A negative regulatory element in the promoter region of the rat α_2 A-adrenergic receptor gene overlaps an SP1 consensus binding site

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Three subtypes of α_2 -adrenergic receptors (α_2 A, α_2 B and α_2 C) have been described that differ in their primary sequence and tissue-specific expression and are encoded by three distinct genes. Previous work has shown that the human α_2 A-adrenergic receptor gene promoter consists of a TATA-box (TATAAA), palindromic sequence (CCCACGTGGG) and GC-box (GGGGCGG) motif. Sequence analysis of the putative promoter region of the rat α_2 A-adrenergic receptor gene showed that these promoter regions are conserved in their sequence and relative location. We analysed the transcriptional activity of these regions using RINm5F, a rat insulinoma cell line that expresses the endogenous α_2 A-adrenergic receptor gene. These results showed

that the region from –484 to –92 has a negative effect on transcription, as deletion of this region in α_2 A-adrenergic receptor gene–chloramphenicol acetyltransferase reporter constructs increased reporter gene activity. This region included the GC-box sequence which is a consensus binding site for the nuclear factor SP1, which is a positive activator of transcription. Gel-mobility-shift assays and supershift assays with an antibody that recognizes SP1 showed binding of the SP1 nuclear factor as well as other nuclear factors to this GC-box region. Additional nuclear factors bind to the downstream palindromic region. We suggest that positive- and negative-acting nuclear factors contribute to the activity of the α_2 -adrenergic receptor promoter.

INTRODUCTION

The α_2 -adrenergic receptors (α_2 -ARs) are a heterogeneous group of receptors that bind to the naturally occurring ligands, adrenaline (epinephrine) and noradrenaline (norepinephrine), and mediate their effects through the G_i class of G-proteins [1–3]. α_2 -ARs have been shown to play a role in such diverse physiological events as peripheral vasoconstriction, platelet aggregation and dense granule release, insulin secretion, Na^+ reabsorption in the kidney and the control of the central sympathetic outflow. Recent molecular analysis of α_2 -AR genes and transcripts have shown that in both human and rat there are three distinct α_2 -AR subtypes that are unique in sequence and ligand-binding properties (reviewed in [4]). Previous work has shown that these receptor subtypes have distinct but overlapping patterns of tissue-specific expression. The α_2 A-AR and α_2 C-AR are the major subtypes found in brain, whereas the α_2 A-AR and α_2 B-AR are the major subtypes in the periphery [5,6]. The α_2 A-AR is the subtype expressed on platelets and in the pancreas.

We have previously defined the promoter region of the human α_2 A-AR gene [7]. The promoter consists of a TATA-box motif, lacks a CCAAT-box but includes two additional sites of nuclear factor binding. One of these sites includes a GC-box sequence, GGGGCGG. The GC-box is a consensus binding site for the nuclear factor SP1 [8]. The other nuclear factor binding site contains a 10 bp palindrome, CCCACGTGGG, which is a unique site for nuclear factor binding.

In this report we analyse the promoter region of the rat α_2 A-AR gene and show that the location of the transcription initiation site as well as the location and sequence of the promoter sequences are highly conserved between the rat and human α_2 A-AR genes. We also show that in RINm5F cells, a rat insulinoma cell line

that expresses the rat α_2 A-AR gene, the GC-box region has a negative effect on transcription.

MATERIALS AND METHODS

Cell culture conditions

RINm5F cells, a rat insulin-producing cell line, were maintained in RPMI 1640 medium supplemented with 10 % fetal calf serum (INTERGEN Company, Purchase, NY, U.S.A.) as described in [9]. HT29 cells, a human colon carcinoma cell line that expresses the human α_2 A-AR [10], were maintained in McCoy's 5A medium with 10 % (v/v) fetal calf serum.

