We recently cloned cDNAs encoding three subtypes of human α1a-adrenergic receptors (α1ARs), α1a, α1b, and α1d (Schwinn, D. A., Johnston, G. L., Page, S. O., Mosley, M. J., Wilson, K. H., Worman, N. P., Campbell, S., Fidock, M. D., Furness, L. M., Parry-Smith, D. J., Peter, B., and Bailey, D. S. (1995) J. Pharmacol. Exp. Ther. 272, 134–142) and demonstrated predominance of α1aARs in many human tissues (Price, D. T., Lefkowitz, R. J., Caron, M. G., Berkowitz, D., and Schwinn, D. A. (1994) Mol. Pharmacol. 45, 171–175). Several lines of evidence indicate that α1aARs are important in clinical diseases such as myocardial hypertrophy and benign prostatic hyperplasia. Therefore, we initiated studies to understand mechanisms underlying regulation of α1aAR gene transcription. A genomic clone containing 6.2 kb of 5′-untranslated region of the human α1aAR gene was recently isolated. Ribonuclease protection and primer extension assays indicate that α1aAR gene transcription occurs at multiple initiation sites with the major site located 696 base pairs upstream of the ATG, where a classic initiator sequence is located. Transfection of luciferase reporter constructs containing varying amounts of 5′-untranslated region into human SK-N-MC neuroblastoma cells indicate that a region extending 125 base pairs upstream from the main transcription initiation site contains full α1aAR promoter activity. Furthermore, distinct activator and suppressor elements lie 2–3 and 3–5 kilobase pairs upstream, respectively. Although the α1aAR promoter contains neither TATA or CAAT elements, gel shift mobility assays targeting three GC boxes immediately upstream of the main transcription initiation site confirm binding of Sp1. Activity of the α1aAR promoter is cell-specific, demonstrating highest activity in cells endogenously expressing α1aARs. The human α1aAR gene also contains several cis regulatory elements, including several insulin and cAMP response elements. Consistent with these observations, we provide the first evidence that treatment of SK-N-MC cells with insulin and cAMP elevating agents leads to an increase in α1aAR expression. In conclusion, these data represent the first characterization of the α1aAR gene; our findings should facilitate further studies designed to understand mechanisms regulating α1AR subtype-specific expression in healthy and diseased human tissue.

α1-Adrenergic receptors (α1ARs) are members of the larger family of G protein-coupled receptors, mediating various sympathetic nervous system responses such as smooth muscle contraction and myocardial inotropy (1). α1ARs couple predominantly via Gαs to activation of phospholipase C-β and hydrolysis of membrane phospholipids. Resultant formation of inositol triphosphate leads to release of calcium from intracellular stores and ultimately to muscle contraction (2), while diacylglycerol formation results in activation of protein kinase C. cDNAs encoding three subtypes of α1ARs (α1a, α1b, and α1d) see Ref. 3 for new IUPHAR α1AR subtype nomenclature) have been cloned, expressed in cells, and characterized pharmacologically (4–9). We have previously demonstrated species heterogeneity (rat, rabbit, and human) and distinct tissue distribution for each α1AR subtype, with α1aARs predominating in many human tissues (10–12). Clinically, α1aARs have been shown to be important in the dynamic component of benign prostatic hyperplasia, with α1aAR-mediated prostate contractions correlating with urinary symptoms (13, 14), and in the development of myocardial hypertrophy (15).

α1AR stimulation is known to have trophic and long term hypertrophic effects on cardiac and vascular smooth muscle structure and function (16–18). In cardiac myocytes, α1AR activation induces selective transcriptional activation of a “hypertrophic gene program,” including expression of embryonic genes (such as atrial natriuretic factor (ANF), β-myosin heavy chain, and smooth muscle α-actin), activation of Raf-1 kinase/mitogen-activated protein kinase pathways, and increases in cell size without cellular proliferation. While the precise signaling mechanisms through which α1AR-stimulated transcriptional activation occurs are not known, multiple

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28237

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) U72653 (6.2-kb 5′-UTR human α1a-AR) and L31774 (coding region of human α1a-AR (old name α1a)).

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The abbreviations used are: AR, adrenergic receptor; αARTF, α-adrenergic receptor transcription factor; ANF, atrial natriuretic factor; bp, base pair(s); CAT, chloramphenicol acetyltransferase; CRE, cAMP response element; CREF, cAMP response element-binding protein; DHFR, dihydrofolate reductase; IBMX, 3-isobutyl-1-methylxanthine; 111H-HEAT, 2-[(2H-4-hydroxy-3-[125I]iodophenyl)ethylaminoethyl]tetrahydroisoquinoline; ICR, initiator; kb, kilobase pair(s); PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PERE, phenylephrine response element; TAF, TBF-associated factors; TEP, TATA-binding protein; TF, transcription factor; 5′-UTR, 5′-untranslated region.
pathways involving Gαs and several protein kinases have been implicated (16, 17, 19–21). These enzymes presumably alter gene transcription via activation of transcription factors such as serum response factor, AP-1, and perhaps some cell-specific nuclear proteins, which in turn bind to distinct regulatory sequences in the 5'- untranslated region (5'-UTR) of ANF and other α1AR-responsive genes such as α3-actin, and β-myosin heavy chain. While some of these pathways are common to other receptors and agents that activate protein kinase C, at least one pathway is specific for α1ARs. This is demonstrated by characterization of the α1AR-sensitive phenylephrine response element (PERE) found in the 5'-UTR of the ANF gene, a cis regulatory element that is not responsive to hypertrophic agents that activate only the protein kinase C signaling pathway (e.g. phorbol esters) (22). This finding emphasizes that α1AR stimulation provides a unique pathway for inducing muscle hypertrophy.

