Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 6995–6999, October 1987
Biochemistry

Structure of the gene for human β_2-adrenergic receptor: Expression and promoter characterization

(glucocorticoid induction/transcription factor Sp1 binding sites)

L. J. EMORINE*, S. MARULLO, C. DELAVIER-KLUTCHKO, S. V. KAVERI, O. DURIEU-TRAUTMANN, AND A. D. STROSBERG

Department of Biotechnology, Pasteur Institute and University Paris VII, 25, rue du Docteur Roux, 75724 Paris Cedex 15, France

Communicated by Harden M. McConnell, June 5, 1987

ABSTRACT **The genomic gene coding for the human β_2-adrenergic receptor (β_2AR) from A431 epidermoid cells has been isolated. Transfection of the gene into eukaryotic cells restores a fully active receptor/GTP-binding protein/adenylate cyclase complex with β_2AR properties. Southern blot analyses with β_2AR-specific probes show that a single β_2AR gene is common to various human tissues and that its flanking sequences are highly conserved among humans and between man and rabbit, mouse, and hamster. Functional significance of these regions is supported by the presence of a promoter region (including mRNA cap sites, two "TATA boxes," a "CAAT box," and three G+C-rich regions that resemble binding sites for transcription factor Sp1) 200–300 base pairs 5′ to the translation initiation codon. In the 3′ flanking region, sequences homologous to glucocorticoid-response elements might be responsible for the increased expression of the β_2AR gene observed after treatment of the transfected cells with hydrocortisone. In addition, 5′ to the promoter region, an open reading frame encodes a 251-residue polypeptide that displays striking homologies with protein kinases and other nucleotide-binding proteins.**

Peripheral catecholamine-responsive systems are mainly characterized by four types of cell surface receptors: the α_1-, α_2-, β_1-, and β_2-adrenergic receptors (α_1-, α_2-, β_1-, and β_2AR), which are coupled to different effector systems via specific GTP-binding proteins (G proteins). The α_1AR modulates levels of inositol trisphosphate through phospholipase C activation, whereas α_2AR and βAR act on cAMP levels by modulating adenylate cyclase activity (1). Cellular responses to catecholamines depend on the type and ratio of each receptor. In particular tissues, this ratio may be controlled by external factors, such as temperature, and by the action of thyroid, glucocorticoid, or estrogen hormones, which exert their action by modulating the number of receptors (2, 3).

In view of the complexity of the adrenergic systems, it is necessary to define the various components involved in the tuning of cellular responses to catecholamines. This requires characterization of the receptor, transducer, and effector components; identification of the interacting regions of each of these components; and localization of DNA elements responsible for the regulated expression of receptor genes. Molecular cloning and eukaryotic cell expression of specific genes constitute a powerful approach to these problems. Several cDNAs coding for the three subunits of various G proteins have been characterized (6). Turkey erythrocyte β_1AR cDNA and hamster lung and human placenta and brain β_2AR cDNAs have also been studied recently (7–10).

Here we describe the human gene coding for the β_2AR of the A431 epidermoid cell line.† Expression of this gene in rabbit TP3 cells, which are devoid of a β_2-agonist-responsive system, restores a functional, agonist-dependent β_2AR/G-protein/adenylate cyclase complex. The β_2AR proximal promoter has been characterized, and potential DNA regulatory elements such as binding sites for glucocorticoid receptor have been defined.

MATERIALS AND METHODS

Cell Lines. A431 human epidermoid carcinoma cells express high levels of the β_2AR (11), whereas CCL137 cells (HEL299 from the American Type Culture Collection) from human embryonic lung are devoid of βAR (C.D.-K., unpublished data). C6 murine glioma cells contain mainly β_1AR (12). TP3 is a rabbit splenocyte line (13) selected for hypoxanthine (guanine) phosphoribosyltransferase deficiency.

