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GENOMIC STRUCTURE OF HUMAN ADRENOMEDULLIN GENE

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SUMMARY: Adrenomedullin (AM) is a potent hypotensive peptide recently discovered from human pheochromocytoma tissue by its stimulating activity of platelet cAMP production. In this study, we have isolated the gene for human AM from a human genomic library and determined its structure. The genomic DNA of human AM consists of 4 exons and 3 introns, and the 5' flanking region contains TATA, CAAT and GC boxes. There are also multiple binding sites for activator protein-2 (AP-2) and a cAMP-regulated enhancer element. Southern blot analyses revealed that the AM gene is situated in a single locus of chromosome 11. These indicate that the human AM gene has components for its functional expression and that the expression may be subject to the activity of protein kinase C and the feedback from cAMP level.

Adrenomedullin (AM) is a newly discovered peptide from human pheochromocytoma tissue by monitoring its stimulating action on platelet cAMP production (1). The peptide consists of 52 amino acids with an intramolecular disulfide bond forming a ring structure of 6 residues, and shares slight homology with calcitonin gene-related peptide (CGRP), a potent hypotensive peptide. Like CGRP, iantravenous injection of AM elicits a strong and long-lasting hypotensive effect

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Abbreviations : AM, adrenomedullin; preAM, preproadrenomedullin; PAMP, proadrenomedullin N-terminal 20 peptide; CGRP, calcitonin gene-related peptide; AP-1, activator protein-1; AP-2, activator protein-2; CRE, cAMP-regulated enhancer; Sp1, promoter-specific transcription factor.

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attributable to a vascular resistance reduction in anesthetized rats (2). The vasodilator action of AM has been also demonstrated in the ex vivo experiment using perfused mesenteric vessels (3).

As expected from the origin of tissue in which AM was discovered, normal adrenal medulla abundantly contains this peptide and prominently expresses its mRNA (1,4). In addition, besides the adrenal medulla, considerable mRNA expression has been recognized in much larger organs such as the heart, kidney, and lung (4). Moreover, a significant concentration of AM has been identified in human plasma by means of specific radioimmunoassay coupled with liquid chromatography (5).

These findings suggest the possibility of AM as a new circulating hormone participating in the regulation of cardiovascular system. Further studies are needed in order to understand the implication of AM in the regulation of cardiovascular system. Among them, analysis of the gene structure is expected to provide information concerning the control mechanism of AM production. In the present study, we isolated the genomic DNA clone encoding AM in human, and determined the complete sequence.

MATERIALS AND METHODS

Isolation of genomic clones:

A human genomic library from the liver constructed in λ phage charon 4A was screened by plaque hybridization at high stringency using 1.4 kb human AM cDNA as a probe (6). One out of 3 positive clones was isolated for further analysis. The clone contained approximately 17 kb genomic DNA fragment. Southern blot analysis indicated that a 6.8 kb EcoRI fragment was hybridized with the probe. This DNA fragment was ligated into the plasmid vector Bluescript (Stratagene), and was amplified in E. coli strain JM109 (Takara Shuzo Co., Ltd.).

Determination of DNA sequence:

The restriction enzyme map of this 6.8 kb DNA fragment was constructed from the digestion patterns by HindIII, KpnI, PstI, SacI, SacII, SpeI, XbaI, and XhoI. Then, the PstI fragments were subcloned into Bluescript plasmid. They were sequenced by a dideoxynucleotide chain terminating method using the automated DNA sequencing system (Model 373A, Applied Biosystems). When necessary, the DNA fragment was shortened to various length using the Exo III / Mung Bean Nuclease Deletion Kit (Stratagene), and was subjected to nucleotide sequencing (7). Remaining gaps between the fragments were closed by PCR amplification using synthetic oligonucleotide primers.

Southern blot analysis:

High molecular weight genomic DNA was isolated from peripheral white blood cells of a healthy male subject using the G NOME DNA Isolation Kit (BIO 101, Inc.). This genomic DNA, 20 μg each, was thoroughly digested with BamHI, EcoRI, or PstI. They were electrophoresed on an 1% agarose gel and was transferred to a nylon membrane. The genomic DNA fragments were hybridized with [^{32}P]-labeled AM cDNA in 5x SSPE [50 mM phosphate buffer (pH 7.4), 0.75 M NaCl, 2.5 mM EDTA], 5x Denhardt's solution, 0.5% (w/v) SDS, and 40% (v/v) formamide at 42°C overnight. The membrane was washed with 2x SSC and 0.1% SDS at 50°C. Then, the blot was exposed to a X-ray film for 24 h at -80°C.

