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Evolution of aspartyl proteases by gene duplication: the mouse renin gene is organized in two homologous clusters of four exons

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Overlapping recombinant clones that appear to encompass the entire renin gene, named *Ren* 1, have been isolated from a library of BALB/c mouse genomic DNA fragments. Based on restriction endonuclease mapping and DNA sequence analysis, *Ren* 1 spans 9.6 kb and contains nine exons interrupted by eight intervening sequences of highly variable size. The first exon, encoding the signal peptide of preprorenin, is separated from the eight following exons by a 3-kb intron. These eight exons are organized into two clusters of four separated by a 2-kb intron. DNA stretches encoding the aspartyl residues, which are part of the active site of renin, are located at homologous positions in both clusters. Our results show that aspartyl protease genes have arisen by duplication and fusion of an ancestral gene containing five exons. The estimated date of the duplication event of the mouse renin genes *Ren* 1 and *Ren* 2 is discussed.
Key words: kidney renin/protein glycosylation/rate of divergence/exon-intron boundaries

Introduction

Aspartyl protease represents one of the protein families most completely characterized by primary amino acid sequence and crystallographic studies. The aspartyl proteases active site contains two aspartate residues localized in two short amino acid stretches with sequence homology to one another, and separated by a distance of about half of the polypeptide chain (Sepulveda *et al.*, 1975). Aspartyl proteases such as penicillopepsin have a bilobal structure in which the two lobes are related by a 2-fold symmetry axis (Hsu *et al.*, 1977). These observations have led to a hypothesis that aspartyl protease genes have evolved by duplication and fusion of an ancestral gene coding for a 15 000–20 000 dalton polypeptide having a fold similar to that of one lobe of pepsin (Tang *et al.*, 1978).

In contrast to the other aspartyl proteases, renin has an optimal activity at neutral pH and a substrate specificity restricted to the cleavage of the prohormone angiotensinogen. The primary source of renin is the kidney where its concentration is extremely low. However, in some mouse strains, high levels of renin activity are found in the submaxillary gland (SMG) of males (Wilson *et al.*, 1977). The amino acid sequence of the SMG renin has been recently reported (Panthier *et al.*, 1982a; Misono *et al.*, 1982). Comparison of the amino acid sequence of renin and pepsin shows ~42% homology. The two most homologous regions are those surrounding the active sites aspartates. These results strongly suggest that renin and pepsin genes derive from a common ancestor.

Recent biochemical and genetic studies have shown that SMG and kidney renins are not the products of the same gene. The glycosylated thermostable kidney renin is encoded by the *Ren* 1 gene present in all mouse subspecies while the unglycosylated thermolabile SMG renin of high producer strains is encoded by a second copy of the gene, *Ren* 2 (Inagami *et al.*, 1980; Wilson and Taylor, 1982; Panthier *et al.*, 1982b; Mullins *et al.*, 1982; Piccini *et al.*, 1982; Panthier and Rougeon, 1983). We report here the structure of the *Ren* 1 gene and present new arguments concerning the origin of aspartyl proteases by gene duplication and fusion.

Results

Isolation and structural analysis of the renin gene

The cloned renin cDNA sequence pRn 1-4 (Panthier *et al.*, 1982b) was used as a probe to isolate the renin gene from a library of BALB/c mouse embryo DNA fragments cloned in the vector Charon 4A (Ollo *et al.*, 1981). Independent overlapping clones containing the renin gene *Ren* 1 were identified. One of them yields four *Eco*RI fragments of 8.8, 3.9, 3.2 and 0.5 kb. Southern blot analysis of this clone showed that only the 3.9-kb and the 8.8-kb *Eco*RI fragments hybridize to the renin cDNA probe. We have previously shown that the 5′ and the 3′ ends of the renin gene are located on *Eco*RI fragments of 8.8 and 3.9 kb, respectively (Panthier *et al.*, 1982b). The *Eco*RI fragments, which appear to contain the entire renin gene as well as 5′- and 3′-flanking sequences, were subcloned in the plasmid pBR322 for structural analysis. The structural organization of the renin gene was determined by restriction endonuclease mapping and DNA sequence analysis according to the strategy outlined in Figure 1.