Recent studies demonstrate that α1ARs themselves are regulated by agonist. Furthermore, regulation by agonist appears to be subtype-specific. Chronic norepinephrine stimulation of α1ARs in neonatal rat ventricular primary myocyte cultures leads to repression of α1a and α1AR mRNA expression, while concurrently transcription of α1aAR mRNA is induced. These changes are not explained by altered mRNA stability, and are accompanied by increased α1AR subtype-specific protein expression, as well as myocardial hypertrophy (15). This phenomenon of differential up-regulation of cardiac α1AR subtypes is also exhibited by other hypertrophic agonists such as endothelin-1, prostaglandin F2α, and phorbol esters, which increase α1AR transcription while repressing α1b and α1dAR mRNA expression (15). Hence, α1ARs appear to play a critical role in the induction of myocardial hypertrophy, and are themselves transcriptionally induced by catecholamines and other agonists commonly found in clinical settings of myocardial hypertrophy.

Despite the importance of α1ARs in pathophysiological states, no studies have examined mechanisms underlying α1AR transcriptional regulation. Understanding mechanisms involved in transcriptional regulation of human α1ARs is important, given that these receptors play a role in prostate disease and cardiac hypertrophy. Therefore, we cloned 6.2 kb of 5'-UTR of the human α1AR gene and, in the present study, we delineate various elements involved in its transcription. Our data indicate that the human α1AR has multiple transcription initiation sites and contains a TATA-less promoter within 125 bp upstream of the main transcription initiation site in a region where we document Sp1 binding. The α1AR promoter is cell-specific, and the receptor is up-regulated by cAMP and insulin. Finally, we identify the presence of two PERE consensus sequences upstream of transcription initiation sites, a finding that may explain the observed agonist-induced up-regulation of α1ARs in rat heart (15).

**Experimental Procedures**

**Cloning Human α1AR Genomic DNA**

To isolate 5'-UTR sequence in the human α1AR gene, a human genomic library (female peripheral blood leukocytes in EMBL3 SP6/T7, average insert size 15 kb, CLONTECH, Palo Alto, CA) was screened. A probe containing 450 bp of 5'-UTR (DNA immediately upstream from the initiator ATG) was generated from EcoRI/Not1 digestion of a human α1AR cDNA (5) clone generously provided by Dr. Julie Tseng-Crank (Glaxo-Wellcome Inc., Research Triangle Park, NC), and radiolabeled with [α-32P]UTP via random priming; approximately 2 x 10^6 phage were screened (100 plates of 150 mm size, containing about 2 x 10^9 phage clones/plate). Positive clones were initially characterized by restriction analysis, followed by southern hybridization (23). DNA sequencing of the entire 5'- regulatory region of each distinct clone was performed using Sanger dideoxy chain termination methods (fmod™ DNA sequencing system; Promega, Madison, WI). Ligation of overlapping clones produced a final genomic construct containing 6.2 kb of 5'-UTR, which was subcloned into pGEM-5ZF (+) (Promega) at SacI/PstI sites using standard molecular biology methods. This final construct provided a template for further 5'-deletion reporter constructs used in examining transcriptional regulation of the human α1AR gene.

**Cell Culture**

SK-N-MC (human neuroblastoma cell line containing endogenous α1ARs (see Ref. 10)), Chang liver (human liver cells, DU145 (human prostate adenocarcinoma (epithelial) were from ATCC, Rockville, MD). Neuroblastoma cells were grown in monolayers utilizing minimum essential medium (SK-N-MC, DU145), Dulbecco's modified Eagle's medium (rat-1 fibroblasts), and basal medium-Eagle's (Chang liver) medium (Life Technologies, Inc.) supplemented with 15% (SK-N-MC) or 10% (all other cell lines) fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 μg/ml) in 5% CO2 at 37 °C. In addition, SK-N-MC cells were supplemented with sodium pyruvate (1 mM) and nonessential amino acids (100 μM). Control rat 1 fibroblasts stably expressing each human α1AR subtype (5) were grown under identical conditions as untransfected rat-1 fibroblasts except for selection with G418 (400 μg/ml).

**RNase Protection Assays**

**RNA Isolation**—Total RNA was extracted from cells (SK-N-MC, Chang liver, DU145, rat-1 fibroblast cells (wild type and stably expressing each human α1AR subtype) using the RNeasy mini kit (RNase-free, Qiagen, Inc., Fridley, MN, TX). Each RNA sample was quantitated spectrophotometrically at 260 and 280 nm and stored at −70 °C as an ethanol precipitate.