Chromosomal mapping:

Chromosomal assignment of human AM gene was determined by southern blot analysis using BIOSMAP Somatic Cell Hybrid Products (BIOS Laboratories, Inc.) (8). Five μg of DNA

from 20 human-hamster somatic hybrid cell lines carrying certain human chromosomes, and genomic DNA of human and hamster as controls were digested with PstI, and electrophoresed on a 1% agarose gel. Hybridization with AM cDNA was performed following the same procedure described above.

RESULTS AND DISCUSSION

The upper part of Figure 1 shows the restriction enzyme map of 6.8 kb EcoRI fragment from a positive genomic DNA clone. This 6.8 kb DNA clone was further digested with PstI, and the fragments were subjected to nucleotide sequencing. The whole cDNA sequence of AM was contained in 2 of the 6 PstI fragments. The lower part of Figure 1 presents the exon-intron structure of AM gene. The gene for AM consists of 4 exons and 3 introns. Figure 2 shows nucleotide sequence of human genomic DNA for AM and its flanking regions. The sequences of 4 exons are underlined. They are exactly identical with the reported sequence of human AM cDNA (4). All introns commence with GT and end with AG, conserving the consensus splicing sequences (9). The initiation codon of preproadrenomedullin (preAM) is located in the second exon, 22-24 bases downstream from the 5' end of the exon. This ATG codon is assigned nucleotide numbers 1 to 3. The preAM sequence is terminated by the TAG codon at nucleotide 939-941, in the fourth exon, 307 bases downstream from the 5' end of the exon. Whole nucleotide sequence corresponding to the 52 amino acid residues of mature AM is included in the fourth exon,