From the complete sequence of the SMG renin mRNA, we were able to establish the organization of the gene and to predict the amino acid sequence of the kidney renin. The approximate location of the exons was established by Southern blot analysis with the pRn 1-4 probe and these regions were sequenced using the Maxam and Gilbert method (1980). The intron-exon junctions were located by comparing the sequences of the exons with the sequence of the SMG renin mRNA. The exact locations of the splice junctions have been assigned on the basis of the GT....AG rule (Breathnach *et al.*, 1978). The renin gene *Ren* 1 coding for a mRNA of ~1600 nucleotides spans 9.6 kb and is split into nine exons by eight intervening sequences of various lengths. The nine exons are 91, 151, 124, 119, 197, 120, 145, 100 and 341 bp respectively, and the eight intervening sequences are ~3120, 510, 690, 710, 1910, 450, 260 and 570 bp respectively. The nucleotide sequence of the nine exons of the renin gene *Ren* 1 and the predicted amino acid sequence of the kidney renin precursor are shown in Figure 2.

Comparison between SMG and kidney renins

The sequences of the *Ren* 1 and *Ren* 2 mRNAs have been

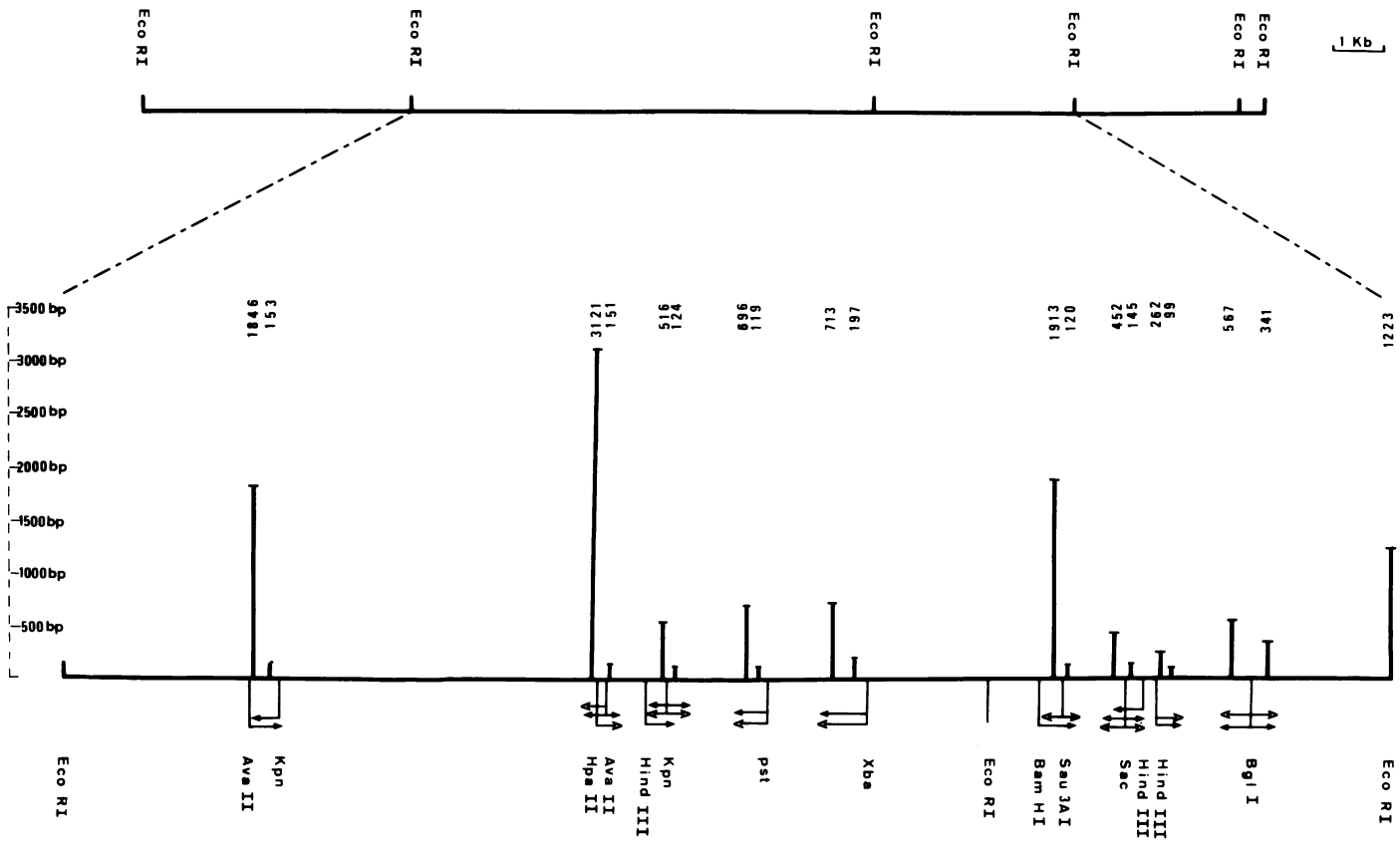

Fig. 1. Restriction map and sequencing strategy for the mouse *Ren* 1 gene. The restriction map of the two subcloned *Eco*RI renin fragments was determined as described in Materials and methods. Vertical lines indicate the length and the position of the nine exons on the two *Eco*RI fragments. The lower part shows the strategy for DNA sequencing of the nine exons. The direction and extent of nucleotide reading are indicated by horizontal arrows. Open and solid arrow heads specify fragments labeled at their 5′ and 3′ ends, respectively.

aligned over 1427 nucleotides. The two sequences differ by 47 nucleotide substitutions and one codon insertion at position 27 of the kidney renin precursor.

The 45 base substitutions found in the protein coding regions account for 21 replacement substitutions and 24 silent substitutions. There are only two base substitutions in the 180 nucleotide long 3′-untranslated (3′ UT) region. This relatively low rate of base substitution in this region could be explained by a gene conversion mechanism in which the 3′ UT region of *Ren* 2 has been homogenized by the corresponding region of the adjacent *Ren* 1 gene. The most important difference between the two renins is the presence of three potential glycosylation sites in the kidney enzyme located at amino acid positions 69, 139 and 319 (Figures 2 and 3).

