th cells found within the diffuse cortical regions. The activated Th cells differentiate into more mature helper cells (effector Th cells) that can provide help to appropriate B cell clones. This occurs following the uptake of antigen by B cell clones that can now, themselves, function as antigen-presenting cells. This process results in the activation of B cell proliferation. Some of the B cell blasts and effector Th cells migrate into a follicle where B cell expansion and differentiation continues. Direct cellular interactions important during B cell activation occur in the diffuse cortex (between antigen-presenting macrophages and naive Th cells and between antigen presenting-B cells and effector Th cells) and within the follicle (between antigen-presenting B cell blasts and effector Th cells).

proliferative cycle. Subsequently, most of the proliferating B cells and some of the effector Th cells migrate from the diffuse cortex into a follicle where memory and effector B cells develop.

One of the remarkable events that occurs during this process is that the appropriate antigen-reactive B and Th cells find each other in order to form a conjugate. Although the frequency varies for each antigen, only one in a million or so B or Th cells will express an antigen receptor specific for any particular antigen. Also, most of the clones that can bind the antigen early in the response do so with intermediate to low affinity, which further hinders the ability of appropriate antigen-reactive lymphocytes finding one another. These odds are markedly reduced by structural and functional features of secondary lymphoid organs. First, sites at which lymphocytes localize (such as the diffuse cortex of the lymph node) structurally consist of dense networks of reticular fibers.

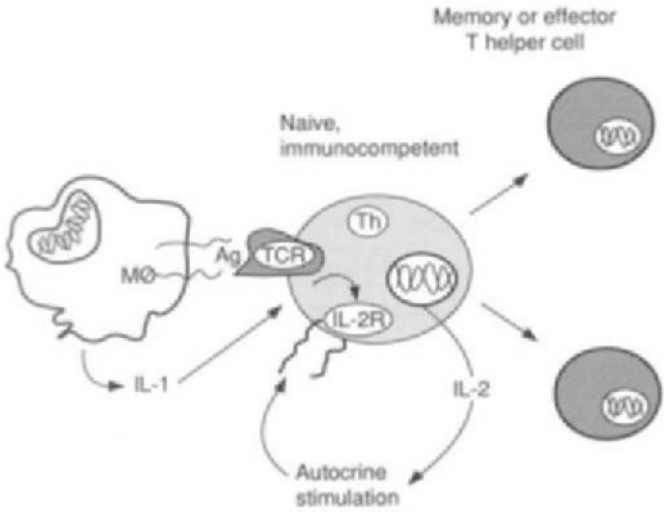

FIG. 2. Activation of naive Th cells. Naive, immunocompetent Th cells are activated by antigen-presenting cells such as macrophages. Growth and differentiative signals are conveyed to the Th cells as a consequence of direct interaction with the APC as well as by soluble mediators, such as IL1, released by the presenting cell. The Th cell enters the early stages of the cell cycle, produces IL2, and expresses high-affinity IL2 receptors allowing IL2 to function as an autocrine growth factor. The Th cells subsequently proliferate, with some of the cells differentiating into effector Th cells.

These fibers retard the movement of cells through the lymphocyte-enriched regions, increasing the probability that cells of like specificity find one another. In addition, during an active response in a lymph node there is an increase in blood flow into the node and a retardation of lymphocyte exodus from the node. Both of these physiologic changes increase the likelihood of antigen-mediated cellular interactions and are probably initiated after the release of neuropeptides from sensory and autonomic neurons that innervate the lymph node (reviewed by Snow, 1990). The trigger for this process may be the stimulation of unmyelinated sensory neurons by chemical mediators released from actively phagocytosing macrophages (the first cellular activity found in the node after antigen entry). Some of these neuropeptides are vasodilators that affect both the increased blood flow into the node as well as the increased extravasation of lymphocytes into the nodal cortex. Autonomic feedback loops triggered after stimulation of the sensory neuron can constrict the efferent lymphatic vessel, thus decreasing the ability of cells to leave the node. The consequence is that more cells are confined at the

site of antigen challenge, resulting in the familiar enlargement of active nodes. Because of these structural and functional features, migrating B and T cells "percolate" through the lymph node and bounce off one cell after another. The slow movement of lymphocytes through the node allows them to form low-affinity contacts continuously with other cells (Noelle and Snow, 1990, 1991). This surveillance activity enhances the chance of Th cells finding their appropriate APC (see Sections III and IV).

III. Activation of Th Cells

The model previously presented suggests that Th cells become activated, through interacting with a "professional" APC such as a macrophage, prior to serving a helper role during B cell activation. These antigen naive, immunocompetent Th cells express surface membrane receptors for antigen (TCR), which are associated with the CD3 complex of proteins (together these proteins comprise the TCR complex), CD4 molecules, and the high molecular weight form of the leukocyte common antigen, CD45 ($CD45R^{hi}$) (Bottomly, 1989). Activation of these naive Th cells requires the delivery of multiple signals by an APC along with the involvement of the soluble mediator interleukin 1 (IL1) (Mackay, 1991; Jenkins and Miller, 1992) and eventually results in the conversion of the T cells into ones expressing the low molecular weight form of CD45 ($CD45R^{lo}$). These activated $CD45R^{hi}$ Th cells are capable of secreting only IL2, which functions as an autocrine growth and differentiation factor (see Fig. 2). The cells that result from this first round of antigen contact are referred to as memory or fully differentiated effector cells. Upon second exposure to antigen, these effector Th cells synthesize an array of lymphokines determined, partly, by the nature of the APC itself (Street and Mossmann, 1991). Interestingly, these effector Th cells display activation requirements that differ from those shown by naive Th cells (Luqman and Bottomly, 1992). For example, the presentation of antigen by B cells to naive Th cells results in the induction of T cell anergy, a form of immune unresponsiveness or tolerance (Parker and Eynon, 1991; Fuchs and Matzinger, 1992). B cells function quite nicely as APC for effector Th cells, and in the process they initiate the sequence of events that results in activating their own proliferation and differentiation. As detailed previously, the physiologic changes occurring in the lymph node help the surveilling naive and effector Th cells to locate the appropriate antigen presenting macrophage or B cell (see Fig. 1).

