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Four additional members of the *ras* gene superfamily isolated by an oligonucleotide strategy: Molecular cloning of YPT-related cDNAs from a rat brain library

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ABSTRACT **Several oligonucleotide mixtures corresponding to a 6-amino acid sequence that is strictly conserved in all the ras and ras-related proteins (from various organisms) were tested for their ability to hybridize to 11 cloned members of the *ras* gene superfamily. Among these mixtures, a combination of two sets of partially complementary oligomers were able to hybridize to all the tested sequences. To identify members of the *ras* superfamily, we screened a rat brain cDNA library with these probes and isolated four genes, denoted *rab1*, *-2*, *-3*, and *-4*, encoding proteins homologous to the yeast YPT protein. Amino acid homology scores with YPT range from 75% for *rab1* to 37% for *rab4*, whereas the homologies with p21 ras and other ras-related proteins are ≈30%, and homologous residues were clustered in the regions involved in GTP/GDP binding. Another striking similarity shared by the rab and the other ras-related proteins is the conservation of at least one cysteine residue near the carboxyl-terminal end involved in the membrane binding of the ras proteins. *rab1* is a mammalian homolog of the yeast *YPT* gene, and the four *rab* genes constitute an additional branch of the *ras* gene superfamily that to our knowledge has not been described in higher eukaryotes.**

The c-Ha-*ras* (1) and c-Ki-*ras* (2) genes were first characterized as the cellular genes transduced in the Harvey and Kirsten sarcoma viruses. Later, these two genes as well as a third closely related one, N-*ras* (3), were found to be frequently activated to a transforming potential in mammalian tumors (for a review see ref. 4). Highly homologous genes have been isolated from a wide variety of organisms including *Drosophila*, *Dictyostelium*, and yeast (5–8). Two other genes, *ral* and R-*ras*, have been identified in mammals (9, 10), and the corresponding proteins share 50% homology with the transforming ras proteins. The *ras* gene family has also been extended by fortuitous discoveries such as the *YPT* gene, found as an open reading frame between the tubulin and actin genes of yeast (11), and the *rho* genes, first identified in an *Aplysia* cDNA isolated for other purposes and later characterized in human, rat, and yeast genomes (12, 13). Rho and YPT proteins share ≈30% homology with the ras proteins.

In mammals, all the known *ras* or *ras*-related genes code for 21- to 24-kDa proteins that share structural and biochemical homologies with the guanine nucleotide-binding regulatory (G) proteins (14) involved in mediation of signal transduction in a variety of receptor/effector systems. ras proteins bind GTP and GDP and exhibit a low GTPase activity, as do the G proteins (15, 16). Four homology boxes corresponding to the GTP binding site are highly conserved in all the ras-related proteins. Among them, a stretch of six residues: Asp-Thr-Ala-Gly-Gln-Glu (in positions 57–62 of Ki-ras) is strictly conserved. A computer search in a protein databank* did not detect any other protein possessing this sequence even among the G proteins or other nucleotide binding proteins; thus, this sequence could be specific for ras-related proteins. According to the genetic code there are 512 distinct nucleotide sequences encoding Asp-Thr-Ala-Gly-Gln-Glu. A probe that could reveal all of them was required to search for additional members of the *ras* superfamily.

We describe here an improved oligonucleotide strategy based on the use of two partially complementary oligonucleotides designed to take advantage of weak G·T base pairings. This low-complexity oligonucleotide mixture hybridized to all the tested *ras* sequences and enabled us to isolate, from a rat brain library, four cDNAs encoding ras-related proteins that share extensive homologies with the yeast YPT protein. Nucleotide† and derived amino acid sequences of these four cDNAs are presented.

