Molecular Structure and Transcriptional Function of the Rat Vascular AT<sub>1a</sub> Angiotensin Receptor Gene

Kazuhisa Takeuchi, R. Wayne Alexander, Yasuyuki Nakamura, Takeshi Tsujino, T.J. Murphy

Rat vascular angiotensin receptors (AT<sub>1a</sub> receptors) are encoded by two mRNA transcripts sharing an identical receptor coding sequence but differing in their 5’ and 3’ untranslated sequences. We screened male Sprague-Dawley rat genomic libraries to clone the vascular AT<sub>1a</sub> receptor gene. Two sets of overlapping clones were isolated that encode over 90 kb of genomic sequence around the AT<sub>1a</sub> receptor gene. Four overlapping clones were identified from the 5’ flanking portion of the gene. These contain the promoter region and two exons, 141 bp and 89 bp in size, respectively, encoding the alternately spliced 5’ untranslated mRNA sequence. Six additional clones overlap each other but do not overlap the set of clones from the 5’ flanking region of the gene. These contain a single 1977-bp exon that encodes 900 bp of the 5’ and 3’ untranslated sequences in addition to a 1077-bp open reading frame identical to that found in vascular smooth muscle cell AT<sub>1a</sub> receptor cDNAs. Primer extension and RNase protection studies indicate that the transcription start site for this gene begins 9 bp upstream from the most 5’ sequence found within the AT<sub>1a</sub> receptor cDNAs. Our mapping studies of the cloned gene, which so far includes an uncloned gap within the second intron, indicate that the transcription start site is no less than 67 kb upstream from the receptor coding exon. Promoter-reporter assays were performed by transfection of vascular smooth muscle cells with deletions of a 3.2-kb promoter region fused to a luciferase cDNA reporter plasmid. Relatively strong basal transcriptional activity is observed from the 5’-most 2 kb of the promoter and diminishes markedly with deletions within 1 kb of the early promoter region, suggesting strong promoter elements in the more upstream regions of the gene. Deletion of a 53-bp early promoter region containing the transcription start site and a putative TATA box completely abolishes the ability of upstream elements to drive transcription of the luciferase cDNA. These results indicate that we have isolated the AT<sub>1a</sub> receptor gene and its functional promoter. (Circ Res. 1993;73:612-621.)

**Key Words** • AT<sub>1a</sub> receptors • transcriptional activity • vascular smooth muscle cells • gene • angiotensin

The circulating hormone angiotensin II is a principal determinant of blood pressure and fluid volume in vertebrates. Its diverse physiological and biochemical actions are mediated in part by activation of cell surface GTP-protein-coupled receptors, termed AT<sub>1</sub> receptors. AT<sub>1</sub> receptors are expressed in a variety of tissues including vascular smooth muscle, kidney, adrenal gland, liver, and reproductive organs and within discrete regions of the central nervous system. DNA clones encoding AT<sub>1</sub> receptors have been isolated from rat, bovine, and human sources. Two isoforms of AT<sub>1</sub> receptors, termed AT<sub>1a</sub> and AT<sub>1b</sub> receptors, which are 95% identical in amino acid sequence, are expressed in the rat and mouse. These are encoded by distinct genes under separate regulatory control, as evidenced by their tissue-specific expression pattern.

We have previously isolated two independent cDNA clones from a rat vascular smooth muscle cell (VSMC) cDNA library having identical open reading frames encoding AT<sub>1a</sub> receptors. However, the two cDNAs, termed pCa18b and pBa23,i401, differ in their 5’ and 3’ untranslated sequences. Identified within the 5’ untranslated region of clone pCa18b is an 89-bp sequence not present in the corresponding region of clone pBa23,i401, although the two cDNAs share an otherwise identical sequence in their 5’ untranslated region. Furthermore, an additional 1 kb of cDNA sequence is found in the 3’ untranslated region of clone pBa23,i401 after the corresponding terminal polyadenylated stretch of clone pCa18b. These observations suggest that the differences in sequence between the two AT<sub>1a</sub> receptor cDNAs arise from alternate splicing of exons from a common gene. In support of this, preliminary evidence has been presented suggesting that the promoter region of the AT<sub>1a</sub> receptor gene is separated from the receptor coding region by at least one intron. However, this study did not present other evidence of the AT<sub>1a</sub> receptor gene structure or functional activity of the putative promoter region. To clarify this issue more completely and to begin to explore the regulated expression of the AT<sub>1a</sub> receptor gene, we screened Sprague-Dawley rat...
Materials and Methods