RNAse protection assay

RNA was isolated from rat spleen or RINm5F cells by a modification [11] of the $CsCl_2$ gradient method [12]. RNase protection was performed as previously described [13]. Antisense probes were constructed in psp65 or psp64 vectors (Promega, Madison, WI, U.S.A.) and linearized before RNA synthesis. Antisense RNA was synthesized from the SP6 promoter in the presence of SP6/T7 quality [α - ^{32}P]cytidine triphosphate (Amersham, Arlington Heights, IL, U.S.A.). RNA duplexes were formed between the radiolabelled probe and RNA samples by overnight hybridization at 45 °C in 80 % formamide, 0.4 M NaCl, 1 mM EDTA, 40 mM Pipes, pH 6.7. Single-stranded RNA was digested by a mixture of RNase A and T1 (Boehringer Mannheim, Indianapolis, IN, U.S.A.) at 37 °C for 1 h. Protected fragments were visualized on a denaturing 6 M urea/6 % poly-

acrylamide gel. End-labelled pBR322 *Hinf*I fragments were used as size markers.

Genomic clones and sequencing

Genomic clones were obtained from a partial *Sau*3A-digested Wistar rat genomic library in λ Dash (Stratagene, La Jolla, CA, U.S.A.) as previously described [14]. Three overlapping genomic clones that contained regions of the α_2 A-AR gene were isolated. A 400 bp *Eco*RI/*Pst*I fragment that contained 380 bp of the 5' untranslated region of the rat α_2 A-AR cDNA was used to probe Southern blots of the genomic clones. A 4 kb *Eco*RI/*Kpn*I fragment that contains 3.4 kb of the region upstream of the protein coding region was cloned into puc18. Fragments were sequenced in double-stranded vectors or in single-stranded M13 vectors following exonuclease III deletion. Sequenase (United States Biochemical, Cleveland, OH, U.S.A.) was used for all sequencing reactions. Sequence was determined from both strands. Analyses of nucleotide sequences were performed with the Mac-Vector/AssemblyLIGN software package (International Biotechnologies, New Haven, CT, U.S.A.).

α_2 A-AR gene constructs

To compare the transcriptional efficiency of potential regulatory regions, gene constructs were made that placed regions of the rat α_2 A-AR gene upstream of the bacterial chloramphenicol acetyltransferase (CAT) gene in the vector pCAT enhancer (Promega, Madison, WI, U.S.A.). These resulting constructs are named according to the positions of their insert fragments relative to the CAP site, i.e. pCATE -824/+477 contains the fragment from -824 bp upstream (-) of the CAP site to 477 bp downstream of the CAP site, where the CAP site is +1 and translation starts at +989.

Restriction endonuclease sites were used to clone the rat α_2 A-AR fragments into the pCATE vector as follows: the *Sst*I/*Sst*I (-824/+477) and *Sal*I/*Sst*I (-92/+477) fragments of the rat α_2 A-AR gene were ligated to the pCATE vector at the *Sal*I site after insert and vector ends were made blunt by Klenow enzyme. Utilizing restriction endonuclease sites in the polycloning region of the vector (*Hind*III-*Pst*I-*Sal*I-*Acc*I-*Xba*I) and the *Hind*III site at -480 in the insert, a *Hind*III digestion of the -824/+477 construct removed the -824/-481 fragment producing the -480/+477 construct. Similarly, using the downstream *Xba*I site within the polylinker, an *Xba*I digestion of the -824/+477 construct removed the -131/+477 fragment which was subcloned at the *Xba*I site of the pCATE vector. The -39/+477 fragment was subcloned in two steps. First, a *Bst*NI/*Bst*NI fragment (-39/+409) was cloned into the pCATE vector at the *Sal*I site after the insert and fragment ends were filled in with Klenow enzyme. Then, the *Hind*III/*Sst*II fragment from the -39/+409 construct was substituted for the *Hind*III/*Sst*II fragment of the -480/+477 construct, allowing for the replacement of the -480/+179 insert fragment by a -39/+179 fragment, resulting in the final -39/+477 construct. The orientation of all constructs was confirmed by restriction enzyme analysis and sequence analysis using primers located upstream (5'-GAATTGTGAGCGGATAAC) or downstream (5'-CCATTTAGCTTCCTTAG) of the polylinker region of the pCATE vector.

