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Characterization of the α_{1B}-adrenergic receptor gene promoter region and hypoxia regulatory elements in vascular smooth muscle

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ABSTRACT **We previously demonstrated that α_{1B}-adrenergic receptor (AR) gene transcription, mRNA, and functionally coupled receptors increase during 3% O_2 exposure in aorta, but not in vena cava smooth muscle cells (SMC). We report here that α_{1B}AR mRNA also increases during hypoxia in liver and lung, but not heart and kidney. A single 2.7-kb α_{1B}AR mRNA was detected in aorta and vena cava during normoxia and hypoxia. The α_{1B}AR 5′ flanking region was sequenced to −2,460 (relative to ATG +1). Transient transfection experiments identify the minimal promoter region between −270 and −143 and sequence between −270 and −248 that are required for transcription of the α_{1B}AR gene in aorta and vena cava SMC during normoxia and hypoxia. An ATTAAA motif within this sequence specifically binds aorta, vena cava, and DDT$_1$MF-2 nuclear proteins, and transcription primarily initiates downstream of this motif at approximately −160 in aorta SMC. Sequence between −837 and −273 conferred strong hypoxic induction of transcription in aorta, but not in vena cava SMC, whereas the *cis*-element for the transcription factor, hypoxia-inducible factor 1, conferred hypoxia-induced transcription in both aorta and vena cava SMC. These data identify sequence required for transcription of the α_{1B}AR gene in vascular SMC and suggest the atypical TATA-box, ATTAAA, may mediate this transcription. Hypoxia-sensitive regions of the α_{1B}AR gene also were identified that may confer the differential hypoxic increase in α_{1B}AR gene transcription in aorta, but not in vena cava SMC.**

Three subtypes of the G protein-coupled α_1-adrenergic receptors (AR) designated as the α_{1A}, α_{1B}, and α_{1D} have been identified (1). Native and cultured rat aorta and vena cava smooth muscle cells (SMC) express both the α_{1B}AR and α_{1D}AR subtypes (2, 3). Among α_1AR subtypes, α_{1D}AR mediates constriction of the rat aorta (4, 5), iliac artery (6), and arterioles in skeletal muscle (7), whereas the α_{1B}AR signals constriction of the vena cava (8) and skeletal muscle venules (7). Sympathetic constriction of arterioles and venules mediated by these vascular α_1AR shows a prominent difference in sensitivity to inhibition by metabolic signals including hypoxia and acidosis (α_{1D}AR sensitive, α_{1B}AR insensitive), which we have proposed serves to simultaneously maximize autoregulation and reflex control of venous return (9). Vascular α_1AR also have been shown to couple to additional cellular functions, including SMC hypertrophy signaled by the α_{1D}AR (10) and modulation of glucose metabolism in liver and heart mediated by α_{1B}AR (11, 12). Consistent with this association of the α_{1B}AR with altered metabolic states, hypoxia (3% O_2) transcriptionally up-regulates α_{1B}AR, but not α_{1D}AR, gene expression in aorta, but not in vena cava SMC, examined *in vitro* and *in vivo* (3). However, it is not known if hypoxic induction of α_{1B}AR expression is shared by other nonvascular tissues.

Although nothing is known concerning how oxygen levels regulate transcription of the α_{1B}AR gene, control depends on *trans*-acting factors binding to the transcription promoter region and regulatory elements within the DNA. To begin to examine transcriptional control, knowledge of gene sequence upstream to the translation start is required. However, from the coding region of the rat α_{1B}AR upstream to −610 (translation start +1) there is 98% similarity (13–15) but upstream of −611 to the end of the reported sequences [GenBank accession L28752 (14) and D32045 (15)] there is no similarity. Thus, functional analysis of α_{1B}AR promoter regulation requires resequencing the 5′ flanking region.

There is also significant disagreement in the location of the site of transcription initiation of the α_{1B}AR in rat cells (14, 15). Kanasaki and coworkers (15) describe a single transcription start site at −173 similar to that reported for the human α_{1B}AR (16). In contrast, Gao and Kunos (14) proposed the existence of three different transcription initiation sites in rat liver. Certain hypoxia-induced genes, under the control of multiple promoters, have preference for a particular promoter and/or transcription start site during hypoxia (17). Therefore, it is important to determine α_{1B}AR sequence required for transcription initiation in both aorta and vena cava SMC, and to identify whether this changes during hypoxia. Selection of an alternative promoter for the α_{1B}AR during hypoxia in aorta, but not in vena cava, could explain differential induction of transcription by reduced oxygen (3).

Erythropoietin, the primary humoral regulator of increased erythropoiesis during chronic hypoxemia, is the best characterized mammalian gene for hypoxic regulation of expression (18). Several *cis*-elements and their binding factors have been shown to be essential for hypoxic increase in erythropoietin transcription in *in vitro* model systems, notably hypoxia-inducible factor 1 (HIF-1) (18). The consensus HIF-1 binding site is present in the α_{1B}AR gene (3). However, it is not clear whether HIF-1 is involved in the hypoxic up-regulation of α_{1B}AR in aorta SMC. Although specific and hypoxia-induced nuclear protein binding to HIF-1 has been demonstrated in all cell types tested (19), whether HIF-1 or other hypoxia-inducible enhancers are capable of mediating hypoxic regulation of α_{1B}AR in SMC remains to be determined.

