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ras GENES[1]

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CONTENTS

PERSPECTIVES AND SUMMARY

Scientists attempting to establish the molecular basis of neoplasia have repeatedly encountered the members of a small gene family known as *ras*. This acronym is derived from the words *ra*t *s*arcoma because these genes were first identified as the transforming principle of the Harvey and Kirsten strains of rat sarcoma viruses (1, 2), two acute transforming retroviruses generated by transduction of the rat H-*ras*-1 and K-*ras*-2 cellular genes, respectively (3, 4). The H-*ras*-1 locus has been transduced into retroviruses on at least two additional occasions during the generation of the Rasheed strain of rat sarcoma virus (5) and the BALB strain of mouse sarcoma virus (6). *ras* genes were inadvertently "rediscovered" when scientists, using gene transfer assays, established the existence of dominant oncogenes in human (7–10) and carcinogen-induced animal tumors (11–14). Most of these transforming genes have been identified as mutated alleles of cellular *ras* genes (11–18). More recently, *ras* oncogenes have been found in tumors induced by retroviruses that lack oncogene sequences. These viruses can integrate in the vicinity of cellular proto-oncogenes, disrupting their normal regulatory elements (19). Activation of *ras* genes by retroviral insertional mutagenesis has recently been described in tumors of avian and mammalian origin (20, 21; J. Ihle, personal communication).

ras genes have been the focus of intense research since 1982 when their transforming alleles were first identified in human tumors. Unveiling the role of *ras* oncogenes in neoplastic development should have a major impact on our understanding of the pathogenesis of human cancer. However, research on *ras* genes is justified on its own merits. *ras* genes are likely to play a fundamental role in basic cellular functions based on their high degree of conservation throughout eukaryotic evolution. Independently of their phylogenetic origin, they code for proteins that bind guanine nucleotides (22–25), have GTPase activity (25–29) and are associated with the plasma membrane (30–32). These properties, along with their significant sequence homology with G proteins (33–36), suggest that *ras* proteins may participate in the transduction of signals across the cellular membrane.

Evolution has preserved their basic biological functions. *ras* proteins of yeast can transform mammalian cells (37) and mammalian *ras* proteins can support the growth of mutant yeast cells (37, 38). Yet, *ras* proteins appear to

participate in multiple, often distinct, biological processes. In mammals, they have been implicated in cellular proliferation (39) and terminal differentiation (40–42). In yeast, they can be required for survival (43, 44) or mating (45). Therefore, it is likely that *ras* proteins may function at a critical crossroads of signal transduction pathways. The study of *ras* genes and their oncogenic alleles is certainly a most fascinating field that has attracted scientists interested in disciplines as diverse as yeast sporulation, control of cell proliferation, neural differentiation, carcinogen-DNA interactions, and human cancer.

During the last couple of years, reviews dealing with the structure and biochemical properties of *ras* proteins (46) and with the involvement of *ras* genes in human cancer (47–49) and in carcinogen-induced tumors (50) have been published. In this review, I have compiled current information on the different areas of *ras* gene research in an attempt to convey an overall view of the fundamental role that *ras* genes play in normal and neoplastic cellular processes.

PRIMARY STRUCTURE

ras genes are an ubiquitous eukaryotic gene family. They have been identified in mammals, birds, insects, mollusks, plants, fungi, and yeasts. Sequence analysis of these genes and their products has revealed a high degree of conservation, which suggests that they may play a fundamental role in cellular proliferation. In this section, I present an overview of the most significant structural features of this gene family.

Mammalian ras *Genes*

To date, three *ras* genes have been identified in the mammalian genome (3, 4, 15–17, 51, 52). They have been designated H-*ras*-1, K-*ras*-2, and N-*ras*. Two pseudogenes, H-*ras*-2 and K-*ras*-1, have been identified and characterized in rats and humans (3, 4, 53, 54) and are likely to exist in most, if not all, mammals. Several mouse and hamster subspecies possess additional *ras* pseudogenes, probably due to a relatively recent germ line amplification (55). Each of the three functional *ras* genes has been cloned and sequenced in at least two mammalian species. They include human H-*ras*-1 (10, 56–59), K-*ras*-2 (54, 60, 61), and N-*ras* (51, 52, 62, 63); rat H-*ras*-1 (3, 12, 64) and K-*ras*-2 (exons I and II only) (65); and mouse K-*ras*-2 (66) and N-*ras* genes (67, 68). Their location in both human and rodent chromosomes has also been determined (reviewed in 69). N-*ras* has been assigned to the short arm of human chromosome 1 (1p22–p32), whereas H-*ras*-1 and K-*ras*-2 have been assigned to the short arms of chromosomes 11 (11p15.1–p15.5) and 12 (12p12.1–pter), respectively. The chromosomal location of the two human *ras* psuedogenes (H-*ras*-2 maps in the X chromosome and K-*ras*-1 in 6p12–

p23) is also known. In the mouse, H-*ras*-1 has been mapped in chromosome 7, whereas K-*ras*-2 and N-*ras* have been assigned to chromosomes 6 and 3, respectively. Finally, in rats, the H-*ras*-1 gene has been mapped in chromosome 1, K-*ras*-2 in chromosome 4, and the H-*ras*-2 pseudogene in the X chromosome (70).

The three functional *ras* genes code for highly related proteins generically known as p21 (71). The p21 coding sequences of each of these genes are equally distributed in four exons except for the K-*ras*-2 gene, which possesses two alternative fourth coding exons (exons IVA and IVB) that allow the synthesis of two isomorphic p21 proteins of 188 and 189 residues that differ in their carboxy terminal domains (54, 60, 72). Although the spliced junctions of all mammalian *ras* genes correspond precisely, suggesting a common origin from one ancestral gene, their intron structures vary greatly. As a consequence, *ras* genes exhibit distinct genetic complexities ranging from the 4.5 kbp size of H-*ras*-1 to the 50 kbp of K-*ras*-2. Mammalian *ras* genes contain an additional 5' noncoding exon (54, 60, 61, 73) located immediately downstream from their respective promoters (74, 74a). These promoters do not possess the characteristic TATA and CAT boxes commonly found in other eukaryotic genes. Instead, they are rich in G/C boxes, which are presumably involved in the binding of Sp1 proteins, a characteristic of the promoters of housekeeping genes (75).

Comparison of the deduced amino acid sequences of mammalian H-*ras*-1, K-*ras*-2, and N-*ras* p21 proteins has helped to define four domains within these molecules (Figure 1*A*). The first domain encompasses the amino terminal third of p21 proteins and is a highly conserved region. For instance, the first 85 amino acid residues of mammalian p21 *ras* proteins of known sequence (human H-*ras*-1, K-*ras*-2, and N-*ras*, rat H-*ras*-2, and mouse K-*ras*-2 and N-*ras* genes) are identical (Figure 1*A*). The next 80 amino acid residues define a second domain where the structures of the different mammalian p21 *ras* proteins diverge slightly from each other (85% homology between any pair of human *ras* genes). A highly variable region encompasses the rest of the molecule except for the last four amino acids, where the sequence Cys^{186}-A-A-X-COOH (where A is any aliphatic amino acid) is present in all members of the *ras* gene family (Figure 1*A*).

ras *Genes of Other Eukaryotes*

ras genes have been highly conserved during evolution (76). They have been identified in chickens (H-*ras* gene) (20), fruit flies (*Drosophila melanogaster* D*ras*1, D*ras*2/64B and D*ras*3 genes) (77–79), mollusks (*Aplysia* Apl-*ras* gene) (80), slime molds (*Dictyostelium discoideum* Dd*ras* gene) (81), plants *(Allium cepa)* (L. Serrano, J. Avila, personal communication), and yeasts (*Saccharomyces cerevisiae RAS*1 and *RAS*2 genes and *Schizosaccharomyces*

pombe SPRAS gene) (82–84). Comparative analysis of the deduced amino acid sequence of the products of these *ras* genes with mammalian p21 *ras* proteins shows a high degree of homology and the same structural domains (Figure 1). Although some of the *ras* gene products of invertebrate species have additional amino terminal residues, they are at least 84% homologous to the highly conserved amino terminal domain of mammalian p21 *ras* proteins. This homology decreases substantially in the second, less conserved domain, and completely disappears in the variable carboxy terminal region with the exception of the conserved carboxy terminal Cys-A-A-X-COOH sequence.

The most striking property of the evolutionary conservation of *ras* genes is their ability to function in heterologous systems. Mammalian *ras* genes under the appropriate control of yeast promoters can complement nonviable *ras*1$^-$*ras*2$^-$ yeast mutants (37, 38). Moreover, mammalian *ras* oncogenes can induce phenotypic alterations in yeast cells (66, 85,). Similarly, chimeric yeast-mammalian *ras* genes and a yeast RAS gene that carries a deletion in its long hypervariable domain are able to efficiently transform mouse NIH3T3 cells in gene transfer assays (37). These results represent the first report of interchangeability between functional genes of yeast and mammals and are the best example to illustrate the high degree of conservation of *ras* genes during evolution.

ras *Oncogenes*

Mammalian *ras* genes acquire transformation-inducing properties by single point mutations within their coding sequences (86–88). Mutations in naturally occurring *ras* oncogenes have been localized in codons 12 (86–88), 13 (89), 59 (90, 91), and 61 (62, 63, 92, 92a). In vitro mutagenesis studies have shown that mutations in codons 63 (93), 116 (94), and 119 (95) can also confer transforming properties to *ras* genes. Missense mutations in the corresponding codons of the *ras* genes of *S. cerevisiae* also induce pronounced phenotypic changes that will be discussed in the section on yeast RAS genes.

The presence of a glycine residue at position 12 appears to be necessary for the normal function of *ras* proteins. Substitution of Gly^{12} by any other amino acid residue (with the exception of proline) results in the oncogenic activation of these molecules (96). A similar affect is observed if Gly^{12} is deleted or if additional amino acids are inserted between Ala^{11} and Gly^{12} (97). Substitution of the neighboring amino acid, Gly^{13}, also has transforming consequences for the harboring cells, although in this case not all substitutions appear to have the same activating effect (89, 93). Whereas Val^{13} and Asp^{13} substitutions clearly yield *ras* oncogenes, replacement of Gly^{13} by Ser^{13} has little effect on the transforming activity of *ras* proteins (93). Miscoding mutations in the domain surrounding codon 61 also play a very important role in the generation of *ras* oncogenes (62, 63, 92). Substitution of Gln^{61} by any

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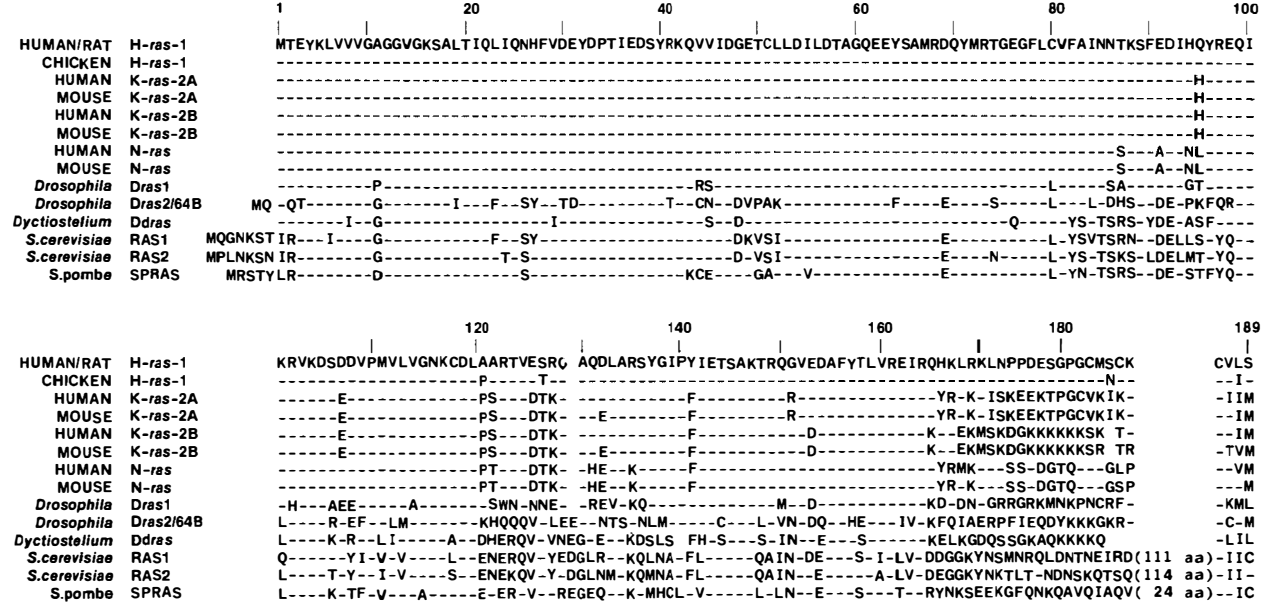