Recombinant DNA Techniques. A library of size-selected *Eco*RI fragments of A431 genomic DNA was constructed in EMBL4 phages (14) and probed (15) with oligonucleotides derived from a hamster lung β_2AR cDNA sequence: base pairs (bp) 869–906 and 926–963 on the coding strand and bp 898–935 on the opposite strand (8). For Southern (16) blots, hybridization and washing were as described (17).

The DNA fragment to be sequenced was subcloned in both orientations in bacteriophage vector M13mp18. Continuous overlapping subclones were obtained as described (18) and sequenced (19). To avoid electrophoretic band compression due to secondary structure in G+C-rich regions, dITP or 7-deaza-dGTP was used in place of dGTP (20).

For nuclease S1 mapping of the mRNA cap site, M13 deletion subclones were used as templates to generate high-specific-activity single-stranded probes (21) HβR-9, HβR-10, and HβR-11 (Fig. 1*b*), which begin, respectively, at positions 1303, 1243, and 1026 and extend to restriction sites at positions 962 (*Nae* I), 887 (*Sma* I), 245 (*Pvu* II). Probe (5×10^5 cpm) was mixed with 50 μg of RNA and processed as described (14), except that formamide was omitted and hybridization was at 65°C for 14 hr.

Transfection of Eukaryotic Cells and Assays of Receptor Binding and cAMP. DNA containing the gene to be expressed was cotransfected into TP3 cells with the selection plasmid pSV2gpt by the calcium phosphate method (23). After 2 weeks in selective medium, resistant cells were screened for gene expression by mRNA dot blot assays. Cells from

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Abbreviations: αAR (α_1AR or α_2AR), α-adrenergic receptor(s) (of the α_1 or α_2 subtype); βAR (β_1AR or β_2AR), β-adrenergic receptor(s) (of the β_1 or β_2 subtype); CGP-12,177 (CIBA–Geigy product 12,177), 4-(3-*t*-butylamino-2-hydroxypropoxy)benzimidazol-2-one; ICYP, 3-iodocyanopindolol; ORF, open reading frame; G protein, GTP-binding protein.

*To whom reprint requests should be addressed.

†The sequence reported in this paper is being deposited in the EMBL/GenBank data base (Bolt, Beranek, and Newman Laboratories, Cambridge, MA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J02960).

