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Cloning, Cell-Type Specificity, and Regulatory Function of the Mouse α_{1B}-Adrenergic Receptor Promoter

MICHAEL J. ZUSCIK, MICHAEL T. PIASCIK, and DIANNE M. PEREZ

Department of Molecular Cardiology, The Lerner Research Institute, The Cleveland Clinic Foundation, Cleveland, Ohio (M.J.Z., D.M.P.); and Department of Pharmacology and the Vascular Biology Research Group, University of Kentucky College of Medicine, Lexington, Kentucky (M.T.P.)

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ABSTRACT

The functionality of a 3422-base pair promoter fragment from the mouse α_{1B}-adrenergic receptor (α_{1B}AR) gene was examined. This fragment, cloned from a mouse genomic library, was found to have significant sequence homology to the known human and rat α_{1B}AR promoters. However, the consensus motif of several key *cis*-acting elements is not conserved among the rat, human, and mouse genes, suggesting species specificity. Confirming fidelity of the murine promoter, robust in vitro expression of a chloramphenicol acetyltransferase (CAT) reporter was detected in known α_{1B}AR-expressing BC_3H1, NB41A3, and DDT_1MF-2 cells transiently transfected with a promoter-CAT construct. Conversely, minimal CAT expression was detected in known α_{1B}AR-negative RAT-1 and R3T3 cells. These findings were extended by transfecting the same promoter-CAT construct into various primary cell types. In support of the hypothesis that α_1ARs are differentially expressed in the smooth muscle of the vasculature, primary cultures of superior mesenteric and renal artery vascular smooth muscle cells showed significantly stronger CAT expression than did vascular smooth muscle cells derived from pulmonary, femoral, and iliac arteries. Primary osteoblastic bone-forming cells, which are known to be α_{1B}AR negative, showed minimal CAT expression. Indicating regulatory function through *cis*-acting elements, RAT-1, R3T3, NB41A3, BC_3H1, and DDT_1MF2 cells transfected with the promoter-CAT construct all showed increased CAT production when challenged with forskolin or hypoxic conditions. Additionally, tissue-specific regulation of the promoter was observed when cells were simultaneously challenged with both forskolin and hypoxia. These results collectively demonstrate that a 3.4-kb *Pvu*II fragment of the murine α_{1B}AR gene promoter can: 1) drive tissue-specific production of a CAT reporter in both clonal and primary cell lines; and 2) confer tissue-specific regulation of that CAT reporter when induced by challenge with forskolin and/or hypoxic conditions.

α_1-Adrenergic receptors (α_1ARs) are a group of heterogeneous but related members of the G-protein-coupled receptor superfamily, which prototypically exhibits seven transmembrane-spanning domains. Extensive effort has been spent classifying the three known α_1AR subtypes (α_{1A}, α_{1B}, α_{1D}) via molecular cloning techniques (Cotecchia et al., 1988; Lomasney et al., 1991; Perez et al., 1991, 1994) and pharmacological analyses (Guarino et al., 1996). Results from these studies show that, like other α_1ARs, the α_{1B} subtype mediates the effects of the sympathetic nervous system evoked by the catecholamine agonists epinephrine and norepinephrine. The best characterized cardiovascular effects of α_{1B}AR activation include contraction, growth, and proliferation of vascular smooth muscle (VSM) cells (Chen et al., 1995; Leech and Faber, 1996; Hrometz et al., 1999) and increased cardiac contractility (Anyukhovsky et al., 1992). In other α_{1B}AR-expressing tissues, including brain, liver, and kidney, the function of the receptor is not so well defined. Limited data indicate that the proposed function of the receptor is to regulate metabolic processes in the liver (Kunos and Ishac, 1987), whereas its function is to regulate sodium and water reabsorption in the kidney (Kopp and Dibona, 1992). These responses, which are normally evoked by agonist binding to α_{1B}ARs, are believed to be transduced primarily via receptor coupling to phospholipase C (Guarino et al., 1996), which leads to the subsequent activation of downstream signaling molecules, including protein kinase C and inositol-1,4,5-trisphosphate.

Because α_{1B}ARs are not ubiquitously expressed, the devel-

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[1] Signal Scan, a search engine maintained by the Advanced Biosciences Computing Center, University of Minnesota, is available on the Internet at a site maintained by the Bioinformatics and Molecular Analysis Section of the National Institutes of Health: http://bimas.dcrt.nih.gov/molbio/signal/.

ABBREVIATIONS: α_1AR, α_1-adrenergic receptor; CAT, chloramphenicol acetyltransferase; VSM, vascular smooth muscle; HBSS, Hanks' buffered salt solution; β-gal, β-galactosidase; CRE, cAMP response element; HIF-1, hypoxia-inducible factor 1.

opment of appropriate physiological responses to activation of second messengers depends in part on receptor tissue distribution. One way that this pattern of expression can be controlled is via regulation of the receptor's transcription by the gene's promoter. Therefore, to examine the regulatory characteristics of the α_{1B}AR promoter, several studies have been performed to analyze its function in both humans and rats. The human 5′-flanking region, which has been sequenced as far 5′ of the translation start codon as −923, was found to contain multiple Sp1 binding sites and a putative cAMP response element (CRE; Ramarao et al., 1992). Comparatively, the more extensively characterized rat promoter, which has been sequenced even further 5′ to −2460, was found to contain three distinct promoter regions (Gao and Kunos, 1994). The middle promoter, denoted P2 by Gao et al., is located between −813 and −432 and is hypothesized to be responsible for driving transcription of the α_{1B}AR gene in most rat tissues, especially in the liver (Gao et al., 1995). Multiple transcription factor binding sites have been identified in this P2 promoter region including a CRE, an hypoxia-inducible factor 1 (HIF-1) site, sites for liver-specific factors [C/EBP, hepatic nuclear factor 1 (HNF-1), HNF-5], and sites for cardiac-specific factors (M-CAT and an E-box) (Gao et al., 1995; Eckhart et al., 1997). Despite these studies of the human and rat promoters, in neither species have cell-type specificity issues been explored.

As a prelude to the development of transgenic models, this report describes our effort to clone, sequence, and analyze a 3.4-kb mouse α_{1B}AR gene promoter fragment. Sequence analysis of this murine promoter fragment and comparison with known rat and human α_{1B}AR promoter sequences has led us to hypothesize that, whereas there is substantial similarity between the three promoters, sufficient divergence has occurred to envision some level of species specificity. We have also confirmed that the mouse promoter fragment is able to regulate chloramphenicol acetyltransferase (CAT) gene expression in response to the competency of a given cell to express α_{1B}ARs. Furthermore, we have observed regulation of promoter function by cAMP (via stimulation with forskolin) and hypoxia (via exposure to 1.5% O_2). These data establish the fidelity of the 3.4-kb murine α_{1B}AR promoter, suggesting that it may be a powerful tool for driving selective expression of a target gene in α_{1B}AR-positive cells or tissues.