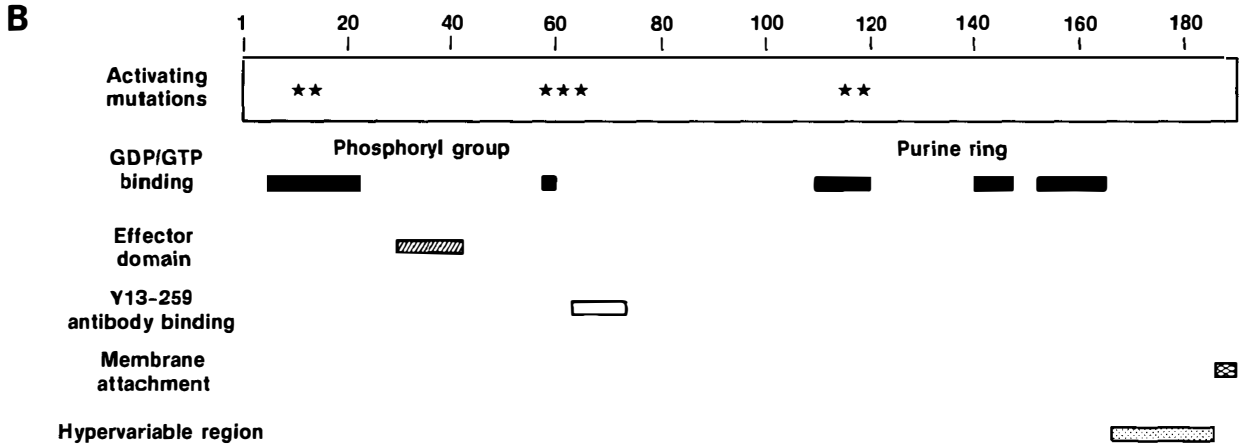

Figure 1 *A*. Comparative amino acid sequence of *ras* proteins. Residues identical to the human H-*ras*-1 gene product are designated by a dash. *B*. Schematic representation of the structural and functional domains defined within mammalian p21 *ras* proteins.

other amino acid residue, except Pro^{61} or Glu^{61} (and to a lesser extent Gly^{61}), yields *ras* oncogenes (98). Substitutions in residue 59 have only been observed in retroviral (90, 91) or in vitro mutagenized *ras* oncogenes (93). In all cases, substitution of the normal Ala^{59} by Thr^{59} has been observed. The effect of these mutations on the structure and biochemical properties of *ras* proteins will be discussed in the following section.

Whereas all cellular *ras* oncogenes carry a single activating mutation, each of the four known retroviral *ras* oncogenes exhibits two mutations. The *ras* oncogenes of the Harvey and Kirsten strains have replaced both Gly^{12} and Ala^{59} residues by Arg^{12} and Thr^{59} or Ser^{12} and Thr^{59}, respectively (90, 91). The BALB strain exhibits two G → A transitions in codon 12, leading to a Gly^{12} (GGA) → Lys^{12} (AAA) substitution (99). Finally, the *ras* gene product of the Rasheed strain, p29 *ras*, carries a Gly^{12} → Arg^{12} substitution as well as 59 additional amino terminal residues derived from the helper virus *gag* gene p15 protein (5). The biological significance of these double mutations is not clear. It could be argued that they reflect selection of those viruses with more malignant properties. However, *ras* oncogenes carrying mutations in codons 12 and 59 are no more transforming than those carrying either mutation alone (93). Moreover, in vitro studies have shown that substitution of Gly^{12} by Lys^{12} (as seen in BALB-MSV) generates a weakly transforming *ras* oncogene (96). Therefore, it is possible that the observed secondary mutations in retroviral *ras* oncogenes may be an evolutionary safeguard against excessive transforming properties.

ras-*Related Genes*

Emerging evidence suggests that *ras* genes are members of a super gene family. Genes exhibiting limited sequence homology to the *ras* gene family have been identified in a variety of eukaryotic organisms. For instance, a new class of genes, designated *rho*, code for proteins that share 30–40% homology with *ras* p21 proteins (100). *rho* genes have been identified in a variety of species, including such highly diverged organisms as snails *(Aplysia)* and humans. The human and *Aplysia rho* genes share 85% sequence homology, indicating that they are as well conserved in the phylogenetic scale as the *ras* genes (100). At least four additional *ras*-related genes that share 35–55% sequence homology with the *ras* gene family have been identified in humans and rodents (R-*ras*) (100a) primates *(ral)* (100b), fruit flies (D*ras*3) (79), and yeast (YP2) (101). The YP2 yeast gene has been recently shown to be involved in microtubule organization (101a). Whether any of these genes are members of evolutionarily conserved gene families remains to be determined. Finally, some of these *ras*-related genes share significant sequence homology with at least one of the two domains (codons 12–13 and 59–61) frequently involved in the malignant activation of *ras* oncogenes. Whether any of these

ras-related genes can become oncogenes, either in vivo or by in vitro manipulation, remains to be determined.

BIOCHEMICAL PROPERTIES

ras proteins, independently of their phylogenetic origin, have been shown to bind guanine nucleotides (GTP and GDP) (22–25) and possess intrinsic GTPase activity (25–29). The relevance of these activities to the biological function of *ras* proteins has been demonstrated by three independent lines of evidence: (*a*) microinjection of anti-*ras* antibodies that inhibit guanine nucleotide binding (102) reverses the malignant phenotype of NIH3T3 cells transformed by *ras* oncogenes (103); (*b*) *ras* mutants that have lost their ability to bind guanine nucleotides do not transform NIH3T3 cells (104, 105); and (*c*) the GTPase activity of *ras* genes is severely impaired in their transforming alleles (25–29). In addition to GTP/GDP binding and GTPase activity, *ras* proteins carrying an $Ala^{59} \rightarrow Thr^{59}$ mutation exhibit an autophosphorylating activity of an, as yet, unknown biological significance (23). In all cases, Thr^{59} has been found to be the phosphate receptor site (106). No transphosphorylating activity has been detected with any *ras* protein, including those carrying Thr^{59} mutations.

The biochemical properties of *ras* proteins closely resemble those of the G proteins involved in the modulation of signal transduction through transmembrane signaling systems (107). In fact, certain domains of *ras* proteins exhibit significant sequence homology with the α subunit of G proteins such as G_s, a protein that activates adenylate cyclase in response to β adrenergic stimuli; G_i, which inhibits this enzyme and perhaps activates phospholipase C; G_o, a protein of as yet unknown function; and transducin, a protein that regulates cGMP phosphodiesterase activity in visual signal transduction (33–36). In addition to G proteins, other nucleotide-binding proteins such as the bacterial elongation factor T_u (EF-T_u), the β subunit of ATP-synthase, adenylatekinase, phosphofructokinase, and tubulin also exhibit certain sequence homology to *ras* proteins (108–110).

In this section, the biochemical properties of *ras* proteins will be discussed in conjunction with genetic studies that have made it possible to assign certain functions to specific domains within these molecules.

Guanine Nucleotide Binding

The homology of *ras* genes with G proteins and EF-Tu is basically limited to regions encompassing amino acid residues 5–22 and 109–120 (108, 109). Direct experimental evidence implicating these domains in guanine nucleotide binding has been obtained recently. Antibodies directed against epitopes located within the amino terminal region of *ras* proteins inhibit GTP binding

by purified p21 *ras* proteins (102). Conversely, the ability of these antibodies to bind to *ras* proteins is inhibited by preincubation with GTP or GDP (102). X-ray crystallography studies of the GDP binding domain of EF-Tu also support the concept that residues around codon 12 form part of the guanine nucleotide–binding site (109). Based on EF-Tu and *ras* sequence homology, the 12th amino acid residue (Gly) of mammalian *ras* proteins should be located in the phosphoryl binding loop (109, 111). Early studies have predicted that replacement of Gly^{12} by any other amino acid residue (except proline) would disrupt the α-helical structure of the amino terminal domain of *ras* proteins, causing a conformational change that would prevent its proper folding (112–114). Thus, replacement or elimination of Gly^{12} may create a rigid domain that cannot efficiently interact with the phosphoryl region of the GTP molecule, reducing the GTPase activity of *ras* proteins. Two additional residues in this domain, Gly^{15} and Lys^{16}, are present in other guanine nucleotide–binding proteins (109, 111). Substitution of Lys^{16} by Asn^{16} significantly reduces GTP/GDP affinity without affecting base specificity, an observation consistent with the idea that these residues are also part of the phosphoryl group (95).

Crystallographic studies of the GDP·EF-T_u complex predict that the guanine-binding pocket may interact with two noncontiguous segments of EF-T_u that are present in *ras* proteins (Asn^{116}-Lys-Cys-Asp^{119} and Ser^{145}-Ala-Lys^{147}) (109, 111). *ras* deletion mutants within residues 109–120 or 130–145 do not bind detectable levels of guanine nucleotides and cannot induce efficient transformation of NIH3T3 cells (104). Moreover, two of three GTP-binding-defective *ras* mutants isolated by random mutagenesis ($Asp^{119} \rightarrow Asn^{119}$ and $Thr^{144} \rightarrow Ile^{144}$) map within these regions (115). Direct biochemical evidence supporting the concept that these regions interact with the guanine ring has been obtained by introducing mutations in residues 116 and 119 (94, 95). Substitution of normal Asn^{116} by a variety of amino acids including isoleucine, lysine, tyrosine, and histidine decreases the binding of guanine nucleotides by several orders of magnitude to below detection levels (94, 115a, 115b). However, only a 10-fold decrease in binding was observed if asparagine was replaced by a related amino acid residue such as glutamine (94). These observations are consistent with the prediction from EF-Tu·GTP crystallographic studies that Asn^{116} may form a hydrogen bond with the O^6 residue of guanine (116). Substitution of Asp^{119} by alanine leads to a 50-fold increase in the dissociation rate of guanine nucleotides (95). In contrast, the $Asp^{119} \rightarrow Ala^{119}$ mutation has no effect on the dissociation rate of inosine diphosphate, a molecule identical to GDP except for the absence of the amino residue in position 2. These results suggest that Asp^{119} may form a hydrogen bond with the 2-NH_2 group of guanine nucleotides (95).

The *ras* protein domain encompassing residues 59–63 does not show

significant sequence homology to other guanine nucleotide–binding proteins. However, miscoding mutations in residues 59, 61, or 63 result in the oncogenic activation of *ras* genes (62, 90–93). The $Ala^{59} \rightarrow Thr^{59}$ mutation is of particular interest because Thr^{59} is the substrate for the autophosphorylating activity of those *ras* oncogenes in which it has been identified (106). These observations suggest that residue 59 may be adjacent to the γ-phosphate of the guanine nucleotide, thus favoring the phosphorylation of the hydroxyl group of the mutant Thr^{59} residue (111). However, this hypothesis must await genetic studies in which amino acid substitutions other than Thr are introduced at this position. The interaction of Gln^{61} with guanine nucleotide is not well understood. Substitution of Gln^{61} by 17 different amino acid residues invariably results in decreased GTPase activity (25, 117). However, there is no quantitative correlation between the reduction in this enzymatic activity and the extent of transformation induced by these mutants, suggesting that additional factors might play a role in the malignant activation of *ras* proteins (117).

Another domain implicated in guanine nucleotide binding has been identified by in vitro mutagenesis. Deletion of residues 152–165 completely abolishes guanine nucleotide binding and transforming activity (105). This sequence includes Arg^{164}, a residue conserved in all *ras* genes (Figure 1). Substitution of Arg^{164} by Ala^{164} also results in loss of GTP binding activity (105). The lack of homology between this domain and EF-Tu has made it impossible to predict whether these residues in general, or Arg^{164} in particular, are part of the GDP binding site. Independent studies have shown that substitution of *ras* oncogene residues 164 to 174 by the sequence Pro-Asp-Gln does not result in loss of transforming activity (118). Thus, it is possible that the domain surrounding the conserved Arg^{164} residue plays an indirect role in the binding of guanine nucleotides.

The Effector Domain

Extensive work on deletion mutants of mammalian *ras* genes has led to the definition of five noncontiguous domains (residues 5–63, 77–92, 109–123, 139–165, and the carboxyl terminal sequences Cys^{186}-A-A-X-COOH) that are absolutely required for *ras* function (104). Whereas some of these domains have been clearly implicated in guanine nucleotide binding, others might play a role in effector recognition. Amino acid substitutions at positions 35 (Thr → Ala), 36 (Ile → Ala), 38 (Asp → Ala), and 40 (Tyr → Lys) have been shown to reduce the biological effect of *ras* proteins in assays utilizing both mammalian (NIH3T3 focus formation) and yeast (complementation of growth of *RAS1ras2* mutants on nonfermentable carbon sources and stimulation of adenylate cyclase) cells (119). These mutations do not disrupt the known biochemical activities of these molecules, suggesting that residues

35–40 might be implicated in the effector activity of *ras* proteins (119). Additional support for this hypothesis comes from the observation that all indispensable domains of *ras* proteins lie in internal hydrophic regions except for the domain corresponding to residues 30 to 42, which is hydrophilic and presumably located in the external surface of the molecule (104). These findings suggest that this domain may be involved in the interaction of *ras* proteins with their putative cellular targets (Figure 1*B*). Recently, a $Gln^{43} \rightarrow Arg^{43}$ substitution has been found to be responsible for the temperature-sensitive phenotype of the 371 strain of Kirsten-MSV (119a). Although the structural consequences of this mutation have not been established, these results add further evidence to the importance of this domain for proper *ras* protein function.

