Cloning and Characterization of the Mouse α1C/A-Adrenergic Receptor Gene and Analysis of an α1C Promoter in Cardiac Myocytes: Role of an MCAT Element That Binds Transcriptional Enhancer Factor-1 (TEF-1)

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This paper is available online at http://molpharm.aspetjournals.org

ABSTRACT

α1-Adrenergic receptor (AR) subtypes in the heart are expressed by myocytes but not by fibroblasts, a feature that distinguishes α1-ARs from β-ARs. Here we studied myocyte-specific expression of α1-ARs, focusing on the subtype α1C (also called α1A), a subtype implicated in cardiac hypertrophic signaling in rat models. We first cloned the mouse α1C-AR gene, which consisted of two exons with an 18 kb intron, similar to the α1B-AR gene. The receptor coding sequence was >90% homologous to that of rat and human. α1C-AR transcription in mouse heart was initiated from a single Inr consensus sequence at −588 from the ATG; this and a putative polyadenylation sequence 8.5 kb 3′ could account for the predominant 11 kb α1C mRNA in mouse heart. A 5′-nontranscribed fragment of 4.4 kb was active as a promoter in cardiac myocytes but not in fibroblasts. Promoter activity in myocytes required a single muscle CAT (MCAT) element, and this MCAT bound in vitro to recombinant and endogenous transcriptional enhancer factor-1. Thus, α1C-AR transcription in cardiac myocytes shares MCAT dependence with other cardiac-specific genes, including the α- and β-myosin heavy chains, skeletal α-actin, and brain natriuretic peptide. However, the mouse α1C gene was not transcribed in the neonatal heart and was not activated by α1-AR and other hypertrophic agonists in rat myocytes, and thus differed from other MCAT-dependent genes and the rat α1C gene.

α1-Adrenergic receptors (α1-ARs), one of three families of receptors for the endogenous catecholamines norepinephrine and epinephrine, are transcribed at different levels in different tissues. For example, α1-AR transcription is robust in rat heart muscle but low or nondetectable in skeletal muscle (Rokosh et al., 1994). Differential transcription is further evident within the rat heart, where α1-ARs are expressed only in cardiac myocytes, and not in cardiac parenchymal nonmyocytes or fibroblasts (Stewart et al., 1994). In adult mouse heart also, α1-ARs are functional in myocytes but not fibroblasts (X. F. Deng, D. G. Rokosh, T. D. O'Connell, S. Cotecchia, and P. Simpson, in preparation). The myocyte-specific transcription of α1-ARs in heart contrasts distinctly with β-ARs, which are expressed by both cardiac myocytes and nonmyocytes (Lau et al., 1980). Thus, catecholamine signaling in the myocardium activates α1-ARs only on myocytes, but β-ARs on both myocytes and nonmyocytes, which has important implications for catecholamine biology in the heart, and for cardiac hypertrophy and failure.

Surprisingly little is known about the mechanisms that control cell-specific transcription of ARs. Previous studies defined cell-specific promoters for the α1B, the α1C (also called the α1A-AR[2]), and the α2C (Saulnier-Blache et al., 1996; Razik et al., 1997; Gao and Kunos, 1998; Zuscik et al., 1999). However, only for the α1B in hepatocytes have further studies documented specific functional DNA sequence elements and their corresponding transcription factors (Gao and Kunos, 1998).

Here we investigated mechanisms for transcription of the α1C-AR in cardiac myocytes. We focused on the α1C, rather

[2] Throughout this article, we use the name α1C-AR to avoid potential ambiguities with the name α1A (Graham et al., 1996). For example, the cloned α1D-AR was named originally the α1A (Lomasney et al., 1991) and the accession numbers in GenBank for the α1D sequence (M60654 and M60655) still refer to this gene as the α1A.

ABBREVIATIONS: AR, adrenergic receptor; TEF-1, transcriptional enhancer factor 1; PCR, polymerase chain reaction; BAC, bacterial artificial chromosome; bp, base pair(s); kb, kilobase pair(s); RPA, ribonuclease protection assay; PMA, phorbol-12-myristate-13-acetate; EC, enhancer core; PGF2α, prostaglandin F2α; CAT, chloramphenicol acetyltransferase; tk, thymidine kinase; CRE, cAMP response element.

than the other two subtypes, the $\alpha 1B$ and $\alpha 1D$, because the $\alpha 1C$ subtype mediates cardiac myocyte hypertrophy and hypertrophic gene induction in cultured rat myocytes (Knowlton et al., 1993; Autelitano and Woodcock, 1998). Furthermore, the $\alpha 1C$ is itself induced by $\alpha 1$-AR and other hypertrophic agonists in cultured rat myocytes and the intact rat (Rokosh et al., 1996; Autelitano and Woodcock, 1998).

To study myocyte-specific expression of the $\alpha 1C$-AR, we first cloned and characterized the mouse gene and its transcription in mouse heart. We then defined a mouse $\alpha 1C$ promoter in cultured neonatal rat cardiac myocytes, and examined the role of MCAT elements in activation of this promoter. MCAT DNA sequences share a consensus 5′-CATNC(C/T)(T/A) and bind members of the family of transcription factors called transcriptional enhancer factor-1 (TEF-1) (Larkin and Ordahl, 1998). We focused on MCAT elements because they are required for transcription of several other cardiac myocyte genes in culture and in the intact heart (α- and β-myosin heavy chain, skeletal α-actin, cardiac troponin T, and B-type natriuretic peptide) (Larkin and Ordahl, 1998). In addition, MCATs are required for increased transcription during cardiac hypertrophy induced by $\alpha 1C$-AR and other hypertrophic agonists (Kariya et al., 1994; MacLellan et al., 1994; Karns et al., 1995; Gupta et al., 1997; Thuerauf and Glembotski, 1997; He and LaPointe, 1999). However, no receptor gene in any tissue has so far been shown to require an MCAT for activity. Therefore, we tested the idea that MCATs might be a common transcriptional regulatory element shared by the $\alpha 1C$-AR and the other cardiac genes regulated by this receptor.

Materials and Methods

Cloning of the Mouse $\alpha 1C$-AR Gene. Initially, a λFIX II 129SV mouse spleen genomic library (Stratagene, La Jolla, CA) was screened using rat $\alpha 1C$-AR cDNA probes to the first exon (*Pst*I probe, −450 to 732 relative to ATG) or the second exon (*Sma*I probe from 1267 to 1827) (Stewart et al., 1994). Restriction fragments from positive clones were subcloned into pBluescript (Stratagene) and sequenced using Thermo-sequenase PCR-based DNA sequencing (Amersham Pharmacia Biotech, Cleveland, OH).

Additional 5′-flanking sequence of the mouse $\alpha 1C$ gene was obtained from a 129SV mouse bacterial artificial chromosome (BAC) library (Genome Systems, St. Louis, MO) using the first exon *Pst*I probe. A positive BAC clone was isolated and digested with *Hin*dIII for a Southern blot using the *Pst*I fragment as a probe; a positive fragment was isolated from an agarose gel, cloned into pBluescript, and sequenced.

Northern Blot. Total RNA was isolated from adult mouse heart, brain, and liver using guanidinium thiocyanate and extraction with phenol:chloroform:isoamyl alcohol. Thirty micrograms of total RNA from each tissue was separated on a 2.2% agarose/formaldehyde gel, transferred to a nylon membrane (Nytran plus; Schleicher & Schuell, Keene, NH), and cross-linked with UV irradiation. The blot was probed with an RNA probe to the first 494 bp of the first exon, and then reprobed with a 250 bp β-actin RNA probe. Template DNA for the $\alpha 1C$-AR probe was amplified from the BAC clone containing the first exon. RNA probes were transcribed (Maxiscript T7 kit; Ambion, Austin, TX) using $[\alpha$-$^{32}P]UTP$ 3000 Ci/mmol (PerkinElmer Life Science Products, Boston, MA) and then gel-purified. Blots were hybridized in Church buffer (0.1 M Na_2HPO_4 pH 7.2, 1% bovine serum albumin, 1 mM EDTA, 7% SDS, and 35% formamide) for 20 h at 65°C, washed (twice with 0.1 M Na_2HPO_4/1% SDS for 15 min at 65°C, twice with 33 mM Na_2HPO_4/0.33% SDS for 15 min at 65°C, and finally twice with 3.3 mM Na_2HPO_4/0.03% SDS for 15 min at 65°C), and exposed to film (Biomax-MR; Eastman Kodak, Rochester, NY) at −70°C with an intensifying screen.