MATERIALS AND METHODS

Design and Synthesis of the Oligonucleotide Probes. Four 20-base oligodeoxyribonucleotide probes were synthesized corresponding to the sequence Asp-Thr-Ala-Gly-Gln-Glu-(Glu or Asp) (Fig. 1). The oligonucleotide oligo-512 is a mixture of all the possible icosamers that might encode this sequence. The oligonucleotides oligo-61 and oligo-61-COMPL were designed to take advantage of the weak G·T base pairings. Thus, oligo-61 (which hybridizes with the noncoding strand) possesses, in positions corresponding to the third bases of each codon, guanosine instead of guanosine or adenosine and thymidine instead of cytidine or thymidine. The oligo-61-COMPL was synthesized according to the same principle to hybridize with the coding strand. The oligo-In that hybridizes with the coding strand was synthesized by incorporating inosine (described as hybridizing weakly with the four naturally occurring bases) at the highly degenerated positions and a mixture of two deoxynucleotides at the other variable positions. Syntheses were performed manually on a silica gel solid phase, using a modification of the phosphoramidite method developed by Beaucage and Caruthers (17). The 20-mer oligonucleotide mixtures were purified from shorter sequences by electrophoresis on a 20% acrylamide/7 M urea gel and eluted. DNA synthesis kits were purchased from New England Biolabs.

Hybridization Conditions. Each of the oligonucleotide probes was 5′-end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP and tested on the following large sample of known

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Abbreviation: G protein, guanine nucleotide-binding regulatory protein.

*EMBL/GenBank Genetic Sequence Database (1986) EMBL Nucleotide Sequence Data Library (Eur. Mol. Biol. Lab., Heidelberg).

†These sequences are being deposited in the EMBL/GenBank data base (Bolt, Beranek, and Newman Laboratories, Cambridge, MA, and Eur. Mol. Biol. Lab., Heidelberg) (accession nos. J02998, J02999, J03000, and J03001).

Conserved amino-acids sequence	57 Asp	58 Thr	59* Ala	60 Gly	61* Gln	62 Glu	63* Glu Asp
H - ras	GAT	ACC	GCC	GGC	CAG	GAG	GAG
K- ras	GAC	ACA	GCA	GGT	CAA	GAG	GAG
N - ras	GAT	ACA	GCT	GGA	CAA	GAA	GAG
Dm - ras	GAC	ACG	GCT	GGC	CAA	GAG	GAG
Y - RAS1	GAT	ACT	GCT	GGA	CAA	GAA	GAG
Y - RAS2	GAT	ACT	GCA	GGG	CAG	GAA	GAA
YPT	GAC	ACT	GCA	GGT	CAA	GAA	CGT
rho 6	GAC	ACG	GCG	GGC	CAG	GAG	GAC
rho 12	GAC	ACA	GCT	GGG	CAG	GAA	GAC
Apl - rho	GAC	ACA	GCG	GGA	CAA	GAG	GAC
ral	GAT	ACA	GCT	GGG	CAG	GAG	GAC
OLIGO 512 20-mer (mix of 512)	5' GAT	ACA	GCA	GGA	CAA	GAA	GA 3'
	C	C	C	C	G	G	
		G	G	G			
		T	T	T			
OLIGO 61 20-mer G/T degenerated (mix of 8)	5' GAT	ACG	GCG	GGG	GCA	GGA	GGA 3'
		T	T	T			
OLIGO 61 COMPL 20-mer complementary sequence G/T degenerated (mix of 8)	3' CTG	TGG	CGG	CCG	GTT	CTT	CT 5'
		T	T	T			
OLIGO IN 20-mer complementary sequence inosine included (mix of 8)	3' CTA	TGI	CGI	CCI	GTC	CTC	CT 5'
	G				T	T	

FIG. 1. (*Upper*) Alignment of the nucleotide sequences encoding Asp-Thr-Ala-Gly-Gln-Glu from the 11 *ras* or *ras*-related genes tested in this study. (*Lower*) Four 20-mer oligodeoxynucleotide mixtures. The amino acid sequence is indicated on the upper line. *, Positions where a single amino acid variation activates p21 ras to a transforming potential. Positions are numbered according to Ki-ras.