Neutralizing Antibodies

One of the reagents most commonly used to characterize *ras* proteins is a rat monoclonal antibody designated Y13–259 (120). This antibody must bind to a highly conserved domain of *ras* proteins as deduced by its ability to recognize the products of each of the mammalian *ras* genes (62, 120) as well as the *ras* proteins of invertebrate species (24, 121, 122). More importantly, this antibody has been recently shown to specifically block the serum-induced mitogenic response of certain cells in culture and to inhibit morphologic transformation induced by mutated *ras* proteins (39, 123). Binding studies utilizing the products of *ras* deletion mutants have localized the epitope recognized by this antibody within residues 70–89 (124) (Figure 1*B*). More refined analysis has established that the Y13-259 antibody interacts with the side chains of residues Glu^{63}, Ser^{65}, Ala^{66}, Met^{67}, Gln^{70}, and Arg^{73} (119). Substitution of any of these residues completely abolished the interaction of *ras* proteins with the Y13-259 antibody in immunoblotting assays. In contrast, substitution of residues 61, 62, 64, 68, 71, 72, 75, 78, or any other residue outside the 60–80 region of *ras* proteins had no detectable effect on their affinity for the Y13-259 antibody (119).

Residues critical for Y13-259 binding are presumably located in the exposed, hydrophilic site of an α helix that forms a structure about 20 Å long, a distance consistent with the dimensions of an antibody-binding site (119). Binding of Y13-259 to *ras* proteins does not affect any of their known biochemical properties (124). Moreover, the Y13-259 antibody-binding domain lies in a dispensable hydrophilic region (104, 119). Therefore, it is likely that the biological activities observed by binding of Y13-259 antibodies to *ras* proteins are exerted through indirect conformational changes.

Membrane Attachment

ras proteins have been localized in the inner side of the plasma membrane (30–32). The primary translational product of *ras* oncogenes is synthesized in

the cytosol (32, 125). Attachment to the plasma membrane requires a post-translational modification that involves the acylation of Cys^{186} by palmitic acid (32, 126–128). Genetic studies have demonstrated that this posttranslational modification is necessary for the biological function of *ras* proteins. Mutants lacking Cys^{186} code for proteins that remain in the cytosol and cannot induce morphological transformation of NIH3T3 cells (31, 129).

Processed *ras* proteins attached to the plasma membrane exhibit a faster migration rate in SDS-polyacrylamide gel electrophoresis than their unmodified cytosolic counterparts (32, 127, 128). It was presumed that this change in mobility was the result of the acylation of the Cys^{186} residue. However, it is likely that an as yet unidentified posttranslational modification may take place prior to fatty acid acylation. In yeast, RAS proteins are converted to forms with faster electrophoretic mobility before the attachment of palmitic acid (32). Studies with mammalian p21 *ras* proteins indicate that removal of lipids does not restore the mobility of the mature protein to that of the precursor form (127). Moreover, the products of *ras* deletion mutants with a slow rate of acylation exhibit an altered electrophoretic mobility independently of whether they contain palmitate (membrane fraction) or not (cytosolic fraction), indicating that the change in electrophoretic migration is not a consequence of acylation (130). Thus, it is likely that *ras* proteins may undergo two steps of posttranslational modification before they become attached to the plasma membrane.

Dispensable Domains

At least five noncontiguous domains of *ras* proteins have been shown to be dispensable for their biological function. They include the amino terminal end, three internal domains, and the hypervariable region. Evidence that the first few amino acid residues are not important for *ras* function comes from the variability in both the type and number of residues found in this region in the different *ras* gene products (Figure 1*A*). Moreover, H-*ras* p21 proteins in which the first three amino acids were replaced by unrelated residues retained their biological properties (131).

In vitro mutagenesis studies utilizing viral *ras* oncogene DNA clones have indicated that three internal domains (residues 64–76, 93–108, and 124–138 in mammalian proteins) can be deleted without drastically affecting their transforming properties (104). Each of these domains corresponds to hydrophilic regions that are presumed to be located in the external surface of *ras* proteins (104). It is possible that they represent long hinge regions that can be deleted without serious consequences to the overall structure of the molecule. However, in vitro mutagenesis studies must be performed with *ras* oncogenes whose products do not require external signals for activation. Therefore, it is conceivable that a putative receptor domain might be found to

be dispensable in these assays. Whether any of these domains are involved in receptor signaling remains to be determined.

Deletion of the entire hypervariable region (residues 166–185 in mammalian *ras* proteins, see Figure 1) does not affect any of the known biochemical properties of *ras* proteins nor their ability to transform NIH3T3 cells (105, 118). It is likely that this long stretch of amino acids, which has been maintained throughout evolution, plays an important role in *ras* function. It is possible that *ras* proteins utilize this domain to interact with other proteins. Its intrinsic variability may serve to confer different functional properties to each of the members of this gene family. Development of biological assays for *ras* proteins other than cellular transformation (e.g. complementation of *S. cerevisiae* ras$^-$ mutants) might shed light on whether these apparently dispensable domains play any role in the biological function of *ras* proteins.

A Model for the Function of ras *Proteins*

The biochemical and biological properties of *ras* proteins, along with their strong resemblance to G proteins, have led to the proposal that *ras* proteins may be involved in signal transduction. A schematic diagram of the currently favored model is shown in Figure 2. This model proposes that *ras* proteins exist in equilibrium between an active and an inactive state. Most of the *ras* molecules in a given cell would exist in their inactive state, which is characterized by a conformation that allows binding of GDP. Normal *ras* proteins will remain in their inactive state until they receive a stimulus from another protein (a receptor?) upstream of a putative pathway of signal transduction. This stimulus would result in the exchange of GDP for GTP followed by a conformational change of the *ras* protein to its active state. Active *ras* proteins would then be able to interact with their putative effector molecules. Once the interaction between the active *ras* proteins and the effector has taken place, they would be immediately deactivated. This can be accomplished by their intrinsic GTPase activity, which would catalyze the hydrolysis of GTP returning the active *ras* protein to the inactive GDP-bound state (Figure 2).

This model takes into account that mutations known to confer transforming properties to *ras* genes must reverse the normal equilibrium between the inactive and active forms. Stabilization of *ras* proteins in their active state would cause a continuous flow of signal transduction, which will result in malignant transformation (Figure 2). Theoretically, this process can be achieved by mutations that inhibit the intrinsic GTPase activity of *ras* proteins (Figure 2*a*), increase the exchange rate between GDP and GTP (Figure 2*b*), or induce an active conformational change that does not require binding of guanine nucleotides (Figure 2*c*). This model also takes into account that normal *ras* genes induce malignant transformation if highly overexpressed (see next section). High levels of normal *ras* proteins may produce enough

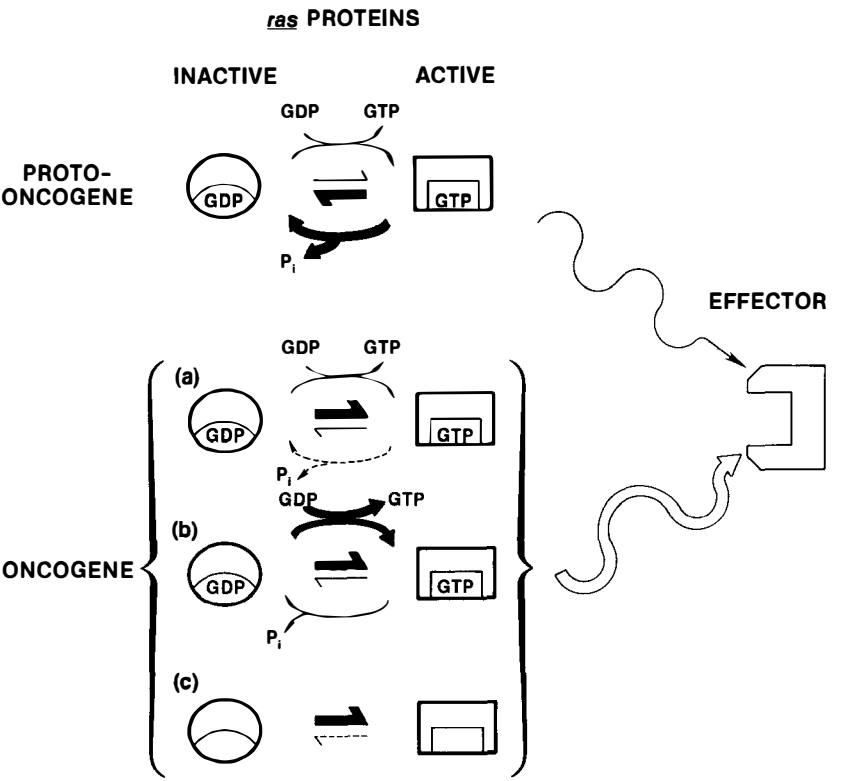

Figure 2 Current model of the mechanism of action of normal and transforming *ras* proteins.

molecules in their GTP-bound active state to induce malignant transformation without affecting the equilibrium between inactive and active forms characteristic of normal *ras* proteins.

Experimental evidence indicates that inhibition of their GTPase activity is the preferred mechanism of activation of oncogenic *ras* proteins (25–29, 117). *ras* oncogenes carrying $Asp^{116} \rightarrow Ile^{116}$ or $Asp^{119} \rightarrow Asp^{119}$substitutions may exert their transforming properties by mechanisms involving increased dissociation rates of the complex between guanine nucleotides and *ras* proteins (94, 95) (Figure 2*b*). Such an increase in the dissociation rate would result in a significant reduction of the affinity of GDP and GTP for *ras* proteins. This would favor the formation of the active [*ras* protein·GTP] complex due to the higher availability of GTP molecules in the cell. However, in the case of the transforming *ras* gene carrying the $Asp^{116} \rightarrow Ile^{116}$ mutation, its gene product does not bind detectable levels of GTP (94). Whereas this observation might be due to the unusually high dissociation rate

(and therefore low affinity) of the [p21·GDP/GTP] complexes, it is conceivable that substitution of aspartic acid by a hydrophobic residue such as isoleucine may induce a conformational change that can activate the *ras* p21 protein without participation of guanine nucleotide binding (Figure 2*c*).

A discrepancy appears to exist regarding the mechanism by which the $Ala^{59} \rightarrow Thr^{59}$ mutation activates *ras* oncogenes. Whereas some authors have reported that these mutants possess low GTPase activity (25, 26), other investigators have found a normal GTPase (132) and increased GDP/GTP dissociation rates (132a). The reasons for these discrepancies remain to be determined.

ras PROTO-ONCOGENES: BIOLOGICAL PROPERTIES

Unveiling the cellular role of *ras* genes requires the use of model systems amenable to genetic analysis. In mammalian cells the extent of genetic manipulation is rather limited. Availability of *ras* DNA clones has allowed a wide range of in vitro mutagenesis studies. However, the only mutants capable of inducing identifiable phenotypes have been the *ras* oncogenes. So far, no experiments involving the genetic manipulation of *Drosophila ras* genes have been reported. Only yeast cells have produced significant genetic information (43–45) that has resulted in the linkage of the *ras* genes to the adenylate cyclase pathway (133). However, these results cannot be extrapolated to other eukaryotic organisms in spite of the conserved structural and functional properties of *ras* genes.

ras *Genes of Yeast*

S. cerevisiae contains two *RAS* loci, *RAS*1 and *RAS*2, that code for proteins of 40,000 (309 amino acids) and 41,000 (322 amino acids) daltons, respectively. *RAS*1 and *RAS*2 are structurally and functionally related to mammalian *ras* genes (82, 83, 134). Genetic analysis of the *RAS*1 and *RAS*2 genes of *S. cerevisiae* has established that neither of them are essential for the viability of these cells (43, 44). Cells containing disruptions in either of these genes can undergo both mitotic and meiotic cell division cycles. However, $ras1^{-}ras2^{-}$ spores of doubly heterozygous diploids are not capable of vegetative growth (43, 44). These findings clearly illustrate the importance of RAS genes for normal cellular proliferation.

Yeast *RAS* gene products exhibit the same biochemical properties as their mammalian counterparts. They bind GDP and GTP and have an intrinsic GTPase activity (24, 25). RAS proteins carrying $Ala^{66} \rightarrow Thr^{66}$ (equivalent to mammalian position 59) and $Gln^{68} \rightarrow Leu^{68}$ (equivalent to mammalian position 61) mutations bind normal levels of guanine nucleotides but have an impaired GTPase activity (25). Moreover, the Thr^{66} mutant exhibited auto-

phosphorylating activity (25). These findings suggest that *S. cerevisiae RAS* proteins may also function as G proteins according to the model depicted in Figure 2.