Materials and Methods

Cloning and Sequencing of Murine α_{1B}AR Promoter. A mouse genomic library (129SVJ female liver, 9- to 23-kb insert size; Stratagene, Inc., La Jolla, CA) was screened to isolate putative promoter sequence from the mouse α_{1B}AR gene. Screening by plaque hybridization was performed with an [α-^{32}P]CTP probe (Random Primed DNA Labeling Kit; Boehringer Mannheim, Mannheim, Germany) derived from exon 1 of the human α_{1B}AR gene (Ramarao et al., 1992). Several rounds of screening facilitated identification of a 15-kb insert, which, after restriction mapping and sequencing, was found to contain approximately 10 kb of 5′-flanking sequence plus exon 1 of the murine α_{1B}AR gene (Fig. 1). Based on work with the rat α_{1B}AR promoter showing functionality of a 3.6-kb *Pvu*II fragment just upstream of the gene's transcription start site (Eckhart et al., 1997), an analogous 3.4-kb *Pvu*II fragment was isolated from our recovered mouse promoter clone, which is located 66-base pairs (bp) upstream of the murine coding region. This putative promoter fragment was subcloned into the *Sal*I site of the pCAT basic vector (Promega Biotech, Madison, WI) to generate the α_{1B}AR promoter-pCAT plasmid. Because promoter insertion into pCAT was accomplished via blunt-end ligation, clones with sense and antisense promoter orientation were distinguished by sequencing with the dideoxy-chain termination method (Sequenase kit; Amersham Corp., Arlington Heights, IL). Large-scale preparations of plasmid DNA were purified with a kit (Wizard Maxipreps; Promega). Sequencing of the entire 3.4-kb promoter fragment was carried out by the Cleveland Clinic Foundation Sequencing Core with a series of nested primers with an average run length of 600 bases (Fig. 1). All screening, subcloning, transformation, and other molecular biology procedures were performed with standard techniques described elsewhere (Sambrook et al., 1989). Sequence alignments were performed with GeneWorks version 2.3, and searches for possible promoter response elements were carried out using Signal Scan, an on-line database and search engine designed and maintained by the Advanced Biosciences Computing Center, University of Minnesota.[1]

Cell Culture. All clonal cell lines used in this study (BC_3H1, DDT_1MF-2, NB41A3, RAT-1, and R3T3) were obtained from American Type Culture Collection (Rockville, MD) and grown in a 37°C, 5% CO_2 incubator with the culture medium recommended by the supplier. Primary cultures of rat VSM cells derived from superior mesenteric, renal, pulmonary, femoral, and iliac arteries were generated with a variation of a previously published method (Gunther et al., 1982). Briefly, the arteries were removed from the animal, aseptically dissected free from fat and connective tissue, cut longitudinally, and spread out flat in a sterile culture dish. Endothelial cells were removed by shearing with a cotton swab, and the arteries were cut into small fragments. These fragments were incubated with gentle agitation at 37°C for 90 min in Hanks' balanced salt solution (HBSS; Sigma Chemical Co., St. Louis, MO) containing 0.1% collagenase (lot 46A034; Worthington Biochemical Corp., Freehold, NJ), 0.125 mg/ml elastase (Worthington), and 2 mg/ml BSA (Sigma). After digestion, samples were centrifuged at 500*g* for 5 min, and the resulting cell pellets were washed once by resuspension with HBSS. After another 5-min spin at 500*g*, cell pellets were resuspended in culture medium composed of a Dulbecco's modified Eagle's medium base (BioWhittaker, Inc., Walkersville, MD) supplemented with 3.6 g/liter $NaHCO_3$, 1% penicillin/streptomycin (BioWhittaker), and 10% fetal bovine serum (Gibco Laboratories, Grand Island, NY). Suspended cells were dispersed in 250-ml tissue culture flasks and were grown in a 37°C, 5% CO_2 incubator. Culture medium was changed every 2 or 3 days, and cells were split at confluency. Primary rat calvarial osteoblasts (obtained from Dr. J. Edward Puzas, University of Rochester, Rochester, NY) were grown in the same culture medium and under the same conditions described for primary smooth muscle cells. Preparation of osteoblastic cells was carried out with a

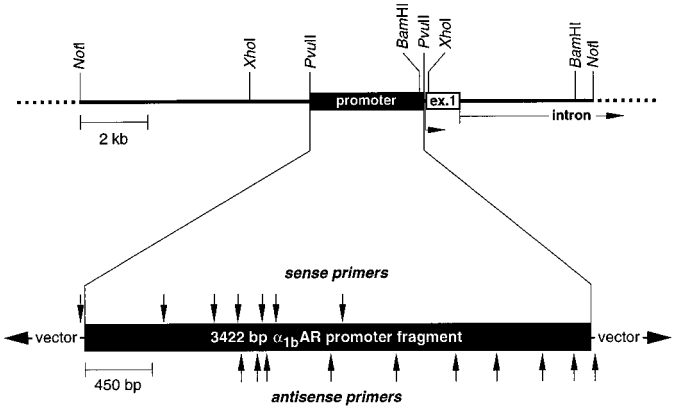

Fig. 1. Restriction map of the original genomic clone of the mouse α_{1B}AR gene and location of sequencing primers. A restriction map of the recovered 15-kb genomic clone and the relative location of sense and antisense primers used to complete sequencing of the 3422-bp fragment.

previously described method (Martinez et al., 1995). For promoter studies, confluent clonal or primary cells were split into p60 dishes and grown to 70% confluency. Several experiments involved culturing of cells under hypoxic conditions. The hypoxic environment (1.5% O_2/5% CO_2, balance N_2) was established in an O_2-regulated incubator.

Transient Transfection. Transient transfections were carried out via a lipofection method with the commercially available Transfectam reagent (Promega) according to the instructions provided by the manufacturer. Briefly, 70% confluent cells in p60 dishes were washed twice with serum-free culture medium and then bathed for 2 h in 1.5 ml serum-free culture medium containing DNA and the Transfectam reagent. The 1.5-ml transfection solution for all clonal cell lines contained 1 μg of sense or antisense (negative control) α_{1B}AR promoter-pCAT plasmid, 1 μg pSV-β-galactosidase (β-gal) plasmid (Promega), and 4 μl Transfectam reagent. Comparatively, the transfection solution for primary cell types contained 1 μg of sense or antisense α_{1B}AR promoter-pCAT plasmid, 1 μg β-gal plasmid, and 12 μl Transfectam reagent. After 2 h, the cells were overlayed with 4 ml of complete medium. Cells were assayed 60 h after transfection.