**Human α1AR DNA Constructs and Radiolabeled Probe Synthesis**—RNase protection assays were used to confirm the site(s) of transcription initiation and to measure α1AR subtype mRNA concentrations in various cells. Two probes were utilized to determine the transcription initiation site, probes (−485, +4) and (−898, −481). Probe (−485, +4) consists of a 0.48-kb Sall/Not1 fragment of the human α1AR 5'-UTR (−485 to +4 relative to the human α1AR translational initiation site) (GenBank™ accession no. U72653) (24) subcloned into pGEM3Zf(+) (all pGEM vectors are from Promega). Probe (−898, −481) consists of a 0.42-kb BamHI/Sall fragment of the human α1AR 5'-UTR (−898 to −481 relative to the human α1AR transnational initiation site) (GenBank™ U72653) (24) subcloned into pGEM3Zf(−). To determine expression of α1AR subtype mRNA in various cell lines, coding region probes for each α1AR subtype were used. The human α1AR coding region probe consists of a 0.33-kb PvuII (blunted)/HindIII fragment subcloned into pGEM-4Z (Smal/HindIII sites), corresponding to nucleotides 955–1293 (GenBank™ L31772) (5). The human α1AR probe consists of a 0.670-kb XhoI/BamHI fragment in pGEM-4Z (Sall/BamHI sites), corresponding to nucleotides 94–766 (GenBank™ L31773) (5). The human α1AR cDNA probe consists of a 0.38-kb EcoRI/FspI fragment in pGEM-4Z (EcoRI/FstI sites), corresponding to nucleotides 520–896 (GenBank™ L31773) (5). A human cyclophilin control probe was obtained from Ambion, Inc. (Austin, TX) and consists of a 0.103-kb KpnI/EcoRI fragment in pTREL (GenBank™ L31772) (5). The human α1AR probe consists of a 0.485-kb PvuII (blunted)/HindIII fragment in pGEM-4Z (Smal/HindIII sites), corresponding to nucleotides 520–896 (GenBank™ L31777) (5). The human α1dAR cDNA probe consists of a 0.33-kb EcoRI/FspI fragment in pGEM-4Z (EcoRI/FstI sites), corresponding to nucleotides 958–1283 (GenBank™ L31774) (5). The human α1bAR probe consists of a 0.38-kb PvuII (blunted)/HindIII fragment in pGEM-4Z (Smal/HindIII sites), corresponding to nucleotides 958–1283 (GenBank™ L31774) (5). The human α1bAR probe consists of a 0.38-kb PvuII (blunted)/HindIII fragment in pGEM-4Z (Smal/HindIII sites), corresponding to nucleotides 94–766 (GenBank™ L31773) (5). The human α1bAR cDNA probe consists of a 0.38-kb EcoRI/FspI fragment in pGEM-4Z (EcoRI/FstI sites), corresponding to nucleotides 958–1283 (GenBank™ L31774) (5). The human α1bAR probe consists of a 0.38-kb PvuII (blunted)/HindIII fragment in pGEM-4Z (Smal/HindIII sites), corresponding to nucleotides 958–1283 (GenBank™ L31774) (5). The human α1dAR cDNA probe consists of a 0.38-kb EcoRI/FspI fragment in pGEM-4Z (EcoRI/FstI sites), corresponding to nucleotides 958–1283 (GenBank™ L31774) (5).

**RNase Protection Assay Methods and Quantitation of Final Product**—RNase protection assays were conducted as described previously (27) with a few modifications using the RPA II kit (Ambion). To determine the α1AR transcription initiation site, 30 μg of total RNA from SK-N-MC cells was dissolved in 20 μl of RPA II kit hybridization buffer containing >20-fold excess of radiolabeled probe (−485, +4) or probe (−898, −481) (3 x 10^5 cpm/reaction) and control calf thymus (1 x 10^4 cpm/10 μg of total RNA), and incubated at 55 °C (α1AR, α1dAR) and 65 °C (α1bAR) overnight. After digestion with RNase A (50 μg/ml) and RNase T1 (700 units), protected RNA fragments were separated on a 6% polyacrylamide gel; radiolabeled RNA size markers and a DNA sequencing ladder using the same primer and genomic DNA were loaded on the same gel. The dried gel was exposed to X-Omat AR film.
pGL2-Basic and pGL2-Enhancer vectors, respectively. pLl (ligating a human blunted original vector. pL labs, Beverly, MA), and religating the blunted fragment into the 5'-UTR immediately upstream of the 1 initiation site in each construct is listed immediately following the A sign. pΔ-5498 was generated by ligating two human α1AR fragments, a 4468-bp SacI/Xhol (Δ-5498, +1030) fragment and a 1991-bp XhoI/AatII (Δ-1030, +961) fragment from the final genomic construct (Fig. 1), into SacI/Xhol sites in the pGL2-Basic vector; hence pΔ-5498 contains the entire cloned 5498-bp untranscribed regulatory region, 696-bp untranslated 5'-UTR, and 5498-bp 5'-UTR. Varying amounts of 5'-UTR immediately upstream of the 1 transcription initiation site in each construct was used for luciferase assays, 50 μl for CAT assays, and 10 μl to determine protein concentration with BCA reagents (Pierce).