Preliminary evidence indicates that *S. cerevisiae RAS*1 and *RAS*2 genes do not have the same cellular function. Extensive genetic analysis of the *RAS*2 locus indicates that this gene is necessary for a normal response to nutrient limitation. *ras*2$^-$ mutants, although viable, have a defect in gluconeogenic growth, accumulate excessive levels of storage carbohydrates, and sporulate prematurely (133, 135, 136). In general, disruption of the *RAS*2 locus results in an overall premature starvation response. The multiple phenotypes observed in *ras*2$^-$ mutants may not necessarily be interconnected. For instance, the inability of *ras*2$^-$ mutants to grow in media containing nonfermentable carbon sources appears to be a consequence of the decrease of *RAS*1 gene expression under these culture conditions (137). Inhibition of *RAS*1 gene transcription coupled with the *ras*2$^-$ mutation may lead to a cell cycle arrest comparable to that observed in *ras*1$^-$*ras*2$^-$ mutants (43, 44). Support for this hypothesis comes from the observation that suppressors of the *ras*2$^-$ defect in gluconeogenic growth led to a significant increase in *RAS*1 mRNA levels (137, 137a). However, direct suppression of the *ras*2$^-$ phenotype in nonfermentable carbon sources by chimeras capable of constitutively expressing *RAS*1 proteins remains to be demonstrated. In contrast, the *RAS*1 product does not seem to play any role in the hypersporulating properties of *ras*2$^-$ mutants, since the same suppressors that allow these cells to grow on nonfermentable carbon sources have no effect on the hypersporulation phenotype (137). Recent studies have suggested that *RAS*1 may play an indirect role in the regulation of glucose-induced inositolphospholipid turnover (137b). These observations indicate that the *RAS* genes of *S. cerevisiae* play different cellular roles in spite of their high sequence homology and genetic complementarity.

Detailed genetic analysis of the *RAS*2 locus has made it possible to identify the cellular pathways in which this gene is involved. Introduction of a Gly^{19} → Val^{19} substitution (equivalent to mammalian 12 position) in *RAS*2 results in a dominant gene capable of inducing a series of phenotypic changes in yeast cells (43, 133). *RAS*2 Val^{19} mutants exhibit a very low sporulating efficiency and can arrest at any phase of the cell cycle upon nutritional stress (43, 133). It it worth noting that this phenotype is the opposite of that induced by elimination of the *RAS*2 gene (133–136), an observation consistent with the idea that mutations in the critical Gly^{19} codon lead to an enhancement of the normal properties of the *RAS*2 product. Comparison of the characteristic phenotype of *RAS*2 Val^{19} mutants with other known yeast mutants has provided fundamental clues regarding the cellular function of the *RAS*2 gene (133). It was observed that *bcy*1, a suppressor of adenylate cyclase–deficient

(*cyr*$^-$) yeasts (138), exhibited an almost identical phenotype to *RAS*2 Val^{19} cells. *bcy*1 is a recessive mutant that affects the regulatory subunit of the cAMP-dependent protein kinase (139). As a consequence, *bcy*1 strains bypass this regulatory step and induce the constitutive activation of the catalytic subunit of this kinase. Genetic crosses between *bcy*1 and several *ras* mutant strains have conclusively demonstrated that *bcy*1 suppressed the lethality induced by the *ras*1$^-$*ras*2$^-$ genotype (133). Similar results have recently been obtained with an extragenic suppressor mutant designated *sra*1 (137a), which is likely to be an allele of *bcy*1. Other mutations that allow *ras*1$^-$*ras*2$^-$ cells to grow have been identified as alleles of adenylate cyclase that produce elevated levels of cAMP (137a, 139a). These observations indicate that *RAS* genes must play a role in the adenylate cyclase signal transduction pathway somewhere upstream from the step involved in the regulation of the cAMP-dependent protein kinase activity. As indicated above, adenylate cyclase is regulated by G proteins whose biochemical properties closely resemble those of *ras* proteins (107). Thus, these studies have raised the interesting possibility that *S. cerevisiae RAS* genes may be regulatory G proteins that control adenylate cyclase.

Subsequent biochemical studies have supported these genetic observations. *S. cerevisiae* cells carrying the dominant *RAS*2 Val^{19} mutant exhibit a 4-fold increase in the levels of cAMP (140). In contrast, *RAS*1*ras*2$^-$ and *ras*1$^-$*ras*2$^-$*bcy*1 strains have 4- and 20-fold lower levels of cAMP than wild-type cells, respectively (140). The fact that *ras*1$^-$*RAS*2 strains only have slightly depressed cAMP levels adds further support to the concept that *RAS*1 and *RAS*2 genes play different cellular roles. In vitro studies utilizing purified *RAS*2 proteins and crude membrane extracts indicate that the increased levels of cAMP are due to stimulation of adenylate cyclase activity by *RAS*2 proteins (140). This stimulation is dependent on the presence of guanine nucleotides, an expected finding considering the structural and functional homologies between *RAS* genes and regulatory G proteins. GTP was found to be 50% more efficient than GDP in the *RAS*2-dependent stimulation of cAMP synthesis (140). Moreover, a *RAS*2 Val^{19} protein that has a defective GTPase is about 2-fold more efficient than a wild-type *RAS*2 molecule in stimulating cAMP synthesis. However, if GTP is substituted by a nonhydrolyzable derivative, thus making this reaction independent of the presence of GTPase activity, wild-type *RAS*2 and *RAS*2 Val^{19} mutant proteins exhibit the same high stimulatory effect (140).

Similar experiments were conducted in *Escherichia coli* cells transformed with *S. cerevisiae* adenylate cyclase (*CYR*1) and *RAS*2 genes (141). Transformants containing the *CYR*1 gene possess a GTP-independent adenylate cyclase activity, which becomes GTP-dependent upon transformation with the *RAS*2 expression plasmid. Substitution of *RAS*2 by *RAS*2 Val^{19} resulted in

both loss of the GTP dependency and increase in the amount of cAMP synthesis. Reconstitution of the GTP-dependent adenylate cyclase was observed by mixing membranes from *CYR1ras1*$^-$*ras2*$^-$*bcy1* yeast with *E. coli* cell extracts containing *RAS2* proteins or by mixing membranes of *cyr1RAS1RAS2bcy1* yeast with *E. coli* extracts containing adenylate cyclase (141). These observations, taken together, indicate that the *RAS2* gene of *S. cerevisiae* participates in the control of adenylate cyclase.

These studies, however, do not resolve whether RAS2 is the regulatory subunit of the catalytic domain of the yeast adenylate cyclase complex. Several lines of evidence argue against this straightforward hypothesis. For instance, yeast *cyr*$^-$ mutants are viable, whereas *ras1*$^-$*ras2*$^-$ are not. Therefore, *RAS* genes must be involved in additional pathways independent of adenylate cyclase. It is possible that the *RAS2* protein activates adenylate cyclase by interacting with its regulatory subunit, forming a G protein cascade. However, *ras* genes and adenylate cyclase are not linked functionally in other eukaryotic organisms. Mammalian epithelial and fibroblastic cell lines transformed with retroviral *ras* oncogenes exhibit reduced adenylate cyclase activity (142). Moreover, purified p21 *ras* proteins do not activate adenylate cyclase in crude mammalian cell membranes (142). Similarly, human *ras* proteins can induce maturation of frog *(Xenopus)* oocytes without affecting their cAMP levels (143). These observations argue against a direct interaction between *RAS* proteins and the regulatory and/or catalytic subunit of the adenylate cyclase enzymatic complex.

Among those eukaryotic organisms in which *ras* genes do not seem to interact with adenylate cyclase is the fission yeast *S. pombe*. *S. pombe* possesses a single *ras* gene, designated *SPRAS*, which codes for a protein of 219 amino acids whose sequence also conforms to the four structural domains of other *ras* proteins (84) (Figure 1*A*). The *SPRAS* protein plays a physiological role completely different from those of *S. cerevisiae RAS* products (45, 143a). Disruption of the *SPRAS* locus does not interfere with either growth rates or with the response to nutritional stress conditions. Instead, *S. pombe spras*−mutants completely lose their ability to mate (45, 143a). In addition, these mutations repress rather than stimulate sporulation and they do not affect the intracellular levels of cAMP. So far, there is no genetic or biochemical information regarding the pathways in which the *SPRAS* gene might be involved in *S. pombe*.

The lack of association between *ras* genes and adenylate cyclase in all the eukaryotic organisms studied so far except for the yeast *S. cerevisiae* represents an evolutionary puzzle. It is possible that *ras* proteins function at a pivotal crossroads of signal transduction pathways. If so, selection of one pathway over another may have changed during evolution depending on the particular physiological necessities of each organism. In support of this view

is the observation that *S. cerevisiae* depends on its intracellular levels of cAMP to exit from G_1 into the S phase and to switch from G_1 into the sporulation pathway (144). In contrast, there is no evidence for the involvement of cAMP in the control of the life cycle of *S. pombe*. Thus, if *ras* proteins were to be involved in the regulation of cell proliferation it is likely that in *S. Cerevisiae* they do so by interacting with adenylate cyclase. However, other organisms in which cAMP does not play such a role in the control of cell growth may not require that their *ras* products interact with the adenylate cyclase pathway.

ras *Genes of Other Invertebrates*

The function of *ras* genes has been investigated in several invertebrate organisms, including the slime mold *D. discoideum,* the fruit fly *D. melanogaster,* and the mollusk *Aplysia.* The *D. discoideum* Dd*ras* gene was accidentally identified during the course of studies aimed at isolating genes differentially regulated during development (81). A 1.2 kbp Dd*ras* mRNA is expressed at high levels in vegetative cells and disappears at the beginning of differentiation. Two species of Dd*ras* mRNA (0.9 and 1.2 kbp) appear again at the end of the aggregation period (about 12 hours), reaching a maximum after 15 hours, and disappear by 22.5 hours, coinciding with midculmination (81). Protein studies indicate that p23, the Dd*ras* gene product, is also expressed at high levels during vegetative growth and decreases during development (82, 121). Whereas some authors have reported that the decrease in p21 expression is a slow and steady process (82), others have shown a remarkable decrease in the amount of p23 with the onset of differentiation followed by a rise during cellular pseudoplasmodial formation, finally decreasing to the lowest level during the late stages of differentiation (121). These latter results support the concept that Dd*ras* p23 expression correlates with the rate of cell proliferation in *D. discoideum.*

Transformation of *D. discoideum* cells with a series of antisense RNA Dd*ras* constructs linked to a neo^R gene led to a low number of geneticin-resistant survivors, none of which carry the antisense Dd*ras* vectors (145). These results have been interpreted as evidence that *ras* genes are required for cellular proliferation. However, it is not known whether these antisense constructs had any effect on endogenous Dd*ras* expression. More recently, the effect of Gly^{19} (equivalent to mammalian Gly^{12}) Dd*ras* mutants (Thr^{19}) have been shown to induce aberrant developmental phenotypes in this microorganism, suggesting an effect of *ras* genes on cAMP-mediated signal transduction (145a). Unfortunately, none of the biochemical parameters involved in the cAMP-mediated signal transduction pathway appear to be significantly affected by the Dd*ras*-Thr^{19} mutant (145a).

In *D. melanogaster,* three D*ras* genes have been identified, mapped, and

molecularly cloned (77–79). D*ras*-1 and D*ras*2/64B are the loci most closely related to the human H-*ras*-1 gene, in spite of some discrepancies in the sequence of the latter (77, 78). D*ras*3, an intronless gene, is more distantly related, although chimeric human H-*ras*-1 and D*ras*3 genes were able to transform NIH3T3 cells with low efficiency (79). To date, no genetic studies have been reported suggesting that these loci are located in chromosomal regions not easily accessible to genetic analysis. Each D*ras* locus appears to code for two or three transcripts of different sizes, which are constantly expressed throughout the development of the fly, although the shorter transcripts appear to be more abundant during the early embryonic stages (78, 146). In situ hybridization studies have shown that transcripts from each D*ras* gene exhibit similar distribution at every developmental stage (147). However, a correlation between D*ras* expression and cell proliferation has been observed. Whereas in embryos the D*ras* transcripts are uniformly distributed, in larvae they are restricted to the dividing cells (147).

This picture completely changes in the adult fly. Here, the highest levels of D*ras* RNA are found in fully differentiated, nondividing cells such as the ovaries and the cortex of brain and ganglia (147). Similar results have been recently observed in the marine mollusk, *Aplysia* (80). In this organism, *ras* proteins are most abundantly expressed in nervous tissue, in the ovotestis, and in fertilized eggs. Immunocytochemical analysis of the nervous tissue revealed that *ras* proteins are present in the neuronal cell bodies, as well as in the axons and in the neuropil (80). These studies suggest that *ras* genes may play a role not only in cell proliferation but also in processes involving terminal cell differentiation.

ras *Genes of Mammalian Cells*

The biological function of *ras* genes in mammalian cells is poorly understood. The existence of mutants (oncogenes) capable of inducing transformation-specific phenotypes suggests that these genes may play a role in cell proliferation. However, *ras* oncogenes can induce terminal differentiation of PC12 neural cells (40–42) (see p. 805). Thus, *ras* genes of mammals may also play a dual role in proliferation and in certain differentiation processes.