MATERIALS AND METHODS

***In Vivo* Hypoxia and Cell Culture.** Adult rats were exposed to 10% inspired O_2 environmental hypoxia for 8 hr as described previously (3). Preparation and culture of adult rat thoracic aorta and vena cava SMC primary cultures were performed as described (2, 3). For hypoxic exposure, cells were placed in an O_2-regulated incubator (Forma Scientific, Marietta, OH) at 1.5% or 3% O_2, 5% CO_2, balance N_2 (3).

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Abbreviations: SMC, smooth muscle cell; AR, adrenergic receptor; RPA, RNase protection assay; HIF-1, hypoxia-inducible factor 1.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. U83985).

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RNA Extraction and Detection. Total RNA was extracted as described (3). Poly(A)$^+$ RNA was selected using Oligotex (Qiagen). Northern analysis was performed using 5 μg (aorta, vena cava) or 2 μg (liver) of poly(A)$^+$ RNA electrophoresed through a 1.2% formamide/formaldehyde agarose gel, transferred to Hybond-N+ membrane (Amersham) by capillary blotting and hybridized with an α_{1B}AR *Xho*I digested [+93 to +527, relative to translation start site (ATG) +1] α^{32}P-CTP-labeled probe (Random PrimeIt, Stratagene). Prehybridization and hybridization were performed at 42°C in Hybrisol I (Oncor). Membranes were then UV crosslinked, washed in 1× standard saline citrate/0.1% SDS 3× 30 min at room temperature and 1 × 30 min at 55°C, and exposed to Kodak X-Omat AR film at −80°C. Size of mRNA was estimated using RNA Millennium markers (Ambion) and Sigma-Gel software (Jandel, San Rafael, CA).

RNase Protection Assays (RPA). RPA were performed as described (3) with one addition. 10^4 cpm of RNA probe for rat cyclophilin (Ambion) transcribed by T7 RNA polymerase in the presence of [α^{32}P]CTP was added to the same RPA reaction to serve as an internal control. Experiments were performed to confirm that low O_2 did not change cyclophilin mRNA levels. The riboprobe for determination of transcription start site was generated by a *Bam*HI/*Xho*I digest (−602 to +93) of the α_{1B}AR gene and subcloned into pBluescript SK (Stratagene) and transcribed by T3 polymerase.

Sequencing. A positive α_{1B}AR clone from a primary screen of a rat FRTL-5 genomic library was generously provided by D. M. Perez (Cleveland Clinic). Southern hybridization was with a *Bam*HI/*Xho*I (−602 to +93) α_{1B}AR α^{32}P-CTP-labeled probe (Random PrimeIt, Stratagene). DNA sequencing was performed in both directions (Sequenase, United States Biochemical).

Plasmid Construction. The *Pvu*II fragment of the α_{1B}AR genomic DNA (−3,500 to +93) was subcloned into the *Sma*I site of pGL$_3$basic luciferase reporter vector (Promega) with 5′ to 3′ orientation (designated −3,500). Promoter deletion plasmids were obtained by digesting the −3,500 plasmid with various restriction enzymes to produce the constructs illustrated in Fig. 3*A* (*Kpn*I −3,200, *Sac*I −2,378, *Spe*I −2,161, *Nsi*I −1,619, *Nhe*I −999, *Apa*I −644, *Bam*HI −602, *Bss*HII −270, and *Sma*I −127). The −248, −209, −195, and −185 constructs were obtained by digesting the −270 plasmid with exonuclease III (New England Biolabs). The reaction was terminated at timed intervals, blunt-ended with mung bean nuclease, and ligated with T4 ligase (New England Biolabs). Except as noted, all other constructs were produced with restriction enzyme digests (New England Biolabs, sites determined using Wisconsin Package version 9.0, Genetics Computer Group, Madison WI). Sequential deletions of P2 were generated using exonuclease III digestion. *Bgl*II linkers were added to both the wild-type and mutated −262 to −242 oligonucleotides, which then were subcloned into the *Bgl*II site of pGL$_3$basic and pGL$_3$promoter luciferase reporter vectors. The −860 to −724 plasmid was made with PCR amplification using primers to α_{1B}AR sequence (primers: −860 to −840 and −724 to −744) and *Bgl*II linkers. The PCR product was agarose gel-purified (GeneClean, Bio101) and subcloned in both orientations into pGL$_3$ promoter. The HIF-1 oligonucleotide (sequence given below) was subcloned into pGL$_3$promoter. Multiple copies of the HIF-1 oligonucleotide (wild type and mutated) were generated as described (20). All constructs were purified by double CsCl gradient centrifugation and verified by sequencing.

Transfection and Luciferase Assay. Cells in passage four were split onto 6-well plates (Falcon) and transfected at 100% confluence for 6 hr with 5 μg/well of luciferase reporter plasmid and 1 μg/well of β-galactosidase normalization plasmid (Promega) using calcium phosphate (21). After two washes, cells were exposed to normoxia or hypoxia, and extracts were prepared 12 hr later. Luciferase analysis was performed using a luciferase assay kit (Promega), and activity was determined with a Wallac Microbeta 1450 counter/luminometer. All experiments were performed in duplicate, with a minimum of three replications.

