

Targeted integration of the *Ren-1D* locus in mouse embryonic stem cells

(gene targeting/renin genes)

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ABSTRACT We have introduced a *Ren-1D* targeting vector into embryonic stem cells containing the two highly homologous mouse renin genes *Ren-1D* and *Ren-2*. Using a polymerase chain reaction (PCR) screen designed to detect targeted integration at *Ren-1D* and *Ren-2*, we isolated 15 targeted embryonic stem cell clones, all of which had undergone a gene conversion event at the *Ren-1D* locus. We did not isolate any clones in which the incoming DNA had recombined with *Ren-2*. Over the region encompassed by our transgene, *Ren-1D* and *Ren-2* display >95% homology. Our results suggest that the machinery driving gene targeting by means of homologous recombination in mammalian cells is capable of distinguishing between these two sequences. Construction of transgenic mice with the embryonic stem cells reported here carrying a mutated renin gene will permit a greater understanding of the functions of the *Ren-1D* and *Ren-2* gene products and their relative contribution to cardiovascular homeostasis.

Renin is an aspartyl protease that catalyzes the initial and rate-limiting step in the conversion of angiotensinogen to the potent vasoactive hormone angiotensin II. The primary site of synthesis of circulating renin is the juxtaglomerular cells of the kidney. However, a number of other extrarenal sites of renin biosynthesis have now been described, including the adrenal gland, liver, and testes (1–3). The function of this extrarenal renin is not known.

In mice, high levels of renin and renin mRNA can also be found in the submandibular gland (4, 5). Mice are polymorphic for the number of renin genes, certain inbred strains harboring one gene (*Ren-1C*) and others containing two genes (*Ren-1D* and *Ren-2*). The mouse renin genes are located on chromosome 1 (6), with the *Ren-2* gene situated ≈20 kilobases (kb) upstream of *Ren-1D* (relative to the transcriptional direction) in two-gene animals; both genes are transcribed in the same direction (7). The presence of the *Ren-2* gene appears to be the result of a duplication of the renin locus ≈3–10 million years ago (8, 9). DNA sequence features unique to *Ren-2* include the presence of a proviral intracisternal A particle located downstream and a B2 repetitive element situated upstream of *Ren-2* (10, 11). However, *Ren-1D* and *Ren-2* display a high degree of homology across the structural region of the gene having the same genomic organization (9, 12, 13) and sharing >96% sequence identity at the level of the transcript (9). At the genomic level (including intronic sequences) *Ren-1D* and *Ren-2* share ≈95% sequence identity (ref. 13; unpublished results). The three mouse renin genes display similar but distinct tissue specificities (1, 3, 14, 15) and similar developmental expression profiles (16).

Gene targeting by means of homologous recombination in embryonic stem (ES) cells is a powerful technology whereby

predetermined mutations can be engineered into mice (see refs. 17 and 18). A number of genes (expressed and silent in ES cells) have now been mutated in ES cells by this route and transgenic mice have been created from these manipulated clones. In this investigation we have introduced a *Ren-1D* targeting vector into mouse ES cells harboring *Ren-1D* and *Ren-2*. We have then determined with which of the two renin genes our incoming DNA has recombined. Our results suggest that the machinery driving gene targeting in mammalian cells is capable of discriminating between these two highly homologous genes. Construction of transgenic mice from these targeted ES cells will enable a more complete understanding of the physiological roles of the *Ren-1D* and *Ren-2* gene products.

MATERIALS AND METHODS

Gene Targeting Construct. The *Ren-1D* targeting vector was constructed by inserting a 2040-base-pair (bp) *Pvu* II fragment containing the herpes simplex virus 1 thymidine kinase (*HSV-Tk*) gene into the *Hind*III site of pUC18, which was end-repaired with T4 polymerase to create pTK. A 5579-bp *Sph* I to *Bam*HI *Ren-1D* fragment (positions 3173–8752 in ref. 13) spanning exons two to five from *Ren-1** (3) was then cloned into the *Bam*HI site of pTK to create pTK-Ren by end-repairing fragment and insert with T4 polymerase. *Ren-1** contains an engineered additional 30-bp tag in exon 2 that contains an *Xho* I site that is unique to this gene (3). The *neo* gene under the control of the mouse phosphoglycerate kinase 1 gene (*Pgk-1*) promoter with 3' termination sequences from *Pgk-1* was then inserted into this *Xho* I site by means of *Xho* I linkers to create pTK-Ren-*neo* (Fig. 1). The *Pgk-1*-*neo* cassette was obtained from the plasmid pKJ1 (see refs. 19 and 20 for further details). The *neo* and *Tk* cassettes transcribe in the same direction as the renin DNA. Prior to transfection, pTK-Ren-*neo* was linearized with *Ssp* I, which cuts within the pUC18 vector sequences.