CAT assays

The calcium phosphate method of transfection was used to co-transfect 20 μ g of each CAT construct and 5 μ g of a β -

galactosidase-containing plasmid into RINm5F cells. To reduce phosphate in the cell culture medium prior to transfection, the RPMI medium was replaced by a 9:1 (v/v) mixture of RPMI 1640 with no phosphate/RPMI 1640. Fetal calf serum was maintained at 10% (v/v). Cells were harvested 48 h after transfection, lysed by freeze-thawing, and assayed for β -galactosidase and CAT enzyme activity [15,16]. In order to correct for transfection efficiencies, CAT assays were performed on cell extracts that contained equivalent amounts of β -galactosidase activity [16]. CAT activity was measured as a percentage of acetylated chloramphenicol after a 4 h incubation at 37 °C and standardized to the activity of pCATE -824/+477. Each construct was tested in a minimum of three transfections. Values were compared by ANOVA followed by Student-Neuman-Keuls pairwise comparisons.

Gel-mobility-shift assay

Nuclear extracts were prepared according to published methods [17]. The gel-mobility-shift assay was performed essentially as described elsewhere [18]. Briefly, 1-5 μ l of nuclear extract was mixed with 0.5 ng of radiolabelled probe (10000-20000 c.p.m.) in a final reaction volume of 15 μ l that contained 20 mM Tris, pH 7.6, 50 mM KCl, 1 mM MgCl₂, 0.2 mM EDTA, 0.01% (v/v) Triton X-100, 5% (v/v) glycerol and 0.2 μ g/ μ l poly(dI-dC), plus or minus specific competitor DNA. Mixtures were incubated on ice for 20 min. Supershift was done by adding 1 μ l of an SP1 antibody or egr₁ antibody (1 μ g/ μ l) to this mixture and incubating for an additional 1 h as recommended by the manufacturer (Santa Cruz Biotechnologies, Santa Cruz, CA, U.S.A.). DNA-protein complexes were analysed on a 3.5% non-denaturing polyacrylamide gel in 0.4 $\times$ TBE, where 1 $\times$ TBE is 0.09 M Tris-borate/0.002 M EDTA, pH 8.3. The probes used were from -131 to -92, which overlapped the GC-box, and from -92 to -41, to overlap the palindrome. Double-stranded competitors include the SP1 competitor (GATCGATCGGGCGGGGCG-ATC) from Stratagene, a double-stranded oligonucleotide competitor that contains the human palindromic region (TCGGCTTCCACGTGGGGCTGCAGC) and the egr family consensus competitor (GGATCCAGCGGGGAGCGGG-GGCGA) from Santa Cruz Biotechnology.

RESULTS

The promoter regions of the rat and human α_2 A-AR genes are highly conserved

Three overlapping genomic clones were isolated from the rat Wistar genomic library in λ DASH. These clones were shown to be overlapping by restriction endonuclease digestions and Southern blot hybridizations with a probe derived from the rat α_2 A-AR cDNA that was cloned from a rat brain cDNA library [5]. A 4 kb *Eco*RI/*Kpn*I fragment that contained about 3.1 kb of sequences upstream of the protein coding region was subcloned for further analysis.

Since the protein coding regions of the human and rat α_2 A-AR genes are highly conserved, it seemed likely that the promoter region and the transcription initiation site (TI) were likely to be conserved between the rat and human α_2 A-AR genes. In the human α_2 A-AR gene the transcription initiation site is located about 1 kb upstream from the translation initiation codon ATG [7]. Therefore, to locate possible promoter regions, a 2 kb fragment upstream of the ATG in the rat α_2 A-AR gene was sequenced. Figure 1 shows this sequence.

A comparison with the human α_2 A-AR sequences showed high sequence similarity especially in the region about 1 kb