RNase Protection Assays (RPAs). To locate the transcription initiation site of the $\alpha 1C$ gene, total RNA from adult mouse heart was analyzed by RPA (RPA II kit; Ambion) using antisense RNA probes to the 5′-flanking sequence (probes 1 and 2: −650/+1 and −801/−302, where ATG is +1). Template DNA for the $\alpha 1C$-AR probes was amplified from the BAC clone containing the first exon. RNA probes were transcribed (Maxiscript T7 kit; Ambion) using $[\alpha$-$^{32}P]UTP$ 800 Ci/mmol (PerkinElmer Life Science Products) and then gel-purified. Purified probes (1×10^6 cpm) were hybridized with 50 μg of total RNA (or tRNA as a control) at 45°C overnight in 20 μl of 80% deionized formamide, 100 mM sodium citrate pH 6.4, 300 mM sodium acetate pH 6.4, and 1 mM EDTA. Nonhybridized RNA was digested with RNase A (1 U/ml) and RNase T1 (40 U/ml). RNA duplexes were precipitated and separated on an 8 M urea/6% acrylamide gel. Gels were then exposed to film (Biomax-MR; Eastman Kodak) at −70°C with an intensifying screen.

To study $\alpha 1$-AR subtype mRNAs in neonatal and adult mouse heart, probes described previously (Rokosh et al., 1994) were used in the same RPA protocol, except nonhybridized RNA was digested with only RNase T1.

Primer Extension Assays. A 20-bp antisense oligonucleotide primer was designed complimentary to the 5′-flanking sequence from −520 to −540 (relative to ATG) of the mouse $\alpha 1C$ gene. The primer was end-labeled with $[\gamma$-$^{32}P]ATP$ (PerkinElmer Life Science Products) using T4 polynucleotide kinase (Life Technologies, Gaithersburg, MD). Labeled primer was annealed for 10 min at 65°C to 2 μg of mouse heart poly(A) RNA (purified from total RNA using the Poly(A) Pure kit; Ambion). Annealed RNA was then reverse transcribed with avian myeloblastosis virus reverse transcriptase for 30 min at 41°C according to the Primer Extension kit (Promega, Madison, WI), with the exception that the reverse transcriptase reaction was in the presence of 25 μCi of $[\alpha$-$^{32}P]dCTP$. The same 20 bp primer was used to sequence DNA from the mouse $\alpha 1C$-AR gene (Thermo-sequenase, Amersham). Primer extension and sequencing reactions were run on an 8 M urea/6% acrylamide gel, and gels were dried and exposed to film (Biomax-MR, Eastman Kodak) at −70°C with an intensifying screen.

Mouse $\alpha 1C$-AR Gene Promoter Constructs. Promoter constructs were cloned into a pUC9-CAT reporter plasmid (Kariya et al., 1994), containing the multicloning site from pBluescript (KS). 5′-Flanking sequences were amplified from the *Hin*dIII BAC DNA clone by PCR using primers with unique restriction sites on each primer (*Sal*I on the 5′ primers and *Xba*I on the 3′ primer). The 5′ primers containing a *Sal*I restriction site started at −4417, −3009, and −1307 bp relative to the transcription start site. A common 3′ primer containing an *Xba*I restriction site started at +14 relative to the transcription start site. Amplified products were digested sequentially with *Sal*I and *Xba*I and cloned directionally into p0CAT. The resulting plasmids were named p4417-$\alpha 1$CAR-CAT, p3009-$\alpha 1$CAR-CAT, and p1307-$\alpha 1$CAR-CAT. A 79-bp reporter construct was cloned in a similar manner. In this case, the 5′-flanking sequences were amplified from the *Hin*dIII BAC DNA clone by PCR using primers (5′ primer −79 bp and 3′ primer +586 bp relative to the transcriptional start site) to amplify a blunt-ended product that was cloned into the *Sac*I site in p0CAT.

Intronic sequences were cloned into the −46tkCAT plasmid that contains 46 bp of the thymidine kinase promoter (Karns et al., 1995). Three constructs were made spanning a total of 5.7 kb of proximal intron sequence. Intronic DNA was digested with *Ava*I and *Sal*I to release a 5.7 kb fragment, which was subsequently digested with *Hin*dIII to generate three fragments that were cloned into the −46tkCAT plasmid. The plasmids were 1.8AH-tkCAT, a 1.8-kb *Ava*I to *Hin*dIII fragment; 1.8H3-tkCAT, a 1.8-kb *Hin*dIII fragment; and 2.1HS-tkCAT, a 2.1-kb *Hin*dIII to *Sal*I fragment.

Mutations to the p4417-$\alpha 1$CAR-CAT Plasmid. Mutations to MCAT910 and MCAT1041 were made in the p4417-$\alpha 1$CAR-CAT plasmid using a PCR-based mutagenesis protocol. Two primers around the site to be mutated, both 5′ to 3′, with one primer complementary to each strand, incorporated the mutated base pairs. One primer of each pair

was phosphorylated on the 5′ end to facilitate ligation after amplification. The p4417-α1CAR-CAT plasmid was used as the template. After amplification, the PCR products were treated with Klenow Fragment (Life Technologies) to create a blunt-ended product, digested with Dpn I (Roche Molecular Biochemicals, Indianapolis, IN) to remove the supercoiled template DNA, and ligated with T4 DNA Ligase (Roche Molecular Biochemicals) overnight at 16°C. DH5α competent cells (Life Technologies) were transformed with the ligation products. Mutations were confirmed using Thermo-sequenase PCR-based DNA sequencing (Amersham Pharmacia Biotech).

Cell Culture and Transfection. Neonatal cardiac myocytes were obtained from the hearts of day-old rats by serial trypsinization and cultured at low density in 60-mm dishes using defined medium as described previously (Long et al., 1991). Myocytes were transfected in duplicate by the calcium phosphate precipitation method with 5 pmol (10–20 μg) of one of the α1C-AR promoter constructs or p0CAT, 0.01 pmol (50 ng) of RSV-luciferase (RSV-LUX) as an internal control and differing amounts of pUC19 to adjust total DNA to 25 μg/dish (Kariya et al., 1994). In experiments with hypertrophic agonists, cells were treated with the following (from Sigma Chemical, St. Louis, MO, except as noted): norepinephrine bitartrate (RBI/Sigma, Natick, MA), timolol maleate, prazosin HCl, phenylephrine HCl, endothelin-1, phorbol-12-myristate-13-acetate (PMA), prostaglandin F-2α (R&D Systems, Minneapolis, MN), or vehicle (100 μM ascorbic acid; Sigma Chemical, or 0.05% dimethyl sulfoxide for PMA and PGF2α). CAT and luciferase activities were assayed 24 h later as described previously (Kariya et al., 1994). In myocytes, luciferase activity was increased by cotransfection with the α1C-AR reporter plasmids (data not shown), and thus CAT activity was not normalized to luciferase in any experiment.

Cardiac fibroblasts were obtained from myocyte culture preplates as described previously (Long et al., 1991), plated at 50,000 cells/60-mm dish, and transfected in duplicate by adenoviral-mediated transfection (Forsayethe and Garcia, 1994), instead of calcium phosphate precipitation, because the nonmyocytes were more difficult to transfect than myocytes. Fibroblasts were transfected with 5 pmol of the α1C-AR promoter constructs and 0.2 pmol (1 μg) RSV-LUX (internal control) by exposure to the adenovirus/DNA cocktail for 2 h, and then washed with and maintained in minimal essential medium with Hanks' salts with 5% calf serum. CAT and luciferase activities were determined as for myocytes. In fibroblasts, luciferase activity was not affected by cotransfection with the α1C-AR reporter plasmids (data not shown), but CAT activities were not normalized to luciferase.