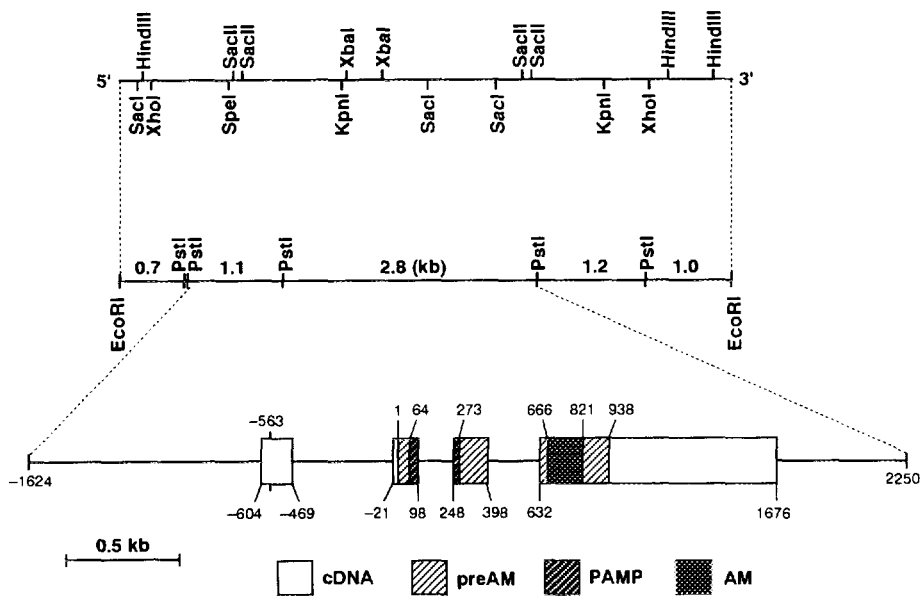
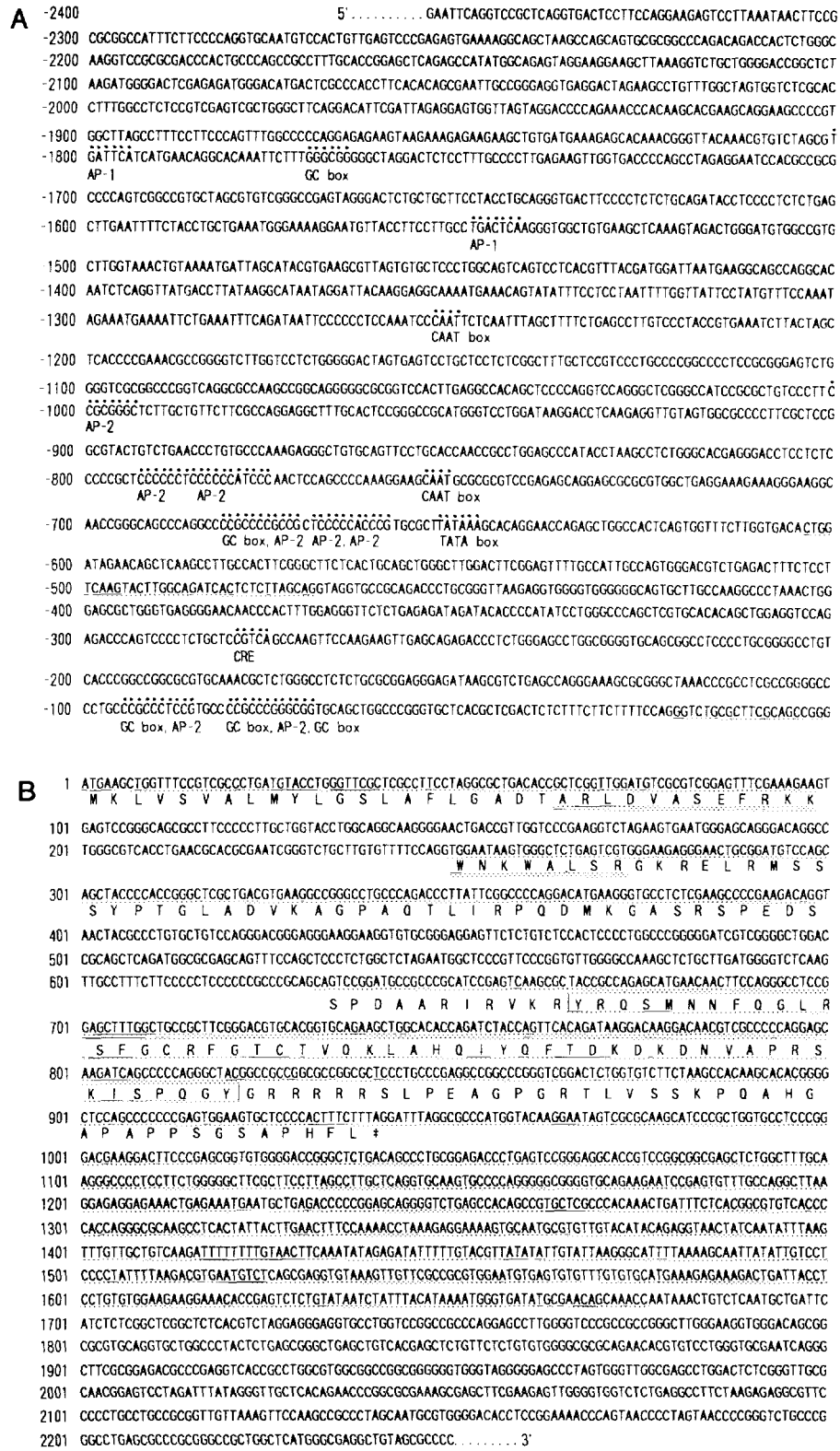


Figure 1. Restriction enzyme map of 6.8 kb EcoRI fragment of human genomic DNA clone including AM gene (upper part) and exon-intron map of human AM gene (lower part). The initiation codon of ATG is assigned nucleotide numbers 1 to 3. Portions of exon corresponding to AM, preproadrenomedullin (preAM) and proadrenomedullin N-terminal 20 peptide (PAMP) are solid, lightly-hatched and heavily-hatched, respectively.


