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Structure and Activation of the Human N-*ras* Gene

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Summary

The normal human N-*ras* gene has been cloned. In structure and sequence it closely resembles the human H-*ras* and K-*ras* genes. The three genes share regions of nucleotide homology and nucleotide divergence within coding sequences and have a common intron/exon structure, indicating that they have evolved from a similarly spliced ancestral gene. The N-*ras* gene of SK-N-SH neuroblastoma cells has transforming activity, while the normal N-*ras* gene does not, the result of a single nucleotide change substituting lysine for glutamine in position 61 of the N-*ras* gene product. From previous studies we conclude that amino acid substitutions in two distinct regions can activate the transforming potential of *ras* gene products.

Introduction

Oncogenes have been found both in RNA tumor viruses and in tumor cells (for review, see Cooper, 1982). The RNA tumor viruses have acquired their oncogenes by transduction (Spector, Varmus, and Bishop, 1978; Ellis et al., 1981), while the oncogenes of tumor cells probably have arisen by somatic mutation of normal cellular genes (Tabin et al., 1982; Reddy et al., 1982; Taparowsky et al., 1982). Considerable overlap has been observed between these two classes of oncogenes. The *ras* genes were first detected as the oncogenes of the Harvey and Kirsten sarcoma viruses. Many of the transforming genes of human and rodent tumor cells detected using the NIH/3T3 transfection assay are also members of the *ras* gene family (Der, Krontiris, and Cooper, 1982; Parada et al., 1982; Santos et al., 1982; Shimizu et al., 1983b; Hall et al., 1983). The Harvey and Kirsten *ras* genes are highly conserved in vertebrates and encode structurally and immunologically related guanine nucleotide binding proteins of approximately 21,000 daltons (Ellis et al., 1981).

We have previously described the structure and sequence of the human H-*ras* and K-*ras* genes, the cellular homologs of the Harvey and Kirsten sarcoma viruses (Shimizu et al., 1983a; Fasano et al. 1983). Here we describe the structure and sequence of the human N-*ras* gene, found to be the transforming gene of the SK-N-SH neuroblastoma cell line (Shimizu et al., 1983b). The H-*ras*, K-*ras*, and N-*ras* genes all have a similar exonic structure, and therefore all probably derive from a common, spliced ancestral gene. Moreover, the three genes share regions of nucleotide homology and have regions of nucleotide divergence which we believe reflect the different physiologic roles each gene plays.

The oncogene of the T24 human bladder carcinoma cells, H-*ras*, differs from the normal human H-*ras* gene in at least one significant way. As a result of a single nucleotide base substitution, the T24 H-*ras* gene encodes valine at position 12, while the normal gene encodes glycine (Tabin et al., 1982; Reddy et al., 1982; Taparowsky et al., 1982). The resulting mutant protein is 100 to 1000 times more potent at inducing transformation (Taparowsky et al., 1982). Here we present evidence that N-*ras*, the transforming gene of SK-N-SH cells, has also undergone mutational activation. The N-*ras* gene of SK-N-SH differs from the normal N-*ras* by at least one significant nucleotide substitution, resulting in an altered amino acid at position 61 of the encoded protein. This difference is sufficient to activate the transforming potential of the N-*ras* gene. Thus there appear to be at least two sites for the mutational activation of the transforming potential of the *ras* genes.

Results and Discussion

Structure of the N-*ras* Gene

DNA from the human neuroblastoma cell SK-N-SH is capable of inducing foci of morphologically transformed NIH-3T3 cells following DNA-mediated gene transfer (Perucho et al., 1981). A clone of the transforming gene of SK-N-SH was obtained by the method of suppressor rescue, as previously described (Shimizu et al., 1983c). We have found this gene to be a newly discovered member of the *ras* gene family, which is now called N-*ras* (Shimizu et al., 1983b). To study the structure of this gene and the nature of its mutational activation, we obtained a genomic clone, λNP13, of the N-*ras* gene from a λ Charon 4a library constructed from human placenta DNA. To facilitate the analysis of gene structure, we also cloned a partial cDNA copy of the SK-N-SH N-*ras* transcript in pBR322 (p6a1) by screening cDNA libraries constructed from poly(A)$^+$ RNA of an NIH/3T3 cell transformed with the N-*ras* gene. Restriction maps of the genomic and cDNA clones are shown in Figure 1.