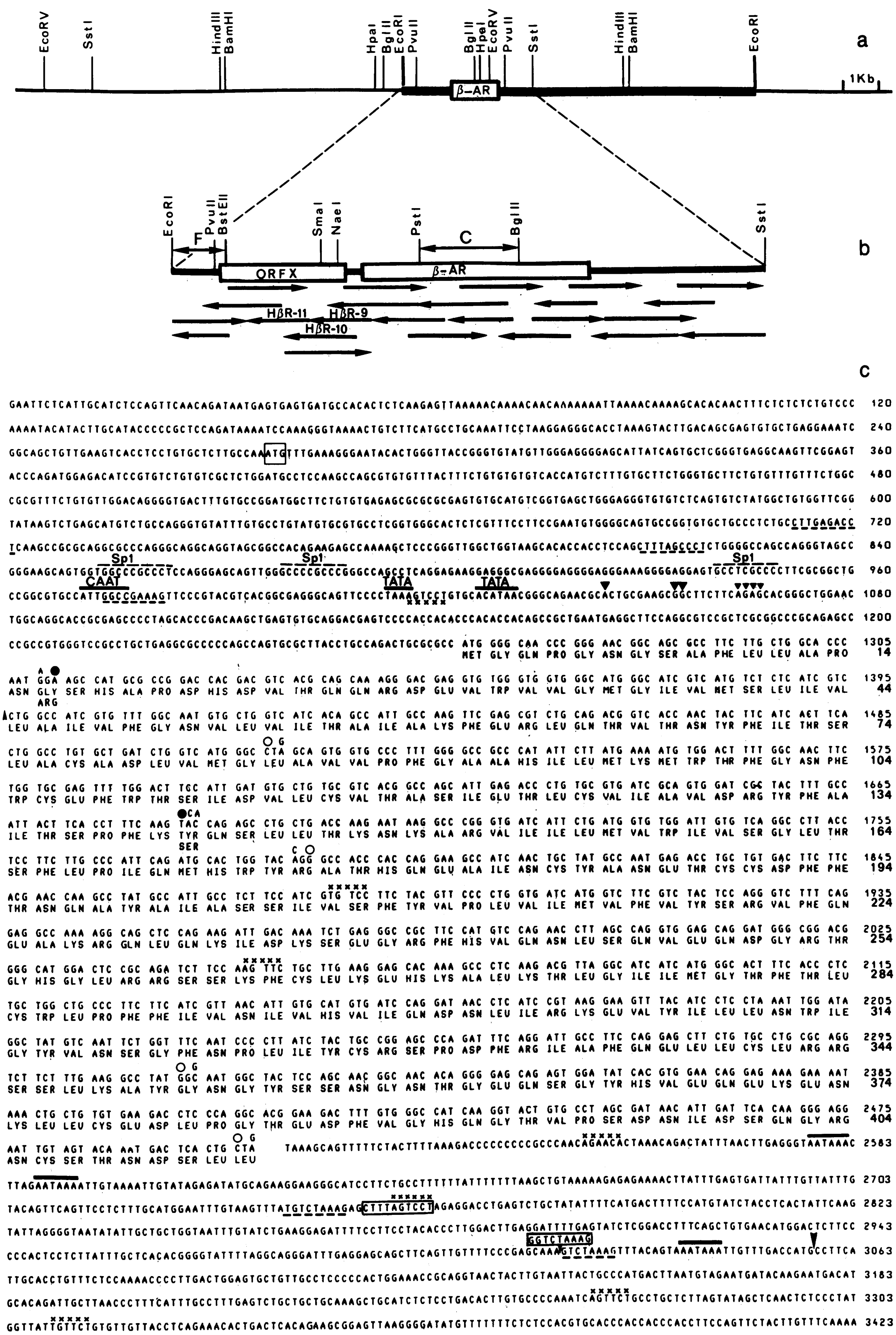

FIG. 1. Structural characterization of the β_2AR locus. (*a*) Map of the β_2AR locus showing conserved restriction sites (cf. Fig. 2). (*b*) Sequencing strategy: each arrow indicates the origin, direction, and extent of sequence determination for individual M13 subclones. HβR-9, HβR-10, and HβR-11, used for mRNA cap site localization, are indicated. Double-headed arrows F and C represent the flanking- and coding-region probes used. (*c*) Nucleotide sequence of the 3.5-kb *Eco*RI-*Sst* I fragment. In the 5′ region, the first methionine codon of ORFX is boxed. Its TAA stop codon is contained in the TATA box at position 1030. Black triangles represent β_2AR mRNA initiation sites. In the 3′

positive wells were subcloned in selective medium on feeder cells at a density of 0.5–1 cell per well in 96-well plates.

Receptor binding assays were performed as described (11), with 3-[^{125}I]iodocyanopindolol ([^{125}I]ICYP, 15–500 pM) or with 4-(3-*t*-butylamino-2-hydroxypropoxy)-[5,7-^{3}H]benzimidazol-2-one ([^{3}H]CGP-12,177, 0.03–2 nM). In competition experiments, cells were incubated with 60 pM [^{125}I]ICYP and various concentrations of antagonists or agonists. For cAMP assays (11), aliquots of 2×10^4 preconfluent cells were incubated for 2 min at 37°C with various concentrations of (*l*)-isoproterenol. Inhibition of adenylate cyclase stimulation was assayed in the presence of different antagonists at a final concentration of 100 μM. The level of cAMP was determined using the Amersham cAMP kit.

