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Analysis of the α_{2C}-Adrenergic Receptor Gene Promoter and Its Cell-Type-Specific Activity

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SUMMARY

As an initial approach to define the regulatory elements and transcriptional factors that account for cell-restricted expression of the α_{2C}-adrenergic receptor (AR) gene, we isolated and characterized the receptor gene and identified regions of the gene conferring cell-specific expression. A 4300-nucleotide (nt) fragment of the 5′-flanking region of the rat α_{2C}-AR gene was isolated from a genomic library. The genomic sequence contained the uninterrupted sequence of the 5′-untranslated region of a previously isolated α_{2C}-AR cDNA clone indicating the lack of introns in the 5′ gene segment. RNase protection assays and/or RNA blot analysis indicated the expression of α_{2C}-AR mRNA in rat brain but not in kidney or liver, which is consistent with the major expression of this gene in neuronal tissue. The 5′ gene segment was used to identify sites of transcriptional initiation and promoter activity by RNase protection assays and transient transfection of reporter gene constructs. With the use of RNA probes progressively upstream of the translational start site, RNase protection assays with rat brain total RNA indicated multiple sites of transcriptional initiation within a ~70-nt span (−660 to −730 nt 5′ to the translational start codon). The zone of transcriptional initiation was part of a larger GC-rich area of the 5′ gene segment that is a characteristic of genes initiating transcripts at multiple sites. The promoter activity of this zone of transcriptional initiation and the influence of gene segments 5′ to this area were addressed using chloramphenicol acetyl transferase reporter gene constructs. Transient transfection of reporter gene constructs indicated that a 96-nt gene fragment (−699/−603 relative to the translational start codon) was sufficient to direct transcription in the neuroblastoma X glioma hybrid cell line NG108-15, a cell line expressing the endogenous α_{2C}-AR. Promoter activity was not observed in constructs lacking the zone of transcriptional initiation. The promoter segment was inactive when introduced into the rat glioma cell line C6B4, the rat submandibular cell line RSMT-A5, and the rat pancreatic β cell line RIN-5AH, all of which do not express the endogenous α_{2C}-AR gene. Upon incubation with nuclear extracts, a 129-nt fragment encompassing the promoter exhibited a gel mobility shift pattern that was specific for cells expressing the receptor protein and involved a nuclear protein that recognized a Sp1 oligonucleotide. These data indicate that a 96-nt gene promoter segment of the α_{2C}-AR gene functions in a cell-type-specific manner.

Tissue-specific responses to the catecholamines norepinephrine and epinephrine are primarily achieved through the selective expression and regulation of AR subtypes in responsive tissues (1, 2). The cell response mediated by the different subgroups of ARs (α_1, α_2, and β) are often antagonistic to each other, as occurs with the adrenergic regulation of lipolysis or in peripheral vascular beds, in which activation of β_2-ARs relax and α-ARs contract the vascular smooth muscle. Antagonism between the AR subtypes is apparent at an early step in the signal transduction cascade, as indicated by the inhibitory-versus-stimulatory coupling of α_2-ARs and β-ARs, respectively, to adenylyl cyclase. Thus, the final cell response elicited by the catecholamines is determined by the types of ARs expressed in the cell. Once the receptor subtypes are expressed, there are several mechanisms that fine-tune the cell response, including agonist concentration, receptor density, receptor coupling efficiency, and receptor subtype-specific regulatory mechanisms such as receptor phosphorylation, sequestration, and internalization.

Although the upstream regions of the genes encoding the α_{1b}-, α_{2A}-, and β_{1-3}-ARs in various species are sequenced and in some cases their promoter elements partially characterized (3–13), the *cis* elements directing cell-specific expression of a particular subtype are poorly understood. As a first approach to this question, we initiated studies to characterize the transcriptional regulatory elements of the rat α_{2C}-AR

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ABBREVIATIONS: AR, adrenergic receptor; nt, nucleotide(s); pSK$^+$, pBluescript SK$^+$; CAT, chloramphenicol acetyl transferase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

gene (RG10) (14). The α_{2C}-AR is one of nine receptors through which epinephrine elicits cellular responses. Three α_2-AR subtypes are encoded by distinct genes. In humans, the genes are *α_2-C10* (α_{2A}), *α_2-C2* (α_{2B}), and *α_2-C4* (α_{2C}); and in rats, the genes are *RG20* ($\alpha_{2A/D}$), RNGα_2 (α_{2B}), and *RG10* (α_{2C}) (15). As is the case with many members of the superfamily of G protein-coupled receptors, α_2-AR couple to multiple heterotrimeric G proteins to regulate various effector molecules in a cell-type-specific manner (16, 17). Each α_2-AR subtype is expressed in a tissue-specific manner as determined through analysis of both receptor mRNA and protein distribution. In rat, α_{2C}-AR mRNA is primarily expressed in the central nervous system, whereas the receptor distribution in human may extend to peripheral tissues (14, 18–27). In the rat central nervous system, α_{2C}-AR mRNA and protein are localized to discrete areas in the hippocampus, basal ganglia, and olfactory system (22–24). To identify elements that might direct the neural-enriched expression of this α_2-AR subtype, we isolated the receptor gene and characterized the functionality of the receptor gene promoter in neuronal and non-neuronal cells.

Experimental Procedures

Materials. [^{32}P]CTP, [α-^{35}S]ATP, and [^{32}P]dCTP were purchased from DuPont-New England Nuclear (Boston, MA). [^{14}C]Chloramphenicol was purchased from Amersham (Arlington Heights, IL). The gene reporter construct encoding CAT was provided by Dr. Arthur Lee (Harvard University, Boston, MA) and Dr. Tom Quertermous (Vanderbilt University, Nashville, TN). pCMV.CAT was provided by Dr. David Kurtz (Medical University of South Carolina, Charleston, SC). The neuroblastoma X glioma mouse/rat hybrid cell line NG108-15 and the parent C6B4 glioma (28) were obtained from Dr. Marshall Nirenbirg (National Institutes of Health, Bethesda, MD). The RSMT-A5 (29) and RIN-5AH (30) cell lines were obtained from Dr. R. B. Wellner (Navy Research Hospital, Washington, D.C.) and Dr. A. Lernmark (University of Washington, Seattle, WA), respectively. Reagents for RNase protection were obtained from Promega (Madison, WI) or Ambion (Austin, TX). Oligonucleotides were synthesized by the core facility at the Medical University of South Carolina.

Cell culture. The neuroblastoma X glioma mouse/rat hybrid cell line NG108-15 was grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 0.1 mM hypoxanthine, 10 μM aminopterin, and 16 μM thymidine. The rat pancreatic β cell RIN-5AH and the rat submandibular gland cell line RSMT-A5 were grown in RPMI 1640 medium supplemented with 10% fetal calf serum. The rat glioma C6B4 was grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. All culture media contained 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml fungizone. Cultures were maintained at 37° in a humidified atmosphere of 5% CO_2.

Plasmid constructs. Phage containing the rat α_{2C}-AR gene (RG10) fragment were previously isolated from a rat genomic library in Charon-4A phage using a 62-mer oligonucleotide derived from a highly conserved sequence in the coding regions of this receptor family (14). A 6300-bp fragment (*Bgl*II/*Bam*HI) was cloned into the *Bam*HI site of the pGEM7 vector. This fragment consists of the receptor protein coding region (1374 nt), a 3′-flanking region (590 nt) with a signal sequence for polyadenylation (AAUAAA) 472 nt 3′ to the termination codon of the open reading frame, and a ~4300-nt segment 5′ to the translational start AUG. The coding region and the sequence 3′ to the translational stop codon were removed through digestion with *Nco*I (site located at the translation start site) and *Hind*III (in pGEM7 linker) and the plasmid religated after a fill-in reaction for sequence analysis of the insert. The resulting construct contained ~4300 nt corresponding to the 5′-flanking region.

Various fragments of the 5′-flanking region were subcloned in pSK$^+$ (Stratagene, La Jolla, CA) to generate riboprobes for RNase protection assay or in pO.CAT (31) for CAT reporter gene assays. The orientation of the fragments in each construct was checked by both sequencing and restriction mapping. Five probes were used for RNase protection assays (numbered relative to the translational start AUG): probe 1 (+3 to −278), ligated into *Sma*I site of pSK$^+$; probe 2 (−363/−660), *Nsi*I/*Sma*I ligated into *Pst*I/*Sma*I site of pSK$^+$; probe 3 (−862/−1112), *Pst*I; probe 4 (−363/−772), *Nsi*I/*Not*I; and probe 5 (−363/−915), *Nsi*I/*Xba*I. Probes 4 and 5, as well as probe 2 in some experiments, were generated from the *Nsi*I/*Xba*I gene fragment ligated into *Pst*I/*Xba*I restricted pSK$^+$.