A
-2400 5'.....GAATTCAGGTCCGCTCAGGTGACTCCTTCCAGGAAGAGTCCTTAAATAACTTCCG
-2300 CGCGGCCATTTCTTCCCCAGGTGCAATGTCCACTGTTGAGTCCCGAGAGTGAAAAGGCAGCTAAGCCAGCAGTGCGCGGCCCAGACAGACCACTCTGGGC
-2200 AAGGTCCGCGCGACCCACTGCCCAGCCGCCTTTGCACCGGAGCTCAGAGCCATATGGCAGAGTAGGAAGGAAGCTTAAAGGTCTGCTGGGGACCGGCTCT
-2100 AAGATGGGGACTCGAGAGATGGGACATGACTCGCCCACCTTCACACAGCGAATTGCCGGGAGGTGAGGACTAGAAGCCTGTTTGGCTAGTGGTCTCGCAC
-2000 CTTTGGCCTCTCCGTCGAGTCGCTGGGCTTCAGGACATTCGATTAGAGGAGTGGTTAGTAGGACCCCAGAAACCCACAAGCACGAAGCAGGAAGCCCCGT
-1900 GGCTTAGCCTTTCCTTCCCAGTTTGGCCCCCAGGAGAGAAGTAAGAAAGAGAAGAAGCTGTGATGAAAGAGCACAAACGGGTTACAAACGTGTCTAGCGT
-1800 GATTCATCATGAACAGGCACAAATTCTTTGGGCGGGGGCTAGGACTCTCCTTTGCCCCTTGAGAAGTTGGTGACCCCAGCCTAGAGGAATCCACGCCGCG
AP-1 GC box
-1700 CCCCAGTCGGCCGTGCTAGCGTGTCGGGCCGAGTAGGGACTCTGCTGCTTCCTACCTGCAGGGTGACTTCCCCTCTCTGCAGATACCTCCCCTCTCTGAG
-1600 CTTGAATTTTCTACCTGCTGAAATGGGAAAAGGAATGTTACCTTCCTTGCCTGACTCAAGGGTGGCTGTGAAGCTCAAAGTAGACTGGGATGTGGCCGTG
AP-1
1500 CTTGGTAAACTGTAAAATGATTAGCATACGTGAAGCGTTAGTGTGCTCCCTGGCAGTCAGTCCTCACGTTTACGATGGATTAATGAAGGCAGCCAGGCAC
-1400 AATCTCAGGTTATGACCTTATAAGGCATAATAGGATTACAAGGAGGCAAAATGAAACAGTATATTTCCTCCTAATTTTGGTTATTCCTATGTTTCCAAAT
-1300 AGAAATGAAAATTCTGAAATTTCAGATAATTCCCCCCTCCAAATCCCAATTCTCAATTTAGCTTTTCTGAGCCTTGTCCCTACCGTGAAATCTTACTAGC
CAAT box
-1200 TCACCCCGAAACGCCGGGGTCTTGGTCCTCTGGGGGACTAGTGAGTCCTGCTCCTCTCGGCTTTGCTCCGTCCCTGCCCCGGCCCCTCCGCGGGAGTCTG
-1100 GGGTCGCGGCCCGGTCAGGCGCCAAGCCGGCAGGGGGCGCGGTCCACTTGAGGCCACAGCTCCCCAGGTCCAGGGCTCGGGCCATCCGCGCTGTCCCTTC
-1000 CGCGGGCTCTTGCTGTTCTTCGCCAGGAGGCTTTGCACTCCGGGCCGCATGGGTCCTGGATAAGGACCTCAAGAGGTTGTAGTGGCGCCCCTTCGCTCCG
AP-2
-900 GCGTACTGTCTGAACCCTGTGCCCAAAGAGGGCTGTGCAGTTCCTGCACCAACCGCCTGGAGCCCATACCTAAGCCTCTGGGCACGAGGGACCTCCTCTC
-800 CCCCGCTCCCCCCTCCCCCCATCCCAACTCCAGCCCCAAAGGAAGCAATGCGCGCGTCCGAGAGCAGGAGCGCGCGTGGCTGAGGAAAGAAAGGGAAGGC
AP-2 AP-2 CAAT box
-700 AACCGGGCAGCCCAGGCCCCCGCCCCGCGCGCTCCCCCACCCGTGCGCTTATAAAGCACAGGAACCAGAGCTGGCCACTCAGTGGTTTCTTGGTGACACTGG
GC box, AP-2 AP-2, AP-2 TATA box
-600 ATAGAACAGCTCAAGCCTTGCCACTTCGGGCTTCTCACTGCAGCTGGGCTTGGACTTCGGAGTTTTGCCATTGCCAGTGGGACGTCTGAGACTTTCTCCT
-500 TCAAGTACTTGGCAGATCACTCTCTTAGCAGGTAGGTGCCGCAGACCCTGCGGGTTAAGAGGTGGGGTGGGGGGCAGTGCTTGCCAAGGCCCTAAACTGG
-400 GAGCGCTGGGTGAGGGGAACAACCCACTTTGGAGGGTTCTCTGAGAGATAGATACACCCCATATCCTGGGCCCAGCTCGTGCACACAGCTGGAGGTCCAG
-300 AGACCCAGTCCCCTCTGCTCCGTCAGCCAAGTTCCAAGAAGTTGAGCAGAGACCCTCTGGGAGCCTGGCGGGGTGCAGCGGCCTCCCCTGCGGGGCCTGT
CRE
-200 CACCCGGCCGGCGCGTGCAAACGCTCTGGGCCTCTCTGCGCGGAGGGAGATAAGCGTCTGAGCCAGGGAAAGCGCGGGCTAAACCCGCCTCGCCGGGGCC
-100 CCTGCCCGCCCTCCGTGCCCGCCCCGGGCGGTGCAGCTGGCCCGGGTGCTCACGCTCGACTCTCTTTCTTCTTTTCCAGGGTCTGCGCTTCGCAGCCGGG
GC box, AP-2 GC box, AP-2, GC box
B
1 ATGAAGCTGGTTTCCGTCGCCCTGATGTACCTGGGTTCGCTCGCCTTCCTAGGCGCTGACACCGCTCGGTTGGATGTCGCGTCGGAGTTTCGAAAGAAGT
