Structure and Expression of the Mouse Angiotensinogen Gene

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SUMMARY

Angiotensinogen is a precursor of the multifunctional octapeptide hormone, angiotensin II. We have isolated the overlapping clones containing angiotensinogen gene locus from C57BL/6 mouse genomic DNA library and analyzed them by restriction enzyme mapping. The gene exhibited a structural organization similar to those of the human, rat and balb/c mouse angiotensinogen genes. Using a genomic DNA fragment of the mouse angiotensinogen gene as a probe, we have investigated the tissue distribution of angiotensinogen messenger RNA (mRNA) in C57BL/6 mouse. The angiotensinogen mRNA was highest in the liver and detectable in such tissues as brain, kidney, submandibular gland, ovary and heart. However, it was undetectable in lung and spleen under the condition used. Optimal alignments of the 5'-flanking regions among the human, rat and mouse angiotensinogen genes disclosed several deletions in the mouse sequence. To assay the promoter activity, the 5'-flanking region of the mouse angiotensinogen gene was ligated to the bacterial chloramphenicol acetyltransferase (CAT) gene, then transfected into different cultured cells. The angiotensinogen gene sequences elicited preferential expression of CAT activity when introduced into HepG2 cells derived from liver and 293 cells from kidney but not in HeLa cells from uterus, suggesting the presence of a cell type-specific promoter within the sequences. These findings on the structure and expression of the mouse angiotensinogen gene should prove useful in studying the function and control of the angiotensin.

Key Words:
Messenger RNA  Promoter  Cell lines  Genomic DNA library
Tissue distribution

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THE sequential processing of circulating angiotensinogen by renin (EC 3.4.23.15) and angiotensin-converting enzyme (ACE) (EC 3.4.15.1) to yield the octapeptide angiotensin II (AII) is initiated by the release of the enzyme renin, primarily from the kidney. AII formed in this way has multiple diverse physiological effects on adrenal function, systemic blood pressure regulation, neurotransmitter synthesis and release, neovascularization, induction of drinking and ovulation. The diverse cellular roles of AII are presumably all mediated by interactions between the effector peptide and its specific receptors for the peptide in the plasma membrane of the target organs including blood vessel, kidney, adrenal, brain, anterior pituitary, uterus, ovary and heart. In addition to the circulating renin-angiotensin system, accumulating data reveal that components of the system also exist in these target organs and their presence is the result of tissue-specific gene expressions. Thus, an emerging concept is that AII may be produced locally in multiple tissues, independent of the circulating system, and that local synthesis of AII may be a significant autocrine or paracrine modulator of tissue function.

The production of transgenic mice by pronuclear microinjection has provided an especially powerful new tool not only for studying the regulation of gene expression in mammalian development, but also for studying the pathogenesis of human diseases as well as for developing and testing new therapies. Recently we have, therefore, generated the transgenic mice carrying the human renin and angiotensinogen genes and analyzed tissue- and cell-specific expression of these transgenes. Although the recombinational reactions of such transfer of the foreign gene into mice are usually nonhomologous, many cell types also possess the enzymatic machinery required for homologous recombination. The homology-dependent recombination between chromosomal DNA and exogenous sequences is referred to as gene targeting, and it offers an additional dimension to transgenic technology. Gene targeting permits the transfer of genetic alterations created in vitro to precise sites within the cellular genome. Particularly, mouse blastocyst-derived embryonic stem (ES) cells that are pluripotent provide us with an excellent opportunity to investigate gene function in the context of the developing and adult animals. This strategy of creating animals with specific genomic changes has immense potential in medicine and in furthering our understanding of the genetic control of mammalian development.

In the present study, as a first contribution to the analysis of mutants carrying lesions within the renin-angiotensin system, we have cloned the mouse angiotensinogen gene from C57BL/6, and characterized its gene structure and promoter activity.
Materials and Methods

Materials

Restriction and DNA modifying enzymes were purchased from TAKARA Shuzo, Nippon Gene and Toyobo. [$\alpha$-$^{32}$P]dCTP (>$3,000$ Ci/mmol) and [$^{14}$C]chloramphenicol (50–60 mCi/mmol) were obtained from Amersham Corp. and DuPont-New England Nuclear, respectively.