Reporter gene constructs are numbered according to the translational start codon. pα_{2C}(−4300/−197).CAT, pα_{2C}(−1289/−197).CAT, pα_{2C}(−772/−197).CAT, pα_{2C}(−915/−363).CAT, pα_{2C}(−588/−278).CAT, and pα_{2C}(−1289/−773).CAT correspond to blunt-ended fragments ligated in the blunt-ended *Xba*I or *Bam*HI cloning site of pO.CAT. pα_{2C}(−716/−589).CAT was generated by ligation of a blunt-ended *Xho*I/*Hind*III gene segment from pSK$^+$ (*Nar*I/*Nar*I inserted into *Cla*I restriction site in polylinker) into the blunt-ended *Xba*I site of pO.CAT. The gene segment (−698/−604) was subcloned into pO.CAT restricted with *Bam*HI and *Xba*I after DNA amplification with an antisense primer harboring a *Xba*I restriction site (5′-GCTCTAGACGCTCGCCGGTGCAGCGTCTC-3′) and a sense primer harboring a *Bam*HI restriction site (5′-CGCGGATCCCGCTGCGCT-ACTGGACTCAAGTT-3′).

Nucleotide sequence analysis. DNA was sequenced by the dideoxy chain termination method using primers T7, Sp6, or T3 or rat α_{2C}-AR-specific primers. The sequences were analyzed by FASTA or FINDPATTERNS (University of Wisconsin GCG program) accessing the Genbank.EMBL, transcription factor site (TFsite) data base or eukaryotic promoter databases (32).

Isolation and analysis of RNA. Total RNA or poly(A)$^+$-enriched RNA was extracted from tissues or cells using RNAzol (Tel-Test, Friendswood, TX) or the Stratagene mRNA enrichment kit. The 5′ termini of the α_{2C}-AR mRNA were mapped with the use of the RNase protection assay. Antisense riboprobes were generated after linearization of the pSK$^+$ constructs described above with the appropriate enzyme. The linearized plasmids (0.5 μg) were incubated for 60 min at 25° in a total volume of 25 μl containing 20 mM dithiothreitol, 0.5 mM ATP, 0.5 mM UTP, 0.5 mM GTP, 0.05 mM CTP, 50 μCi of [^{32}P]CTP, 40 mM Tris·HCl, pH 7.5, 6 mM $MgCl_2$, 5 mM NaCl, and 50 units of T3 or T7 RNA polymerase. The DNA template was degraded by the addition of RNase-free DNase I (Boehringer-Mannheim Biochemicals, Indianapolis, IN) in a total volume of 100 μl. The radiolabeled riboprobe was precipitated, gel purified and either used on the same day or diluted and stored at −20° in buffer H (80% formamide, 40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), pH 6.7, 400 mM NaCl, 1 mM EDTA) at a concentration of ~50,000 cpm/μl. Total RNA (50–100 μg) was mixed with ~100,000 cpm riboprobe in 30 μl of buffer H, heated for 5 min at 90°, and incubated for 16 hr at 55°. Hybridization was terminated by the addition of 300 μl of RNase solution (5 mM EDTA, 300 mM NaCl, 10 mM Tris, pH 7.4, 40 μg/ml RNase A, 400 units/ml RNase T1) and incubation at 37° for 90 min. RNases were denatured by the addition of 350 μl of solution D (4 M guanidium thiocyanate, 0.5% sodium *N*-lauryl sarkosine, 25 mM sodium citrate, pH 7.0, and 100 mM β-mercaptoethanol). The protected riboprobe was isopropanol precipitated and electrophoresed on an 8 M urea/5-6% polyacrylamide gel in Tris/borate/EDTA buffer. The gel was dried and exposed to film for 2–6 days. Translated RNA probes of known size were used as size markers. In some experiments, the Ambion RNase protection kit was used according to the manufacturer's instructions. RNA samples prepared from cells transfected with pCAT receptor constructs were treated with 2 units of DNase I for 30 min at 37° before RNase protection assays to eliminate plasmid contamination of the RNA samples. Yeast tRNA

was used as a control for effectiveness of RNA hydrolysis and the specificity of the observed signals. Experimental conditions were such that probe hydrolysis was largely complete.

Transfection and promoter activity assays. Cell lines were cotransfected with a CAT reporter gene vector and the β-galactosidase reporter gene vector pSV.β-GAL. The efficiency of transfection was also determined by transfection with CAT reporter gene vectors where the CAT gene was driven by viral promoters (simian virus 40, RSMT-A5 and RIN-5AH; cytomegalovirus, NG108-15 and C6B4). In a series of preliminary experiments, the different cell types were transfected with the viral promoter CAT reporter genes by calcium phosphate/DNA coprecipitation, DEAE-dextran, or electroporation to determine the optimal procedure for introducing gene constructs into the different cell types.

NG108-15 cells and C6B4 glioma cells were transfected by calcium phosphate/DNA coprecipitation. Cells at 50–60% confluence were cotransfected with 3–10 μg of pSV.β-GAL vector and 10 μg of pCAT construct/100-mm plate. One milliliter of DNA-calcium precipitate was prepared for each plate in HEPES-buffered saline solution as previously described (14, 33) and added dropwise onto each plate containing 10 ml of regular media. After a 16-hr incubation at 37°, the cells were washed with phosphate-buffered saline and incubated with fresh media for 24–48 hr.

RIN-5AH and RSMT-A5 cell lines were transfected by electroporation using a BTX (San Diego, CA) electropulser. Cells from two to five confluent 100-mm culture dishes were harvested in phosphate-buffered saline, pelleted, resuspended in 400 μl of serum-free media (RPMI 1640 or Dulbecco's modified Eagle's medium), and mixed with 25 μg of reporter gene construct and 10 μg of pSV.β-GAL. Cells were electroporated (960 μF, 230 V) in a volume of 400 μl in a 2-mm gap electroporation chamber (BTX), incubated at 24° for 3–4 min, plated onto one 100-mm culture dish, and incubated in fresh media for 24–48 hr.

Transfected cells were harvested, pelleted, and resuspended in 100 μl of 20 mM Tris·HCl, pH 8.0, and 2 mM $MgCl_2$. Each preparation underwent three freeze/thaw cycles to maximize cell lysis, and the cell extract was used for determination of both β-galactosidase activity (34) and CAT activity (33, 34a). CAT activity was determined using 10–35 μl of heated cell extract (65° for 10 min) by incubation for 30 min to 12 hr at 37° in a final volume of 50 μl containing 100 mM Tris·HCl, pH 8.0, 0.5–1 mM *n*-butyryl CoA, and 0.125–0.3 μCi of [^{14}C]chloramphenicol (Amersham). The reaction was stopped by the addition of 200 μl of 2:1 tetrapentadecane/xylene mixture. The labeled enzyme product was extracted into the organic phase and quantified in a liquid scintillation counter. Data were corrected for transfection efficiency as determined with pSVβ-GAL. Essentially identical results were obtained when the radiolabeled product was analyzed with thin layer chromatography.

Gel mobility shifts. Crude nuclear extracts were prepared according to a modification of the technique described by Andrews and Faller (35). Briefly, 100 mg of fresh tissue or ~100 million cells was homogenized with a Dounce homogenizer at 4° in 10 ml of buffer A (10 mM HEPES-KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride). The homogenate was transferred into microfuge tubes and centrifuged for 10 sec at 12,000 rpm at 4°. The supernatant was discarded, and the pellet was resuspended in 1 ml of buffer A. After a second centrifugation, the supernatant was discarded, and the pellet was resuspended in 100 μl of buffer B (20 mM HEPES-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 4°) and incubated for 20 min at 4°. The samples were then microfuged for 2 min at 12,000 rpm, and the supernatant was stored at −70°. Protein concentration was determined according to the method of Lowry (36). The integrity of the nuclear extracts was verified by gel shift assay using Sp1 (5′-ATTCGATCGGGGCGGGGCGAGC-3′) and Ap1 (5′-AGCTTGGTGACTCATCCG-3′) double-strand oligonucleotide probes labeled with ^{32}P.

The 5′ gene segment in pSK$^+$ was digested with *Nar*I to generate a 129-nt fragment that contained the active gene promoter and labeled with ^{32}P-dCTP by the addition of Klenow fragment of DNA polymerase I. The reaction product was loaded onto a nondenaturing 6% polyacrylamide gel, and the probe was purified from the gel through overnight elution at 37° in 0.1% sodium dodecyl sulfate, 0.5 M ammonium acetate, 10 mM magnesium acetate, and 1 mM EDTA. The end-labeled probe was precipitated by isopropanol, washed with 70% ethanol, and resuspended in water. Approximately 1 fmol of end-labeled probe was incubated for 30 min at 25° with nuclear extracts (4–6 μg of protein) in a final volume of 20 μl containing 20% glycerol, 40 mM HEPES-NaOH, pH 8, 60 mM KCl, 50 mM NaCl, 1 mM EDTA, 0.5 mM dithiothreitol, 3 μg of poly(dI/dC), and 0.5 mg/ml bovine serum albumin (Fraction V). The optimal concentrations of bovine serum albumin and poly(dI/dC) were determined in a series of preliminary experiments. Competing oligonucleotides were preincubated with the nuclear extract for 10 min before the addition of the labeled probe. The reaction was electrophoresed on a 5% polyacrylamide gel containing 2.5% glycerol and Tris/borate/EDTA buffer. The gel was dried and autoradiographed for 12–24 hr.