-1035 CAGCTCTTCATAAAATATCTGTGCCACAACAAATGCTCAACGGTTCCTCAGTAGTTGAAAGCTGTGCAAGCAAAGAGGAAGGTCCGGGACG
 -945 CTAACATCTGCAGAAGGTTCCGAGCCTCACTCATCGGAGGTAGATATTTCCAACCTGAAGATTCTGCATCATCGCAGTGCCTGTTGA
 -855 AATCTGTTCCTCGCTTACCCACCCCGAGCTCAGTCCAAAAGTCCCAAGACAGGAAAAAAGGGGAGGAGGGGTTCAGAA
 -765 GGCACCTGGAGCCCTTAAGTCGCTTTCCAGCCGTGACGACCAGCGGCCAGCAGGTGTCACTGCGCTCTCGTTCTGTCAAGCTCAGGGCC
 -675 TGCCCTCCAGGACCCCTTCCCAACGCGGTTCAAGGGGGCGCAGGGCAGACTCCAGGCCCCGAGAGTCCCGCAGACAGCTCGTGGCTGC
 -585 AAGGATACGTGGCTTCTCTTCCCTAAGTATGGATAACTCCGGCTGGTCTTCTCGCTGGGTGCTGGTCCATTCCGCTCCAGCGGTTC
 -495 TTAGGAACCTGCACAAGCTTACCTCTTCAGGATCCCTTTTCATCCAGCCACCAGAGCAACTGCGCTGCGCTGGGGCGGGGTCTTCGC
 -405 CAGCGACTCTTATGCCACCTGCATCTCTAATTTCGGGTCCCAACACCTTGGCACCCTCTCTGCTAGAACCCAGCTAGATGGGTGTA
 -315 GCTGGGTGCCCGCTACTGTCTCTGGGCCAGCTAGTAGTGTCCAGCAGGCCAATCCAGCAGGGGCGCTCCCACTCCCTAGGCTCT
 -225 CTCTTAAAGCCCTGCTCCCGTACCCGCTTTCCAAAAGGCGGTCCAGCTTGCAGGCGAGCGCTCTCTGGGGTCCGACGCGGTGCG
 -135 GGCTCTAGACCTAAGAGGGGAGGCGCGAAGGGGAGGAGGGTTCGACTTCCACGTTGGGCTGACGCTAGCTGCTCAGCAACGCGGCG
 -45 TTCTTCTCGGGGTATATAAATCCAGTCCAGCGTTGAGAGGCCGAGCAGCAGCGGCTCCGCTTCCGTGCAGACGCCAGCAGCGCGGCAC
 +46 ACCACCCAGCCACCCAGCGCCCCAGACCAAGTGTCTTCGGTCCAGCTAAACTCAGTGGTCTCCGACCCCGCGGGTCTGCGCTC
 +136 AGCGCCGCCAGCTCGCGGTAGCGGCCACCTTGGCTCCGCGCGGCTCTGAGAGCCGATCGCTCACCTAGCTTGGCTCTGAGGACC
 +226 GAGGTGGGGGGCGGCTTCATCGCGCCCCACACCTTTACCCACCGCGCGCGCGCCCGAAGCTCTGTGCGCAGCGTCCAGCCCCA
 +316 GCAGGCGCACAACCTTGAAGTCCCTTGGGGCTTCTGAGGAGGCGAGTCCGCGCTTCCGCGGAGGTCCGCGCCAGCTCGCGCACCG
 +406 CGCTTGGAGGTAGCAACGAGTCCGAAATCTCTTCGGGGTATAAGAAGACAGACCTAGGACCCCGAGCTCGCAACGACACCCCTCGGC
 +496 TGCTCTTGGGGTAAAGACTTCTGCTTTCTCTCAGCTTAAGAGTCAAGGGATAGATGGCTAGCTAGATACATTTGTTTAAATTTCTGACA
 +586 TCTTTCCAAATCATAGGCCACCGATAATTTCTTTCTTTCTTTGAGAATAAATCTCCAGCCACAGCTGGCCCCACTCGATCTGTC
 +676 CGCTTAGAATAAAACTTGACTGTGTTTGTGGAGCTGGGAGAGAGAAAGCACCACCGAGCATCGAAAGACGAACCGGTGCCAGTTC
 +766 GCGGGCTCCAGTCCATGGACCTAGCGGTCCCGAGCTTGGGCCGCTCCCTGCGGCCCTCTTCCCTATGTGAGGCGCGCGGGACCA
 +856 GCCGCGCACCGGAGGAAGAGGAAGGACCCAGGACGCGGGGCGGAAAGCAGCTGGCAGCAGGCCAGGAGAACCAGCACCGCGTTCAT
 +946 GTTCCGCCAGGAGCAGCGCTGGCGAGGGCAGCTTTGCGCCC ATG GGC TCC CTG CAG