**CAT and Luciferase Activity**

CAT assays were performed by the phase-extraction method as described previously (23). The final xylene phase was placed in a scintillation vial and resultant tritium activity counted by a scintillation counter. Luciferase activity was measured as described previously using the Luciferase Assay System (Promega) (26, 29). Luciferase reaction buffer (44 μl; 0.125 mM MgSO4, 42 mM ATP, 4.5 mg/ml bovine serum albumin) and 156 μl of 50 mM glycine (pH 7.8) were added to 100 μl of cell extract (200 μg protein) at 20°C. The reaction was initiated upon the addition of 100 μl of a 200 μM luciferin solution; measured luminescence was stable within 20–30 s. Final luciferase activity was normalized to CAT activity to control for transfection efficiency, and results were reported as fold-over basal control (pGL2-Basic or pGL2-Enhancer; Promega).

**Electrophoretic Gel Mobility Shift Assay**

Gel mobility shift assays were performed using two double-stranded oligonucleotides corresponding to DNA sequence immediately upstream of the human α1AR main transcription initiation site (–74 to –3 (probe 1), and –146 to –89 (probe 2)) in buffer consisting of 12 mM Tris, pH 7.9, 12% glycerol, 35 mM KCl, 0.07 mM EDTA, 1 mM dithiothreitol, 7.5 mM MgCl2, with 2 μg of poly(dI-dC) (to inhibit nonspecific protein binding). Probes 1 and 2 were created with 4–8 bp of 5'-overhanging sequence at the terminal ends; each probe was radiolabeled by filling in the 5'-overhanging DNA sequence using Klenow in the presence of [α-32P]dGTP and other nonradiolabeled nucleotides. Radiolabeled probes 1 and 2 (0.2 ng each) were incubated with purified Sp1 (1 footprinting unit; Promega, Milwaukee, WI) in 20 μl of gel mobility shift assay buffer (40 mM HEPES, pH 7.9, 10 mM MgCl2, 50 mM NaCl, 0.2 mM, phosphodiesterase inhibitor) were incubated with SK-N-MC cells for 12 or 24 h. In cAMP experiments, forskolin (25 μM) and non-radiolabeled DHFR oligonucleotide (5'-AGTGTATCGGAAAAGGGCGGATCTA-3') (30); a 50–100-fold excess was used to compete for binding to probe 1 and probe 2 (added at the same time as labeled probe). To test for specificity, 50- and 100-fold molar excesses of a mutant Sp1 DHFR oligonucleotide (5'-AGTGTATCGGAAAAGGGCGGATCTCA-3') (30) were also utilized. Resultant oligonucleotides (and associated proteins) were separated according to size by electrophoresis on a 4% nondenaturing gel (30:1 acrylamide/bisacrylamide ratio, containing 2% glycerol) in Tris glycine buffer (0.025M Trizma (Tris base), 0.19 M glycine) at 4 °C. The gel was dried and exposed to Kodak X-Omat AR film for 4–12 h.

**Effect of Insulin and cAMP on Regulation of α1AR Expression**

SK-N-MC cells were grown in monolayers (described above) using heat-inactivated fetal bovine serum to 90% confluence. After initial time-course studies, insulin (1 μg/ml in water) was incubated with SK-N-MC cells for 12 or 24 h. In CAMP experiments, forskolin (25 μM, activator of adenyl cyclase) and 3-isobutyl-1-methylxanthine (IBMX, 0.2 mM, phosphodiesterase inhibitor) were incubated with SK-N-MC cells for 12 or 24 h. Control cells were incubated with the same concentration of solvent as treated cells (water for insulin experiments, ethan-ol and dimethyl formamide for CAMP experiments). At the end of the treatment period, total RNA was isolated from SK-N-MC cells and utilized for RNase protection assays (using human α1AR and control human cyclophilin probes) as described above. To confirm whether mRNA changes in α1AR expression were present at a protein level, saturation binding was performed using the radiolabeled α1AR antagonist [3H]-HEAT (300 pM) as described previously (5); prazosin 1 μM was used for nonspecific binding. Analysis of α1AR subtypes was determined using competition analysis with a Kd concentration of [3H]-HEAT (130 pm) and non-radiolabeled α1AR subtype selective ligand 5-methylura- pidil as described previously (5, 31).