All chemicals were purchased from Sigma Chemical Co, St Louis, Mo, unless otherwise indicated. Forskolin was purchased from Calbiochem Corp, San Diego, Calif. Restriction enzymes, cell culture media, and supplements were obtained from GIBCO-BRL, Gaithersburg, Md. Avian reverse transcriptase and T7 DNA polymerase (Sequenase) and sequencing reagents were purchased from United States Biochemical Corp, Cleveland, Ohio. Reagents for riboprobe synthesis and RNase protection assays were from Amobion, Inc, Austin, Tex. HATF nitrocellulose membranes were from Millipore Corp, Bedford, Mass, and nylon Hybond N blotting membranes and radiochemicals ([α-32P]dCTP, >3000 Ci/mmol; [α-32P]UTP, 800 Ci/mmol; [γ-32P]ATP, >3000 Ci/mmol; and [α-35S]dATP, 1200 Ci/mmol) were purchased from Amersham Corp, Arlington Heights, Ill. Oligonucleotides were synthesized by the Emory University Microchemical Facility. Random nonamer primer DNA labeling kits (Prime-It) and pBluescript II [pBS(SK) and pBS(SK)] were purchased from Stratagene Inc, La Jolla, Calif. Plasmids pOLuc and pSVLuc were generous gifts from Dr Allan R. Brasier through the Laboratory of Molecular Endocrinology, Massachusetts General Hospital, Harvard Medical School, Boston, with permission of Dr Suresh Subramani, University of California, San Diego. Plasmids pGEM4 and pSV8gal vectors were purchased from Promega Corp, Madison, Wis.

Library Screening

Approximately 1 x 10^8 plaques from a male Sprague-Dawley rat genomic library in λDASHII (Stratagene) were hybridized on duplicate nitrocellulose membranes with a 984-bp Kpn I–Aha II restriction fragment, corresponding to nucleotides 94 through 1078, from cDNA pCa18b (Reference 5) labeled with [α-32P]dCTP using random nonamer primers and T7 DNA polymerase. Hybridization conditions were for 10 hours at 42°C in HYB buffer (50% formamide, 6x standard saline citrate, 5x Denhardt’s solution, 100 μg/mL sheared denatured salmon testes DNA, and 0.1% sodium dodecyl sulfate).13 The membranes were washed for 1 hour at 52°C with three changes of 2x standard saline citrate and 0.1% sodium dodecyl sulfate. Positive plaques were identified by overnight autoradiography on XAR film with a single intensifying screen. These were purified by successive rounds of screening under the same conditions. Under identical hybridization and washing conditions, duplicate lifts of approximately 5 x 10^9 clones from a cosmid pWE15 Sprague-Dawley rat genomic library (Clontech, Palo Alto, Calif) were screened with a 0.8-kb [α-32P]UTP-labeled riboprobe prepared using T3 RNA polymerase from the 3' end of Rsa I–digested clone ARGL10, which was isolated from the λDASHII library (see below).

Mapping, Subcloning, Sequencing, and Southern Analysis

Restriction sites for Xba I, EcoRI, and BamHI in the genomic clones were mapped from both ends by comparing hybridization patterns for [32P] end-labeled T3 and T7 primers to restriction enzyme fragment sizes after partial and complete digestion of the genomic clones. After incubation with enzymes, the cloned DNA was separated electrophoretically on 0.7% agarose/Tris-acetate-EDTA horizontal gels. Identical duplicate gels were Southern-blotted onto Hybond N nylon membranes and hybridized in HYB buffer with either T3 or T7 oligonucleotide primers labeled with [γ-32P]ATP and T4 DNA kinase. Standard molecular cloning protocols were used for subcloning and analysis of gene fragments using pBS shuttle vectors. Selected regions of the gene were sequenced on both strands by the dyeoxy chain termination method using [α-35S]dATP with reagents and protocols supplied by the Sequenase V2.0 kit (United States Biochemical). Sequence reactions were resolved on 5% polyacrylamide/8 M urea sequencing gels run at 65 W in 1x Tris-borate-EDTA buffer. Data were analyzed using GENEPRO and the Wisconsin ggc package run on the Emory University Vax computer.

Primer Extension and Ribonuclease Protection Assays

For primer extension assays, the primer 5'–GCT-GAGAGAGAGCCGCAGCGCTGGTACCGGCC-3' was labeled with [γ-32P]ATP and T4 DNA kinase and hybridized overnight at 42°C in 40 mM/L PIPES (pH 6.4), 1 mM/L EDTA (pH 8.0), 0.4 mM/L NaCl, and 80% formamide with total RNA prepared from cultured rat aortic VSMCs. The samples were precipitated with ethanol and resuspended in a 20 μL buffer containing 50 mM/L Tris-HCl (pH 7.6), 60 mM/L KCl, 10 mM/L MgCl2, 1 mM/L of each dNTP, 1 mM/L dithiothreitol, 20 U RNasin (Amersham), 1 μg actinomycin D, and 50 U avian reverse transcriptase and incubated for 2 hours at 42°C. The products were analyzed by resolution on a 5% polyacrylamide/8 M urea sequencing gel and overnight autoradiography on XAR film with a single intensifying screen. The sizes of extended products were measured using both a radio-labeled Hae III–digested φX174 ladder and a sequencing reaction ladder from the M13mp18 template using the –40 primer supplied in the Sequenase kit. RNase protection assays were performed using a riboprobe spanning both exon 1 and the proximal portion of the promoter region. A 3.3-kb Spe I–Kpn I restriction fragment of the promoter region was subcloned into compatible sites in pBS(SK) and linearized with Pst I, and the ends were filled in with the Klenow fragment. This was used as a template for synthesizing an [α-35S]UTP–labeled 185-base riboprobe using T7 RNA polymerase with reagents and protocols supplied in the MAXiscript Kit (Ambion). Total RNA (10 μg) prepared from rat tissues was hybridized overnight with 2.3 x 10^5 cpm of gel-purified riboprobe and then digested with RNase A and RNase T1 using reagents and protocols supplied with the RPAII kit (Ambion). The reaction products were resolved on 5% polyacrylamide/8.3 M/L urea sequencing gels and analyzed after 48-hour autoradiography on XAR film with a single intensifying screen.
Luciferase Reporter Vector Constructions