Figure 1 Sequence of the rat α_2 A-AR gene 5' flanking regions

Reported are the 2038 nucleotides of sequence. Numbers on the left-hand margin refer to the first nucleotide on that line. The proposed transcription initiation site (TI) is indicated by the arrow. Numbers upstream of the TI are negative, numbers downstream are positive. Boxed are the promoter sequences referred to in the text. The series of GGAGG repeats are indicated by triangles. The protein coding region is italicized.

Figure 2 Comparison of the 5' flanking regions of the rat and human α_2 A-AR genes

Shown is a partial alignment of 5' flanking regions. Mismatches are indicated by lower-case letters. Dashes have been inserted to maximize alignment.

upstream of the ATG (Figure 2) that contains the putative promoter region. The TATA-box, GC-box and palindrome are conserved in sequence and location.

To determine whether the TI of the rat α_2 A-AR gene was located downstream of the putative promoter regions, an *Xba*I/*Sst*I fragment (−131 to +477 in Figure 1) was used in an RNase protection assay using rat spleen RNA, which has an abundance

of α_2 A-AR gene transcripts [5]. If the rat α_2 A-AR gene was like the human α_2 A-AR gene, then the 600 nucleotide antisense probe would protect a fragment of about 480 nucleotides (Figure 3a). The full-length probe appears as a faint band in the RNase protection assays using rat spleen RNA (Figure 3b, lanes 1 and 2) or using tRNA as a control (lane 3). Only in lanes 1 and 2 can the specific band of 475 nucleotides be detected. These results are consistent with the proposal that the TI is located downstream of the TATA-box, and maps to within 989 bp upstream of the ATG, in nearly the same location as the TI of the human α_2 A-AR gene.

The GC-box region has a negative effect on transcription in RINm5F cells

In order to study the transcriptional activity of these potential promoter regions, RINm5F cells were used for transient transfections of reporter gene constructs. This rat insulin-secreting cell line has been shown to express an α_2 -AR subtype that has low affinity for prazosin, suggesting the α_2 A-AR subtype is expressed in this cell line [19]. To test this, we used RNase protection analysis and the α_2 A-AR-specific probe. Figure 4 shows that the RINm5F RNA has the same specific protected fragment as the control cell line (CHOA) that expresses the α_2 A-AR cDNA.

A variety of CAT constructs were co-transfected along with the β -galactosidase gene into RINm5F cells. In order to control for transfection efficiencies, CAT activity was calculated on extracts that contained equivalent amounts of β -galactosidase

Figure 3 Mapping of α_2A -AR transcripts

(a) Map of the probe used for RNase protection. The line represents the *Pst*I fragment (-933/+1003). The X and S represent the *Xba*I and *Sst*I sites, respectively, (-131/+477) that were used to subclone a fragment for the RNase protection assay. If the TI is located near the arrowhead then the mRNA would protect a band of about 480 nucleotides (broken line) from the approximately 600 nucleotide antisense RNA probe. (b) RNase protection assay. Lanes 1 and 2, 20 μ g of rat spleen RNA; lane 3, 20 μ g of tRNA; lane 4, pBR322 *Hinf*I markers (sizes, in nucleotides, are shown). Full-length probe and specific protected band are indicated.

Figure 4 RNase protection assay of RINm5F cells with the rat α_2A -AR-specific probe

A 333 bp fragment (*Kpn*I/*Xmn*I) of the rat α_2A -AR cDNA [5] was used to synthesize an antisense RNA probe that is specific for the rat α_2A -AR mRNA. Lane 1, pBR322 *Hinf*I fragments (sizes in nucleotides are shown); lane 2, RNase protection with 20 μ g of tRNA; lane 3, RNase protection with 1 μ g of RNA from transfected CHO cells that are expressing the rat α_2A -AR; lanes 4-6, RNase protection with 20 μ g of RNA from RINm5F cells. Full-length probe and specific protected bands are labelled.