**RESULTS**

**Human α1AR Genomic Clone—Four genomic clones (16.4 kb) were isolated with identical restriction maps (Fig. 1). Each clone contained a 6.0-kb BamHI fragment (consisting of 899 bp of 5'-UTR, the entire 0.88 kb of the first exon encoding the human α1AR, and 4.3 kb of intron sequence; Fig 1A), a 5.4-kb SacI fragment (consisting exclusively of α1AR 5'-UTR; Fig.
Identification of the Transcription Initiation Site—As recently described by our laboratory (24), sequence analysis of the human α1a-AR 5'-UTR (GenBank™ U72653) reveals a TATA-less promoter. Therefore, location of the putative transcription initiation site was investigated using RNase protection and primer extension assays. RNase protection assays were performed with total RNA isolated from SK-N-MC cells (the only human cell line expressing endogenous α1aARs); the location of RNA probes used (relative to the translation initiation site ATG) is shown in Fig. 2A. As shown in Fig. 2B, probe (−485, +4) is generally fully protected (lanes 3–5, 489-bp fragment), indicating that the main transcription initiation site is >489 bp upstream of the translation initiation site. In addition, a partially protected fragment (110 bp) is located 106 bp upstream of the translation initiation site (Fig. 2B, lanes 7–9), representing a minor transcription initiation site. Note there are several other fragments, which can be attributed to probe self-protection since they are present in the tRNA lane in multiple (n = 6) experiments (despite rigorous prehybridization denaturation at 95 °C, and hybridization at temperatures up to 65 °C). To further define the main transcription initiation site, a second RNase protection assay probe was utilized. Experiments with probe (−898, −481) show four partially protected fragments (lanes 12–14); the largest and most prominent is 200 bp in size (corresponding to −681 bp upstream from the ATG; also denoted by an arrow in Fig. 2A) as well as minor fragments (195, 144, and 131 bp in size, corresponding to −676,
FIG. 3. Elucidation of the transcription initiation site using primer extension assays. Total RNA isolated from SK-N-MC cells was used for primer extension analysis with a 36-bp antisense oligonucleotide complementary to the region (~530 to ~566 from the ATG). Resultant cDNA products are shown in the left lane. DNA sequencing reactions on genomic 3'-UTR DNA, performed with the identical primer used in the primer extension assays, are shown in the right four lanes. From the DNA ladder, the main transcription initiation site was determined to be CAA (~696 from the ATG, large upper arrow); a second minor transcription initiation site occurs at GCC (~689, small lower arrow). See "Results" for details.

FIG. 4. Luciferase reporter experiments. Constructs containing varying amounts of human α1a-AR 5'-UTR fused to the promoterless luciferase gene were transiently transfected into SK-N-MC cells and resultant luciferase activity measured (n = 5–12 independent transfections for each construct). The left half of the figure schematizes each construct (white box = promoterless luciferase, while the right half presents luciferase results. Luciferase activity is normalized for cotransfected CAT activity, and expressed as fold over control (black box, relative to pGL2-Basic in A and B; gray box, relative to pGL2-Enhancer in B) (mean ± S.E.). The main site of transcription initiation is defined as +1. Restriction enzymes are as follows: S, SacI; H, HindIII; A, AvrII. A, constructs in A utilize a pGL2-Basic vector (which does not contain a SV40 enhancer); luciferase results reveal the presence of an endogenous activator in human α1a-AR 5'-UTR between ~1927 and ~2869, and a suppressor of basal transcription located upstream of ~2869. B, constructs in B utilize pGL2-Enhancer (which contains a SV40 enhancer in the vector). These results demonstrate that the first 125 bp upstream from the transcriptional initiation site is sufficient for basal transcription (contains entire promoter sequence). See "Results" for details.

human α1a-AR gene occurs approximately 680 bp upstream from the ATG, a 36-bp oligonucleotide complementary to the region ~530 to ~566 was used in primer extension assays with total RNA isolated from SK-N-MC cells. Primer extension assays reveal three transcription initiation sites; Fig. 3 shows two of the transcription initiation sites furthest upstream from the ATG, one major site (~689, larger upper arrow) contains an initiation sequence of CAA in agreement with other initiators (34–36), while a second site contains the sequence GCC (~689, smaller lower arrow). The third initiation site was found further downstream (~625, data not shown).

Both primer extension and RNase protection assays demonstrate a major transcription initiation site for the human α1a-AR gene between ~680 and ~700, and minor transcription initiation sites 5–7 bp and 69–71 bp further downstream; however, initiation sites identified with these two methods are not identical. Although discrepancies as large as 17 bp between primer extension and RNase protection assays have been reported previously for other genes (37), to ensure the discrepancy was not due to the presence of a small intron, alternative splicing in the 5'-UTR, or RNA tertiary conformation, we se-
Delineation of the Promoter Region and Sequences Involved in Modulating Basal Transcription—Serial deletions of the human α1AR 5′-UTR ligated to a luciferase reporter gene were used to determine the general location of the promoter and investigate regulatory regions involved in α1AR expression in human SK-N-MC cells. Each construct was transfected into SK-N-MC cells, and luciferase activity was measured (normalized for cotransfected CAT activity) and expressed as fold increase over control plasmid (pGL2-Basic for panel A and pGL2-Enhancer for panel B). As shown in Fig. 4A, in the absence of vector SV40 enhancer sequences (pGL2-Basic vector), transcription occurs with as little as 125 bp of 5′-UTR immediately upstream from the transcriptional initiation site. Sequences located between −1927 and −2869 (relative to the transcription initiation site +1) enhance basal transcription. Further upstream, DNA sequences between −2869 and −5498 repress basal transcription, indicating the possible presence of a suppressor or silencer (38). The presence of SV40 enhancer sequences in the vector (pGL2-Enhancer vector; Fig. 4B) increases luciferase activity in pLSΔ−125 and pLSΔ−1927 to the level of pLSΔ−2869. These results confirm that DNA sequences present in the first 125 bp upstream from the transcription initiation site are sufficient for basal transcription of the human α1AR gene, and thus contain the basic promoter.