IV. Activation of Naive B Cells

The activation of small resting, naive B cells by TD antigen requires a multistep process involving signals derived by antigen binding to the mIg receptor as well as direct Th cell/B cell contact (Figs. 3–6). The naive B cell leaves the bone marrow expressing both mIgM and mIgD as its antigen-specific receptors along with high levels of MHC class II molecules and a high molecular weight form of CD45 (referred to as B220). Both the mIgM and mIgD receptors display similar abilities to deliver either activation or tolerogenic signals to B cells (Brink *et al.*, 1992). These naive B cells circulate between lymphatics and blood until they either encounter their specific antigen or die. The vast majority of peripheral B cells exhibit a lifespan of less than 2 weeks (Freitas *et al.*, 1986; Udhayakumar *et al.*, 1988). The B cell repertoire is continuously replenished throughout life, with as many as 5×10^7 new B cells produced each day within the bone marrow (Opstelten and Osmond, 1983). The clones that ultimately participate as the functional repertoire are selected as a consequence of the antigens to which an individual becomes exposed.

A. The Membrane Immunoglobulin Receptor Complex

Membrane immunoglobulin (mIg) molecules are devoid of intrinsic signaling capabilities. Therefore, as seen with the TCR complex, mIg must associate within the plane of the membrane with transducer elements capable of informing the cell that its antigen receptors are occupied (see Reth *et al.*, 1991; Reth, 1992). Both mIgM and mIgD associate with disulfide-linked heterodimers composed of a subunit encoded by the *mb-1* gene (referred to as Igα) and a second subunit encoded by the *B29* gene (referred to as Igβ/Igγ). The Igα/Igβ heterodimer functions to both facilitate transport of mIg to the cell surface, as well as a signal-transducing element coupling mIg to the appropriate signaling pathways, partly by linking the receptors to various members of the Src family of protein tyrosine kinases (Lin and Justement, 1992).

Membrane immunoglobulin belongs to the family of protein tyrosine kinase (PTK) receptors. Some members of this family, exemplified by the epidermal growth factor and insulin receptors, possess intrinsic PTK activities (Moolenaar, 1991). Since mIg are devoid of intrinsic PTK, they acquire kinase activity by associating with soluble PTK belonging to the Src family, such as *lyn, fyn, blk,* and *lck* tyrosine kinases (Burkhardt *et al.*, 1991; Yamanashi *et al.*, 1992; Campbell and Sefton, 1992; Leprince *et al.*,

1992). Which PTK associates with the mIg receptor complex at various times is important since these kinases may have nonoverlapping substrate specificities. These details have not yet been addressed experimentally.

The PTK receptors signal cells through the induction of phosphatidylinositol (PI) metabolism (Ullrich and Schlessinger, 1990; Klausner and Samelson, 1991; Cantley *et al.,* 1991; Bolen, 1991), a mechanism clearly utilized by the mIg complex (Cambier and Ransom, 1987; DeFranco, 1987; Snow, 1991). This is initiated by the binding of antigen to mIg resulting in microaggregation of occupied receptors in the plane of the membrane along with other integral membrane proteins such as CD45 (Justement *et al.,* 1991). The CD45 protein is a phosphotyrosine phosphatase that dephosphorylates a phosphotyrosyl residue found at the carboxyl terminus of PTK. Subsequently, the aggregated PTK transphosphorylate each other within their kinase domains, completing the activation of kinase activity. The phosphotyrosyl residue within the kinase domain serves as a binding site for proteins containing Src homology 2 (SH2) domains (Carpenter, 1992). This includes phospholipase C gamma (PLC-γ), which is phosphorylated and activated after binding to a mIg-associated PTK (Hempel *et al.,* 1992; Coggeshall *et al.,* 1992). The metabolism of PI by PLC-γ releases the second messengers inositol trisphosphate (IP_3) and diacylglycerol (DAG), which elicit calcium mobilization and membrane localization and activation of protein kinase C (PKC). The PTK receptors, such as the mIg receptor, also initiate a serine/threonine kinase cascade starting with the Raf-l kinase and ending up with the activation of mitogen-activated protein (MAP) kinase (Pelech and Sanghera, 1992; Woodgett, 1992; Roberts, 1992). Among the other proteins that MAP kinase phosphorylates and activates is the 90-kDa S6 kinase known as RSK (Casillas *et al.,* 1991). Finally, some of the receptor-associated PTK and PKC can phosphorylate cytoskeletal elements (Aderem, 1992) enabling the antigen-induced, microaggregated mIg complexes to physically associate with the cytoskeleton (Braun *et al.,* 1982; Gupta and Woda, 1988; Albrecht and Noelle, 1988). These complexes coalesce into larger aggregates that eventually cap to one pole of the cell, facilitating the uptake of antigen necessary for the B cell to function ultimately as an APC (Watts *et al.,* 1991).