Preparation of Cell Extracts. Cell extracts were prepared from cotransfected cells via cell lysis with a commercially available Reporter Lysis Buffer (Promega). Briefly, culture medium was removed, and the cells were washed twice with HBSS. After the second wash, HBSS was removed, 0.4 ml Reporter Lysis Buffer was added to each dish, and the dishes were slowly rocked at room temperature for 15 min. The cell layer was then scraped, and cellular debris was transferred to a microcentrifuge tube. Tubes were vortexed and then centrifuged at top speed in a microcentrifuge for 2 min. Supernatants were recovered and split into two aliquots, one for determination of β-gal activity, the other for CAT activity. Lysates to be used for CAT determination were heated to 60°C for 10 min to inactivate endogenous deacetylase activity.

Determination of β-gal Activity. Transfection efficiency in each cell line was determined by measuring the amount of β-gal activity in cell extracts with a colorimetric assay system (Promega). Aliquots of cell extract were mixed with 150 μl assay buffer (supplied with kit) and sufficient Reporter Lysis Buffer to make a final volume of 300 μl. Reaction mixtures were incubated between 0.5 and 2.5 h at 37°C and were terminated by adding 500 μl of 1 M sodium carbonate. Absorbance was read at 420 nm, and absorbance readings were translated into units of activity via a standard curve. Standard curves for β-gal enzyme activity were generated for between 0 and 6 mU of standard enzyme activity with purified β-gal enzyme according to instructions supplied by the manufacturer. The amount of cell extract used in the experimental determination of β-gal activity was varied as necessary to be certain that 420-nm absorbance readings were within the limits of the standard curve.

Determination of CAT Activity. Subsequently, α_{1B}AR promoter function was determined by measuring the amount of CAT enzyme activity present in cell extracts via a liquid scintillation counting assay system (Promega). One hundred-microliter aliquots of cell extract were mixed with 3 μl [^{14}C]chloramphenicol (0.05 mCi/ml; New England Nuclear, Boston, MA), 5 μl *n*-butyryl coenzyme A, and 17 μl distilled H_2O. Reaction mixtures were incubated for 1 to 5 h at 37°C and were terminated by adding 300 μl of mixed xylenes. The reaction product generated by CAT activity in the cell extract, *n*-butyryl chloramphenicol, partitioned mainly into the xylene phase, whereas unmodified chloramphenicol remained in the aqueous phase. Samples were back-extracted twice with 100 μl of 250 mM Tris-HCl (pH 8.0) to completely remove unmodified chloramphenicol from the xylene phase. A fixed 250-μl volume of the final xylene phase from each sample was transferred to a scintillation vial with 6 ml Ecoscint A (National Diagnostics, Inc., Manville, NJ). The cpm measured in each sample was corrected for transfection efficiency (estimated by β-gal activity measured in parallel aliquots), and actual CAT activities were determined by extrapolation from a standard curve. The amount of cell extract used in the experimental determination of CAT activity was varied as necessary to be certain that the measured radioactivity was within the limits of the standard curve. One unit of CAT activity was defined as the amount of CAT enzyme required to transfer 1 nM of the acetate moiety from *n*-butyryl coenzyme A to chloramphenicol in 1 min at 37°C. Statistical significance ($p < .05$) in all CAT assays was identified via one-way ANOVA followed by a Neuman-Keuls multiple-comparison test.

Results

Cloning and Sequence Analysis. A positive plaque from the murine genomic library, identified by hybridization with a probe from exon 1 of the human α_{1B}AR gene, was found to contain an approximately 15-kb insert. After restriction mapping (Fig. 1) and Southern blot analysis with the same exon 1 probe, a 5-kb *Bam*HI fragment containing exon 1 was subcloned into pBluescript. Sequencing of this *Bam*HI fragment confirmed the identity of the murine α_{1B}AR gene and identified the start of the 5′-untranslated region. Overall, the recovered phage insert was found to contain approximately 10 kb of 5′-flanking DNA, 949 bp of the murine α_{1B}AR coding region in exon 1, and approximately 4 kb of intronic sequence.

Previous work has shown that an approximately 3.6-kb *Pvu*II promoter fragment of the rat α_{1B}AR gene (−3500 to +93, relative to the adenosine of the translation start codon) is sufficient to drive expression of the promoterless pGL_3basic luciferase gene when transiently transfected into primary rat aorta and vena cava VSM cells (Eckhart et al., 1997). To accommodate a comparison of rat, mouse, and human α_{1B}AR promoter sequences and to examine the fidelity of the mouse promoter in vitro, an analogous 3422-bp *Pvu*II fragment (−3490 to −68) was isolated from the original 15 kb-insert and subcloned into the *Sal*I site of the promoterless pCAT basic vector. Reported in Fig. 2 are sequence data from −1721 to the *Pvu*II site of the promoter fragment, along with sequence 3′ of the *Pvu*II site to the start codon.

The fully sequenced 3422-bp mouse α_{1B}AR gene promoter fragment was aligned with the known rat and human α_{1B}AR promoters, and the sequence was searched for possible *trans*-acting factor binding sites. Of the previously characterized 3.6-kb *Pvu*II fragment of the rat promoter, sequence has been determined as far 5′ as −2460 (Gao and Kunos, 1994; Eckhart et al., 1997). Alignment of this known rat sequence with overlapping regions of the mouse promoter showed 78% identity between the two species. Comparatively, alignment of the human α_{1B}AR promoter, which has been sequenced as far 5′ as −923 (Ramarao et al., 1992), showed 65% identity with overlapping regions in the mouse promoter. It is also of interest that, when aligning the 5′-flanking region of the mouse α_{1B}AR gene with an overlapping 3500-bp domain of the related human α_{1A}AR gene, poor similarity (18% identity) was observed (Razik et al., 1997). Subsequent sequence analysis of the mouse α_{1B}AR promoter revealed the presence of multiple potential sites for binding of various factors (Table 1). Included among those sites is confirmation of a site for HIF-1 and a CRE, both identified in the rat α_{1B}AR gene (Gao et al., 1995; Eckhart et al., 1997).