To create promoter/reporter chimeric constructs, a series of nested restriction fragment deletions of the AT<sub>1</sub> receptor gene promoter region was subcloned into pLuc, a modified pGEM vector containing the cDNA for firefly luciferase.<sup>14</sup> The following restriction fragments from the 5′ end of λARGL3 were subcloned by directed ligation into pBS(SK): SpeI-KpnI (−3231/+104), XbaI-KpnI (−558/+104), HindIII-KpnI (−331/+104), PstI-KpnI (−54/+104), EcoRI-KpnI (−987/+104), and EcoRI-PstI (−987/−54) (see Figs 1 and 2). To create a 2.1-kb EcoRI-KpnI fragment (−1969/+104) from λARGL3, first replace EcoRI-ApaI linker with a 1.2-kb ApaI–KpnI fragment (−1074 to +104 bp) from λARGL3 by ligation, and then place by ligation into pBS. To subclone these fragments linearized with SpeI and recircularized by ligation to a phosphorylated, self-complementary SpeI linker containing an internal BamHI site (5′-CGGATCCGAGCT-TGTTCG),<sup>10</sup> to subclone the 1.1-kb EcoRI–KpnI fragment (−987 to +104 bp) into pLuc, this fragment was isolated from λARGL3, flushed with the Klenow fragment, ligated with a Smal–BamHI adapter (purchased from Stratagene), digested with BamHI, and then ligated into the BamHI site of pLuc. pBS clones containing the +104-bp KpnI site were linearized with KpnI and recircularized by ligation with a phosphorylated synthetic self-complementary KpnI linker containing an internal BamHI site (5′-CGGATCCGAGCT-TGTTCG) to place a BamHI restriction site at the 3′ end of each of these fragments. All inserts were then excised with BamHI and ligated to the BamHI site of pLuc upstream from the luciferase cDNA. The correct 5′ to 3′ orientation of the promoter fragments relative to the luciferase cDNA was confirmed by restriction mapping analysis.

VSMC Transfections

Promoter–luciferase reporter plasmid DNA for VSMC transfections were prepared by alkaline lysis of 500-mL bacterial cultures and purified using a differential precipitation protocol with LiCl and polyethylene glycol according to standard methods.<sup>13</sup> Rat VSMCs (strain 0413, passages 11 through 19) grown in a humidified 5% CO<sub>2</sub> atmosphere were seeded onto 6-cm culture dishes 1 day before transfection in Dulbecco’s modified Eagle medium/F-12 media supplemented with 10% heat-inactivated calf serum and penicillin/streptomycin. Transfections were performed on cells at approximately 70% confluence using a modification of the CaPO<sub>4</sub> coprecipitation method. A 25–μg mixture of plasmid DNA in 450-μL deionized H<sub>2</sub>O was mixed with 500 μL of 2× HBS (mmol/L: NaCl, 280; KCl, 10; Na<sub>2</sub>PO<sub>4</sub>, 1.5; dextrose, 12; and HEPES, 50; pH 7.05 to 7.10) and then gently mixed with 50 μL of 2.5 mol/L CaCl<sub>2</sub>. This was allowed to sit for 10 minutes at 22°C until a fine precipitate formed. The mixture was then added dropwise to VSMCs, which were then returned to the incubator for 6 hours. After this, the medium was aspirated, and the cells were rinsed once with 15% glycerol in phosphate-buffered saline (PBS), pH 7.4, washed twice with PBS, and incubated an additional 48 hours in the culture medium. DNA mixtures for each transfection included equimolar amounts of AT<sub>1</sub> receptor promoter–luciferase reporter plasmid [equivalent to 15 μg of p(−3231/+104)Luc], 10 μg pSVβgal, and a mass balance of pGEM4 to account for the differences in mass of the promoter sequences in the reporter constructs.

