expect that other proteins, including Fos (*21, 26*), may participate in effecting this and other AP-1–dependent responses. Our results do not rule out the possibility that AP-1 can also play a role in mitogenic responses, as has been suggested by Ryseck *et al.* (*11*) and by Lamph *et al.* (*9*). In JB6 cells, however, promotion and mitogenesis have been dissociated (*27*). Under the (log phase) conditions of our experiments, TPA and, EGF are not mitogens but they are transformation promoters (*27, 28*). These experiments thus measure parameters of AP-1 function related to promotion and do not address mitogenesis-related events.

This report provides evidence for an association between AP-1–induced function and promotion of neoplastic transformation, and suggests that a defect in AP-1 activity may render the cell unresponsive to promotion stimuli. Recent experiments indicate that transfer of an activated *pro* gene to a P^- cell can reconstitute, not only the P^+ phenotype (*4*), but also AP-1–dependent transactivation of CAT gene expression induced by TPA (*19*). This suggests that *pro* genes can execute control over the activity of AP-1. Such investigations promise to shed light upon mechanisms of the signal transduction pathway for promotion of neoplastic transformation by TPA, EGF, and other tumor promoters.

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Selective Amplification and Cloning of Four New Members of the G Protein–Coupled Receptor Family

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An approach based on the polymerase chain reaction has been devised to clone new members of the family of genes encoding guanosine triphosphate–binding protein (G protein)–coupled receptors. Degenerate primers corresponding to consensus sequences of the third and sixth transmembrane segments of available receptors were used to selectively amplify and clone members of this gene family from thyroid complementary DNA. Clones encoding three known receptors and four new putative receptors were obtained. Sequence comparisons established that the new genes belong to the G protein–coupled receptor family. Close structural similarity was observed between one of the putative receptors and the 5HT1a receptor. Two other molecules displayed common sequence characteristics, suggesting that they are members of a new subfamily of receptors with a very short nonglycosylated (extracellular) amino-terminal extension.

THE INITIAL DISCOVERY THAT THE β-adrenergic receptor is structurally and evolutionarily related to the visual pigment opsin (*1*) has led to the identification of a growing number of members of this gene family. These have in common the presence of seven transmembrane segments and the ability to interact with G proteins. To clone the thyrotropin receptor [which is coupled to adenylyl cyclase via Gs (*2*)], we have devised a method based on the polymerase chain reaction (PCR) (*3*).

Polyadenylated [poly(A)$^+$] RNA prepared from human thyroid tissue was reverse transcribed and the resulting cDNAs were subjected to amplification by PCR with the use of a set of highly "degenerate" primers (Fig. 1). These were devised from the compilation of sequences corresponding to the third and sixth transmembrane segments of the following receptors: β_1-, β_2-, and α_2-adrenergic receptors (*4–6*); M1 muscarinic receptor (*7*); substance K receptor (*8*); and the serotonin receptor subtype G-21 (*9*) [now known as the 5HT1a receptor (*10*)]. The sequence similarity between any two of the receptors in this region ranged from 52 to 80% (Fig. 1A). Therefore, each primer consisted of a mixture of oligonucleotides with a number of degeneracies allowing a 78% match, or better, with any of the receptors. Nevertheless, the choice of the

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primer composition was oriented arbitrarily toward β_1- and β_2-adrenergic and the 5HT1a receptors to avoid excessive degeneracy. After 55 amplification cycles, agarose gel electrophoresis revealed a clear pattern of cDNA species (Fig. 1B). Individual cDNAs were cloned directly in M13 bacteriophage derivatives for sequencing. Of 80 clones analyzed, 40 were found to contain sequences with a strong similarity to the receptors. These could be classified into seven categories, of which five corresponded to sequences encoding unknown receptors and two contained the sequences of the β_2-adrenergic and the 5HT1a receptors. Considering the expected scarcity of the corresponding mRNAs in the thyroid tissue, the proportion of clones with the characteristics of receptors is a measure of the enrichment achieved by the procedure. For comparison, the abundance of the β_2-adrenergic receptor in cDNA libraries of placental or A431 cells is approximately $1/5 \times 10^5$ (*5*).

