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THE GENOMIC ORGANIZATION OF HUMAN ANGIOTENSIN II TYPE 1 RECEPTOR

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SUMMARY: As a step toward the elucidation of human Ang II type 1 receptor gene expression, the genomic organization of the human AT_1 receptor was investigated. Comparison of the genomic DNA and cDNA sequences revealed that it consists of at least five exons. The length of the AT_1 receptor gene is greater than 55 kb, and the size of the exons ranges from 59 to 2,014 base pairs. Four of the exons encoded 5'-untranslated sequences. Multiple transcription initiation sites were observed by primer extension experiment. The promoter function was examined by using luciferase as a report gene in transfected human vascular smooth muscle cells.

Angiotensin II (Ang II) exerts a wide variety of physiological effects on the cardiovascular, renal endocrine, and central and peripheral nervous system (1). Reflecting the versatility of the biological effects mediated by Ang II, its receptors are present in a wide variety of tissues (2). Cloning of human AT_1 receptor cDNA and genomic DNA has been reported (3-8). However, its genomic structure is unknown. In order to elucidate the mechanism of transcriptional regulation of the human AT_1 receptor gene and to facilitate analysis of a possible genetic disorders of the human AT_1 receptor, we have isolated and analyzed the human AT_1 receptor gene. In this study, we report the genomic organization of the human AT_1 receptor gene with evidence for the presence of five exons, the first four encoding 5'-untranslated sequences. The promoter/luciferase constructs were used to determine the promoter function in transfected human vascular smooth muscle cells (VSMCs).

MATERIALS AND METHODS

Cloning of the cDNA library. A human liver cDNA library in lambda ZAP was obtained from Stratagene (La Jolla, CA). A total of 4 X 10^6 lambda phages were screened on nylon membranes. The *Eco*RI DNA fragment (0.8 kb) of rat AT_{1A} cDNA, that contained the coding region, was used as a probe. Positive DNA clones were subcloned into a Bluescript KS(+) vector for further analysis.

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Plasmids. Four different human AT_1 cDNAs were isolated and used in this study. They were called pC1, pC2, pC3 and pC4, respectively (Fig. 1). The 5'-flanking region of the human AT_1 gene, spanning the first exon was subjected to double restriction enzyme digestion with *Hin*dIII and *Cla*I, subcloned in the Bluescript vector (pDF) and used to construct the promoter/luciferase fusion gene.

Cloning of the genomic library. A human lymphocyte genomic library in lambda DASH II was obtained from Stratagene (La Jolla, CA). A total of 4 x 10^6 lambda phage plaques were screened on nylon membranes. For the isolation of DNA fragments encoding the human exon 1, 2 or 3 , fragments digested with *Eco*RI and *Acc*I from the plasmids, pC1, pC2 and pC3, were used as combination probes. For the isolation of DNA fragment encoding exon 4, the *Eco*RI-*Rsa*I fragment from the plasmid pC4 was used as a probe.
DNA sequence. The nucleotide sequence of the 5'-flanking region of the human AT_1 gene was determined by the dideoxy chain termination method. Computer analysis of the sequence data was carried out by using the software of IntelliGenetics, Inc.

Primer extension. The 21-mers antisense oligonucleotide, 5'-CAGACGTCCTGT CACTCGCTG-3' (Fig. 4) was end-labeled with γ-^{32}pATP. Total RNA isolated from human placenta and liver were used. The primer-extended DNA products were run on a sequencing gel alongside a dideoxy sequencinf ladder prepared with the same primer.

Expression of human AT_1 promoter/luciferase constructs. Eight human promoter fragments were deleted from pDF plasmid, and inserted into pGL2 basic lusiferase vector. Ten µg of each constructs was transfected into 1x10^6 human vascular smooth mascle cells by DEAE-dextran transfection method. After transfection 48h, the luciferase activity was mesured by using TROPIX, a leader in luminescence (MGM INC., Hamden, CT).

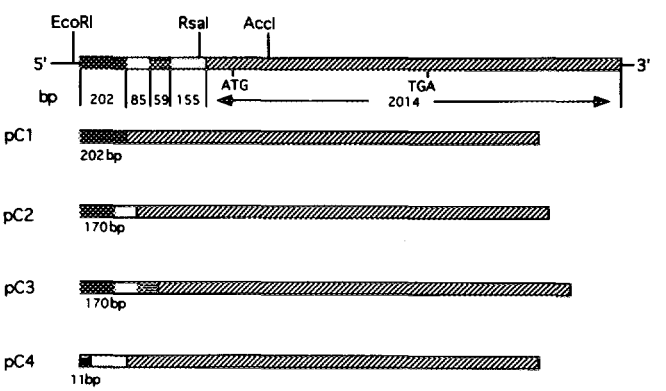

Fig.1. Schematic illustration of human AT1 receptor cDNAs. The four human AT_1 cDNA clones (pC1, pC2, pC3 and pC4) are shown. Each contained different exons which are indicated by (▓) for exon 1, (□) for exon 2, (▒) for exon 3, (▒) for exon 4 and (▨) for exon 5, respectively. The restriction enzymes used in this study are marked. The location of the start site codon ATG and the termination codon TGA are also marked. The length of each exon is indicated by base pair numbers. The cDNA on the top is the composite full length cDNA.