Results

Analysis of α_{2C}-AR gene sequence 5′ to translational start site. The rat α_{2C}-AR gene was isolated from a phage library, and a 1500-nt segment 5′ to the translational initiation site was sequenced (Fig. 1). The sequence of a rat brain α_{2C}-AR cDNA clone (25) was contained uninterrupted in the genomic sequence.[3] Sequence analysis of the 5′-flanking region indicated the absence of a TATAA box, an element that serves to localize the transcription start site in many genes. However, the gene segment was enriched in GC nt (Figs. 1 and 2). The percentage of GC nt reached 78% in the gene segment from −584 to −818, and this area contained numerous methylation-sensitive enzyme restriction sites (e.g., *Hpa*II, *Hha*I), generating CpG islands (37) (Fig. 2). Many genes that lack a TATAA element use GC-rich regions, a GC box or boxes, or an initiator element to align the transcription complex (38, 39). Because no consensus pyrimidine-rich initiator sequence was identified in the area of transcriptional initiation, the α_{2C}-AR promoter apparently belongs to the GC-rich group. Sequence homology searches of a transcription factor data base (TFsite) and a eukaryotic promotor data base revealed several consensus recognition sequences for transcription regulatory proteins (Fig. 1). The gene segment contained three putative recognition sites for Sp1 nuclear protein, a *trans*-regulatory protein that often plays an important role in the transcription of genes using GC-rich promoters. To characterize the promoter elements of the receptor gene, the receptor mRNA was first analyzed by RNase protection to define a zone of transcriptional initiation. The role of this region in transcription regulation was then investigated using a heterologous expression system.

Identification of a zone of transcriptional initiation. To identify regions of transcriptional initiation, antisense RNA probes derived from the 5′-flanking region of the receptor gene were generated and hybridized with total RNA isolated from rat brain (Fig. 3A). RNA probes 1 and 2 span sequences identified in cDNA clones, whereas probe 3 is located further upstream 5′ to the end of isolated cDNA clones (25). In RNase protection assays, probes 1 and 2 were

[3] The α_{2C}-AR gene sequence to the −915 *Xba*I restriction site was previously determined by Voigt *et al.* (25) in rat, and the two sequences exhibit 96% nt identity.

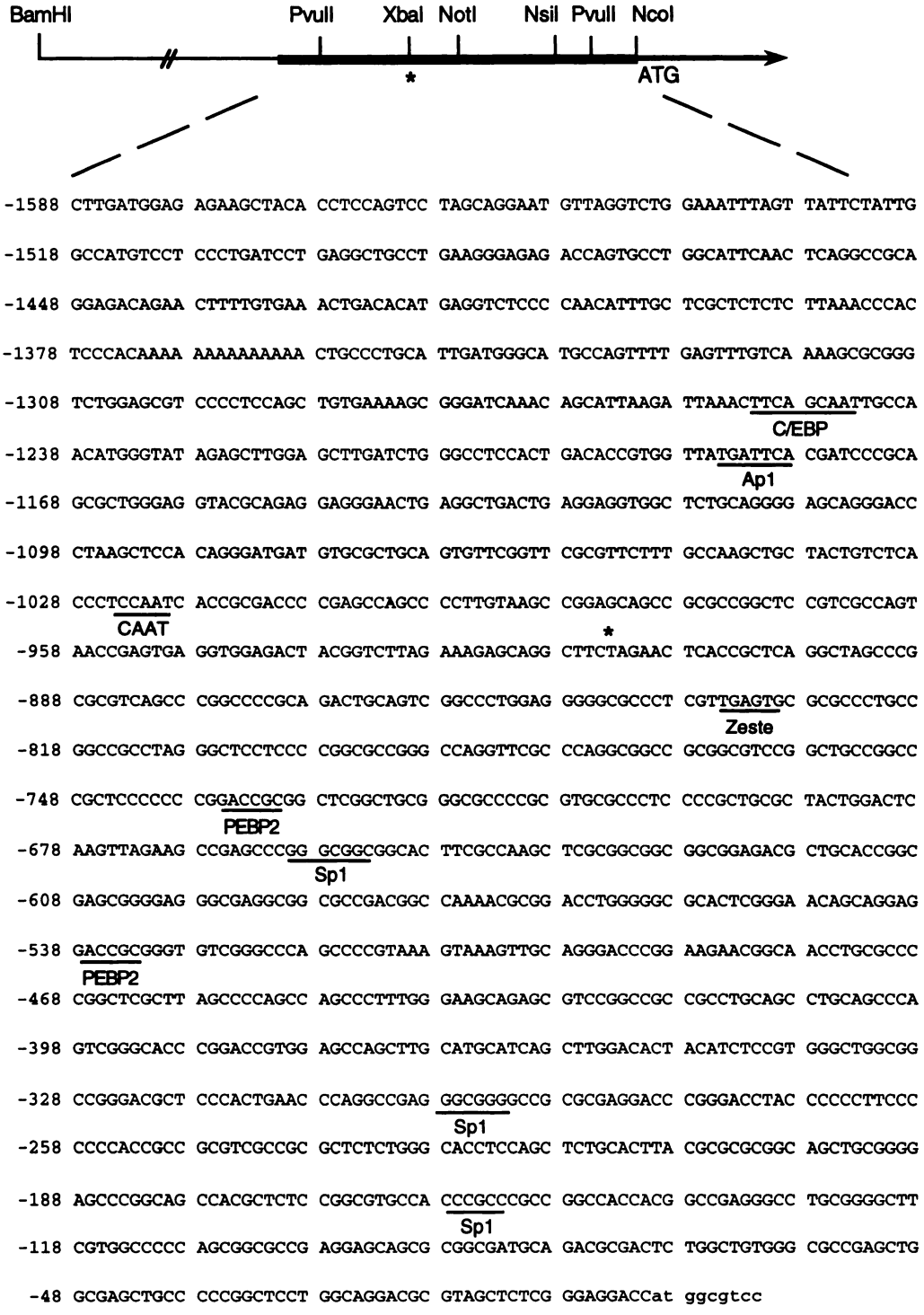

Fig. 1. The nt sequence of the rat α_{2C}-AR gene upstream of the protein coding region. A genomic clone encompassing the α_{2C}-AR coding sequence was isolated from a Charon 4A library and characterized as described in Experimental Procedures. The subcloned gene segment consisted of the α_{2C}-AR coding region (1374 nt), a 3′-flanking region (~600 nt), and a 5′-flanking region (~4300 nt). *Underlined sequences*, consensus sites for transcription regulatory factors identified as described in the text. *, The 5′ end of a previously isolated α_{2C}-AR cDNA clone (25). *ATG*, start of the open reading frame encoding the α_{2C}-AR protein.

fully protected, whereas no protection of probe 3 was observed. These data indicated that the transcript was initiated 5′to nt −660 (Figs. 1 and 3). The protected species was absent in liver and kidney, which is consistent with the major expression of the α_{2C}-AR in the central nervous system (Fig. 3B). To further localize the transcription initiation site, we generated probes that encompassed the region between nt −915 and −363. Probes 4 and 5 extended 112 and 255 nt from the 5′ end of probe 2, which was fully protected in RNase protection assays (Figs. 3 and 4). RNase protection experiments with probes 4 and 5 indicated that there are multiple start sites within a zone of transcriptional initiation (nt −660 to −730). Although probe 2 is fully protected and appears as a single, intense signal, several protected species were observed with probes 4 and 5 resulting in signals of lower intensity (Fig. 4).[4] None of the protected species were

[4] The fastest migrating protected species identified with probes 4 and 5 is slightly smaller than the size of the protected species identified with probe 2. The reason for this is unclear, but it is likely due to the high percentage of GC nt in this region (Figs. 1 and 2). Indeed, analysis of the secondary structure of the 5′-flanking region with the use of MFOLD (University of Wisconsin GCG Sequence Analysis Program) indicated that the region from nt −558 to −788

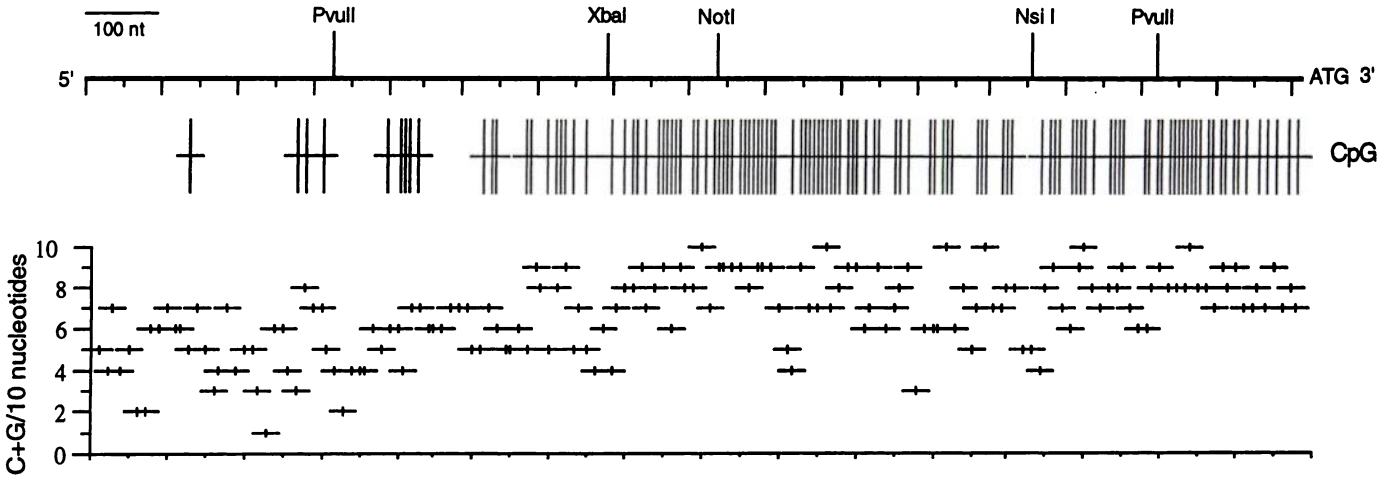

Fig. 2. Identification of GC-rich regions and CpG islands in the α_{2C}-AR gene 5′ to the site of translational initiation. CpG islands are potential sites of nt methylation (37). *ATG*, start of the open reading frame encoding the α_{2C}-AR protein.