The difficulty of direct genetic manipulation of *ras* loci within mammalian cells has been partially overcome by microinjection studies using monoclonal antibodies directed against p21 *ras* proteins. Early studies have shown that microinjection of purified mouse *ras* Lys^{12}, human *ras* Val^{12}, or retroviral *ras* Arg^{12} Thr^{59} mutant p21 proteins into NIH3T3 mouse fibroblasts led to transient morphologic transformation and cell proliferation (148–150). Similarly, anti-p21 monoclonal antibodies were able to induce transient reversion of the malignant phenotype of rodent cells transformed by *ras* oncogenes (103, 123). These studies have been taken one step further by examining the

effect of Y13-259, a monoclonal antibody capable of recognizing all known *ras* proteins (120), in NIH3T3 cells. Microinjection of this antibody into the cytoplasm of NIH3T3 cells prior to serum stimulation leads to a significant decrease in the number of cells capable of tritiated thymidine uptake (39). These results indicate that cells injected in G_0 were blocked from entering the S phase. Time course experiments suggest that *ras* gene proteins are not required once the cells have entered the S phase. Instead, they appear to be necessary at about eight hours after serum induction, a time that is estimated to coincide with the beginning of the S phase (39). The validity of these observations has been confirmed by showing that NIH3T3 cells transformed by a *ras* mutant that cannot bind the Y13-259 antibody are not prevented from entering the S phase (151). Studies utilizing antibodies specific for each of the mammalian *ras* products may provide additional information on the role of each of these proteins in the proliferation of mammalian cells.

The location of *ras* proteins in the inner surface of the cell membrane (30), along with their similarity to G proteins (107), has raised the possibility that *ras* proteins may participate in the transduction of mitogenic signals. The direct effect of *ras* proteins in the dynamics of cellular membranes has recently been investigated by microinjection experiments (151a). Both normal and transforming *ras* p21 proteins induce the appearance of increased surface ruffles and fluid-phase pinocytosis. Whereas the effect of the normal protein is short-lived, that of its transforming counterpart remains for at least 15 hr after injection (151a). Increased ruffling and pinocytosis are also characteristic membrane responses to certain hormones and mitogenic compounds. These observations support the view that *ras* proteins participate in signal pathways initiated at the cellular surface.

Identification of the receptor and effector systems that interact with *ras* proteins has, so far, been elusive. It has been reported that the GDP binding activity of *ras* proteins in rodent cells transformed by *ras* oncogenes is stimulated by the addition of epidermal growth factor (EGF) (152). These observations, along with the increased production of TGFα, an EGF-like growth factor, by *ras*-transformed cells, have suggested a certain degree of biochemical interaction between *ras* proteins and the EGF receptor pathway.

More recently, it has been suggested that *ras* genes may play a regulatory role in the phosphatidylinositol pathway. Rodent fibroblasts transformed by different *ras* oncogenes exhibit elevated steady-state levels (2- to 3-fold) of phosphatidylinositol-4,5-biphosphate (PIP_2) and their breakdown products, the second messengers 1,2-diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP_3) (152c). Similar results have been obtained when NIH3T3 cells carrying an inducible N-*ras* proto-oncogene were treated with several growth factors such as bombesin and bradykinin but not with EGF or PDGF (152d). These observations have led to the proposal that N-*ras* p21 proteins

may be identical to Gp, the putative G protein that mediates the activation of phospholipase C, the enzyme responsible for the breakdown of PIP_2 into DAG and IP_3 (152d). However, in related experiments in which *ras* p21 proteins were microinjected into REF52 rat fibroblasts, stimulation of phospholipase A_2 was observed instead (151a). Additional experiments are needed to determine whether these observations have physiological relevance or are merely due to an overflow effect caused by the high levels of *ras* proteins present in these cells.

Studies on the effect of Y13-259 antibodies on the proliferative properties of NIH3T3 cells have provided some additional clues. Cells transformed by oncogenes whose normal alleles have been identified as growth factor receptors (*fms*) or that are known to interact with the plasma membrane (*fes* and *src*) cannot enter the S phase if microinjected with Y13-259 antibodies (153). In contrast, this antibody has no effect on cells transformed by two oncogenes whose products are known to be located in the cytoplasm (*mos* and *raf*). These results have been interpreted as evidence that *ras* genes are involved in signal transduction from a variety of receptors and other membrane-associated molecules (153). However, other interpretations are also possible. Further studies with additional oncogenes as well as with antibodies directed against other proto-oncogenes and membrane proteins such as growth factor receptors, protein kinase C, phospholipases, etc, will be necessary to assess the usefulness of this experimental approach.

Studies on the expression of *ras* genes in mammalian cells indicate that they are expressed at low levels in most, if not all, cell lineages. Unlike other proto-oncogenes, *ras* genes are consistently expressed throughout development of the mouse embryo (154, 155). Increased expression (up to eightfold) of *ras* genes has been reported in actively proliferating tissues such as regenerating rat liver (156). However, increased levels of *ras* expression do not always correlate with cellular proliferation. Studies aimed at determining the levels of *ras* proteins in different rat organs have found the highest p21 *ras* expression levels in brain, whereas proliferating tissues only show limited expression (157). A similar study in the mouse revealed the highest levels of expression in heart, another nondividing tissue (158).

More recently, an extensive immunohistochemical survey of p21 *ras* expression in normal fetal and adult human tissue has been conducted (M. Furth, C. Cordon-Cardo, personal communication). In each case, immunoblots of tissue lysates were used to corroborate the immunohistochemical data. The basic conclusions from these studies are (*a*) almost every fetal and adult tissue expresses detectable levels of p21 proteins; (*b*) in most cell lineages, the level of p21 expression was significantly higher in immature than in differentiated cells; and (*c*) certain terminally differentiated cells, including epithelial cells of endocrine glands and the neurons of the

central nervous system, expressed high levels of p21 proteins. These findings add further support to the concept that *ras* genes play a dual role in basic cellular proliferation and in certain specific functions of terminally differentiated cells.

ras *ONCOGENES*

Mechanisms of Activation

ras genes can acquire transforming properties by qualitative and quantitative mechanisms. As indicated throughout this review, missense mutations within certain domains yield highly efficient transforming *ras* genes. However, increased expression of normal *ras* proto-oncogenes can also induce certain manifestations of the malignant phenotype. Linkage of normal *ras* genes to retroviral regulatory elements (LTR) results in the malignant transformation of NIH3T3 cells (3, 159, 159a). Similar results have been obtained by integration of multiple copies of a DNA clone of the normal human H-*ras*-1 gene (160). These tumorigenic cells invariably show 30- to 100-fold higher levels of *ras* gene expression than either their normal counterparts or cells transformed by *ras* oncogenes activated by single point mutations (159, 159a, 160). In general terms, the neoplastic properties induced by highly overexpressed *ras* proto-oncogenes are more limited than those induced by their mutated alleles even when driven by their own promoters. Combination of qualitative and quantitative alterations of *ras* genes results in oncogenes (e.g. retroviral *ras* oncogenes) capable of inducing a more complete spectrum of neoplastic pheonotypes.

In vitro Transforming Properties

Cellular *ras* oncogenes transform established rodent cell lines in a dominant fashion (47–49). Neither the resident proto-oncogenes nor cotransfection with their respective normal alleles affects the transformation efficiencies of *ras* oncogenes. However, their levels of expression appear to modulate their transforming potency (161). *ras* oncogenes are necessary not only for initiation, but also for maintenance of the transformed phenotype. This has been demonstrated by the existence of a Kirsten-MSV ts mutant (162) and by in vitro experiments in which *ras* oncogenes were linked to inducible promoters (163) or used to induce reversible transformation of normal rat fibroblasts (161).

Cellular *ras* oncogenes cannot transform rat primary embryo cells (164–166). Tranformation of these cells by *ras* oncogenes requires the cooperation of nuclear oncogenes (167) such as c-*myc* (164), adenovirus E1A (165), polyoma large T (164), N-*myc* (168), or p53 (169, 170). Alternatively, *ras* oncogenes can transform rat embryo cells if the surrounding cells are elimin-

ated, for instance, by cotransfection with the *neo* gene followed by selection with geneticin (171–173). Linkage of *ras* oncogenes to transcriptional enhancers increases the percentage of cells that become transformed under these experimental conditions (171).

The molecular basis for the inhibitory effect of the surrounding cells remains to be elucidated. Reconstitution experiments indicate that normal cells can inhibit the transformed phenotype of *ras*-transfected cells only if plated at high cell density (174). These results explain early observations indicating that *ras*-containing retroviruses can transform rat primary embryo cultures (175) but only at high multiplicity of infection (L. Parada, personal communication). This inhibitory effect can be partially overcome by treating the *ras*-containing primary embryo cells with tumor promoters such as 12-0-tetradecanoyl-phorbol-13-acetate (TPA) (172). However, most TPA-treated or geneticin-resistant rat embryo cells carrying *ras* oncogenes alone will only divide a few times and will not become established as cell lines (172, 173). Quantitative experiments have indicated that primary embryo cells transfected with *ras* oncogenes are one or two orders of magnitude less likely to acquire long-term proliferative properties than those carrying a nuclear (e.g. c-*myc*, p53, adenovirus E1A, etc) oncogene (172, 173). These experiments show that *ras* oncogenes are highly inefficient in rescuing cells from senescence. Moreover, they indicate that induction of malignant transformation by *ras* oncogenes requires the establishment of a proliferative stage in the targeted cells. It is possible, however, that in vitro establishment may not be sufficient criteria to allow the phenotypic expression of *ras* oncogenes. Transformation of rat REF52 cells by these oncogenes requires complementation by adenoviruses E1A (176). Therefore, in vitro transformation of fibroblastic cells by *ras* oncogenes may require additional changes, often associated with, but not necessary for continuous proliferation.

Evaluation of the neoplastic properties of *ras* oncogenes in cells other than cultured fibroblasts has been somewhat hampered by the limited cell types that can be efficiently transfected by these oncogenes. The use of retroviruses carrying *ras* oncogenes has somewhat circumvented this problem. *ras*-containing retroviruses efficiently transform rodent cells of hematopoietic origin, including those of erythroid, lymphoid, and myeloid lineages (177–180). In the case of erythroid cells, the H-*ras* containing Harvey-MSV can induce their malignant transformation without affecting their differentiation program as these cells retain their property to synthesize hemoglobin upon induction (177). Infection of murine mast cells by this virus enhances growth properties of these cells, leading to the establishment of long-term cell lines (178). However, these Harvey-MSV-transformed mast cells require the continuous presence of interleukin-3 (IL-3) for growth, indicating that *ras* oncogenes do not participate in the IL-3 signal transduction pathway (178).

Transformation of myeloid cells results in the generation of both mature macrophage-like and immature myelomonocytic cell lines (179). Both could differentiate either spontaneously in the case of the more mature cells, or by treatment with phorbol esters in the case of the myelomonocytic cells (179). Finally, infection of mouse lymphoid cells with BALB and Harvey-MSVs yields transformed early progenitor cells as determined by the absence of markers specific for the B or T cells (180). Unlike in the case of erythroid and myeloid cells, these early progenitor lymphoid cells cannot be induced to differentiate into more mature lymphoid lineages (180).

Neither cellular nor retroviral *ras* oncogenes have been able to induce stable transformation of normal human fibroblasts (181). However, transfection of normal human bronchial epithelial cells by a DNA clone of the Harvey-MSV *ras* oncogene has yielded colonies of transformed cells (182). These cells are obtained at very low frequency and after careful and long-term culture. They exhibit all the characteristics of malignant cells, including a highly aneuploid karyotype, which suggests that multiple genetic changes have occurred during in vitro selection (182). Other authors have been able to transform human epidermal keratinocytes (183), embryonic kidney cells (184), and normal fibroblasts (185) by *ras*-containing retroviruses. However, transformation of these cells required prior acquisition of indefinite life-span by either infection with DNA tumor viruses such as AD12-SV40 (183) and BK (184) or by treatment with ^{60}Co α-rays (185).

ras oncogenes can also confer additional neoplastic properties to cell lines derived from human tumors. Infection of a human osteosarcoma cell line, HOS, with Kirsten-MSV not only induces drastic morphologic changes but confers on them the property of anchorage-independent growth and of producing tumors in nude mice (186). Transfection of MCF-7 breast carcinoma cells with a DNA clone of Harvey-MSV causes loss of estrogen dependence for neoplastic growth (187). However, bypassing estrogen dependence does not appear to be an intrinsic property of *ras* oncogenes since MCF-7 cells transfected with cellular H-*ras*-1 oncogenes remain hormone dependent (S. Sukumar, personal communication). Thus, it is likely that *ras* oncogenes can only induce the loss of hormone dependence in MCF-7 cells when driven by powerful transcriptional enhancers such as retroviral LTRs.

ras *Oncogenes and Experimental Metastasis*

The ability of *ras* oncogenes to induce metastatic phenotypes has been investigated. NIH3T3 and primary rat embryo cells transfected with *ras* oncogenes can form metastatic nodules in the lung when injected subcutaneously in nude mice (173, 188–190). Other investigators, however, have only observed metastatic foci when *ras*-transformed NIH3T3 cells were retransfected with DNAs isolated from certain metastatic human tumor cell

lines (191) or when these cells were injected intravenously (191, 192). In the case of 10T1/2 mouse cells expression of the metastatic phenotype (as determined by intravenous injection) correlates with the levels of expression of transfected H-*ras*-1 oncogenes (192a). In a different system however, transfection of MT1 Cl 5/7 mouse mammary carcinoma cells with the same human H-*ras*-1 oncogene only increased their capacity to metastasize when injected subcutaneously but not intravenously (193). These results suggest that the primary effect of the H-*ras*-1 oncogene may relate to their ability to escape the primary tumor site rather than to colonize new sites (193).

