Identification of a Negative Cis-regulatory Element and Trans-acting Protein That Inhibit Transcription of the Angiotensin II Type 1a Receptor Gene*

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The rat angiotensin II type 1a receptor (AT1a-R) gene is expressed in a cell-specific manner. We demonstrated that the negative regulatory element (NRE) between -489 and -331 is active in PC12 cells (Murasawa, S., Matsubara, H., Urakami, M., and Inada, M. 1993 J. Biol. Chem. 268, 26996-27003). Gel retardation assays confirmed that PC12 cells have a trans-acting factor bound to the NRE. By means of a DNase I footprint assay we identified the core of the NRE as an (A + T)-rich sequence (TAATCTTTATTTTA) located at nucleotides -456 to -442. Oligonucleotides corresponding to the NRE core directed mutagenesis at nucleotides -451 to -448 eliminated the specific protein/DNA binding and restored expression of the AT1a-R in transient transfection assays (2.7-fold increase). The NRE did not negatively affect the thymidine kinase promoter. No homology was found with known NREs, suggesting that this is a novel NRE. Southwestern blotting revealed a 53-kDa, specific binding protein in PC12 cells and the rat brain, but not in the liver, spleen, adrenal gland, and kidney. These findings demonstrate that the core sequence of the rat AT1a-R is an (A + T)-rich sequence located at nucleotides -456 to -442 and the 53-kDa protein is a specific binding protein, and suggest that this protein may be a trans-acting factor which determines the neuron-specific down-regulation of the AT1a-R gene.

Angiotensin II has multiple physiological effects in the cardiovascular, endocrine, and nervous systems that are initiated by binding to specific receptors located on the plasma membrane (1). Two major subtypes (type 1 and type 2) of angiotensin II receptors have been revealed by their differential affinity for nonpeptide drugs (2). Angiotensin II type 1a receptor (AT1a-R)1 cDNAs have been cloned from rat vascular smooth muscle cells (3), bovine adrenal zona glomerulosa cells (4), and rat kidney (5). Angiotensin II mRNA is expressed in a variety of cells and tissues including vascular smooth muscle cells, liver, kidney, and spleen, while the mRNA abundance is low in other tissues such as heart, brain, thymus, and testis. AT1a-R gene expression is regulated in an ontogenic manner (6). Thus, the rat AT1a-R gene is cell-specifically and developmentally regulated.

We characterized one negative and three positive cis-regulatory elements in the 5'-flanking region of the rat AT1a-R gene (7). The negative cis-regulatory element (NRE) was located between -489 and -331 and inhibited the promoter activity of the 590-bp 5'-flanking region by a factor of 10. The trans-acting factor that binds to the element was present in PC12, not in vascular smooth muscle and glial cells. This suggested that the trans-acting factor is a major determinant which regulates the expression of the rat AT1a-R gene in PC12 cells. However, the NRE was located within 159 nucleotides (nt) from -489 to -331 and the core sequence has not been mapped in detail.

Here, we identified the core sequence to clarify the negative cis-regulation, using the gel retardation assay and the DNase I footprint analysis. We showed that the core sequence is (A + T)-rich (TAATCTTTATTTTA, nt -456 to -442). Southwestern blotting revealed that a nuclear protein of about 53 kDa bound to the NRE in PC12 cells and the rat brain, but not in vascular smooth muscle cells, glial cells, kidney, spleen, adrenal gland, and liver.

MATERIALS AND METHODS

Gel Retardation Assays—The gel retardation assay was performed and nuclear extracts from culture cells were prepared as described in Ref. 8. The final protein concentration was 1-0.5 mg/ml. Nuclear extracts from the rat brain, liver, adrenal gland, and kidney were prepared essentially as reported by Gorski et al. (9). The final protein concentration was 1-2 mg/ml.