observed when similar experiments were performed using yeast tRNA instead of brain RNA. Two major mRNA species from rat brain of ~298 and ~325 nt were observed with both probe 4 and probe 5, indicating that two preferred sites of transcriptional initiation were located at ~−660 and ~−687 nt 5′ to the translational start site. It is not clear whether the multiple start sites are observed in the same cell or reflect the cellular heterogeneity of the brain RNA preparation. However, the clustering of multiple start sites is characteristic of the transcription of genes that use a GC-rich promotor. Repeated attempts to characterize the transcriptional start sites by primer extension were unsuccessful, likely due to the inherent difficulty of such experiments with a low abundance of transcripts that are GC rich and in which transcription is initiated from multiple sites. Thus, to further characterize this area of the receptor gene and localize a zone of transcriptional initiation, we evaluated the promotor activity of 5′-flanking gene segments.

Localization of the α_{2C}-AR gene promoter. As an initial approach to localizing the α_{2C}-AR gene promoter elements, we evaluated the ability of 5′-flanking fragments to activate transcription of a CAT reporter gene in a neuronal-like cell line that expressed the endogenous receptor gene as well as in three cell lines that did not express detectable receptor mRNA. The neuroblastoma X glioma hybrid cell line NG108-15 expressed transcripts encoding both α_{2B}- and α_{2C}-AR subtypes (Fig. 5) (Refs. 21 and 40).[5] The NG108-15 cell line was generated from a rat glioma cell and a murine neuroblastoma cell line. The α_{2C}-AR mRNA is apparently of murine origin because it was detected in NG108-15 cells by RNA blot analysis using total RNA but not by RNase protection assays (Figs. 3B and 5). The inability to detect α_{2C}-AR mRNA in RNase protection assays likely reflects mismatches in the nt sequence of the murine transcript and the probe derived from the rat gene. Fragments of the 5′-flanking region were inserted into a CAT reporter gene vector (pO.CAT), and their promoter activity was determined after transient transfection. pα_{2C}(−1289/−197).CAT contained the 5′-end of a cDNA clone isolated by Voigt *et al.* (25) and the zone of transcriptional initiation identified above. The pα_{2C}(−1289/−197).CAT transfectants exhibited 5–8-fold greater CAT activity than that observed with a promotorless CAT construct. The −1289/−197 gene segment functioned as a cell-type-specific heterologous promoter in that it was active in cells expressing the endogenous receptor gene (i.e., NG108-15) but not in cells lacking the receptor transcript (i.e., C6B4 glioma cells, RIN-5AH pancreatic β cells,[6] RSMT rat submandibular cells) (Fig. 6).

Additional reporter constructs were generated to identify the region of the active segment required for promoter activity and to determine the gene segments driving transcription in a cell-type-specific manner. The reporter construct containing a large segment of the 5′ upstream region [pα_{2C}(−4300/-197).CAT] exhibited minimal activity, and the low activity of this construct is consistent with the relatively low levels of α_{2C}-AR mRNA in NG108-15 cells (Fig. 5). The gain in activity obtained on truncation of this larger 5′ segment to nt −1289 suggests the presence of negative regulatory elements in the deleted segment. The pα_{2C}(−1289/−197).CAT construct was then split so that one construct contained the 5′ end of the longest cDNA clone previously isolated (25), and another construct contained the zone of transcriptional initiation identified through RNase protection analysis of brain α_{2C}-AR mRNA. The former construct [pα_{2C}(−1289/−773).CAT] exhibited low activity, whereas the latter [pα_{2C}(−772/−197)] increased CAT activity 10–15-fold (Fig. 7) in a cell-type-specific manner. In addition, the active reporter constructs used a site of transcription initiation in NG108-15 cells that is similar to that used by the intact gene in brain.[7] The pα_{2C}(−466/−196).CAT construct was progressively truncated at the 5′ and 3′ ends. A 96-nt construct encompassing the zone of transcriptional initiation increased CAT activity 15–20-fold in NG108-15 cells (Fig. 7). No significant CAT induction was observed with constructs lacking the transcription initiation site [pα_{2C}(−588/−278).CAT,

was characterized by a strong stem structure motif. The resulting secondary structure of either the probe or mRNA may subtly influence sites of annealing at the 5′ end of the mRNA and/or hydrolysis of the annealed complex.

[5] Although NG108-15 cells express both α_{2B}- and α_{2C}-AR mRNA, photoaffinity labeling and partial receptor protein purification indicate the predominant expression of the α_{2B}-AR protein (54).

[6] The RIN-5AH cell line expresses the $\alpha_{2A/D}$-AR as determined by radioligand binding assays and/or RNA blot analysis (55).

[7] J. D. Sherlock, J. Saulnier-Blache, and S. M. Lanier, unpublished observations.

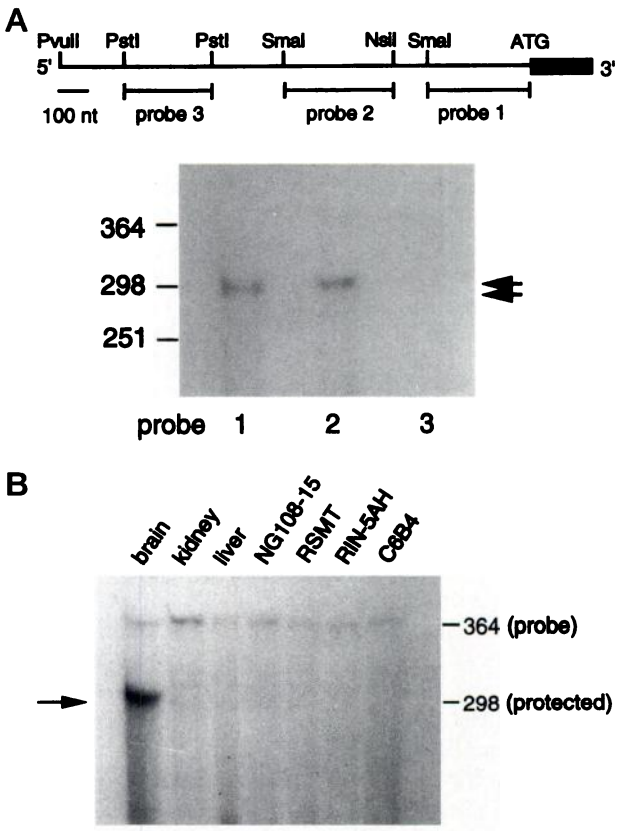

Fig. 3. Analysis of α_{2C}-AR mRNA in rat brain. A, total RNA was isolated from rat brain, and the 5′ region of the α_{2C}-AR mRNA was characterized in RNase protection assays as described in Experimental Procedures. In this experiment, 100 μg of total RNA was used for hybridization with each probe. *Lines on left*, migration of radiolabeled RNA of known size. *Probe 1*, undigested, 372 nt; complete protection, 282 nt. *Probe 2*, undigested, 364 nt; complete protection, 298 nt. *Probe 3*, undigested, 317 nt; complete protection, 251 nt. Similar results were obtained in three separate experiments using different preparations of RNA. *Arrows on right*, protected species observed with probes 1 and 2. *Top*, location of the probes in the 5′ upstream gene region. B, expression of α_{2C}-AR mRNA in tissues and cell lines. RNA from liver, kidney, and the cell lines NG108-15, RSMT-A5, RIN-5AH, and C6B4 was evaluated for expression of the α_{2C}-AR mRNA with the use of RNase protection assays. Total RNA (~100 μg) was prepared from tissues and cells and hybridized with probe 2 for analysis in RNase protection assays as described in Experimental Procedures. *Line on the right*, migration of the protected species (298 nt). Undigested probe, 364 nt. The results are representative of two experiments using different RNA preparations.