The following control experiments were conducted: Similar data to those reported were obtained (*i*) when conducted in the absence of β-galactosidase cotransfection and normalized to protein content, (*ii*) using DOTAP [*N*-(1-(2,3-dioleoyloxy)propyl)-*N*,*N*,*N*-trimethylammonium methyl sulfate] (Boehringer Mannheim) instead of calcium phosphate, and (*iii*) β-galactosidase activity was not altered during hypoxia. In addition, we verified in aorta (with similar results in vena cava) that the activity of the luciferase vectors were sufficient to study increases and decreases in transcriptional activity. When luciferase activity was normalized to pGL$_3$basic (luciferase vector lacking a promoter/enhancer), pGL$_3$promoter (simian virus 40 promoter) activity was 55-fold ± 3-fold greater and pGL$_3$control (simian virus 40 promoter and enhancer) was 340-fold ± 41-fold greater than pGL$_3$basic alone.

Gel-Shift Analysis. Nuclei were isolated from approximately 4×10^7 aorta, vena cava, or DDT$_1$MF-2 cells at 80% confluence (20). Sequences for the oligonucleotides used were as follows: −262 to −242 (5′-TGGACCATTAAACTCGAGCCG-3′), −262 to −242 mutated (5′-TGGACCA*CTCAG*CTCGAG CCG-3′) (Life Technologies, Grand Island, NY); HIF-1 (5′-CAGGCGACGTGCTGCCGGGT-3′), HIF-1 mutated (5′-CAGG CGA*AAA*GCTGCCGGGT-3′) (Macromolecular Resources, Fort Collins, CO), consensus cAMP response element binding (5′-AGAGATTGCCTGACGTCAGAGAGCTAG-3′) (Promega); the sequence unrelated oligonucleotide for both gel-shifts (see Figs. 3*A* and 5) was (5′-CGAACTGATGGTCAAGGTGATTCA-3′) (Life Technologies). The double-stranded −262 to −242 and HIF-1 probes were end-labeled with [γ^{32}P]ATP and gel-purified on a 5% nondenaturing polyacrylamide gel. Ten micrograms of nuclear protein was incubated with 0.5 ng of 2–5 × 10^4 cpm of labeled probe in a 20-μl reaction mixture containing 4 μg of polydI-dC, 10 mM Hepes at pH 7.9, 10% glycerol, 2% Ficoll-400, 40 mM NaCl, and 2 mM DTT. The binding reactions were carried out on ice for 30 min, and DNA-protein complexes were separated on a 5% nondenaturing polyacrylamide gel. For competition experiments, 10- to 200-fold molar excess of unlabeled annealed oligonucleotides were added to the binding reaction mixtures before addition of the labeled probes.

RESULTS

Previously, we showed that 8 hr of low O_2 produced a 2- to 5-fold increase in α_{1B}AR mRNA, protein, and functional receptor response in aorta, but not in vena cava SMC (3). To determine whether this also occurs in other tissues, rats were exposed to 10% inspired O_2 (hypoxic hypoxia) for 8 hr. Tissues were harvested, RNA extracted, and RPAs performed. α_{1B}AR mRNA levels were normalized to cyclophilin levels after confirming that cyclophilin levels were unchanged during hypoxic exposure (data not shown). α_{1B}AR mRNA increased 40 ± 28% in liver ($P = 0.03$) and 30 ± 7% in lung ($P = 0.02$) during hypoxia but not in heart or kidney.

In contrast to α_{1B}AR mRNA in liver that has been purported to be under the control of three promoters (14), Northern analysis revealed that aorta and vena cava express only one α_{1B}AR mRNA approximately 2.7 kb in size (Fig. 1*A*). It is thus likely only one promoter and transcription start site is used in normoxic vascular SMC. Hypoxia (3%, 8 hr) did not change α_{1B}AR mRNA size (data not shown), suggesting that the same promoter and transcription start site direct expression of the α_{1B}AR during hypoxia. The size of the α_{1B}AR mRNA, exclusive of the 5′ untranslated end, has been estimated at approximately 2.3 kb (14). Therefore, these data suggest that the transcription start site resides between −200 and −600 bp.

To define the minimal promoter area used by vascular SMC, a 3.5-kb *Pvu*II-digested fragment was isolated and partially

sequenced. The results confirm the sequence reported by Gao and Kunos (14) and extend it further upstream (Fig. 1*B*).

Restriction enzyme and exonuclease III digests were performed on a construct containing 3.5 kb of α_{1B}AR sequence (5′ to ATG) that was subcloned into a luciferase vector lacking promoter (pGL$_3$basic), to functionally define sequence within the 5′ region required for α_{1B}AR gene transcription. The constructs shown in Fig. 2*A* were transiently transfected into aorta SMC, and luciferase and β-galactosidase activity was measured. Sequence between −270 and −248 is required for α_{1B}AR gene transcription in aorta (Fig. 2*A*). These same constructs were transfected into vena cava SMC and yielded a similar pattern, including the abrupt loss of transcription with deletion of sequence downstream of −270 (data not shown). To better identify a minimal promoter region and remove the putative P1 region identified in liver (14), we constructed a plasmid subcloned into pGL$_3$basic that ranged from −270 to −143. This construct showed robust transcriptional activity with an 880 ± 75% increase in luciferase activity as compared with the promoterless vector. This suggests that we have functionally identified a promoter region within the α_{1B}AR gene that is used in aorta and vena cava SMC. This data also corroborates other previously identified transcription start sites in rat (15) and human (16) at approximately −175. A similar pattern was obtained for the same constructs transfected into aorta SMC and exposed to hypoxia, where deletion constructs downstream of −270 once again resulted in loss of transcription (data not shown). These data strengthen the conclusion from our Northern data (Fig. 1*A*) that the same promoter and transcription start site is used in vascular SMC during hypoxia.

