Isolation of the Mouse *Ren-1C* Gene and Characterization of Renin Gene Expression in Both ES-D3 Cells and Their Parental Mouse Strain

Keiji TANIMOTO, Kouichi TAMURA,
Fumihiro SUGIYAMA¹⁾, Kazuo MURAKAMI and
Akiyoshi FUKAMIZU

Institute of Applied Biochemistry and
¹⁾*Laboratory Animal Research Center,*
University of Tsukuba, Tsukuba, Ibaraki 305, Japan

Abstract. Renin is the rate-limiting enzyme in the renin-angiotensin system, the only known biochemical cascade that produces the potent octapeptide effector molecule angiotensin II that controls, to a large degree, cardiovascular homeostasis and reproductive function in mammals. In the present study, we have cloned the *Ren-1C* gene from the C57BL/6 mouse and determined its genomic structure. In order to provide the molecular basis for the application of the gene targeting method to the renin gene, we also characterized the structure and expression of the renin gene in embryonic stem D-3 (ES-D3) cells and in its parental mouse strains, 129/Sv mouse. The *Ren-1C* gene is 12 kilobases long and is composed of 9 exons interrupted by 8 introns. Renin mRNA was undetectable in ES-D3 cells but was found to be highly expressed in the submandibular gland of the 129/Sv mouse, in addition to the kidney. Southern blot analysis reveals that ES-D3 cells carry the additional duplicated renin gene, *Ren-2*, consistent with the classification of the 129/Sv mouse as a two-renin gene strain.

Key words: Renin, ES-D3 cells, Gene structure, Gene expression.

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Renin, an aspartyl proteinase synthesized mainly in the kidney, is the rate-limiting factor in the renin-angiotensin system, which is well known for its role as a systemic regulator of blood pressure and fluid homeostasis [1]. This system operates *via* the cleavage of circulating angiotensinogen by renin to produce angiotensin I which is in turn acted upon by angiotensin-converting enzyme to produce the active octapeptide, angiotensin II. In addition to its endocrine role, the renin-angiotensin system may also function in a paracrine manner, as components of the renin biochemical cascade have recently been reported in many organs, particularly in those concerned with cardiovascular and reproductive function [2].

Reproductive organs in which renin-angiotensin

system components have been described include the ovary, testis, uterus and prostate gland [3–5]. High levels of angiotensin II have been found in the rat ovary and various specific receptors have been localized to several ovarian structures [5]. Furthermore, administration of the angiotensin II antagonist, saralasin, has been shown to block rat ovulation, suggesting the possible involvement of the renin-angiotensin system in the ovulatory process [6]. Thus renin, through the control of angiotensin II synthesis, is thought to play an important role in many physiologic processes.

Although the renin gene for rat and human exists in the genome as a single copy, mice have been known for some time to be polymorphic in respect to the number of renin genes. All inbred strains of mice commonly carry the *Ren-1* renin gene and some strains of mice such as DBA/2,

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Correspondence: A. Fukamizu

AKR and Swiss possess two renin genes, *Ren-1* and *Ren-2*. The 2 common alleles of the *Ren-1* locus are designated by their origin: *Ren-1C* is the renin gene in strains containing a single renin locus and *Ren-1D* is the gene in strains bearing 2 renin loci. Despite the fact that *Ren-1C* and *Ren-2* compared to *Ren-1D* display 98% and 96% [7, 8] sequence identity at the cDNA level, respectively, each renin gene exhibits a unique pattern of tissue-specific expression. *Ren-1C* is expressed mainly in the kidney, with only weak expression in the submandibular gland (SMG) and none in the adrenal [9]. Although *Ren-1D* and *Ren-2* are expressed at approximately equal levels in the kidney and adrenal, *Ren-2* gene expression in the SMG is approximately 100-fold higher than that of the *Ren-1D* gene [9, 10].

