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The Role of Non-*ras* Transforming Genes in Chemical Carcinogenesis

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DNA transfection experiments using the NIH 3T3 mouse fibroblast cell line have demonstrated that chemically induced tumors and chemically transformed cell lines frequently contain dominant transforming genes. Although many of the genes detected using the NIH 3T3 transfection-transformation assay are activated versions of H-*ras*, K-*ras*, and N-*ras*, in some experimental systems activated forms of genes such as *met* and *neu* that are unrelated to *ras* have been observed. The activated *met* gene was originally detected in a human cell line that had been transformed by exposure to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Subsequent studies demonstrated that the *met* proto-oncogene encodes a novel growth factor receptor and that gene activation involves the production of a chimeric gene in which the regions of *met* encoding the extracellular and transmembrane domains of the receptor are replaced by the 5'-region of an unrelated gene called *trp*. The activated *neu* gene was detected in tumors of the nervous system that arose in mice following transplacental exposure to *N*-ethyl-*N*-nitrosourea. The *neu* gene also encodes a novel growth factor receptor but, in contrast to *met*, its activation involves a single T:A → A:T point mutation in the region of the *neu* gene encoding the receptor transmembrane domain.

The presence of genetic alterations in chemically induced malignancies has also been assessed in cytogenetic studies and by Southern analysis of DNA from neoplastic cells. These studies have demonstrated the presence of altered versions the c-*myc* and *mos* genes in plasmocytomas induced in mice following exposure to pristane or mineral oil and of activated *pim*-1 and c-*myc* genes in thymomas that arise in AKR mice following treatment with *N*-methyl-*N*-nitrosourea. Analyses of the mechanisms of activation of these non-*ras* genes has provided important insights into the different ways in which genes may become activated following chemical exposure.

Introduction

The new technologies of DNA transfections and molecular biology have resulted in major advances in our understanding of the molecular mechanisms of chemical carcinogenesis. Indirect support for the idea that DNA is the critical target during chemical carcinogenesis was originally provided *a*) by the discovery, for particular classes of chemical carcinogens, of correlations between carcinogenicity and the extents of covalent binding to DNA in target tissue; *b*) by the discovery of correlations between carcinogenicity and mutagenicity; and *c*) by the identification of karyotypic abnormalities in cells from chemically induced malignancies (*1–5*).

The first direct support for the concept that chemical transformation may involve the generation of activated transforming genes (oncogenes) by alteration of normal cellular genes (proto-oncogenes) was, however, provided by the observation that DNA from lines of chemically transformed cells could be used to transform a line of NIH 3T3 mouse fibroblasts in DNA transfection experiments (*6*). The DNA transfection procedure and other techniques that can now be used for detecting activated cellular genes have been applied to at least a dozen model systems of tumor induction and cell transformation, and it has become apparent that the identity of the activated gene detected using these procedures depends upon the experimental system under investigation.

In many studies, all of the genes detected are members of the *ras* gene family (H-,K-, and N-*ras*) that are usually activated by point mutations in codons 12 or 61. For example, H-*ras* is activated in *N*-methyl-*N*-nitrosourea (MNU)-induced rat mammary tumors and in dimethylbenz[*a*]anthracene (DMBA)-initiated mouse skin papillomas and carcinomas, while both N-*ras* and K-*ras* are activated in thymomas that arise in MNU-treated RF/AKR mice (*7–12*). However, genes that are unrelated to *ras* are also frequently detected, and in a minority of cases (e.g., for *met* and *neu*), the mechanism of gene activation of these non-*ras* genes has been examined in detail (*13–16*). Since the role of *ras* gene activation in chemical carcinogenesis has been adequately discussed elsewhere (*17–19*), this review will deal entirely with the non-*ras* genes that are activated in chemically induced tumors and chemically transformed cell lines.

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The *met* Gene

The *met* gene was originally detected by transfection of DNA from a transformed human cell line, called MNNG-HOS (*20,21*), that was derived by treating HOS cells with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). The HOS cell line is, as its name implies, derived from a human osteosarcoma. HOS cells exhibit a flat morphology when grown in tissue culture and do not induce tumors when injected into nude mice. They can, however, be converted into morphologically transformed cells that form tumors when injected into nude mice by treatment with chemical carcinogens, such as MNNG and DMBA (*22, 23*). In DNA transfection experiments, the activated *met* gene was detected in MNNG-HOS cells but not in the parent HOS cell line, indicating that treatment of HOS cells with MNNG had given rise to a dominant transforming gene (*20*).