Receptor-induced PI metabolism elicits defined changes in cellular function. The receptor-associated PTK and PKC regulate numerous cellular enzymes and proteins (Cantley *et al.,* 1991; Nishizuka, 1992). Increases in intracellular calcium likewise influence numerous cellular biochemical processes. Also, PKC, MAP kinase, and RSK kinase all affect gene expression by phosphorylating transacting factors (Hunter

and Karin, 1992; Karin and Smeal, 1992), a function associated with the occupancy of mIg (Chiles *et al.*, 1991; J. Liu *et al.*, 1991; Pollok and Snow, 1991; Tilzey *et al.*, 1991). It is important to stress that the receptor-mediated turnover of PI does not deliver a complete growth stimulus to resting B cells (Cambier *et al.*, 1982; Snow *et al.*, 1983; Pike and Nossal, 1984, Alderson *et al.*, 1987; Krusemeier and Snow, 1988). This is in accordance with findings in all the other mammalian systems studied to date (Rozengurt, 1986; Whitman and Cantley, 1988; Schwartz, 1990; Weaver and Unanue, 1990; Moolenaar, 1991). This does not imply that the cellular changes elicited after mIg occupancy are not important to the cell but rather emphasizes that induction of cell cycle progression is a complicated process involving many signaling cascades that turn on and off over a 24-hour period (Hartwell and Weinert, 1989; Murray and Kirschner, 1989; Pardee, 1989; Millar and Russell, 1992). A basic premise of the model being proposed in this chapter is that the induction of cell cycle progression is best accomplished through the direct, physical association and communication between two cells. The conversion of an antigen-binding B cell into an antigen-presenting cell enables that cell to initiate the process by which it becomes activated by interacting with an appropriate antigen-reactive Th cells (Fig. 3). In the following section we present a model describing how the physical association between a Th cell and B cell enables the Th cell to communicate important growth-inducing signals to the B cell.

B. The Role of Th Cells

There is evidence suggesting that migrating T cells continuously form low-affinity contacts with other cells (lymphocytes and macrophages) as they percolate through secondary lymphoid tissues (van Seventer *et al.*, 1991a; Noelle and Snow, 1991). This can be considered as a surveillance function for Th cells and involves the reversible binding of adhesion molecules, such as lymphocyte function-associated antigen (LFA)-l and intercellular adhesion molecule (ICAM)-l, to their respective counterreceptors. These interactions are therefore antigen nonspecific and MHC nonrestricted in nature. As the cells briefly associate with each other, the TCR is closely juxtaposed to surface proteins expressed upon the opposing cell. If the TCR recognizes a peptide presented in association with an MHC class II molecule the dynamics of the cellular contact change dramatically. This process occurs during the initial activation of a naive Th cell by an APC (Fig. 2) and is the means by which the effector Th cell initiates the process leading toward specific B cell activation (Fig. 3).

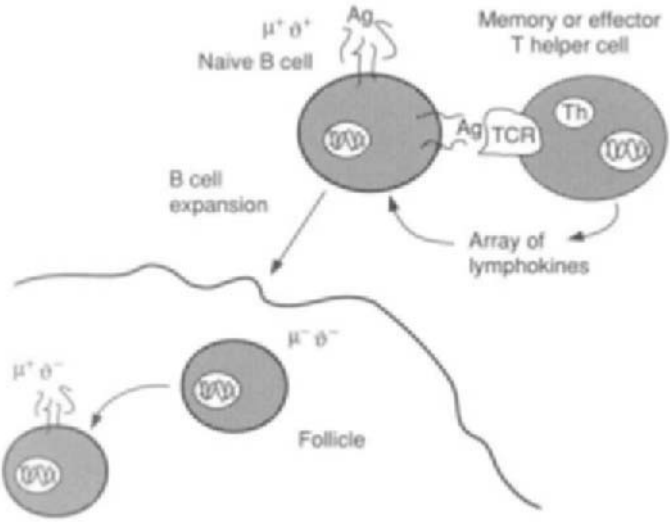

FIG. 3. Activation of naive B cells. B cells expressing specific receptors for antigen (in the form of membrane immunoglobulin) capture antigen, resulting in the antigen being processed and presenting on the B cell surface in association with MHC class II molecules. This process is identical to that mediated by the macrophage, except that only B cells expressing the appropriate antigen receptors are selected to participate in the response. The antigen-presenting B cells form conjugates with an effector Th cell for up to 18 hours during which the B cell is induced to proliferate. Some of the B cell blasts and effector Th cells move into a follicle where the process of B cell expansion and differentiation continues. The naive B cell uses both mIgM and mIgD as antigen receptors. As the cell begins to proliferate, it removes all its antigen receptors and later reexpresses only mIgM.

The physical association between an effector Th cell and a B cell via the TCR recognition of MHC class II-presented peptide on the surface of the B cell initiates the cognate phase of B cell activation (Fig. 4). This cellular interaction can last for up to 18 hours during which time the two cells continuously communicate with each other (Figs. 4–6). This communication occurs through the action of signal-transducing molecules present at the point of opposition. The ability of the two cells to communicate is limited, therefore, by the counterreceptors available upon their surface membranes and by the overall affinity of such receptors for their ligand (i.e., a counterreceptor found upon the opposing membrane). Thus, the high-affinity binding of a set of counterreceptors elicits at least a undirectional biochemical signal. For the communication to continue, this biochemical signal must either alter the binding affinity of a preexisting counterreceptor (via a posttranslational modification) or induce the expression of a new counterreceptor upon the surface membrane.

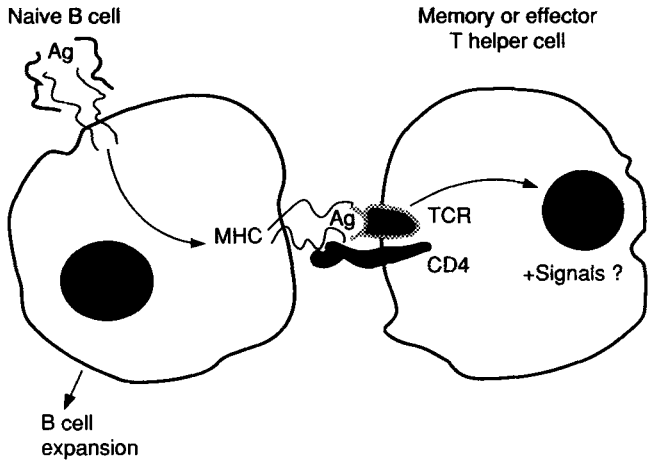

FIG. 4. Step I in the communication process, initiation of conjugate formation between a naive B cell and Th cell. Antigen is captured by a membrane immunoglobulin receptor. The antigen is internalized, processed, and reexpressed in association with MHC class II molecules. The Th cell antigen receptor recognizes the antigenic peptide/MHC class II complex. CD4 is recruited to the point of opposition and binds to a monomorphic determinant on the class II molecule. This results in a signal being delivered to the Th cell.