We localized the coding regions of the genomic N-*ras* gene by Southern blot hybridizations, using as probes our cDNA clone and a clone of the partially homologous Harvey sarcoma virus *ras* gene, pHB-11 (Ellis et al., 1981). We then sequenced the genomic coding regions using the strategy shown in Figure 1. A comparison of this sequence with the known viral (Dahr et al., 1982; Tsuchida, Ryder, and Ohtsubo, 1982) and human K-*ras* (Shimizu et al., 1983a) and H-*ras* (Fasano et al., 1983; Capon et al., 1983) sequences enabled us to locate the coding exons of the N-*ras* gene. The complete nucleotide sequence of the N-*ras* coding exons and the predicted amino acid sequence of its encoded protein are shown in Figure 2. For comparison, the predicted amino acid sequences of the human H-*ras* gene from T24 bladder carcinoma cells and the human K-*ras* gene from Calu-1 lung carcinoma

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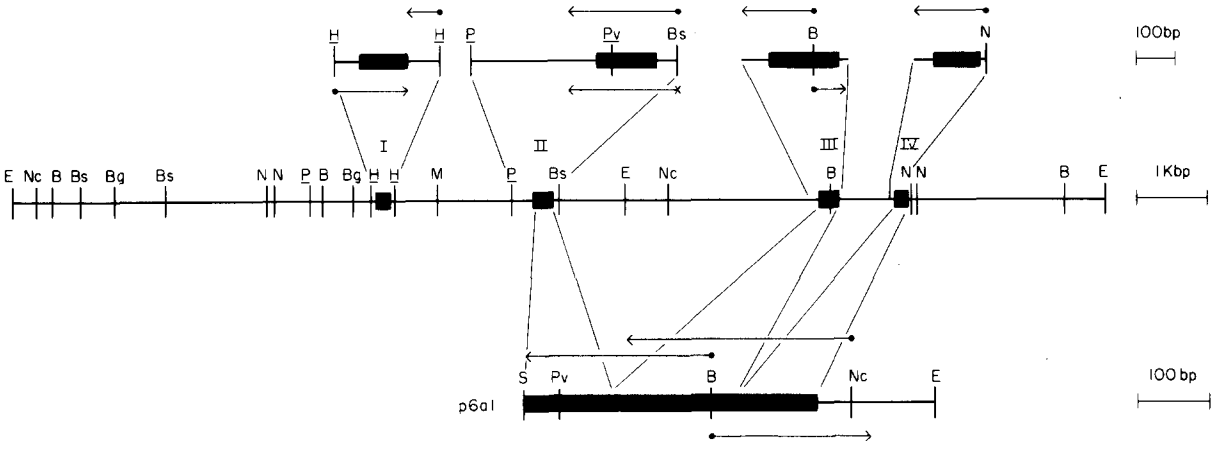

Figure 1. Restriction Map and Sequencing Strategy for the Genomic and cDNA (p6a1) Clones of the N-*ras* Gene

Coding regions are indicated as solid blocks. Restriction endonuclease sites are as follows: B, Bal I; Bg, Bgl II; Bs, BstE II; H, Hind III; M, Mst II; N, Nde I; Nc, Nco I; P, Pst I; Pv, Pvu II; E, Eco RI; S, Sal I. The map is not complete for the restriction sites that are underlined. The Sal I and Eco RI sites of p6a1 are molecular linkers. The areas of the two clones which were sequenced by the Maxam and Gilbert method are represented by arrows. (●) indicates restriction sites which were 3′-OH labeled with E. coli DNA polymerase I large fragment; (×) indicates sites which were 5′-OH labeled with T4 polynucleotide kinase.

cells are also shown, as are the viral H-*ras* and K-*ras* genes.

If we assume that the N-*ras* gene utilizes the same ATG start codon as the H-*ras* gene, then N-*ras* has four coding exons, which together encode a 189 amino acid protein, the same length as the H-*ras* and one of the two alternate K-*ras* gene products (Shimizu et al., 1983a). Indeed, the mature form of the SK-N-SH N-*ras* product, which is immunoprecipitable with a broad-spectrum anti-*ras* monoclonal antibody, migrates in SDS polyacrylamide gels with a mobility similar to the H-*ras* and K-*ras* products (Shimizu et al., 1983b).