ES Cells and Cell Manipulations. EK-CP1 ES cells (21) were maintained either on mitomycin-treated STO feeders that had been rendered resistant to G418 by transfection of the plasmid pMC1neo (22) or on recombinant leukemia inhibitory factor (Amrad, Melbourne, Australia) as described (23). EK-CP1 cells are derived from 129/Sv/Ev mice, which contain *Ren-1D* and *Ren-2* (ref. 24 and our Southern analyses). Cells were electroporated using a Bio-Rad Gene Pulser. Briefly, 10⁷ trypsinized ES cells resuspended in 1 ml of phosphate-buffered saline (PBS) were electroporated with 25 μg of linearized pTK-Ren-*neo* at 500 μF and 250 V in a 0.4-cm pathlength electroporation chamber (Bio-Rad). These cells were then plated out on ten 9-cm Petri dishes. Selection with G418 (250 μg/ml) and gancyclovir (2 μM) was applied after

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Abbreviation: ES, embryonic stem.

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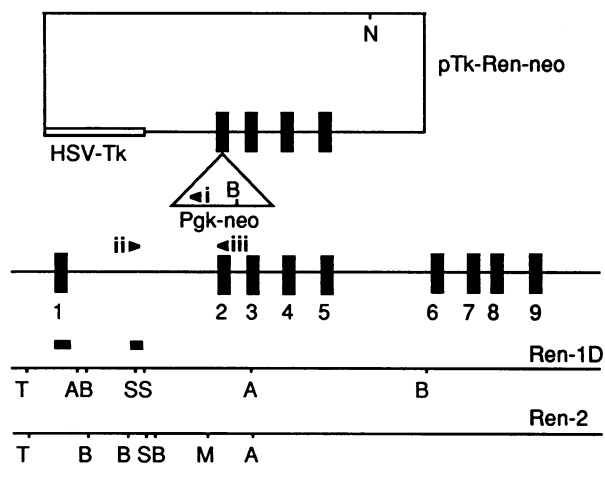


FIG. 1. Targeted integration into the mouse renin genes. The *Ren-1D* targeting vector pTk-Ren-neo is shown at the top. The site of insertion of the Pkg-neo cassette in exon 2 is shown with the *HSV-Tk* gene displayed as an open box. The structure of the mouse renin genes with exons (1–9) displayed as solid boxes is shown below. The relevant restriction sites in *Ren-1D* and *Ren-2* are shown at the bottom. pUC18 vector sequences are not to scale. The positions of PCR primers i–iii are indicated by arrowheads. Heavy lines indicate the probe DNA used for studying the structure of the *Ren* genes. A, *Asp*718; B, *Bam*HI; S, *Sph* I; T, *Sst* I; N, *Ssp* I; M, *Sma* I. Scale bar = 1 kb.

40 hr. Clones were isolated after 9–13 days as described (25). Targeting enrichment values for gancyclovir were calculated by placing plates under G418 selection only.

PCR and Southern Analyses of Cells. Clones were analyzed by PCR initially in pools of four essentially as described (26). Briefly, cells were pelleted at $650 \times g$ and resuspended in 5 μ l of PBS, and 55 μ l of distilled water was then added. Following a 10-min heat treatment at 95°C, 10 μ g of proteinase K in 1 μ l of distilled water was added and the samples were incubated at 55°C for 30 min. The proteinase K was then inactivated at 95°C for 10 min. Thirty microliters of this DNA preparation was used in a 100- μ l PCR reaction using Cetus Taq polymerase according to the manufacturer's instructions. Amplifications were carried out for 40 cycles of 1 min at 95°C, 2 min at 65°C, and 10 min at 72°C.