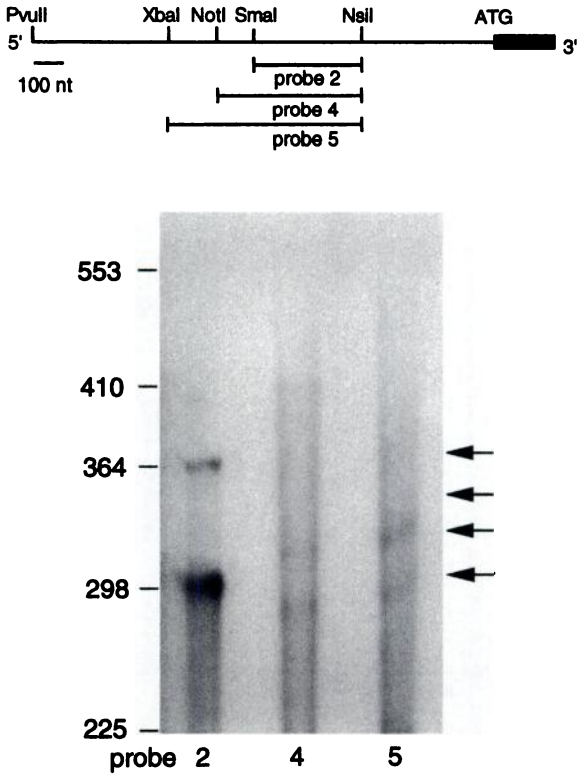

Fig. 4. Analysis of the 5′ end of the α_{2C}-AR mRNA in rat brain. Poly(A)$^+$-enriched RNA was prepared from brain, and ~50 μg was hybridized with each probe for analysis in RNase protection assays as described in Experimental Procedures. *Arrows*, major protected species observed with *probes 4 and 5*. *Probe 2*, undigested, 364 nt; completely protected, 298 nt. *Probe 4*, undigested, 476 nt; completely protected, 410 nt. *Probe 5*, undigested, 619 nt; completely protected, 553 nt. This experiment was repeated three times with similar results. *Top*, location of the probes in the 5′ upstream gene region.

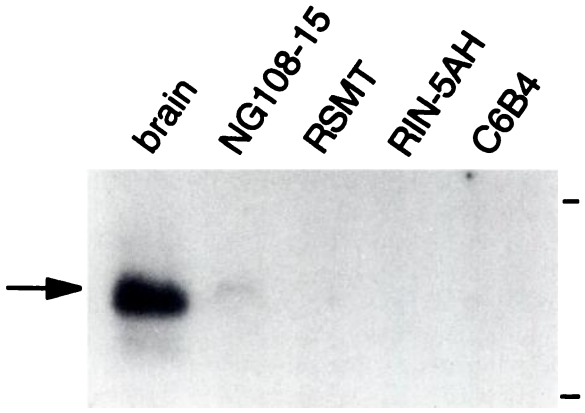

Fig. 5. Expression of α_{2C}-AR mRNA in rat brain and cell lines. Total RNA was isolated from brain tissue and cell lines, and 50 μg was gel electrophoresed and transferred to a nylon membrane for hybridization with a random-primed probe generated from the coding region of the rat α_{2C}-AR (RG10) gene. *Lines on the right*, migration of 28 S and 18 S ribosomal RNA. *Arrow*, migration of the hybridized message. Similar results were obtained in two or three separate experiments.

pα_{2C}(−1289/−773).CAT]. Each of the reporter constructs were inactive in the non-receptor-expressing cell line RSMT, indicating that *cis*-acting elements involved in the transcriptional initiation of the α_{2C}-AR gene in a cell-type-specific manner are present in the fragment −698 to −604 (Figs. 1 and 7).

Cell-type-specific DNA-binding proteins interacting with the receptor promoter. As an initial approach to identifying the factors involved in the cell-type-specific expression of the α_{2C}-AR gene, we performed gel shift analysis with the active promoter segment (−716/−589) and nuclear extracts prepared from NG108-15 cells (α_{2C}-AR-expressing cell line) and RSMT-A5 cells (α_{2C}-AR-nonexpressing cell line). The entire active promoter fragment was used instead of short oligonucleotides to provide a template for the generation of the transcription complex. Although the use of the longer fragment results in the generation of several nonspecific gel-shifted species, its use may allow detection of species that are dependent on interactions with various "accessory" proteins within the immediate environment. Gel shift assays indicated the presence of three shifted species (Fig. 8A, *I, III, and IV*) with NG108-15 cell nuclear extracts that were not

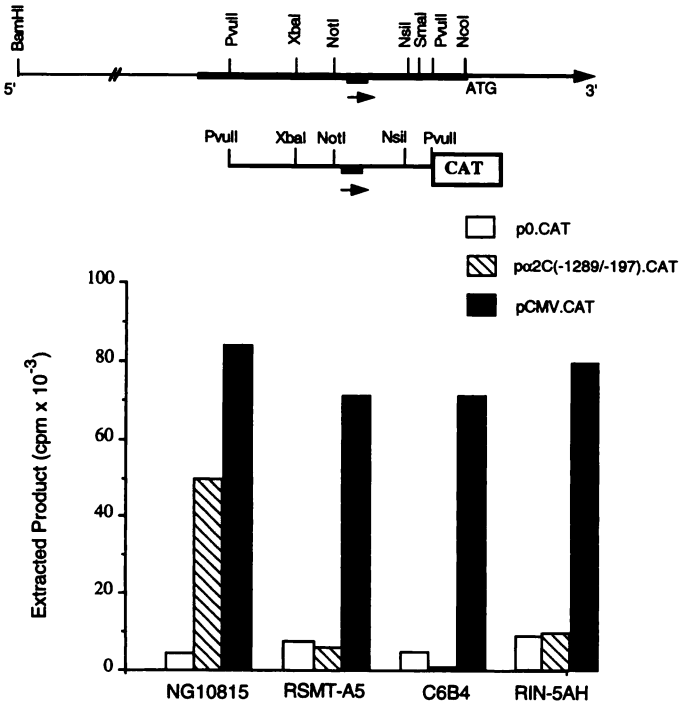

Fig. 6. Analysis of α_{2C}-AR gene promoter in neuronal and non-neuronal cell lines. NG108-15, RIN-5AH, RSMT-A5, and C6B4 cells were transfected with pα_{2C}.CAT constructs as described in Experimental Procedures. Cells were harvested at 48 hr for analysis of CAT activity. The radiolabeled enzymatic product was measured through liquid scintillation counting after organic extraction of cell extract. Data were corrected for transfection efficiency and are the average of two experiments that were representative of the results generated in larger number of experiments for RSMT-A5 and NG108-15 cells and presented in Fig. 7. *ATG*, start of the open reading frame encoding the α_{2C}-AR protein. *Top, thickened segment in diagram*, sequence indicated in Fig. 1. *Arrow*, area of transcriptional initiation identified in RNase protection assays.

observed with nuclear extracts prepared from the receptor-nonexpressing cell line RSMT-A5. The generation of species IV was insensitive to the presence of increasing concentrations of the gene segment used to prepare the radiolabeled probe and was considered nonspecific, whereas the generation of species I, II, and III was inhibited by the competing gene segment. As indicated (Fig. 8A), the active promoter segment contained a consensus recognition site for the *trans*-regulatory nuclear protein Sp1. Generation of species I was not observed when an oligonucleotide encoding the Sp1 consensus recognition site was included in the incubation (Fig. 8A). Species II was present with both cell nuclear extracts, but the Sp1 oligonucleotide only inhibited the generation of this species in NG-10815 nuclear extracts. The generation of species III was not altered by inclusion of the Sp1 oligonucleotide. Although the *trans*-regulatory protein Sp1 is ubiquitous, corresponding Sp1-sensitive gel-shifted species were not observed with the active promoter segment when the probe was incubated with nuclear extract prepared from the receptor-nonexpressing cell line RSMT-A5, despite the clear expression of nuclear proteins capable of interacting with labeled oligonucleotides encompassing putative recognition sites for Sp1 or Ap1 (Fig. 8, *bottom*).

Discussion

Based on the results of RNase protection assays and sequence analysis of genomic and cDNA clones, the α_{2C}-AR mRNA consists of ~660-730 nt 5′ to the translational AUG start codon, 1374 nt of protein coding sequence, and 474 nt of 3′-untranslated sequence, generating a calculated message size of ~2508-2578 nt plus the polyadenylation tract. The calculated size is similar to that of the major receptor mRNA species identified through RNA blot analysis but smaller than the 2752-nt α_{2C}-AR cDNA clone identified by Voigt *et al.* (25), the sequence of which is contained uninterrupted in the genomic clone.[3] The 2752-nt α_{2C}-AR cDNA clone isolated from brain may be a less-abundant transcript that was not detectable by RNase protection or it may be localized to discrete brain areas not represented in the tissue samples used in the current report. Within the 5′ segment of the α_{2C}-AR gene, there exists a 96-nt fragment that is GC rich, encompasses a zone of transcriptional initiation, and drives transcription of a heterologous gene in a cell-type-specific manner. The functionality of the α_{2C}-AR gene promoter segment in the neuronal-derived cell line NG108-15 but not in the non-neuronal cell lines is consistent with the primary expression of the receptor message in the central nervous system of the rat.