Gel Mobility Shift Assays. Rat TEF-1ε for the binding reactions was produced in vitro using a reticulocyte lysate expression system (TnT Quick Coupled Transcription/Translation System; Promega). Nuclear extracts from cardiac myocytes were prepared as described previously (Kariya et al., 1993). The radiolabeled probe for the gel shift assays was the 22-bp enhancer core/MCAT (EC/MCAT) sequence (−215/−194) from the rat β-myosin heavy chain promoter (Kariya et al., 1993, 1994). Cold competitor probes from the α1C-AR promoter were designed encoding MCAT910, a mutant MCAT910, and MCAT1041 (Fig. 4A). Binding reactions and analysis were as described previously (Kariya et al., 1993, 1994), except that the gel contained 6% acrylamide, not 4%. Binding affinities for each competing oligonucleotide were compared by IC_{50} values estimated from a logarithmic equation used to describe the competition curve.

Data Analysis. Results are given as mean ± S.E.M. for the number of experiments indicated in each figure. Treated versus control values were tested for deviation from unity by calculation of confidence limits. Mean values were compared by the paired, two-tailed Student's t test.

Results

Mouse α1C-AR Gene Cloning. We previously cloned the rat α1C-AR cDNA (Stewart et al., 1994). To obtain the mouse α1C-AR gene, we screened a mouse spleen genomic library with rat α1C-AR cDNA probes to exons 1 and 2. We isolated two genomic clones (Fig. 1A, i and ii). The first (Fig. 1A, i) contained 1.2 kb of 5′-flanking sequence, the entire 0.88 kb of coding sequence for the first exon of the mouse α1C-AR, and 11.9 kb of intron sequence. The second clone (Fig. 1A, ii) contained approximately 6 kb of intron sequence, the entire 0.52 kb of coding sequence for the second exon and 8.5 kb of 3′-flanking sequence. To obtain additional 5′-flanking sequence, we screened a mouse BAC library with the first exon *Pst*I probe. We isolated a 7.7 kb *Hin*dIII fragment that contained 5 kb of 5′-flanking sequence, the entire 0.88 kb of coding sequence for the first exon and 1.8 kb of intron sequence (Fig. 1A, iii). Analysis of the mouse α1C-AR primary sequence showed that the gene contained two exons separated by a large intron of at least 18 kb, inserted between the coding sequences for the sixth transmembrane domain and the third extracellular loop. This gene structure was identical to that of the α1B-AR (Ramarao et al., 1992; Gao and Kunos, 1993).

Mouse α1C-AR Amino Acid Sequence. Figure 1B compares the mouse α1C-AR deduced amino acid sequence with the rat and human α1C-AR sequences. There was a 97% homology between the mouse and rat sequences and a 92% homology between the human and mouse. Most of the amino acids important for agonist binding or G protein coupling were conserved in the mouse α1C-AR (Fig. 1B legend).

α1C-AR Gene Transcription Initiation Site in Mouse Heart. We located the transcription initiation site of the α1C in adult mouse heart, using RPAs with probes from the cloned gene, followed by primer extension. As shown in Fig. 2A, RPAs were done using 50 μg of total RNA from adult mouse heart and two different probes. Probe 1 (−650 to 1 relative to the ATG) gave a specific (absent in tRNA lane) 600-bp fragment (Fig. 2A). Based on these results, probe 2 was designed further upstream (−801 to −302 relative to the ATG) and gave a specific 275 bp fragment (Fig. 2A). Together, these results suggested the presence of a single major transcription initiation site, located between ~577 and ~600 bp upstream from the ATG.

To localize the transcription initiation site exactly, a 20 bp oligonucleotide complementary to the sequence from −520 to −540 (relative to the ATG) was used in primer extension assays with 2 μg of poly(A) RNA from adult mouse heart. A single band was detected and aligned against 5′-flanking sequence of the mouse α1C (Fig. 2B). This result defined a single transcription initiation site 588 bp upstream of the ATG. The mouse α1C promoter did not have a TATA box, but the initiation site contained an Inr initiator consensus sequence TCAGATA (6/7 match to consensus sequence Py Py A-1 N A/T Py Py) (Fig. 2B) (Lo and Smale, 1996). The human α1C promoter is also TATA-less, and in a human neuroblastoma cell line has a major initiation site at an Inr consensus approximately 100 bp farther upstream of the ATG (at −696 bp) (Razik et al., 1997). This difference in start site locations between the mouse and human α1C-AR genes might be due to different positions of the same Inr sequences. The human gene in neuroblastoma cells also has minor initiation sites closer to the ATG (Razik et al., 1997).

α1C-AR mRNA Transcription in Adult Mouse Heart. To compare α1C mRNA size and tissue distribution with that in the rat (Rokosh et al., 1994; Stewart et al., 1994), and to confirm the cloned gene structure, we did a Northern blot with 30 μg of total RNA isolated from adult mouse heart,

brain, and liver, using an RNA probe directed against the first 500 bp of exon 1 (Fig. 2C). Mouse heart had a predominant α1C mRNA of 11 kb, with minor transcripts of 7.5 and 3.5 kb. Brain had similar mRNAs, and liver had none. Actin mRNA was detected in all tissues (Fig. 2C). Thus, mouse α1C-AR mRNA size and tissue distribution were similar to that found in the rat (Rokosh et al., 1994; Stewart et al., 1994). Notable is the much larger size of α1C mRNA compared with α1B or α1D ($\sim$2 kb) (Fig. 2C; Stewart et al., 1994).

Sequencing identified four potential poly-adenylation sites in the 3'-flanking sequence of the mouse α1C gene, two $\sim$2 kb 3' of the second exon and two more $\sim$8 to 8.5 kb 3' (Fig. 1A). Therefore, the size of the predominant 11 kb α1C-AR mRNA could be accounted for by $\sim$600 bp of 5'-untranslated region,

the 1.4 kb of coding sequence, and 8.5 kb of 3'-untranslated region before the final poly-adenylation signal (Fig. 1A).

α1C-AR mRNA Transcription in Mouse Heart Development. In the rat, all three α1-AR subtype mRNAs are expressed in the neonatal heart (Stewart et al., 1994). To study expression during postnatal cardiac development in the mouse, we used RPA to detect the subtype mRNAs in mouse hearts of various ages (Fig. 2D). As shown in Fig. 2D, the α1C was not detected in newborn (day 1) mouse heart of three different strains (C57BL/6, CD-1, and FVB), but was seen by weaning (day 21) in all strains. The α1B and α1D were present in the mouse heart at each time point (Fig. 2D); and all three subtypes were present in the newborn rat, confirming prior results (Stewart et al., 1994). Thus, the α1C

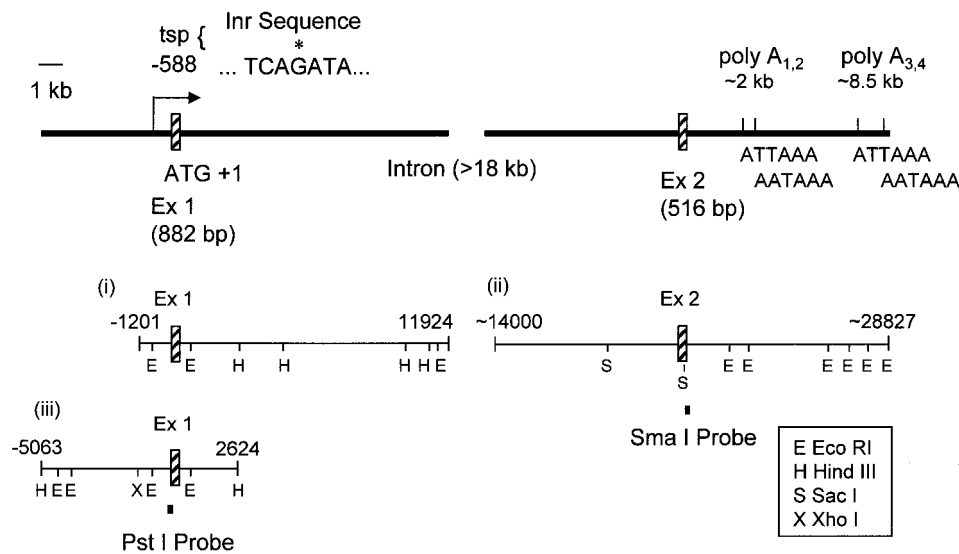## A. Structure of the Mouse α1C/A-AR Gene