Sp1 binds to GC Boxes in Close Proximity to the Human α1AR Transcription Initiation Site—Since the human α1AR promoter contains neither TATA nor CAAT box consensus sequences (24), the presence of three GC boxes ((G/T)(G/A)GGCG(G/T)(G/A)(G/A)(G/T)) (39) located immediately upstream (−139, −100, −12 bp) from the predominant transcription initiation site suggests that binding of Sp1 in these regions might play a role in basal and/or activated transcription in this promoter. To investigate this possibility, we performed gel mobility shift assays utilizing radiolabeled oligonucleotides corresponding to the smallest amount of human α1AR 5′-UTR with full promoter activity; see Fig. 4B) into each of the cell lines. Luciferase activity is expressed as luciferase activity/control pGL2-Enhancer (which contains a SV40 enhancer in the vector). Cell-specific transcription of the human α1AR gene is apparent.
\( \alpha_{1a} \)-Adrenergic Receptor Gene Regulation

\[ \text{IRE IRE IRE} \]

\[ +\text{696} \]

\[ \text{ATG} \]

**Fig. 7.** Schematic highlighting selected cis regulatory elements present in the human \( \alpha_{1a} \)-AR gene. Locations of consensus sequences for putative cis regulatory elements in the human \( \alpha_{1a} \)-AR 5′-UTR are shown. Sp1 binding sites (GC box sequences, rectangles with rightward diagonal lines) are present immediately upstream of the TATA-less transcription initiation site in SK-N-MC cells (+1, arrow). A second GC-rich region (consistent with Sp1 binding; rectangle with thin rightward diagonal lines, +550 to +690) surrounds the downstream minor transcription initiation site. Putative insulin response elements (IRE, gray rectangles) and cAMP response elements (CRE, black ovals) are highlighted in addition to other known transcription factor binding sequences (Ap1, white ovals; Ap2, rectangles with leftward diagonal lines). Two PERE (checkered rectangle) consensus sequences are present in the human \( \alpha_{1a} \)-AR gene (+1405 bp upstream of the main transcription initiation site in SK-N-MC cells (+1) and +194 bp upstream of a minor transcription initiation site located 106 bp 5′ to the translation initiation site). This raises the possibility that the PERE could act as a cis regulatory element enhancing \( \alpha_{1a} \)-AR transcription in the presence of agonist. For more details and identification of other consensus sequences present, see Ref. 24.

**Fig. 8.** Induction of human \( \alpha_{1a} \)-AR gene transcription by cAMP and insulin. Results from an RNase protection assay examining human \( \alpha_{1a} \)-AR and control cyclophilin mRNA concentrations in total RNA (30 \( \mu \)g) isolated from SK-N-MC cells treated with insulin (1 \( \mu \)g/mL, A) or cAMP (25 \( \mu \)M forskolin and 0.2 mM IBMX, B). Although no significant changes in \( \alpha_{1a} \)-AR mRNA expression occur at 12 h for either drug, modest increases in \( \alpha_{1a} \)-AR mRNA expression with insulin (20–30% increase) and significant increases with forskolin/IBMX (2.2-fold increase) occur at 24 h.

**Effect of Insulin and cAMP on \( \alpha_{1a} \)-AR Transcription and Expression—**As schematized in Fig. 7, multiple putative cis regulatory elements for binding of insulin and cAMP response element-binding protein (CREB) are present in the human \( \alpha_{1a} \)-AR gene. We therefore investigated whether these agents affected human \( \alpha_{1a} \)-AR gene transcription. For more details and competition analysis with the \( \alpha_{1a} \)-AR subtype selective ligand 5-
methylurapidil reveals that increased α1AR binding is due to increases in the α1aAR subtype (Table I).

**DISCUSSION**

The α1aAR is the predominant α1AR in most human tissues including prostate and heart and appears to play an important role in benign prostatic hyperplasia and cardiac hypertrophy, yet nothing is known about transcription of the α1aAR gene. In the present study we report cloning of the human α1aAR gene, determine its overall genomic structure including the presence of an intron (>9 kb) in the encoded protein at the junction of the sixth transmembrane and third intracellular loop (identical location as bovine α1aAR (4), rat/human α1b (32, 33)), and identify several transcription initiation sites, the most prominent located 696 bp upstream from the translation initiation codon in human SK-N-MC cells. Furthermore, we demonstrate that 125 bp immediately upstream of the transcription initiation site serves as the functional promoter, show Sp1 binding in this GC-rich region, identify several transcription initiation sites, and identify type-specific increased transcription and protein expression of human α1aARs in the presence of insulin and cAMP in SK-N-MC cells. This represents the first identification and characterization of mechanisms underlying transcriptional regulation of α1aARs.