RESULTS

Structural Characteristics of the Cloned DNA Fragment. Extensive similarities exist between mammalian βAR (1, 24). To search for the human β_2AR gene we derived three oligonucleotide probes from a hamster lung β_2AR cDNA sequence (8). The probes, hybridized to blots of A431 DNA, revealed a single 9.8-kilobase (kb) *Eco*RI fragment. Restriction mapping analysis of this fragment, cloned in EMBL4 phages, showed that the sequences homologous to the probes lie in a 3.5-kb *Eco*RI–*Sst* I fragment whose sequence is presented in Fig. 1*c*.

Translation in the six phases revealed two long open reading frames (ORFs). The longest ORF (Fig. 1*c*) and its 5′ and 3′ untranslated sequences are highly homologous to corresponding cDNA sequences for turkey β_1AR (56% identity; ref. 7) and for hamster (8) and human (9, 10) β_2AR (76% and 99%, respectively). In the coding region, few differences exist between the three human sequences (Fig. 1*c*). Most prominent is the presence, in the 3′ untranslated region of the A431 and placental (9) genes, of a 10-bp sequence that is absent at homologous positions in the brain cDNA sequence (10). Almost the same sequence is inserted in the complementary strand of the brain cDNA at a position corresponding to our bp 3021. Surprisingly, the insertion/deletion event preserves in both sequences a tandem repeat of this 10-bp sequence (Fig. 1*c*). This decamer contains a consensus sequence for the glucocorticoid receptor binding site WGTYCT (W = A or T; Y = C or T) (25). Such glucocorticoid-response elements and close approximations of the decamer are found at several other positions in the coding and flanking regions of the gene (Fig. 1*c*). Homology between the A431 and the brain β_2AR genes falls from 99% to random homology after the mRNA polyadenylylation site. The sequences responsible for this lack of homology seem to be unique to the published sequence of the brain gene, since its 3′ end contains *Eco*RI and *Bam*HI restriction sites that do not exist at corresponding places in the β_2AR gene from A431 or other human cells, including neuroblastoma cells (Fig. 2).

The second ORF (ORFX) translates into a 251-residue polypeptide of 26,681 Da (Fig. 3) and possesses four methionine codons, none of which fit Kozak's criteria for effective translation initiation (27). A close approximation of a consensus polyadenylylation signal is contained in the ACATAA sequence constituting the final two codons (positions 1027–1032 in Fig. 1*c*). Analyses of total RNA from A431 (β_2AR-positive) and CCL137 (βAR-negative) cells with the F probe (Fig. 1*b*) did not detect any hybridization signal (data not shown).

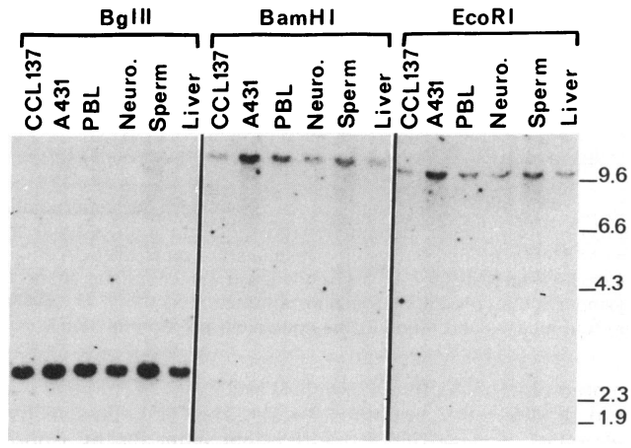

FIG. 2. Conservation of the β_2AR gene locus. Southern blots of human DNA from various tissues [CCL137 and A431 cells, peripheral blood lymphocytes (PBL), IMR32 neuroblastoma cells (26), semen, and liver] from unrelated individuals were hybridized to the C probe (Fig. 1*b*). Results are shown only for *Bgl* II, *Bam*HI, and *Eco*RI restriction enzymes, but the other conserved sites are mapped in Fig. 1*a*. Molecular size markers (in kb) are indicated at right.