ras gene sequences: Ha-*ras* (18), Ki-*ras* (19), N-*ras* (20), Dm-*ras1* (15), Y-*RAS1* and Y-*RAS2* (21), *YPT* (11), *Aplysia rho*, *rho6*, and *rho12* (12), and *ral* (9) (see Fig. 1). An equimolecular quantity of each plasmid, linearized at a unique restriction site, was spotted on nitrocellulose. Filters were prehybridized for 4 hr at 65°C and hybridized for 12 hr at 45°C or 50°C in a solution containing 5× SSPE (1× SSPE = 3.6 M NaCl/200 mM NaH_2PO_4, pH 7.4/20 mM EDTA, pH 7.4), 5× Denhardt's solution (0.1% polyvinylpyrrolidone/0.1% Ficoll/0.1% bovine serum albumin), and 0.5% Nonidet P-40. Filters were washed with a solution containing 2× SSC (0.3 M NaCl/30 mM sodium citrate, pH 7.0) and 0.1% $NaDodSO_4$ twice for 30 min at 20°C and once for 30 min at each of the following temperatures: 45°C, 47°C, 50°C, or 52°C. Blots were then autoradiographed.

Screening of the cDNA Library and DNA Sequence Analysis. The λgt10 rat brain cDNA library was kindly provided by D. Anderson (California Institute of Technology, Pasadena, CA). About 5×10^4 recombinant plaques were screened by plaque hybridization with 300,000 cpm per filter of an equimolar mixture of 5'-end-labeled oligo-61/oligo-61-COMPL. Prehybridization was for 4 hr at 65°C, and hybridization was for 12 hr at 45°C. Blots were washed twice for 30 min at 20°C and once for 30 min at 47°C in the buffers described above. Nucleotide sequences were determined by using phage M13 vectors and the dideoxy chain-termination method (22).

RESULTS

Probes, Assays, and Determination of Hybridization Conditions. The four probes separately plus the equimolecular combination oligo-61/oligo-61-COMPL were tested on 11 *ras* gene sequences under several hybridization and washing conditions. The results are shown in Fig. 2. Oligo-512 hybridized with every tested sequence, but a background was observed when a nonspecific sequence such as phage λ was

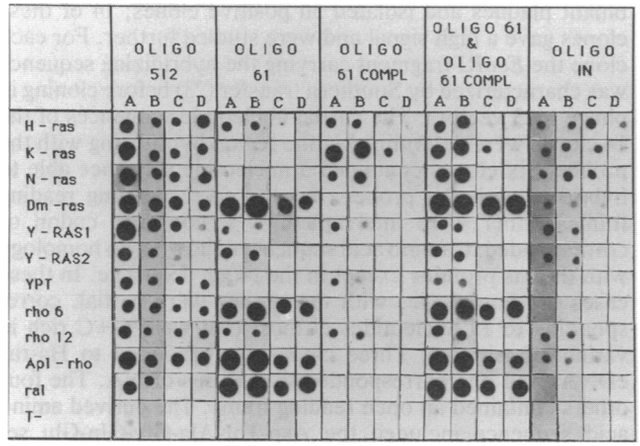

FIG. 2. Hybridization signals obtained with the five mixtures tested on 11 known *ras* sequences. The line T− represents phage λ DNA used as control. Stringency conditions were as follows. Columns: A, hybridization at 45°C and washes at 45°C; B, hybridization at 45°C and washes at 47°C; C, hybridization at 50°C and washes at 50°C; D, hybridization at 50°C and washes at 52°C.

used. Neither oligo-61 nor oligo-61-COMPL used alone hybridized with every tested sequence, but, as expected, all the sequences that gave a low signal with one of these two probes gave a high signal with the other. A mixture of oligo-61/oligo-61-COMPL used under the optimum stringency conditions (Fig. 2) hybridized with every sequence tested. The intensities of the signals were quite comparable to those obtained with the oligo-512; however, nonspecific hybridization with phage λ DNA as control was lower. It was observed that the signal intensities varied with the sequences. Oligo-In was expected to yield more constant intensity signals, and, actually, comparable intensities were observed for all the sequences that hybridized, but some sequences gave very low or even no signals.