Isolation and characterization of mouse angiotensinogen gene

Genomic DNA was obtained from C57BL/6 mouse liver and the DNA library was constructed in $\lambda$ phage vector Charon 28 from a partial Sau3AI digest of DNA. Approximately $5 \times 10^5$ phages from the library were screened by plaque hybridization using a 1096-bp AccI fragment excised from rat angiotensigen cDNA (kindly supplied by Dr. Shigetada Nakanishi, Kyoto University).12) Phage DNAs of positive clones were subcloned into pUC119, and the DNA sequences were determined using the dideoxynucleotide chain termination procedure.13)

RNA isolation and Northern hybridization analysis

Total RNA was isolated from various tissues of C57BL/6 mouse by the method of Chirgwin et al.14) A 383-bp AatI-AatII genomic DNA fragment was used as a hybridization probe after labelled with [$\alpha$-$^{32}$P]dCTP and DNA polymerase I. RNA blotting, hybridization and washing were conducted as described previously.15)

Plasmid constructions, cell culture, DNA transfection and CAT assay

pUCSV3cat and pUCSV0cat were used as positive and negative controls, respectively.16) pUCmAGcat425 was constructed by inserting the 442-bp Sau3AI-HinPI genomic DNA fragment into pUCSV0cat.

HepG2 and HeLa cells were maintained in minimum essential medium (MEM) containing 10% fetal bovine serum. Human embryonic kidney 293 cells were cultured in MEM supplemented with 10% heat-denatured horse serum. These cell lines were kept in 5% $CO_2$ and plated approximately 24 hr before transfection at a density of $5 \times 10^5$ cells in 6 cm diameter plastic dishes.

Transfections and CAT assay were performed essentially, as described previously,15,17) using 3 $\mu$g of plasmid DNA in a CaPO$_4$ coprecipitate. Cells were harvested 42 h after transfection and assayed for CAT activity.
RESULTS

The C57BL/6 mouse genomic DNA library used was a collection of recombinant phage that contain mouse liver DNA fragments generated by partial digestion with Sau3AI and joined to the BamHI sites of λ Charon 28 arms. The DNA library was screened for the presence of recombinant phage-carrying sequences that are homologous to the rat angiotensinogen cDNA probe. From 5×10^5 phages two positive clones, λmAG-2 and λmAG-4, were isolated and plaque-purified, and their DNAs were extracted and subjected to BamHI restriction enzyme digestion. The BamHI restriction map showed that λmAG-2 overlaps the 3' ends of λmAG-4 by about 1.3 kb (Fig. 1). A more detailed mapping revealed that the position of various restriction sites of the C57BL/6 mouse angiotensinogen gene is almost identical to that of the gene from balb/c mouse and the location of the exons of the C57BL/6 gene was, therefore, confirmed by analogy from the restriction enzyme sites of the balb/c mouse gene as shown in Fig. 1. Furthermore, DNA sequence analysis (Fig. 3) showed that a highly conserved area extending from nucleotide +22 to −501 of the mouse angiotensinogen gene was found, with a homology between sequences of C57BL/6 and balb/c mice of about 99% (data not shown). From these results, as can be seen in Fig. 1, we concluded that λmAG-4 contains genomic DNA sequences that are 5' to the angiotensinogen gene while λmAG-2 contains genomic DNA sequences that are 3' to the gene.

The tissue specificity of angiotensinogen gene expression was assessed by Northern blot analysis using a cloned genomic DNA fragment of the mouse

![Restriction map of the mouse angiotensinogen genes. Two overlapping phage clones, λmAG-2 and λmAG-4, were isolated from mouse genomic DNA library. Exons are represented by boxes and the intron and flanking regions by thin lines. The filled boxes indicate the coding regions and the open boxes indicate the 5'- and 3'-untranslated regions. The maps of restriction enzyme recognition sites are shown by vertical bars.](image-url)
Fig. 2. Tissue distribution of mouse angiotensinogen mRNA. Total RNA (lane 1, 10 μg; lanes 2 to 7, 20 μg; lane 8, 2.5 μg; lane 9, 4.4 μg) and poly (A)+RNA (lane 10, 7 μg) derived from various tissues of C57BL/6 mice were subjected to Northern blot analysis. Lanes 1, liver; 2, kidney; 3, submandibular gland; 4, brain; 5, spleen; 6, lung; 7, heart; 8, adrenal; 9, ovary and 10, heart.