Transcriptional initiation of most mammalian functional genes involves three general classes of promotor elements: a TATAA box, an initiator sequence, and GC-rich segments or GC box elements (38, 39, 41–44). In some instances, the individual elements are found in various combinations. These structural entities direct the formation of a transcription complex positioning RNA polymerase II in the required position and guide the initiation of transcript synthesis. The TATAA box is perhaps the best understood element involved in transcriptional initiation and exhibits consistent spatial behavior relative to transcription start sites. The GC box element operates via a poorly understood mechanism. The GC-rich segments are often characteristic of ubiquitously expressed housekeeping genes, but they clearly drive the transcription of several cell-type-specific genes. The GC-rich group exhibits several common properties, such as a high GC content in the promoter region and clustered sites of transcriptional initiation. The 5′-flanking region of the α_{2C}-AR contains features that are reminiscent of the latter group of gene promoters: rich in GC and no TATAA box. As often occurs with such genes, several CpG islands are found in the GC-rich region and are potential sites for methylation/demethylation events. The concentration of CpG islands in the promoter region of the α_{2C}-AR gene suggests that gene expression may be regulated by altered methylation of this region influencing the chromatin-complexed gene structure. As occurs with the α_{2C}-AR gene, several members of the G protein-coupled receptor superfamily are also transcribed via GC-rich promoter segments lacking a TATAA box. Because several of these genes are also intronless, they present good models with which to investigate mechanisms of tissue-specific gene expression via such promoter elements.

Several factors are involved in the cell-type-specific expression of genes, including chromatin structure and the interaction of negative and positive *trans*-regulatory proteins. Among the group of genes that use GC-rich promoter elements, the *trans*-regulatory nuclear protein Sp1 often plays

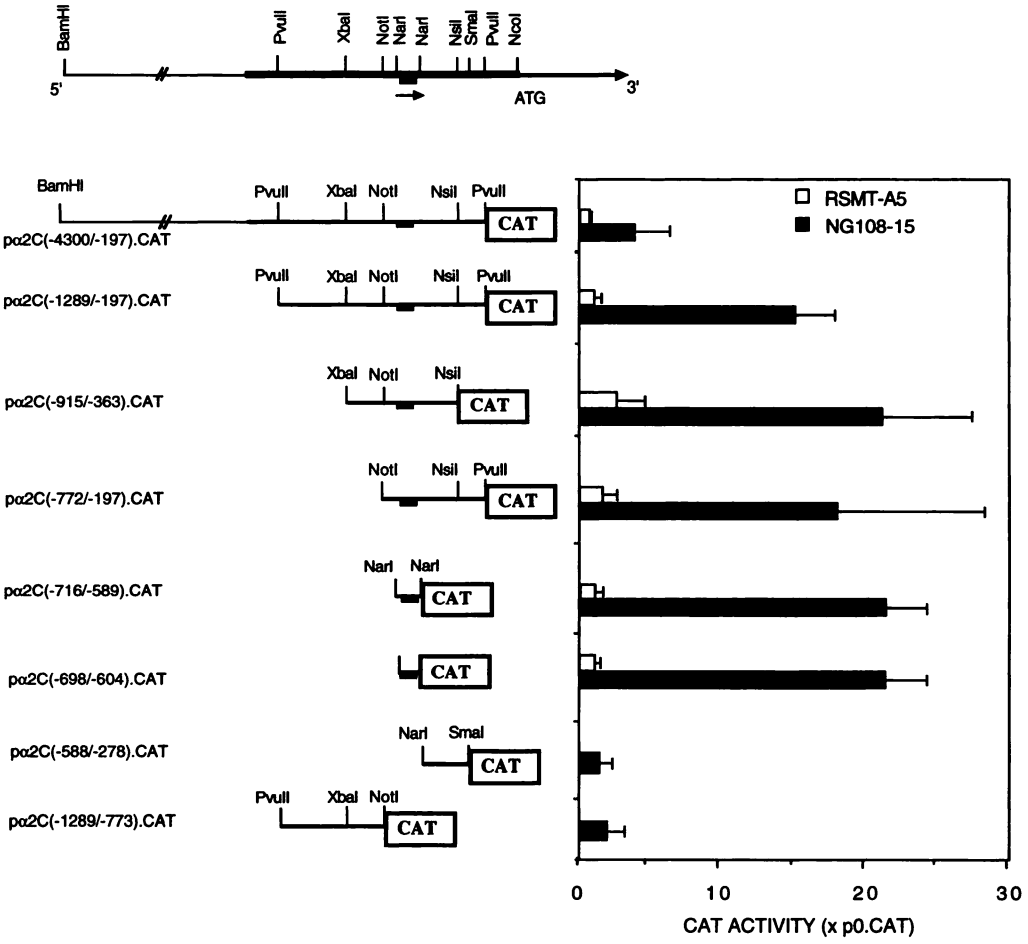

Fig. 7. Transcriptional activity of α_{2C}-AR gene fragments in the neuroblastoma X glioma hybrid cell line NG108-15 and the rat submandibular cell line RSMT. The 5′ segment of the receptor gene was progressively truncated and inserted into the reporter gene construct for determination of promoter activity. Data are corrected for transfection efficiency and expressed as the fold increase (mean ± standard error of three to eight transfection experiments) in CAT activity obtained in cells transfected with a promoterless CAT vector (pO.CAT). In each experiment, cells were transfected with both the pCAT construct and pSV.β-gal to control for transfection efficiency. In parallel experiments, cells were also transfected with the pCMV.CAT or pSV40.CAT, which increased CAT enzymatic activity by 50–80-fold above background levels observed with promoterless vector constructs in both cell types. *ATG*, start of the open reading frame encoding the α_{2C}-AR protein. *Top, thickened segment in diagram*, sequence indicated in Fig. 1. *Arrow*, area of transcriptional initiation identified in RNase protection assays.

an important role in transcription initiation and may also contribute to cell-type-specific transcription of certain genes (45–48). The putative Sp1 site at −654 to −660 nt 5′ to the translational start codon in the α_{2C}-AR gene may subserve such a function. Although both receptor-expressing and -nonexpressing cells possess nuclear proteins that are capable of recognizing oligonucleotides encompassing a consensus recognition site for Sp1, the oligonucleotides compete with the 126-nt receptor promoter fragment for gel-shifted species only when nuclear extracts from the receptor expressing cell line are used. Such an observation may reflect the expression of cell-type-specific DNA-binding proteins that recognize a core motif similar to that recognized by the transcription factor Sp1. If Sp1 is required for transcription of the receptor gene, expression of other nuclear proteins in receptor-nonexpressing cells that also recognize this site may compete for DNA/Sp1 binding, repressing gene transcription. Alternatively, within the context of the transcription complex assembled on the longer promoter fragment, the specific proteins that bind DNA may be influenced by interactions with other cell-specific accessory proteins.

Although the α_{2C}-AR is primarily expressed in discrete neurons in the central nervous system, its promoter segment does not share homology with the promoter of various neuron-specific genes, such as neuromodulin, SCG10, peripherin, aldolase c, synapsin, nerve cell adhesion molecule, nerve growth factor receptor, and neuronal sodium channels (47, 49–53). The 96-nt promoter fragment of the α_{2C}-AR gene may contain elements required for gene expression in the olfactory system or the outer granular layer of the dentate gyrus, areas in which the receptor mRNA and protein are enriched. Detailed analysis of such elements may allow the selective manipulation of neural gene expression in these brain regions. Although the genes encoding other members of the AR family contain *cis*-recognition elements for glucocorticoids and cAMP-response factors, such elements are not clearly identified in the upstream gene segment of the α_{2C}-AR. Activation of protein kinase C by phorbol esters increases α_{2C}-AR mRNA levels in NG108-15 cells (40). Although it is not known which gene segment is required for the effect of phorbol esters on the levels α_{2C}-AR mRNA, there is a consensus Ap1 recognition site in the gene 5′ to the area of transcription initiation. Analysis of the genes encoding other AR subtypes do not indicate any general features that are shared with the α_{2C}-AR. Transcription of the α_{1b}-AR gene is initiated from multiple start sites involving three putative promoters, one of which includes a TATAA box (6, 10). The upstream gene promoter segment of the α_{2A}-AR contains both a GC box and a TATAA box for transcriptional initiation (5, 7, 8). The upstream gene segment of the β_1-AR lacks a TATAA box and is GC rich, as is the case for two of the putative promoters of the α_{1b}-AR (3, 6, 10). TATAA boxes are also present in the β_2-AR and β_3-AR genes (4, 9, 13). The lack of shared features among the different groups of AR genes indicate that it would be possible to selectively modulate the