The appearance of either a new counterreceptor or the enhancement of a preexisting receptor's ability to bind its counterligand results in the elicitation of a biochemical signal in the opposite direction. This ordered delivery of biochemical signals to each cell results in the expression of genes whose protein products are required for both cellular proliferation and differentiation. It is hard to imagine any single ligand–receptor pair being able to accomplish such an important and intricate task as the induction of cell cycle progression.

Assuming the validity of the previous assumptions, the binding of the TCR to the peptide-presenting MHC class II molecules not only increases the stability of the B/Th cell conjugate but must also induce a fundamental change in the counterreceptors present at the point of opposition. The first such change is the recruitment of CD4 molecules into the TCR complex. The CD4 molecule is a T cell integral membrane protein involved during the strengthening of binding between a Th cell and an antigen-presenting B cell. By binding to monomorphic domains of MHC class II proteins (Gay *et al.*, 1987, 1988; Doyle and Strominger, 1987), CD4 helps to increase the avidity of the TCR complex for antigen/MHC class II on the B cell (Janeway *et al.*, 1988) (Fig. 4). The recruitment of CD4 molecules occurs after the initial ligation of the TCR

by class II-presented antigen (Diazani *et al.*, 1992), and the MHC class II molecules promote the aggregation of CD4 proteins to the site of opposition (Mecheri *et al.*, 1991). Because CD4 molecules become temporally and spatially associated with the TCR during antigen activation, Janeway (1988) suggests that they function as coreceptors. The importance of the coreceptor activity of CD4 is highlighted by the observation that the *lck* PTK associates with the cytoplasmic domain of CD4 (Veillette *et al.*, 1988; Rudd *et al.*, 1988). The cross-linking of CD4 activates this kinase (Veillette *et al.*, 1989), and this represents a key biochemical event associated with T cell activation (Glaichenhaus *et al.*, 1991; Oyaizu *et al.*, 1992). Thus, the physical interaction between an antigen-presenting, naive B cell and an effector Th cell promotes the assembly, at the point of opposition, of a complete TCR signaling complex. This elicits a strong signal into the T cell, one of the consequences of which is the cell surface expression of gp39, a newly described counterreceptor (Fig. 5).

The counterreceptor gp39 is the ligand for CD40 (Armitage *et al.*, 1992; Noelle *et al.*, 1992a) and is a 260 amino acid type II membrane protein that exhibits homology to tumor necrosis factor (TNF) (Armitage *et al.*, 1992; Hollenbaugh *et al.*, 1992). The CD40 molecule is a 45- to 50-kDa type I membrane protein constitutively expressed on B cells

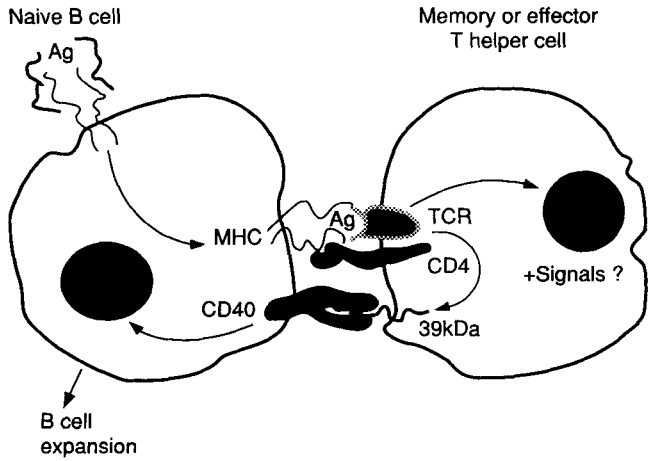

FIG. 5. Step II in the communication process, the production of gp39 by the Th cell. As a consequence of the signal delivered through the TCR (see Fig. 4), the Th cell produces gp39, a new cell surface counterreceptor. The ligand for gp39 is CD40, a counterreceptor constitutively expressed upon B cell membranes. As a result of these changes at the point of opposition, a biochemical signal is returned to the B cell.

(Uckun *et al.*, 1990) and is homologous to members of the nerve growth factor receptor family (Stamenkovic *et al.*, 1989). The cross-linking of CD40 on human B cells by antibody (Valle *et al.*, 1989; Gordon *et al.*, 1989a) or soluble recombinant gp39 (Hollenbaugh *et al.*, 1992) delivers comitogenic signals that help drive resting B cells into the cell cycle. At this time, the biological ramifications of CD40 occupancy are unclear. Although this signal-transducing molecule cannot deliver a complete growth signal (Noelle *et al.*, 1992b), the biochemical pathway(s) elicited from the action of this counterreceptor is intergrally involved with the cell cycle progression of resting B cells. This set of counterreceptors represents one of the fundamental changes at the point of opposition that occurs as a consequence of TCR occupancy. In this case, signals off the TCR elicit the synthesis of a new counterreceptor (gp39) (Fig. 4). The cell surface expression of gp39 allows the Th cell to talk back to its B cell partner by signaling through CD40 expressed upon the B cell membrane (Fig. 5).

The conversation between the B and Th cell continues by the induction of B cell expression of an activation antigen referred to as B7 (Fig. 6). The B7 antigen is a member of the immunoglobulin superfamily (Freeman *et al.*, 1989) and is a 44- to 54-kDa type I membrane protein.