The 159-bp NRE fragment (nt -489 to -331) obtained by Xhol and HindII digestion was labeled with [α-32P]dATP using Klenow fragment (Takara Shuzo, Kyoto, J. apam) and purified as reported (8). Oligonucleotides corresponding to the NRE (5'-TAATCTTTATTTTA-3'), mutation 1 (5'-TAATCGGGATTTTA-3'), and mutation 2 (5'-TAATCGGGATTTTA-3') were synthesized, labeled with [γ-32P]ATP using T4 polynucleotide kinase (Takara Shuzo, Kyoto), and annealed to make double-strand DNA. Nuclear extracts were incubated for 15 min on ice in a 30-μl reaction mixture containing 12 mM Hepes, pH 7.9, 60 mM KCl, 0.1 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 12% glycerol, and 2 μg of double-stranded poly(dI-dC) in the presence or absence of excess competitor DNA. A radiolabeled DNA probe was added (0.1-0.5 ng, 15,000 cpm), and the incubation was continued for 30 min at room temperature. Thereafter, the mixture was loaded on a 6% polyacrylamide gel in 1 x TBE (90 mM Tris-HCl, pH 8.0, 89 mM borax, 2 mM EDTA), and electrophoresed at 140 V for 3 h followed by autoradiography.

DNase I Footprint Analysis—DNase I footprinting was performed using a modification of the procedure described in Ref. 8. The 159-bp NRE fragment (nt -489 to -331) was labeled only at the Xhol site (Fig. 2A) or BssHII site (Fig. 2B) in the polylinker site of the pBluescript vector with Klenow fragment and [α-32P]dCTP, then digested with HindII (sense probes). The sizes of these sense probes were 159 and 211 bp, respectively. To obtain the antisense probe, the NRE fragment was...
was subcloned into pGEM vector and labeled at the NotI site in the polylinker site of the vector. After gel purification, the probe (~40,000 cpm) was incubated with nuclear extracts in 100 µl containing 12 µm Heps, pH 7.9, 60 mM KCl, 4 mM MgCl2, 0.1 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10% glycerol, and 4 µg of dl-dC. The mixture was incubated for 30 min at room temperature then 50 µl of 12 µm Heps, pH 7.9, containing 5 mM CaCl2, 5 mM MgCl2, and 5 ng (Fig. 2A) or 20 ng (Fig. 2B) of DNase I (Life Technologies, Inc.) was added and the incubation continued for 1 min at room temperature (Fig. 2A) or 15°C (Fig. 2, B and C). The reaction was stopped with 100 µl Tris-HCl, pH 8, 100 µM NaCl, 1% Sarkosyl, 10 mM EDTA, and 1 µg/ml RNA. The RNA was extracted with phenol/chloroform, precipitated with ethanol, and separated on a 6% polyacrylamide gel, 8 µm urea sequencing gel. To define the position of the protected region, G ↔ A sequence ladders were prepared (8).

Plasmid Construction—A chloramphenicol acetyltransferase (CAT) plasmid with the 159-bp NRE fragment (nt -489 to -331) deleted was constructed as follows. The AT1a 331-CAT construct (7) was digested with HindIII at the 5'-end of the 331 bp of 5'-flanking region, blunt-ended with Klenow, and further digested with Clal at the upstream linker site in the Bluescript vector. The 5'-flanking region (nt -980 to -489) was obtained from AT1a 980-CAT (7) by digestion with Clal and Xhol, the 3'-end of which was blunt-ended with Klenow and subcloned between the Clal and blunt-ended sites of the AT1a 331-CAT construct.