The production of transgenic animals by pronuclear microinjection has provided a particularly powerful approach not only for studying the regulation of gene expression in mammalian development, but also in studying reproductive endocrinology [11–13]. The use of homology-dependent recombination between chromosomal DNA and exogenous DNA sequences in the method of gene targeting has offered a further dimension to transgenic technology. This approach employs pluripotent blastocyst-derived mouse embryonic stem (ES) cells [14] which are utilized for the introduction of selected targeted mutations into the mouse germ-line. As a first step in the *in vivo* analysis of mutants carrying lesions within the *Ren-1C* gene we have chosen the C57BL/6 strain as a cloning source and have subsequently isolated the *Ren-1C* gene. Furthermore, we have characterized the structure of this renin gene and have examined its expression in ES-D3 cells, which are commonly used in gene targeting experiments [15, 16], as well as in the donor 129/Sv strain by blot hybridization analyses.

Materials and Methods

Isolation of *Ren-1C* genomic clones

A mouse genomic DNA library was constructed as a partial *Sau3AI* digest of C57BL/6 DNA cloned into bacteriophage λ Charon 28. Plaque hybridization was performed using a ^{32}P -labelled 816-base pair (bp) *KpnI/NcoI Ren-2* cDNA fragment [17] as

a probe. DNA restriction fragments were subcloned into plasmid vectors and sequenced by the dideoxy-chain termination method [18].

Northern and Southern blot analyses

Total RNAs were extracted from liver, SMG and kidney of the 129/Sv mouse and the ES-D3 cell line and were denatured with 1 M glyoxal and 50% dimethyl sulfoxide followed by electrophoresis on a 1.2% agarose gel and transferred to a GeneScreen Plus membrane. Mouse genomic DNAs were extracted from C57BL/6 and 129/Sv strains and were digested by *EcoRI*, electrophoresed on a 0.7% agarose gel, and transferred to a GeneScreen Plus membrane. Hybridization was performed using a ^{32}P -labelled mouse *Ren-2* cDNA probe [17].

Results

Isolation of the *Ren-1C* gene

The C57BL/6 mouse genomic library generated represents a collection of recombinant phages produced by inserting partial *Sau3AI* digest products of liver genomic DNA between the *BamHI* sites of the arms of λ Charon 28. Through plaque screening by the use of a mouse *Ren-2* cDNA probe [17], we have isolated 3 overlapping phage clones, designated as $\lambda\text{mRn-1}$, $\lambda\text{mRn-2}$, and $\lambda\text{mRn-3}$ (Fig. 1), that in total contain the entire *Ren-1C* gene. Detailed restriction endonuclease mapping and partial sequence analysis revealed that the positions of various restriction endonucleases including *EcoRI* and *HindIII* as well as *KpnI* of the C57BL/6 *Ren-1C* gene are identical to those of the *Ren-1D* and *Ren-2* genes from the DBA/2 mouse strain [7] and that the 5'-flanking region extending from nucleotides -187 to +18 in the *Ren-1C* gene exhibits striking sequence similarity to those of the *Ren-1D* (99.5% identity) and *Ren-2* (91.2% identity) genes (data not shown). The locations of the exons and introns of the *Ren-1C* gene were able to be deduced both by comparison with the restriction enzyme sites of the DBA/2 mouse renin genes (Fig. 1) and by partial sequence analysis (data not shown).

Northern blot analysis

As ES-D3 cells, derived from murine strain 129/Sv, are now commonly used for establishing

Fig. 1. Restriction map of the mouse *Ren-1C* gene. Three overlapping phage clones, λ mRn-1, λ mRn-2 and λ mRn-3, were isolated from a C57BL/6 mouse genomic DNA library. Exons are depicted by the filled-in boxes and intronic sequences are represented by the joining thin horizontal line. The locations of accompanying restriction enzyme recognition sites are shown by vertical bars.

cell lines that contain a mutation at a specific site within a chromosome by the gene targeting approach [15, 16], we have attempted to determine the level of renin gene expression in these cells in addition to investigating the expression levels in the SMG and kidney of the donor mouse strain, 129/Sv. The latter investigation is prompted by the observation that there exist characteristic marked differences in tissue- and sex-specific expression of the renin genes in strains bearing two renin genes. In such strains, *Ren-2*-derived mRNA accumulates in the male SMG to an approximately 50-fold higher level than that of the female with the level of renin gene expression in the kidney being 2-fold greater in the female than in the male [19]. Therefore, we have extracted total RNAs from liver (negative control), SMG and kidney of the 129/Sv mouse as well as from ES-D3 cells and have performed Northern blot analysis. Fig.2 shows that the level of renin mRNA in the 129/Sv strain is considerably higher in the male SMG (lane 5) than in that of the female (lane 2). In the same strain, however, the level of kidney renin mRNA is approximately 2-fold higher in the female (lane 3) than that in the male (lane 6). On the other hand, ES-D3 cells did not display any synthesis of renin mRNA (lanes 7 and 8). Under similar Northern hybridization conditions, SMG RNA from the C57BL/6 mouse did not produce any detectable hybridization signal (data not shown).