The DNA sequence of cDNA clones prepared from transcripts of the *met* proto-oncogene revealed that the normal cellular mouse *met* genes encode a 1380 amino acid protein with the characteristics of a growth factor receptor (*24*) (Fig. 1). The N-terminal 18 amino acids of this protein is rich in hydrophobic residues, suggesting that this region of the protein is a signal peptide used for insertion into the membrane. A second hydrophobic domain is found at residues 930 to 954. This domain has the characteristics of a membrane-spanning region and is followed by a highly basic stretch of residues that may function as a "stop transfer" sequence. The putative transmembrane domain divides the *met* protein into two regions that correspond to the extracellular and intracellular portions of the protein. The amino-terminal extracellular domain of 929 amino acids contains many cysteine domains, including a small cysteine-rich region, and 10 consensus sequences for asparagine-linked N-glycosylation (Asn-Xaa-Ser/Thr). The cytoplasmic domain of 426 amino acids contains a protein tyrosine kinase (PTK) region that has a unique domain of 127 amino acids between the transmembrane and PTK domains that is much longer than the corresponding domains found in other tyrosine kinase receptors (*24*) (Fig. 1). The human *met* protein has an almost identical structure (*25*).

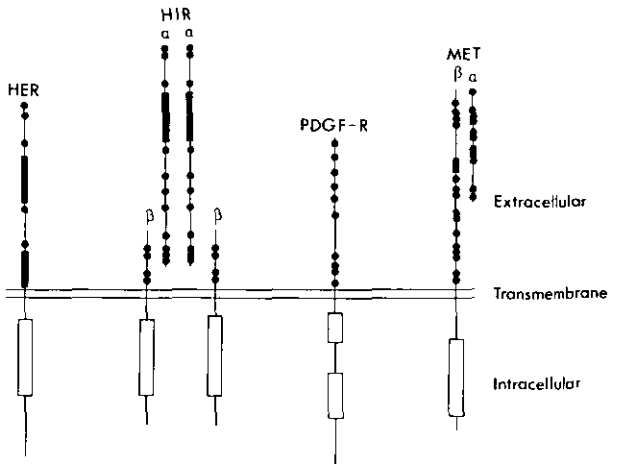

FIGURE 1. Diagram comparing the structures of epidermal growth factor receptor (HER, class I receptor), the insulin receptor (HIR, class II receptor), the platelet-derived growth factor receptor (PDGF-R, class III receptor) and the *met* receptor protein (MET). The tyrosine kinase domain (▭), cysteine residues (●), and cysteine-rich regions (▬) are shown. The *neu* protein has a structure similar to that shown for the epidermal growth factor receptor.

Examination of the predicted amino acid sequences of the mouse *met* protein revealed the presence of a potential proteolytic cleavage site with the sequences Lys-Arg-Arg-Lys-Arg-Ser 302 amino acids from the amino terminals (*24*). This basic sequence is similar to the sequence Arg-Lys-Arg-Arg-Ser found at the cleavage site of the insulin receptor precursor and to the sequence Arg-Lys-Arg-Arg-Asp found at the cleavage site of the precursor of the insulinlike growth factor I receptor (*26,27*). In the precursors of the insulin and insulinlike growth factor I receptors, this is the site for cleavage of the precursor into α and β subunits, which in the mature receptor are joined by disulfide bonds in an $\alpha_2\beta_2$ configuration (Fig. 1) (*28*). Cleavage at the basic sequence present in the *met* protein and removal of the signal peptide would generate and N-terminal peptide of 282 amino acids that might become associated with the remaining membrane-bound portion of the *met* protein in a manner similar to that observed for the insulin and insulinlike growth factor I receptors (*24,29*).

To test this hypothesis, the structure of the *met* protein was examined directly using antibodies raised against synthetic peptide corresponding to the carboxy terminus of the *met* protein. When proteins were extracted, immunoprecipitated, and subjected to gel electrophoresis under nonreducing conditions, a 190-kDa protein was observed. However, when this 190-kDa protein was excised from the gel and treated with β-mercaptoethanol, it yielded subunits of 145 kDa and 50 kDa. These results demonstrate that the *met* protein is indeed a heterodimer in which a 145-kDa β-subunit is joined by disulfide bonds to a 50-kDa α-subunit (*29–31*) (Fig. 1).