A mutation corresponding to NRE mutation 2 was created by PCR overlap extension mutagenesis (10). Briefly, two DNA fragments having overlapping ends were first amplified using two sets of primers (A and B, C and D), from a AT1a 980-Bluescript construct into which a 1242-bp EcoRI-Sacl fragment containing the 980-bp 5'-flanking region (nt -980 to +262) had been subcloned (7). Primers A and D were T3 and T7 primers for pBluescript KS(-), respectively. Primers B and C had the following sequences: B, 5'-TTTACACCGCTGAAATACCATAGATT-3' (antisense: nt -432 to -456); C, 5'-TAATTACCGCTGCTAAGA-3' (sense: nt -448 to -432). Primer B contained an oligo-NRE mutation 2 (Fig. 2A) at the 3' end, and primers B and C were designed to overlap at the 5' end (underlined). The two respective PCR products were mixed and amplified again with primers A and D. The resultant PCR product was subcloned into the pGEM-T vector (Promega, Madison, WI), and sequenced using T7 and T3 primers to confirm the mutated NRE and other DNA sequences. The PCR product was further subcloned into the 5' end of the CAT gene.

To construct a NRE-thymidine kinase (TK)-CAT fusion gene, the NRE fragment (nt -489 to -331), obtained by digestion with Xhol and BamH1, was blunt-ended at Xhol site by Klenow, and then subcloned into the pGEM T-vector (Promega, Madison, WI), and sequenced using T7 and T3 primers to confirm the mutated NRE and other DNA sequences. The PCR product was further subcloned into the XhoI site of CAT vector to construct the NRE-TK-CAT construct.

Negative cis-Regulation of Angiotensin II Receptor Gene

Reagents and Statistical Methods—All reagents were purchased from Sigma unless otherwise indicated below. Results are expressed as mean ± S.E. Analysis of variance and the Dunnet’s test were used for multigroup comparisons (12). Values of p < 0.05 were considered significant.

RESULTS

Gel Retardation Analysis of Negative Cis-regulatory Element—Our previous study demonstrated that three positive cis-regulatory elements and one strong NRE are present in the 980-bp 5'-flanking region of the rat AT1a-R gene (7). However, the NRE core sequence has not been mapped and the transacting factor has not been identified.

Fig. 1 shows the result of gel retardation analyses using 159 bp of the NRE fragment (nt -489 to -331) as a probe. The NRE fragment formed five retarded bands (arrows A, B, C, D, and E) in Fig. 1) upon incubation with cellular nuclear extract from PC12 cells. Although these bands differed in mobility, the addition of a 100-fold molar excess of the same unlabeled NRE fragment completely competed with the slowest band (arrow A). Other retarded bands (arrows C and D) also competed with the unlabeled NRE fragment, whereas the inhibition was not complete even with a 100-fold molar excess of the competitor.

The arrow B and E bands were not inhibited with an excess of the competitor. The retarded band corresponding to arrow A was not detected in glial cells, A10 cells, and vascular smooth muscle cells. No specific band complex was observed when the nuclear extract from glial cells, A10 cells, or vascular smooth muscle cells was incubated with the NRE fragment.

Mapping the Core Sequence of the NRE—To better define the protein-binding site in the region responsible for the transcriptional inhibition, the NRE fragment (nt -489 to -331) was analyzed by DNase I footprinting using a nuclear extract from PC12 cells. A protected region between nt -456 and -442 (5'-TAATCTTTTATTTTATTTA-3') was detected when a sense strand of the NRE fragment was labeled (Fig. 2A). Since the protected region was at the very end of the tested fragment, we extended the 5' end of the NRE fragment by 52 bp using the nucleotide sequences of the vector and move the protected region to the middle of the gel. As shown in Fig. 2B, a protected region was identical to that observed in Fig. 2A, and other protected regions were not clearly characterized in this study. To confirm the sequences protected by the sense probes (Fig. 2, A and B), an antisense strand of the NRE fragment was used as a probe and partially digested with DNase I. Fig. 2C shows that the nucleotide sequences between -456 and -442 were protected, which corresponded to the region identified by the sense probes.