We have proposed a model for the maturation of the SMG renin precursor (Panthier *et al.*, 1982a). The signal peptide is first cleaved to produce the prorenin. Prorenin is then converted into active renin containing two chains, A and B, by proteolytic cleavages after dibasic residues. The two dibasic residues Lys_{63} Arg_{64} and Arg_{353} Arg_{354}, which are involved in the maturation of the SMG renin, are found at identical positions in the kidney renin sequence. These results suggest that active kidney renin is a glycosylated protein built up of two polypeptide chains linked by a disulphide bridge. It is also likely that the differences in thermostability of the two renins result from some of the numerous amino acid differences observed.

Structural organization of aspartyl protease gene

Aspartyl proteases have a bilobal structure in which the two topologically similar domains are related by a 2-fold symmetry axis. The two lobes are connected by a short peptide (Hsu *et al.*, 1977; Tang *et al.*, 1978). The tri-dimensional structure of renin may be similar to that of other aspartyl proteases (Blundell *et al.*, 1983). Because of the great similarity between aspartyl proteases and renin, and because all the sequences can be aligned with only few insertions and deletions, we can establish with confidence the relationships between renin gene organization and the three-dimensional structure of renin (Figure 4).

The two lobes of the aspartyl proteases are connected by a short peptide (residues 172–176) located on the surface of the protein (Tang *et al.*, 1978). Examination of the stereoview of the three-dimensional structure of the SMG renin indicates that the two lobes are connected by a short peptide found at an equivalent position, the numbering of the residues being based on the primary sequence of the pepsin.

Examination of the sequence alignments of aspartyl proteases shows that the connecting peptide 172–176 of the pepsin corresponds to residues 244–248 in the sequence of the preprorenin (Panthier *et al.*, 1982a). This peptide is encoded by the first half of exon 6. Together with results shown in Figure 4, this shows that each domain of the renin is encoded by each of the two blocks of four exons (exons 2–5 and ex-