M K L V S V A L M Y L G S L A F L G A D T A R L D V A S E F R K K
101 GAGTCCGGGCAGCGCCTTCCCCCTTGCTGGTACCTGGCAGGCAAGGGGAACTGACCGTTGGTCCCGAAGGTCTAGAAGTGAATGGGAGCAGGGACAGGCC
201 TGGGCGTCACCTGAACGCACGCGAATCGGGTCTGCTTGTGTTTTCCAGGTGGAATAAGTGGGCTCTGAGTCGTGGGAAGAGGGAACTGCGGATGTCCAGC
W N K W A L S R G K R E L R M S S
301 AGCTACCCCACCGGGCTCGCTGACGTGAAGGCCGGGCCTGCCCAGACCCTTATTCGGCCCCAGGACATGAAGGGTGCCTCTCGAAGCCCCGAAGACAGGT
S Y P T G L A D V K A G P A Q T L I R P Q D M K G A S R S P E D S
401 AACTACGCCCTGTGCTGTCCAGGGACGGGAGGGAAGGAAGGTGTGCGGGAGGAGTTCTCTGTCTCCACTCCCCTGGCCCGGGGGATCGTCGGGGCTGGAC
501 CGCAGCTCAGATGGCGCGAGCAGTTTCCAGCTCCCTCTGGCTCTAGAATGGCTCCCGTTCCCGGTGTTGGGGCCAAAGCTCTGCTTGATGGGGTCTCAAG
601 TTGCCTTTCTTCCCCCTCCCCCCGCCCGCAGCAGTCCGGATGCCGCCCGCATCCGAGTCAAGCGCTACCGCCAGAGCATGAACAACTTCCAGGGCCTCCG
S P D A A R I R V K R Y R Q S M N N F Q G L R
701 GAGCTTTGGCTGCCGCTTCGGGACGTGCACGGTGCAGAAGCTGGCACACCAGATCTACCAGTTCACAGATAAGGACAAGGACAACGTCGCCCCCAGGAGC
S F G C R F G T C T V Q K L A H Q I Y Q F T D K D K D N V A P R S
801 AAGATCAGCCCCCAGGGCTACGGCCGCCGGCGCCGGCGCTCCCTGCCCGAGGCCGGCCCGGGTCGGACTCTGGTGTCTTCTAAGCCACAAGCACACGGGG
K I S P Q G Y G R R R R R S L P E A G P G R T L V S S K P Q A H G
901 CTCCAGCCCCCCCGAGTGGAAGTGCTCCCCACTTTCTTTAGGATTTAGGCGCCCATGGTACAAGGAATAGTCGCGCAAGCATCCCGCTGGTGCCTCCCGG
A P A P P S G S A P H F L ‡
1001 GACGAAGGACTTCCCGAGCGGTGTGGGGACCGGGCTCTGACAGCCCTGCGGAGACCCTGAGTCCGGGAGGCACCGTCCGGCGGCGAGCTCTGGCTTTGCA
1101 AGGGCCCCTCCTTCTGGGGGCTTCGCTTCCTTAGCCTTGCTCAGGTGCAAGTGCCCCAGGGGGCGGGGTGCAGAAGAATCCGAGTGTTTGCCAGGCTTAA
1201 GGAGAGGAGAAACTGAGAAATGAATGCTGAGACCCCCGGAGCAGGGGTCTGAGCCACAGCCGTGCTCGCCCACAAACTGATTTCTCACGGCGTGTCACCC
1301 CACCAGGGCGCAAGCCTCACTATTACTTGAACTTTCCAAAACCTAAAGAGGAAAAGTGCAATGCGTGTTGTACATACAGAGGTAACTATCAATATTTAAG
1401 TTTGTTGCTGTCAAGATTTTTTTTGTAACTTCAAATATAGAGATATTTTTGTACGTTATATATTGTATTAAGGGCATTTTAAAAGCAATTATATTGTCCT
1501 CCCCTATTTTAAGACGTGAATGTCTCAGCGAGGTGTAAAGTTGTTCGCCGCGTGGAATGTGAGTGTGTTTGTGTGCATGAAAGAGAAAGACTGATTACCT
1601 CCTGTGTGGAAGAAGGAAACACCGAGTCTCTGTATAATCTATTTACATAAAATGGGTGATATGCGAACAGCAAACCAATAAACTGTCTCAATGCTGATTC
1701 ATCTCTCGGCTCGGCTCTCACGTCTAGGAGGGAGGTGCCTGGTCCGGCCGCCCAGGAGCCTTGGGGTCCCGCCGCCGGGCTTGGGAAGGTGGGACAGCGG
1801 CGCGTGCAGGTGCTGGCCCTACTCTGAGCGGGCTGAGCTGTCACGAGCTCTGTTCTCTGTGTGGGGCGCGCAGAACACGTGTCCTGGGTGCGAATCAGGG
1901 CTTCGCGGAGACGCCCGAGGTCACCGCCTGGCGTGGCGGCCGGCGGGGGGTGGGTAGGGGGAGCCCTAGTGGGTTGGCGAGCCTGGACTCTCGGGTTGCG
2001 CAACGGAGTCCTAGATTTATAGGGTTGCTCACAGAACCCGGCGCGAAAGCGAGCTTCGAAGAGTTGGGGTGGTCTCTGAGGCCTTCTAAGAGAGGCGTTC
2101 CCCCTGCCTGCCGCGGTTGTTAAAGTTCCAAGCCGCCCTAGCAATGCGTGGGGACACCTCCGGAAAACCCAGTAACCCCTAGTAACCCCGGGTCTGCCCG
2201 GGCCTGAGCGCCCGCGGCCGCTGGCTCATGGGCGAGGCTGTAGCGCCCC.....3'