mRNA Cap Site and RNA Polymerase II Promoter Site. The three probes used in these experiments were annealed either to themselves or to RNA from A431 or from β_2AR-negative cells (Fig. 4). None of the probes was self-protected. HβR-11 was not protected with any RNA, whereas HβR-9 and HβR-10 were protected with A431 RNA, yielding three fragments, whose lengths correspond to three mRNA initiation regions (Fig. 1*c*). As in several eukaryotic RNA polymerase II promoters, a reversed "CAAT box" and a "TATA box"-like sequence are found about 80 and 30 bp upstream, respectively, from the mRNA start regions. The absence of a true TATA box, and the presence instead of two weakly homologous sequences separated by roughly 10 bp (as are the two main mRNA cap sites), might be responsible for the observed low specificity of RNA initiation. Variations from the consensus sequence (KGGGCGGRRY, where K = G or T, R = A or G, and Y = C or T) for the binding site of transcription factor Sp1 (28) occur in three such sequences found in reverse orientation in the A431 βAR gene (Fig. 1*c*).

Transfection and Expression in Eukaryotic Cells. To verify that the 3.5-kb *Eco*RI–*Sst* I fragment indeed contains the functional elements for βAR expression, the fragment was transfected into eukaryotic cells in plasmid constructs lacking any foreign promoters. One of the transfectant clones, TP3-15, binds both ICYP and CGP-12,177 with apparent K_d values of 35 pM and 170 pM, respectively (Fig. 5*a*). At saturation, the maximum number of sites (B_{max}) per 10^6 cells is 39 fmol for ICYP and 31 fmol for CGP-12,177. In contrast, the original TP3 cells do not bind CGP-12,177, and low levels of ICYP binding are observed. In competition experiments (Fig. 5*c*), various agonists inhibit ICYP binding with the following order of potency: procaterol > (*l*)-epinephrine > (*l*)-norepinephrine (respective IC_{50} values are 6.9 ± 1.5, 31 ± 7, and 540 ± 110 μM). For antagonist competitors (Fig. 5*d*), the order is (*l*)-propranolol > (*d*)-propranolol > butoxamine

region, three polyadenylylation signals are identified by overbars, and the mRNA polyadenylylation site is indicated by an arrowhead. The boxed decanucleotide beginning at bp 2754 is deleted at the corresponding position but inserted on the complementary strand of the human brain cDNA sequence at a position corresponding to bp 3021. Close approximations of this decamer (in either orientation) are highlighted by broken underlining. Sequences that resemble glucocorticoid-response elements (in either orientation) are indicated by "×" symbols. In the β_2AR coding region, discrepancies between the A431 and published cDNA sequences (9, 10) are shown with open circles for silent substitutions and with black circles for substitutions leading to replacement by the residue indicated below the translated sequence.

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MFEREYTGLPGVCWEGSIISARVRQVRSTQMETSVSVSLWMPPSQRVFTF   50
CVCHHVFVLLGASVFVSGRVSVLDRGDFVPDGFCVRARASVHVGELGGCV  100
SVSMAVVRYKSEHVCQGVFVPVCACLGGHSRFLPNVGQCRCAALCLETSS  150
RAGAQGRQVAATEEPKAPGLAGKHTTSSFSPLGPARVAGKQWWPALQGAV  200
GPRPGQPQEKEGEGRGGKGEECLAPSRLPACHWPK*VPVRHGEGSSPKVLCT 251
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FIG. 3. ORFX amino acid sequence (standard one-letter amino acid symbols). Three methionines (potential initiation codons) are marked with triangles. Cysteine residues are underlined. A phosphoryl-binding-site consensus sequence is boxed; a star marks a lysine residue, essential for phosphorylation activity of several protein kinases and found at the equivalent position in ORFX.