To look for additional members of the *ras* superfamily, we decided to use the combination of oligo-61 and oligo-61-COMPL in equimolecular quantities under the stringency conditions described in Fig. 2 for columns B. This mixture successfully hybridized with λgt10 ral clone and λEMBL4 Ha-ras clone plaque (data not shown); this combination of probes, therefore, appeared to be appropriate for screening libraries.

Hybridization Study Using a Mixture of Two Partially Complementary Oligonucleotides. From our first results (Fig. 2), it appeared that for most of the tested sequences the signal intensity given by the combination oligo-61/oligo-61-COMPL was higher than the intensity obtained with each oligonucleotide alone. We tested the two probes separately and together on a ral clone, a sequence that mainly hybridized with oligo-61, on a Ki-ras clone, a sequence that mainly hybridized with oligo-61-COMPL, and on a Ha-ras clone, a sequence that hybridized with both. The hybridizations were performed under low-stringency conditions (42°C), and the washings were at increasing temperatures (from 42°C to 60°C). Melting curves were drawn (data not shown), and in each case the curve obtained with the mixture of the two complementary probes could be superimposed on the sum of the curves obtained with oligo-61 alone and oligo-61-COMPL alone. Thus, even though two partially complementary oligonucleotide probes were present in the same mixture during the hybridization process, there was no competition for hybridization with DNA fixed on nitrocellulose.

Screening of the cDNA Library from Rat Brain and Characterization of the Positive Clones. The mixture of the two 20-mer probes oligo-61/oligo-61-COMPL was used to screen a λgt10 rat brain cDNA library. We screened 50,000 recom-

binant plaques and isolated 50 positive clones; 14 of these clones gave a high signal and were studied further. For each clone the *Eco*RI fragment carrying the hybridizing sequence was characterized by Southern transfer (23) before cloning in phage M13 vectors. The partial nucleotide sequences of the 14 clones were determined in the region hybridizing with the probes. Six clones contained a nucleotide sequence able to hybridize with the probes, but the corresponding reading frames either were interrupted by a nonsense codon or corresponded to amino acid sequences showing no homology with the ras proteins except in the target sequence. In these cases the homologies with the probes were partial, corresponding to 15 nucleotides at most (but were G+C rich in variable positions). Three clones corresponded to Ha-ras cDNAs and one corresponded to a Ki-ras cDNA. The four others contained an open reading frame. The derived amino acid sequence included the Asp-Thr-Ala-Gly-Gln-Glu sequence and protein sequence homologies with the yeast YPT protein. These were named *rab1*, *-2*, *-3*, and *-4* (for *ras* genes from *rat brain*). Their nucleotide and deduced amino acid sequences are shown in Fig. 3. Assignment of the initiator AUG codons is based on DNA sequence analysis and comparison with YPT of the protein sequences encoded by the open reading frames. Although the conserved sequence features make these assignments very likely, this has yet to be formally demonstrated. *rab1* possesses two ATG codons at its 5′ end, both preceded by a eukaryotic ribosome binding site, thus the real ATG initiation site could not be determined. The same structure was also found in a human rab1 cDNA (data not shown). Preliminary results suggest that the *rab* genes are expressed in brain at higher levels than the *rho* or *ral* genes. This might explain why rho or ral cDNAs were not isolated by the screening we used.