Table 1. Sequences of all known G protein–coupled receptors were aligned to maximize homology, and scores were calculated with the matrix of Dayhoff (*18*). To allow unambiguous alignment of all receptors, only the regions showing conservation in length were considered (*18*). The COOH- and NH_2-terminal and, depending on the receptors, most of the loops separating the transmembrane domains IV, V, VI, and VII were thus excluded from the computation. The references contain the original description of each sequence. Adrenergic, ad.; muscarinic, musc.

Receptor (source)	Ref.	α2B	α2A	β1	β2	M1	M2	M3	M4	SKR	5HT1c	5HT1a	MAS	RDC8	RDC7	α1	RDC1	RDC4
α_2B-ad. (human)	(*19*)	1369																
α_2A-ad. (human)	(*6*)	1130	1360															
β_1-ad. (human)	(*4*)	550	531	1348														
β_2-ad. (human)	(*20*)	514	509	1000	1329													
Musc M1 (human)	(*21*)	492	450	455	458	1349												
Musc M2 (human)	(*21*)	472	455	394	400	1069	1337											
Musc M3 (human)	(*21*)	437	425	368	377	1025	1197	1310										
Musc M4 (human)	(*21*)	456	455	432	457	1143	1062	1009	1315									
SKR (bovine)	(*8*)	374	382	359	382	328	334	337	309	1352								
5HT1c (rat)	(*22*)	535	527	489	443	465	472	442	452	306	1328							
5HT1a (human)	(*9*)	621	597	557	559	534	540	494	561	362	520	1314						
MAS (human)	(*23*)	81	62	41	31	87	83	54	79	71	81	31	1350					
RDC8 (dog)		353	384	322	275	333	338	323	340	319	324	469	119	1327				
RDC7 (dog)		364	370	319	260	352	347	326	353	286	376	421	114	804	1314			
α_1-ad. (hamster)	(*11*)	660	653	593	584	503	522	502	496	393	576	670	122	444	425	1344		
RDC1 (dog)		194	191	167	156	216	237	236	300	374	241	263	113	192	198	265	1374	
RDC4 (dog)		676	679	565	552	524	527	513	538	358	548	837	40	361	342	668	258	1308