> practolol (respective IC_{50} values are 23.4 ± 6.2 nM and 1.32 ± 0.18, 4.5 ± 0.9, and 46 ± 2 μM). The forskolin-sensitive adenylate cyclase of TP3 cells is not inducible by isoproterenol. However, in the transfected TP3-15 cells, isoproterenol is able to stimulate the adenylate cyclase activity by a factor of 5 (Fig. 5*e*). Again, inhibition of the process follows the same antagonist potency order.

To investigate glucocorticoid effects on β_2AR synthesis in TP3-15 cells, dialyzed fetal bovine serum was used in the culture medium, resulting in slightly modified B_{max} (18 fmol per 10^6 cells) and K_d (9 pM). Addition of 5 μM hydrocortisone nearly doubled receptor number (Fig. 5*b*).

DISCUSSION

Despite extensive studies of β_1AR and β_2AR (1, 24), it is not clear whether these two classes of receptors are encoded by a single gene specifically processed toward the β_1 or β_2 subtype or by distinct genes. Using probes derived from hamster β_2AR cDNA, we cloned a human gene encoding a 46,404-Da peptide that is highly homologous to mammalian β_2AR and to a lesser extent to avian β_1AR. Upon transfection of this gene in eukaryotic cells, a protein was produced that displayed β_2AR rather than β_1AR properties. Using probes derived from the A431 gene, we studied RNA from several sources by cap site analysis (Fig. 4) and blot hybridization (data not shown). Positive signals were detected in A431 cells, which express β_2AR. No signals were obtained with C6 cells, which express mostly β_1AR. These results indicate that we have cloned a gene coding for β_2AR and that distinct genes code for β_1AR and β_2AR.

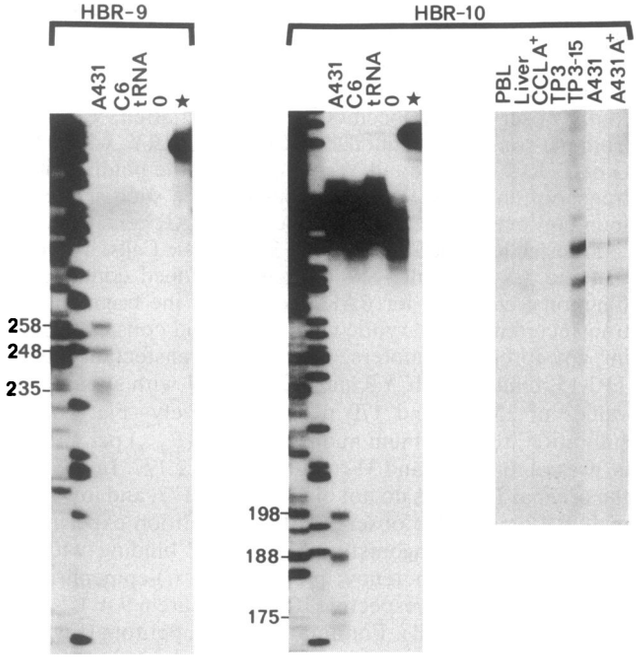

FIG. 4. Nuclease S1 mapping of mRNA cap site. Single-stranded probes (HβR-9 and HβR-10) were annealed to themselves (lanes 0); to tRNA from yeast; to total RNA from A431 cells, C6 glioma cells, peripheral blood lymphocytes (PBL), liver, TP3 cells, or TP3-15 transfectants; or to poly(A)$^+$ RNA from CCL137 or A431 cells (CCLA$^+$ or A431A$^+$). Products of nuclease S1 digestion were analyzed by electrophoresis and autoradiography. Stars indicate undigested probes. Molecular size markers (in bp) are shown at left.