Although these findings do not support the use in SMC of the putative promoters P1, P2, and P3 previously hypothesized to function in the rat liver (14), we made constructs surrounding these regions and transiently transfected them into aorta (Fig. 2*B*) and vena cava SMC (data not shown) during normoxia (Fig. 2*B*) and hypoxia (data not shown) to further test this hypothesis. Minimal transcriptional activity, as compared with the constructs that ranged from +93 to −270 and further upstream, was evident for all but one construct. The region from −746 to −273 showed robust transcriptional activity, whereas −659 to −273 showed virtually none. Gao and Kunos (14) also reported that sequence between −813 and −675 was capable of driving transcription in human liver Hep3B cells and hamster DDT$_1$MF-2 cells. However, loss of this sequence in our full-length deletion analysis resulted in no loss of transcriptional activity (Fig. 2*A*). Therefore, it appears that, at least in these transfection studies, in the context of α_{1B}AR sequence ranging from −3,500 to +93, this region does not drive endogenous transcription in vascular SMC to a significant degree although its involvement cannot be conclusively ruled out with this data.

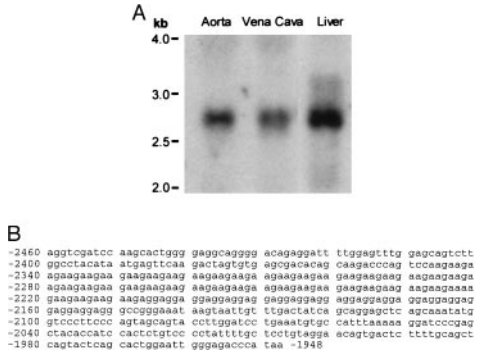

FIG. 1. Cultured aorta and vena cava SMC express a single α_{1B}AR mRNA whereas liver expresses two α_{1B}AR mRNA. (*A*) Northern analysis using a radiolabeled probe that spanned +93 to +527 was performed on 5 μg (cultured aorta and vena cava SMC) or 2 μg (liver) poly(A)$^+$ RNA. (*B*) Sequence of the 5′ region of the α_{1B}AR gene confirmed sequence previously reported (14) (−2,050 to −1,947) and extended it further upstream to −2,460 [all orientations relative to met (ATG) +1].

Based on the data from our full-length deletion analysis (Fig. 2*A*), we focused on the sequence between −270 and −248 that is critical for α_{1B}AR gene transcription in vascular SMC. An ATTAAA motif within this sequence has been shown to function as an atypical TATA-box in the epidermal growth factor gene (22). Gel-shift analyses were conducted to test whether this motif was capable of binding aorta and DDT$_1$MF-2 SMC nuclear proteins. The migration of a radiolabeled double-stranded oligonucleotide corresponding to sequence within −262 to −242 was slowed upon incubation with aorta SMC nuclear proteins (Fig. 3*A*). The formation of these complexes was competitively inhibited in the presence of 10× and to a greater extent with 50× excess unlabeled −262 to −242 oligonucleotide. A double-stranded −262 to −242 oligonucleotide mutated to ACTCAG was used to determine if these specific complexes were binding to the ATTAAA motif. This mutated oligonucleotide was not capable of competing aorta nuclear protein binding. The pattern was similar in DDT$_1$MF-2 (Fig. 3*A*) and vena cava SMC, as well as during hypoxia (data not shown). These results indicate that protein(s) are binding to the ATTAAA motif and perhaps, similar to epidermal growth factor, this sequence acts as an atypical TATA-box. A putative cap site is located 53 bp (−202) 3′ to ATTAAA (as determined using the transcription factor