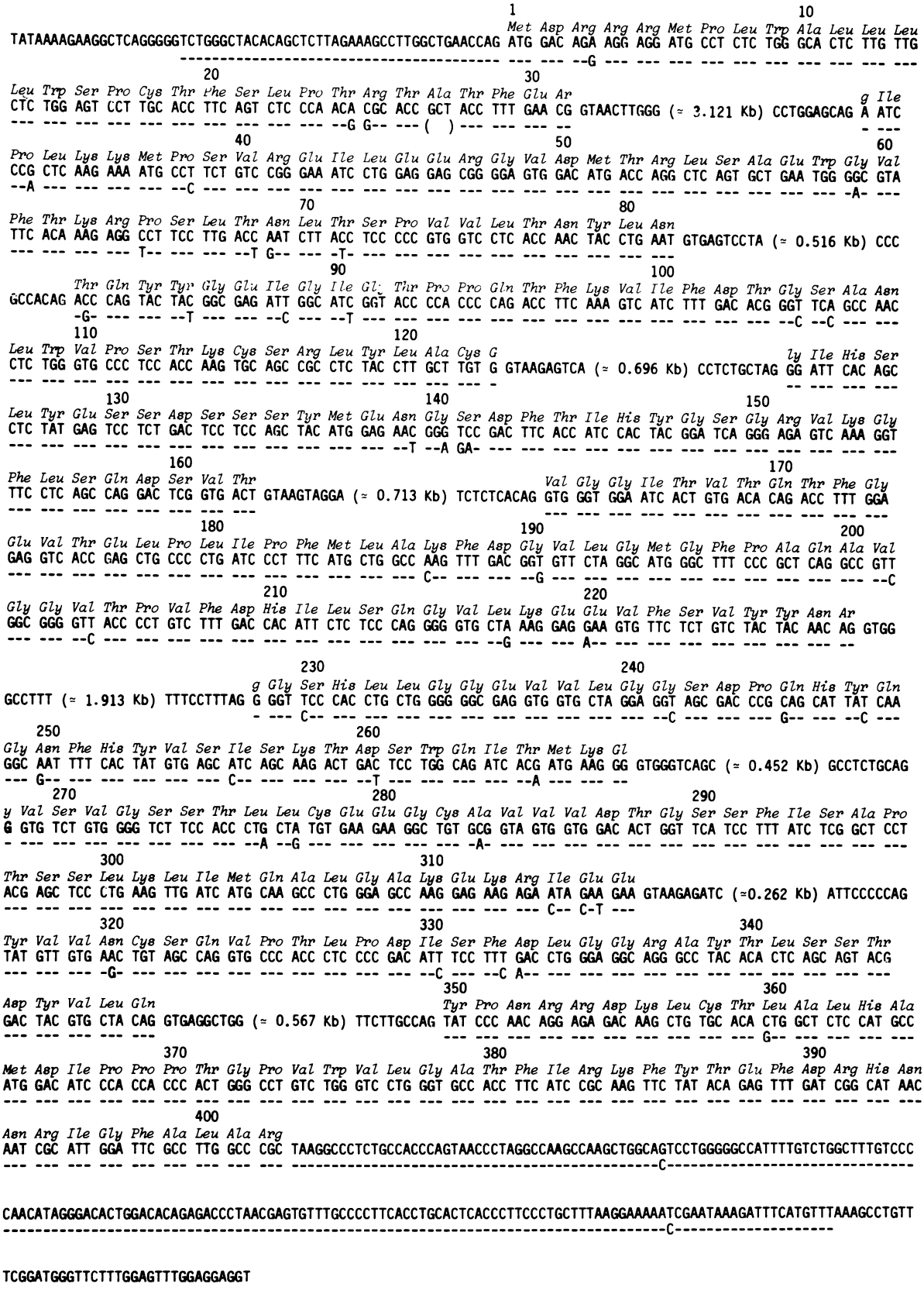

Fig. 2. Nucleotide sequence of the coding regions and of the 5′- and 3′-flanking regions of the mouse *Ren* 1 gene. The DNA sequence of the coding regions and the predicted amino acid sequence of the *Ren* 1 gene are shown. Amino acids are numbered from the NH_2 terminus of the preprorenin. Dashes indicate positions where the *Ren* 1 sequence is identical with the sequence of the SMG mRNA encoded by the *Ren* 2 gene (Panthier *et al.*, 1982).

ons 6–9), and that the two active site aspartates are located at equivalent positions in the two clusters. Such an organization strongly supports the hypothesis of Tang (1979) in which aspartyl proteases have evolved by gene duplication and fusion of an ancestor gene encoding a single polypeptide chain with a size and fold similar to one lobe of pepsin.