activity. All CAT values are calculated relative to the activity of the pCATE -824/+477 construct which was averaged and set to 100% for each assay. These values are reported in Table 1. Figure 5 shows a representative CAT assay. The pCATE -824/+477 is weakly expressed, only 2-fold over the negative control constructs such as the pCATE vector with no insert. However, the activity of pCATE -92/+477 is about the same as

Table 1 Activity of CAT constructs in RINm5F

Relative activity $\pm$ S.E.M. are calculated relative to the pCATE -824/+477 construct. Pairwise comparisons show that the means of pCATE -824/+477, pCATE -480/+477, pCATE -39/+409 and pCATE -39/+477 are not significantly different and the means of the control and pCATE -92/+477 are not significantly different. All other pairwise comparisons are significantly different at $P < 0.05$ as determined by the Student-Neuman-Keuls test. Control refers to the pCAT control vector which contains SV40 promoter and enhancer.

CAT construct	Relative activity
-824/+477	100.1 $\pm$ 5.80
-480/+477	103.5 $\pm$ 9.31
-131/+477	161.7 $\pm$ 8.75
-92/+477	245.5 $\pm$ 16.05
-39/+409	105.8 $\pm$ 6.49
-39/+477	103.3 $\pm$ 11.56
No insert	48.9 $\pm$ 6.08
Control	251.3 $\pm$ 33.95

Figure 5 CAT assay in RINm5F cells

The Figure shows a representative CAT assay after transfection of rat gene α_2A -AR/CAT constructs into RINm5F cells. CAT activity was determined on extracts containing equivalent amounts of β -galactosidase activity. The CAT reaction mixture was extracted with ethyl acetate and chromatographed on silica-gel thin layers in 5% methanol/95% chloroform as described. No insert indicates pCATE vector with no insert, control indicates pCAT control vector which has an SV40 promoter and enhancer, numbers refer to α_2A -AR gene fragments. Note that the -39/+409 construct, which lacks the GC-box and palindrome, has the same activity as the -39/+477 construct (Table 1), which has an additional 68 base pairs of 3' sequence.

a positive control CAT construct which contains strong promoter and enhancer sequences. Deletion of the regions from -824 to -480 has no significant effect on CAT activity whereas deletion of the region from -480 to -131 causes a significant increase ($P < 0.05$) in CAT activity. Deletion of the region from -131 to -92, which overlaps the GC-box, causes a significant increase in CAT activity to nearly 2.5 times the level of the pCATE -824/+477. On the other hand, deletion of the region from -92 to -39, which overlaps the palindromic region, causes a decrease in CAT activity to the level of the pCATE -824/+477.

Nuclear factor binding to the GC-box and palindromic regions

To show that nuclear factors from RINm5F cells could bind to both the GC-box and the palindrome, probes that overlapped

Figure 6 Gel-mobility-shift assay of the $-92/-49$ probe with RINm5F and HT29 nuclear extracts

Lane 1, probe alone; lanes 2–6, probe plus HT29 nuclear extract and the following: lane 3, unlabelled $-92/-49$, lane 4, unlabelled $-92/-67$; lanes 5 and 6, SP1 competitor. Lanes 7–11, probe plus RINm5F extract and the following: lane 8, unlabelled $-92/-49$; lane 9, unlabelled $-92/-67$; lanes 10 and 11, SP1 competitor. Dashes indicate bands that compete only with the $-92/-49$ piece; arrowheads indicate bands that compete with the $-92/-67$ piece.

these regions were used in gel-mobility-shift assays with nuclear factors from HT29 and RINm5F cells. Figure 6 shows a gel-mobility-shift assay with the $-92/-49$ probe that overlaps the palindromic region. Nuclear extracts from either HT29 (lanes 2–6) or RINm5F cells (lanes 7–11) shift the mobility of the radiolabelled probe, indicating the formation of nuclear factor–DNA complexes. The pattern of nuclear factor binding is similar but not identical with extracts from HT29 and RINm5F cells. Addition of unlabelled fragments from $-92/-49$ eliminates all the nuclear factor binding (Figure 6, lanes 3 and 8), whereas addition of a double-stranded oligonucleotide that only overlaps a portion of the probe from -92 to -67 (including the palindromic region) competes only for the lower bands (Figure 6, lanes 4 and 9; bands are labelled P in the Figure). On the other hand, addition of the SP1 consensus site does not compete for any nuclear factor binding with the palindromic probe (lanes 5, 6, 10 and 11). This suggests that these band shifts represent specific nuclear factor–DNA interactions that include the palindromic region and other regions downstream from -68 to -49 .