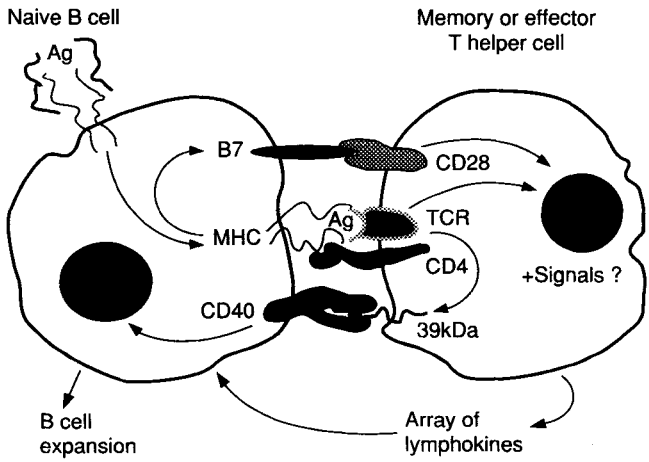

FIG. 6. Step III in the communication process, induction of B7 expression. As the two cells communicate with one another, the B cell is induced to express B7. The ligand for B7 is CD28, a constitutively expressed counterreceptor found upon Th cell membranes. The resultant biochemical signal regulates the synthesis, by the Th cell, of an array of lymphokines. These soluble mediators act as both B cell progression and differentiation factors.

The mRNA for B7 appears 4–12 hours after the induction of B cell activation (Freeman *et al.*, 1989) and parallels the expression of the protein upon the B cell membrane. The expression of B7 is elicited after the cross-linking of MHC class II proteins (Koulova *et al.*, 1991) apparently as a consequence of class II-mediated increases in cAMP (Nabavi *et al.*, 1992). This observation clarifies earlier results that demonstrated that cells transfected with class II proteins with truncated cytoplasmic domains required the exogenous addition of cAMP to effectively present antigen to Th cells (St-Pierre *et al.*, 1989; St-Pierre and Watts, 1991). As mentioned previously cross-linking of MHC class II induces increases in cAMP (Cambier *et al.*, 1987), which also occurs after the direct interaction of paraformalderhyde-fixed, activated Th cells and resting B cells under conditions favoring the cell cycle progression of the B cells (Pollok *et al.*, 1991). These results are consistent with the cell contact-mediated expression of B7 on B cells that are in conjugate with Th cells. The B7 antigen binds to CD28 (Fig. 6) (Linsley *et al.*, 1990), a constitutively expressed homodimer that is a member of the immunoglobulin superfamily (Aruffo and Seed, 1987). The B7 antigen also binds CTLA-4, an integral membrane protein found on T cells, which is a homolog of CD28 (Linsley *et al.*, 1991a). The binding of B7 to CD28 and/or CTLA-4 delivers a comitogenic signal to Th cells (Koulova *et al.*, 1991; Reiser *et al.*, 1992) resulting in an increased synthesis of IL2 (Jenkins *et al.*, 1991; Linsley *et al.*, 1991b). This occurs by causing both an increase in IL2 mRNA stability (Lindsten *et al.*, 1989) and the induction of NF-kB transcription factor activity that binds to an enhancer found 5′ to the *IL2* gene (Fraser *et al.*, 1991; Verweij *et al.*, 1991). This enhancer region is found in front of a number of cytokine genes (Fraser and Weiss, 1992) suggesting that B7 binding of CD28 regulates a number of lymphokines produced by Th cells. Thus, the reciprocal signal to the Th cell elicited after B7 expression on the B cell regulates the synthesis of soluble mediators that can influence both B cell proliferation and differentiation. Signaling through CD28 also elicits an increased expression of the IL2 receptor alpha chain (Cerdan *et al.*, 1992), thus complementing the effects of increased IL2 upon the Th cells. The cross-linking of CD28 mediates the foregoing effects at least in part by activating a PTK distinct from those activated through the TCR, which phosphorylates a 100-kDa cellular substrate (Vanderberghe *et al.*, 1992; Lu *et al.*, 1992). The optimal expression of this CD28-stimulated kinase activity requires a previous signal through the TCR, consistent with this signaling pathway being activated downstream from antigen binding, but as a consequence of B cell–Th cell communication.

An additional set of counterreceptors involved in B cell–Th cell

interactions are CD2, a 50-kDa glycoprotein expressed on all T cells and its ligand LFA-3 (CD58), a 55- to 70-kDa PI-linked membrane protein found on all cells, including B cells (Selvaraj *et al.*, 1987). Optimal signaling through CD2 requires a previous signal through the TCR (Bockenstedt *et al.*, 1988; Alcover *et al.*, 1988). Similar to the TCR, signaling through CD2 activates PI metabolism (Yang *et al.*, 1986; Samelson *et al.*, 1990; Ley *et al.*, 1991). The consequences of signaling through CD2 versus the TCR appear to be quantitative rather than qualitative, suggesting that the cell contact-mediated biochemical process induced through CD2 serves to propagate the same pathway induced after antigen binding to the TCR. This may amplify the signal to levels that exceed background noise (Koshland *et al.*, 1982) and/or extend it in intensity so as to activate important components of the signaling pathway continuously, i.e., various isoforms of PKC. Accordingly, this represents another means for the antigen-presenting B cell to communicate with the Th cell.