RESULTS AND DISCUSSION

Cloning of human AT_1 cDNA. Four human AT_1 cDNAs, differing in their 5' untranslated regions, were cloned from human liver cDNA library. They were named each as pC1, pC2, pC3 and pC4 (Fig. 1). Of those, clones pC1 and pC2 were identical to two previously reported cDNAs, respectively (5,8). On the other hand, clones pC3 and pC4 were found to contain additional exons which have not been reported to date. Clone pC3 contained a part of exon 1 (170 bp), full

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catcatccttgctgccgtcaatatcccgagagggaggaggttgggccgggagggtctccggggcggggcggaggagg
agggaatgcaaaacagagcctcgtccccggaacccaagaagcagcaacgcccctcactataaattcggagctgcctc
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.....(intron 1).....
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.....(intron 2).....
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.....(intron 3).....
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.....(intron 4).....
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tatgaatatatttttatttaaattttgatacaactgatagtttaatactattggttatagatttttatcctgacat
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Fig. 2. The intron/exon nucleotide sequences of the human AT_1 gene. Exon sequences are shown in capital letters. The first base of exon 1 is the 5' end of cDNA.

length of exons 2 (85 bp), 3 (59 bp) and 5 (2014 bp). Clone pC4 was different from pC1, pC2 and pC3 in that it contained an 11 bp partial sequence of exon 3 and full length exons 4 (155 bp) and 5 (2014 bp). The sequence of each exon is summarized in Fig. 2. In the present study, we have found two cDNA clones contained additional sequences which had not been reported so far (pC3 and pC4, see Fig.1). These two additional sequences arise by alternative splicing, and the length were 59 and 155 bp, respectively.

Cloning and characterization of human AT1 genomic clones. we succeed to clone five different clones which contain distinct exons of human AT1 receptor from a human lymphocyte genomic library by using combination of ^{32}P-labeled pC1, pC2, pC3 and pC4 cDNA probes. Five different clones which contained different exons were used to construct the map of human AT_1 gene with sites for restriction endonuclease *Eco*RI, *Bam*HI and *Hin*dIII (Fig. 3). In addition, the sequence of exon/intron junctions from human AT_1 gene are shown in Fig. 2. The 5' intron border invariably starts with GT, and the 3' border terminates with AG. These features are consistent with reported donor and acceptor consensus sequences. The human AT_1 gene consists of five exons divided by four introns, whereas the rat AT_{1A} and AT_{1B} receptor genes contained four exons and three exons, respectively (9,10). In addition, the rat AT_{1A} and AT_{1B} gene had 2 exons in the 5' untranslated region and third exon contained the entire open reading frame, the human AT_1 receptor gene contained 4 exons in the 5' untranslated region, the fifth exon contained the entire coding region. Independent of our work, Bergsma et al. (6) reported that one clone of human cDNA had a 23 bp sequence which had not been found in our cDNA in the 5' untranslated region. It is possible that the additional exon containing this 23 bp

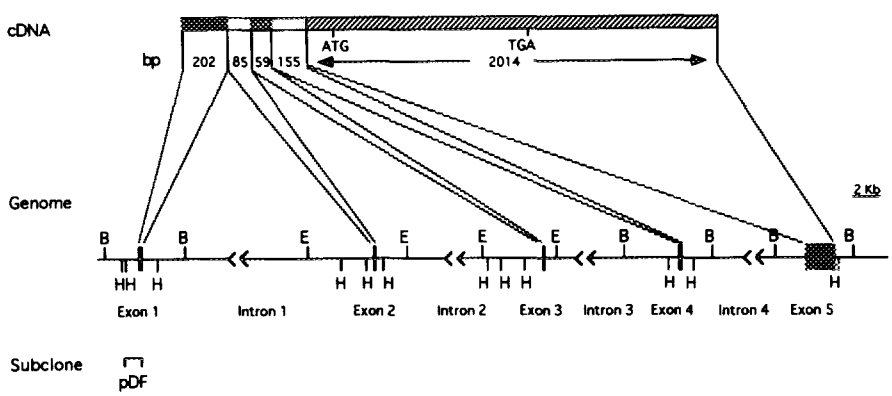


Fig. 3. Structure of the human AT_1 receptor gene. The AT_1 receptor gene consists of five exons and four introns. The fifth exon contains the entire coding region from the translation start codon ATG to the stop codon TGA. Exons are indicated by solid boxes. Intron regions are numbered as intron 1 through intron 4. The restriction map and subcloning strategy are also shown. E: *Eco*RI; B: *Bam*HI; H: *Hin*dIII.