The rat middle promoter (P2), located in the region between −813 to −432 (Gao and Kunos, 1994), is believed to be

essential for transcription of the α_{1B}AR gene in most rat tissues (Gao et al., 1995). The alignment of this rat α_{1B}AR gene P2 promoter with analogous overlapping regions in the mouse and human gene showed 90% identity between rat and mouse and 55% identity between rat and human (Fig. 3A). Several DNA consensus sequence motifs, including the above-mentioned CRE, a GC box, a CACCC domain, an activator protein 2 (AP-2) site, and binding sites specific for liver factors (C/EBP, HNF-1, and HNF-5) and heart factors (M-CAT, E-box), have been identified by Gao et al. (1995) in the rat α_{1B}AR gene P2 promoter. Of these reported sites, the CRE and the GC box are exactly conserved in both the mouse and human, and the CACCC domain, the C/EBP site, the M-CAT site, and the E-box are exactly conserved in the mouse (Fig. 3B). In addition to these common transcription factor binding sites, Gao et al. (1995) identified the binding of what was thought to be a novel factor believed to be α-adrenergic receptor specific. This factor, which binds to two distinct sites in the rat α_{1B}AR gene P2 promoter, was later shown to be nuclear factor 1 (NF-1) (Gao et al., 1996). The putative NF1 binding sites identified in these previous studies, which comprise half the NF1 consensus sequence (see Table 1, footnote *b*), are also exactly conserved between both the mouse and human genes.

Promoter Function in Clonal Cell Lines. The ability of the 3.4-kb mouse α_{1B}AR promoter to drive expression of a CAT reporter gene was examined. This was accomplished by cotransfecting the α_{1B}AR promoter-pCAT plasmid and the β-gal plasmid into two α_{1B}AR-negative and three α_{1B}AR-positive cell lines. The ability of these cells to activate the promoter was then assessed by measuring CAT enzymatic activity, which was corrected for transfection efficiency (β-gal). Figure 4 shows that CAT activities were less than 3.3-fold over the antisense control in α_{1B}AR-negative RAT-1 cells (rat fibroblast) and R3T3 cells (mouse embryonic fibroblast). Comparatively, CAT activities were greater than 15-fold over control in α_{1B}AR-positive NB41A3 cells (mouse neuroblastoma) and greater than 35-fold over control in α_{1B}AR-positive BC_3H1 cells (mouse brain tumor). The most robust production of CAT activity, greater than 40-fold over the antisense control, was detected in α_{1B}AR-positive DDT_1MF-2 cells (hamster leiomyosarcoma). CAT activity produced by DDT_1MF-2, NB41A3, and BC_3H1 cells was statistically different from activity produced by RAT-1 and R3T3 cells ($p < .05$). Also, CAT activity produced by DDT_1MF-2 and BC_3H1 cells was statistically different from activity produced by NB41A3 cells ($p < .05$).

Promoter Function in Primary Cell Cultures. Because the promoter-reporter studies discussed above confirm the fidelity of the 3.4-kb mouse α_{1B}AR promoter in established cell lines, the α_{1B}AR promoter-pCAT plasmid was used as a tool to compare the competency of several primary cell types to activate CAT expression. As in the above experiments with clonal cell lines, primary cell cultures were cotransfected with the α_{1B}AR promoter-pCAT plasmid and the β-gal plasmid. The ability of primary cells to activate the promoter was then determined by measuring CAT enzymatic activity, which was corrected for transfection efficiency. Whereas α_{1B}AR-negative rat calvarial osteoblasts (bone cells) did not significantly activate the promoter compared with the antisense promoter control, several rat VSM cell types exhibited competency (Fig. 5). VSM cells derived from vessels such as iliac, femoral, and pulmonary artery produced CAT activity that was 3- to 6-fold over antisense controls. Comparatively, VSM cells derived from vessels such as renal and superior mesenteric artery showed more robust production of CAT activity that was greater than 10-fold over antisense controls. Not only did all of the VSM cells tested produce a significantly greater amount of CAT activity compared with the osteoblasts ($p < .05$), but VSM cells from renal and superior mesenteric artery produced significantly greater CAT activity than VSM cells from iliac, femoral, or pulmonary artery ($p < .05$).

Promoter Regulation by cAMP and Hypoxia. The presence of the aforementioned CRE and HIF-1 binding site on the mouse 3.4-kb *Pvu*II promoter fragment suggests pos-

Fig. 2. Promoter sequence. Sequence data from −1721 to the *Pvu*II site of the promoter fragment along with sequence 3′ of the *Pvu*II site to the α_{1B}AR start codon is shown (→). The *Pvu*II site, which marks the 3′ end of the recovered promoter fragment, is indicated by a shaded box. Regions of this mouse promoter that overlap with important domains identified in the rat α_{1B}AR promoter (P1, P2, and P3) are denoted. The entire sequence of the 3422-bp promoter fragment, including unreported sequence 5′ of −1721, has been submitted to Genbank (accession no. AF116943).

sible regulatory roles for cAMP and hypoxia in the mouse α_{1B}AR gene. To examine these putative regulatory functions, RAT-1, R3T3, NB41A3, BC_3H1, and DDT_1MF2 cells that were transiently cotransfected with the β-gal plasmid and the α_{1B}AR promoter-pCAT plasmid were challenged for 12 h with 100 μM forskolin, hypoxic conditions, or both. After the 12-h challenge, cell extracts were harvested for determination of CAT activity. Compared with control cells that were transfected with the promoter-reporter construct but not treated, CAT production was significantly stimulated in all five cell types by forskolin or by exposure to hypoxia (Fig. 6). Forskolin and hypoxia evoked a roughly similar percent increase in all cell types, with R3T3 cells and RAT-1 cells exhibiting the most robust response (190 and 145% increases, respectively; Fig. 6A). Comparatively, NB41A3, BC_3H1, and DDT_1MF2 cells showed less robust but significant increases in CAT production (83, 34, and 49% increases, respectively; Fig. 6B). Interestingly, when cells were challenged with both forskolin and hypoxia, cell-type-specific regulation was observed. In both the RAT-1 and R3T3 lines, dual challenge evoked a significant increase in CAT production relative to levels measured when either one challenge or the other was applied (107 and 70% increases, respectively; Fig. 6A). However, NB41A3, BC_3H1, and DDT_1MF2 cells did not exhibit this additive effect. Dual exposure in these cells resulted in a level of CAT production that was not significantly different from levels seen with either forskolin or hypoxia treatment alone (Fig. 6B). These novel findings establish the functionality and cell-type specificity of CRE and HIF-1 consensus sites in the 3.4-kb *Pvu*II fragment of the α_{1B}AR gene and combine with our earlier data to confirm the in vitro fidelity of this promoter.

TABLE 1

Sites for common DNA binding factors in the mouse α_{1B}AR promoter

Sequence analysis of our 3422-bp mouse α_{1B}AR gene promoter fragment revealed the presence of multiple consensus sites on the sense strand that are specific for binding of various trans-activating factors. Of these, several common factors/sites are listed. Sites conserved between the mouse and rat promoters are italicized, and sites found only in the mouse are boldfaced. The location of elements that occur more than 5 times are not reported (NR). Locations reported are relative to the adenosine of the initial ATG codon.