The splice junctions of the N-*ras* gene correspond precisely to those of both the H-*ras* and K-*ras* genes (see Figure 2 and Table 1). Thus all three genes have evolved from a similarly spliced ancestral gene that contained a minimum of four coding exons. In both humans and rats, however, the K-*ras* gene has an additional, diverged fourth coding exon, resulting in the potential to encode two alternate products (Shimizu et al., 1983a). We searched for such an alternate fourth coding exon within the 7.0 kb Eco RI fragment of N-*ras* by low stringency hybridization using viral H-*ras* and K-*ras* probes, and none was found.

A comparison of the predicted amino acid sequence of N-*ras* with that of H-*ras* and K-*ras* shows a remarkable sequence conservation between amino acid positions 1–120 and 129–166. A maximum of 12 differences is noted between any two known *ras* gene products in these regions (see Table 2). Nevertheless, 40%–60% of conserved amino acid positions utilize different codons (data not shown). This indicates that the maintenance of amino acid sequence is the result of evolutionary pressure.

There is one major and one minor region of amino acid divergence for the *ras* gene products. In the minor region, positions 121–128, the H-*ras* differs from the K-*ras* in five of eight positions. Here the N-*ras* gene resembles K-*ras*, differing at only one position.

In amino acid positions 171–185, the N-*ras*, H-*ras*, and K-*ras* gene products show little similarity. We therefore call this major region of divergence the variable region. Nevertheless, the variable regions of the viral *ras* genes, which originate from the rat genome (Ellis et al., 1981), closely resemble their respective human homologs (see Table 2). It thus appears that selective pressure has maintained this level of conservation. Other features of the amino acid sequence provide clues for the function of the variable region. For all *ras* proteins, the variable region is preceded by an amino acid sequence BNBB, where B is a basic amino acid and N is any amino acid. BNBB is a potential proteolytic cleavage site often used during processing in viral glycoproteins and secreted cellular products (Rice and Strauss, 1981; Ullrich et al., 1977; Perler et al., 1980). Following the variable region, all *ras* genes encode the amino acid sequence cysteine AAX, where A is an aliphatic amino acid and X is the carboxy-terminal amino acid. We have interpreted this structure to mean that, after processing, the variable regions form protein domains which may determine the physiologic specificity of the *ras* proteins (Shimizu et al., 1983a).

Activation of the N-*ras* Gene in SK-N-SH Cells

To examine the nature of the activating mutation in the N-*ras* gene of SK-N-SH cells, we cloned this gene directly from λ Charon 4a libraries constructed from SK-N-SH DNA. The two clones obtained, λNS12 and λNS35, had restriction endonuclease maps identical with the insert of the placental λNP13 clone (see Figure 1). In transfection experiments, DNA from λNS35 could induce foci of morphologically transformed NIH/3T3 cells, while DNA from λNS12 and λNP13 could not.