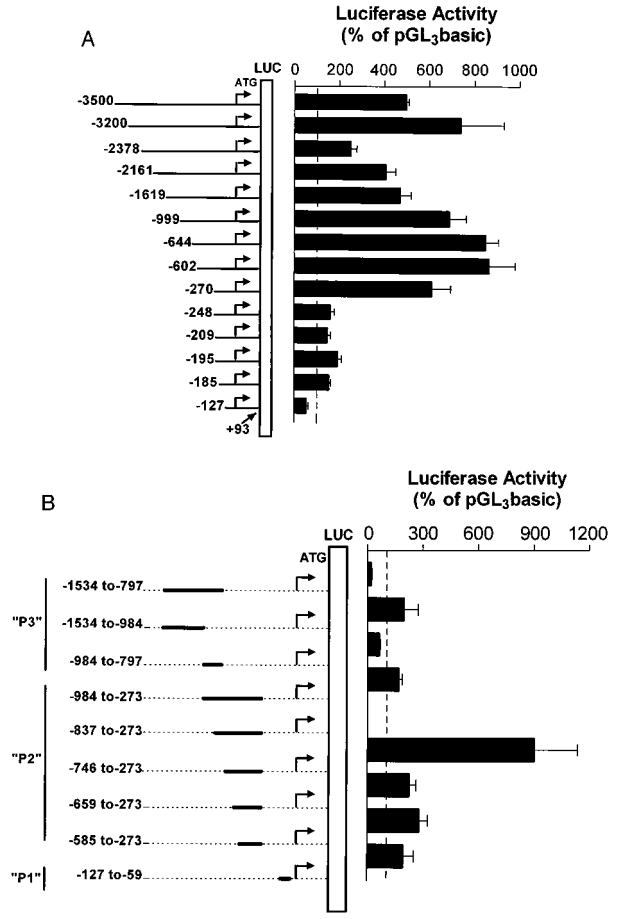

FIG. 2. Sequence between −270 and −248 is essential for transcription of the α_{1B}AR gene. (*A*) Sequential restriction digest and exonuclease III deletion of the 5′ region of the α_{1B}AR gene subcloned in the pGL$_3$basic (luciferase vector lacking a promoter) were obtained and transiently transfected in aorta SMC. (*B*) Constructs, designed based on three previously reported promoters, P1, P2, and P3 (14), were made using restriction enzyme and exonuclease III digestion. Duplicate transient transfection assays for each construct described above were performed in aorta SMC (n = 3). Data were normalized to β-galactosidase activity and presented as mean ± SEM and a percentage of pGL$_3$basic. Broken line at 100% indicates no change from pGL$_3$basic luciferase activity.

binding data set in the Wisconsin Package version 9.0, Genetics Computer Group), also consistent with the ATTAAA motif serving as an atypical TATA-box. In addition, transcription initiation from this region would yield a 5′ untranslated segment of approximately 200 bp, confirming our Northern data (Fig. 1*A*).

To ascertain the α_{1B}AR gene transcription start site, we performed RPAs using a probe to sequence ranging from −602 to +93 (Fig. 3*B*). The 290-bp protected fragment that resulted upon analysis of liver and kidney total RNA suggests that the major transcription start site for the α_{1B}AR gene is at approximately −200 in these tissues. In aorta, the 250-bp protected species corresponds to an α_{1B}AR gene transcription start site at approximately −160 (Fig. 3*B*).

The preceding results indicate that the α_{1B}AR promoter lies between −270 and −143 and likely encompasses the atypical TATA-box sequence, ATTAAA located at −256. This same promoter appears to be used by aorta and vena cava SMC and remains unchanged during low O_2 exposure. We next wanted to determine *cis*-elements within the α_{1B}AR gene that influence this promoter and confer increased transcription during low O_2 (3).

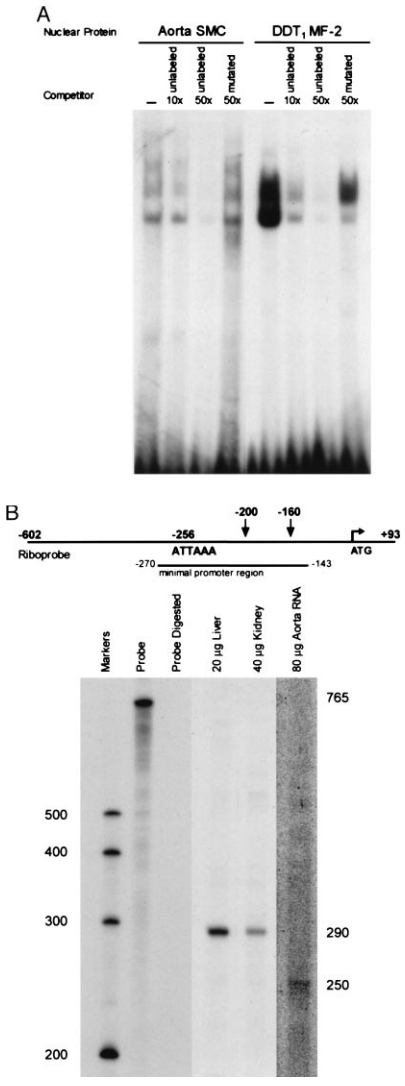

FIG. 3. A specific nuclear protein(s) from aorta SMC and DDT_1MF-2 binds to an oligonucleotide (−262 to −242) surrounding the ATTAAA motif and the α_{1B}AR gene transcription start site is located between −160 and −200. (*A*) Representative gel-shift analysis was performed using an α^{32}P-ATP radiolabeled double-stranded oligonucleotide (−262 to −242) and 10 μg of nuclear protein isolated from cultured aorta SMC or DDT_1MF-2 cells. Competition was performed using unlabeled oligonucleotide and an oligonucleotide mutated at the ATTAAA motif to ACTCAG. (*B*) Representative RPA to determine transcription start site. The 765-bp riboprobe ranged from −602 to +93 and included additional pBluescript vector sequence. It encompassed the minimal promoter region we identified and the ATTAAA motif. Probe digested lane consisted of 80 μg tRNA hypbridized with probe. Other tissue/cells used and amount of total RNA were as indicated.