Conjugated B and Th cells also communicate through CD5 (Van de Velde *et al.*, 1991), a 67-kDa differentiation antigen expressed by most Th cells (Wang *et al.*, 1980), and its ligand CD72 (Lyb-2), a 45-kDa type II membrane protein expressed by all mature B cells (Sato and Boyse, 1976; Nakayama *et al.*, 1989). The CD5 molecule associates in the membrane with components of the TCR/CD3 complex and with the *lck* and *fyn* protein tyrosine kinases (Burgess *et al.*, 1992). Cross-linking CD5 delivers a comitogenic signal to T cells as a result of increases in tyrosine phosphorylation (Burgess *et al.*, 1992), calcium mobilization (Ledbetter *et al.*, 1985), increased secretion of IL2 and expression of the high-affinity IL2 receptor (Ceuppens and Baroja, 1986; June *et al.*, 1987). Cross-linking CD72 on B cells delivers costimulatory signals that participate in B cell cycle progression (Subbarao and Mosier, 1983, 1984; Snow *et al.*, 1986). Occupancy of CD72 elicits PI metabolism (Grupp *et al.*, 1987) and may be involved in a cell contact-mediated propagation or amplification of the PI response induced after antigen binding to the mIg receptor found upon the B cell. The recruitment, therefore, of this pair of counterreceptors at the point of opposition provides another example of bidirectional communication between the B and Th cells.

Finally, a number of cell surface adhesion molecules participate both in strengthening the bound between the B and Th cells and in delivery of biochemical signals to both cells. The first direct evidence for this was provided by Dustin and Springer's (1989) demonstration that occupancy of the TCR results in the transient increase in the affinity of LFA-1 molecules for their counterligand, ICAM-1. Since this seminal observation, occupancy of additional membrane-associated molecules such as

CD2 (Van Kooyk *et al.*, 1989), MHC class II (Mourad *et al.*, 1990) and CD44 (Koopman *et al.*, 1990) have all been shown to be capable of transiently increasing LFA-1 avidity for ICAM-1. Similiarly, the binding of antigen by mIg receptors on the B cell can elicit an increase in B cell-expressed LFA-1 affinity (Hedman and Lundgren, 1992). Thus, LFA-1 molecules found on both cells in the conjugate may participate in strengthening the bond between them. Occupancy of the B and Th cell antigen receptors mediate this change in LFA-1 affinity by the PKC-dependent phosphorylation of the LFA-1 beta subunit (Hibbs *et al.*, 1991; Pardi *et al.*, 1992), leading to the association of the LFA-1 heterodimer with the cytoskeleton (Pardi *et al.*, 1992). The attachment of these integrins to the cytoskeleton may serve to redistribute LFA-1 molecules to the point of opposition, therefore increasing the number of adhesion molecules available at the site of opposition. Finally, the elevated affinity of LFA-1 for its ligand can be maintained long term in a cAMP-dependent manner (Haverstick and Gray, 1992). The physiologic significance of this can be found by the observation that binding of the TCR to B cell-expressed MHC class II molecules elicits production of cAMP (Cambier *et al.*, 1987; Bishop, 1991). This together with results indicating that cross-linking class II proteins increases LFA-1 affinity for its ligands (Mourad *et al.*, 1990) suggest that signaling through class II is responsible for maintenance of high-affinity LFA-1 binding potential. The ability of both LFA-1 and ICAMs to serve as signal-transducing molecules (Geissler *et al.*, 1990; van Seventer *et al.*, 1991b; Moy and Brian, 1992; Damle *et al.*, 1992; Fischer *et al.*, 1992; Galandrini *et al.*, 1992) suggests that their occupancy contributes to biochemical processes required for B cell cycle progression (Mazerolles *et al.*, 1988; Tohma *et al.*, 1991; Owens, 1991).

This section has dealt with a number of integral membrane proteins that are found on B and Th cells and function as counterreceptors. The sequence in which these receptors are recruited into the process of B and Th cell activation remains to be clarified, but one possible scenerio for some of the receptors is shown in Figs. 4–6. There are undoubtedly additional pairs of counterreceptors to be discovered. They will participate at some point during the 16–18 hours that the B and Th cells remain in conjugate. The ability of these pairs of counterreceptors to bind each other will be transient in nature but may occur multiple times during the activation cascade. In this way, the signaling pathways controlled by one or the other counterreceptor can affect the expression of genes at more than one point during the process. One possible outcome of this is that there will be an ever-changing cast of transacting factors temporally available during growth induction. This means that the elic-

itation of the same signaling pathways at these different points in the process has dramatically different effects upon which genes are transcribed. Any interruption in this dialogue between the two cells will result in either the development of an anergic state or the initiation of apoptosis. As described earlier, regulating cellular entrance into the cell cycle by direct communication between two cells offers many advantages over such a critical commitment being induced as a consequence of occupancy of a single receptor, such as mIg. The most important advantage is protection of the biological system from the spurious recruitment of resting cell expansion. Finally, as the B cells enter the G_1 stage of the cell cycle, lymphokines released by Th cells are required for the movement of the cells through G_1 and into the S stage of the cell cycle (Fig. 6). The lymphokines release is dependent on how antigen is presented to the Th cell, and this in turn will determine both the extent of proliferation of the B cell clones and the isotypes ultimately secreted by the B cells differentiating into antibody-secreting cells. For example, in murine systems optimal proliferation of Th cell-activated B cells requires the presence of IL4 (Killar *et al.*, 1987; Boom *et al.*, 1988; Hodgkin *et al.*, 1990; Noelle *et al.*, 1991). Interleukin 4 can be considered, therefore, as a progression factor during at least the initial pass of B cells through the cell cycle.

V. Th Cell Regulation of Follicular B Cell Survival

The proliferating B cell blasts move from the diffuse cortex into a follicle (Fig. 3) where they form a mass of proliferating cells referred to as a germinal center (GC). These dividing cells are devoid of mIg (Butcher *et al.*, 1982; George and Claflin, 1992) and have begun the process of hypermutation at the immunoglobulin variable region locus (Berek and Milstein, 1987). Clearance of the original antigen receptors from the membrane during the process of somatic mutation prevents the cell from simultaneously expressing receptors differing in their variable regions. These proliferating cells eventually replace their mIgM but not mIgD upon their surfaces (Fig. 3 and 7); however, the mIgM is expressed at lower levels (Butcher *et al.*, 1982). The loss of mIgD as a coreceptor, along with the reduced level of mIgM probably contributes to the process of maintenance of only high-affinity clones within the response (see below; MacLennan and Gray, 1986, George and Claflin, 1992).