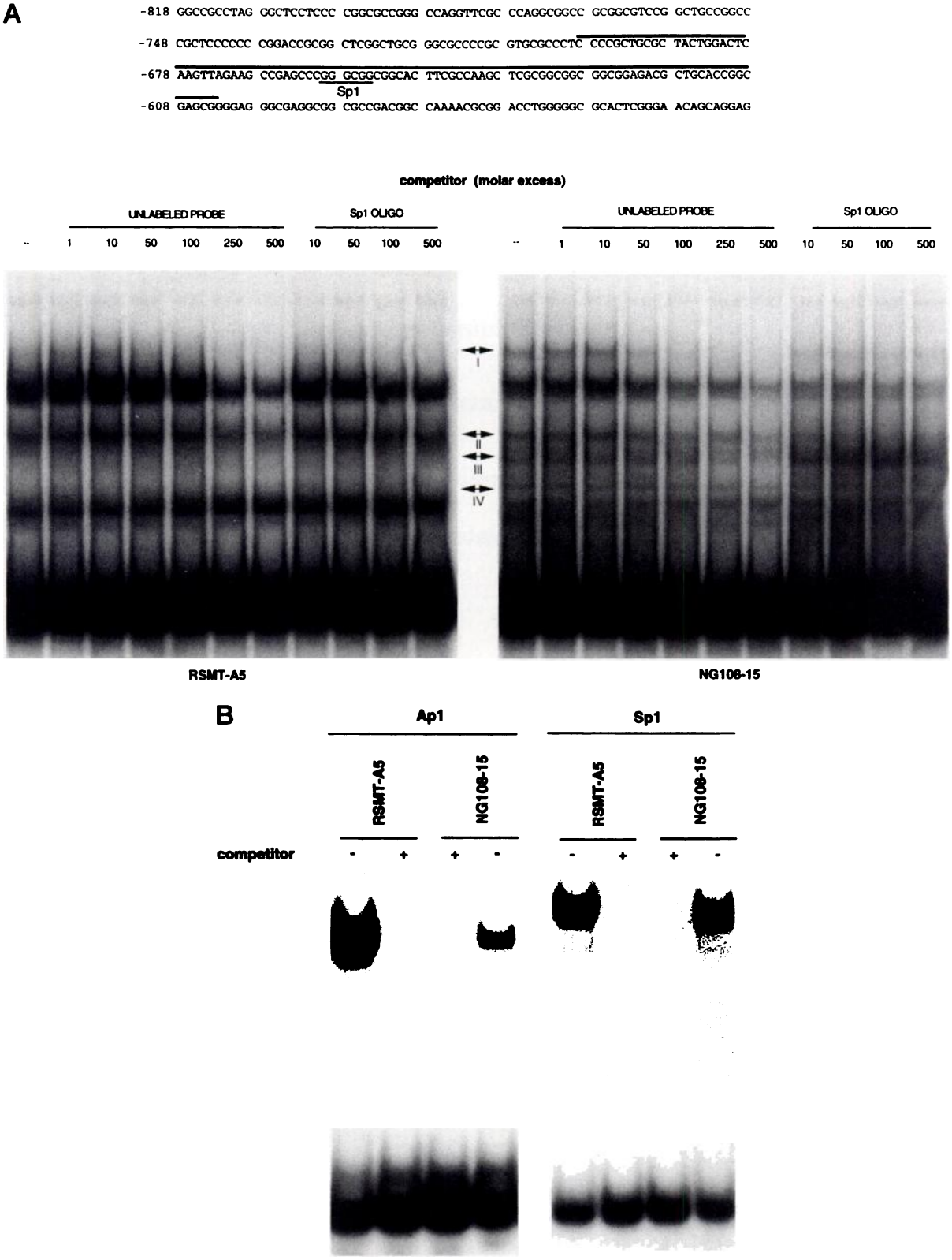

Fig. 8. Interaction of the α_{2C}-AR gene promoter with nuclear proteins in RSMT-A5 and NG108-15 cells. A, a 129-nt gene fragment (*NarI*/*NarI*, nt −587 to −716) encompassing the major sites of transcriptional initiation and cell-type-specific promoter elements (*underlined*) was end-labeled with ^{32}P and incubated with nuclear extracts (4–6 μg of protein) from NG108-15 (receptor-expressing) and RSMT-A5 (receptor-nonexpressing) cells as described in Experimental Procedures. Incubations were performed in the presence and absence of unlabeled probe or oligonucleotides encoding a recognition site for the nuclear protein Sp1. DNA/protein interactions were detected by gel shift analysis as described in Experimental Procedures. *Arrows*, gel-shifted species described in the text. B, analysis of nuclear extract using labeled oligonucleotides encoding Sp1 or Ap1 transcription factor recognition sequences. +, Samples in which the nuclear extract was preincubated with a 50-fold molar excess of unlabeled oligonucleotides.

expression of a particular subgroup to alter the final response of the cell to the adrenergic agonist.