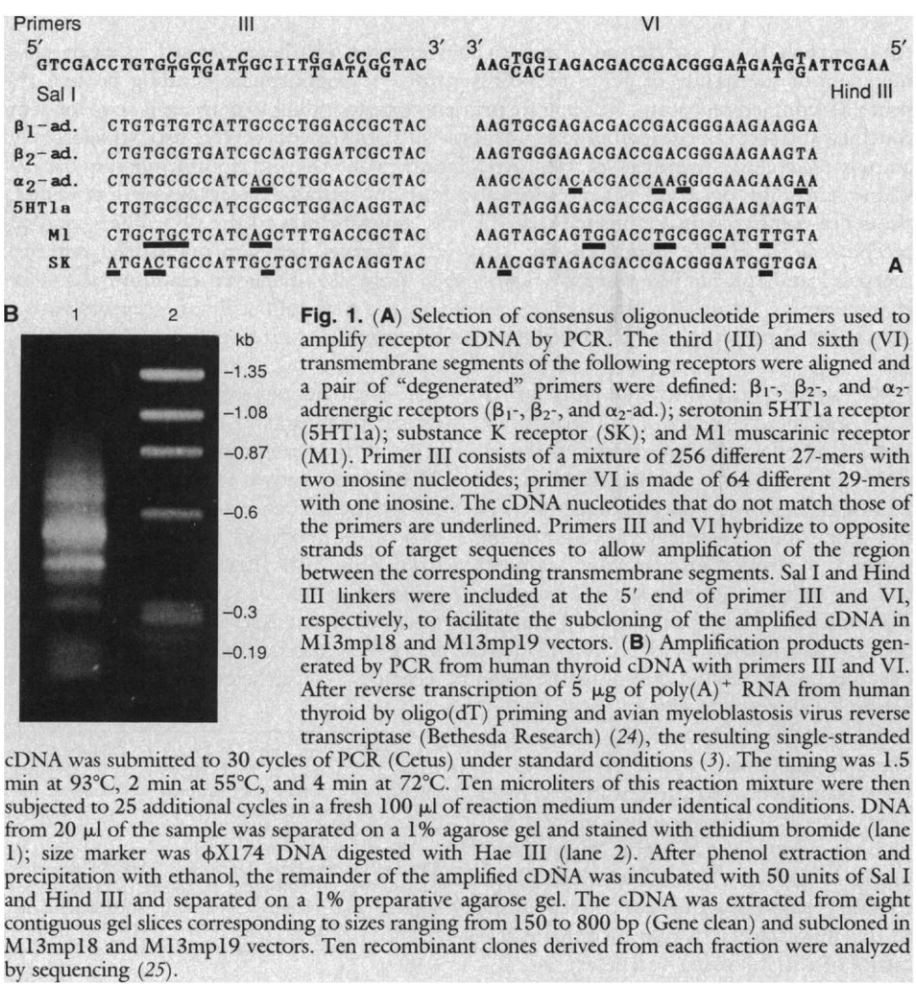

Fig. 1. (**A**) Selection of consensus oligonucleotide primers used to amplify receptor cDNA by PCR. The third (III) and sixth (VI) transmembrane segments of the following receptors were aligned and a pair of "degenerated" primers were defined: β_1-, β_2-, and α_2-adrenergic receptors (β_1-, β_2-, and α_2-ad.); serotonin 5HT1a receptor (5HT1a); substance K receptor (SK); and M1 muscarinic receptor (M1). Primer III consists of a mixture of 256 different 27-mers with two inosine nucleotides; primer VI is made of 64 different 29-mers with one inosine. The cDNA nucleotides that do not match those of the primers are underlined. Primers III and VI hybridize to opposite strands of target sequences to allow amplification of the region between the corresponding transmembrane segments. Sal I and Hind III linkers were included at the 5′ end of primer III and VI, respectively, to facilitate the subcloning of the amplified cDNA in M13mp18 and M13mp19 vectors. (**B**) Amplification products generated by PCR from human thyroid cDNA with primers III and VI. After reverse transcription of 5 μg of poly(A)$^+$ RNA from human thyroid by oligo(dT) priming and avian myeloblastosis virus reverse transcriptase (Bethesda Research) (*24*), the resulting single-stranded cDNA was submitted to 30 cycles of PCR (Cetus) under standard conditions (*3*). The timing was 1.5 min at 93°C, 2 min at 55°C, and 4 min at 72°C. Ten microliters of this reaction mixture were then subjected to 25 additional cycles in a fresh 100 μl of reaction medium under identical conditions. DNA from 20 μl of the sample was separated on a 1% agarose gel and stained with ethidium bromide (lane 1); size marker was ϕX174 DNA digested with Hae III (lane 2). After phenol extraction and precipitation with ethanol, the remainder of the amplified cDNA was incubated with 50 units of Sal I and Hind III and separated on a 1% preparative agarose gel. The cDNA was extracted from eight contiguous gel slices corresponding to sizes ranging from 150 to 800 bp (Gene clean) and subcloned in M13mp18 and M13mp19 vectors. Ten recombinant clones derived from each fraction were analyzed by sequencing (*25*).

To obtain the complete primary structure of the putative receptors, thyroid cDNA libraries were screened for full-length clones. The amino acid sequences of four such clones obtained from a dog library (RDC1, RDC4, RDC7, and RDC8) are shown in Fig. 2. The sequence of the fifth clone (RDC5) remains incomplete at its NH_2-terminus, as a result of the extreme rarity of the corresponding cDNA in the available libraries (Fig. 2). The dog and human sequences were more than 90% similar in the region between transmembrane segments III and VI. The alignment of the candidate receptors with the sequence of the β_2-receptor, taken as the archetype, clearly indicates that they all belong to the same multigene family. This conclusion is supported by the fact that the hamster α_1-adrenergic sequence (*11*) is 91% identical to that of clone RDC5. We therefore consider RDC5 to represent the dog α_1 receptor.