Luciferase and β-Galactosidase Assays

After a 48-hour culture, the cells were washed in situ twice with PBS, and enzyme assays were performed. Luciferase assays are a modification of the method by deWet et al.<sup>15</sup> Washed cells were lysed for 10 minutes at 22°C by adding 0.2 mL lysis buffer (25 mmol/L Tris-P0<sub>4</sub> [pH 7.8], 2 mmol/L dithiothreitol, 2 mmol/L 1,2-diaminocyclohexane-N,N,N',N''-tetraacetic acid, 10% glycerol, and 1% Triton X-100). The lysates were scraped with a rubber policeman, transferred to 1.5-mL microcentrifuge tubes, and spun at 13,000 rpm in a microcentrifuge for 10 minutes. Supernatant (50 μL) was added to 350 μL assay buffer (mmol/L: Tris-P0<sub>4</sub>, 25 [pH 7.8]; MgSO<sub>4</sub>, 20; and ATP, 5) in an 0.8×50-mm polypropylene tube. The tube was placed in a luminometer (model TD-20e, Turner Designs, Sunnyvale, Calif). 100 μL of 1 mmol/L luciferin was injected, and integrated peak luminescence was measured over a 10-second window after a 5-second delay. When standard reagents were used under these conditions, the lower limit of detection was 0.4 fg firefly luciferase (purchased from Sigma) with an intra-assay variation of 6.8% at 40 fg firefly luciferase (data not shown). β-Galactosidase activity was used to normalize for variations in transfection efficiency and was determined by a 150-minute incubation of 50 μL cell extract with 150 μL of 2× β-galactosidase assay buffer (120 mmol/L Na<sub>2</sub>PO<sub>4</sub>, 80 mmol/L Na<sub>2</sub>PO<sub>4</sub>, 2 mmol/L MgCl<sub>2</sub>, 100 mmol/L 2-mercaptoethanol, and 13.3 mg/mL o-nitrophenol-β-d-galactopyranoside) in a final volume of 300 μL. The reaction was stopped by the addition of 500 μL Na<sub>2</sub>C<sub>2</sub>O<sub>4</sub>, and the absorbance at 420 nm was measured on a spectrophotometer.

Results

Genomic Cloning, Mapping, and Analysis

Seven clones belonging to two sets of overlapping genomic fragments were isolated from a ADASHII male Sprague-Dawley rat genomic library (Fig 1). Restriction enzyme sites for XbaI, BamHI, and EcoRI were mapped from both ends in clones ARGL3, ARGL10, ARGL8, ARGL1, and ARGL5. This latter clone was isolated using a riboprobe prepared from the 3′ end of ARGL1. Restriction fragments corresponding to overlapping domains of the genomic clones and those that hybridized to probes prepared from the AT<sub>1</sub> receptor-encoding cDNAs pCa18b and pBa23:i401 were isolated and sequenced. The 5′ clone set includes λARGL3 (14 kb), λARGL10 (15 kb), and λARGL12 (15 kb). Analysis of these indicates that λARGL10 and λARGL12 are nearly identical and overlap with λARGL3, which extends upstream into the 5′ flanking region an additional 6.0 kb. Clone λARGL3 contains a 3.6-kb XbaI 1 restriction fragment, not found in λARGL10 or λARGL12, which hybridizes to a cDNA probe encoding the most 5′ untranslated
sequence of the vascular AT1a receptor cDNA pCa18b (data not shown). These three clones do not hybridize with cDNA probes containing only the AT1a receptor coding sequence. However, clones that do hybridize with probes containing the AT1a receptor coding sequence include λRGL8 (20 kb), λRGL1 (17 kb), λRGL4 (16 kb), and λRGL5 (13 kb). Analysis of these indicates that each encodes the receptor coding sequence but not the sequence from the most 5' untranslated domains of the cDNAs. A 2.2-kb Xba I restriction fragment from λRGL8, whose 3' end was donated by the λDASHII vector sequence, hybridized to AT1a receptor cDNA probes. This fragment was subcloned into the expression vector pCDM8 and has been shown to express angiotensin II binding activity when expressed in COS-7 cells (data not shown).

These data indicated that an uncloned gap existed in the cloned AT1a receptor gene between the 5' flanking region and the region containing the receptor coding exon. Several probes were prepared from the 3' end of λRGL10 and λRGL12 and the 5' end of λRGL8 in order to fill in this gap region by a genomic walking strategy. However, Southern hybridization studies using Sprague-Dawley rat genomic DNA indicated that only a single riboprobe prepared from the 3' end of Rsa I-digested ARGL10 would likely yield informative results, because all other probes tested appeared to contain one or more highly repetitive elements (data not shown). Using this riboprobe, a 34-kb cosmid clone (cosRGL3) was isolated from a pWE15 Sprague-Dawley rat cosmid library and was shown by restriction mapping and sequence analysis to overlap over a 2-kb region with the 3' end of clone λRGL10. Additional attempts to further clone the gap region were unsuccessful.