Amino Acid Sequence Homologies Among the rab, ras, ral, rho, and YPT Proteins. The amino acid sequences of rab1, -2, -3, -4, Ki-ras, ral, rho, and YPT are aligned in Fig. 4. The percentage of amino acids strictly conserved between YPT and the rab proteins is 75% for rab1, 38% for rab2, 40% for rab3, and 37% for rab4. Four regions corresponding to residues 10–17, 57–63, 113–120, and 143–149 of Ki-ras and located in the GTP binding site are highly conserved in rab, YPT, ral, rho, and ras proteins. Amino acids 64–84 are highly conserved among the rab proteins and YPT and might be considered to be a distinctive characteristic of the *rab* family. The same region is also conserved among the ral and ras proteins. It is, however, different between these two groups. As in all the ras proteins a cysteine near the carboxyl-terminal end is found in the rab proteins. It should be noted that the last five carboxyl-terminal residues of the rab1 and rab2 proteins, Gly-Gly-Gly-Cys-Cys, are identical to those of YPT, whereas rab3 ends with Asp-Cys-Ala-Cys, and rab4 ends with Glu-Cys-Gly-Cys. These features clearly indicate that the *rab* genes belong to a third branch of the *ras* superfamily distinct from the *ras* protooncogenes and from the *rho* family.

DISCUSSION

Oligonucleotide probes are powerful tools for the isolation of cDNAs when at least a partial amino acid sequence of a protein is known (24, 25). Each protein poses a specific problem for the choice of a target region and the design of the oligonucleotide mixture. We developed an oligonucleotide strategy to search for additional members of the *ras* superfamily. Alignment of the ras-related proteins reveals a stretch of six amino acids Asp-Thr-Ala-Gly-Gln-Glu-(Glu or Asp) that appears to represent the best target region for two reasons. (*i*) It is found only in ras or ras-related proteins (and not in G proteins or in other nucleotide binding proteins). (*ii*) Half of the Asp-Thr-Ala-Gly-Gln-Glu residues are coded only by two triplets, and this reduces the number of possible coding sequences. Our purpose was to obtain a probe that could hybridize to every nucleotide sequence coding for this peptide. Three strategies were used: very complex oligonucleotide mixtures, G/T-degeneracy mixtures, and probes containing deoxyinosine (26). The use of a very complex