To investigate the existence of tissue-related or genetic variations in the β_2AR gene locus, DNA from various tissues derived from unrelated individuals was analyzed by Southern blot hybridization (Fig. 2). No tissue or individual restriction polymorphism was detected over a 20-kb chromosomal region. However, our DNA sequencing results showed that minor individual variations exist among human β_2AR sequences. The drastic decrease in the homology between our sequence and that of the brain cDNA (10) after the mRNA polyadenylylation site might correspond to a rare individual example of genetic exchange. The Southern blot analyses show that both the 5′ and 3′ flanking regions of the β_2AR gene are highly conserved. In line with this observation, the 5′-flanking-region probe F (Fig. 1*b*) detects the corresponding rabbit, mouse, and hamster sequences under stringent conditions (data not shown). Indeed, these flanking sequences contain the promoter region used in various human tissues as well as in the rabbit TP3 spleen cells (Fig. 4). Because of its high G+C content this promoter region is able to form strong secondary structures (as evidenced by the difficulties encountered during DNA sequencing of this particular region). The methylation pattern of these regions and/or their secondary or chromatin structure might affect the level of gene expression (29). Also, we have shown that functional glucocorticoid-response elements are certainly present in the cloned fragment, since hydrocortisone treatment of the TP3-15 transfected cells nearly doubled β_2AR number. In particular, a glucocorticoid-response-element sequence is contained in the decamer whose position varies from the brain β_2AR cDNA to our clone but still occurs as a tandem repeat in both sequences (Fig. 1*c*). Since temperature or stress, and any of several steroid hormones, is able to modulate β_2AR expression, other distal regulatory elements might be discovered in the vicinity of the β_2AR gene. Thus, conservation of the sequences flanking the β_2AR gene could reflect the presence of regulatory DNA elements modulating the expression of the β_2AR.

The potential product of ORFX (Fig. 3) contains 14 cysteine residues, 8 of which are concentrated within a region of about 60 amino acids, and it harbors the Gly-Xaa-Gly-Xaa-Xaa-Gly-Lys phosphoryl-binding-site consensus sequence found in nucleotide-binding proteins (22). Moreover, residues 98–212 of ORFX constitute a region of significant homology (22% or 40% if one considers conservative substitutions) with one of these proteins: yeast elongation factor EF-1α (5). In various growth factor receptors, a lysine residue, essential for protein kinase activity, is found toward the COOH terminus, 11–20 residues from this consensus sequence (4). ORFX encodes a lysine at a corresponding position (Fig. 3). Several genes for such receptors map in the same region of chromosome 5 as the β_2AR gene (9).

We wish to thank Dr. T. J. Kindt for oligonucleotide synthesis and Ms. C. Peyrat for editorial assistance. This work was supported by grants from the Centre National de la Recherche Scientifique and the Institut National de la Santé et de la Recherche Médicale.