The GC-region probe ($-131/-92$) also forms nuclear factor complexes with both HT29 and RINm5F nuclear extracts (Figures 7a and 7b). Addition of a competitor specific for the SP1 nuclear factor consensus binding site eliminates all but one band when either nuclear extract is used (Figures 7a and 7b, lanes 3 and 4). To show that these bands relate to the SP1 nuclear factor and are not just due to other nuclear factors that can bind to the GC-box sequence, an antibody that recognizes SP1 from human, rat or mouse was added to the DNA/nuclear factor mix. The SP1 antibody causes a supershift of one of the middle bands (Figure 7, lanes 5 and 6), indicating that this nuclear factor–DNA complex contains SP1. However, several of the bands eliminated by the SP1 competitor do not shift with the SP1 antibody. These bands may represent additional complexes that form between the GC-box and other nuclear factors.

It has been suggested that nuclear factors of the *egr* family of zinc finger proteins may function by binding to a GC-rich region

Figure 7 Gel-mobility-shift assays of the $-131/-92$ probe

(a) With RINm5F extracts, (b) with HT29 extracts. Lane 1, probe alone; lanes 2–13, probe plus extract and the following: lanes 4 and 5, SP1 competitor; lanes 6 and 7, SP1 antibody; lanes 10 and 11, *egr* consensus competitor; lanes 12 and 13, *egr*₁ antibody. Arrowheads indicate bands that are eliminated by the SP1 competitor, large arrowhead specifies the band that is also supershifted by the SP1 antibody, open arrows indicate the supershifted bands.

overlapping the GC-box and interfering with the function of SP1 [20]. In addition NGF1A, which has also been called *egr*₁, has been reported to be expressed in RINm5F cells grown in fetal calf serum [21]. To test whether *egr*₁ could bind to the GC-box of the α_2 A-AR gene, the *egr* consensus competitor (Figure 7, lanes 10 and 11) and an antibody specific for *egr*₁ (Figure 7, lanes 12 and 13) were used in the gel-shift assay. No bands are eliminated by the *egr*₁ competitor and no bands are shifted by the specific antibody. Thus, in these cells there is no detectable binding of *egr*₁ nuclear factors to the GC-box region of the α_2 A-AR.

DISCUSSION

The α_2 -AR are members of a family of G-protein-linked receptors that mediate the cellular response to catecholamines. Both the human and rat have three α_2 -AR genes. The human genes have been referred to as α_2 -C10, α_2 -C4 and α_2 -C2, on the basis of their location on human chromosomes 10, 4 and 2, respectively. It is generally agreed that these genes encode for the human α_2 A-AR (α_2 -C10), α_2 B-AR (α_2 -C2) and α_2 C-AR (α_2 -C4). Some differences in nomenclature have been used to describe the rat genes, with some groups referring to the rat homologue of α_2 -C10 gene as an

α_2 D-subtype receptor due to differences in antagonist binding affinities of the expressed receptors [22]. However, as shown in the study of the mouse α_2 -AR, key amino acid differences can alter the affinities for antagonists [23]. Sequence analysis shows that the rat α_2 A-AR is 89–90% homologous with the human α_2 A-AR at the amino acid level; therefore any difference in ligand-binding properties would be due to few amino acid changes.

The analysis of promoter sequences in this report provides further evidence for the similar origins of these genes. The rat α_2 A-AR transcript, like the human α_2 A-AR transcript, is 3.8 kb in size [11]. The work presented here shows that the apparent TI is similarly located about 1 kb upstream of the ATG in both the human and rat α_2 A-AR genes. In addition, the promoter region of these two genes is highly conserved; the TATA-box, GC-box and palindromic region are nearly identical. Both genes have an alternative promoter structure that includes a TATA-box but contains other nuclear factor-binding regions in place of a CCAAT-box.