Comparative studies using different mouse cell lines indicate that *ras* oncogenes will confer metastatic properties to NIH3T3 cells, but not C127 cells (190). These results are independent of the levels of expression of p21 *ras* proteins (190). Thus, induction of the metastatic phenotype is not an intrinsic property of *ras* oncogenes. This concept is further supported by the similar incidence of *ras* oncogenes in primary and metastatic tumors (47–49).

ras *Oncogenes and Terminal Differentiation*

In addition to their transforming properties, *ras* oncogenes can also induce terminal differentiation of a rat pheochromocytoma cell line designated PC12. PC12 cells can differentiate into neuron-like cells if infected with *ras*-containing retroviruses or microinjected with oncogenic, but not normal, p21 *ras* proteins (40–42). Treatment of PC12 cells with nerve growth factor (NGF) or cAMP has been shown previously to promote neurite outgrowth (194, 195). Differentiation of PC12 cells induced by *ras* oncogenes clearly proceeds by a mechanism independent of cAMP. However, *ras* oncogenes induce differentiation markers very similar to those induced by NGF. Moreover, both *ras* oncogenes and NGF have a lag of 15–24 hours and require RNA and protein synthesis to induce the neural differentiation of PC12 cells (40–42). Thus, it is possible that *ras* oncogenes may participate in a signal transduction pathway commonly used by NGF. This hypothesis has been strengthened by a recent report showing that microinjection of PC12 cells with antibodies (Y13-259) against p21 *ras* proteins inhibits neurite formation induced by NGF but not by cAMP (196).

At least one other oncogene, v-*src*, has been shown to promote neural differentiation in PC12 cells (197). The effect of v-*src* is highly reminiscent of the effect of *ras* oncogenes. Interestingly, linkage between *src* and *ras* oncogenes has been suggested by two independent lines of evidence. As described above, NIH3T3 cells transformed by v-*src* cannot enter the S phase when microinjected with anti-*ras* Y13-259 antibodies (153). In the next section, I discuss the existence of flat revertants of Kirsten-MSV cells that can suppress the transforming phenotype induced by v-*src* (198). Thus, it is possible that *ras* and *src* genes participate in the same or in very interconnect-

able pathways. Unfortunately, it is not yet known whether microinjection of anti-p21 antibodies into PC12 cells also inhibits v-*src*-induced neural differentiation. Such experiments may shed light on a possible functional relationship between *ras* and *src* genes in these cells.

Suppressor Genes

One of the advantages of studying *ras* genes in microorganisms is the possibility of identifying genes that can suppress the abnormal phenotypes induced by *ras* genes carrying mutations equivalent to those that activate mammalian *ras* oncogenes. The first of these suppressors (*sup* H) has been recently identified (198a). *sup* H is an allele of *ste* 16, a yeast mutant that does not produce functional a-factor, a mating pheromone. The carboxy terminus of the a-factor is Cys-Val-Ile-Ala-COOH, a sequence similar to the consensus carboxy terminus of *ras* proteins, Cys-A-A-X-COOH (see section on *Membrane Attachment*). Thus, it is likely that *sup* H (now designated *RAM*, for *RAS* protein and a-factor maturation function) represents a mutation in a gene coding for an enzyme responsible for the attachment of fatty acid to the carboxy terminal Cys residue of the *RAS*2 Val^{19} protein (198a).

In mammalian cells there is also genetic evidence for the existence of suppressors of *ras* oncogenes. Flat revertants of NIH3T3 cells transformed with Kirsten-MSV have been described (198, 199). These revertants express high levels of biochemically active p21 protein, have rescuable transforming viruses, and cannot be retransformed by infection with Kirsten-MSV (198, 199). These cells are not tumorigenic and exhibit a very low frequency of retransformation even after long periods of culture. Cell hybridization studies have shown that the revertant phenotype is dominant in hybrids between revertant cells and cells transformed by several *ras* oncogenes (198). These results suggest that the revertant phenotype is not due to the absence of a cellular protein (e.g. a protein located downstream in the putative *ras* signaling pathway). Recently, it has been possible to transmit this suppressor phenotype to other *ras*-transformed NIH3T3 cells by standard gene transfer technology (M. Noda, personal communication).

Hybridization studies between these flat revertants of Kirsten-MSV NIH3T3 cells and cells transformed by other oncogenes indicate that the *ras* suppressor mutation can also suppress transformation induced by the v-*fes* and v-*src* oncogenes but not by v-*mos*, v-*fms*, or v-*sis* (198). These results are in good agreement (with the exception of those for v-*fms*) with those obtained by microinjection of anti-p21 monoclonal antibodies (153). Although the evidence is still mostly circumstantial, it is possible that *ras*, *fes*, and *src* genes are part of the same or interconnected signaling pathways.

Additional experimental evidence supports the existence of *ras* suppressor genes. Syrian hamster tumors induced by cells transformed by cooperating

ras and *myc* oncogenes exhibit the nonrandom loss of chromosome 15 (200). Fusion of human tumor cell lines carrying *ras* oncogenes (e.g. HT-1080 and EJ cells) with normal human fibroblasts yields nontumorigenic cells (201, 202). Similarly, fusion of Chinese hamster cells transformed with *ras* oncogenes to nontransformed Chinese hamster cells results in loss of tumorigenicity (203). Most of the hybrids, generated by fusion between normal and tumorigenic cells, are morphologically transformed, indicating that the putative gene(s) involved in the suppression of tumorigenicity must be different from those responsible for the flat phenotype of the Kirsten-MSV revertants. Moreover, some human tumor cell lines containing *ras* oncogenes (e.g. T24 bladder carcinoma cells) are "nontumorigenic" in nude mouse assays (204, 205). Thus, it is possible that those genes involved in the suppression of tumorigenicity of *ras*-transformed cells may not exert their inhibitory action by direct interaction with *ras* oncogenes.

ras ONCOGENES IN ANIMAL TUMORS

ras oncogenes have been implicated in the development of a variety of tumors. They have been found in four strains of acute transforming retroviruses of rodents (3–6) and are occasionally activated in tumors induced by retroviruses that do not carry oncogenes (20; J. Ihle, personal communication). *ras* oncogenes, however, have been more frequently identified in tumors of nonviral etiology. In particular, animal tumors induced by chemical or physical carcinogens have been an abundant source of this class of oncogenes (150). Moreover, *ras* oncogenes are present in about 10% of the most common forms of human neoplasia, thus making them the most frequently identified oncogene family in human cancer (47–49).

As documented in the previous section, the transforming properties of *ras* oncogenes have been extensively documented in in vitro cell culture systems. However, cancer is a multistage disease that probably results from accumulation of independent genetic and epigenetic errors. The progressive generation of aneuploidy during tumor development suggests that most of the aberrations present in tumor cells may occur after malignancy has been irreversibly established. Therefore, the basic question posed by the frequent identification of *ras* oncogenes in spontaneous tumors such as those of humans is whether *ras* oncogenes participate in the induction of neoplastic development or are a consequence of it. Some experimental evidence suggests that *ras* oncogenes can, in fact, become activated after cells have acquired neoplastic properties (206–208). However, recent results obtained in animal model systems indicate that *ras* oncogenes are more likely to participate in the initiation of tumor development. These findings will be reviewed in this section.

Carcinogen-Induced Tumors

ras oncogenes have been found to be reproducibly activated in a variety of carcinogen-induced animal model tumor systems (Table 1). In rats, induction of mammary carcinomas by a single dose of nitroso-methylurea (NMU) during puberty leads to the activation of H-*ras*-1 oncogenes in 86% of the tumors (12, 209). Substitution of this carcinogen by dimethyl-benz(a)anthracene (DMBA) also results in the malignant activation of the H-*ras*-1 locus although in only one fourth of the tumors (209). Activation of the K-*ras*-2 locus has been observed in 40% of kidney mesenchymal tumors induced by a single dose of methyl(methoxymethyl)nitrosamine (DMN) (210) and in 74% of lung tumors (adenocarcinomas and squamous cell carcinomas) by chronic exposure to tetranitromethane (TNM) (J. Stower, M. W. Anderson, personal communication).

In mice (Table 1), H-*ras*-1 oncogenes have been found to be reproducibly activated in 90% of skin papillomas and carcinomas of Sencar mice initiated by DMBA or dibenz(c,h)acridine (DBACR) treatment and followed by promotion with TPA (211–213). Similarly, H-*ras*-1 oncogenes have been found in each of 4 mammary tumors arising from hyperplastic alveolar nodules implanted in mice treated with DMBA (214). Exposure of AKRxRF hybrid mice to X-rays or to repeated NMU treatment leads to the induction of lymphomas, most of which (over 60%) contain activated K-*ras*-2 or N-*ras*

Table 1 Activation of *ras* oncogenes in carcinogen-induced animal tumors

Species	Carcinogen	Tumor	Oncogene	Incidence	Reference
Rat	NMU	Mammary carcinoma	H-*ras*-1	86%	12,209
	DMBA	Mammary carcinoma	H-*ras*-1	23%	209
	DMN	Kidney mesenchymal	K-*ras*-2	40%	210
	TNM	Lung carcinoma	K-*ras*-2	74%	a
Mouse	DMBA	Skin carcinoma	H-*ras*-1	90%	211–213
	DBACR	Skin carcinoma	H-*ras*-1	80%	213
	DMBA	Mammary carcinoma	H-*ras*-1	100%	214
	X-rays	Lymphoma	N-*ras*,K-*ras*-2	57%	14, b
	NMU	Lymphoma	N-*ras*,K-*ras*-2	85%	14, b
	MCA	Thymic lymphoma	K-*ras*-2	83%	214a
	MCA	Fibrosarcoma	K-*ras*-2	50%	215
	HOAFF	Hepatocellular carcinoma	H-*ras*-1	100%	217
	VC	Hepatocellular carcinoma	H-*ras*-1	100%	217
	HODE	Hepatocellular carcinoma	H-*ras*-1	100%	217
	Furfural	Hepatocellular carcinoma	H-*ras*-1	85%	a
	TNM	Lung carcinoma	K-*ras*-2	100%	c

[a] S. Reynolds, M. W. Anderson, personal communication.
[b] A. Pellicer, personal communication.
[c] J. Stowers, M. W. Anderson, personal communication.

oncogenes (14). K-*ras*-2 oncogenes have also been observed in 10 out of 10 lung tumors induced by TNM (J. Stowers, M. W. Anderson, personal communication), and in 10 out of 12 thymic lymphomas (214a) and 2 out of 4 fibrosarcomas induced by MCA (215). Finally, H-*ras*-1 oncogenes have been found in almost 100% of hepatocellular carcinomas of $B6C3F_1$ mice that either arose spontaneously (216) or were induced by carcinogens such as N-hydroxy-2-acetyl-aminofluoride (HOAAF) (217), vinyl carbamate (VC) (217), 1'-hydroxy-2'-3'-dehydroestragole (HODE) (217), or furfural (S. Reynolds, M. W. Anderson, personal communication). The frequent and reproducible activation of *ras* oncogenes in these animal tumors strongly supports the concept that *ras* oncogenes play a causative role in neoplastic development (Table 1).

ras oncogenes have also been randomly (<10% incidence) identified in certain animal model systems such as rat fibrosarcomas induced by either 1,6- or 1,8-dinitropyrene (217a, M. Nagao, personal communication) or liver carcinomas induced by methyl(acetoxymethyl)nitrosamine (DMN-OAc) (J. Rice, personal communication). Finally, *ras* oncogenes have been identified in certain rodent cell lines transformed by in vitro exposure to chemical carcinogens. Whereas the K-*ras*-2 oncogene was found in mouse cells treated with 3-methyl-cholanthrene (MCA) (218), N-*ras* was found to be activated in four tumorigenic guinea pig cell lines initiated by nitroso compounds and polycyclic hydrocarbons (219; J. Doniger, personal communication).

ras *Genes as Targets of Carcinogens*

It is generally accepted that most carcinogens are mutagens. A significant number of chemical carcinogens are known to form adducts with DNA bases (reviewed in 220). Whereas some of these adducts are highly mutagenic due to their miscoding properties (221, 222), others can lead to mutations due to the generation of apurinic sites or because of the limited fidelity of repair polymerases (reviewed in 223). Whereas most of these mutations will have no consequence to the host, a small number of them can trigger neoplastic development. Identification of these critical mutations has eluded scientists for many years.

The reproducible activation of *ras* oncogenes in carcinogen-induced tumors has made it possible to correlate their activating mutations with the known mutagenic effects of certain carcinogens (Table 2). Each of the H-*ras*-1 oncogenes present in rat mammary carcinomas induced by NMU but not DMBA became activated by G → A transitions are the most common mutations induced by NMU (221, 222). These mutations result from the miscoding properties of O^6-methylguanosine, one of the adducts generated by the methylating activity of NMU. In contrast, DMBA forms large adducts with adenine and guanine residues whose repair very seldom leads to the

generation of G → A substitutions (220). These findings have led to the proposal that NMU is directly responsible for the malignant activation of H-*ras*-1 oncogenes in these mammary tumors (209).