B. Deduced Amino Acid Sequences

Fig. 1. Cloning of the mouse α1C-AR gene. A, structure of the mouse α1C-AR gene. The genomic organization of the mouse α1C-AR gene is represented in the upper portion of the figure, where the striped boxes represent the two exons, which are separated by an intron at least 18 kb long. The transcriptional initiation site, 588 bp upstream of the ATG (Fig. 2, below), is indicated by an arrow, and four potential polyadenylation signals are marked 3' to the second exon. The three genomic clones used to characterize the gene are labeled i, ii, and iii. The *Pst*I ($-$450 to 732 of exon 1) and *Sma*I (1267–1827 of exon 2) probes used in the cloning are shown beneath clones ii and iii. Enzyme abbreviations are given in the inset. B, alignment of α1C-AR deduced amino acid sequences from mouse, rat, and human. Dots indicate homology in all species. Nonconserved changes in amino acid sequence that could have structural significance are in boldface. Amino acids conserved with the α1B-AR and implicated in intracellular signaling are underlined, including residues important for agonist binding (C109 and S188/192) (Hwa and Perez, 1996; Perez et al., 1996) and G protein coupling (V209 to A243 and K268/A271) (Cotecchia et al., 1992; Wu et al., 1995; Perez et al., 1996). N13 is a potential site for N-linked glycosylation, and is also conserved among α1 subtypes and across species in the α1C. The location of the intron is noted by double arrows. The seven transmembrane (TM) spanning regions and three intracellular loops are indicated. Asterisks are spaced every 10 amino acids. Rat sequences are from Stewart et al. (1994), and human are from Schwinn et al. (1990).

was not an early developmental mRNA in the mouse heart, in contrast with the rat.

Identification of a Mouse α1C-AR Promoter in Cardiac Myocytes and the Role of an MCAT Element in Promoter Activity. We showed previously that the α1C-AR is transcribed in rat cardiac myocytes, and not in cardiac nonmyocytes (fibroblasts) (Stewart et al., 1994). α1-ARs are also functional in myocytes but not fibroblasts in the adult mouse heart (X. F. Deng, D. G. Rokosh, T. D. O'Connell, S. Cotecchia, and P. Simpson, in preparation). However, little is

known about mechanisms that might regulate AR transcription in specific cells such as cardiac myocytes. Here we tested fragments of the cloned mouse α1C gene for promoter activity using transfection in cultured neonatal rat cardiac myocytes. We used rat myocytes rather than mouse as a test system, because the α1C was not transcribed in neonatal mouse myocytes (Fig. 2D).

We focused on the possible role of MCAT elements in promoter activity, because MCATs are required for myocyte-specific transcription of several structural genes and for in-
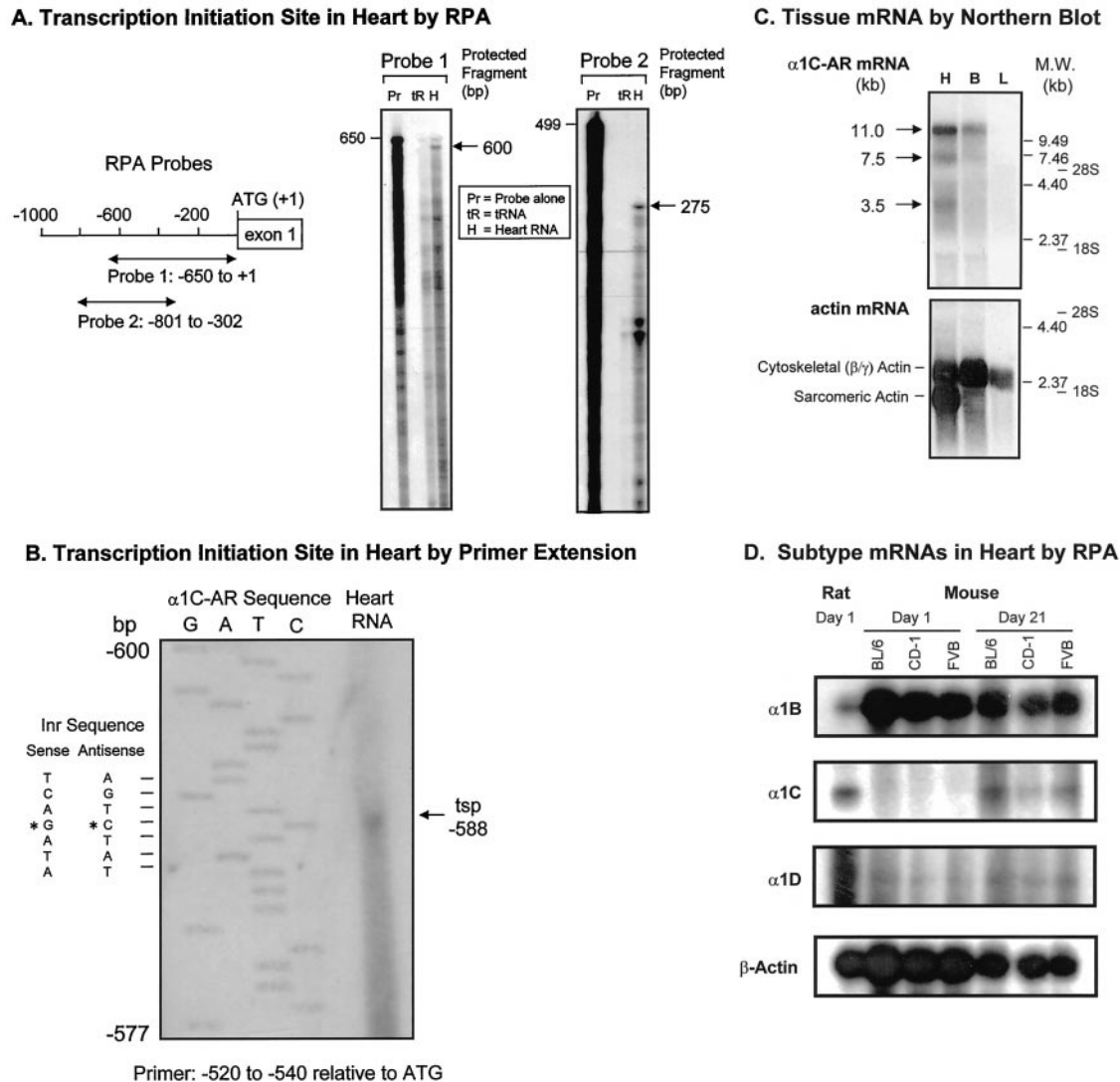

Fig. 2. Transcription of the α1C-AR in mouse heart. A, identification of the mouse α1C-AR transcriptional initiation site in heart by RPA. Antisense RNA probes from −650 to +1 and −801 to −302 (ATG +1) of the 5′-flanking sequence of the mouse α1C-AR (shown on left) were labeled with [α-^{32}P]dUTP and used in a RPA with 50 μg of total RNA from adult mouse heart or 50 μg of control tRNA. The gel lanes show the probe alone (Pr), tRNA control (tR), and heart RNA (H). Protected fragments in the heart RNA lane are labeled by approximate size, 600 bp with probe 1 and 275 bp with probe 2. These results indicate a single major transcriptional start site located approximately 577 to 600 bp upstream of the ATG. B, identification of the mouse α1C-AR transcriptional initiation site in heart by primer extension. Primer extension was used to localize the transcription initiation site precisely. Poly(A) RNA (2 μg) from mouse heart was used in a primer extension assay with a 20 bp antisense oligonucleotide primer against the sequence from −520 to −540 (ATG +1). The same primer was used to sequence DNA from the 5′-flanking sequence of the mouse α1C gene. The sequence is shown in the figure aligned with the primer extension reaction. The transcription initiation site is 588 bp upstream of the ATG and encompasses an Inr sequence TCAG(−588)ATA (sequence shown on the left). C, identification of mouse α1C-AR by Northern blot. Total RNA (30 μg) from adult mouse heart (H), brain (B), and liver (L) was probed with an [α-^{32}P]dUTP-labeled antisense RNA probe encompassing nucleotides 3 to 497 of the first exon of the α1C-AR gene (top). The same blot was stripped and reprobed with a 250 bp β-actin probe (bottom); sarcomeric (seen only in heart) and cytoskeletal actin are labeled. The molecular weights of the α1C-AR mRNAs are shown on the left. Molecular weight size markers and ribosomal RNA mobility are shown on the right. D, α1-AR subtype mRNAs in mouse heart development by RPA. Antisense RNA probes to the rat α1-AR subtypes α1B, α1C, and α1D (Rokosh et al., 1994), and to β-actin, were labeled with [α-^{32}P]dUTP and used in RPA with 25 μg of total RNA from a newborn rat heart (day 1), newborn mouse heart (day 1; one litter pooled), and weanling mouse heart (day 21). RNA was made from hearts of three different mouse strains: C57BL/6J (BL/6), CD-1, and FVB/NJ (FVB). The α1C was not detected in the newborn heart in any mouse strain.

duction of these genes by α1-AR and other hypertrophic agonists (see Introduction). However, MCAT dependence of a receptor gene has never been observed.