Fig. 2. Analysis of renin gene expression. Tissue preparations from female (lanes 1 to 3) and male (lanes 4 to 6) 129/Sv mice along with extracts from ES-D3 cells (lanes 7 and 8) were used as sources of RNA. Total RNA (lanes 1 to 7, 15 μ g; lane 8, 30 μ g) was subjected to Northern blot analysis using a 32 P-labelled mouse *Ren-2* cDNA probe [17]. The positions of the 28S and 18S ribosomal RNA markers are indicated.

Southern blot analysis

The above Northern blot analysis is consistent with the existence of an additional renin gene, *Ren-2*, in strain 129/Sv. As ES-D3 failed to synthesize any significant amount of renin mRNA we performed Southern blot analysis on genomic DNA extracted from this cell line in order to establish whether or not ES-D3 cells possess 1

Fig. 3. (A) Organization of mouse renin genes. Hatched boxes represent *Sau*3AI DNA fragments inserted into λ Charon 28 forming λ mRn-1 and λ mRn-3. The nine exons are shown by the filled-in boxes with adjoining intronic sequences represented by the thin horizontal line. The *Eco*RI (R) DNA fragments detected by Southern analysis are depicted by the double arrow lines with the fragment sizes as indicated. (B) Southern blot analysis of mouse renin genes. Two micrograms of tail DNA (lanes 1 and 3) and ES-D3 cell DNA (lane 2) and 0.2 ng of recombinant phage DNA (lanes 4 and 5) were digested with *Eco*RI followed by electrophoresis on a 0.7% agarose gel and transferred to a nylon membrane. Lane 1, DBA/2 DNA (two-renin gene strain); lane 2, ES-D3 cell DNA (derived from strain 129/Sv); lane 3, C57BL/6 DNA (one-renin gene strain); lane 4, λ mRn-1 DNA; lane 5, λ mRn-3 DNA. Sizes of detected bands are shown on the right side of each gel.

renin gene or 2 renin genes. This method of approach was employed since on the basis of *Eco*RI hybridization patterns strains of mice bearing either 1 or 2 renin genes can be readily distinguished (Fig. 3A) [20]. For comparison, we used genomic DNAs from DBA/2 and C57BL/6 mice and phage DNAs from λ mRn-1 and λ mRn-3. As shown in Fig. 3B, genomic DNA extracted from the one-gene mouse, C57BL/6, gave rise to 2 bands of 8.8 and 3.9 kb (lane 3) that represent the *Ren-1C* gene, while DNA extracted from the two-gene mouse, DBA/2, and ES-D3 cells gave rise to 2 additional hybridization bands of 9.2 and 4.4 kb (lanes 1 and 2) representing the *Ren-2* gene. The cloned *Ren-1C* gene carried on phages λ mRn-

1 and λ mRn-3 also exhibited 2 bands of 8.8 and 3.9 kb (Fig. 2A, and lanes 4 and 5 of Fig. 3B), although additional bands of 10 and 7.2 kb derived from the cloning vector were also observed. These results are consistent with the fact that ES-D3 cells possess 2 renin genes, *viz.* *Ren-1D* and *Ren-2*. Therefore, these data in combination with Northern analyses support the conclusion that the 129/Sv mouse strain carries both the *Ren-1D* and *Ren-2* genes.