Cellular processes that involve selection typically utilize apoptosis as a means for eliminating cells that do not fit criteria established for survival (Williams, 1991). Thus, GCs are sites that exhibit high rates of cellular

death (Nieuwenhuis and Opstelten, 1984). In support of this are the observations showing that only a small percentage of cells entering the GC rejoin the recirculating pool (Gray *et al.*, 1990) and that cells obtained from the GC quickly undergo apoptosis *in vitro* (Liu *et al.*, 1989). In summary, the GC is the site at which clones bearing receptors of the highest affinity for antigen are propagated, while all other clones initially recruited into the response undergo apoptosis.

The selection of B cell clones for survival within a GC depends on both the mIg receptors expressed on the B cells as well as the effector Th cells present within the follicle (Fig. 7). The reappearance of mIgM enables antigen to participate during this selection process. In the case of Th cells, their presence is required for the development of GC both *in vivo* (Jacobsen *et al.*, 1974; Klaus and Kunkl, 1982; Vonderheide and Hunt, 1990) and *in vitro* (Tew *et al.*, 1990; Kosco *et al.*, 1992). In the *in vitro* system described by Tew and colleagues, soluble Th cell-derived factors do not replace intact Th cells, suggesting a requirement for direct cellular interactions during GC formation. This possibility is supported by experimental findings showing that the prolonged growth and

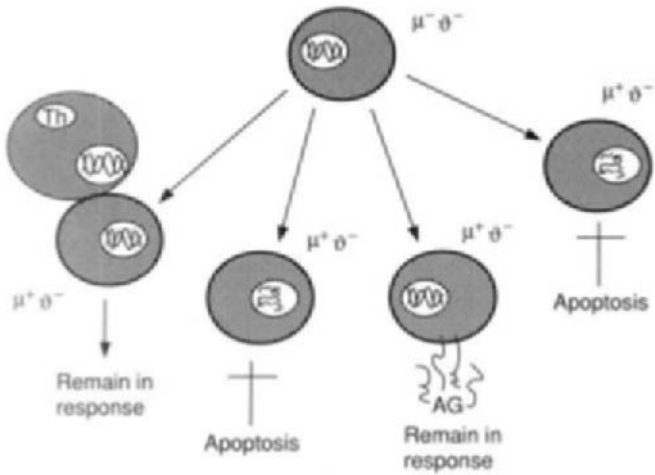

FIG. 7. The fate of B cell blasts within the follicle. The membrane immunoglobulin negative B cell blasts that enter the follicle eventually reexpress mIgM but not mIgD. This represents a key decision point for the B cell. If no further signals are received, the B cell blast undergoes programmed cell death (apoptosis). For the B cell blast to survive requires the cell to be able to capture antigen, process and reexpress antigenic peptides in association with MHC class II molecules, and interact with an effector Th cell. The resultant signals either continue the B cell expansion or elicit B cell differentiation. This process allows for the selection of only high-affinity B cell clones for maintenance in the response.

differentiation of activated B cells requires the regular delivery of signals through contact with effector Th cells (Rajasekar *et al.*, 1987, 1988; Finkelman *et al.*, 1992). Presumably, mechanisms similar to those described in the previous section are involved during this stage of Th cell regulation of B cell survival, expansion, and differentiation.

The *in vitro* cultivation of GC B cells isolated from human tonsils has proven useful to study the signals involved with survival of activated B cell clones (Liu *et al.*, 1989). As mentioned previously, these cells spontaneously undergo apoptosis unless they are rescued by appropriate environmental signals. The addition of immobilized, but not soluble, anti-IgM (or anti-IgG) blocks apoptosis for several days (Liu *et al.*, 1989). Cell survival can be extended further if the anti-IgM-stimulated blasts are simultaneously stimulated through their CD40 surface glycoproteins. In addition, the engagement of CD19 provides a modest survival signal to GC B cells (Holder *et al.*, 1992). Soluble CD23 (the low-affinity IgE receptor) and IL1 not only promote survival of GC B cells, but they also direct a portion of cells to differentiate into antibody secreting cells (Y. J. Liu *et al.*, 1991). During Th cell–B cell interactions, CD23 expression is induced upon B cell membranes as a consequence of signaling through CD72 (the CD5 counterreceptor) (Katira *et al.*, 1992). The counterreceptor for CD23 is CD21, the complement-2 receptor (Aubry *et al.*, 1992). There is evidence for a role of B cell-expressed CD23 interacting with Th cell-expressed counterreceptor (presumably CD21) during Th cell control of B cell proliferation (Shields *et al.*, 1992).

Although much remains to be revealed, one possible interpretation of these studies follows. After reexpression of mIg receptors (early in the response in the form of mIgM) the proliferating B cells that migrate into the follicle must receive additional signals to survive. The initial signal is antigen binding to the newly reexpressed antigen receptors resulting in further stimulation of only the higher-affinity clones. This probably occurs as a consequence of B cells binding to antigen presented upon the surface of follicular dendritic cells (FDC) (Tew and Mandel, 1978, 1979). This physical interaction with the FDC also provides the opportunity for B cells to engage CD23 expressed by the FDC (Gordon *et al.*, 1989b). Subsequently, further signals are delivered by Th cells that have relocalized into the GC. Some of the Th cells physically interact with the GC B cells, utilizing counterreceptors to communicate with the B cell blasts similar to that described previously for Th cell–naive B cell interactions. Although the details are still poorly understood, the presence of Th cells within the GC provides circumstancial evidence for a role for these cells in regulating GC B activation. This involvement of Th cells

occurs after antigen engagement of the newly reexpressed mIgM receptors and is probably initiated by B cell blasts acting as APC. Importantly, Th cells that physically associate with antigen-presenting GC B cell blasts release lymphokines that help to determine the isotypes of the specific antibody ultimately produced.