Factor/Site	Occurrence	Location
AP-1	>5	NR
AP-2	>5	NR
CACCC site	1	*−615*
CCAAT-box	2	*−1150,* −2991
C/EBP	5	*−656, −1178,* **−1569**, −1688, −2884
CRE	1	*−441*
E box	1	*−770*
GC box	1	*−447*
HIF-1	1	*−168*
HNF-5	>5	NR
LBP-1	>5	NR
M-CAT	1	*−737*[a]
NF-1	2	*−532,*[b] *−560*[b]
NF-E1	2	**−1538**, −3189
NF-κB	1	*−795*
Spl	>5	NR

[a] As reported in the rat promoter (Gao et al., 1995), the mouse promoter contains a 1-bp mismatch (CATGGCT) of the consensus for the cardiac factor M-CAT (CATNC[C/T][T/A]).

[b] These two binding domains (TGGCT and TGGCG), which have been shown to facilitate binding of NF1 in the rat (Gao et al., 1996), represent half of the reported consensus sequence motif for NF1 (MTGGNNNNNNGCCA) (Shaul et al., 1986).

Discussion

Genomic Structure. The genomic structure of the murine α_{1B}AR gene is believed to be similar to the reported rat (Gao and Kunos, 1993) and human (Ramarao et al., 1992) α_{1B}AR gene structures. Specifically, a large intron was found in the murine gene located at the end of the transmembrane 6 coding region, the exact location of which is conserved in the human and rat α_{1B}AR genes as well as in the human and bovine α_{1A}AR genes (Razik et al., 1997). Because we did not clone exon 2 of the murine α_{1B}AR gene, we do not know the size of the putative intervening intron, which we predict could be as extensive as 10 to 20 kb in length. Because other adrenergic receptors are either intronless or have not conserved their intronic structure, current theories pertaining to the origin of genes (Gilbert et al., 1997) suggest that the conservation of intronic structure in the α_1AR family make it the youngest phylogenetic member of the adrenergic receptor superfamily (Feng et al., 1997).

Comparison of Mouse, Rat, and Human α_{1B}AR Promoter Regions. The sequence of the 3.4-kb mouse α_{1B}AR gene promoter fragment was compared with previously published promoter sequence in both human and rat α_{1B}AR genes. This comparison showed that the mouse fragment was 65% identical with overlapping domains in the human gene and 78% identical with overlapping domains in the rat gene. These alignments indicate that, although there is significant similarity between overlapping putative promoter domains from each species, there is sufficient sequence divergence to raise the possibility that regulation of promoter function may vary from species to species.

Three functionally separate promoter domains (P1, P2, and P3) have been identified in the rat gene (Gao and Kunos, 1994). The proximal P1 promoter and the distal P3 promoter (−455 to −49 and −1608 to −1107, respectively), which were shown to be strong enough to evoke expression of a CAT reporter, were thought to be responsible for driving production of 2.3- and 3.3-kb mRNA transcripts (Gao and Kunos, 1994). These two transcripts, found to be present only in the liver, have unknown functional relevance. Compared with P1 and P3, the P2 promoter was more extensively characterized in this earlier study. Located between −813 and −432, P2 was implicated as the domain responsible for generating the major 2.7-kb transcript in most rat tissues, particularly in the liver. Figure 3A shows sequence alignments of the rat P2 domain with overlapping domains in the mouse and human promoter. Analysis of these alignments indicated that there is 90% identity between rat and mouse and 55% identity between rat and human. These findings support the previously mentioned possibility that regulation of α_{1B}AR promoter function may be at least partially species specific. As shown in Fig. 3B, this hypothesis is further strengthened by the finding that several *cis*-acting elements present in the rat α_{1B}AR gene P2 promoter are not exactly conserved in the mouse or human genes.

Contrary to our search for rat-specific DNA elements in the mouse P2 promoter, the full rat promoter was examined for elements that are found to be present in the mouse. A computer-based search of the entire 3.4-kb mouse α_{1B}AR gene promoter fragment revealed the presence of multiple possible regulatory elements, a subset of which is shown in Table 1. Whereas several of these sites are exactly conserved between

overlapping regions of the mouse and rat sequence, several sites are mouse specific. Conserved sites include a CRE and an HIF-1 site, supporting the hypotheses that cAMP (Gao et al., 1997) and induction of hypoxia (Eckhart et al., 1997) are important universal regulators of α_{1B}AR promoter function. Note that both of these sites are conserved in the human promoter as well. Suggestive of some divergence between the rat and mouse, several mouse-specific elements were identified, including two possible sites for binding of the liver, factor C/EBP and an NF-E1 site. Not noted in the table, several AP-1, AP-2, HNF-5, and leader binding protein 1 (LBP-1) sites found in the mouse are also not exactly conserved in overlapping regions of the rat. Note that our identification of the presence/absence of possible response-element consensus motifs does not imply their functionality; functional analysis of such sites must be performed to confirm the transcriptional role of any such putative element.

Because α_1ARs are thought to be involved in cell growth and proliferation, a search of our murine promoter fragment for elements known to control gene transcription during growth or proliferative phases was performed. In particular, an α_1AR regulatory sequence denoted as a phenylephrine response element (PERE), which has been identified in the hypertrophy-specific atrial natriuretic factor promoter region (Ardati and Nemer, 1993), is not present in the 3422-bp α_{1B}AR promoter. Furthermore, neither the cardiac hypertrophy-specific NF-AT3/GATA4 binding site (Molkentin et al., 1998) nor the developmental regulator of smooth, skeletal, and cardiac muscle proliferation referred to as the CArG box (Karns et al., 1995) was found to be conserved in the α_{1B}AR promoter. However, proliferative-specific elements such as AP-1 (Angel and Karin, 1991) and NF-κB (Baeuerle and Baltimore, 1996) and hypertrophy-specific elements M-CAT and Sp1 (Karns et al., 1995) are present (see Table 1).