Transcriptional activity of the luciferase vectors pGL_3basic or pGL_3promoter alone were unaffected by hypoxia, as expected for these controls (Fig. 4*B*). Hypoxia increased transcriptional activity of the −602 to +93 construct, and this was lost with the −270 to +93 and −127 to −49 constructs (Fig. 4*B*). A consensus HIF-1 transcription factor binding site at −164 and its corresponding constitutive binding partner CACAG (23) at −334 are present in the 5′ region flanking the α_{1B}AR coding sequence (Fig. 4*A*). The loss of this CACAG in the −270 to +93 construct may attenuate activity elicited by HIF-1 (23). However, unlike endogenous α_{1B}AR sensitivity to hypoxia (3), use of 3% O_2 may not have been low enough to activate detectable increases in transcription in these reporter assays (24). Likewise, cAMP levels increase during hypoxia (25), and there are four putative AP2 sites between −602 and −270 (Fig. 4*A*) that could link increases in cAMP to the hypoxic increase in transcription of the −602 to +93 construct and the α_{1B}AR gene.

Strong hypoxia-induced transcription in aorta SMC was conferred by the −837 to −273 construct, with a 700% increase in luciferase activity. This induction was lost with the −746 to −273 construct. In contrast, the −837 to −273 construct transfected

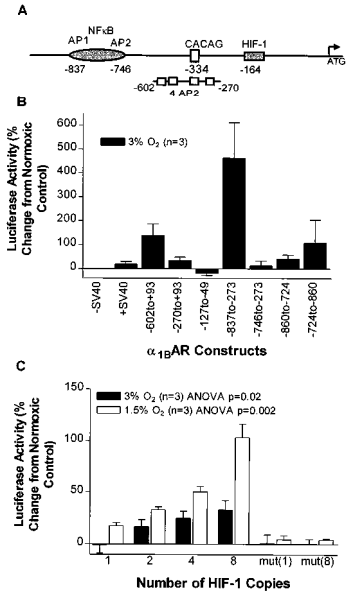

FIG. 4. Specific hypoxic regulation exists for the α_{1B}AR gene. (*A*) There is a consensus HIF-1 binding site at −164 and its companion constitutive element (CACAG) at −334. α_{1B}AR sequence between −837 and −746 contains putative binding sites for a number of transcription factors including AP1, NFκB, and AP2 (as defined by the transcription factor data set in the Genetics Computer Group program), which have been implicated in the control of hypoxic upregulation of other genes (18). (*B*) Characterization of induction of transcriptional activity in aorta SMC by 3% O_2 exposure using pGL_3basic for all except −860 to −724 and −724 to −860, which were subcloned into pGL_3promoter. (*C*) One, two, four, or eight copies of α_{1B}AR sequence surrounding the putative consensus HIF-1 binding site (GACGTGCT) or one or eight copies of an oligonucleotide mutated at base pairs critical for HIF-1 binding (22) (GAAAAGCT) were subcloned into pGL_3promoter. For both *B* and *C*, constructs were transiently transfected in duplicate into aorta SMC. After 12 hr of low O_2 (3% or 1.5% O_2) or 21% O_2, cells were harvested and luciferase and β-galactosidase activity were determined. Data are normalized to β-galactosidase activity and expressed relative to pGL_3basic (−simian virus 40 promoter) or promoter (+simin virus 40 promoter) activity (dependent on vector backbone) and as a percent of time-matched normoxic controls, mean ± SEM, $n = 3$ for all constructs.

into vena cava SMC increased transcription less (240%). Sequence between −837 and −746 contains putative AP1, NFκB, and AP2 sites for binding transcription factors that have been implicated in mediating hypoxia-induced transcription of other genes (18). It is possible that these *trans*-acting factors or other *cis*-elements within this region are responsible for conferring the hypoxic increase in transcription of α_{1B}AR in aorta, liver, and lung, but not in vena cava, kidney, and heart. To further test the hypoxia-inducing capacity of the −837 to −746 region, we subcloned this segment into pGL$_3$promoter (−860 to −724). There was minimal hypoxia-induced transcription off of this region when oriented in the forward direction. However, hypoxia doubled luciferase activity when this region was present in the reverse orientation (−724 to −860) (Fig. 4*B*). While these data suggest that this region is capable of binding *trans*-factors that increase transcription in aorta but less so in vena cava SMC during hypoxia, protein–protein interactions may extend between this region and −724 to −273 that confer the more robust hypoxia induction.

Because the HIF-1 binding site is essential for hypoxic induction of the erythropoietin gene (18), we examined whether HIF-1 alone can confer transcriptional increases in α_{1B}AR mRNA. One, two, four, or eight copies of the α_{1B}AR HIF-1 binding site, GACGTGCT, and 6 bp flanking this region in both directions, were subcloned into pGL$_3$promoter, transfected into aorta SMC and assessed for luciferase activity during hypoxia. Because mutation of this binding site to GAAAAGCT has been shown to completely attenuate HIF-1 binding (23), we also subcloned one and eight copies of the mutated oligonucleotide into pGL$_3$promoter. Both 3% and 1.5% hypoxia-induced transcription at successively greater magnitudes as the number of HIF-1 copies was increased (Fig. 4*C*). This induction was lost when the HIF-1 binding site was mutated. This pattern also was seen in vena cava cells (data not shown). Therefore HIF-1 is capable of increasing α_{1B}AR transcription in an O_2 tension-dependent manner in both cell types.