Since the AT1a receptor cDNA pBa23.i401 has an additional 1 kb of sequence positioned downstream from the 3' end of pCa18b, this may be encoded by one or more additional exons that should be found 3' flanking exon 3. Several attempts to clone the genomic sequence encoding this region of the cDNA were unsuccessful. Database sequence analysis of the 3' untranslated sequence of pBa23.i401 reveals that its first and last 180 bp of sequences are 95% to 99% identical to B2 repetitive elements, and probes prepared from these regions of the cDNA hybridize to several restriction fragments from the genomic clones. Further, probes prepared from the middle region of this cDNA sequence hybridize strongly to DNA fragments within the phage vector sequence. Because of these technical limitations, further exploration of the genomic origin of the additional 1 kb of cDNA sequence found in pBa23.i401 proved fruitless.

Our analysis of the genomic clones isolated in this study yield a model of the physical structure of the rat AT1a receptor gene (Fig 1). Clone λRGL3 contains the most 5' flanking DNA of the gene and includes approximately 6 kb of the gene upstream from exon 1, which encodes 141 bp of the most 5' untranslated sequence common to both cDNAs pCa18b and pBa23.i401.
sequence within a 2.0-kb EcoRI fragment in λARGL3 that is also found in λARGL10 and λARGL12. Exon 2 is separated from exon 1 by an 8.3-kb intron. The AT1a receptor protein coding region, in addition to 900 bp of the 5' and 3' untranslated sequences, is encoded within 1977 bp of exon 3. Exon 3 is found within clones λARGL8, λARGL4, λARGL5, λARGL1, and λARGL53. The size of intron 2, which separates exons 2 and 3, could not be determined precisely since our clones do not overlap in this region, but intron 2 is no less than 59 kb in size.

**Promoter Region Sequence Analysis**

Fig 2A presents 3.4 kb of the genomic DNA sequence including 3231 bp that begins 11 bp upstream from the sequence common to the most 5' untranslated regions of both vascular AT1a receptor cDNAs. A computer-assisted search was performed on this sequence to look for exact matches with well-defined transcriptional control elements. Sites for interaction with the RNA polymerase II transcription complex including TATA and GC boxes (Sp1) are found proximally upstream from the transcription start site at positions −30 and −95 bp, respectively. A pair of tandem Sp1 elements are also present much further upstream beginning at −2446 bp. Other notable sequences in this region of the gene that match perfectly the consensus elements include a protein kinase C-responsive element (AP-1) found at position −387 bp, a protein kinase C-responsive element (APRE-1) were identified with the query sequence TCAC 25 bp and 20 bp, four LF-A1 liver-associated elements at positions −2879, −2599, −2513, and −1445 bp, and an anterior pituitary-specific enhancer element at −1700 bp. An enhancer corelike element is found in the distal 5' flanking region of the promoter at position −3045 bp. Seven potential alternative phase response elements (APRE-1) were identified with the query sequence TCAC 24 at positions −3088, −2991, −2745, −2062, −1930, −1162, and −968 bp. An element identical to the chicken lysozyme silencer 1, which lacks position and orientation dependence in that gene, is observed within exon 1 at +37 bp. Also downstream from the transcription start site at position +86 of the first exon is an AP-2 enhancer element, which, like AP-1 elements, is responsive to phorbol esters and protein kinase C. The β-globin promoter is a typical example of an AP-2 element.

**Determination of the Transcription Start Site**

The beginning of the AT1a receptor cDNA sequences is located 39 bp downstream from the putative TATA box and is shown in Fig 2 as beginning at base +11 and ending at base +141. Primer extension analysis and RNase protection assays were performed to determine the mRNA transcription start site. The 30-base oligonucleotide primer used in primer extension assays is a reverse complement of the sequence shown in Fig 2 from position +124 to +96. The largest extended radiolabeled DNA product we observed was 125 bp in size (Fig 3A). This corresponds to a transcription start site located 11 bp upstream from the 5'-most sequence found in the AT1a gene.
receptor cDNAs and is indicated as position +1 in Fig 2. Evidence of primer-mediated extension was not observed when yeast RNA was substituted as control for VSMC RNA in these experiments (data not shown). However, since a comparatively abundant amount of incorporated radioactivity was observed in shorter extended products, which are approximately 99 and 88 bp in size, RNase protection assays were performed to see if these bands represented transcription start sites (Fig 3B). The riboprobe used in this assay is a 186-base antisense RNA runoff from a Pst I-digested 3.2-kb Spe I–Kpn I genomic DNA template subcloned into pBluescript SK+. This Pst I site is located at position −54 in the promoter sequence, which is 158 bp upstream from the Kpn I site (+105 bp) found in exon 1. If transcription begins at position +1 in the sequence presented in Fig 2, as the primer extension data suggest, this probe would protect a 105-bp fragment following digestion with RNases after hybridization to tissues expressing AT1a receptor mRNA. Fig 3B demonstrates this result showing a protected fragment running at approximately 105 bp following RNase protection of total RNA prepared from VSMCs, kidney, and liver. Protected bands were not evident when yeast RNA was used as a control (data not shown). Further, no additional protected fragments of shorter or longer lengths are evident in these exper-
Sequence of the Rat AT1<sub>a</sub> Receptor Gene
Exon/Intron Junctions

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<thead>
<tr>
<th>Junction</th>
<th>Upstream</th>
<th>Downstream</th>
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<tr>
<td>Exon 1–Intron 1</td>
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</tr>
<tr>
<td>Exon 3–Polyadenylation site</td>
<td>TTATGTGAATGT</td>
<td>...</td>
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The exon sequence is capitalized, and the intron sequence is in lowercase letters. The GT/AG splice junction sequences of introns 2 and 3 are underlined. The 3’ end of exon 3 is either spliced to additional exons or serves as substrate for polyadenylation.