The biosynthesis of the *met* protein has been examined in detail (*31*). Following metabolic labeling of cells in the presence of tunicamycin, an inhibitor of co-translational N-glycosylation, anti-*met* antibodies immunoprecipitated a protein of 150 kDa; the molecular weight of this protein is an agreement with the size of the *met* protein calculated from its protein sequence.

In pulse-chase experiments carried out in the absence of tunicamycin, a protein with an apparent molecular weight of 170 kDa appears first. This early precursor is already glycosylated but probably does not function as an active receptor since it is not expressed at the cell surface nor phosphorylated on tyrosine. The 170-kDa protein appears to rapidly undergo a conformational change, probably as a consequence of modification of intra-chain disulfide bands, to form a protein species with an apparent molecular weight of 180 kDa. Subsequently, this single polypeptide precursor is cleaved

to form the 145-kDa β-subunit. Although it has not been unequivocably demonstrated, it is believed that this precursor also gives rise to the 50-kDa α subunit. In the mature receptor, the β subunit may be phosphorylated on tyrosine, serine, and threonine. The α and β subunits are both detected when cells are labeled with ^{125}I under nonpermeating conditions and are therefore both exposed at the cell surface (*31*).

Growth factor receptors possessing a protein tyrosine kinase domain are currently classified into different groups on the basis of common structural motifs (Fig. 1). Receptors belonging to class I (epidermal growth factor [EGF] receptor and *neu*) are monomeric and are characterized by the presence of two cysteine-rich regions within the extracellular domain. Class II induces the insulin and insulinlike growth factor I receptors, which have a tetrameric ($\alpha_2\beta_2$) subunit structure, while class III receptors (colony-stimulating factor [CSF]-I and platelet-derived growth factor [PDGF] receptors) are monomeric but have a split tyrosine kinase domain. The *met* protein seems to be the prototype of a new class of receptors that have a unique αβ subunit structure. The ligand that is presumed to bind to the *met* receptor has not been identified, and represents a primary goal of future studies must be to identify this ligand.

Activation of the *met* gene in MNNG-HOS cells involves a chromosomal rearrangement in which the region of the *met* gene encoding the extracellular and transmembrane domains is replaced by 5′ region of an unrelated gene designated *trp* (translocated promoter region) (*13,14*). This chimeric gene is transcribed to produce a unique 5.0-kb hybrid *tpr-met* mRNA that is in turn translated to form a 60- to 65-kDa fusion protein in which the protein tyrosine kinase domain of met is fused to the amino-terminal region of the trp protein (*13,14,29,33*). The region of the trp protein present in the trp-met fusion protein exhibits weaker homology to several structural proteins, including laminin and lamin, indicating that the normal trp protein may also encode a structural protein (*32*). Although the identity and subcellular location of the normal product of the *trp* gene have not been determined, it is possible that formation of the fusion protein may confer transforming potential in the met protein tyrosine kinase (PTK) domain by redirecting its subcellular location, thus altering the spectrum of proteins phosphorylated by the kinase. In addition, the modification of the structure of *met* may alter its response to normal cellular control mechanisms.

The mechanism of activation of *met* is reminiscent of that observed for the *trk* and *abl* genes. Activation of c-*abl* occurs in chronic myelogenous leukemia, where the Philadelphia translocation results in the substitution of the 5′ sequences at the c-*abl* gene with *bcr* gene sequences. The protein encoded by the activated gene retains the PTK domain and exhibits enhanced PTK activity when compared to the normal c-*abl* protein (*34*). Similarly, during activation of the *trk* gene, the carboxyl-terminal tyrosine kinase domain of a putative transmembrane receptor became attached to the amino-terminal 221 amino acids of nonmuscle tropomyosin (*35*). Thus, in each case, the 3′-end of the activated gene encodes a PTK domain, while initiation of transcription occurs in a separate DNA domain that comprises the 5′-end of the gene.