To test whether hypoxia induces binding to the HIF-1 oligonucleotide, we performed gel-shift analysis. The double-stranded HIF-1 oligonucleotide was radiolabeled and incubated with aorta nuclear protein from control cells and cells exposed to 4 hr of either 3% or 1.5% O_2. A constitutive binding (Fig. 5, bracketed region) could be competed away almost completely with 200× excess unlabeled oligonucleotide (data not shown). In addition, this binding was competed by 100× excess cAMP response element binding (CREB) oligonucleotide, similar to what others have shown for the erythropoietin HIF-1 (26), but not by an oligonucleotide of unrelated sequence (data not shown). This binding was not competed by the mutated HIF-1 oligonucleotide because *cis*-sequence for CREB and HIF-1 overlap, and the mutation also inhibits CREB binding (Fig. 5). Three percent O_2 induced a new binding activity that was further increased by 1.5% O_2 (Fig. 5, arrows). This binding was competed by 10× and to a greater extent by 50× excess unlabeled oligonucleotide. A similar pattern was obtained in DDT$_1$MF-2 SMC (data not shown). Therefore, the data in Figs. 4 and 5 suggest that low O_2 induces specific binding to the HIF-1 element that can confer a functional increase in α_{1B}AR gene transcription.

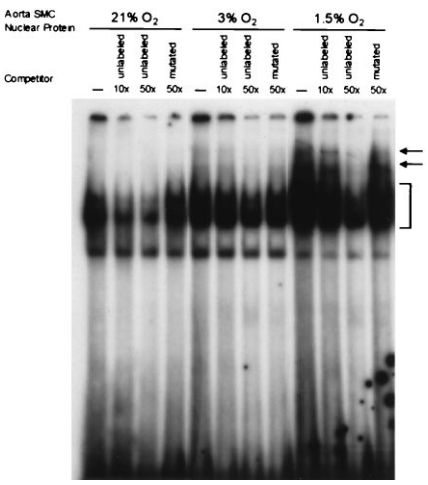

FIG. 5. Binding to the α_{1B}AR HIF-1 sequence is induced during hypoxia in aorta SMC. A radiolabeled double-stranded oligonucleotide surrounding the α_{1B}AR consensus HIF-1 binding site was tested for binding with nuclear protein isolated from control aorta SMC or cells exposed to 4 hr of 3% or 1.5% O_2. Competition was performed with unlabeled oligonucleotide and an oligonucleotide mutated at critical HIF-1 binding sites. Arrows indicate inducible binding, and bracketed area indicates constitutive binding.

DISCUSSION

We have identified sequence required for transcription initiation of the α_{1B}AR and regulation of transcription by hypoxia-responsive *cis*-acting elements in vascular SMC. A minimal promoter region was located between −270 and −143, and sequence between −270 and −248 was required for transcription initiation in vascular SMC, leading to the production of a single 2.7-kb transcript that was unchanged by hypoxia. This −270 to −143 sequence is 92% similar with the human α_{1B}AR, and a putative cap site is present in both sequences at −202, and RPAs suggest that the major transcription start site resides between −160 and −200 for the α_{1B}AR in aorta, liver, and kidney. Within this sequence, a putative atypical TATA-box, ATTAAA, bound nuclear proteins from aorta, vena cava, and DDT$_1$MF-2 SMC. While this binding activity was unaffected by hypoxia, sequence between −837 to −273, but not −746 to −273, mediated hypoxic increase in transcription in aorta but not vena cava SMC. On the other hand, an oligonucleotide containing the hypoxia-sensitive transcription factor HIF-1 element surrounded by α_{1B}AR sequence demonstrated both constitutive and hypoxia-induced binding activities, and conferred hypoxic induction of transcription in aorta, vena cava, and DDT$_1$MF-2 SMC.

The 2.7-kb rat α_{1B}AR transcript is widely expressed (1), is the only mRNA found in heart (27, 28), brainstem, cerebral cortex, kidney, and aorta (28), and was the only transcript we detected in aorta and vena cava SMC. A 2.8-kb α_{1B}AR mRNA is also the only transcript reported for human tissues (16). However, three additional transcripts (2.3, 3.3, and 4.0 kb) have been described for the rat α_{1B}AR in certain tissues (14, 27–29). The existence of multiple promoters and transcription start sites has been proposed to account for this reported heterogeneity of rat α_{1B}AR transcript size (14). However, a prominent transcription start site was identified at −178 for the α_{1B}AR gene in four different human tissues/cells (16). Likewise, Kanasaki and coworkers (15) described a single transcription start site at −173 for the α_{1B}AR in the rat FRTL-5 thyroid cell line. In contrast, Gao and Kunos (14) have proposed that rat liver uses three α_{1B}AR transcription start sites (−54, −443, and −1,107). Our Northern data, transcription reporter assays, and RPAs indicate that aorta and vena cava SMC transcribe α_{1B}AR from a single start site that is unchanged during hypoxia. Our evidence in rat for the 2.7-kb size of the α_{1B}AR transcript and location of the start site around −160 to −200 bp is consistent with Kanasaki *et al.* (15) and Ramarao *et al.* (16). Furthermore, our results identify the minimal promoter region (−270 to −143) and sequence (−270 and −248) required for transcription initiation.