The analysis of promoter function in this report has further localized the necessary transcriptional sequences to a region that includes the palindrome and the TATA-box. These data also suggest that a region that overlaps the GC-box has a negative effect on transcription. This is surprising since the SP1 nuclear factor, which usually has a positive effect on transcription, can be shown to bind to this area in supershift assays. The analysis of nuclear factor binding did suggest that additional nuclear factors may also bind to the GC-box. It has been reported that members of the *egr* transcription factors may function, in part, by blocking SP1 binding, and that NGF1A (*egr*₁) is found in the RINm5F cell line [21]. However, a competitor for the *egr* consensus binding site did not compete for nuclear factor binding to the –131/–92 probe, despite the similarity of this sequence to the GC-box region. As might be expected given the lack of competition with the *egr* sequence, the anti-*egr*₁ antibody did not cause a supershift of this probe. Thus, other nuclear factors may be responsible for the negative effect of the region from –131/–92.

There have been several reports of negative-acting transcriptional elements that overlap the GC-box. In the muscle-specific enhancer of the acetylcholine receptor α -subunit gene, the binding of a nuclear factor to an overlapping GC-homopolymer has been proposed to contribute to the muscle-specific expression of this gene by interfering with SP1 binding in non-myogenic cell lines [24]. In the D₂-dopamine receptor gene, a GC-box region that is surrounded by simple sequence repeats has an overall negative effect on transcription due to nuclear factors that interact with a GTGG motif [25]. In the rat α_2 A-AR gene, the GC-box is surrounded by overlapping GGAGG (four identical GGAGG segments and one CGAGG repeat within 28 nucleotides). The GGA sequence is similar to the core GGA(A/T) sequences of the *ets* gene family, which includes *ets*₁, *ets*₂ and *Net*, nuclear factors that can be repressors of gene expression [26,27]. The importance of these repeat sequences remains to be tested.

The interaction of both positive-acting (SP1) and potential negative-acting nuclear factors with the GC-box and overlapping sequences suggests a complex mechanism of transcriptional regulation that may depend on the activities of competing suppressor or activator elements. Recent work indicates that SP1 is a member of a group of zinc finger proteins that bind to GC-box sequences [28,29] and at least one of these proteins (SP3) can function as an inhibitor of gene transcription [30,31].

In contrast to the GC-box region, the palindromic region appears to have a positive effect on expression, as deletion of this region reduces CAT activity. In our previous work with the

human gene, deletion of both the GC-box and palindromic region reduced CAT activity to background levels [7]. In the rat gene, deletion of both of these regions does not completely abrogate transcriptional activity. However, the human gene (–61/–17) construct previously used [7] and the rat gene construct used in this report (pCATE –39/+477) differ in several regards. Most significant is the lack of sequences downstream of the TATA-box in the construct lacking the GC-box and palindrome from the human gene [7]. As previously shown, deletion of these downstream regions can significantly reduce transcription [7]. In addition, the rat construct (–39/+477) contains only a few nucleotides upstream of the TATA-box motif, whereas the human gene construct contained additional upstream sequences. It is possible that the lack of upstream sequences may allow the TATA-box of the rat gene to function as a stronger basal promoter in the context of the enhancer-containing pCATE vector. Finally, the CAT assays with the rat and human gene constructs were tested in different cell lines.

The nuclear extracts from HT29 and RINm5F extracts appeared to have different patterns of binding to the rat palindromic region. Two DNA–nuclear factor bands appeared to be specific for the palindromic sequence with the HT29 nuclear extract, whereas only one DNA–nuclear factor complex was detected with the RINm5F nuclear extract. The patterns of nuclear factor binding to regions downstream of the palindrome also differed between extracts from the two cell lines. The HT29 cells and RINm5F cells are derived from different tissues and species. These data suggest that there may be tissue- and/or species-specific differences in nuclear factors that control the expression of the α_2 A-AR gene. Further studies may show whether tissue-specific patterns of nuclear factor interaction with these key regions are responsible for differences in transcription.

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