Primers used for the reaction were derived from Pkg-neo sequence (5'-GCTGCTAAAGCGCATGCTCCAGAC-3'), which primes on the *Pgk-1* promoter (primer i), and from renin intron 1 sequence (5'-GAAGAGGTTAGTGAATGCTCAGAAC-3') (primer ii). The renin primer flanks the incoming vector DNA and is derived from sequence that is identical in *Ren-1D* and *Ren-2*. The renin primer (primer ii) thus primes on *Ren-1D* and *Ren-2*. This was formally demonstrated by sequence analysis and restriction mapping of PCR products derived from EK-CP1 cell DNA isolated and processed as above using primer ii and a primer derived from exon 2 (5'-TTCAGCACTGAGCCTGGTCATGTCCACTCC-3') (primer iii). Primer iii is derived from sequence that is again identical in *Ren-1D* and *Ren-2* and is situated 21 bp upstream of the site of insertion of the Pkg-neo cassette in the targeting vector. A PCR product of ≈ 1630 bp is generated with primers ii and iii. When this product was cloned and 12 of these clones were sequenced, 5 were found to contain *Ren-1D* DNA and 7 contained *Ren-2* DNA. When the product was digested with *Sma* I, which cuts *Ren-2* but not *Ren-1D* amplified DNA to release fragments of 1416 bp and 215 bp (Fig. 1), the 1639-bp fragment diagnostic of *Ren-1D* amplification and the 1416-bp *Ren-2* fragment were both generated (Fig. 2). Conversely, in PCR reactions using primer ii and primers derived from sequences near primer iii that are gene specific (by having

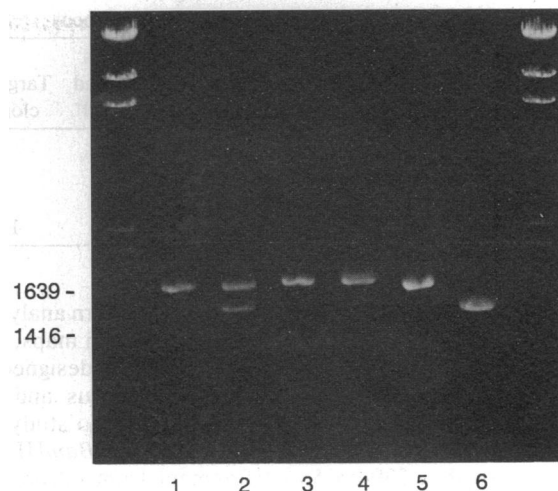


FIG. 2. PCR reactions demonstrating the specificity of primer ii used to detect targeted integration at the *Ren* loci. Lanes 1 and 2 are with primer ii and a primer derived from exon 2 (primer iii) that also primes on both renin genes. Following digestion with *Sma* I, which cuts *Ren-2* but not *Ren-1D* amplified DNA, fragments of 1639 bp and 1416 bp (diagnostic of *Ren-1D* and *Ren-2* amplification) are produced. When primer iii is replaced with primers specific for either *Ren-1D* (lanes 3 and 4) or *Ren-2* (lanes 5 and 6), gene-specific fragments are amplified. Lanes 1, 3, and 5 are uncut DNA and lanes 2, 4, and 6 are following digestion with *Sma* I. *Hind*III-digested λ markers flank lanes 1–6.

mismatches at the 3' termini), fragments were generated that following the diagnostic *Sma* I digestion were clearly either *Ren-1D* or *Ren-2* DNA (see Fig. 2). Following targeted integration of the incoming DNA by gene replacement, the PCR assay with primers i and ii produces a fragment of ≈ 1700 bp, irrespective of which gene is targeted.

PCR products were separated by electrophoresis on 0.6% agarose gels, transferred to Hybond N membranes (Amersham) using a Pharmacia Vacublotter, and hybridized with a random-primed (27) 1624-bp *Sph* I to *Stu* I fragment of *Ren-1D* (positions 3173–4797 in ref. 13) that is contained within the PCR product obtained from a targeted clone. This probe hybridizes to *Ren-1D* and *Ren-2* amplified DNA at high stringency due to the close sequence identity of the two *Ren* genes. However, pools containing targeted clones usually gave rise to PCR products that were visible after ethidium bromide staining. Clones from positive pools were then analyzed individually in PCR reactions and positives were expanded.