Although the H-*ras*-1 locus can be activated by G → A substitutions in either of the two guanine residues present in the 12th codon (GGA), only those affecting the middle position (G^{35}) have been observed in NMU-induced mammary carcinomas (209). Such a strong positional effect adds further support to the concept that NMU is directly involved in the generation of these mutations. A possible explanation for the reproducible occurrence of $G^{35} \rightarrow A^{35}$ transitions in these tumors has recently been obtained. Analysis of the rate of repair of O^6-methylguanosine residues in the ampicillanase gene of a bacteriophage f1/pBR322 plasmid chimera has revealed a DNA consensus sequence around unrepaired $\overset{*}{G}$ residues (224). This sequence, GCTGGTCGCCA$\underline{\text{GG}}\overset{*}{\text{A}}$GG, where $\overset{*}{G}$ is the unrepaired O^6MeG residue, is 75% homologous (12 out of 16 residues) to the rat H-*ras*-1 sequence around the critical 12th codon (underlined in the consensus sequence). The probability of a random match with this stringency is 0.0017. Moreover, no other sequence in the entire H-*ras*-1 locus exhibits a similar match with this consensus sequence (224). Therefore, it is possible that NMU may induce mammary carcinomas in rats by interacting with a DNA sequence within a critical proto-oncogene domain that cannot be efficiently repaired.

The concept that *ras* oncogenes can be the targets of chemical carcinogens has been further supported by recent experimental evidence obtained with other animal tumor model systems (Table 2). Induction of skin carcinomas in mice by DMBA and phorbol esters involves the specific activation of H-*ras*-1 oncogenes by A → T transitions in the second base of codon 61 (212, 213). However, this mutation has not been seen when the initiating carcinogen,

Table 2 Specific mutagenesis of H-*ras*-1 oncogenes in chemically induced tumors

Species	Type of tumor	Carcinogen	Activating mutation	Incidence	Reference
Rat	Mammary carcinoma	NMU	$G^{35} \rightarrow A$	61/61	209
		DMBA	$A^{182}, A^{183} \rightarrow N$	5/5	209
Mouse	Skin papilloma or carcinoma	DMBA	$A^{182} \rightarrow T$	33/37	212,213
Mouse	Hepatocarcinoma	None	$C^{181} \rightarrow A$	6/11	a
			$A^{182} \rightarrow T$	3/11	a
			$A^{182} \rightarrow G$	2/11	a
		HO-AAF	$C^{181} \rightarrow A$	7/7	217
		VC	$A^{182} \rightarrow T$	6/7	217

[a] S. Reynolds, M. W. Anderson, personal communication.

DMBA, was replaced by *N*-methyl-*N*-nitro-*N*-nitroso-guanidine (MNNG), an alkylating carcinogen (212). Similar observations have been reported in hepatomas of male $B6C3F_1$ mice. In this model system, H-*ras*-1 oncogenes present in spontaneous tumors of old age mice (216) are activated by random mutations in the first two nucleotides of codon 61 (S. Reynolds, M. W. Anderson, personal communication). However, when these hepatocellular carcinomas are induced by carcinogenic treatment, the activating mutations conform to a specific pattern (217). Treatment of $B6C3F_1$ mice with a single dose of HO-AAF generates H-*ras*-1 oncogenes activated by C → A transversions in the first base of codon 61 (217). Although the mutagenic specificity of HO-AAF is not known, both *N*-hydroxy-*N*-2-aminofluorene and *N*-acetoxy-acetylaminofluorene (AAAF) are known to induce G-C → T-A transversions (225, 226). Moreover, in vitro treatment of H-*ras*-1 proto-oncogene DNA with AAAF leads to its oncogenic activation by $C^{181} \rightarrow A^{181}$ transitions (227). Substitution of HO-AAF by VC as the initiating carcinogen results in a completely different mutagenic spectrum. H-*ras*-1 oncogenes present in hepatocellular carcinomas induced by VC consistently exhibit missense mutations (mostly A → T transversions) in the A^{182} residue (217). These findings, taken together, indicate that *ras* oncogenes can be directly activated by the mutagenic properties of the initiating carcinogens.

Multistep Carcinogenesis

Activation of *ras* oncogenes by the initiating carcinogens in tumors induced by a single carcinogenic insult implies that these oncogenes must participate in the initiation of neoplastic development (209, 212, 217). This concept is supported by the identification of transforming H-*ras*-1 genes in the majority of skin papillomas induced by topical application of DMBA or DBACR followed by treatment with tumor promoters (213, 228). Most of these papillomas will regress spontaneously and only a few of them will develop as malignant carcinomas. These results provide independent biological evidence that *ras* genes can be activated during the early stages of tumor development (213, 228).

Activation of *ras* oncogenes is not sufficient to trigger tumorigenesis. Emerging evidence indicates that they must cooperate with secondary genetic events and/or with specific developmental programs in order to elicit neoplastic development. For instance, it has been shown that the active stage of proliferation of the developing mammary gland at the time of the carcinogen insult plays a fundamental role in tumor development (reviewed in 229). Recent results, however, indicate that activation of *ras* oncogenes does not have to be concomitant with mammary gland development (S. Sukumar, personal communication). Treatment of newborn rats with a single dose of NMU leads to the development of mammary carcinomas 2–3 months after the

animals have reached sexual development. Most of these tumors carry H-*ras*-1 oncogenes, each activated by the G → A transition diagnostic of NMU-induced mutagenesis. Treatment of these animals with antiestrogen drugs followed by ovariectomy eliminates the occurrence of mammary carcinomas (S. Sukumar, personal communication). These observations suggest that whereas H-*ras*-1 oncogenes might become activated early in life, manifestation of their malignant properties requires the hormone-induced proliferation and/or differentiation that takes place in the mammary gland during sexual maturation.

A similar situation may occur in the skin carcinogenesis model of mice. Initiated cells are known to remain dormant in the skin of DMBA-treated mice for long periods of time until they are treated with tumor promoters (230). Considering that most of the papillomas and carcinomas generated in this model system contain H-*ras*-1 oncogenes (211–213, 228), it is likely that these initiated cells already carry activated *ras* oncogenes. Direct support for this hypothesis has been recently obtained (231). Infection of mouse epidermal skin cells with Harvey-MSV results in the generation of initiated cells that do not express discernible neoplastic properties unless they are treated with TPA. These results indicate that in this model system *ras* oncogenes also require cooperation with cellular proliferation to allow expression of their neoplastic properties.

Induction of skin carcinomas by Harvey-MSV has provided direct experimental evidence for the existence of secondary genetic events in *ras*-induced tumors (231). Whereas Harvey MSV-induced papillomas appear to be polyclonal, the malignant carcinomas are clonal in origin. Papillomas are known to regress to small hyperkeriotic lesions before a few of them begin their invasive growth characteristic of skin carcinomas. Therefore, a second genetic event distinct and independent from *ras* oncogene activation is required for development of skin carcinomas (231). Recently, it has been shown that skin grafts of cultured keratinocytes infected with Harvey-MSV lead to the generation of papillomas but not carcinomas (231a), thus adding further evidence to the concept that *ras* oncogenes are not sufficient for the development of the full malignant phenotype in, at least, this model system.

Similar conclusions have been obtained by independent studies utilizing transgenic mice. Mice carrying in their germ line H-*ras* oncogenes linked to the promoter region of the mouse whey acidic protein (WAP) gene (N. Hynes, B. Groner, personal communication) or to the LTR of the mouse mammary tumor virus (MMTV) (P. Leder, personal communication). These animals develop single mammary tumors after a relatively long latent period, suggesting that a secondary event is necessary for tumor development. Mating transgenic mice carrying MMTV-*ras* chimeras with those containing MMTV-

myc genes (232), results in offspring that develop neoplastic outgrowths in most of their breasts (P. Leder, personal communication). These results suggest that animals carrying two resident oncogenes may not require additional genetic events for neoplastic development.

The requirement of a second genetic event may not be necessary if *ras* oncogenes are activated during the early stages of development. Transgenic mice carrying *ras* oncogenes under the transcriptional control of the elastase promoter die of pancreatic tumors a few days after birth (R. Palmiter, personal communication). In mice, the pancreas evolves from the gut at day 11 of gestation. At day 14 to 15, elastase begins to be expressed in the acimer cells of the pancreas and by day 16 tumors can already be detected. In contrast, the pancreas of transgenic mice carrying the normal *ras* proto-oncogene linked to the elastase promoter developed normally and the mice survived up to one year of age (R. Palmiter, personal communication). These results dramatically illustrate that under certain circumstances expression of a single *ras* oncogene is sufficient to induce tumor development.

Retrovirus-Induced Tumors

To date, four *ras*-containing retroviruses have been identified. They include Harvey-MSV isolated by inoculation of rats with Moloney murine leukemia virus (MuLV) followed by injection of the rat-passaged virus into newborn BALB/c mice (1), Kirsten-MSV derived from rats inoculated with cell-free filtrates from thymic lymphomas of old C3H mice (2), BALB-MSV isolated from an hemangiosarcoma of a BALB/c mouse induced by transmission of a cell-free extract of blood from a spontaneous chloroleukemia of an 18-month-old BALB/c mouse (233, 234), and Rasheed-MSV generated by in vitro cocultivation of a spontaneously transformed rat cell line (235). These viruses primarily cause erythroleukemias and fibrosarcomas when injected into newborn rodents. However, the pathogenic spectrum of these viruses can be altered by manipulations involving changes in dose, route of administration, strains, age of infected animals, etc. These studies have been extensively reviewed in the past (175, 236) and will not be considered here any further.

Introduction of the human H-*ras*-1 oncogene isolated from the T24/EJ bladder carcinoma cell line into appropriate vectors has also yielded transforming retroviruses. These viruses can also induce erythroleukemias and sarcomas when injected into newborn mice (236a). These experiments illustrate the wide malignant spectrum of human *ras* oncogenes. However, they should be interpreted with caution as the levels of expression of these human *ras* oncogenes are much higher in retrovirus-infected cells that they would normally be in human tumors.

Construction of similar retroviruses carrying the normal human H-*ras*-1

proto-oncogene did not yield transforming viruses (236a). These results are somewhat surprising considering the in vitro transforming properties of LTR-*ras* proto-oncogene chimeras (3, 159). In related experiments, the miscoding mutations of codons 12 and 59 of the H-*ras* oncogene present in Harvey-MSV have been eliminated (73). The resulting Gly^{12}/Ala^{59} Harvey-MSV mutant virus exhibits decreased transforming properties, although it retains the capacity to transform NIH3T3 cells and rat embryo cells (73). Other authors have reported that whereas transcriptionally enhanced *ras* proto-oncogenes cannot transform primary rat embryo cultures, they can confer long-term proliferative properties to the transfected cells (171). The reasons for these discrepancies probably lie in the different constructs and assay conditions used in these experiments.

Replication-competent retroviruses that do possess oncogene sequences may also exert their neoplastic properties by transcriptional activation of cellular *ras* genes. Induction of a chicken nephroblastoma by a myeloblastosis-associated retrovirus resulted in elevated levels (25-fold) of a novel H-*ras* transcript that contained retroviral LTR U5 sequences (20). Viral integration did not occur adjacent to the H-*ras* locus but probably next to a distant splicing donor site. Sequence analysis of a DNA clone complementary to the hybrid LTR U5-H-*ras* transcript showed no mutations in any of the critical *ras* codons (20). Thus, it is likely that transcriptional activation of the normal chicken H-*ras* locus contributed to the development of this tumor.

A related event may have accounted for the highly elevated (30-fold) expression of p21 *ras* proteins in a myeloid cell line (416B) derived from a mouse bone-marrow culture infected with the Friend retrovirus complex (237, 238). In this case, a 3.5 kbp segment of the Friend helper virus was inserted within the first intron of the mouse K-*ras*-2 locus (21). As a result, K-*ras*-2 transcription was initiated at a new site (presumably within the retroviral LTR) that excluded the first K-*ras*-2 noncoding exon located upstream from the viral integration site (21). The original 416B cells were nontumorigenic (237, 238). However, these cells have now become malignant, presumably as a consequence of continuous passage in vitro (21). These observations suggest that transcriptional activation of the K-*ras*-2 locus in this mouse hematopoietic cell line may have contributed, but was not sufficient to induce tumorigenic properties in these cells. Similar findings have been recently obtained in DA-2 cells, a cell line derived from a Moloney murine leukemia virus (MLV)–induced tumor. In these cells, Moloney-MLV sequences became integrated 5' of the first coding exon of the H-*ras*-1 locus (J. Ihle, personal communication). This genomic rearrangement is likely to be responsible for the high levels of expression of p21 *ras* proteins observed in DA-2 cells and it is presumed to have contributed to tumor development.

ras ONCOGENES IN HUMAN CANCER

Incidence

ras oncogenes exist in a variety of human cancers. They have been identified in carcinomas of the bladder, breast, colon, kidney, liver, lung, ovary, pancreas, and stomach; in hematopoietic tumors of lymphoid (acute lymphocytic leukemia, B-cell lymphoma, Burkitt's lymphoma) and myeloid (acute and chronic myelogenous leukemias, promyelocytic leukemia) lineage; and in tumors of mesenchymal origin such as fibrosarcomas and rhabdomyosarcomas. Other tumors, including melanomas, teratocarcinomas, neuroblastomas, and gliomas have also been shown to possess *ras* oncogenes. Specific references to these studies can be found in Ref. 49 and will not be included here. Recently, a H-*ras*-1 oncogene has been identified in 1 of 5 keratoacanthomas, a well-characterized human benign tumor of the skin (J. Leon, A. Pellicer, personal communication). Keratoacanthomas exhibit an initial aggressive growth followed by spontaneous regression. These findings resemble those observed in the mouse skin model tumor system (see previous section) in which H-*ras*-1 oncogenes were shown to participate in the early stages of tumor development.