An oligonucleotide (oligo-NRE) was designed to encompass the protected 15-bp sequences. With oligo-NRE as the labeled
DNase I as described under “Materials Methods.” Five sense of nuclear extract from PC12 cells, and partially digested with antisense probe. These probes were incubated in the presence or absence of BssHII site (Fig. 3A) as the sense probes, and at the NdeI site (C) as an antisense probe. These probes were incubated in the presence or absence of nuclear extract from PC12 cells, and partially digested with DNase I as described under “Materials Methods.” Five μg of nuclear extract was used in panel A and increasing amounts of nuclear extract were used in panel B and C. G + A lane indicates the sequence ladder by the Maxam-Gilbert reactions. The protected portion is shown in the left side of panels.

Gel retardation analyses were performed using the promoter NRE fragment as a probe, to determine whether or not the retarded band could be competed by the oligo-NRE. Excess oligo-NRE interfered with the protein-NRE binding and reduced the amount of a slowest retarded product (Fig. 3B). These findings suggest that the slowest retarded band (arrow A in Fig. 1) is due to the interaction between nuclear protein and oligo-NRE sequence.

Site-directed Mutagenesis of the NRE—Two sets of mutations were designed within the NRE core sequence; one (oligo-NRE mutation 1) was mutated at −452 and −447 and the other (oligo-NRE mutation 2) at −451 to −448 (Fig. 5A). Gel retardation analyses using oligo-NRE mutation 1 as a probe showed that the mutation had no effect on protein/oligo binding, whereas an oligo-NRE mutation 2 probe efficiently reduced it (Fig. 4B). Next, we examined whether the oligo-NRE mutation 2 interferes with the wild-type oligo-NRE binding to the nuclear protein. As shown in Fig. 4C, mutation 2 had no effect on the protein/wild-oligo interaction. These results confirmed the assignment of this band to the oligo-NRE, and demonstrated that the T451T450T449T448 sequences in the middle of the NRE core sequence are important for the protein/DNA binding.

Functional Significance of the NRE in Transcriptional Regulation—We previously showed that the NRE fragment (nt −489 to −331) inhibits the transcriptional activity of the promoter region between −489 and −1 about 10-fold (7). Since the relatively strong positive cis-regulatory element was located between nt −560 to −489 (7), we examined the inhibitory effect of the NRE fragment on the 980 bp of promoter region using the NRE fragment-deleted CAT construct. The CAT activity was normalized for transfection efficiency by means of the co-transfected β-galactosidase gene and for cell density by the protein concentration, and expressed as a relative value to that of the promoterless CAT construct (7). The obtained relative CAT value of the wild AT1a 980-CAT construct in PC12 cells was arbitrarily assigned a value of 1.0 for quantitative comparison.

**Fig. 3.** Gel retardation analyses of the oligo-NRE. A, the oligo-NRE (nt −456 to −442) was labeled and used in gel retardation analyses with nuclear extract (2 μg) from PC12 cells. Glia refers to gel retardation using nuclear extract (2 μg) from glial cells. Oligo I, II, and III show gel retardation when oligonucleotides encompassing the more upstream region (oligo I, nt −489 to −457) and the NRE sequences for the human major histocompatibility complex class I gene (oligo II, CCAAAATTTCTGAAAGGTATTTAAA) (18) and the rat prolactin gene (oligo III, TATAATTTTATA) (19) were used as competitors (molar ratio, 100×). B, the NRE fragment (nt −489 to −331) and the oligo-NRE (nt −456 to −442) were used as the probes and the competitors, respectively, and incubated with nuclear extract (2 μg) from PC12 cells. Arrows indicate the retarded products specifically bound to the probe.

**Fig. 4.** Gel retardation analyses of the oligo-NRE mutations. A, the NRE mutation 1 (−452 and −447) was labeled and competed with the unlabeled NRE mutation 1. B, the NRE mutation 2 (−451 to −448) was labeled and reacted with nuclear extract. C, the wild oligo-NRE was the probe and competed with wild oligo-NRE or unlabeled NRE mutation 2. Nuclear extract (2 μg) was prepared from PC12 cells. The arrow indicates the retarded band. Sequences for the wild oligo-NRE and the mutations are shown in Fig. 5.
The results showed that deleting the NRE fragment from the promoter region yielded a 2.7-fold increase (p < 0.01, n = 6) in the relative CAT activity (Fig. 5B). In addition, we constructed an oligo-NRE mutation 2 in the 980 bp of promoter region, and fused it to the CAT gene. As shown in Fig. 5B, the relative CAT activity significantly (p < 0.01, n = 6) increased about 2.2-fold compared with the CAT construct containing the wild NRE, indicating the functional significance of the NRE core sequence in the transcripional inhibition of PC12 cells.