α_{1B}AR Promoter Function in Clonal Cell Lines. To determine whether our 3.4-kb mouse promoter fragment was

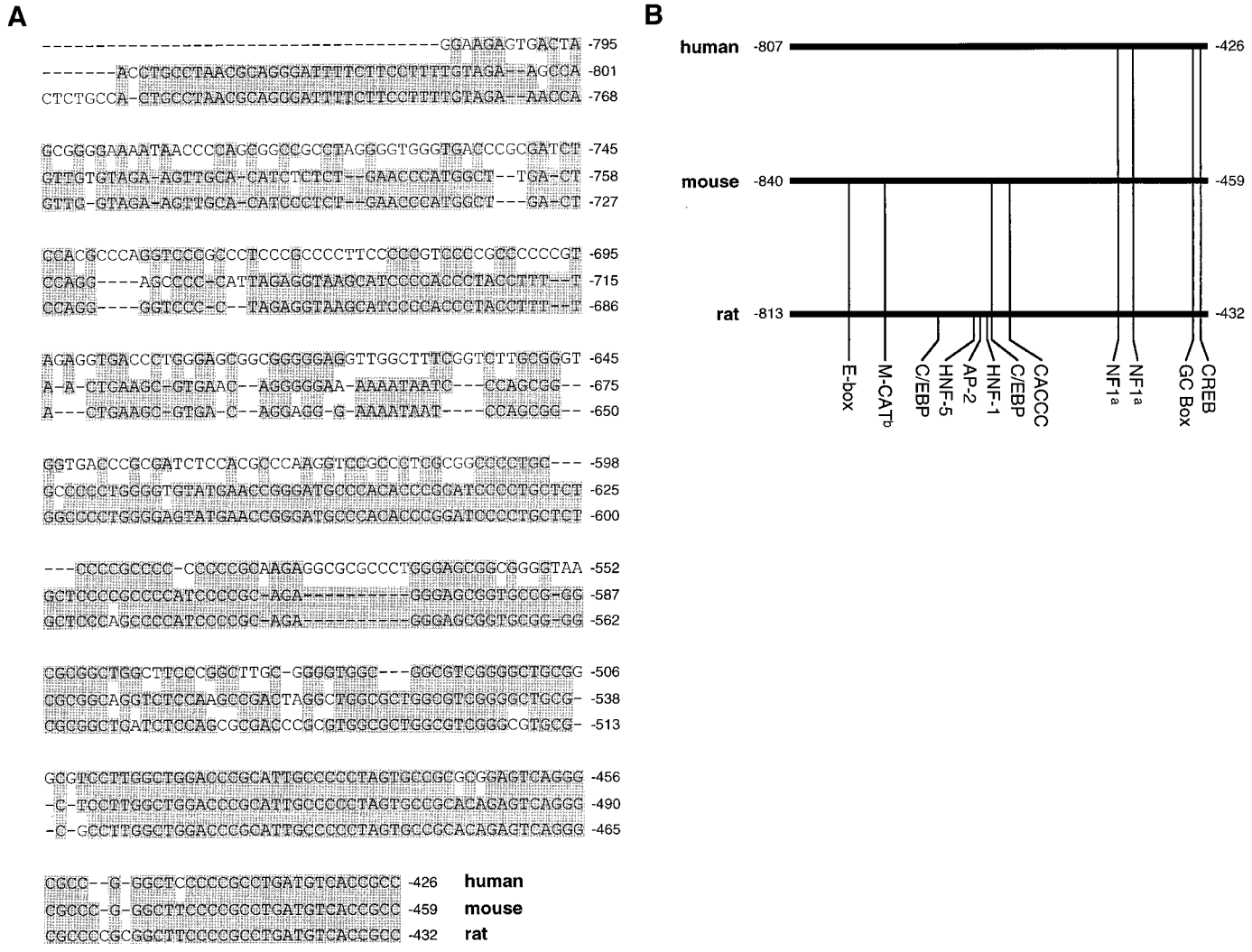

Fig. 3. Comparison of the rat α_{1B}AR gene P2 promoter with overlapping mouse and human sequence. The rat α_{1B}AR gene P2 promoter, proposed by Gao et al. (1995) to be responsible for driving the most widely expressed 2.7-kb mRNA transcript of the α_{1B}AR gene (Gao and Kunos, 1994), was aligned with overlapping sequence from the mouse and human genes. A, sequence exactly conserved between species is shaded. There is 90% identity between rat and mouse and 55% identity between rat and human. B, relative positions of important *cis*-acting elements found in the rat gene are shown. Elements that are conserved in overlapping domains of the mouse and/or human are identified. [a]As reported in the rat promoter (Gao et al., 1995), the mouse promoter contains a 1-bp mismatch (CATG*G*CT) of the consensus for the cardiac factor M-CAT (CATNC[C/T][T/A]). [b]These two binding domains, which have been shown to facilitate binding of NF1 in the rat (Gao et al., 1996), represent half of the reported consensus sequence motif for NF1 (Shaul et al., 1986).

sufficient to maintain appropriate tissue-specific expression, CAT reporter studies were performed in α_{1B}AR-positive and -negative clonal cell lines. Presumably, if sufficient promoter was recovered from our genomic screen to conserve fidelity of expression, α_{1B}AR-negative cells transfected with the α_{1B}AR promoter-pCAT plasmid should not significantly activate the promoter or show expression of the CAT gene. Conversely, α_{1B}AR-positive cells should be able to activate the promoter, which in turn should evoke strong expression of CAT activity. Clonal cell lines utilized for this control experiment that were α_{1B}AR negative included RAT-1 fibroblasts and R3T3 mouse embryonic fibroblasts. The fact that these fibroblastic lines are adrenergic receptor negative has made them common candidates for stable expression of various adrenergic subtypes. For comparison, three α_{1B}AR-positive clonal cell lines were also tested, including BC_3H1 mouse brain tumor cells, NB41A3 mouse neuroblastoma cells, and DDT_1MF-2 hamster leiomyosarcoma cells. In DDT_1MF-2 and BC_3H1 cells, previously reported saturation binding experiments showed B_{max} values of 396 and 118 fmol/mg of protein, respectively (Han et al., 1992). This report also showed competition bind-

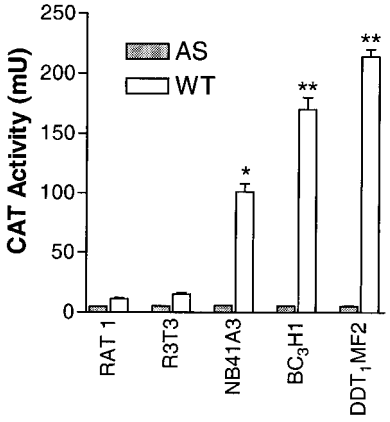

Fig. 4. CAT activity produced by various clonal cell lines. RAT-1, R3T3, NB41A3, BC_3H1, and DDT_1MF-2 cells were transiently cotransfected with the β-gal plasmid and the α_{1B}AR promoter-pCAT plasmid. The α_{1B}AR promoter-pCAT plasmid containing the antisense orientation of the promoter fragment, which should not stimulate production of CAT, provided a negative control. CAT activity produced by each cell line was determined by correcting for transfection efficiency (β-gal activity) and then interpolating the actual CAT activity from a standard curve. *, significantly different from CAT levels detected in α_{1B}AR-negative RAT-1 and R3T3 fibroblasts. **, not only significantly different from CAT levels detected in fibroblastic cells but also from levels in NB41A3 cells. Statistical significance ($p < .05$) was identified via one-way ANOVA followed by a Neuman-Keuls multiple-comparison test ($n = 11$ for each value reported).