The *neu* Gene

A high proportion of offspring of pregnant rats that have been treated with a single dose of *N*-ethyl-*N*-nitrosourea (ENU) during the second half of gestation develop central and peripheral nervous system tumors after a latency of around 200 days (*36–38*). Shih et al. (*39*) demonstrated that DNA from cell lines derived from intracranial tumors induced in BD-IX rats could transform NIH 3T3 cells in the DNA transfection assays. The transforming gene transferred in these experiments was unrelated to *ras* and was associated with the expression of a phosphoprotein of relative molecular mass 185,000 (p185) (*40*). Subsequent studies demonstrated that *neu* was related to, but distinct from, the gene that encodes the EGF receptor (*41,42*). The nucleotide sequence of the *neu* cDNA revealed a 1260 amino acid protein that exhibits 50% amino acid homology to the EGF receptor and possesses the characteristics of a growth factor receptor, including the presence of a extracellular ligand-binding domain, a transmembrane domain, and a cytoplasmic protein tyrosine kinase domain (*43*). When considered together, these observations strongly suggest that *neu* encodes a growth factor receptor, although the identity of the ligand that binds to this putative receptor remains to be determined.

The cell lines examined by Shih et al. (*39*) were believed to be derived from neuroblastomas and glioblastomas that arose in the central nervous system. However, the identification of these tumors was equivocal because no histological examination of the primary tumors' tissue was reported and because schwannomas may also develop intracranially. Indeed, an extensive study of oncogene activation in primary glial tumors and schwannomas that developed in transplacentally exposed F344 rats revealed that *neu* activation occurred exclusively in schwannomas; of 59 gliomas examined, none showed *neu* gene activation (*38*).

Comparisons of the activated and normal versions of the *neu* gene have demonstrated that *neu* gene activation in the cell lines derived from intracranial tumors and in primary schwannomas invariably involves a T:A → A:T transversion mutation in codon 664 (*15,38*). Unexpectedly, this alteration, which changes valine to glutamic acid, falls within the putative transmembrane domain. The presence of this acidic residue in the otherwise hydrophobic transmembrane domain does not alter the subcellular location of *neu* because, like its normal counterpart, the activated *neu* protein is membrane associated (*44*). In addition, the membrane-associated p185 appears to be responsible for transformation because antisera to p185 suppress the transformed phenotype in *neu*-transformed cells (*45*).

In fact, it is now believed that the presence of the glutamic acid residue causes activation of the receptor by promoting deimerization and higher PTK activity in the absence of the ligand (*46*).

Although reactions of ENU with target tissue inflict many different types of damage on cellular DNA, O^6-ethylguanine (O^6-EtG) and O^4-ethylthymine (O^4-EtT) are considered to be the major promutagenic lesions. O^6-EtG and O^4-EtT would be expected to cause, respectively, G:C → T:A and T:A → C:G transition mutations by facilitating mispairing during DNA replication. Indeed, analysis of the types of mutation induced following exposure of bacteria to ENU reveal that the majority of the changes are transition mutations of these types (*47*). A low level of transversion mutation was found, but notably no T:A → A:T transversions are detected. T:A → A:T transversion mutations have, however, been detected in globin genes of the progeny of female mice treated with ENU (*48,49*). To explain how ENU causes T:A → A:T mutations, it may be necessary to search for new promutagenic lesions from among the variety of different products that result from exposure of DNA to ENU.

c-*myc*, *pvt*-1, and *pim*-1 Loci

B-cell neoplasms (called plasmocytomas) can be induced in BALB/c and NZB mouse strains by intraperitoneal injection of either mineral oil or pure alkanes such as prisane (2,6,10,14-tetramethylpentadecane). These agents cannot attack DNA directly but induce a severe inflammatory response at the site of injection, and plasmocytomas are detected as free cells after at least 130 days (*50*). Cytogenetic studies have revealed that the majority of plasmocytomas possess specific chromosomal translocations involving chromosomes 15 and 12 or chromosomes 15 and 6. The translocation observed most frequently involves the c-*myc* locus on chromosome 15 and the immunoglobin heavy chain (IgH) locus on chromosome 12; translocations involving the immunoglobin κ light chain on chromsome 6 and a locus designated *pvt*-1 on chromosome 15 are found less frequently. [For a review see Cory (*16*).]

The immunoglobin heavy chain gene undergoes a series of rearrangements during B-cell development. Initially the region of the gene encoding the immunoglobin variable region is assembled by a series of recombinations involving variable (V), diversity (D), and joining (J) elements leading to the production of a gene encoding a μ-class heavy chain. Subsequently, recombination occurs between switch regions (S), leading to the construction of genes that determine the synthesis of other classes of immunoglobin.