from nucleotide 666 to 821. The sequence for proadrenomedullin N-terminal 20 peptide (PAMP), which is possibly processed from an AM precursor and is assumed to be biologically active (4), is interposed by the second intron. The 3' end of fourth exon is followed by AATAAA sequence, a polyadenylation signal (10). This is in accordance with the reported 3' end structure of cDNA for human AM (4).

Figure 2 also indicates results of search for motif sequence in 5' flanking region and the first intron of human AM gene. A TATA box is located at nucleotide position -653, which is 49 bases upstream from the 5' end of reported AM cDNA sequence (11). A potential CAAT box is seen at -755 (12). A GC box, a binding site of the promoter-specific transcription factor (Sp1), is also adjacent at -682 (13). These elements are regarded as essential to the initiation of transcription by RNA polymerase II and basal expression of the gene. In addition, there are several binding sites of activator protein-2 (AP-2) in the 5' upstream region of exon 1, at -670, -682, -784, -793, and -1001 (14). Because AP-2 is assumed to mediate transcriptional activation induced by protein kinase C and cAMP, expression of AM gene may be subject to these signal-transduction pathways (14). Especially, considering that AM stimulates platelet cAMP production, the multiple AP-2 sites suggest existence of feedback mechanism by cAMP. In intron 1, at -280, there is consensus sequence for cAMP-regulated enhancer (CRE) (15). This may be also involved in such putative feedback regulation of AM gene expression by cAMP. Another CAAT box exists at -1255, and two activator protein-1 (AP-1) binding sites are seen at -1549 and -1801 (16). There is also another GC box at -1771 and the first intron contains multiple GC boxes near its 3' end. Although these motifs are a little distant from 5' end of the first exon, it may be possible that they participate in tissue specific expression of the AM gene. As well as AP-2, AP-1 has been shown to mediate transcriptional activation by phorbol ester (16). Therefore, expression of the AM gene may be influenced in such state as protein kinase C is activated, and thereby, AM may have a role in the process of cell proliferation.