Southwestern Blots of the Trans-acting Factor in PC12 Cells—To characterize the binding factor, the nuclear extract from PC12 cells was resolved by SDS-PAGE and Southwestern blotted with the 32P-labeled NRE 159-bp fragment or oligo-NRE probes. As shown in Fig. 6, three binding proteins with different molecular sizes (53, 35, and 33 kDa) were detected by the NRE fragment probe, while the oligo-NRE probe bound to a single protein (53 kDa). These bands disappeared in the presence of excess cold probe and the oligo-NRE mutation 2 (Fig. 5A) probe did not bind to any protein. On the other hand, the nuclear extract from glial, A10 cells, or vascular smooth muscle cells did not contain the protein bound to the NRE fragment and oligo-NRE (data not shown). We also examined the presence of this nuclear protein in the rat brain, liver, spleen, adrenal gland, and kidney using oligo-NRE as a probe. A single band corresponding to the 53-kDa protein was found in the nuclear extract from the rat brain, but not in the liver, spleen, adrenal gland, and kidney (data for rat brain are shown). Thus, we concluded that the 53-kDa protein specifically binds to the NRE core sequence. The other bands (35 and 33 kDa) detected by the NRE fragment probe may reflect the trans-acting proteins bound to the region other than the NRE core sequence, or the proteins nonspecifically bound to this DNA fragment.

Action of the NRE Fragment upon a Heterologous Promoter—To test whether the NRE fragment could regulate a heterologous promoter, the fragment was subcloned upstream of the thymidine kinase (TK) promoter fused to the CAT gene. In transient assays using PC12 cells, the expression of the fusion gene containing the TK promoter alone appreciably increased compared with the TK-less CAT gene. When the 159-bp NRE fragment was added to the TK-CAT construct and transfected into PC12 cells, the CAT activity was unaffected, although the NRE fragment was placed immediately upstream of the TK gene (Fig. 7). These results suggested that the NRE sequence acts as a negative regulatory element of the rat AT1a gene, rather than as a silencer element, and that it works in junction with the native, rather than the heterologous promoter.

**DISCUSSION**

We demonstrated that three positive cis-regulatory elements and one strong NRE are present in the 5'-flanking region of the rat AT1a-R gene, and suggested that this NRE is one of major determinants that regulate the level of gene expression in PC12 cells (7). Here, we discovered and mapped the core sequence of the NRE (5'-TAATCCTTTATTTTA-3') between −456 and −442. This element reduced AT1a-R gene expression by a factor of 2.7 in transient transfection assays using PC12 cells. Site-directed mutagenesis in this core sequence affected the specific DNA-protein interaction and eliminated the suppression of AT1a-R gene expression in the transient transfection.
assays. These data demonstrated that specific protein-DNA interaction at this sequence down-regulates the gene expression in PC12 cells.

Although NREs have been detected in a variety of genes (13), the molecular mechanisms by which they exert their effects remain obscure. While the NREs can exhibit enhancer-like qualities and function on heterologous promoters in a distance and orientation-independent fashion (14), they often reduce rather than abolish the activity of heterologous promoters and demonstrate a preference for a specific promoter (15, 16). The NRE in the rat AT1a-R gene, when transferred to the TK promoter, had no significant effect on the transcriptional activity, suggesting that the NRE in the AT1a-R gene works more effectively in conjunction with the native AT1a-R gene promoter than with the heterologous promoter.