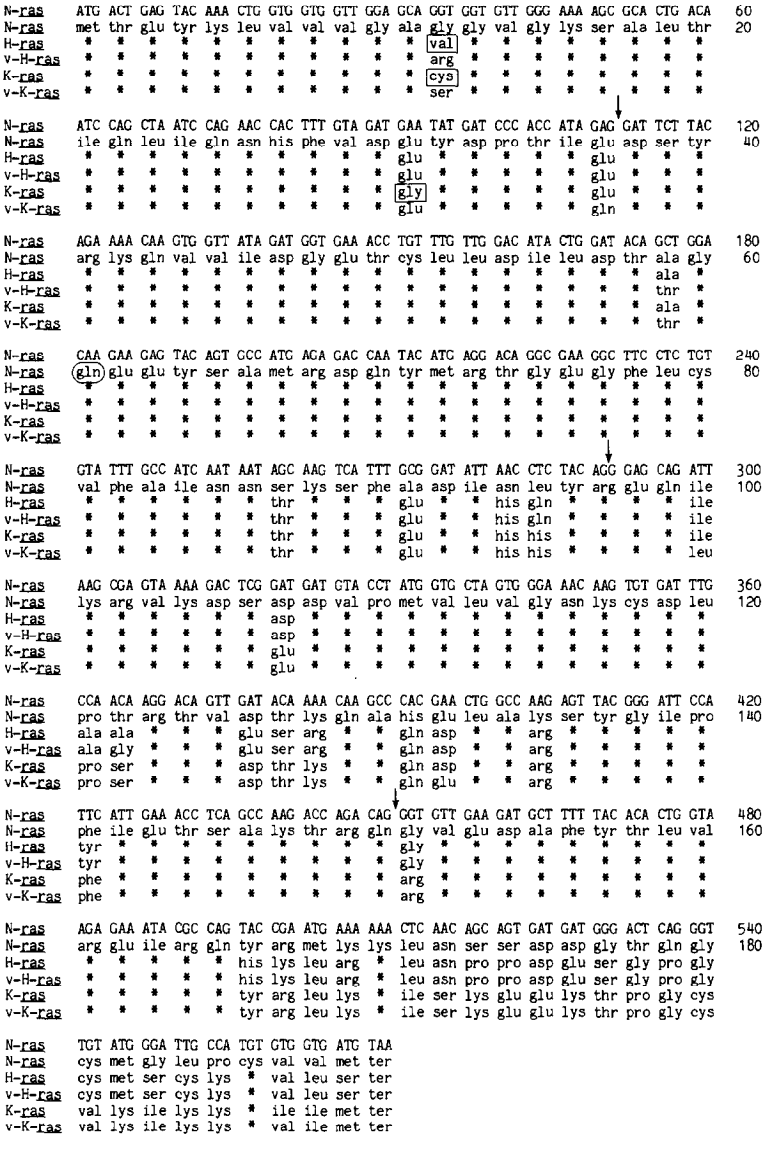

Figure 2. The Nucleotide Sequence of the Coding Region of the N-*ras* Gene and Comparison of the Predicted Amino Acid Sequences for Other Members of the *ras* Gene Family

The nucleotide sequence presented is that of the nontransforming N-*ras* gene (λNP13). The circle around amino acid 61 indicates the only amino acid difference between this gene and the transforming N-*ras* gene (λNS35), which encodes lysine at position 61. The amino acid sequence shown for H-*ras* is that of the transforming gene contained in the human bladder carcinoma cell line T24 (Fasano et al., 1983; Capon et al., 1983). The box about amino acid 12 indicates the only amino acid difference between this gene and the normal H-*ras* gene (Capon et al., 1983) which encodes glycine. The amino acid sequence shown for K-*ras* is that of the transforming gene contained in human lung carcinoma cell line Calu-1 (Shimizu et al., 1983a). The boxes around positions 12 and 31 indicate the only amino acid differences in the first exon between this gene and the normal K-*ras* gene which encodes glycine and glutamine in those positions, respectively (Shimizu et al., 1973a). The sequence for viral H-*ras* and K-*ras* are taken from Dahr et al. (1982) and Tsuchida et al. (1982), respectively. Vertical arrows indicate the position of splice junctions for the cellular *ras* genes.

To determine the location of the functionally significant differences between transformation-competent and incompetent genes, chimeric genes were constructed. In these experiments, summarized in Figure 3, restriction endonuclease fragments were purified from each N-*ras* gene, mixed, ligated, and transfected onto NIH/3T3 cells. When the 250 bp BstE II/Pvu II fragment from the N-*ras* of λNS35 was joined to the complementary fragments of the N-*ras* from λNP13, the resulting chimeric gene had transforming activity. Therefore the 250 bp BstE II/Pvu II fragment from the transforming N-*ras* gene contains mutations sufficient to activate the transforming potential of a normal N-*ras* gene.

The 250 bp BstE II/Pvu II fragment, which contains a portion of the second exon, was sequenced for the transforming and normal N-*ras* genes. Only one nucleotide difference was found. The 61st amino acid encoded by the nontransforming N-*ras* gene of λNP13 is glutamine (CAA), the same amino acid encoded by the H-*ras* and K-*ras* genes (see Figure 2). By contrast, the 61st amino acid encoded by the transforming N-*ras* gene is lysine (AAA). To confirm this nucleotide difference, the plasmid p6a1 containing a cDNA to the mRNA of the transforming N-*ras* gene was sequenced. The cDNA had the same nucleotide sequence as the transforming N-*ras* gene, encoding lysine in position 61. From these observations we conclude that, like the H-*ras* gene of T24 cells, a single nucleotide substitution has altered the product of a N-*ras* gene, thereby activating its transforming potential.