One possible explanation for the difference between our results in rat SMC and those reported for rat liver (13, 14, 30) is tissue specificity. It has been suggested that genes, such as the α_{1B}AR, which contribute to control of liver metabolism, contain alternative liver-specific promoters (31). However, the

proposed use of multiple promoters and transcription start sites in rat liver may be specific to this species, because the α_{1A}AR, rather than the α_{1B}AR, is expressed in rabbit, dog, and human liver (32) and has been detected only as a single transcript (33). Gao and coworkers (30) also have reported that the transcription start site corresponding to the single 2.7-kb α_{1B}AR mRNA in rat heart is at a different, but unspecified, location from the transcription start site they describe for this transcript in rat liver. In addition, Gao and Kunos (14, 30) performed their promoter studies using a rat α_{1B}AR gene transfected into heterologous human Hep3B and hamster DDT_1MF-2 cell lines so perhaps some species–species interaction is occurring, which complicates interpretation.

Several observations suggest that the ATTAAA motif, located at −256, is a functional atypical TATA-box within the α_{1B}AR gene promoter in SMC. Loss of sequence, including this motif, results in attenuation of transcriptional activity, and proteins bind specifically to this motif in aorta, vena cava SMC, and DDT_1MF-2 cells (Figs. 2 and 3). Four other possible transcription start sites just upstream of −178 were identified using primer extension and PCR analysis, suggesting that the location of the transcription start site may lie somewhere between −178 and −227 in the human α_{1B}AR gene (16). There is a potential cap site within 53 bp downstream (−202) of ATTAAA, and our RPAs identified the region between −160 and −200 as being the site of transcription initiation (Fig. 3), which is consistent with the hypothesis that the ATTAAA motif may function as an atypical TATA-box. Importantly, this motif is capable of mediating transcription in other genes, including type IV collagenase (34) and epidermal growth factor (22). Furthermore, the α_{1B}AR region from −270 to −143 is 59% G+C-rich, which is inconsistent with the use of an Sp1-like promoter.

We have identified a region of the α_{1B}AR gene that is induced by hypoxia in aorta, but not in vena cava SMC, which may be important in the hypoxic increase of α_{1B}AR in arteries (Fig. 4). This region between −837 and −746 contains consensus sequences for known hypoxia-sensitive transcription factors: AP1, NFκB, and AP2. As well, there are four AP2 sites further downstream, and we provide evidence (Fig. 4) that additional downstream sequence is likely involved in the hypoxic response (Fig. 4*B*). cAMP increases in aorta SMC during hypoxia (25), and increased cAMP levels promote α_{1B}AR gene transcription (26). Therefore, one possible hypothesis is that a hypoxia-induced increase in cAMP may be mediating at least part of the increase in α_{1B}AR gene transcription. Increased cAMP appears to mediate the hypoxic up-regulation of lactate dehydrogenase A (25, 35), and O_2 sensing by cells has been suggested to involve cAMP-activated protein kinase A (26). However, this hypothesis would require that cAMP levels be differentially regulated in arterial versus venous SMC, and to our knowledge, this has not yet been examined. It has been shown that unlike aorta SMC, cAMP levels decrease in bovine endothelial cells during hypoxia (36) and cAMP activity decreased in rat lung but not in rat liver after 24 hr of hypoxia (37). Although a smaller hypoxic increase in cAMP levels in venous SMC may explain the smaller 240% increase in the −837 to −746 α_{1B}AR construct in vena cava SMC compared with the 700% increase evident in aorta SMC (Fig. 4*B*), additional transcriptional regulatory mechanisms probably are involved. We also identified a potential role for the ubiquitous hypoxic transcription factor HIF-1 element in the hypoxic increase in α_{1B}AR gene transcription. Consistent with other cell types (19), HIF-1 binding activity was induced at both 3% and to a greater degree at 1.5% O_2. However, these experiments did not reveal a substantial difference in the ability of HIF-1 to drive α_{1B}AR gene transcription in aorta and vena cava SMC during hypoxia.

Although the significance of selective hypoxic increase in arterial SMC α_{1B}AR expression, which we have demonstrated occurs *in vivo* (3), remains to be determined, several possibilities are noteworthy. Local metabolic regulatory mechanisms, including reduced O_2 and acidosis, inhibit α_{2D}AR and α_{1D}AR constriction of blood vessels, whereas α_{1B}AR constriction is insensitive (9). Thus, an increase in α_{1B}AR density in an ischemic tissue could impair autoregulatory metabolic inhibition of sympathetic constriction, placing the tissue at risk for excess sympathetic constriction. In chronic systemic hypoxemia, increased α_{1B}AR density could favor preservation of sympathetic SMC tone and oppose hypotension. Stimulation of α_{1B}AR in cardiomyocytes increases glucose uptake, phosphofructokinase activity, and glucose-6-phosphate dehydrogenase mRNA (12), and in liver increases glycogenolysis (11). Consequently, during prolonged hypoxia, an increase in α_{1B}AR may sustain or increase adrenergic constriction and at the same time also increase metabolic capacity of certain cells.

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