Discussion

In the present study, we have cloned the *Ren-1C* gene from a C57BL/6 mouse genomic DNA library and have determined its structure. As expected, the exon-intron arrangement (9 exons and 8 introns) of the *Ren-1C* gene (Fig. 1) is very similar to that of both the *Ren-1D* and *Ren-2* genes [7] as well as that of the rat renin gene [21]. Despite the fact that the *Ren-1D* and *Ren-2* genes have been widely used in the production of a number of transgenic animals [9, 10, 22], the *Ren-1C* gene has not been similarly examined. In the case of *Ren-2*, Mullins *et al.* [22] have demonstrated that transgenic rats carrying this gene exhibit fulminant hypertension, evidence which represents a divergence away from the long standing belief that only the *Ren-1C* and *Ren-1D* genes expressed mainly in the kidney play the major role in blood pressure regulation [23]. These results are suggestive of the possible involvement of the *Ren-2* gene in the pathogenesis of high blood pressure. In contrast to these data, the *Ren-2* gene when introduced into mice with a *Ren-1C* genetic background did not lead to the development of hypertension [9, 10]. At present, the reason for the different effects of *Ren-2* action between the 2 species, rat and mouse, is poorly understood and therefore future investigations aimed at understanding these differences are likely to focus on altering the host's genetic background by the use of the gene targeting approach. One important candidate for this approach is the *Ren-1C* gene which may possibly be mutated by the targeting method and thereby further our knowledge of host regulation in the genesis of hypertension.

It has generally been accepted that the frequency of gene targeting is strongly dependent upon

the extent of homology between the targeting, or mutated DNA construct, and the locus targeted [24–26]. For example, a 2-fold increase in the extent of homology results in a 20-fold increase in the frequency of targeting [24]. Further studies have indicated that constructs prepared with vector DNA isolated from strains of mice from which the recipient ES cell line was derived (isogenic DNA) targeted 4 to 20 times more efficiently than did the same construct bearing vector DNA derived from an unrelated mouse strain (nonisogenic DNA) [25, 26]. Besides the overall degree of homology and vector source, individual basepair variation may, in addition, affect the rate of homologous recombination. In this regard, Letsou and Liskay [27] have shown that the frequency of intrachromosomal gene conversion is sensitive to even a single base-pair mismatch within a 1-kb interval.

Interestingly, Miller *et al.* [28] have recently reported that a *Ren-1D* targeting vector homologously recombined only with the *Ren-1D* locus of EK-CP1 ES cells which possess both the *Ren-1D* and *Ren-2* genes. This is in spite of the fact that both renin loci are highly homologous. It can therefore be anticipated that in the *Ren-1D* targeted EK-CP1 transgenic mouse renin activity may not be greatly altered as compared with the wild-type due to the presence of the fully active *Ren-2* locus. Likewise, one can predict that the *Ren-1D* gene borne on a vector derived from two-renin gene ES cells would poorly recombine with the *Ren-1C* gene of ES cells originating from mice carrying a single renin gene on the grounds of homology constraints and the nonisogenic source of the gene. Therefore, in order to perform efficient *Ren-1C* gene targeting and thus assess the involvement of this gene in blood-pressure homeostasis and reproductive function, it is necessary to prepare mutants of *Ren-1C* on a vector derived from single renin gene ES cells and thereby effectively introduce these constructs into

an ES cell line originating from a one-gene mouse strain.

As judged by Northern blot analysis (Fig. 2), we were unable to detect mouse renin mRNA in ES-D3 cells derived from the 129/Sv mouse. Nevertheless, the present study has shown that in this strain there is a considerable amount of renin mRNA present in the SMG as well as in the kidney. Since a high level of expression of the renin gene in the SMG is diagnostic for the presence of two renin genes, as such is the case with the DBA/2 strain [19], the expression results suggest that like this strain the 129/Sv mouse possesses 2 renin genes, *viz.* *Ren-1D* and *Ren-2*. Southern blot analyses (Fig. 3) are consistent with this concept.

Thus in summary, this study has shown that ES-D3 cells carry both the *Ren-1D* and *Ren-2* genes by Northern and Southern blot analyses. In contrast to the ES-D3 cell line, Ledermann and Bürki [29] have established an ES cell line derived from the C57BL/6 mouse which can be used in gene targeting experiments aimed at examining the functional role of the *Ren-1C* gene. To this end, the cloned *Ren-1C* gene from strain C57BL/6 described in the present study will be of great assistance in that efficient recombination and thus effective gene targeting should be able to be realized on the basis of the ideal homology match.

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