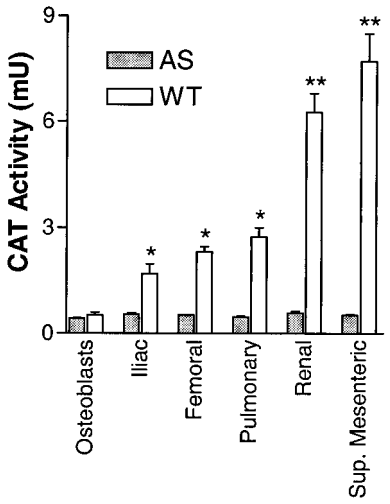

Fig. 5. CAT activity produced by various primary VSM cell types. Rat calvarial osteoblasts and VSMs derived from iliac, femoral, pulmonary, renal, and superior mesenteric arteries were transiently cotransfected with the β-gal plasmid and the α_{1B}AR promoter-pCAT plasmid. The α_{1B}AR promoter-pCAT plasmid containing the antisense orientation of the promoter fragment, which should not stimulate production of CAT, provided a negative control. CAT activity produced by each cell type was determined by correcting for transfection efficiency (β-gal activity) and then interpolating the actual CAT activity from a standard curve. *, significantly different from CAT levels detected in α_{1B}AR-negative rat calvarial osteoblasts. **, not only significantly different from CAT levels detected in osteoblastic cells but also from levels detected in iliac, femoral, and pulmonary artery VSM cells. Statistical significance ($p < .05$) was identified via one-way ANOVA followed by a Neuman-Keuls multiple-comparison test ($n = 5$ for each value reported).

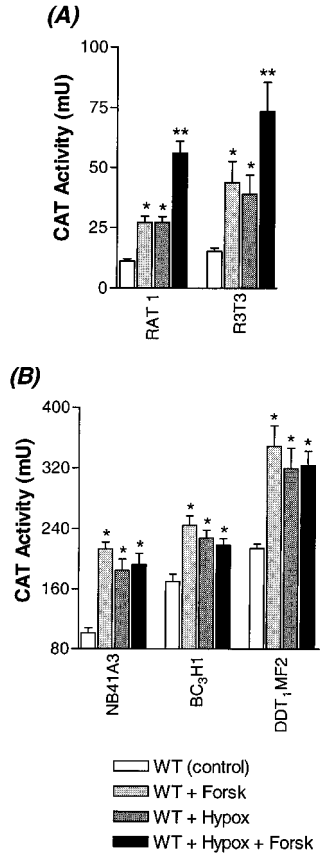

Fig. 6. Stimulation of CAT production with forskolin and hypoxic challenge. RAT-1, R3T3, NB41A3, BC_3H1, and DDT_1MF-2 cells were transiently cotransfected with the β-gal plasmid and the sense-oriented α_{1B}AR promoter-pCAT plasmid. After 48 h, transfected cells were challenged with a 12-h exposure to 100 μM forskolin (Forsk), 1.5% O_2 (Hypox), or both conditions. CAT activity produced by each cell line under each experimental condition was determined by correcting for transfection efficiency (β-gal activity) and then interpolating the actual CAT activity from a standard curve. A, CAT activity produced by RAT-1 and R3T3 cells. B, CAT activity produced by NB41A3, BC_3H1, and DDT_1MF2 cells. Note the difference in the y-axis scale between A and B. Statistical significance ($p < .05$) was identified via one-way ANOVA followed by a Neuman-Keuls multiple-comparison test ($n = 11$ for untreated WT controls, $n = 3$ for all other values reported). *, significantly different from CAT levels detected in unstimulated cells containing the sense-oriented (WT) promoter. **, not only significantly different from CAT levels detected in the unstimulated cells but also from levels detected in WT promoter-transfected cells challenged with either 100 μM forskolin or hypoxia.

ing data from each line that were consistent with the presence of a single population of α_{1B}AR sites. Similar binding experiments in NB41A3 cells showed a B_{max} of 23 fmol/mg of protein, with competition binding data also consistent with the presence of a single population of α_{1B}ARs (Esbenshade et al., 1993). Based on these combined data, all three of these cell lines are considered to express a pure population of the α_{1B} subtype.

Confirming fidelity of the 3.4-kb α_{1B}AR gene promoter fragment, RAT-1 and R3T3 cells produced significantly less CAT activity than α_{1B}AR-positive NB41A3, BC_3H1, and DDT_1MF-2 cell lines (Fig. 4). In addition to this ability to drive tissue-specific expression, the promoter also exerted regulatory control over CAT expression that paralleled the aformentioned differences in α_{1B}AR content seen among the three α_{1B}AR-positive lines tested. DDT_1MF-2 and BC_3H1 cells, which express a large number of α_{1B}ARs, produced the largest amount of CAT activity. NB41A3 cells, which express relatively fewer receptors, produced more CAT activity than the fibroblastic lines but significantly less activity than DDT_1MF-2 and BC_3H1 cells. These findings suggest that, in the case of mouse-derived R3T3, NB41A3, and BC_3H1 cells, our 3.4-kb α_{1B}AR gene promoter fragment is sufficient to not only confer tissue-specific expression but also exert regulatory control that mirrors the function of the endogenous promoter. Note that in the case of RAT-1 and DDT_1MF2 cells, which are derived from rat and hamster, respectively, our finding of apparent tissue-specific expression and regulatory control exerted by the promoter could be partially influenced by both tissue-specific and species-specific factors.

Screening VSM Cells with the α_{1B}AR Promoter-pCAT Plasmid. As a means to understanding the role of α_1ARs in the regulation of blood pressure and hypertrophic cell growth, significant effort has been exerted by numerous investigators to determine the pattern of vascular expression for each of the three α_1AR subtypes. Regarding the pattern of α_{1B}AR expression in particular, some studies have approached this question by quantitating mRNA levels in several different rat blood vessels. However, these RNase protection and reverse transcriptase-polymerase chain reation experiments showed no significant difference in α_{1B}AR mRNA content between various vessels (Guarino et al., 1996). Other recent experiments have involved immunohistochemistry along with antisense oligonucleotide techniques in aortic, caudal, femoral, iliac, mesenteric resistance, renal, and superior mesenteric arteries. In agreement with the earlier mRNA experiments, these studies showed that antisera raised against the α_{1B}AR (Fonseca et al., 1995) exhibited roughly equal immunoreactivity in each of the blood vessels examined (Piascik et al., 1997; Hrometz et al., 1999). From a functional perspective, however, the magnitude of developed tension evoked by phenylephrine or naphazoline in α_{1B}AR antisense-treated vessels was not found to be uniform, with only the mesenteric resistance artery exhibiting an antisense-evoked reduction in contractile responses to agonist (Piascik et al., 1997; Hrometz et al., 1999). Because these results suggest a contractile role for α_{1B}ARs in mesenteric arteries only, the possibility arises that α_{1B}ARs in general may be more functionally relevant in other aspects of vascular function. As an example of this idea, α_1ARs are also thought to be involved in catecholamine-induced proliferation and hypertrophy of VSM (Nakaki et al., 1990).