Recently, many cis-acting, negative transcriptional elements in mammals have been identified (13), some of which include (A + T)-rich sequences. These include human Ig heavy chain (AAATTTTTT) (17), human major histocompatibility complex class I (CCAAAATTCGTGAAAAAGTATTAAAA) (18), rat prolactin (AAATAA, TATAAAATTTT) (19), and mouse Ig (ATTAATTTT) (20). However, the (A + T)-rich NRE sequence identified in the rat AT1a-R gene did not match these (A + T)-rich sequences and was not competed with the NRE oligos for the human major histocompatibility complex class I and rat prolactin (Fig. 3A), indicating that this NRE is a novel negative element.

The rat AT1a-R gene has a subtype gene, AT1b-R, which has very high homology (96%) in the coding sequence (21–24). Although the receptor-mediated signal was similar to that in the AT1a-R, the profile for the tissue distribution between the AT1a-R and AT1b-R mRNAs was quite distinct. The AT1a-R is the dominant form expressed in the liver, kidney, vasculature, lung, ovary, testis, and heart, whereas the AT1b-R is expressed in greater quantities in the adrenal gland, anterior pituitary, and uterus (21–23). Very recently, Guo and Inagami (25) have sequenced the rat AT1b-R promoter region, in which the homology between both subtypes was low and the (A + T)-rich NRE sequence observed in the rat AT1a-R gene was not detected. Since the abundant expression of the AT1b-R gene is restricted in a few organs compared with that of the AT1a-R gene, a much stronger NRE may be present and regulate a tissue-specific expression. In the 5-flanking region of the human AT1a-R gene, Guo et al. (26) and Takayanagi et al. (27) found that the cis-regulatory region inhibits the gene transcription between –881 to –642, and –962 to –114, respectively. However, the (A + T)-rich NRE sequence observed in the rat AT1a-R gene was not located in these regions, and a similar element (AAATTTTTTTAA) was present more upstream. This study demonstrated that the trans-acting protein bound to the NRE of the rat AT1a-R gene is present in PC12, but not in vascular smooth muscle cells and glial cells. Thus, whether the (A + T)-rich sequence in the human AT1a-R gene can function as a NRE depends on a suitable cell model containing an abundant amount of the specific trans-acting protein.

Southwestern blots suggested the involvement of a 53-kDa protein in the AT1a-R promoter function in PC12 cells. We showed in a previous study (7) that the expression of the AT1-R is very low in PC12 cells: the mRNA level was detectable only by the reverse transcriptase-PCR method, not by the Northern blot, and the AT1-R protein was not quantified by the binding assay. Summers et al. (28) have reported using the neonatal rat brain, that glial cells predominantly express AT1-R, whereas neurons contain a small amount of AT1-R. We also found that the promoter region of the rat AT1a-R gene contains a positive cis-regulatory element only in glial and PC12 cells (7), suggesting that the regulatory mechanism of rat AT1a-R gene expression may differ between the central and peripheral tissues. The finding that a nuclear protein bound to the NRE in the brain, but not in vascular smooth muscle cells, glial cells, the spleen, kidney, adrenal gland, and liver, also supports this contention. The expression of the AT1a-R gene is also inhibited in the adrenal gland and the pituitary gland, in which the AT1b-R subtype is predominant. The 53-kDa protein was not detected in the adrenal gland, suggesting that other regulatory mechanisms to suppress the AT1a-R gene transcription may function in these tissues. Since the NRE has only a 2–3-fold effect in inhibiting the AT1a-R promoter activity and no effect on the heterologous promoter, the inhibitory action of the 53-kDa protein may not be sufficient to determine the neuron-specific down-regulation or tissue-specific regulation of the AT1a-R gene. Recent evidence demonstrates that the blood pressure is effectively reduced in the AT1a-R knock-out mice (29). The cloning of a gene encoding the 53-kDa protein bound to the NRE may help the isolation of a novel nuclear protein that moderately reduces blood pressure by inhibiting the expression of the AT1a-R gene.

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