VI. Th Cell Regulation of B Cell Isotype Switching

At the beginning of a humoral immune response, the predominant isotype expressed is IgM. Later in the primary response there is the progressive appearance of additional Ig isotypes resulting from the responding B cells undergoing isotype switching. This represents an important component of the humoral response because each isotype possesses unique capabilities that serve to enhance the effector capabilities of the organism against extracellular pathogens. During a primary humoral response, isotype switching occurs within an active germinal center and requires the participation of Th cells (Kraal *et al.*, 1982; Butcher *et al.*, 1982; Apel and Berek, 1991).

Class switching occurs after a somatic rearrangement of the heavy chain gene locus such that the VDJ segment is brought 5′ to the newly selected heavy chain isotype locus (Gritzmacher, 1989). Prior to undergoing this rearrangement, the selected heavy chain isotype locus becomes transcriptionally active resulting in the appearance of sterile heavy chain transcripts (Lutzker *et al.*, 1988; Stavnezer *et al.*, 1988; Rothman *et al.*, 1988; Berton *et al.*, 1989; Esser and Radbruch, 1989; Severinson *et al.*, 1990). During the induction of class switching, the chromosomal location of the selected locus appears to open up and become active, allowing the switching machinery access to this region of DNA. This process is regulated by both cell contact between B and Th cells and Th cell-derived lymphokines. At present, there are two possible scenarios explaining how Th cells induce class switching. In the first, cell contact-mediated biochemical signals delivered to B cell blasts in conjugate with a Th cell enable a lymphokine-derived signal to initiate transcription of sterile transcripts of a new heavy chain Ig locus (Noelle *et al.*, 1992c). In other words, the cell contact-delivered signal serves to keep the cells in cycle, thus allowing a lymphokine to induce the differentiation of some of the cycling cells. In at least one experimental system, soluble mediators responsible for providing a differentiative signal to B cells do so by acting upon B cells traversing the G_2 stage of the cell cycle (Brooks *et al.*, 1985). In the second possibility, cell contact delivers the differentiative signals resulting in the induction of transcription of ster-

ile transcripts (Schultz *et al.*, 1992). In both cases, a lymphokine is responsible for inducing the subsequent somatic rearrangement bringing the new heavy chain locus near the variable region locus.

VII. Conclusions

Secondary lymphoid organs are designed to facilitate the efficient capture, processing, and presentation of invading pathogens by cells of the immune system. These organs are compartmentalized, such that certain cell types perferentially localize to descrete regions. For example, phagocytic cells line the subcapsular sinus of lymph nodes and are ideally located to phagocytose antigens coming in from the surrounding tissues. These phagocytes subsequently contribute during the initiation of the humoral response in at least two important ways. First, noxious chemicals released as the cells phagocytose antigen stimulate the central nervous system resulting in physiologic changes to the lymphoid organ's vasculature. As a result, more lymphocytes are brought to the site of the response. The central nervous system also constricts the efferent lymphatic vessel, thus reducing the number of cells exiting the node. Second, the phagocytic cells serve as APC and help to select naive Th cell clones for expansion and differentiation to effector helper cells. This occurs in the diffuse cortex of the lymph node, a compartment situated right next to the subcapsular sinus. The diffuse cortex is structurally defined by a dense network of reticular fibers so that cells cannot easily migrate through. Rather, the migrating cells are continuously detained by the fibers. As the influx of the cells increase and the efflux decreases, these impediments to cellular nodal travel increase the probability that lymphocyte clones sharing reactivity to the captured antigen find one another. Also found in the diffuse cortex are naive B cells. Unlike Th cells, B cells can directly bind soluble antigen, which they process and, similar to phagocytic cells, present to Th cells. However, they can serve only as APC to fully differentiated Th effector cells. As B cell clones are selected to join the developing response, some of the proliferating B and Th cells migrate to a third compartment referred to as a follicle. Follicles are sites of intense B cell proliferation and differentiation. The Th cells and their lymphokines are integrally involved during this final phase of the humoral response.

A central tenet of this discussion maintains that direct cellular communication between an effector Th cell and antigen-binding B cell regulates each stage of the process by which B cells are induced to proliferate and differentiate into antibody-secreting cells. The decision to enter the proliferative cycle is one the B cell cannot make lightly. All cells are

bombarded in a continuous fashion by imputs from their environments. To protect the cell from erroneously entering the cycle, the process must require the delivery of an ordered series of environmental signals. It is hard to imagine how a single ligand–receptor interaction can provide such a growth-inducing series of signals. However, if growth factors promote the physical association between two cells while initiating a process by which pairs of counterreceptors are recruited at the point of opposition, then it's easier to understand how a single factor can seem to regulate cellular growth. In the case of lymphocytes, specific antigen serves as the growth inducer by promoting the binding of an antigen-reactive B cell to an antigen-reactive Th cell. From that point on the cells apparently remain in conjugate for many hours during which continuous dialogue takes place. This occurs by the sequential appearance of counterreceptors at the point of opposition, each capable of eliciting a biochemical signal following binding their respective ligand on the opposing cell. For this communication system to work, the elicited biochemical signal must induce the expression of new sets of genes while simultaneously changing the array of counterreceptors expressed upon the cell's surface membrane. This, then, allows the cell to talk back to its conjugal partner. Such a system enables the ordered delivery, over time, of signals in sequence that results in the ordered expression of genes whose products are required for a resting cell to enter the cell cycle.

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