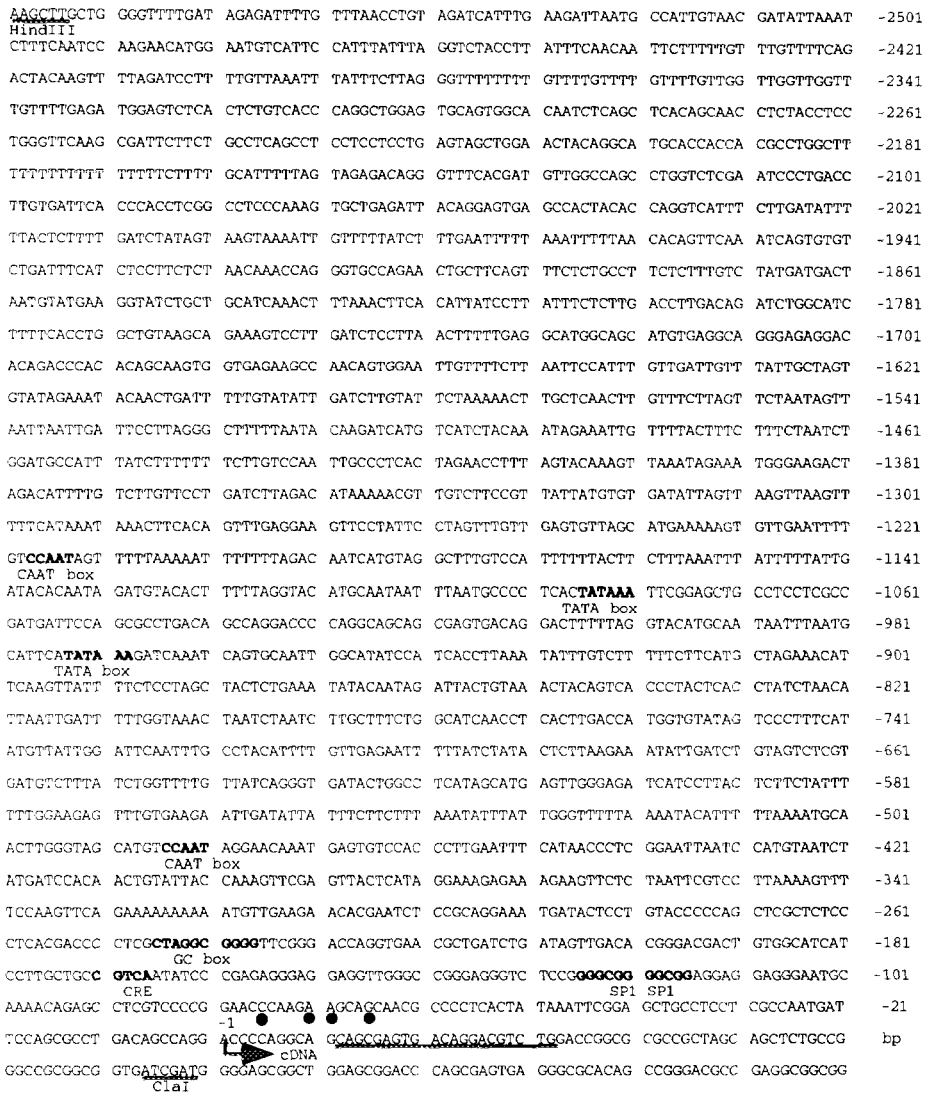


Fig. 4. Sequence analysis of the 5'-flanking region of the human AT_1 receptor gene. To examine the nucleotide sequence of the promoter regions of the human AT_1 receptor gene, a 2659 base-pair *Hin*dIII/*Cla*I fragment spanning exon 1 was subcloned into Bluescript vector (pDF). Sequence analysis was performed as described in Materials and Methods. Recognized consensus sequences are bracketed and labeled: (i) TATA box; (ii) CAAT box; (iii) Sp1 recognition sequence; (iv) CRE sequence; (v) GC box. The transcription initiation sites are indicated by black dots. The primer used in primer extension is indicated by underline.