High molecular weight DNA was prepared from the PCR-positive clones and 4 μ g was digested with restriction enzymes. This resulting product was then fractionated on 0.6% agarose gels, transferred to Hybond N, and hybridized with random-primed probes (27).

RESULTS

We introduced a *Ren-1D* targeting vector into ES cells derived from a mouse strain harboring *Ren-1D* and *Ren-2*. The targeting vector contains ≈ 5.5 kb of *Ren-1D* DNA spanning exons two to five with a neomycin-resistance gene inserted into exon 2. The *HSV-Tk* gene was also included in the vector to permit negative selection with gancyclovir (28). From three transfection experiments (Table 1) $\approx 33,400$ G418-resistant ES cell clones were obtained, of which $\approx 12,500$ were also resistant to gancyclovir; 2840 of these clones were picked and screened for targeted integration of the transgene, initially in pools of four using a PCR assay (26). The assay detects targeted integration at *Ren-1D* and *Ren-2*. Fifteen putative targeted clones were identified.

Table 1. Targeted integration into the mouse renin locus

Exp.	Cells electro-porated	G418-resistant clones	(G418 + Ganc)-resistant clones	Colonies picked and screened	Targeted clones
1	4×10^7	≈14,400	≈4,400	960	5
2	2×10^7	≈9,500	≈4,100	1256	7
3	2×10^7	≈9,500	≈4,000	624	3
Total		33,400	12,500	2840	15

Ganc, gancyclovir.

DNA from these clones was studied by Southern analyses. Since *Ren-1D* and *Ren-2* share similar restriction maps, two separate Southern blots were performed, one designed to detect targeted integration at the *Ren-1D* locus and the second designed to detect targeting at *Ren-2*. To study the structure of *Ren-1D*, DNA was digested with *Bam*HI and hybridized with a 239-bp *Sph* I fragment from intron 1 of *Ren-1D* (positions 2934–3173 in ref. 13). The probe flanks the targeting vector DNA and so does not detect the transgene following random integration. This Southern analysis identifies only a 6656-bp fragment from *Ren-1D* in parental CP1 ES cells and in transfected cells where the transgene has integrated randomly. However, following targeted integration by double crossover and gene replacement, a new *Bam*HI site is introduced into the gene that produces a 4001-bp fragment (Fig. 1). In contrast, the presence of two additional *Bam*HI sites in *Ren-2*, ≈80 bp upstream and 90 bp downstream of the *Sph* I probe, means that a 410-bp fragment is revealed in this gene (Fig. 1). Targeted integration at the *Ren-2* locus is therefore not detected with this Southern analysis except in cases where a crossover has occurred within the 90-bp *Sph* I–*Bam*HI region.

Fig. 3 shows such a Southern analysis on DNA from the 15 clones positive in the PCR assay. DNA from all 15 clones showed the predicted hybridization pattern including the 4001-bp fragment diagnostic of targeted integration by gene replacement at the *Ren-1D* locus.

To study the structure of the *Ren-2* gene by Southern analysis in these targeted clones, DNA was digested with *Sst* I and *Asp*718 and hybridized with a 192-bp fragment spanning exon 1 of *Ren-1D* (positions 1232–1434 in ref. 13). The probe again flanks the transgene and so does not detect randomly integrated vector DNA. In parental CP1 ES cells and in cells

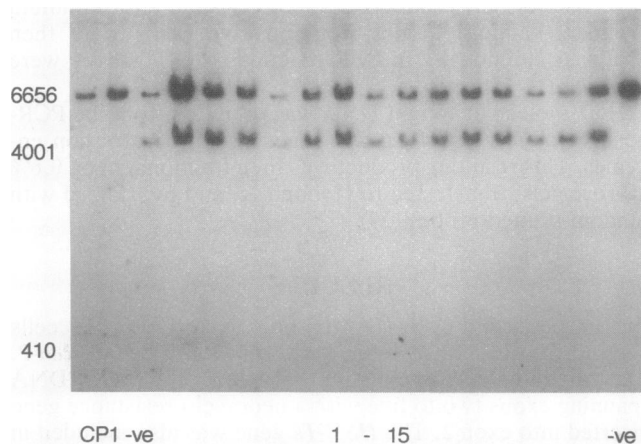


FIG. 3. Southern analysis designed to detect targeted integration at *Ren-1D*. The first two lanes are with DNA isolated from nontransfected ES cells (CP1) and from a nontargeted clone (-ve). Clones (1–15) all show the 4001-bp fragment diagnostic of targeted integration at the *Ren-1D* locus. The last lane (-ve) is with DNA isolated from a further transfected but nontargeted clone. The 410-bp *Ren-2*-derived fragment is only just visible on this exposure but is clearly apparent after longer exposures.