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References

1. Hieble, J. P., W. E. Bondinell, and R. R. Ruffolo, Jr. α- And β-adrenoceptors: from the gene to the clinic. 1. Molecular biology and adrenoceptor subclassification. *J. Med. Chem.* **38:**3415–3444 (1995).
2. Ruffolo, R. R., Jr., W. Bondinell, and J. P. Hieble. α- And β-adrenoceptors: from the gene to the clinic. 2. Structure-activity relationships and therapeutic applications. *J. Med. Chem.* **3:**3681–3716 (1995).
3. Collins, S., J. Ostrowski, and R. J. Lefkowitz. Cloning and sequence analysis of the human β_1-adrenergic receptor 5′-flanking promoter region. *Biochim. Biophys. Acta* **1172:**171–174 (1993).
4. Emorine, L. J., S. Marullo, O. Delavier-Klutchko, S. V. Kaveri, O. Durieu-Trautmann, and A. D. Strosberg. Structure of the gene for human β_2-adrenergic receptor: expression and promoter characterization. *Proc. Natl. Acad. Sci. USA* **84:**6995–6999 (1987).
5. Frazer, C. M., S. Arakawa, W. R. McCombie, and J. C. Venter. Cloning, sequence analysis, and permanent expression of a human α_2-adrenergic receptor in Chinese hamster ovary cells: evidence for independent pathways of receptor coupling to adenylate cyclase attenuation and activation. *J. Biol. Chem.* **264:**11754–11761 (1989).
6. Gao, B., M. S. Spector, and G. Kunos. The rat α_{1B}-adrenergic receptor gene middle promoter contains multiple binding sites for sequence-specific proteins including a novel ubiquitous transcription factor. *J. Biol. Chem.* **270:**5614–5619 (1995).
7. Handy, D. E., and H. Gavras. Promoter region of the human α_{2A}-adrenergic receptor gene. *J. Biol. Chem.* **267:**24017–24022 (1992).
8. Handy, D. E., M. T. Zanella, A. Kanemaru, A. Tavares, C. Flordellis, and H. Gavras. A negative regulatory element in the promoter region of the rat α_{2A}-adrenergic receptor gene overlaps an SP1 consensus binding site. *Biochem. J.* **311:**541–547 (1995).
9. Kobilka, B. K., T. Frielle, H. G. Dohlman, M. A. Bolanowski, R. A. Dixon, P. Keller, M. G. Caron, and R. J. Lefkowitz. Delineation of the intronless nature of the genes for the human and hamster β_2-adrenergic receptor and their putative promoter regions. *J. Biol. Chem.* **262:**7321–7327 (1987).
10. Ramarao, C. S., J. M. Denker, D. M. Perez, R. J. Gaivin, R. P. Riek, and R. M. Graham. Genomic organization and expression of the human α_{1B}-adrenergic receptor. *J. Biol. Chem.* **267:**21936–21945 (1992).
11. Sakaue, M., and B. B. Hoffman. cAMP regulates transcription of the α_2-adrenergic receptor gene in HT-29 cells. *J. Biol. Chem.* **266:**5743–5749 (1991).
12. Shilo, L, M. Sakaue, J. M. Thomas, M. Philip, and B. B. Hoffman. Enhanced transcription of the human α_{2A}-adrenergic receptor gene by cAMP: evidence for multiple cAMP responsive sequences in the promoter region of this gene. *Cell Signal.* **6:**73–82 (1994).
13. van Spronsen, A., C. Nahmias, S. Krief, M. M. Briend-Sutren, A. D. Strosberg, and L. J. Emorine. The promoter and intron/exon structure of the human and mouse β_3-adrenergic-receptor genes. *Eur. J. Biochem.* **213:**1117–1124 (1993).
14. Lanier, S. M., S. Downing, E. Duzic, and C. J. Homcy. Isolation of rat genomic clones encoding subtypes of the α_2-adrenergic receptor. *J. Biol. Chem.* **266:**10470–10478 (1991).
15. Bylund, D. B., D. C. Eikenberg, J. P. Hieble, S. Z. Langer, R. J. Lefkowitz, K. P. Minneman, P. B. Molinoff, R. R. Ruffolo, Jr., and U. Trendelenburg. International Union of Pharmacology nomenclature of adrenoceptors. *Pharmacol. Rev.* **46:**121–136 (1994).
16. Duzic, E., and S. M. Lanier. Factors determining the specificity of signal transduction by guanine nucleotide-binding protein-coupled receptors. III. Coupling of α_2-adrenergic receptor subtypes in a cell type-specific manner. *J. Biol. Chem.* **267:**24045–24052 (1992).
17. Sato, M., R. Kataoka, J. Dingus, M. Wilcox, J. D. Hildebrandt, and S. M. Lanier. Factors determining the specificity of signal transduction by G-protein-coupled receptors. IV. Regulation of signal transfer from receptor to G-protein. *J. Biol. Chem.* **270:**15269–15276 (1995).
18. Berkowitz, D. E., D. T. Price, E. A. Bello, S. O. Page, and D. A. Schwinn. Localization of messenger RNA for three distinct α_2-adrenergic receptor subtypes in human tissues: evidence for species heterogeneity and implications for human pharmacology. *Anesthesiology* **81:**1235–1244 (1994).
19. Blaxall, H. S., N. A. Hass, and D. B. Bylund. Expression of α_2-adrenergic receptor genes in rat tissues. *Receptor* **4:**191–199 (1994).
20. Handy, D. E., C. S. Flordellis, N. N. Bogdanova, M. R. Bresnahan, and H. Gavras. Diverse tissue expression of rat α_2-adrenergic receptor genes. *Hypertension (Dallas)* **21:**861–865 (1993) .
21. Lorenz, W., J. W. Lomasney, S. Collins, J. W. Regan, M. G. Caron, and R. J. Lefkowitz. Expression of three α_2-adrenergic receptor subtypes in rat tissues: implications for α_2 receptor classification. *Mol. Pharmacol.* **38:** 599–603 (1990).
22. Nicholas, A. P., V. Pieribone, and T. Hökfelt. Distributions of mRNAs for α_2-adrenergic receptor subtypes in rat brain: an *in situ* hybridization study. *J. Comp. Neurol.* **328:**575–594 (1993).
23. Rosin, D. R., E. Talley, A. Lee, R. Stornetta, B. Gaylinn, P. G. Guyenet, and K. R. L. Lynch. Distribution of α_2-adrenergic receptor-like immunoreactivity in the rat central nervous system. *J. Comp. Neurol.* **372:**135–165 (1996).
24. Scheinin, M., J. W. Lomasney, D. M. Hayden-Hixson, U. B. Schambra, M. G. Caron, R. J. Lefkowitz, and R. T. Fremeau, Jr. Distribution of α_2-adrenergic receptor subtype gene expression in rat brain. *Mol. Brain Res.* **21:**133–149 (1994).
25. Voigt, M. M., S. K. McCune, R. Y. Kanterman, and C. C. Felder. The rat α_2-C4 adrenergic receptor gene encodes a novel pharmacological subtype. *FEBS Lett.* **278:**45–50 (1991).
26. Meister, B., A. Dagerlind, A. P. Nicholas, and T. Hokfelt. Patterns of messenger RNA expression for adrenergic receptor subtypes in the rat kidney. *J. Pharmacol. Exp. Ther.* **268:**1605–1611 (1994).
27. Eason, M. G., and S. B. Liggett. Human α_2-adrenergic receptor subtype distribution: widespread and subtype-selective expression of α_2-C10, α_2-C4, and α_2-C2 mRNA in multiple tissues. *Mol. Pharmacol.* **44:**70–75 (1993).
28. Hamprecht, B. Structural, electrophysiological, biochemical, and pharmacological properties of neuroblastoma-glioma cell hybrids in cell culture. *Int. Rev. Cytol.* **49:**99–170 (1977).
29. He, X., J. Ship, X. Wu, A. M. Brown, and R. B. Wellner. β-Adrenergic control of cell volume and chloride transport in an established rat submandibular cell line. *J. Cell. Physiol.* **138:**527–535 (1989).
30. Karlsen, A. E., W. Y. Fujimoto, P. Rabinovitch, S. Dube, and A. Lernmark. Effects of sodium butyrate on proliferation-dependent insulin gene expression and insulin release in glucose-sensitive RIN-5AH cells. *J. Biol. Chem.* **266:**7542–7548 (1991).
31. Prost, E., and D. D. Moore. CAT vectors for analysis of eukaryotic promoters and enhancers. *Gene* **45:**107–111 (1986).
32. Ghosh, D. A relational database of transcription factors. *Nucleic Acids Res.* **18:**1749–1756 (1990).
33. Kingston, R. E. Transfection of DNA into eukaryotic cells, in *Current Protocols in Molecular Biology* (Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl, eds.). John Wiley and Sons, New York, 9.1–9.14 (1987).
34. Sambrook, J., E. F. Fritsch, and T. Maniatis. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 16.66–16.67 (1989).
34a. Seed, B., and J.-Y. Sheen. A simple phase-extraction assay for chloramphenicol acyltransferase activity. Gene **67:** 271–277 (1988).
35. Andrews, N. C., and D. V. Faller. A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. *Nucleic Acids Res.* **19:**2499–2501 (1991).
36. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193:**265–275 (1951).
37. Bird, A. The essentials of DNA methylation. *Cell* **70:**5–8 (1992).
38. Javahery, R., A. Khachi, L. Kiersten, B. Zenzie-Gregory, and S. T. Smale. DNA sequence requirements for transcriptional initiator activity in mammalian cells. *Mol. Cell. Biol.* **14:**116–127 (1994).
39. Tijan, R., and T. Maniatis. Transcriptional activation: a complex puzzle with few easy pieces. *Cell* **77:**5–8 (1994).
40. Hu, G., L. A. Querimit, L. A. Downing, and M. E. Charness. Ethanol differentially increases α_2-adrenergic and muscarinic acetylcholine receptor gene expression in NG108–15 cells. *J. Biol. Chem.* **268:**23441–23447 (1993).
41. Blake, M. C., R. C. Jambou, A. G. Swick, J. W. Kahn, and J. C. Azizkhan. Transcriptional initiation is controlled by upstream GC-box interactions in a TATAA-less promoter. *Mol. Cell. Biol.* **10:**6632–6641 (1990).
42. Buermeyer, A. B., N. E. Thompson, L. A. Strasheim, R. R., Burgess, and P. J. Farnham. The H1P1 initiator element plays a role in determining the *in vitro* requirement of the dihydrofolate reductase gene promoter for the C-terminal domain of RNA polymerase II. *Mol. Cell. Biol.* **12:**2250–2259 (1992).
43. He, X., and M. G. Rosenfeld. Mechanisms of complex transcriptional regulation: implications for brain development. *Neuron* **7:**183–196 (1991).
44. Sadowski, C. L., R. W. Henry, S. M. Lobo, and N. Hernandez. Targeting TBP to a non-TATA box *cis*-regulatory element: a TBP-containing complex

activates transcription from snRNA promoters through the PSE. *Genes Dev.* **7**:1535–1548 (1993).
45. Li, R. S., M. S. Abrahamsen, R. R. Johnson, and D. R. Morris. Complex interactions at a GC-rich domain regulate cell type-dependent activity of the ornithine decarboxylase promoter. *J. Biol. Chem.* **269**:7941–7949 (1994).
46. Pecorino, L. T., A. L. Darrow, and S. Strickland. *In vitro* analysis of the tissue plasminogen activator promoter reveals a GC box-binding activity present in murine brain but undetectable in kidney and liver. *Mol. Cell. Biol.* **11**:3139–3147 (1991).
47. Thomas, M., I. Makeh, P. Briand, A. Kahn, and H. Skala. Determinants of the brain-specific expression of the rat aldolase C gene: *ex vivo* and *in vivo* analysis. *Eur. J. Biochem.* **218**:143–151 (1993).
48. Zhang, D. E., C. J. Hetherington, S. Tan, S. E. Dziennis, D. A. Gonzalez, H. M. Chen, and D. G. Tenen. Sp1 is a critical factor for the monocytic specific expression of human CD14. *J. Biol. Chem.* **269**:11425–11434 (1994).
49. Desmarais, D., M. Filion, L. Lapointe, and A. Royal. Cell-specific transcription of the peripherin gene in neuronal cell lines involves a *cis*-acting element surrounding the TATA box. *EMBO J.* **11**:2971–2980 (1992).
50. Li, L., T. Suzuki, N. Mori, and P. Greengard. Identification of a functional silencer element involved in neuron-specific expression of the synapsin I gene. *Proc. Natl. Acad. Sci. USA* **90**:1460–1464 (1993).
51. Mori, N., C. Schoenherr, D. J. Vandenbergh, and D. J. Anderson. A common silencer element in the SCG10 and type II Na^+ channel genes binds a factor present in nonneuronal cells but not in neuronal cells. *Neuron* **9**:45–54 (1992).
52. Nedivi, E., G. S. Basi, I. V. Akey, and J. H. Skene. A neural-specific GAP-43 core promoter located between unusual DNA elements that interact to regulate its activity. *J. Neurosci.* **12**:691–704 (1992).
53. Sauerwald, A., C. Hoesche, R. Oschwald, and M. W. Kilimann. The 5′-flanking region of the synapsin I gene: a G+C-rich, TATA- and CAAT-less, phylogenetically conserved sequence with cell type-specific promoter function. *J. Biol. Chem.* **265**:14932–14937 (1990).
54. Wilson, A. L., K. Seibert, S. Brandon, E. J. Cragoe, Jr., and L. E. Limbird. Monovalent cation and amiloride analog modulation of adrenergic ligand binding to the unglycosylated α_{2B}-adrenergic receptor subtype. *Mol. Pharmacol.* **39**:481–486 (1991).
55. Hamamdzic, D., E. Duzic, J. D. Sherlock, and S. M. Lanier. Regulation of α_2-adrenergic receptor expression and signaling in pancreatic *beta*-cells. *Am. J. Physiol.* **269**:E162-E171 (1995).

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