Because we have established the fidelity of the α_{1B}AR promoter-pCAT reporter plasmid in clonal cell lines, we attempted to use this reporter plasmid to: 1) assess the competency of various primary VSM cell types to activate transcription of the α_{1B}AR gene; and 2) compare these promoter-reporter results with the pharmacological and functional results discussed above. Although all primary VSM cell types tested produced significantly more CAT activity than the negative control, VSM cells from renal and superior mesenteric arteries produced the most robust CAT activity (Fig. 5). The fact that these two VSM cell types produced CAT activity that was significantly greater than that seen in VSM cells from iliac, femoral, or pulmonary artery suggests that they are the most competent of the cell types tested to transcribe the α_{1B}AR gene. The α_{1B}AR-negative osteoblasts, which did not produce a significant amount of CAT activity, served as a negative control for this experiment. Collectively, these data are consistent with previous RNA and immunohistochemical analyses, which showed that the α_{1B}AR is widely expressed in all vascular tissue. However, the more robust CAT activity seen in renal and superior mesenteric arteries suggests differential regulation of the α_{1B}AR subtype in various vessels. Although contraction of renal and superior mesenteric artery does not appear to be mediated by the α_{1B}AR, proliferative and/or growth responses in these cells may be regulated by this subtype. Indeed, the mouse α_{1B}AR promoter contains an M-CAT site and numerous AP-1, Sp1, and NF-$\kappa\beta$ sites (Table 1), suggesting receptor regulation by factors that control hypertrophic and proliferative responses. Therefore, we propose that measuring the competence of a cell to activate transcription of our promoter-reporter plasmid may have predictive value when attempting to screen tissues for α_{1B}AR content or regulation. Given the lack of highly selective ligands for the various adrenergic subtypes, similar promoter-reporter constructs could be valuable tools when screening numerous tissues for adrenergic competency.

Regulation of α_{1B}AR Gene Promoter by cAMP and Hypoxia. Because the rat α_{1B}AR promoter has already been shown to possess functional CRE and HIF-1 sites, our goal became to test for similar response element function in the murine promoter. Thus, the functional fidelity of the 3.4-kb murine α_{1B}AR gene promoter fragment was examined by applying forskolin and/or hypoxic challenge to cells transfected with the α_{1B}AR promoter-pCAT plasmid. As can be seen in Fig. 6, all five cell lines showed increased CAT production after forskolin or hypoxic challenge compared with the unchallenged control. These findings, which are similar to previously published results with the analogous rat promoter (Eckhart et al., 1997; Gao et al., 1997), provide important support for the hypothesis that our α_{1B}AR gene promoter fragment possesses functional fidelity.

Unlike what has been attempted previously, combined activation of the CRE and HIF-1 *cis*-acting sites was carried out to examine promoter function under dual stimulation. We hypothesize that net control of transcription by the α_{1B}AR gene promoter is dependent on a combinatorial mechanism that is comprised of the regulatory effect of multiple factors. Precedent for this idea was established in an earlier study that reported the interaction of an AP-2 site with the CRE in the regulation of both basal and cAMP-evoked activity of the rat α_{1B}AR promoter (Gao et al., 1997). Our initial test of this hypothesis was attempted in this study by exposing the

mouse α_{1B}AR gene promoter fragment to a simultaneous challenge with forskolin and hypoxia. In α_{1B}AR-negative RAT-1 and R3T3 cells, this dual challenge of the promoter evoked a significant increase in CAT production above the level of production seen with either challenge alone (Fig. 6A). The effect was additive in both cell lines, with the sum of each individual treatment roughly equaling the level of production observed with the dual treatment. Interestingly, in α_{1B}AR-expressing NB41A3, BC_3H1, and DDT_1MF2 cells, this additive effect was not present, with dual challenge producing a level of CAT activity that was similar to levels seen with forskolin or hypoxia alone. These results support our hypothesis that α_{1B}AR promoter control is dependent on a combinatorial mechanism by demonstrating that cells competent to express α_{1B}ARs (i.e., NB41A3, BC_3H1, and DDT_1MF2 cells) can exhibit a different promoter response than α_{1B}AR-negative cells to the same conditions. We suspect that the lack of promoter response to the dual challenge seen in α_{1B}AR-competent cells may be due to the net effect of a complement of α_{1B}AR-related positive and negative transcriptional regulators that are always present and functional due to the endogenous expression of the α_{1B}AR gene. In α_{1B}AR-negative fibroblasts, this complement of factors may not be present, leading to divergent responsiveness. Whereas our current data cannot sort out this issue, the novel responses seen to dual challenge of the murine α_{1B}AR gene promoter fragment with forskolin and hypoxia confirm our assertion that this promoter is capable of regulating tissue-specific transcription apparently via the action of one or more functional *cis*-acting domains.

Concluding Remarks. Experiments described in this article 1) compared known sequences from mouse, rat, and human α_{1B}AR promoters and 2) examined the function of the mouse promoter in various clonal and primary cell lines. Sequence analyses indicated that, whereas there is substantial similarity among mouse, rat, and human α_{1B}AR promoter regions, there is some sequence divergence, with several putative response elements not being conserved between species. CAT reporter studies of the mouse α_{1B}AR promoter showed that CAT production was restricted to α_{1B}AR-positive clonal cell lines, supporting our hypothesis that the promoter can regulate tissue-specific expression. Also demonstrating differential regulation of the promoter, VSM cells from vessels such as renal and superior mesenteric artery showed robust CAT production, whereas VSM cells from vessels such as iliac, femoral, and pulmonary artery showed a significantly lower competency to produce CAT activity. Finally, functionality of a CRE and an HIF-1 site in this mouse α_{1B}AR gene promoter was confirmed, and novel tissue-specific effects on promoter function were observed when both of these sites were simultaneously activated. Based on these collective results, we propose that the α_{1B}AR promoter-pCAT plasmid, when used in CAT reporter studies like the ones described herein, may prove to be a valuable tool when screening cell types for competency to activate the α_{1B}AR gene. Our 3.4-kb mouse promoter could also be useful to target a gene systemically to α_{1B}AR-expressing tissues in a transgenic model.

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Send reprint requests to: Prof. Dianne M. Perez, Department of Molecular Cardiology, NB5, The Lerner Research Institute, The Cleveland Clinic Foundation, 9500 Euclid Ave., Cleveland, OH 44195. E-mail: perezd@ccf.org