As diagrammed in Fig. 3A, 4.4 kb of sequence 5′ to the transcription start site contained numerous consensus elements, including seven MCATs, nine GATA elements, two a cAMP response elements (CREs), and single SP1 and AP-1 sites. As shown in Fig. 3B, a CAT reporter plasmid containing this 4.4 kb sequence was active as a promoter in cardiac myocytes. The level of activity (2.3 ± 0.2-fold versus empty reporter vector, $n = 20$, $p < 0.05$) was consistent with the lower relative level of endogenous α1C transcription, as compared with β-myosin heavy chain and skeletal α-actin, two other promoters we have studied in this identical system (Kariya et al., 1994; Karns et al., 1995).

To test whether sequences in addition to the 4.4 kb were active in myocytes, we also studied 5.8 kb of proximal intron sequence. We made heterologous reporter plasmids in thymidine kinase (tk)-CAT containing sequential intron fragments (from the end of the first exon) of 1.8, 1.8, and 2.1 kb. None of these intron reporter plasmids had detectable activity in myocytes (0.9 ± 0.1-, 0.8 ± 0.0-, and 0.9 ± 0.1-fold versus empty vector, $n = 2$).

To further define the DNA elements required for activity of the 4.4 kb fragment in myocytes, deletion mutants were constructed that contained only ~3 or ~1.3 kb of 5′ sequence, and identical 3′ ends (Fig. 3A). Full promoter activity was retained in a 1.3 kb reporter (2.2 ± 0.2-fold, $n = 10$, $p < 0.05$, Fig. 3B), whereas activity was lost in a 79-bp reporter (data not shown).

As a control, we also tested these α1C reporter plasmids in cultured rat nonmyocytes or fibroblasts, where the endogenous α1C is not transcribed. As shown in Fig. 3B, none of the reporter plasmids had detectable activity in nonmyocytes. However, the control RSV-luciferase reporter was expressed in nonmyocytes (data not shown), demonstrating that these cells were transfected. Thus, these results defined a mouse α1C promoter that mimicked the myocyte-specific expression of the endogenous α1C in heart cells (Stewart et al., 1994).

Among the seven consensus MCAT elements present in the 4.4 kb promoter (Fig. 3A), only two remained in the 1.3 kb minimal promoter, MCATs named M1 and M2 (Fig. 3B). To test whether either or both of these MCATs was required for activity, we introduced point mutations into each MCAT. We made the MCAT mutations in the context of the full-length 4.4 kb reporter plasmid, where all other potential regulatory elements were intact (Fig. 3A). As shown in Fig. 3C, mutation of MCAT M1 (at −910 from the transcription start site) reduced CAT activity of the 4.4 kb construct to levels of the empty vector (1.0 ± 0.1-fold versus empty vector, $n = 5$). Mutation of MCAT M2 (at −1041) had no effect (2.2 ± 0.1-fold, $n = 3$), and double mutation of both MCATs was the same as mutation of MCAT M1 alone (1.1 ± 0.1-fold, $n = 5$) (Fig. 3C). Thus, these results indicated that α1C-AR promoter activity in cardiac myocytes required MCAT M1 at −910. Importantly, the active MCAT M1 at −910 is conserved in the human α1C-AR promoter, whereas the inactive MCAT M2 at −1041 is not, although MCAT functionality in the human promoter was not tested (Razik et al., 1997).

TEF-1 Binds the Active α1C Promoter MCAT Element M1. MCAT elements bind members of the family of transcription factors known as TEF-1 (Larkin and Ordahl, 1998), and TEF-1-binding MCATs are required for transcrip-

tion of several contractile protein genes and cardiac BNP (see Introduction). We have studied in detail an MCAT in a 22-bp sequence (−215 to −194) in the rat β-myosin heavy chain promoter, named the proximal EC/MCAT. The EC/MCAT is required for promoter activity in myocytes and binds to TEF-1 in vitro and in vivo (Kariya et al., 1993, 1994; Stewart et al., 1998). To test whether the M1 MCAT required for α1C promoter activity also bound to TEF-1, we did competition gel mobility shift assays, using the EC/MCAT as the probe and both recombinant TEF-1 and endogenous TEF-1.
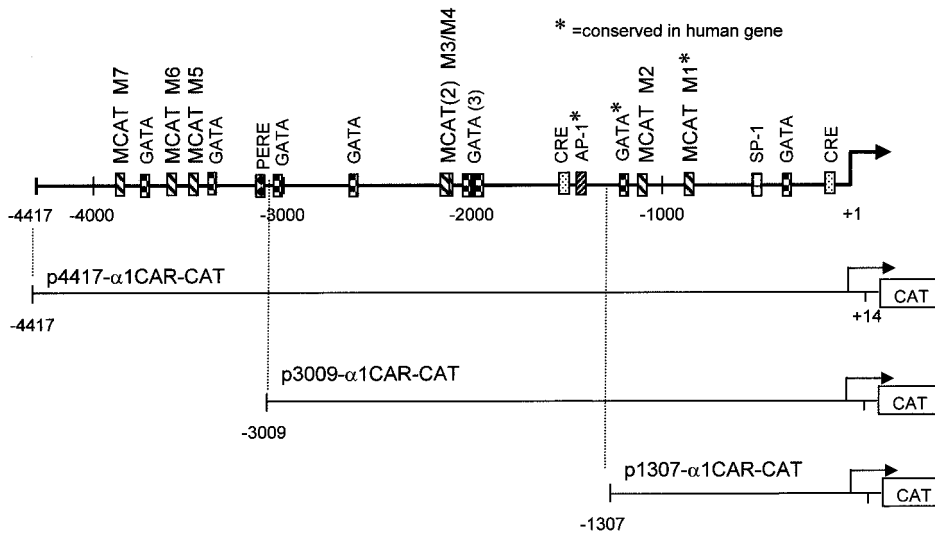
With recombinant TEF-1, the EC/MCAT probe formed one major complex (Fig. 4A, lane 2) that was competed with increasing concentrations of unlabeled EC/MCAT probe (lanes 3–5). The MCAT M1 oligonucleotide (MCAT910) also competed for TEF-1 binding (lanes 6–8), whereas a mutated version of this oligonucleotide (MCAT910 mut) did not compete (lanes 9–11), indicating that TEF-1 bound to MCAT M1. Interestingly, the MCAT M2 oligonucleotide (MCAT1041) also competed for TEF-1 binding (lanes 12–14), and more efficiently than did the EC/MCAT or the MCAT M1 oligonucleotides (lanes 12–14 versus 3–5 and 6–8).