Dzau et al.19) have used RNA blot hybridization to compare the expression of the angiotensinogen gene in mouse and rat. The difference between mouse and rat is that angiotensinogen mRNA is detectable in testis and submandibular gland of mouse, but not in either tissue of rat. It was of interest, therefore, to compare the 5'-flanking regions of the mouse and rat20) genes, since it is well established that most regulatory sequence elements lie in the 5'-flanking region of eukaryotic genes. Alignment of the mouse and rat 5'-flanking region (Fig. 3) revealed a typical TATA box beginning at comparable positions in both genes. Two other features shown in the alignment are particular noteworthy. First, a region extending from nucleotide +22 to −207 in the mouse gene exhibits striking sequence similarity (90% identical) to its rat homolog. On the other hand, a further comparison to the promoter region of the human angiotensinogen gene15) reveals the lower homology (47% identical) in the region. Second, the mouse gene contains a large deletion located between nucleotide positions −209 and −210, where the rat gene contains 28 consecutive thymidine residues.20) One hypothesis arising from this comparison is that these various conserved domains and/or the multiple insertions and deletions could be important in determining the observed pattern of tissue- or species-specific expression of the angiotensinogen gene.

A convenient approach to study control of gene expression in eukaryotic cells and to identify DNA sequences functioning as cis-acting transcriptional signals is to fuse a predetermined DNA segment upstream to the coding sequence of the bacterial enzyme CAT. These constructs are then introduced
into cultured cells by transfection, and expression can be measured as CAT enzymatic activity. Therefore, we initiated an in vitro study to identify functionally important domains in the 5'-flanking region of the mouse angiotensinogen gene. DNA sequences containing the 5'-flanking region of the mouse angiotensinogen gene (425 nucleotides) were fused to the CAT gene. The hybrid CAT gene was introduced into different cell lines, and the transient expression of the CAT gene, mediated by the promoter activity of the angiotensinogen gene fragment, was measured. The results in Fig. 4 showed that the 5'-flanking sequences of the angiotensinogen gene exhibit the promoter activity in hepatoma HepG2 cells and embryonic kidney 293 cells, but not in cervical carcinoma HeLa cells. This suggested the presence of a functional and cell type-specific promoter within this region tested.
Fig. 4. Chloramphenicol acetyltransferase (CAT) enzyme activity in HeLa, HepG2, and 293 cells transfected with pUCmAG425cat. pUCmAG 425cat (mAg) contains the CAT-coding gene and the 425-bp 5'-flanking region (at nucleotide positions -425 to +17; see Fig. 3) of the mouse angiotensinogen gene. pUCSV3cat (SV3) and pUCSV0cat (SV0) are used for positive and negative controls, respectively. CAT assay was performed with extract containing 80 μg of cell lysate protein.

DISCUSSION

Analysis of the mouse angiotensinogen gene structure is an important first step toward analyzing the function and mechanism which regulates its expression. The genomic organization of C57BL/6 mouse angiotensinogen gene predicted from the restriction enzyme mapping is almost identical to that of the balb/c gene, whose structure is very similar to that of the other species.15,21 By genomic Southern blot analysis, Clouston et al22 found that a major difference between the structures of C57BL/6 and balb/c angiotensinogen genes is that an intracisternal A-particle (IAP) gene family is inserted 9 kb upstream from the transcription start site of the C57BL/6 mouse gene. Mouse IAPs are endogenous retrovirus-like structures containing long terminal repeat (LTR)-like sequences. Genes coding for IAPs are members of a dispersed multigene family with approximately 1000 copies per haploid genome in Mus musculus.23 It has been shown that IAP genomes are associated with both the inactivated and activated cellular genes. A recent report has demonstrated that IAP LTRs vary in their promoter activity and some are capable of bidirectional transcription.24 In addition to directing their own transcription, IAP LTRs have the capability of promoting transcription of adjacent cellular sequences as found for LTRs for many retroviruses. Two documented cases are the insertion of an IAP sequence into the intron of the immunoglobulin kappa-light chain gene, thereby inactivating its expression,25 and the activation of the c-mos gene in the plasmacytoma
cells by the insertion of an IAP gene in the 5’ end of the c-mos coding region. However, it is unclear whether the IAP sequence of the C57BL/6 angiotensinogen gene has an ability to activate the gene expression as a regulatory element.

We have determined the sites of synthesis of mouse angiotensinogen by screening for the presence of its mRNA in a variety of tissues. The angiotensinogen mRNA was detected in the liver, kidney, submandibular gland, brain and heart. Additionally, Dzau et al have found that the angiotensinogen mRNA is present in the adrenal and testis in CD-1 mouse, but Clouston et al demonstrated that its testicular mRNA is absent in balb/c and A/J mice. Although the former authors described only the presence of angiotensinogen mRNA in the heart of CD-1 mouse and the latter authors established that both the endogenous angiotensinogen gene and the transgenic mini-balb/c angiotensinogen gene are expressed in the transgenic mouse heart of Swiss strain, no data are available for the expression of cardiac angiotensinogen gene in normal mouse. Thus, our result is the first report of the cardiac expression of the normal mouse angiotensinogen gene.