The extents of similarity among the known and newly isolated potential receptors were computed as described in Table 1. RDC4 appears clearly related to the 5HT1a receptor, whereas RDC7 is closer to RDC8 than to the others. The homology scores for these two couples of sequences (804 and 837, respectively) are not very different from those obtained for structurally and functionally related receptors such as the β-adrenergic (1000) and the muscarinic (1009 to 1197) receptor subtypes. Aside from their high homology score, RDC7 and RDC8 share a very short (if any) NH_2-terminal extracellular domain devoid of potential *N*-glycosylation sites. Together with a recently described nonglycosylated variant of α_2-receptors (*12*), they can therefore be considered as constituting a new subfamily in the G protein–coupled receptors. The last potential receptor, RDC1, showed a low similarity with all other receptors. The highest score was obtained with the substance K receptor. This type of comparison cannot predict the nature of the ligands of the new candidate receptors. Although the similarity of RDC4 with 5HT1a suggests that it could correspond to a member of the large family of serotonin receptors (*13*), it is clear that functional and binding assays will be required to achieve correct identification in each case.

The tissue distributions of the individual candidate receptors were then investigated by Northern blotting of RNA from nine

dog tissues (Fig. 3). None of the transcripts displayed the thyroid specificity of the thyroid-stimulating hormone (TSH) receptor; instead, each probe hybridized to RNA from a different selection of tissues. Analysis of the strong signals indicates that RDC7 and RDC8 were both expressed in the brain, with RDC7 transcripts being also present in the thyroid. RDC1 transcripts were clearly found in the heart, kidney, and thyroid. RDC5 (the α_1-adrenergic receptor) was relatively abundant in most tissues tested, except the thyroid. Three types of α_1 transcripts of different sizes were observed to have a tissue-specific distribution; in the stomach, the primary species was 4.8 kb; in the heart, 4.1 and 2.6 kb; and in the lung, 4.8 and 2.6 kb. This is compatible with the differential use of polyadenylation signals or with the existence of closely related cross-hybridizing α_1 receptor subtypes. The situation for RDC4 remains unclear because of our inability to obtain a significant signal on the blot with RNA from any tissue. Virtually no hybridization was found between thyroid RNA and probes RDC5 and RDC8, despite the fact that these clones resulted from the selective amplification and screening of thyroid cDNA. These observations correlate with the rarity of these clones in the cDNA library and suggest that the amplification and selectivity of the procedure resulted in cloning of receptors belonging to

Fig. 2. Primary structure of four putative G protein–coupled receptors (RDC1, RDC4, RDC7, and RDC8). The sequences were aligned with that of the human β_2-adrenergic receptor (β_2-ad.) and with the dog α_1-adrenergic receptor (RDC5). Of the M13 recombinants containing amplified human cDNA inserts (legend to Fig. 1B), five clones showing strong amino acid sequence similarities with the fourth and fifth transmembrane segments of G protein–coupled receptors were selected (PCRR1, PCRR4, PCRR5, PCRR7, and PCRR8). These were used to screen a λgt11 cDNA library of dog thyroid (*24*). This particular library was used because of the abundance of full-length clones (*26*). Of 8×10^5 clones screened, 120 were positive with PCRR1 probe, 2 with PCRR4, 1 with PCRR5, 17 with PCRR7, and 4 with PCRR8. The phages with longest canine inserts were selected and renamed: RDC1, 2050 bp; RDC4, 1670 bp; RDC5, 2500 bp; RDC7, 2270 bp; and RDC8, 2275 bp. The cDNA inserts were sequences on both strands (*25*) from M13 and pBs single-stranded DNA subclones with a combination of Exonuclease III deletions, directed subcloning, and target-priming with oligonucleotides (Applied Biosystem 381A). The corresponding amino acid sequences were aligned to optimize the homology between individual receptors. Amino acid identities between RDC4 and 5HT1a and between RDC7 and RDC8 are indicated by dots. Amino acids that appear in more than three of the aligned sequences are boxed. Potential glycosylation sites are underlined. Putative transmembrane domains are identified by roman numbers. The DNA sequences of RDC1, RDC4, RDC5, RDC7, and RDC8 have been deposited in the European Molecular Biology Laboratory and GenBank data bases under accession numbers X14048, X14049, X14050, X14051, and X14052, respectively.