To exclude the possibility that other amino acid differences exist between the normal and transforming N-*ras* proteins, we determined the entire coding sequence of the

transforming N-*ras* gene by sequencing the cDNA and genomic clones (see Figure 1). There was complete concordance with the nucleotide sequence of the N-*ras* gene cloned from placenta DNA except at the nucleotide position encoding the altered 61st amino acid. In particular, both the normal and the transforming N-*ras* genes encode glycine at position 12, a position frequently mutated in other transforming *ras* genes (Tabin et al., 1982; Reddy et al., 1982; Taparowsky et al., 1982; Shimizu et al., 1983a).

We sequenced λNS12, the N-*ras* clone from SK-N-SH which could not transform NIH/3T3 cells, and found glutamine encoded at position 61. From this we infer that the SK-N-SH genome contains two N-*ras* genes: one transforming and one normal. This is consistent with the idea that the transforming mutation acts dominantly.

It is now apparent that activation of the transforming potential of the *ras* proteins can result from amino acid substitutions in either of two regions. Our work has shown that substituting lysine for glutamine in position 61 appears to activate *ras* protein. Similarly, work from another laboratory has recently shown the activation of the transforming potential of H-*ras* by a substitution of leucine for gutamine in position 61 (Yuasa et al., 1983). In this regard, it is of interest that both viral H-*ras* and K-*ras* proteins contain threonine in the nearby position 59, while human cellular *ras* genes encode alanine at that position (see Figure 2). We do not know if threonine in position 59 activates the transforming potential of the *ras* proteins. However, threonine in position 59 is the site for autophosphorylation by GTP (Shih et al., 1982), hence the amino acid in position 59 may be near the GTP binding site of the *ras* proteins. If so, position 61 would also be close to the GTP binding site. On the other hand, substituting valine (Tabin et al., 1982; Reddy et al., 1982; Taparowsky et al., 1982), cysteine (Shimizu et al., 1982a), serine, aspartic acid, or arginine (O. Fasano, unpublished) for glycine at position

Table 1. Splice Junctions of *ras* Gene Coding Exons

	EXON I	INTRON	EXON II
N-ras	GAG/GTGAGGC	.....	CCCCCAG/GAT
H-ras	GAG/GTGAGCC	.....	CCTGCAG/GAT
K-ras	GAG/GTAAATC	.....	TTCTCAG/GAT
	EXON II	**INTRON**	**EXON III**
N-ras	CAG/GTACTAG	.....	TTTTTAG/GGA
H-ras	CAG/GTGAACC	.....	CTCTCAG/GGA
K-ras	TAG/GTGGGTT	.....	TTCCCAG/AGA
	EXON III	**INTRON**	**EXON IV**
N-ras	CAG/GTATGGT	.....	TTTATAG/GGT
H-ras	CAG/GTGAGGC	.....	TTCCCAG/GGA
K-ras	CAG/GTAAGTA	.....	AATGCAG/AGA
Consensus	AG/GT	.....	PyPyPyNCAG/N

The locations of the splice junctions in the N-*ras* gene were determined by DNA sequencing (see Figure 1). The position of splice junctions of the human N-*ras* gene are shown in Figure 2. Genomic sequences about the splice junctions for the human K-*ras* gene were determined in previously unpublished work, and for the human H-*ras* gene from Fasano et al. (1983) and Capon et al. (1983). All three cellular *ras* genes have sequences about their splice sites that compare favorably with the consensus sequence for RNA splicing (Sharp, 1981). Py: pyrimidine. N: any nucleotide.