Figure 3 shows the southern blot analysis of human genomic DNA digested with BamHI, EcoRI or PstI. Digestion by any of the three enzymes resulted in an appearance of a single positive band. Accordingly, AM gene is supposed to reside in a single site of the human genome. A 2.8 kb positive fragment is cut out by PstI. This is thought to be identical with the 2.8 kb PstI fragment cloned and sequenced from the human liver genomic library in the current study. This fragment covers 97% of the exons. The single 6.8 kb band seen in the EcoRI lane is also considered as corresponding to the 6.8 kb EcoRI fragment cloned from the library. Whole AM gene is included in

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Figure 2. Nucleotide sequence of human genomic DNA encoding human AM gene. The initiation codon of ATG is assigned nucleotide numbers 1 to 3. Panel A shows the 5' upstream sequence from this ATG codon, and panel B shows the 3' downstream sequence thereafter. Dotted nucleotides indicate motif sites in the 5' flanking region of AM gene. Sequence of AM cDNA is underlined. Amino acid sequence of preproadrenomedullin (preAM) is attached below. Amino acid sequence corresponding to mature AM is framed and that of proadrenomedullin N-terminal 20 peptide (PAMP) is doubly underlined.

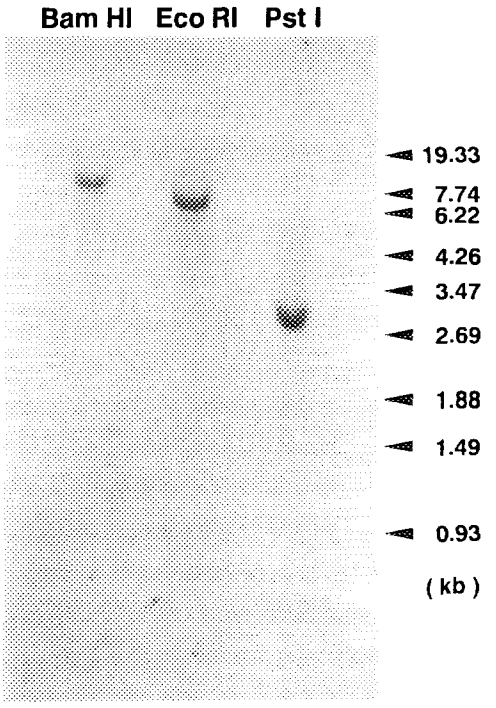

Figure 3. Southern blot analysis of human genomic DNA isolated from peripheral white blood cells. Twenty μg DNA was digested with BamHI, EcoRI or PstI, and was electrophoresed on an agarose gel. Hybridization was performed using AM cDNA as a probe. Arrowheads on the right indicate the positions of size markers in kb.

this fragment. The BamHI lane showed a single band at 11.5 kb. This is also compatible with the absence of BamHI site in the sequenced 6.8 kb EcoRI fragment. These results of genomic southern blot analysis suggest that the AM gene resides in a single locus of the human genome.