The traditional concept of the renin-angiotensin system is a circulating endocrine system whose components are secreted by different organs, i.e., renin from the kidney, angiotensinogen from liver and angiotensin-converting enzyme from lung. Recently, there is increasing recognition that the renin-angiotensin system exerts its modulatory influences on circulating homeostasis not only through its circulating components, but also through generation of these components at a local level in various tissues. Especially in the heart, the biochemical and molecular biological identification of renin, angiotensinogen, AII and its specific receptor has supported the existence of the intrinsic renin-angiotensin system. Although ACE-like activity was reported to be higher in atria than in ventricles, it has been suggested to be different from the enzyme present in lung with regard to a number of parameters, such as isoelectric point, noncovalent cation requirements and substrate specificity. Instead of the known pathway of angiotensin production through ACE, recent studies have suggested that alternative pathways leading to the formation of AII are active in the heart and demonstrated that the major cardiac AII-forming enzyme is chymase, a serine protease, with an ability to convert angiotensin I to AII specifically. The identification of heart chymase adds a new dimension to the cardiac renin-angiotensin system, because this chymase potentially allows for the formation of AII without an inactivation of other regulatory peptides.

Therefore the paracrine and autocrine functions of cardiac angiotensin have received a great deal of attention for several reasons as follows: First,
the cardiac angiotensin may influence coronary vascular tone and therefore blood flow. Second, in addition to its vasoconstricting effect, angiotensin has also shown a positive inotropic property and may contribute to the regulation of cardiac contractility. The third speculative function of the angiotensin is in the cardiac cell development. Since it has been reported that AII induces hypertrophy, not hyperplasia, of cultured aortic smooth muscle cells, it is a logical extension to speculate that angiotensin may be involved in the cardiac hypertrophy. In contrast, of particular interest is the observation that constitutive expression of the introduced proto-oncogene c-myc in the transgenic mouse during development results in enhanced hyperplastic growth of the heart. In fact, Naftilan et al have provided evidence that AII can induce c-myc mRNA in vascular smooth muscle cells. Although there is no direct evidence at present time, it would be likely that angiotensin plays a role in cardiac hyperplasia through the c-myc gene activation.

It is well established that tissue culture cells transfected with recombinant DNA can express cloned genes if the regulatory sequences are present. Comparison of the 5'-flanking sequences of the rat, human and mouse angiotensinogen genes shows that these regions contain several identifiable promoter elements, including TATA box at positions around −30 bp relative to the putative start site of transcription. Clouston et al identified that the 750-bp 5'-flanking sequence of the balb/c mouse angiotensinogen gene is a minimal promoter, being sufficient to confer steroid hormones and bacterial endotoxin inducibility, on a mini-gene construct containing an internal deletion of much of its coding region introduced into the genome of Swiss mice. Our previous result regarding the human angiotensinogen promoter analysis showed that the 1.3-kb 5'-flanking sequences can function in a cell type-specific fashion. Brasier et al also found that there are multiple cis-acting DNA regulatory elements, in the 1.6-kb 5'-flanking region of the rat angiotensinogen gene, mediating its hepatic gene expression. In the present study, we have demonstrated that the DNA flanking sequences which contain 425 nucleotides of the mouse angiotensinogen gene appear to be responsible for its cell type-specific transcription in HepG2 and 293 cells. It has been proposed that the cell- and species-specific activity of flanking DNA sequences may be due to interactions with trans-acting factors. The specific gene activity could, therefore, be determined by the presence or absence of trans-acting factors that interact with the regulatory sequences to activate or repress transcription. Although we cannot rule out that other sequences present in and around the mouse angiotensinogen gene in its native chromosomal location have regulatory functions, we suspect that the
425-bp 5'-flanking sequences are the best locations for detecting DNA elements involved in cell- and species-specific transcriptional control of the angiotensinogen gene. We can now ask if this type of control is mediated through the enhancer, the proximal element, or through as yet unidentified sequences.

In summary, we have cloned the C57BL/6 mouse angiotensinogen gene and localized the functional promoter to the 5'-flanking region of the gene. As mentioned in the introduction of this literature, the gene-targeting strategy has been widely used to transfer mutations created in vitro to specific loci in mouse ES cells. Thus, we believe that the characterization of the angiotensinogen gene structure and the creation of mice with defined mutations within the gene should facilitate further understanding of the in vivo function of angiotensin.

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REFERENCES