One of the most important ways gene expression is regulated is through transcription. To examine basal and inducible transcription in the human α1aAR gene, we began by defining the transcription initiation site in SK-N-MC cells. Both RNase protection and primer extension assays identified several transcription initiation sites, with the main site located 680–700 bp upstream from the translation start site. Since there was a 15-bp discrepancy in the exact transcription initiation site between these two methods, sequencing of cDNA made from α1aAR mRNA in this region was used to resolve this conflict. Although normally primer extension assays are less accurate than RNase protection assays, in the present study sequencing confirmed primer extension results, locating the main transcription initiation site 696 bp upstream from the ATG. One possible explanation for the discrepancy between RNase protection and primer extension assays is the presence of potential palindromic sequence in this region. A tertiary structure loop of DNA can be formed by CAAA (−1 to +3) complementing with TTTG (−16 to +19); a similar palindromic motif has been reported in a plant gene to give a shortened (hence incorrect) RNase protection assay fragment, similar to our findings (37). Taking this information into account, five transcription initiation sites in the human α1aAR gene are located 696 (main), 689, 640, 625, and 106 bp upstream from the ATG. Precedence for multiple transcription initiation sites seen in the human α1aAR gene exists in several catecholamine receptor genes, including the rat α1dAR (which contains three distinct promoters) (33, 40, 41), human α1dAR (32), rat β1AR (42), and the human dopamine1A receptor (43).

The absence of TATA and/or a CAAT box sequence in the human α1aAR gene is interesting. Although TATA-less promoters were initially thought to be associated with housekeeping genes, over the last 5 years it has become apparent that many membrane receptor genes contain TATA-less promoters (35). In the adrenergic receptor family, using strict criteria (TATA/TATA/AT(7/7 matches required), only human α2a, rat α2a/b, and turkey βARs contain a TATA box. CAAT box consensus sequence (GGG(T/C)CAATCT; 7/9 matches required with CAATC intact) is present only in human/rat α1b, and mouse α2bAR genes. In the absence of a TATA box, transcription begins at initiator (Inr) consensus sequences and is often associated with Sp1 binding to GC-rich regions further upstream in the promoter (34–36, 44, 45). Several Inr sequences (34, 35) are present in the human α1aAR gene (24), with a classic Inr consensus sequence (−3CTCA−1) located exactly at the major site of transcription initiation (696 bp upstream from the translation start site); this sequence has been associated with strong promoters in TATA-less genes. In fact, this Inr is found in the terminal deoxynucleotidyl transferase gene and adenovirus IVa2 and major late promoters, where (in the absence of a TATA motif) the sequence appears to be loosely recognized by RNA polymerase II in the context of a preinitiation complex consisting of TATA-binding protein (TBP), TFIIIB, or TFIIIF (45). Sp1 is a transcription factor that binds to at least two GC-rich regions on TATA-less promoters and (with other factors) is necessary for basal transcription (35). Sp1 also binds to TATA-containing promoters to stimulate transcription to higher levels; activation of transcription by Sp1 involves coactivators with the transcription factor TAF110 as well as involvement of TBP, TAF250 and TAF150 (46). Using gel mobility shift assays, we demonstrate specific Sp1 binding to at least two (and possibly more) GC-rich regions located immediately upstream of the main transcription initiation site in the human α1aAR gene. Since only 125 bp is required to obtain full promoter activity in this gene, Sp1 binding to at least two of these sites (−100, −12) may be important in α1aAR gene transcription.

Human α1aAR promoter activity is cell-specific, with highest activity demonstrated in SK-N-MC cells. Although trans-proteins bound to cis-regulatory elements are known to modulate transcription, recent evidence suggests that varying arrangements of basal promoter elements in the transcription complex can modulate the effect of a given activator (47), providing another mechanism for cell-specific differential regulation of transcription. Indeed, while increased basal human α1aAR promoter activity in Chang liver cells may be due to the existence
of multiple cis-regulatory consensus sequences for liver-specific proteins present in the 5′-UTR of this gene, differences between other cell lines may be due to varying compliments of proteins required in the transcription complex. In genes with multiple transcription initiation sites, cell-specific utilization of alternative transcription initiation sites can also affect expression; the human $\alpha_{1d}$AR provides an example in this regard, since a single transcript occurs in kidney, whereas multiple mRNA species (with size corresponding to alternative transcription initiation sites) are present in other tissues (32). The presence of GC-rich sequence 5′ to a minor transcription initiation site (located 106 bp upstream of the translation start site), raises the possibility of alternative promoter activity in different cells/tissues in the human $\alpha_{1d}$AR gene; however, thus far the human $\alpha_{1d}$AR gene appears to have identical initiation sites in every tissue tested (SK-N-MC, prostate, vessels; data not shown). In addition to cell-specific promoter activity, results from experiments utilizing varying amounts of human $\alpha_{1d}$AR 5′-UTR fused to reporter luciferase sequence demonstrate modulation of basal promoter activity in SK-N-MC cells due to the presence of activator sequence 2–3 kb upstream from the transcription initiation site, as well as a strong suppressor/negative modulator sequence >3 kb upstream. Sequence analysis reveals the presence of several putative positive and negative response elements in each corresponding region, although the exact activator and suppressor remain to be determined. Of note, a novel transcription factor, a-adrenergic receptor transcription factor (aARTF), has recently been shown to be essential for transcription of the rat $\alpha_{1d}$AR gene in most tissues (48). Consensus sequences for aARTF binding are present in the human $\alpha_{1d}$AR gene, but are not present in the promoter region; hence, aARTF does not appear to be essential for human $\alpha_{1a}$AR transcription.