Replicate experiments are summarized in Fig. 4B, which quantifies binding affinity and shows the approximate IC_{50} for each competing oligonucleotide. The graph shows that TEF-1 binds with lower affinity to the two active MCATs (157 nM for MCAT M1 at −910 and 24 nM for the EC/MCAT) than to an inactive MCAT (4 nM for MCAT M2 at −1041).
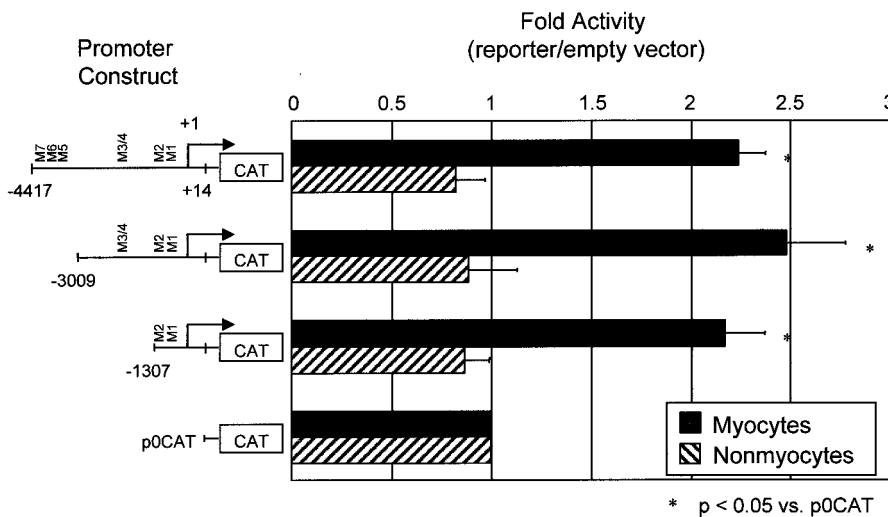
To test whether endogenous TEF-1 bound to these oligonucleotides the same as recombinant TEF-1, the gel shift assay was done with cardiac myocyte nuclear extracts (Fig. 4C). Myocyte nuclear extracts and the EC/MCAT probe produced a shifted duplex band named C2 (Fig. 4C, lanes 2, 4, 6, and 8). Earlier we showed conclusively by immunoblot of a gel shift that this band contains TEF-1 (Kariya et al., 1993, 1994). In further agreement with prior results, this complex was competed by unlabeled probe, showing specificity (Fig. 4C, lane 3). The MCAT M1 oligonucleotide (MCAT910) competed for endogenous TEF-1 binding (lane 5), whereas a mutated version of this oligonucleotide (MCAT910 mut) did not compete (lane 7). The MCAT M2 oligonucleotide (MCAT1041) also competed for endogenous TEF-1 binding (lane 9). The relative order of TEF-1 affinity for the oligonucleotides was the same for endogenous TEF-1 in these nuclear extracts: MCAT1041 > EC/MCAT > MCAT910 (lanes $9 > 3 > 5$), as for recombinant TEF-1 (Fig. 4, A and B).

Activation of the Mouse α1C-AR Promoter by Norepinephrine and Other Hypertrophic Agonists. In the rat, hypertrophic stimuli increase α1C-AR mRNA in cardiac myocytes in culture and in vivo (Rokosh et al., 1996). To test whether hypertrophic agonists stimulated the mouse α1C-AR promoter, rat myocytes were transfected with the full-length 4.4 kb α1C promoter, and then treated with various agonists for 24 h. Norepinephrine increased α1C promoter activity by ~4-fold versus vehicle. However, the norepinephrine effect was mediated entirely through a β-AR, because induction was blocked by the β-AR antagonist timolol and not by the α1-AR antagonist prazosin (Fig. 5). Phenylephrine mildly activated the promoter, but this was blocked by timolol, consistent with a slight β-AR action of 20 μM phenylephrine (Fig. 5). Endothelin and PGF2α had no effect, and PMA induced the promoter only slightly, albeit significantly (Fig. 5).

A. Mouse α1C/A-AR Promoter and Reporter Constructs

* =conserved in human gene

MCAT M7 GATA MCAT M6 MCAT M5 GATA PERE GATA GATA MCAT(2) M3/M4 GATA (3) CRE AP-1 * GATA* MCAT M2 MCAT M1 * SP-1 GATA CRE

-4417   -4000   -3000   -2000   -1000   +1

p4417-α1CAR-CAT
-4417   +14   CAT

p3009-α1CAR-CAT
-3009   CAT

p1307-α1CAR-CAT
-1307   CAT

B. Promoter Activity in Cardiac Myocytes and Nonmyocytes

Promoter Construct

Fold Activity
(reporter/empty vector)

0   0.5   1   1.5   2   2.5   3

M7 M6 M5   M3/4   M2 M1   +1
-4417   +14   CAT   *

M3/4   M2 M1
-3009   CAT   *

M2 M1
-1307   CAT   *

pOCAT   CAT

■ Myocytes
▨ Nonmyocytes

* p < 0.05 vs. pOCAT

C. Activity of Promoter MCAT Mutants in Cardiac Myocytes

Promoter Construct

Fold Activity
(reporter/empty vector)

0   0.5   1   1.5   2   2.5   3

M7 M6 M5   M3/4   M2 M1   +1
-4417   +14   CAT   *

M7 M6 M5   M3/4   M2 M1
M1 MCAT910mut   CAT   †

M7 M6 M5   M3/4   M2 M1
M2 MCAT1041mut   CAT   *

M7 M6 M5   M3/4   M2 M1
M1/2 MCATdblmut   CAT   †
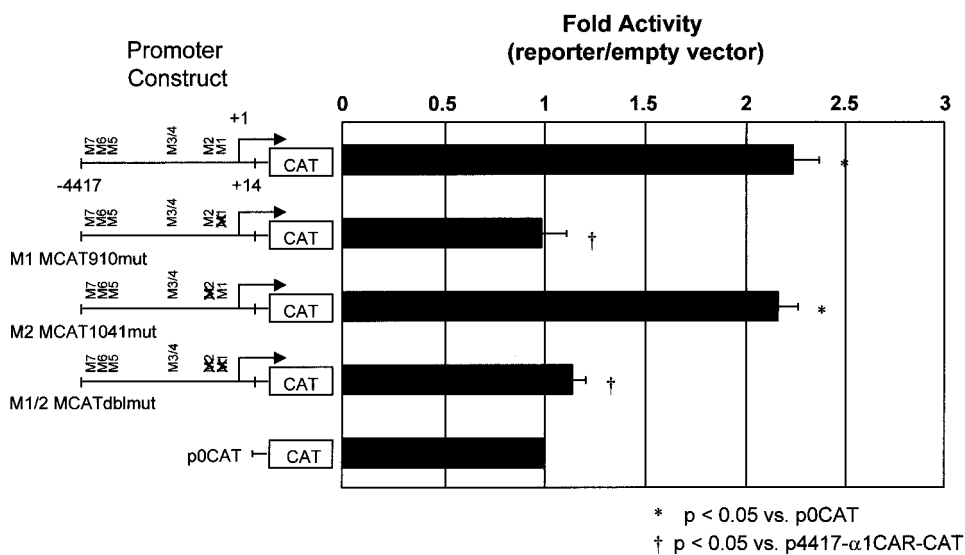
pOCAT   CAT

* p < 0.05 vs. pOCAT
† p < 0.05 vs. p4417-α1CAR-CAT

Fig. 3. Analysis of an α1C-AR promoter in rat heart cells. A, mouse α1C-AR promoter and reporter constructs. The top of the figure shows the locations of potential cis-regulatory elements (relative to the transcription initiation site), including seven consensus MCAT motifs. An asterisk indicates elements conserved with the human sequence (Razik et al., 1997). The bottom of the figure diagrams the three CAT reporter plasmids, containing ~4.4, 3.0, and 1.3 kb of sequence. B, activity of the mouse α1C-AR promoter in cardiac myocytes and nonmyocytes. The three CAT reporter plasmids containing 4417, 3009, and 1307 bp of promoter sequence were transiently transfected into primary cultures of rat neonatal cardiac myocytes and nonmyocytes. Cardiac myocytes were cultured in serum-free medium, whereas nonmyocytes were cultured in serum-supplemented medium to maximize any expression of the reporters. These were exactly the same conditions where myocytes but not fibroblasts have α1-AR mRNAs, proteins, and function (Rokosh et al., 1996, and references therein). CAT activity was measured after 24 h. The graph shows the constructs on the left and the corresponding CAT activity on the right, expressed as fold activation relative to pOCAT (empty vector). The locations of the seven MCAT elements (M1-M7) are indicated. An RSV-luciferase construct was cotransfected as a control, but CAT activities were not corrected for luciferase expression. Each experiment was performed with duplicate dishes, and data are presented as mean $\pm$ S.E.M. (n = 10–20 experiments in myocytes, n = 3 experiments in nonmyocytes). $*p$ < 0.05 versus pOCAT. C, activity of mouse α1C-AR promoter MCAT mutants in cardiac myocytes. Point mutations were made in MCATs M1, M2, or both (double, dbl) in the context of the full-length 4.4 kb α1C promoter (M1 CATGCCA mutated to CgatCgA, M2 CATACTT to CgatCgT, mutations in lowercase; see also Fig. 4A). Plasmids were transfected as in B. Values are mean $\pm$ S.E.M. (n = 3–5 experiments for each mutant construct). $*p$ < 0.05 versus pOCAT; $^\dagger p$ < 0.05 versus p4417-α1CAR-CAT.