...ments, suggesting it is unlikely that the additional intensely radioactive but smaller fragments observed in the primer extension experiments result from alternate transcription start sites within exon 1.

Alternate 3’ Transcript Processing?
The exon/intron junctions surrounding exons 1, 2, and 3 are presented in the Table. The 3’ splice junctions for intron 1 and intron 2 have consensus domains for splicing of group II genes. Both introns 1 and 2 begin with a GT dinucleotide and end with an AG pair, consistent with the GT-AG splice rule.27 The possibility of an unusual splicing event occurring at the 3’ end of exon 3 is presumed from the sequence differences between the two cDNAs. Clone pCa18b ends with the sequence 5'-...TTGTAAATGT[poly(A+)]-3'. The terminal pentanucleotide AATGT found in pCa18b is absent in the corresponding region of clone pBa23;i401, which instead has a 9-base stretch of polyadenylated before continuing with an additional 1 kb of 3’ untranslated sequence. RNase protection studies support the hypothesis of alternate splicing in this region of the AT<sub>a</sub> receptor gene (Fig 4). RNase protection of an mRNA doublet is observed when the probe is used as an antisense prepared from the 3’ end of clone pCa18b that spans the putative splicing polymorphism. The difference in size between these two observed protected fragments is consistent with the observed sequence differences of cDNAs pCa18b and pBa23;i401 in this region. Further, Northern hybridization studies with AT<sub>a</sub> receptor cDNA probes reveal two distinct hybridizing transcripts approximately 2.3 and 3.4 kb in size that are consistent with the sizes of the two AT<sub>a</sub> receptor cDNAs.3 These two transcripts are found in mRNA prepared from tissues wherein hybridization is evident for the AT<sub>a</sub> receptor but not the AT<sub>b</sub> receptor, such as eye and lung.8,11 and are thereby unlikely to reflect cross hybridization during Northern analysis to both AT<sub>a</sub> and AT<sub>b</sub> receptor transcripts. The simplest interpretation of these observations is that the 2.3- and 3.4-kb mRNA transcripts arise from a single gene encoding the AT<sub>a</sub> receptor.

Southern Analysis
Southern hybridization studies were performed using a 100-bp BstXI–KpnI probe prepared from exon 1 (Fig 5). This was performed to compare the hybridization patterns of restriction enzyme fragments in rat genomic DNA and DNA prepared from the genomic clones. The probe hybridizes to a single restriction fragment identically sized in both genomic DNA and in the cloned genomic DNA irrespective of the enzyme or enzyme combinations used to prepare the DNAs. This result indicates that the AT<sub>a</sub> receptor is a single-copy gene and that the cloned DNAs are identical to the native gene in this region.

Promoter/Luciferase Reporter Deletion Analysis
Seven luciferase expression constructs containing deletions over 3.2 kb of the promoter region were prepared by ligation of restriction enzyme fragments into the enhancerless luciferase cDNA reporter plasmid poLuc.14 Molar equivalents of the luciferase constructs were cotransfected into cultured rat VSMCs with the plasmid pSVβgal, so that luciferase activity could be normalized for variations in transfection efficiencies. Basal promoter activities of these fragments, as assessed by their ability to drive luciferase cDNA expression, are presented in Fig 6 as luciferase activity in Turner light units, normalized for spectrophotometric analysis of β-galactosidase activity (absorbance at 420 nm). The 3.2-kb p(–3231/+104)Luc construct promoter activity is 23-fold higher than that observed in cells transfected with poLuc, which lacks any promoter sequence, and
exon 1 genomic DNA were approximated 99-bp exposed gion driving which is activity 1 pg h 50% p(987/-54)Luc and to the HindIII site [p(-331/+104)Luc] markedly attenuates the ability of the cloned promoter to drive luciferase expression. The p(-54/+104)Luc construct removes the putative Sp1 elements but leaves intact the TATA box and transcription start site. Expression from this construct is lower than that for next larger p(-331/+104)Luc fragment and is only twofold to threefold above that found for poLuc and is therefore likely near the minimal promoter unit for this gene. The p(-987/-54)Luc deletion removes the putative AP2 and TATA elements and the transcription start site from the early promoter region and lacks the 105 bp of exon 1 to the Kpn I site. Promoter activity from this construct is indistinguishable from that found for poLuc alone. The lack of promoter activity from this construct supports strongly the notion that this region of the rat vascular AT1a receptor gene is the functional promoter. By contrast, when deletional analysis is performed with these constructs transfected into Hela cells, which do not express measurable AT1a receptors, the promoter activity from each of the AT1a receptor gene luciferase constructs is at best twofold higher than that observed for poLuc and 20-fold less than that observed for pSV2Luc (data not shown).