Discussion

Evolution of renin genes

The divergence rate between homologous genes of different species, or between related genes of a single species is theoretically proportional to the time elapsed from the beginning of the divergence. Nethertheless, no absolute time scale can be applied since rates of gene evolution differ widely depending on the multigenic family considered (Wilson *et al.*, 1978). Furthermore, non-reciprocal transfer of information (gene conversion) in multigenic families can modify the rate of gene evolution at a single locus (Ollo and Rougeon, 1983). Generally, the divergence rate between alleles at a single genetic locus is ~1%. In contrast, we have found that the rate of total substitutions between kidney and SMG renins (4.8%) is higher than the rate of divergence between alleles at a single locus. This suggests that the two non-allelic renin genes, *Ren* 1 and *Ren* 2, could have arisen by duplication before mouse speciation. A more precise indication concerning the time of divergence of both renin genes may be obtained by comparing the *Ren* gene of mouse with the renin gene of a different species having a single gene. We have recently reported the nucleotide and the deduced amino acid sequence analysis of a human kidney renin cDNA fragment and shown that there is a single renin gene in the human genome (Soubrier *et al.*, 1983). Comparison of this amino acid sequence with the mouse kidney renin sequence shows that the proteins are 69.4% homologous for 299 positions compared. Assuming that the mammalian radiation occurred 80 million years ago, this result means that a 1% divergence rate at the

	69	70	71	139	140	141	319	320	321
	Asn	Leu	Thr.....	Asn	Gly	Ser.....	Asn	Cys	Ser
KIDNEY	AAT	CCT	ACT.....	AAC	GGG	TTC.....	AAC	TGT	AGC
SMG	G--	---	-T-	--C	--A	GA-	-G-	---	---
	Asp	Leu	Ile	Asn	Gly	Asp	Ser	Cys	Ser

Fig. 3. Glycosylation sites in kidney renin. Three potential N glycosylation sites corresponding to the typical sequence Asn X Thr or Asn X Ser present in the kidney renin but not in the SMG renin are shown. Residues are numbered from the NH_2 terminus of the preprorenin.

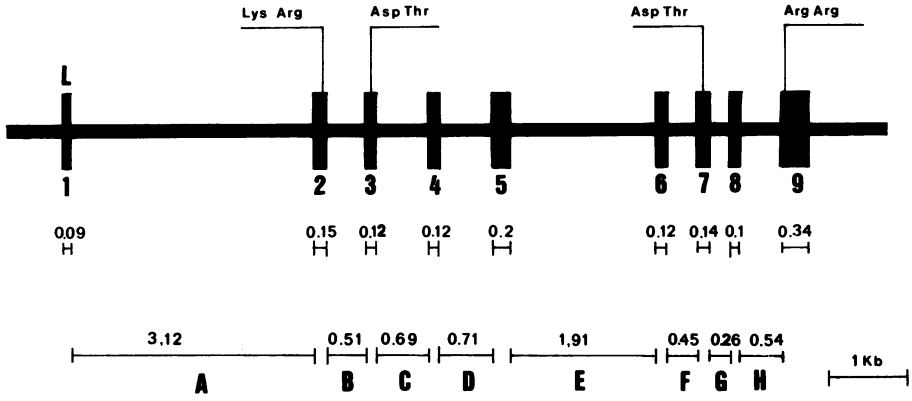

Fig. 4. Organization of the mouse renin gene. The figure shows the location of the coding regions in the renin gene. The protein coding segments are indicated by dark blocks numbered 1–9 in the 5′ to 3′ direction (exon 9 includes the 3′ UT region of the renin mRNA). Intervening sequences are numbered from A to H. The sizes of exons and introns are given below.