So far, transforming *ras* genes are the oncogenes most frequently identified in human cancer. Their overall incidence is estimated to be around 10–15%. This figure is likely to be lower in certain malignancies such as breast carcinoma, while it may be higher in other tumors such as acute myelogenous leukemia (89). The actual incidence of *ras* oncogenes in human tumors is difficult to assess. Available information has been obtained from transfection assays, which are somewhat insensitive, particularly in detecting large oncogenes such as K-*ras*-2. Moreover, many scientists do not conduct parallel experiments to test the sensitivity of their transfection assays and to control for the intactness of the donor DNA. This is of particular importance in laboratories that lack direct access to reliable tumor material. Finally, some authors only report those tumors or tumor cell lines in which they have obtained positive results. Recent studies utilizing the RNA:RNA hybrid mismatch technology (see next section) have shown the presence of K-*ras*-2 oncogenes carrying codon 12 mutations in 22 of 55 colon carcinomas (M. Perucho, personal communication). Considering that mutations at codon 61 of K-*ras*-2 oncogenes have not yet been analyzed and that N-*ras* oncogenes have also been found in colon carcinomas (49), the overall incidence of *ras* oncogenes in this common type of human cancer may have been underestimated. In any case, available information to date indicates that (*a*) *ras* oncogenes are present in a significant percentage (varying from 5 to 40%) of most human tumors; (*b*) they are not associated with specific types of neo-

plasia; and (*c*) their activation does not correlate with the histo-pathological properties of the tumor.

The involvement of *ras* genes in human cancer is not limited to their activation by point mutations. It is likely that expression of abnormally high levels of normal *ras* products may also contribute to malignancy. This could be achieved by perturbation of their regulatory sequences (20, 21) or by gene amplification. In human tumors, there is no evidence for transcriptional activation of *ras* proto-oncogenes by mutations affecting their regulatory elements. In contrast, significant amplification ($\geq$10 fold) of *ras* genes has been observed in a variety of human tumors (160, 239–242). However, the overall incidence of *ras* gene amplification in human neoplasia is estimated to be not higher than 1% (160, 240).

Loss of the normal allele in cells carrying *ras* oncogenes has been observed with relative frequency in tumor cell lines, but only rarely in tumor biopsies (243). Interestingly, loss of *ras* alleles also occurs in tumors that do not carry *ras* oncogenes (240, 244, 245). Whether these deletions play any role in the pathogenesis of human cancer remains to be determined.

Expression Studies

Expression of *ras* genes is often increased in tumors relative to normal tissues. Careful quantitative analysis has indicated that the levels of *ras* transcripts in about 50% of human tumors are 2- to 10-fold higher than in control tissue (246–248). Similar results have been obtained by immunoblot analysis of p21 *ras* expression in a variety of primary carcinomas (249–251). Surprisingly, metastatic tissues derived from primary colon carcinomas consistently exhibited low levels of p21 *ras* proteins (249). The expression of *ras* genes in human tumors has also been studied by immunohistochemical techniques. Immunocytochemistry has the advantage of allowing the evaluation of *ras* gene expression in individual cells. These studies have utilized two monoclonal antibodies, Y13-259 (120) and RAP-5 (252), neither of which discriminates between the different p21 *ras* proteins or between their normal and activated forms. Unfortunately, no clear picture has emerged from these studies. Some authors have consistently identified increased levels of immunoreactivity in malignant cells of mammary (252), colon (253), bladder (254), and prostate (255) carcinomas as compared to those of corresponding benign tumors, dysplastic lesions, or normal epithelium. Other authors, however, have not been able to detect any significant differences between normal and malignant tissues (256–258). Moreover, two independent laboratories have reported that the RAP-5 monoclonal antibody may recognize a cytoplasmic cellular component distinct from p21 (258, 258a). In this regard, it is worthwhile to note that a monoclonal antibody that specifically recognizes Val^{12} p21 *ras* proteins by immunoblotting or even by immuno-

peroxidase staining of human tumor cell lines in culture, reacts nonspecifically with a high percentage (over 50%) of formalin-fixed tissue sections (259; see next section). These observations indicate that further work is needed to validate the use of paraffin-embedded formalin-fixed tissue samples in studying the expression of *ras* genes in human tumors.

The biological significance of results obtained by studying the expression of *ras* genes in human tumors is not clear. Enhanced expression of normal *ras* gene products has been implicated in the development of certain animal tumors (20; J. Ihle, personal communication) and in transformation of cells in culture (21, 159, 159a, 160). However, in these cases a 30- to 100-fold increase in the levels of both mRNA and p21 *ras* protein expression has been consistently observed. On the other hand, increases in *ras* transcripts of up to eight-fold can be associated with normal proliferative processes such as regenerating rat liver (156). Therefore, there is not sufficient experimental basis to implicate moderate variation of *ras* proto-oncogene expression in the neoplastic development of human tumors.

Predisposition to Cancer

Extensive genetic evidence suggests the existence of genes responsible for the predisposition of certain individuals to cancer. *ras* oncogenes may not belong to this class of genes. First of all, *ras* oncogenes become activated in somatic rather than in germ line cells (89, 243, 260, 261). Second, normal cells of patients with high cancer risk syndromes do not contain *ras* oncogenes (262). However, a recent population study on the frequency of H-*ras*-1 alleles in normal individuals versus cancer patients has revealed a statistically significant correlation between the latter and certain unusual H-*ras*-1 alleles (244).

The human H-*ras*-1 locus contains a region of around 800 bp of tandemly repeated sequences located 1000 bp downstream from its polyadenylation signal (58). Genetic variability within this stretch of repeated sequences generates a variety of restriction fragment length polymorphisms (RFLP). Up to 20 polymorphic H-*ras*-1 alleles have been identified, of which only four are commonly found in the human population (244). Of a total of 230 samples of white blood cells of normal donors, only 9 (3.9%) exhibited any of the 16 rare alleles. In contrast, of 298 samples of either normal or tumor tissue from cancer patients, 32 (10.7%) contained rare alleles. No significant difference was seen between normal and tumor samples of cancer patients, indicating that the increased frequency of rare alleles in these patients is an inherited property and not a consequence of somatic events associated with tumor development (244). All tumor types, including myelodysplastic lesions, were associated with rare H-*ras*-1 alleles, suggesting that these RFLPs are indicative of genetic predisposition to most, if not all, forms of cancer. Different results have been obtained in an independent study investigating the

frequency of rare H-*ras*-1 alleles in lung cancer (245). In this study, 132 patients were examined, 66 with small cell lung carcinomas (SCLC) and 66 with non-SCLC. Rare H-*ras*-1 allelic patterns were found in patients with non-SCLC but not in those with SCLC (245). These observations suggest that large numbers of cancer patients must be examined to fully assess the incidence of rare H-*ras*-1 alleles in specific types of malignancies. The need for such studies has been further indicated by recent reports contesting the possible linkage between rare H-*ras*-1 RFLP and the development of myelodysplasia (263) and melanoma (263a).

It is not clear how these rare H-*ras*-1 alleles may predispose to cancer. It has been speculated that the rare alleles representing specific arrays of tandem-repeats may be in linkage disequilibrium with missense mutations in the H-*ras*-1 coding sequences that may confer weakly oncogenic properties to this locus (244). Recently, it has been shown that these polymorphic repeated sequences have enhancer activity in in vitro CAT assays (263b; S. Ishii, personal communication). Thus, it is possible that a somewhat altered regulation over long periods of time of the H-*ras*-1 gene or of a putative neighboring locus may predispose certain cells to neoplastic growth. However, experimental evidence to support any of these hypotheses has, as yet, to be obtained.

New Diagnostic Methods

So far, identification of *ras* oncogenes in human tumors has relied on tedious and insensitive transfection assays. It is evident that any attempt to utilize information on activation of *ras* oncogenes in clinical studies will require the development of faster and more sensitive diagnostic methods. The first step in this direction has involved the use of oligonucleotides of defined sequence to identify single point mutations in genomic DNA. Hybrids between oligonucleotides that form a perfect match with genomic sequences are more stable than those that exhibit a single mismatch (264). Oligonucleotides corresponding to sequences surrounding the critical 12th and 61st codons of *ras* genes have been successfully used to identify the presence of *ras* oncogenes in certain human neoplasias (89, 265). In spite of the significant advantages of oligonucleotide probing over current gene transfer assays, this technique is still somewhat cumbersome. *ras* genes can theoretically be activated by almost 100 different base substitutions, of which over 30 have already been identified in naturally occurring tumors (49). This heterogeneity can be partially overcome by using oligonucleotide mixtures containing each of the three possible substitutions at a given residue (89, 265). Nevertheless, the significant number of oligonucleotide probes required to identify each possible *ras* oncogene limits the general applicability of this technique.

Several new methods capable of identifying single point mutations over

relatively long stretches of DNA sequences may simplify the task of identifying *ras* oncogenes. One of these methods is based on the abnormal electrophoretic migration of DNA heteroduplexes containing single base mismatches in denaturing gradient gels (266). Unfortunately, this method can only identify between 25 and 40% of all possible base substitutions (266). Moreover, considerable technical expertise is required for the preparation of the denaturing gradient gels. The development of [^{32}P]-labeled RNA probes has made possible the identification of single base pair mismatches in RNA:RNA and RNA:DNA hybrids based on their sensitivity to RNAse A digestion (267, 268). Hybridization of tumor DNA or RNA samples with these probes, followed by incubation with RNAse A, cleaves the labeled RNA probes into fragments of defined sizes that can be easily resolved by polyacrylamide gel electrophoresis. These techniques can identify most base substitutions (267, 268). Moreover, the RNA:RNA hybrid method provides useful information regarding the levels of expression of *ras* oncogenes in these tumors. In addition, the relative expression of both normal and transforming alleles can be analyzed (267). Recently, a new method involving the primer-mediated enzymatic amplification of specific genomic sequences has been developed (269). This process, which can easily be automated (K. Mullis, personal communication), should make possible the routine use of oligonucleotide probes to identify *ras* oncogenes in clinical laboratories.

In spite of these technological improvements, the use of nucleic acid hybridization techniques to identify human *ras* oncogenes would always require the use of six different probes (specific for the first and second exons of each of the three *ras* loci) and enough tissue to obtain sufficient amounts of RNA or DNA. In theory, a more practical approach would be to generate monoclonal antibodies capable of discriminating between the putative "active" and "inactive" conformation of p21 *ras* proteins (see Figure 2). Unfortunately, antibodies elicited against peptides containing the missense mutations characteristic of *ras* oncogenes only identify those p21 *ras* proteins carrying the amino acid substitutions present in the immunizing peptide (102, 259, 270). On the other hand, antibodies elicited against bacteria-synthesized p21 *ras* proteins recognize the normal and oncogenic forms with equal efficiency (our unpublished observations). X-ray crystallographic analysis of p21 molecules should help in identifying putative domains that may elicit such diagnostic antibodies.

A potential caveat in using monoclonal antibodies to identify the products of *ras* oncogenes in human tissue sections is the potential lack of specificity (see previous section). Monoclonal antibodies capable of recognizing oncogenic *ras* proteins carrying Val^{12} substitutions by immunoblotting analysis have been shown to be highly specific in immunoperoxidase staining of human carcinoma cell lines in culture (259). However, when this antibody

was used to analyze formalin-fixed tumor biopsies, a significant number gave false positive results (259). Therefore, further work will be required to work out the technical challenges posed by the diagnostic identification of *ras* oncogenes in human tumor biopsies.

Future Avenues

In spite of the extensive documentation on the malignant properties of *ras* oncogenes, their presence in human tumors cannot be considered as sufficient evidence of a causative role in the pathogenesis of human cancer. Results obtained with carcinogen-induced tumors and transgenic mice have indicated that *ras* oncogenes can participate in the initiation of carcinogenesis. However, these observations do not preclude the possibility that *ras* oncogenes may also be generated in cells that have already acquired neoplastic properties. To evaluate the involvement of *ras* oncogenes in human cancer it is necessary to determine what percentage of *ras* oncogenes plays a primordial role in tumor development and what percentage becomes activated as a consequence of it. Unfortunately, this question cannot be easily addressed as in human carcinogenesis it is virtually impossible to backtrack to the early events that led to neoplastic development.

Future progress on the involvement of *ras* oncogenes in human cancer depends on results derived from two independent avenues of research. First, we must continue ongoing efforts aimed at unveiling the molecular and biochemical mechanisms by which *ras* oncogenes disrupt the normal proliferative programs. Second, we must develop fast and reliable diagnostic methods to allow the routine identification of *ras* oncogenes in human biopsies. This information, once applied to a large number of tumors, may reveal hidden connections between the activation of these oncogenes and the etiology and/or pathology of human cancer.

It is evident that the molecular pathways that lead to carcinogenesis may not parallel the histo-pathological criteria that have served to develop current cancer treatment programs. We can only hope that understanding the mechanisms by which oncogenes participate in neoplasia may provide a more rational approach to cancer therapy. The presence of *ras* oncogenes in at least 10% of human cancers certainly justifies the intense interest dedicated to this gene family during the last few years.

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