The major translocation found in plasmocytomas brings together the c-*myc* locus and the 3′-end of the IgH locus in a "head-to-head" configuration. Within the IgH locus the switching regions are the predominant targets for translocation, and it is generally supposed that translocations result from rare aberrant interchromosomal recombinations that occur during B-cell maturation. Within the c-*myc* gene, the majority of translocations occur either 5′-end to the first exon or within the first exon and intron. In plasmocytomas the unrearranged c-*myc* allele is usually transcriptionally silent while the translocated allele is actively transcribed (*51*). This observation indicated that a major consequence of IgH invasion of the c-*myc* locus is the deregulation of c-*myc* expression. In fact, it is the constitutive expression of the rearranged c-*myc* locus that is believed to play a major role in the induction of plasmocytomas. In contrast, the precise role that exposure to mineral oil or pristane plays in generating these translocations still remains to be established.

AKR mice, in contrast to most other mouse strains, develop thymomas spontaneously after 6 months of age. AKR mice express high levels of endogenous murine leukemia viruses (MuLVs), and in some spontaneous AKR thymomas and in some thymomas induced by infecting young mice of other strains with MuLVs, a critical event in tumor development involves modification of cellular c-*myc* and *pim*-1 genes by proviral integration at these loci (*52,53*). The *pim*-1 gene was, in fact, originally isolated as a specific site of integration of MuLVs in mouse thymomas and is a member of a family of genes encoding protein kinases (*54*).

When young AKR mice are treated with a single dose of MNU, thymomas start to appear at 3 months, and all of the treated mice developed thymomas before the first tumors appear in untreated groups (*55,56*). Proviral integrations at the *pim*-1 and c-*myc* loci have also been detected in these MNU-induced thymomas. It is, however, clear that the MNU-induced tumors are quite distinct from the spontaneous thymomas that develop in the AKR mouse strain because they lack a class of recombinant MuLVs (called MCF viruses) that are found in all spontaneous tumors and because, in contrast to spontaneous thymomas, they frequently contain activated *ras* genes (*56*). In fact, it is possible that the development of thymomas in MNU-treated AKR mice involves a cooperation between genes that are activated by chemical exposure (e.g., *ras*) and genes that are activated by proviral integration (e.g., *pim*-1 and c-*myc*).

Cytogenetic studies have revealed that chemically induced thymomas exhibit trisomy of chromosome 15. Although the significance of this abnormality remains to be established, it is conceivable that the modest increase in copy number of genes such as c-*myc* that are located in chromosome 15 may facilitate thymoma development.

The *mos* Gene

Activated cellular *mos* genes have been detected in a small proportion of mineral-oil-induced mouse plasmocytomas (*57–61*). *Mos* was originally identified as a transforming sequence of Moloney murine sarcoma virus and is now believed to encode a cytostatic factor that is responsible for causing meiotic arrest in vertebrate eggs (*62*). *Mos* is normally expressed at high levels in oocytes (*63*), but inappropriate expression of *mos* at

modest levels in certain other cell types can result in cell transformation (*64*).

The altered *mos* genes found in plasmocytomas were originally detected as gene rearrangements by Southern analysis. More detailed molecular analyses demonstrated that the rearrangements resulted from integration of intercisternal A-particle (IAP) genomes within the 5′-end of the coding region of the *mos* gene (*58–60*). IAP's particle genomas are located at 1000 or more sites per haploid genome and are generally considered to represent a class of movable genetic element that is frequently expressed in many murine tumors, including plasmocytomas. The transcriptional activation of *mos* that accompanies IAP integration appears to result from the juxtaposition of *mos* sequences and transcription control elements present in the LTRs of the IAP genome and from the separation of *mos* from *cis*-action negative control elements normally located around 1 kb upstream from the *mos* coding region (*64,65*).