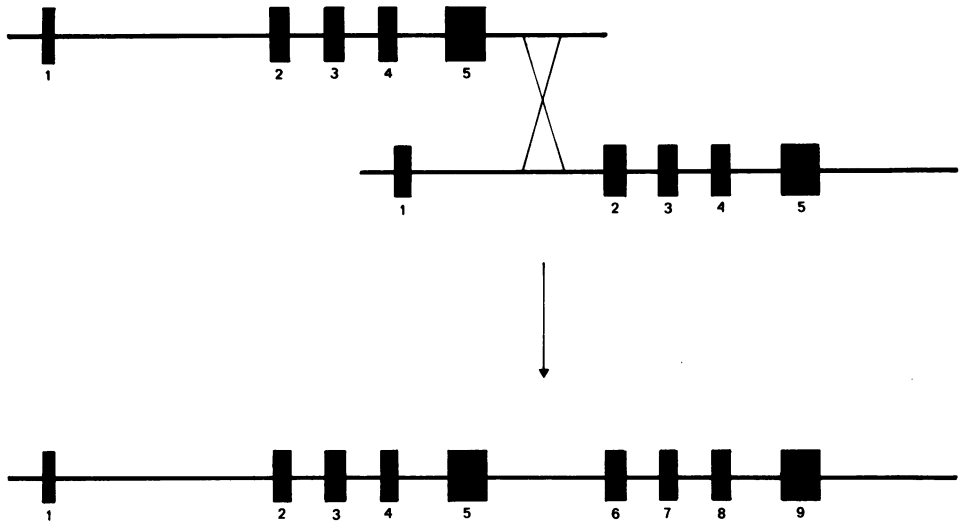

Fig. 5. A model for the evolution of the aspartyl proteases by gene duplication. The primitive gene encoding a 20 000 dalton protein was divided into five exons by four intervening sequences. As a result of a duplication event by unequal meiotic crossing-over by homologous recombination between direct repeats located in the first intervening sequence and 3′ of the ancestral gene, the aspartyl protease ancestral gene is formed. Alternative models involving firstly duplication of the five exons gene and secondly fusion of the two genes by deletion of the intergenic region containing the first exon are equally valid.

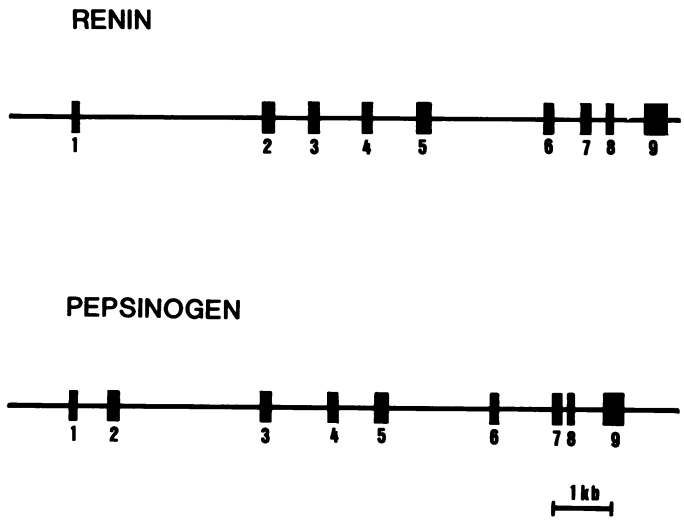

Fig. 6. Comparison of the human pepsinogen gene and the mouse renin gene organization. The figure shows the location of the coding regions in the mouse renin gene (this paper) and in the human pepsinogen gene (Sogawa *et al.*, 1983).

amino acid level corresponds to 80/31.6 = 2.5 million years. It follows that the divergence time between kidney and SMG mouse renins is 2.5 x 4.8 = 12 million years. Human and mouse kidney renin nucleotide sequences are 77.5% homologous in the coding regions, so that a 1% divergence rate at the nucleotide level corresponds to 80/22.5 = 3.5 million years and the divergence time between kidney and SMG mouse renin genes is 3.5 x 3.8 = 13.3 million years. It is generally accepted that species separation of rat and mouse occurred 10 million years ago. Our results suggest that the duplication of *Ren* 1 and *Ren* 2 mouse renin genes could have occurred before the separation of these species. The ancestral species which gave rise to the different subspecies of mouse could have harbored both renin genes, *Ren* 1 and *Ren* 2, and the presence of one or two renin genes in mouse, which we first interpreted as a duplication (Panthier *et al.*, 1982b), could be interpreted as a deletion of the *Ren* 2 gene in some populations of mouse subspecies.