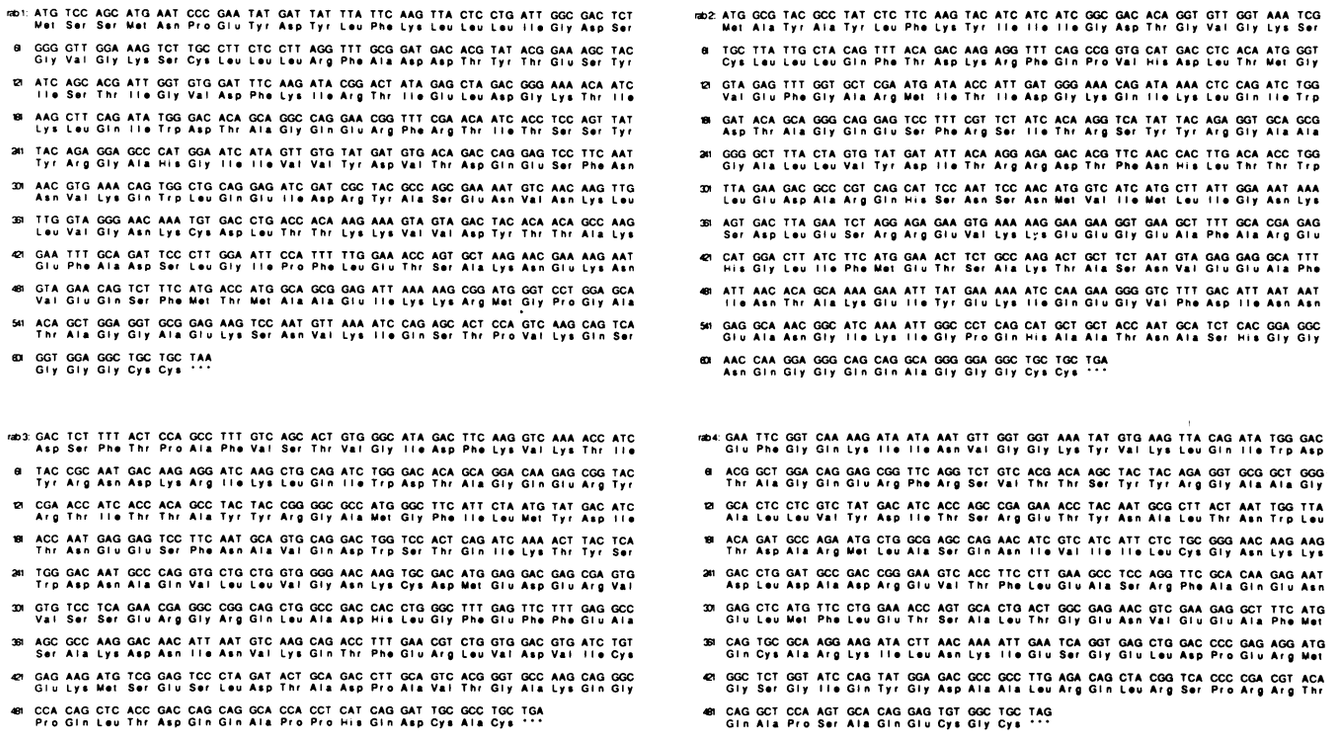

FIG. 3. Nucleotide and predicted amino acid sequences of the *rab1*, *-2*, *-3*, and *-4* open reading frames. Numbers indicate the position of nucleotides starting at the initiation codon (*rab1* and *rab2*) or at the first nucleotide in the open reading frame (*rab3* and *rab4*).