The β-AR activation of the promoter was mediated partly by a CRE 156 bp upstream of the transcriptional start site (CRE156, CCTA<u>CGTCA</u>GGGC, site underlined). Mutation of the CRE (CCTA<u>Cccta</u>GGGC, mutations in lowercase) had

A. Recombinant TEF-1 Binds to α1C/A MCAT Elements

EC/MCAT: 5'-   CATGC<u>CATACC</u>ACAACAATGAC -3'
M1 (MCAT910):   5'- CAAGTCCC<u>CATGCC</u>AGCAAGTCGG -3'
MCAT910mut: 5'- CAAGTCCC<u>CgatCg</u>AGCAAGTCGG -3'
M2 (MCAT1041): 5'- GGGACCTG<u>CATACT</u>TTGTCTTTT -3'
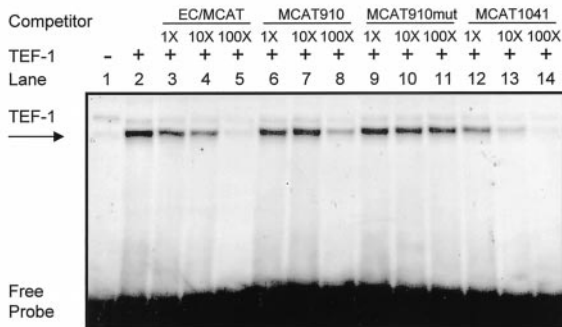
B. TEF-1 Binding Affinity for MCAT Elements
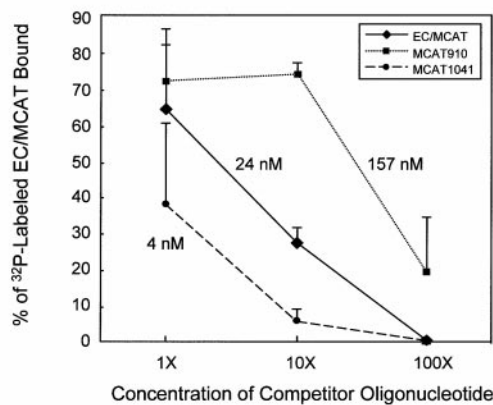
C. Myocyte TEF-1 Binds α1C/A MCAT Elements

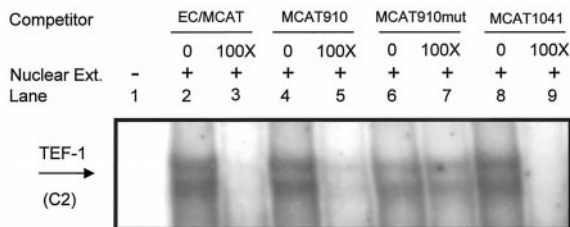**Fig. 4.** Binding of TEF-1 to MCATs in the α1C-AR promoter. A, recombinant TEF-1 binds to the α1C MCAT elements. The 22 bp rat β-myosin heavy chain proximal enhancer core/MCAT (EC/MCAT; −215/-194) (Kariya et al., 1993, 1994) was labeled with [γ-^{32}P]dATP and incubated with in vitro translated TEF-1ε, in the presence or absence of increasing amounts of unlabeled competitor oligonucleotides. Sequences of the proximal enhancer core/MCAT probe and the competitor oligonucleotides are shown at the top of the figure. Bound and free probe were separated on a 6% gel and visualized on film by autoradiography. The shifted TEF-1 band is indicated by the arrow on the left, and the fold concentrations of each competitor are indicated at the top of each lane. B, TEF-1 binding affinity for MCAT elements. Densitometry was used to quantify the gel shift bands in A and in an additional identical experiment. The graph shows the percentage of labeled probe bound to TEF-1 after competition

little effect on basal activity (data not shown), but reduced norepinephrine induction by half (2.3 ± 0.2-fold versus vehicle, $n = 3$). Mutation of MCAT M1 at −910, or of both MCAT M1 and MCAT M2 at −1041, reduced basal activities to levels of the empty vector (Fig. 3C), but did not impair norepinephrine induction (MCAT M1 5.1 ± 0.4-fold, $n = 3$; MCAT M1 and M2 4.2 ± 0.7, $n = 3$).

Interestingly, a phenylephrine ("α1-AR") response element (GGGGAGGG) as defined in the atrial natriuretic factor promoter (Ardati and Nemer, 1993) is found in the human α1C promoter (Razik et al., 1997) and was also present in the mouse promoter (PERE at −3102, Fig. 3A). However, the data in Fig. 5 indicated that this PERE was not sufficient for α1-AR activation. Indeed, there was no major activation by α1-adrenergic or other Gq-coupled agonists, and there was no MCAT-dependent stimulation of the mouse α1C promoter.

Discussion

Here we cloned and characterized the complete structure of the α1C-AR gene and characterized transcription of the gene in adult mouse heart. We defined an α1C promoter that was active in cultured neonatal rat cardiac myocytes but not fibroblasts and showed that activity of the promoter required an MCAT element that bound TEF-1. This is the first example of a receptor gene regulated by an MCAT element, and one of the few cases (Gao and Kunos, 1998) to identify a mechanism for adrenergic receptor transcription in a specific cell.

The structure of the α1C-AR gene was similar to that of the α1B-AR (Ramarao et al., 1992; Gao and Kunos, 1993), in that the gene contained two exons separated by a large intron (at least 18 kb). Similar to the major promoter of the α1B (Gao and Kunos, 1993), the α1C promoter was TATA-less, and transcription in mouse heart was initiated from a single site that contained an Inr consensus. This Inr is possibly the same one used by the human α1C promoter for transcription initiation in neural cells (Razik et al., 1997). The predominant α1C mRNA in both mouse and rat heart, ~11 kb, is much larger than the mRNAs of the α1B or α1D in heart (both ~2 kb) (Fig. 2C; Stewart et al., 1994). Here we were able to account for this long α1C mRNA by transcription from the Inr at −588 upstream of the ATG, 1.4 kb of open reading frame, and a potential polyadenylation sequence 8.5 kb downstream.

We identified a 4.4 kb fragment of mouse α1C-AR 5′-nontranscribed sequence that was active as a promoter in cultured neonatal rat myocytes but not in nonmyocytes, and thus mimicked the myocyte-specific transcription of the endogenous α1C gene in the heart. The magnitude of α1C promoter activity, ~2.5-fold over empty vector, was congruent with the much lower abundance of endogenous α1C transcripts in comparison with the structural genes β-myosin heavy chain and skeletal α-actin (Bishopric et al., 1987;

with the indicated concentration of cold oligonucleotides. The molar concentration of oligonucleotide giving 50% inhibition of binding to the EC/MCAT (IC$_{50}$) was estimated from the competition curves as ~4 nM for MCAT M2 (MCAT1041), ~24 nM for the EC/MCAT, and ~157 nM for MCAT M1 (MCAT910). The mutated version of MCAT M1 (MCAT910 mut) did not compete 50% of TEF binding even at 100×. Points are mean ± range, $n = 2$. C, endogenous TEF-1 in cardiac myocyte nuclear extracts binds to the α1C MCAT elements. Gel shift was done as in A, using 10 μg of cardiac myocyte nuclear extract. The TEF-1 shifted complex (C2) is indicated on the left (see also Kariya et al., 1993), and the unlabeled competitors (100-fold) are indicated at the top of each lane.

Waspe et al., 1990; Stewart et al., 1994; Rokosh et al., 1996); these latter promoters are activated ~10-fold over basal in the same system (Kariya et al., 1994; Karns et al., 1995).