Uncharacterized Transforming Genes

Although the great majority of genes detected using DNA transfection procedures are members of the *ras* gene family, in some studies low frequencies of genes that are not closely related to H-*ras*, K-*ras*, and N-*ras* are also observed. For example, analyses of 113 chemically induced mouse hepatomas revealed that 58 tumors contained activated H-*ras*, 3 tumors transferred activated K-*ras*, 2 tumors yielded activated *raf*, and 3 tumors contained activated genes that were apparently unrelated to *ras* or *raf* (*66–68*). Similarly, in studies on DMBA-transformed mouse urothelial cells, 1 of the 4 activated genes that were detected was not a member of the *ras* gene family (*69*). In other studies, higher incidences of activation of non-*ras* gene have, apparently, been observed. Thus, examination of 4 activated fibrosarcomas induced in rats by 1,8-dinitropyrene (1,8-DNP) revealed that 1 tumor contained K-*ras*, while the other 3 contained activated genes that were unrelated to *ras* (*70,71*). In addition, McMahon et al. (*72*) have provided evidence for transforming gene activation in a high proportion of hepatocellular carcinomas induced in Fischer rats by aflatoxin B_1. Activated K-*ras* was detected in 2 carcinomas, while evidence that 8 of the 11 tumors contained genes that were unrelated to *ras* was also provided.

Garte et al. (*73*) found that DNA from seven nasal squamous cell carcinomas that were induced in rats by inhalation of methylmethanesulfonate (MMS) efficiently transformed NIH 3T3 cells. MMS is an alkylating agent that produces only low levels of O-alkylated bases and would be expected to be only a poor inducer of the point mutations that are required for *ras* gene activation. Accordingly, the genes detected in the MMS-induced tumors were not closely related to H-, K-, and N-*ras*.

Shiner et al. (*74*) examined the mechanism of morphological transformation of a stable immortal hamster cell line (4DH2) following exposure to MMU, ENU, and dimethylsulfate (DMS). In these experiments, treatment with ENU and MNU gave rise to both progressively growing large foci and compact small foci, whereas treatment with DMS produced almost exclusively large foci. Since ENU and MNU are both potent point mutagens, while DMS is only a poor inducer of point mutations, it was assumed that the small foci arose as a consequence of point mutagenic events. Similarly, since each of these three alkylating agents produces similar levels of gross chromosome damage, it was proposed that the generation of large foci involved more substantial genetic alterations and was unlikely to involve *ras* gene activation. In agreement with this prediction, all of the dominant transforming genes detected in large foci using DNA transfection procedures were not related to K-, N-, or H-*ras*.

Concluding Remarks

Several distinct types of genetic alteration have been implicated in the activation of non-*ras* transforming genes. For example, activation of the *met* gene involves a chromosomal rearrangement, activation of *neu* requires a point mutation, and activation of *pim*-1 and c-*myc* in chemically induced thymomas involves proviral integration. There is also some evidence that gene amplification may occur in chemically and radiation-induced tumors. Thus, certain types of chemically induced tumors are known to contain double minute chromosomes, the morphological hallmark of gene amplification (*75*). In addition, Wong (*76*) has detected amplification of the gene encoding the epidermal growth factor receptor (c-*erbB-1*) in oral carcinomas induced by treating hamsters with DMBA, while Sawey et al. (*77*) observed amplification of c-*myc* in radiation-induced mouse skin tumors.

A potentially exciting area for future investigation is the analysis of loss or inactivation of tumor-suppressor genes during chemical carcinogenesis. Since the loss or inactivation of specific chromosomal loci, such as the p53 and *Rb*-1 genes, is a common feature of the development of many types of human cancer, it would be useful to have an animal model that would allow the mechanism of gene loss and its role in carcinogenesis to be studied in more detail. It is perhaps worthy of note that the p53 gene, which is now believed to be a tumor-suppressor gene, can be overexpressed and mutated in chemically transformed cells. Indeed, p53 was originally identified as both a cellular protein associated with the large T-antigen of SV40 and as a tumor-specific transplantation antigen in 3-methylcholanthrene-induced mouse fibrosarcomas (*78–81*).

Carcinogenesis is generally considered to be a multistep process. Evidence for this is provided by analysis of the pathology of cancer development (*82*) and by studies on the kinetics of appearance of cancer (*83*). In addition, it is now well established that transformation of certain types of primary cell may be activated by co-

operation between different classes of activated oncogene; for example, Land et al. (*84*) demonstrated that primary rat fibroblasts can be transformed by cooperation between activated forms of *ras* and *myc*. When considered together, these observations indicate that several genetic changes may be required to activate full transformation. In this regard, the identification of genetic changes that cooperate with, for example, *neu* activation in schwannomas or *myc* activation in plasmocytomas, may provide a fruitful area for future studies.

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