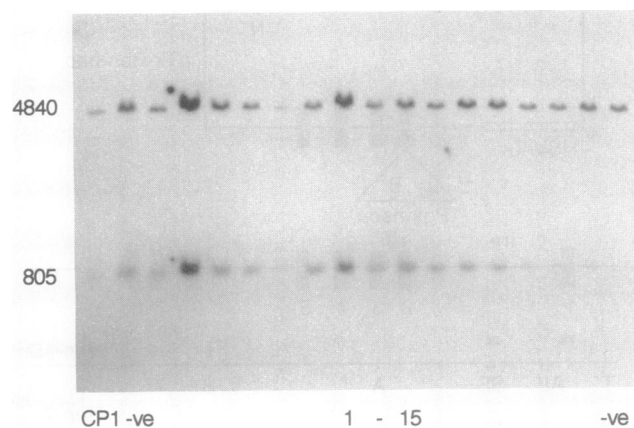


FIG. 4. Southern analysis designed to study the structure of the *Ren-2* gene. The first two lanes are negative controls with DNA isolated from nontransfected ES cells (CP1) or from a transfected nontargeted clone (-ve). Clones 1–15 display an identical hybridization pattern showing that the *Ren-1D* targeting vector has not recombined with the *Ren-2* locus. The last lane (-ve) is with DNA isolated from a further transfected but nontargeted clone.

where the transgene has integrated randomly, *Ren-2* gives rise to an ≈4840-bp hybridizing fragment, but following targeted integration of the transgene at this locus, the introduction of the additional 1.8 kb of *neo* DNA means that a 6640-bp fragment is produced. An additional *Asp*718 site in intron 1 of *Ren-1D* produces an 805-bp hybridizing fragment from this gene. This *Asp*718 site precludes the detection of targeted integration at *Ren-1D* by this Southern analysis.

Fig. 4 shows this Southern analysis on DNA isolated from the 15 clones screened as positive by the PCR assay. All displayed the wild-type hybridization pattern showing that the incoming vector DNA had not recombined with *Ren-2*.

DISCUSSION

We have introduced a *Ren-1D* targeting vector into mouse ES cells containing the two highly homologous renin genes *Ren-1D* and *Ren-2*. Using a PCR screen designed to detect targeted integration at *Ren-1D* and *Ren-2*, we isolated 15 targeted clones, all of which had undergone a gene conversion event at the *Ren-1D* locus. We did not isolate any clones in which the incoming vector DNA had recombined with *Ren-2*.

Sequence comparisons of *Ren-1D* and *Ren-2* show that they share ≈95% homology over the structural region of the gene (13). Similar comparisons over the 5579-bp region encompassed by our transgene show an overall homology of 95.2%. Previous studies have demonstrated that the frequency of gene targeting by means of homologous recombination in mammalian cells increases with the extent of homology between incoming and target DNA (22). However, in these studies, comparisons were made using vectors that contained varying amounts of DNA sequence displaying complete homology with no mismatches. In our experiment, we have used a single targeting vector but the target loci vary due to sequence mismatches.

Utilizing different strains of the *HSV-Tk* gene, Waldman and Liskay (29, 30) have studied the effect of base-pair mismatch on intra- and extrachromosomal homologous recombination in mammalian cells. A 19% evenly dispersed nucleotide mismatch reduces extrachromosomal recombination 3- to 15-fold relative to the rate between regions of perfect homology. The same mismatches cause a >1000-fold reduction in the intrachromosomal rate, indicating that there are at least some mechanistic differences between the two forms of recombination. Gene targeting involves recombination between chromosomal and newly introduced DNA and

as such does not correspond to either of the above situations. Nevertheless, our inability to target to the *Ren-2* gene out of 15 independent *Ren*-targeted clones suggests that the 4.8% mismatch between *Ren-1D* and *Ren-2* over the region of our transgene is capable of reducing the rate of gene targeting by at least 10-fold.