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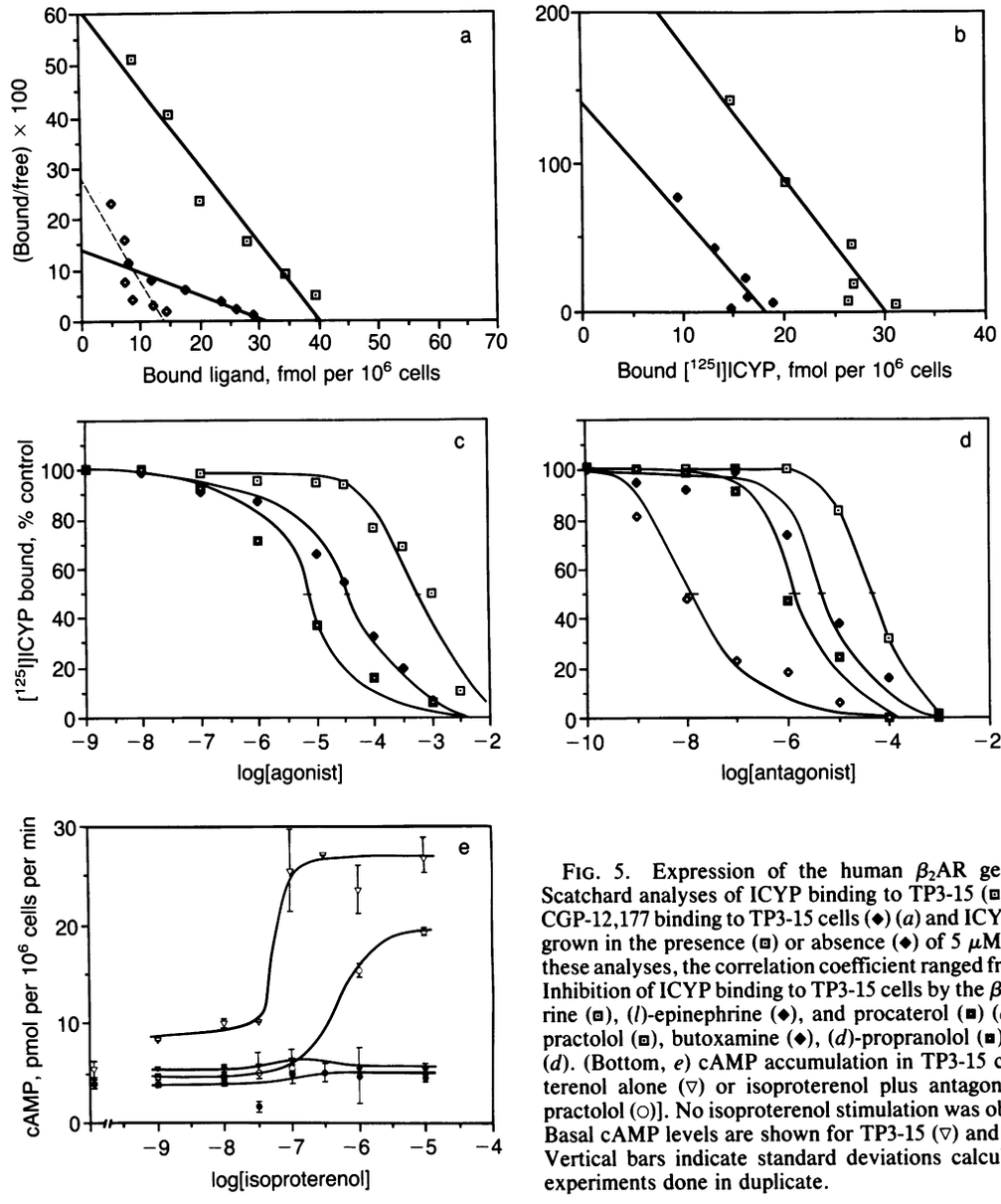

FIG. 5. Expression of the human β_2AR gene in TP3 cells. (*Top*) Scatchard analyses of ICYP binding to TP3-15 (▫) or TP3 (◇) cells and of CGP-12,177 binding to TP3-15 cells (◆) (*a*) and ICYP binding to TP3-15 cells grown in the presence (▫) or absence (◆) of 5 μM hydrocortisone (*b*). For these analyses, the correlation coefficient ranged from 0.82 to 0.99. (*Middle*) Inhibition of ICYP binding to TP3-15 cells by the β-agonists (*l*)-norepinephrine (▫), (*l*)-epinephrine (◆), and procaterol (▪) (*c*) and the β-antagonists practolol (▫), butoxamine (◆), (*d*)-propranolol (▪), and (*l*)-propranolol (◇) (*d*). (Bottom, *e*) cAMP accumulation in TP3-15 cells treated with isoproterenol alone (▽) or isoproterenol plus antagonists [butoxamine (▼) or practolol (○)]. No isoproterenol stimulation was observed for TP3 cells (●). Basal cAMP levels are shown for TP3-15 (▽) and TP3 (●) on the left axis. Vertical bars indicate standard deviations calculated from two or three experiments done in duplicate.

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