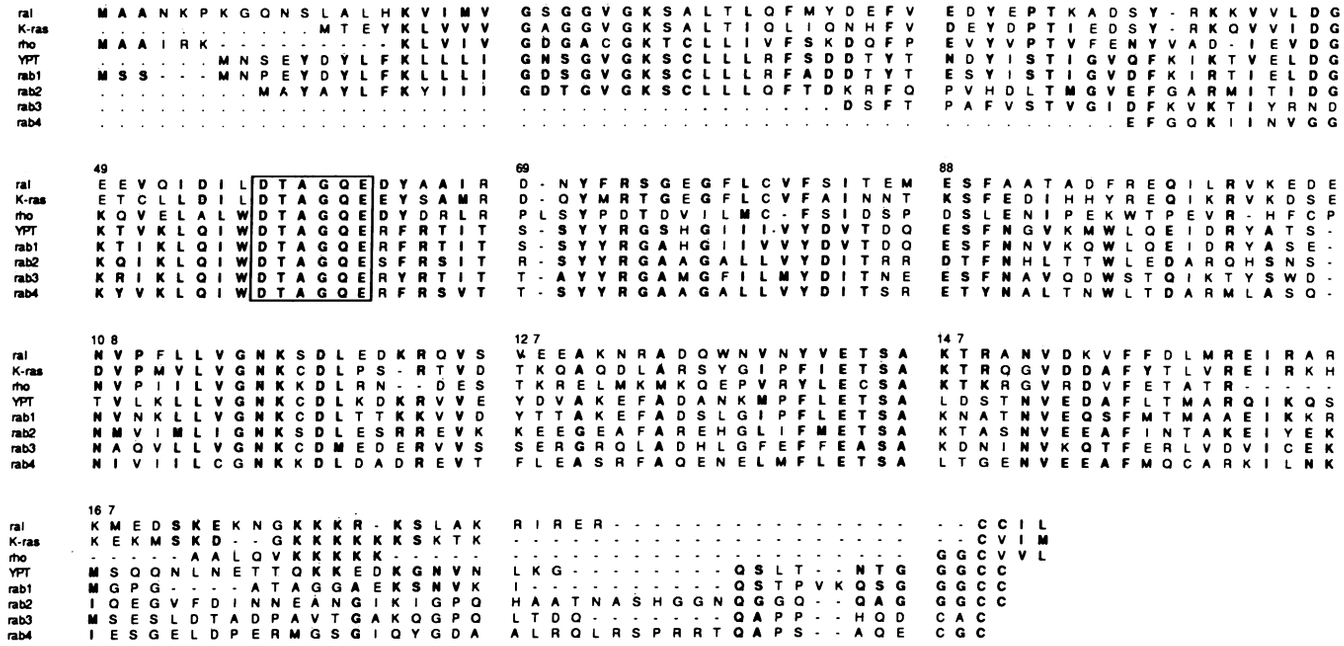

FIG. 4. Alignment of the four rab protein sequences with the human Ki-ras (exon 4B), *Aplysia* rho, and yeast YPT proteins. Dashes indicate gaps introduced for optimal alignment. Sets of identical or conservative residues are indicated by dots. Conservative amino acid substitutions are grouped as follows: cysteine, serine, threonine, proline, alanine, and glycine; asparagine, aspartic acid, glutamic acid, and glutamine; histidine, arginine, and lysine; methionine, isoleucine, leucine, and valine; phenylalanine, tyrosine, and tryptophan. The reference numbering is that of Ki-ras.

mixture corresponding to all the possibilities offered by the genetic code ensures that every nucleotide sequence corresponding to a given protein sequence will be identified; however, a high-complexity probe often leads to nonspecific hybridizations and increases the proportion of irrelevant sequences isolated. A less-complex probe constituted by a mixture of two partially complementary probes containing guanosine, thymidine, or guanosine and thymidine at the third base of each codon yielded the best results in our hands. This approach was tested only on 11 known sequences among the 512 possible ones, although we cannot be sure that all of them could be identified by hybridization. Nonetheless, the most unfavorable conditions were encountered when, for both oligo-61 and oligo-61-COMPL, three out of the six wobble bases hybridized strongly (i.e., G·C or A·T) and three of the six hybridized weakly (i.e., G·T). This was the case for several of the tested sequences, and two of them represented very different situations. (*i*) For Ha-ras the three low base-pairing positions with oligo-61 were placed at a central position, but with oligo-61-COMPL they were located at the two ends. (*ii*) For Y-RAS1 the three low base-pairing positions were placed at one end with oligo-61 and at the other end with oligo-61-COMPL. Both probes gave positive hybridization signals with these two sequences under the stringency conditions used for screening. Thus, the mixture of oligo-61/oligo-61-COMPL might reveal most of the nucleotide sequences coding for the peptide sequence. Perhaps unknown members of the ras superfamily present some variability in the region corresponding to amino acids 57–63, so that the corresponding clones would not be isolated by the methodology we developed. In fact, 6 clones among the 14 clones we isolated had a few mismatches with the oligonucleotide probes (due to the low-stringency conditions) but none of these clones was ras-related. Thus, a strategy similar to the one we developed for the *ras* superfamily should be adaptable to search for more members of other gene families.

We isolated four cDNAs coding for the proteins rab1, -2, -3, and -4 that share, respectively, 75%, 38%, 40%, and 37% homology with YPT and around 30% homology with the ras proteins and the other described ras-related proteins. rab1 and rab2 cDNAs contain full-length coding regions. The rab1 open reading frame encodes a protein of 202 or 205 amino acids (depending on which ATG codon is effective for initiation) with expected molecular weights of 22,500 or 22,800. The predicted rab2 protein is 212 amino acids long with an expected molecular weight of 23,400. Three-dimensional models for p21 ras have been proposed (27, 28). We shall refer to these models for a discussion of the structural homologies between the rab and ras proteins. The homologies are localized in four main regions corresponding to amino acid residues 5–21, 53–62, 112–120, and 141–147 (using the numbering system for Ki-ras) that have been implicated in the binding of GTP/GDP to p21 ras. Two other regions involved in GTP binding, amino acid residues 75–84 (29) and 152–164 (30) are less homologous between the rab and ras proteins, but many of the amino acid changes are conservative. The distances between these six boxes of homology as well as the hydrophobicity curves (data not shown) are almost identical for p21 ras, YPT, and rab proteins. Besides these six regions only a few amino acids are strictly conserved such as Thr-35, Tyr-71, and Cys-192. Thr-35 and Tyr-71 are probably important to maintain structural or functional properties of the ras-related proteins. The role of Cys-192 is discussed below. Amino acids 29–49 that have been shown to be important for the transforming potential of p21 ras (31) are not very conserved between the rab and ras proteins or among the four rab proteins themselves. This region probably corresponds to a large flexible loop interacting with the effector (28, 31). Amino acids 63–75 are highly conserved in the four rab proteins and are very different from the corresponding region of Ki-ras needed for the binding of monoclonal antibody Y13-259 that blocks p21 function (32). No precise role has been assigned to this region, but it might interact with regulatory proteins. These interacting proteins should be different between rab and ras but may be identical or closely related for all the rab proteins.