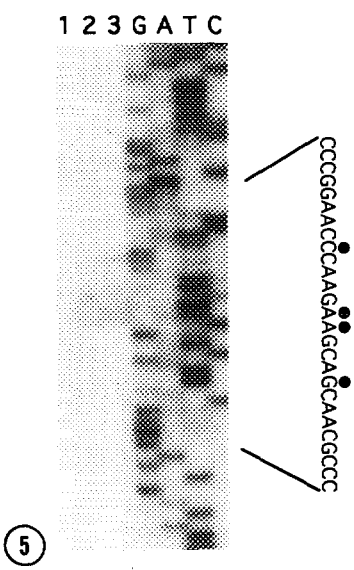
5' untranslated region may exist in the human AT_1 gene and spliced differently. Exons 1 through 4 of the human AT_1 receptor gene are small (<250 bp) and are located in 5' untranslated region. A similar results were observed in rat AT_{1A} and AT_{1B} receptor gene (9,10).

The 5'-flanking region of human AT_1 receptor gene. The 2.6 kb *Hin*dIII-*Cla*I 5'-flanking region was completely sequenced and analyzed using a computer

program that identified known regulatory sequences (Fig. 4). Two putative TATA boxes, two CAAT boxes, two overlapping SP1 recognition sequence, a GC box and a cyclic-AMP induced responsive element were found. It seems that human AT_1 promoter is unusual because it contains characteristics of both housekeeping genes and regulated genes promoters. Most eukaryotic promoters include a TATA box, upstream regulatory sequences such as CAAT boxes, and tissue-specific or hormonal-responsive elements (11,12). Conversely, a typical housekeeping gene does not contain a TATA box, is GC-rich, and its transcription is initiated at several sites (13).

Primer extension. The sites of transcription initiation are shown in Fig. 5. Two major bands were observed at position -70, and -71 bp upstream the 5' end of the cDNA. The other primer extension products were observed at -66, and -76 bp upstream the 5' end of the cDNA. The top band (-76 bp) corresponeded to C, not A or G. It may cause to polymerase stop sequence in the RNA shortly.

Expression of human AT_1 promoter/luciferase constructs in human VSMCs. Total of eight human AT_1 promoter/luciferase chimeric constructs were used to mesure their enzyme activity in human VSMCs (Fig. 6). In experiment with the



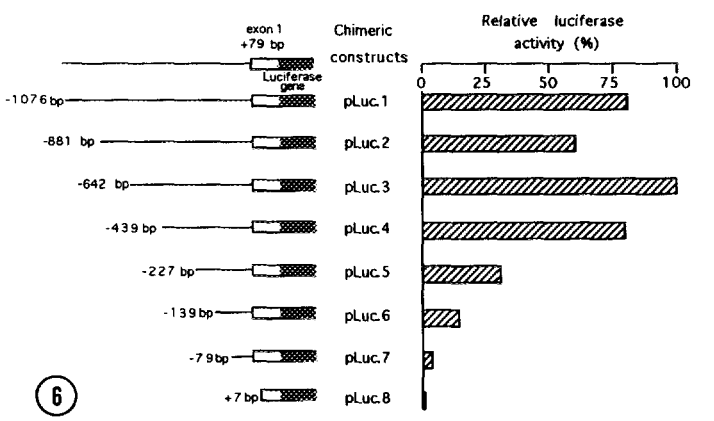


Fig.5. Mapping of the sites of transcription initiation by the primer extension analysis. Lane 1: fifty μg of yeast tRNA, lane 2: two hundred μg of total RNA from the human placenta and fifty μg of total RNA from human liver. Marker lanes G, A, T and C indicate sequencing ladders of the human gene using the same primer.

Fig.6. Expression of human promoter/luciferase constructs in human VSMCs. The chimeric human promoter/luciferase constructs are presented with a line, and the names of the constructs are given. The relative luciferase values are expressed as percentages of the activity obtained with the pLuc.3 (100%). The values are obtained from three individial experiments.

pLuc.3, the highest luciferase activity was obtained, and the luciferase level obtained with this was taken as 100% in each experiment. Transfection with pLuc. 1 and pLuc.4 gave higher luciferase levels that were about 79% of those obtained with pLuc.3. A positive regulatory region may locate between pLuc.1 and pLuc.2, and one negative regulatory region may locate between pLuc.2 and pLuc.3 by comparison of pLuc.1 through pLuc.3 luciferase activity. Transfection with pLuc.8 gave a background luciferase activity. A similar result was observed when transfected with pGL2 basic plasmid itself (data not shown).

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