Discussion

Clones encoding over 90 kb of the rat AT1a receptor gene were isolated from two genomic libraries. Four of the isolated clones encode the 5' portion of the gene, and five overlapping clones encode the receptor-coding exon and the 3' portion of the gene. The conservation of 5' flanking region of the p(-3231/+104)Luc construct. Deletion of an additional 982 bp of the 5' flanking region of the promoter to a second EcoRI site at position -987 bp [the p(-987/+104)Luc construct] reduces this activity by almost 50%. Further deletion to the Xba I site [p(-558/+104)Luc] and to the HindIII site [p(-331/+104)Luc] markedly attenuates the ability of the cloned promoter to drive luciferase expression. The p(-54/+104)Luc construct removes the putative Sp1 elements but leaves intact the TATA box and transcription start site. Expression from this construct is lower than that for next larger p(-331/+104)Luc fragment and is only twofold to threefold above that found for poLuc and is therefore likely near the minimal promoter unit for this gene. The p(-987/-54)Luc deletion removes the putative AP2 and TATA elements and the transcription start site from the early promoter region and lacks the 105 bp of exon 1 to the Kpn I site. Promoter activity from this construct is indistinguishable from that found for poLuc alone. The lack of promoter activity from this construct supports strongly the notion that this region of the rat vascular AT1a receptor gene is the functional promoter. By contrast, when deletional analysis is performed with these constructs transfected into Hela cells, which do not express measurable AT1a receptors, the promoter activity from each of the AT1a receptor gene luciferase constructs is at best twofold higher than that observed for poLuc and 20-fold less than that observed for pSV2Luc (data not shown).

Discussion

Clones encoding over 90 kb of the rat AT1a receptor gene were isolated from two genomic libraries. Four of the isolated clones encode the 5' portion of the gene, and five overlapping clones encode the receptor-coding exon and the 3' portion of the gene. The conservation of
structure among the overlapping regions of each of the isolated clones suggests that the model of the gene structure is uncomplicated by cloning artifacts. The alternate splicing model of the ATa receptor gene predicts two transcripts that correspond to the cDNAs pCa18b and pBa23,i401 (Fig 1). Exons 1 (141 bp), 2 (89 bp), and 3 (1977 bp) are spliced together to form a 2.2-kb transcript represented by clone pCa18b. This appears to be the most abundantly processed transcript in tissues expressing ATa receptors. We propose that exons 1 and 3, but not exon 2, are joined to form a second, less abundant 3.3-kb transcript represented by clone pBa23,i401. Presumably, an additional 3' flanking exon or exons are joined to form the additional 1 kb of sequence found in this clone, but those putative exons could not be identified in this study. It is within the realm of possibilities that exon 2 can be spliced into the larger transcript or spliced out of the smaller transcript, but this has not been tested in this study.

As for many, but not all, of the G protein–coupled receptors, the entire receptor coding region is uninterrupted by introns. Nevertheless, the untranslated regions of the ATa receptor mRNA result from alternate splicing of at least two additional exons, and each of these exons is separated from the others by a considerable distance. Although we have isolated approximately 90 kb of largely contiguous genomic clones around the rat vascular ATa receptor gene, we have still been unable to place a more precise size limit on the gene. Two regions that have proven refractory to cloning include the gap within intron 2 and the additional sequence 3' flanking the receptor open reading frame. Attempts to clone the gap region within intron 2 were complicated by considerable difficulties in making specific probes to this region. It is likely that the existence of several short repetitive elements within this intronic sequence complicated our genomic walking strategy. Presently, we assume that this region of the gene encodes only an intronic sequence, but further experiments will be necessary to determine if this is correct. Notably, these rat ATa gene fragments do not overlap within this gap region with genomic clones encoding over 30 kb of the rat ATb receptor (authors' unpublished observations).

Since a contiguous overlapping gene could not be isolated, we were concerned about the relation of the 5' flanking region found in ARGL3 to the receptor coding exon represented in ARGL8. Southern hybridization restriction analysis using a 100-bp genomic DNA probe encoding a portion of exon 1 demonstrates that the cloned DNA is identical to native genomic DNA (Fig 5). This probe also identifies a single-copy gene in rat genomic DNA, indicating that no other region of the rat genome is similar to that which has been cloned in ARGL3. This supports our conclusion that this is the promoter region of the vascular ATa receptor gene.