Figure 4 presents the southern blot panel of DNA from human-hamster somatic hybrid cell lines. The positive 2.8 kb PstI fragment hybridized with AM cDNA probe is seen in the lane of human genomic DNA but not in the hamster genomic DNA lane. Among the hybrid cell lines tested, only the DNA from the cell line No. 1049 yielded the corresponding band. This cell line contains human chromosomes 5 and 11. Several other cell lines (No. 734, 750, 867, 937, 1006 and 1079) also contain chromosome 5, however, they did not show a positive band. On the other hand, no other hybrid cell lines contain chromosome 11. Therefore, it is concluded that the human AM gene is localized on chromosome 11. Further sublocalization of the AM gene seems of interest, because the chromosome 11 is known to contain a number of genes involved in the endocrinological system including insulin, parathyroid hormone, and calcitonin (17-19). Responsible genes for multiple endocrine neoplasia type 1 and insulin-dependent diabetes mellitus have been found in the chromosome 11 (20, 21). CGRP, which shows partial homology to AM, is

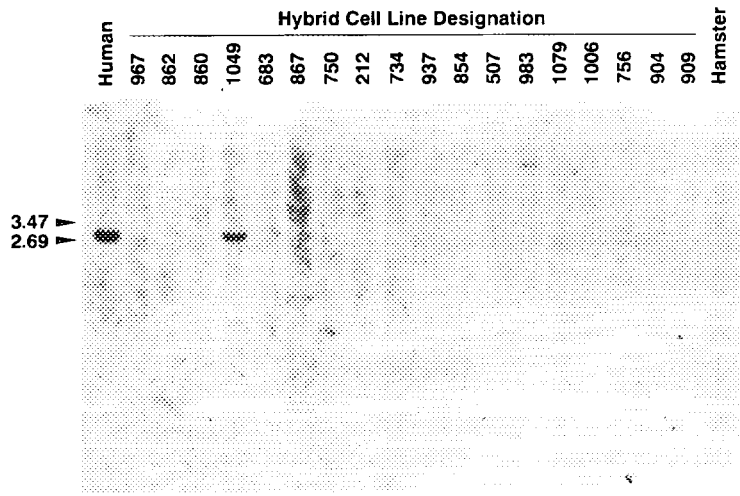

Figure 4. Southern blot panel for chromosomal assignment of AM gene. Genomic DNA from human-hamster somatic hybrid cell lines was digested with PstI and was electrophoresed on an agarose gel. The panel was hybridized with [^{32}P]-labeled AM cDNA. Arrowheads on the left indicate the positions of size markers in kb.

also encoded in this chromosome (22). With regard to the cardiovascular system, the chromosome 11 includes the gene for apolipoprotein A1, a major component of HDL, and the genes responsible for catecholamine metabolism such as dopamine receptors and tyrosine hydroxylase (23-25). Production of AM may be affected in these endocrinological and cardiovascular disorders. In addition, the chromosome 11 also contains a number of oncogenes and tumor suppressor genes relating to the genesis of lung, liver, breast and esophageal carcinomas, and hematological malignancies (26-32). The process of carcinogenesis induced by oncogenes is supposed to involve activation of protein kinase C (33). Therefore, considering that the 5' flanking region of AM gene includes multiple binding sites for AP-2 which is assumed to mediate signal transduction from protein kinase C (14), the expression of AM gene may be modulated in the process of cell proliferation. Several vasodilator substance such as natriuretic peptides and prostaglandins are known to inhibit the proliferation of cells (34, 35). In this context, AM may also have influence on cellular growth and thereby may affect the structure of cardiovascular system including cardiac hypertrophy and vascular wall thickening.

Because AM has a potent vasodilator action and its mRNA is expressed in various cardiovascular organs, the behavior of this peptide in the cardiovascular system is of primary interest. In the present study, we have determined the genomic structure of human AM gene. Analysis of the nucleotide sequence indicates that the AM gene is equipped with essential elements for its functional expression as a housekeeping gene and the expression may be affected by protein kinase C activity and cAMP level. Thus, the information obtained in this study is expected to

provide a step for further understanding of the regulatory mechanism of AM production and the pathophysiological role of this peptide in the cardiovascular and endocrine systems.

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