In *Escherichia coli*, the rate of homologous recombination is strongly influenced by the lengths of perfect homology between two sequences rather than their overall homology (31). This also seems to be the case with intrachromosomal recombination in mammalian cells. Here, mismatches placed so as to reduce the lengths of perfect homology between two sequences appear to have a more profound effect on recombination rates than changes that preserve long stretches of contiguous sequence but cause a greater reduction in overall homology (30). From such studies, it has been concluded that efficient intrachromosomal recombination in mammalian cells requires between 134 and 232 bp of uninterrupted homology. The nucleotide mismatches between our targeting vector and *Ren-2* are fairly evenly spaced with the single longest stretch of complete homology being 217 bp within the first intron. Other major stretches of contiguous sequence are 120 bp, 97 bp, and 86 bp, all of which are associated with exons (unpublished data).

It might be argued that our inability to recover *Ren-2*-targeted clones is not due to the sequence differences between the two renin genes but is the result of a failure of the P_{gk}-neo cassette to function at the *Ren-2* locus. In this situation, recombinant clones would not be rendered resistant to G418. We believe this scenario is unlikely since the two mouse renin genes are both silent in ES cells (as judged by Northern analysis; data not shown), tightly linked on chromosome 1 [being only 20 kb apart (7)], and share similar developmental expression profiles (16). Thus, it is difficult to envisage that the P_{gk}-neo cassette would fail to function when situated in an identical position in *Ren-2* in such a closely spaced region of chromatin. Also, this cassette is known to function well at silent loci in ES cells (see ref. 32).

A further possibility is that there is a lower inherent frequency of homologous recombination at the *Ren-2* locus compared to *Ren-1D*. Though our data do not exclude this possibility, we argue that the high level of homology between the two genes (particularly across the region of our targeting vector) renders it unlikely that *Ren-1D* possesses a recombination hot spot with respect to *Ren-2*. Likewise, the close proximity of the genes suggests that the overall chromatin configuration at the *Ren* loci is conserved. Thus, the simplest explanation for our inability to recover *Ren-2*-targeted clones is that the sequence mismatches between the targeting vector and *Ren-2* and possibly the absence of long stretches of complete homology between the two DNAs significantly reduce the rate of homologous recombination at this locus. Such an explanation is consistent with previous findings on intrachromosomal recombination (30).

The frequency of gene targeting to the *Ren-1D* locus that we observe (calculated here as a ratio of the number of targeted clones recovered to the number of G418- and gancyclovir-resistant clones screened) is 1:189. Negative selection with gancyclovir produced an ≈ 2.7 -fold enrichment. This enrichment is low compared to other reports (28, 33) and may in part be due to the different promoters used to drive the *neo* and *Tk* genes. The stronger P_{gk} promoter might be expected to function at unfavorable integration sites that render the *Tk* promoter silent. Nevertheless, in other targeting experiments using identical promoters to drive the *neo* and *Tk* genes, we (and others) have observed enrichment values of <10 (unpublished observations; see, for example, ref. 20).

Inbred strains of mice contain either one renin gene (*Ren-1C*) or two (*Ren-1D* and *Ren-2*). All three genes display

overlapping but distinct tissue specificities (1, 3, 15). In addition, mouse strains have been created that contain *Ren-1C* and a transgenic *Ren-2* (2, 34) and *Ren-1C* and a transgenic *Ren-1D* (3). Recently, Mullins *et al.* (35) have produced transgenic rats harboring *Ren-2*. These animals are severely hypertensive. Compared to the *Ren-1D* and *Ren-1C* gene products, that of *Ren-2* is thermolabile and also unglycosylated (9, 36), which may effect its processing (37). Homologous recombination between *Ren-1D* and our targeting vector introduces 1.8 kb of P_{gk}-neo DNA into exon 2 of the gene at a position slightly upstream to sequences that encode the site where pro-renin is cleaved to active renin. One copy of the *Ren-1D* gene is thus ablated in the gene-targeted cells. Construction of transgenic mice with the renin-targeted ES cells reported here will permit a greater understanding of the function of the *Ren-1D* and *Ren-2* gene products and their relative contribution to blood pressure homeostasis.

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