A single MCAT element, MCAT M1 at −910 from the Inr, was required for activity of the full-length promoter in myocytes and bound recombinant and endogenous TEF-1 in gel mobility shift assay. Thus, an MCAT element that bound TEF-1 was required for α1C transcription in myocytes. This active MCAT in the α1C-AR promoter is conserved in the human gene, although its activity was not tested (Razik et al., 1997), just as an active MCAT in the rat β-myosin heavy chain promoter is conserved among species (Kariya et al., 1994). It is intriguing that an identical MCAT/TEF-1 mechanism is used in myocytes for transcription of the α1C receptor gene and for transcription of several other cardiac genes (α- and β-myosin heavy chain, skeletal α-actin, cardiac troponin T, and B-type natriuretic peptide) (see Introduction). This observation links an MCAT/TEF-1 signaling mechanism that maintains the cardiac myocyte phenotype to α1C transcription in myocytes, and suggests a possible autoregulatory system for transcription. That is, MCATs and TEF-1 promote transcription of genes that are targets for α1C signaling, such as contractile proteins and cardiac BNP (Kariya et al., 1994; MacLellan et al., 1994; Karns et al., 1995; Gupta et al., 1997; Thuerauf and Glembotski, 1997; He and LaPointe, 1999), and also transcription of the receptor gene itself. The α1B promoter similarly contains at least one MCAT (Saulnier-Blache et al., 1996; Razik et al., 1997; Gao and Kunos, 1998; Zuscik et al., 1999), although it is not

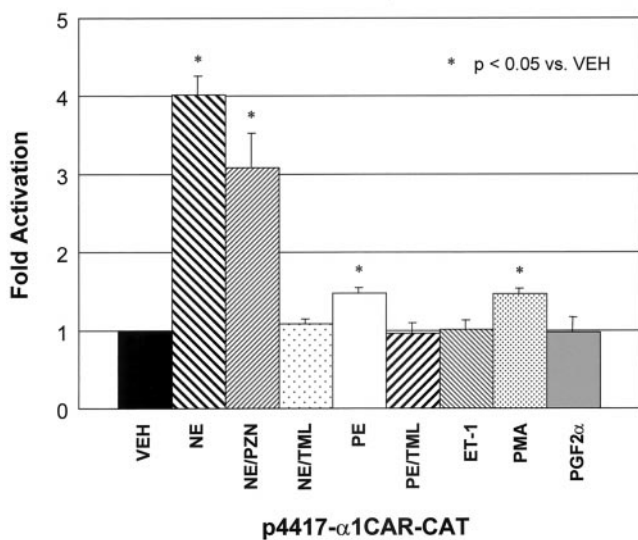## Promoter Activity with Hypertrophic Agonists

Fig. 5. Activation of the mouse α1C-AR promoter and promoter mutants by norepinephrine and other hypertrophic agonists. Cardiac myocytes on culture day 1 were transfected for 2 h with p4417-α1CAR-CAT, and then treated the next day with various hypertrophic agonists. CAT activity was measured after 24 h, and is shown here relative to the vehicle-treated control cells. An RSV-luciferase construct was cotransfected as a control, but CAT activities were not corrected for luciferase expression. Each experiment was performed with duplicate dishes, and data are presented as mean ± S.E.M. *$p < 0.05$ versus vehicle. Abbreviations of agonists and numbers of experiments (in parentheses) are as follows: doses were 2 μM except as noted: VEH, vehicle (20); NE, norepinephrine (16); NE/PZN, NE plus prazosin 0.2 μM (3); NE/TML, NE plus timolol (5); PE, phenylephrine 20 μM (4); PE/TML, PE plus timolol (4); ET-1, endothelin-1 100 nM (3); PMA 100 nM (3); PGF2α 1 μM (3).

known whether the α1B MCAT is functional. We are further exploring a possible connection in myocytes between the α1C receptor and TEF-1 using mice with gene knockouts (O'Connell et al., 2000a,b) and studies of α1C signaling. Consistent with a connection we find that TEF-1 phosphorylation is altered by α1C-AR stimulation (R. Q. To, C. Turck, and P. Simpson, unpublished observations).

It was initially surprising in this regard that the mouse α1C promoter was not activated by α1-AR or other Gq-coupled hypertrophic agonists (Fig. 5), whereas the rat α1C gene is activated robustly by hypertrophic stimuli in culture and in the intact rat (Rokosh et al., 1996; Autelitano and Woodcock, 1998). However, recent data confirm that the endogenous mouse α1C gene also differs from the rat. In the mouse, α1C mRNA in myocytes does not increase with hypertrophy in vivo (Snyder et al., 1999; Wang et al., 2000), in contrast with the rat where it does increase (Rokosh et al., 1996). For example, abdominal aortic banding, which increases α1C mRNA in rat heart (Rokosh et al., 1996), does not do so in the mouse (Snyder et al., 1999; D. G. Rokosh, P. C. Simpson, unpublished observations).

At least two mechanisms could account for the difference in α1C transcription with hypertrophic stimuli between rat and mouse: differences in intracellular signaling and/or differences in gene structure. Our results favor differences in gene structure, because we tested the mouse gene in rat myocytes, where signaling does activate the endogenous α1C. For example, the rat α1C gene might contain an "inducible" MCAT, or some other hypertrophic response element, not present in the mouse gene. In this regard it is interesting that inducible MCATs have an A or a T at the fourth position, rather than the G found in the "noninducible" MCAT M1 at −910 in the mouse α1C-AR promoter (Fig. 4A). A structural gene difference might also explain why β-AR stimulation activates the mouse promoter (Fig. 5) but not the rat (Rokosh et al., 1996). β-AR activation of the mouse α1C promoter required a CRE, rather than an MCAT/E-box as in the α-myosin heavy chain promoter (Gupta et al., 1994), and the rat gene might lack the required CRE found in the mouse. The mechanism(s) require further study, but the results do indicate important differences in transcription activation between the rat and the mouse, comparable with those seen previously with atrial natriuretic factor (Seidman et al., 1991).

Another transcription difference between rat and mouse is more probably explained by differences in signaling rather than gene structure, the delayed maturation of α1C transcription during development in the mouse. In the newborn heart, the α1C is not transcribed in the mouse, but is transcribed in the rat (Fig. 2D). Because we studied the mouse promoter in neonatal rat myocytes, we know that the structure of the mouse gene supports transcription in neonatal cells. Thus absent transcription in neonatal mouse myocytes might reflect some rat-mouse difference(s) in signaling, such as a kinase that activates TEF-1, a TEF-1-interacting protein (see below), or a mechanism that unfolds chromatin over the active mouse MCAT element.

Finally, it was noteworthy that only one of the seven MCAT consensus elements in the mouse α1C promoter was required for activity in myocytes, emphasizing the need for functional studies such as those reported here. Similar observations in other promoters (Kariya et al., 1994; Larkin and Ordahl, 1998) have raised the idea that TEF-1 requires

some additional cofactor for activity in myocytes. Indeed "active" MCATs in myocytes can have apparently lower binding affinity for TEF-1 than inactive MCATs (Kariya et al., 1993, 1994; I. K. G. Farrance and P. C. Simpson, unpublished observations), as we observed in this study with both recombinant and endogenous TEF-1 (Fig. 4). Lower affinity TEF-1 binding at an active MCAT might favor a protein conformation that allows for association with a required coactivator. A few TEF-1 coactivators are known, including Max in the α-myosin heavy chain promoter (Gupta et al., 1997) and poly(ADP-ribose) polymerase in the cardiac troponin T promoter (Butler and Ordahl, 1999). However, the active MCAT M1 at −910 is not flanked by an E-Box (CANNTG; Max binding site) as in the α-myosin heavy chain promoter (Gupta et al., 1997), or a poly(ADP-ribose) polymerase binding site (TGTTG) as in the cardiac troponin T promoter (Butler and Ordahl, 1999). In addition, MCAT element M7 (Fig. 3A) *is* flanked by a poly(ADP-ribose) polymerase-binding site, but was not required for α1C-AR promoter activity. Thus, it is likely that novel TEF-1-interacting proteins exist, such as the human protein TONDU, related to vestigal in *Drosophila* (Vaudin et al., 1999), and several labs are pursuing this idea.

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