An important finding of the present study is that the $\alpha_{1b}$AR is up-regulated by insulin in cAMP. Interestingly, both of these agents have been shown to affect other subtypes of $\alpha_{1}$ARs, although the effect of these agents appears to be subtype-specific. For example, a recent study showed that insulin and insulin-like growth factor increased expression of the $\alpha_{1d}$AR in rat vascular smooth muscle up to 3-fold without affecting expression of the $\alpha_{1b}$ (49). The increase in $\alpha_{1d}$AR expression by insulin in SK-N-MC cells in our study is more modest, despite the presence of several putative insulin response elements present in 5′-UTR of $\alpha_{1b}$AR gene. It is possible that insulin-mediated effects on $\alpha_{1a}$AR expression are cell type-specific and more pronounced effect may occur in other tissues. In contrast to insulin, we demonstrate subtype-specific up-regulation (2.2-fold of $\alpha_{1a}$AR mRNA and protein expression occurs with CAMP in SK-N-MC. This is an important finding and suggests that $\alpha_{1b}$AR may be cross-regulated by receptors that stimulate CAMP formation (e.g. $\beta$ARs, thyroid stimulatory hormone receptors). Since many tissues contain both $\alpha_{1}$AR and $\beta$ARs (e.g. heart, prostate, and adipocytes), regulation of $\alpha_{1b}$AR by CAMP has important clinical implications. Indeed, a recent study in brown adipose tissue suggests that neural stress (cold) and direct $\beta$AR stimulation both result in specific $\alpha_{1a}$AR up-regulation in this tissue (50). The exact mechanism by which CAMP up-regulates $\alpha_{1a}$AR mRNA is not clear; however, it is known that CAMP increases transcription of a number of genes via activation of the CREB family of transcription factors which interact with CAMP response element (CRE) sequence present in the 5′-UTR. Although the main CRE is characterized by the palindromic sequence 5′-TGACGTA-3′, several variations of this sequence also bind CREB and mediate transcription (51). Examination of the human $\alpha_{1a}$AR gene identifies the presence of several putative CREs (24), hence it is conceivable that CAMP increases $\alpha_{1a}$AR expression directly by increasing transcription through CRE sequences; this mechanism is involved in CAMP-mediated up-regulation of the $\alpha_{1d}$AR (52). In addition to directly acting through CREs, CAMP can also increase transcription by interacting with other regulatory sequences such as AP-1 (53), AP-2 (54), Sp1 (55), inverted CCAAT motif (56), and the estrogen response element (57). Since many of these regulatory sequences are present in human $\alpha_{1b}$AR gene (24), it is possible that cis-acting elements other than the CRE are responsible for observed CAMP-mediated up-regulation of $\alpha_{1a}$ARs in SK-N-MC cells. Alternatively, CAMP may up-regulate $\alpha_{1a}$ARs by increasing the stability of its mRNA, as has been observed for phosphoenolpyruvate carboxykinase (58). The exact mechanism by which CAMP up-regulates $\alpha_{1a}$AR will be the subject of our future studies.

An interesting property of the $\alpha_{1b}$AR, not shared by $\alpha_{1b}$ or $\alpha_{1d}$, is that agonist exposure in myocytes leads to $\alpha_{1b}$AR up-regulation (15). In the current study, we find two PERE consensus sequences (22) in the 5′-UTR of the human $\alpha_{1d}$AR gene located upstream of transcription initiation sites in SK-N-MC cells (~1405 from the main (+1) transcription initiation site; ~194 from a minor transcription initiation site located 106 bp upstream of the translation initiation site) (Fig. 7). It is intriguing to speculate that subtype-specific up-regulation of $\alpha_{1d}$ARs in the presence of agonist in rat neonatal myocyte cultures might be mediated by the binding of a cardiac specific transcription factor to the PERE cis regulatory element, resulting in increased $\alpha_{1d}$AR transcription in myocytes. Whether agonist-induced up-regulation of $\alpha_{1d}$ARs is specific to myocardium or more generalizable to other non-cardiovascular tissues (such as prostate in benign prostatic hyperplasia) remains to be determined.

In summary, we present the first description of the human $\alpha_{1d}$AR gene, initial characterization of its promoter, and up-regulation by cAMP. Based on the finding of two PERE consensus sequences in the human $\alpha_{1b}$AR gene, we also suggest the tantalizing possibility that PERE-mediated induction of $\alpha_{1b}$AR expression provides a novel mechanism underlying cell-specific up-regulation of $\alpha_{1d}$ARs in myocardial hypertrophy.

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α1a-Adrenergic Receptor Gene Regulation

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