Table 2. Sequence Conservation of *ras* Genes and *ras* Gene Products

Amino Acid Positions 1–120 and 129–166

	N-*ras*	H-*ras*	v-H-*ras*	K-*ras*	v-K-*ras*
N-*ras*	1.0 1.0	0.937 0.778	0.930 0.785	0.930 0.795	0.924 0.802
H-*ras*		1.0 1.0	0.987 0.871	0.956 0.768	0.937 0.766
v-H-*ras*			1.0 1.0	0.949 0.768	0.943 0.772
K-*ras*				1.0 1.0	0.962 0.941
v-K-*ras*					1.0 1.0

Amino Acid Positions 171–185

	N-*ras*	H-*ras*	v-H-*ras*	K-*ras*	v-K-*ras*
N-*ras*	1.0 1.0	0.400 0.489	0.400 0.489	0.0 0.311	0.0 0.311
H-*ras*		1.0 1.0	1.0 0.955	0.067 0.333	0.067 0.333
v-H-*ras*			1.0 1.0	0.067 0.311	0.067 0.311
K-*ras*				1.0 1.0	1.0 0.978
v-K-*ras*					1.0 1.0

For each pair of *ras* genes, the number at left represents the fraction of conserved amino acids and the number at right represents the fraction of conserved nucleotides in the region under comparison. Sequence data for the transforming H-*ras* gene of T24 were obtained from Fasano et al. (1983), for the transforming K-*ras* gene of Calu-1 from Shimizu et al. (1983a), and for the viral H-*ras* and K-*ras* genes from Dahr et al. (1982) and Tsuchida et al. (1982), respectively. The amino acid sequences for all the *ras* genes are presented in Figure 2.

The top shows the data for the conserved region of the *ras* genes, spanning amino acid positions 1–120 and 129–166 (a total of 158 amino acids, 474 nucleotides). The bottom shows the data for the variable region, spanning amino acid positions 171–185 (a total of 15 amino acids, 45 nucleotides).

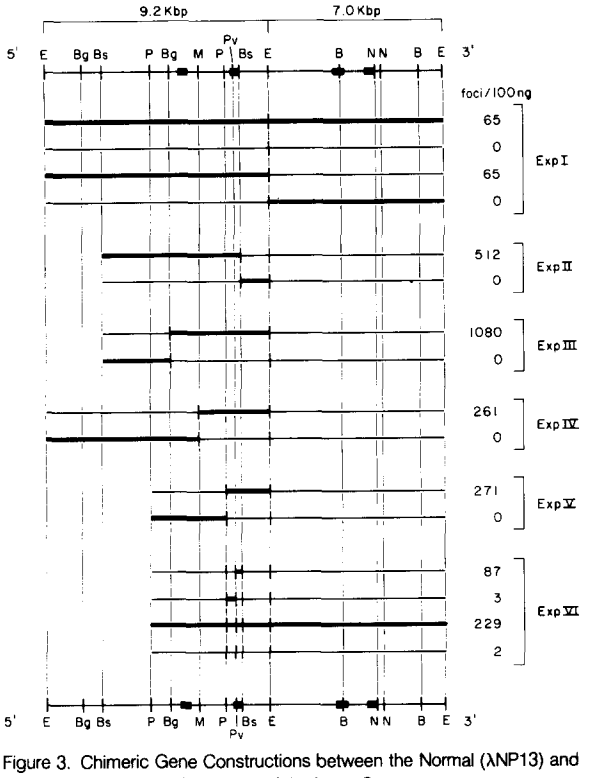

Figure 3. Chimeric Gene Constructions between the Normal (λNP13) and the Transforming (λNS35) Alleles of the N-*ras* Gene

Restriction site abbreviations are the same as in Figure 1. Coding exons are represented as solid blocks. Constructions between the transforming (▬) and nontransforming (—) alleles were made using T4 DNA ligase and gel-purified restriction fragments from Eco RI subclones of λNS35 and λNP13. The short vertical lines in each construct indicate restriction sites used for ligation. An estimate of the amount of the appropriate chimeric construction in each ligated mixture was determined by gel electrophoresis. Transfections were performed using ~100 ng of each construct. Plates were scored for foci after 14 days. Separate experiments were performed for the sets of constructions shown. Because of variation in transfection efficiencies, numbers between experimental groups should not be compared. In experiment VI, a low number of spontaneously arising foci was observed.

12 activates the transforming potential of the *ras* proteins. Recent theoretical studies of the *ras* protein amino acid sequence suggest the region about the glycine of position 12 might be involved in guanine nucleotide binding (Wierenga and Hol, 1982). If so, the two regions where amino acid substitutions lead to activation of transforming potential may both neighbor the guanine nucleotide binding site of *ras* proteins.