We presumed that the sequence encoding the additional 1 kb of the 3' untranslated sequence found in the pBa23,i401 cDNA but not the pCa18b cDNA would be found in the 3' flanking region of the cloned gene. However, this does not seem to be the case. If this additional sequence is encoded by one or more additional exons, they must be more than 10 kb downstream from exon 3, since they are not present in these clones. Analysis of the 3' untranslated region of pBa23,i401 reveals that it is composed of at least three domains. The first and last 180 bp are virtually identical to B2 repetitive elements, which are highly interspersed elements (>106 copies per genome) that have evolved by retrotransposition.16,28 Probes prepared from this region of pBa23,i401 hybridize to several restriction fragments along the length of the cloned ATa receptor gene at both 5' and 3' of the receptor coding exon and uniformly to Southern blots of endonuclease-treated genomic DNA (data not shown). The middle 646 bp of the pBa23,i401 sequence does not correspond to any other sequence reported in the nucleotide databases. Probes made from this region of the 3' untranslated region fail to hybridize to any fragments of our cloned rat DNA but strongly hybridize to cosmids and phage genomic cloning vector sequences (data not shown), thus rendering any library screening strategy using this fragment.

B2 repetitive elements have been suggested to function in regulation of gene expression by modulation of mRNA transport and stability.29,30 This may provide clues as to the mechanisms that may affect ATa receptor gene expression and ultimately the regulation of ATa receptor expression at the cell surface. Further experiments to test these possibilities are needed. However, it remains possible that the additional 3' untranslated region found in pBa23,i401 is simply a cloning artifact that occurred in the construction of the original VSMC cDNA expression library. We feel that this is unlikely since Northern analysis indicates that ATa cDNA probes hybridized to both 2.2- and 3.2-kb mRNA species in those tissues in which only the ATa receptor subtype is expressed. This larger transcript corresponds in size to the insert in pBa23,i401. It is unlikely that this larger transcript comes from the ATa receptor gene since riboprobes specific for that gene product fail to reveal measurable ATb mRNA expression in tissues such as liver and VSMCs, in which the 3.2-kb transcript is abun-dantly expressed and which only appear to express the ATa receptor transcript.11 Additionally, the RNase protection assay using an ATa receptor–specific riboprobe presented in this manuscript demonstrates two protected fragments, consistent with the differences observed in the two cDNAs, suggesting that either alternative splicing or polyadenylation occurs at the 3' region of exon 3.

The point at which transcription is initiated in the vascular ATa receptor gene was determined by both primer extension studies and RNase protection assays. The results of these two approaches are in reasonable agreement and position the transcription start site 30 bp downstream from the putative TATA box, consistent with the known orientation of the TATA box relative to the transcription start site in most genes.10 The sequence of the transcription start site (dA after dC) is also consistent with that common to transcription start sites.18 Further supporting these data is the failure of p(-987/-54)Luc to drive the expression of luciferase activity when transfected into VSMCs. This construct includes a deletion of a 158-bp Pr 1-Kpa I fragment encoding 54 bp of the promoter region containing the TATA box and transcription start site and 104 bp of exon 1. Loss of promoter activity from this deletion suggests this 158-bp early promoter fragment contains elements essential for transcriptional activity in VSMCs.
The ability of regions of the promoter to drive transcription in rat VSMCs was tested by measuring basal transcriptional activity from promoter region restriction enzyme deletions fused to a luciferase cDNA. Each of several deletions of over 3 kb of the promoter region is capable of efficiently driving the expression of the luciferase reporter cDNA in VSMCs but not in Hela cells. This and previously discussed data strongly support our conclusion that this is the promoter region of the vascular AT\(_1\) receptor gene. The smallest construct tested, p(-351/+104)Luc, consistently drives luciferase transcription to a level threefold over that seen from the promoterless construct pOluc and is therefore transcriptionally active. Our deletion analysis suggests that more distal elements are required for enhanced transcription of the AT\(_1\) receptor gene. When an additional sequence from the promoter region is added in front of the luciferase cDNA, the transcription steadily increases to a maximum of 30-fold from the p(-1969/+104)Luc construct over that from pOluc control. Luciferase expression is slightly suppressed (23-fold above pOluc activity) from the largest construct tested in these studies, p(-3231/+104)Luc, compared with that seen for p(-1969/+104)Luc. Although slight, this suppression is consistently observed and suggests that negative regulatory elements may reside in the -3231- to -1969-bp region. Further experiments will be necessary to explore these issues. It will be of interest to perform these experiments in cells expressing AT\(_1\) receptors from other tissues to determine if this pattern of promoter activity is restricted to smooth muscle cells. Such studies may provide insights into tissue-specific factors modulating AT\(_1\) receptor gene transcription in smooth muscle and non-smooth muscle tissues. Additional studies are in progress to explore interactions between well-characterized signal transduction pathways and their potential roles in regulating AT\(_1\) receptor gene expression. Such studies may provide insight into the role of alterations in transcriptional activity in mechanisms of upregulation and downregulation of AT\(_1\) receptors in smooth muscle cells.

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