The most striking differences between all the members of the *ras* superfamily are located in the carboxyl-terminal

region from amino acid residue 166 to the end. However, a cysteine residue located four amino acids before the carboxyl-terminal end of Ha-ras and involved in membrane anchoring (33) is found in all the ras proteins. Deletions and point mutations in v-Ha-*ras* gene (34) suggest that several carboxyl-terminal aliphatic residues in addition to this cysteine might also be important for biological activity. Such a structure with a cysteine followed by aliphatic residues is found in the ras, ral, and rho proteins, but in YPT and the rab proteins the cysteine is the last carboxyl-terminal residue and one other cysteine is found immediately before (YPT, rab1, and rab2) or with one intervening amino acid (rab3 and rab4). rab1 and rab2 possess exactly the same five carboxyl-terminal residues as YPT. The conservation of this sequence from yeast to humans suggests an important functional role for this region. Several residues upstream from the cysteine, the ras, ral, and rho proteins share a sequence of basic amino acids that is not found in the rab proteins. These variations in the carboxyl-terminal region of the rab and ras proteins might correspond to different sublocalizations in the cellular membrane systems. The precise position of the rab proteins and the nature of the interacting proteins remain to be characterized.

rab1 was first identified in a rat cDNA, but we also isolated a human rab1 cDNA and the encoded protein is identical to the rat protein in the sequenced region (data not shown). As for Y-RAS1 and Y-RAS2, which are the yeast counterparts of the classical *ras* genes, the overall homology between YPT and rab1 proteins is around 70%, and two domains can be discriminated. The first one corresponding to amino acid residues 1–104 shows 95% homology between YPT and rab1, whereas the second one from amino acid residues 104–167 shows around 60% homology. Downstream from amino acid residue 167, rab1 and YPT diverge radically except for the last five carboxyl-terminal amino acids Gly-Gly-Gly-Cys-Cys. rab2, -3, and -4 share ≈40% homology with rab1 and thus are in the same situation compared to rab1 as ral or R-ras compared to Ha-ras, Ki-ras, and N-ras. rab2, -3, and -4 proteins share ≈40% homology with YPT, and the presence of their counterparts in yeast remains an open question.

The identification of an increasing number of ras-related proteins raises one question: What specific function might be exerted by each of them? It has been reported (35) that the YPT protein might be involved in microtubule organization and is required for cell viability, but the functional relationships existing between the vertebrate and the yeast ras-related proteins remain to be defined. In mammals we may speculate that, as with the G proteins, the ras proteins are involved in mediation of signal transduction between membrane receptors and cytoplasmic effectors. The ability to transduce the signal is probably regulated by GTP binding and hydrolysis—essential properties determined by internal highly conserved regions—whereas some external regions are specific in each protein. Thus, the different proteins of the *ras* superfamily may share common properties that are used in various biological pathways by interacting specifically with various receptors, effectors, or regulatory proteins. We postulate that the presence of a large number of different proteins of the *ras* superfamily, at least in some cell types such as neural cells, might reflect a role in the integration of many different or even in some cases antagonist signals.

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