Despite the possible proximity of activating mutations to the GTP binding site of the *ras* proteins, both mutant and normal cellular *ras* proteins bind GTP (Papageorge, Lowy, and Scolnick, 1982). We propose a model which accounts for this. We suggest that, normally, the *ras* proteins interact about the guanine nucleotide binding site with a regulatory protein, which thereby prevents the activation of the *ras* proteins by GTP. Mutant *ras* proteins, on the other hand, altered about the guanine nucleotide binding site, may interact less efficiently with this putative regulatory protein. As a result, the mutant *ras* proteins may be more readily activated by guanine nucleotides. A precedent exists for these kinds of interactions. The regulatory component of the adenyl cyclase complex consists of a dimer of a 35,000 dalton protein and a 45,000 dalton protein (Northup et al., 1980). The 45,000 dalton protein will activate adenyl cyclase when it in turn is activated by GTP (Northup, Smigel, and Gilman, 1982). The 35,000 dalton protein competitively inhibits the GTP-induced activation of the 45,000 dalton component (Northup, Sternweis, and Gilman, 1983).

Experimental Procedures

Cells and Nucleic Acids

NIH/3T3 cells were used for transfection assays, and SK-N-SH cells were grown as previously described (Perucho et al., 1981). NP-2-1, NIH/3T3 cells transformed with SK-N-SH DNA, were described by Shimizu et al., 1983c. Cellular DNAs were prepared by SDS lysis, proteinase K digestion, and phenol extraction as described before (Perucho et al., 1981). Poly(A)$^+$ cytoplasmic RNAs were prepared as described by Taparowsky et al., 1982. Human placenta was frozen in liquid N_2 and pulverized, and DNA was extracted as described above. Plasmid DNAs were prepared by lysis of chloramphenicol-amplified cultures of E. coli DH1 and λ phage DNA prepared after CsCl phage banding as described elsewhere (Tanaka and Weisblum, 1975; Kaiser and Hogness, 1960). Plasmids pHB-11, which contains the viral H-*ras* gene, and pKBE-2, which contains the viral K-*ras* gene, were as described (Ellis et al., 1981) and were the generous gift of E. Scolnick.

Plasmid and λ Phage Libraries

Genomic libraries of SK-N-SH DNA and human placental DNA were constructed in λ Charon 4a from DNA partially digested with Eco RI restriction endonuclease. Phage libraries were screened for N-*ras* sequences using the method of Benton and Davis (1977) and using as probes fragments "R" and "L" from the N-*ras* gene as described in our previous work (Shimizu et al., 1983c). A cDNA library cloned into pBR322 was constructed from poly(A)$^+$ cytoplasmic RNA of NP-2-1 cells. The cDNA library construction utilized the double linker insertion method of Kurtz and Nicodemus (1981). Molecular linkers containing Eco RI and Sal I restriction endonuclease sites (Collaborative Research) were added simultaneously after rendering both ends of cDNA molecules blunt. This library was screened by colony filter hybridization (Grunstein and Hogness, 1975) using fragments of the N-*ras* gene as probe.

Other Procedures

The 9.2 and 7.0 Eco RI fragments of the N-*ras* genes were subcloned into pAT 153 (Twigg and Sheratt, 1980). These were used for restriction endonuclease mapping by electrophoretic analysis of double digestion products in agarose gels. Restriction endonuclease fragments were separated by agarose gel electrophoresis. DNA ligations for chimeric gene constructions utilized restriction fragments, purified from agarose gels, and T4 DNA ligase in buffer recommended by supplier (Bethesda Research Labs) at a final DNA concentration of 200 μg/ml. For use as probes, DNA fragments were nick translated with ^{32}P-dXTPs by published methods (Maniatis, Jeffrey, and Kleid, 1975). Blot hybridizations were done as described before (Shimizu et al., 1983b) at either high or low stringency. High stringency was aqueous hybridization in 6× SSC at 71°C with a final blot wash in 0.5× SSC at 71°C. Low stringency was aqueous hybridization in 6× SSC at 55°C with a final blot wash in 2× SSC at 55°C. DNA fragments were sequenced by the method of Maxam and Gilbert (1980) after 3′-OH end labeling with E. coli polymerase I large fragment (Bethesda Research Labs) or 5′-OH labeling with T4 polynucleotide kinase (Bethesda Research Labs).

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