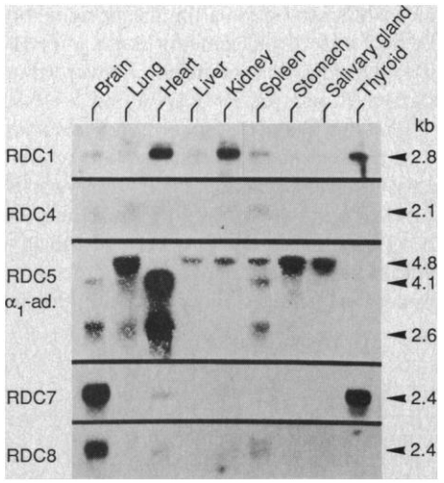

Fig. 3. Tissue-specific expression of four putative G protein–coupled receptors RDC1, RDC4, RDC7, RDC8, and the α_1-adrenergic receptor (RDC5). RNA blots were prepared with poly(A)$^+$ RNA extracted from nine different dog tissues (top) and probed with individual putative receptor sequences (left). The sizes were deduced from λ and φX174 DNA markers digested with Hind III and Hae III, respectively (right). The amount of RNA per lane was 5 μg (blots RDC1 and RDC8) and 20 μg (RDC4, RDC5, and RDC7). Selective precipitation in urea-LiCl medium (*24*) was used to isolate RNA from tissues of adult dogs. Poly(A)$^+$ RNA was extracted by oligo(dT) cellulose as described (*24*). Poly(A)$^+$ RNA were treated with glyoxal, fractionated on 1% agarose gels (*27*), and transferred to nylon membrane (Pall-biodyne). Hybridizations were performed with ^{32}P-labeled deoxyadenylate triphosphate probes (3×10^6 cpm/ml, 10^8 cpm/μg, random priming method). RDC1 and RDC8 probes were hybridized in the presence of 10% dextran sulfate.

minor cell populations present in the thyroid gland.

The approach described in the present study offers many advantages over homology cloning methods that are based on the screening of libraries with cross-hybridizing probes. The amplification provided by PCR together with the specificity achieved by two criteria of sequence similarity yield a low background compatible with the direct identification of clones by DNA sequencing. Careful selection of the primer sequences and degeneracies should make it possible to orientate the amplification process to specific subfamilies of genes. This will prove invaluable in cloning the many dozens of G protein–coupled receptors, the existence of which has been inferred from physiological and pharmacological evidence (*13–16*).

Note added in proof. After the present study was completed, a similar cloning approach was published (*17*).

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Murine MHC Polymorphism and T Cell Specificities

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The major histocompatibility complex (MHC) genes are polymorphic in mouse and man. The products of these genes are receptors for peptides, which while bound, are displayed to T lymphocytes. When bound peptides from antigens are recognized by T lymphocytes, an immune response is initiated against the antigens. This study assessed the relation of the polymorphic MHC molecules to their peptide specificity. The results indicate that although an individual of the species has a limited ability to recognize antigens, the species as a whole has broad reactivity. This rationalizes the extreme polymorphism observed.

A T HELPER LYMPHOCYTE, THROUGH its antigen-specific receptor, recognizes a peptide bound to a class II molecule, is activated, and stimulates an immune response to the antigen from which the peptide was derived (*1*). The primary structure of the NH_2-terminal polymorphic domain of the MHC molecule is essential for both T cell recognition and providing specificity for the binding of peptides (*2, 3*). Hence the immune responsiveness of the individual is determined in large part by the amino acid sequences of the class II molecule. The diversity of antigenic peptides recognized within the bacteriophage lambda repressor protein, cI (residues 1 to 102), was evaluated as a function of the polymorphisms of the class II molecules of the mouse species. We screened 13 different strains of mice; specific T cell hybrids generated from mice immunized with 1–102 were tested for with a panel of overlapping peptides spanning the entire protein (Fig. 1). Some strains (C57BL/6, B6.C-H-2^{bm12}, B10.D2, BALB/c, SM/J, P/J, SAF, and CLA) had only one target peptide, whereas others (B10.M, B10.RIII, B10S, B10.BR, and C3H.JK/Sn) had more than one. In the strains that had multiple targets, one was always immunodominant. The frequency of hybrids that recognized nondominant targets was usually about one-tenth that of hybrids recognizing the immunodominant target. Multiple MHC alleles can recognize the same target peptide. The class II molecules of different MHC haplotypes can display different T cell epitopes within the larger target peptide. For example, the part of 12–26 that is responsible for binding to I-A^d consists of residues 12 to 24, whereas the part of this same peptide which binds to I-E^k consists of residues 15 to 26 (*4–6*).

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