The above interpretation is, of course, open to criticism since kidney and SMG renins are likely not to be subjected to the same selective pressures. If we take another multigenic family as a reference, the duplication may appear a more re-

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                Met Asp Arg Arg          Ala Thr Phe Glu Ar
GCTGAACCAG      ATG GAC AGA AGG ..... GCT ACC TTT GAA CG  GTAACTTGGG
-AA-----(  )    --- A-G T-G CT- ..... TGC -T- A-G T-C AA  --G-G-CC--
                Met Lys Trp Leu          Cys Ile Met Tyr Ly

              g Ile Pro Leu Lys          Asn Tyr Leu Asn
CCTGGAGCAG    A ATC CCG CTC AAG ..... AAC TAC CTG AAT     GTGAGTCCTA
-AAACCA---    G G-- --C --- -TC ..... --- --- --- G--     ------GTGC
              s Val Pro Leu Ile          Asn Tyr Leu Asp

                Thr Gln Tyr Tyr          Tyr Leu Ala Cys G
CCCGCCACAG      ACC CAG TAG TAC ..... TAC CTT GCT TGT G   GTAAGAGTCAAGCC
G--TGG----      -TG G-- --C -T- ..... AGT --- --C --C A   ---(  )-(  )--
                Met Glu Tyr Phe          Ser Leu Ala Cys T

             ly Ile His Ser Leu          Asp Ser Val Thr
ACCCTCTGCTAG GG ATT CAC AGC CTC ..... GAC TCG GTG ACT     GTAAGTAGGA
GT---(  )--(  )- CC -AC --- -A- -G- ..... --- A-T --C CAG --GG-C-CCT
             hr Asn His Asn Arg          Asp Thr Val Gln

                Val Gly Gly Ile          Val Tyr Tyr Asn Ar
TCTCTCACAG      GTG GGT GGA ATC ..... GTC TAC TAC AAC AG  GTGGGCCTTTGA
C-C-A-C---      --T --A --C --- ..... --- --- CT- -G- GC  --AA-(  )---
                Val Gly Gly Ile          Val Tyr Leu Ser Al

              g Gly Ser His Leu          Ile Thr Met Lys Gl
CTTTCCTTTAG   G GGT TCC CAC CTG ..... ATC ACG ATG AAG GG  GTGGGTCAGC
------( AC)-- C -A- GA- --G AGT ..... --- --C G-- G-C A-  ---A-A-T--
              a Asp Asp Gln Ser          Ile Thr Val Asp Se

              y Val Ser Val Gly          Arg Ile Glu Glu
TAGCCTCTGCAG  G GTG TCT GTG GGG ..... AGA ATA GAA GAA     GTAAGAGATC
-T---(  )-(  )--  C A-C A-C A-- AAC ..... TC- GAT -GC --C     --G--TCCAG
              r Ile Thr Met Asn          Ser Asp Gly Asp

                Tyr Val Val Asn          Tyr Val Leu Gln
ATTCCCCCAG      TAT GTT GTG AAC ..... TAC GTG CTA CAG     GTGAGGCTGGG
C-CTTT----      ATG --G --C -G- ..... --- A-C --G ---     ------(  )A--C
                Met Val Val Ser          Tyr Ile Leu Gln

                Tyr Pro Asn Arg          Ala Leu Ala Arg
GCTTCTTGCCAG    TAT CCC AAC AGG ..... GCC TTG GCC CGC     TAAGGCCCTC
TT----(  )----  AGC GAG GGG --C ..... C-- G-- --T (  )    ----C-TAAG
                Ser Glu Gly Ser          Pro Val Ala
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Fig. 7. Comparison of nucleotide sequences at the exon-intron junctions in human pepsinogen and mouse renin genes. Nucleotide sequences of mouse renin gene and human pepsinogen gene (Sogawa *et al.*, 1983) at the splicing sites were compared. Identical nucleotides are indicated by dashes.

cent event. The Cκ genes of rat and mouse are 88.5% homologous at the nucleotide level, which corresponds to a divergence rate of ~1% in 0.8 million years (Sheppard and Gutman, 1981). With such a value the duplication of renin genes would have occurred 3 million years ago. Systematic study of the renin locus in different species of rodents and in different subpopulations of wild mice, or the structural analysis of renin genes of rodents, should allow us to date the duplication event with respect to phylogenesis.

Organization and evolution of aspartyl proteinase genes

The 1-4-4 organization of the renin gene in which the two active site aspartates are located at equivalent positions in the two clusters of four exons strongly supports the duplication fusion hypothesis of Tang (1979). We suggest that the ancestral gene was divided into five exons by four intervening sequences and that aspartyl protease genes have been generated by unequal meiotic cross-over between two direct repeats of DNA located respectively in the first intervening sequence and 3′ of the ancestral gene (Figure 5). The relatively large size of the fifth intervening sequence which separates the two clusters can be explained by this model. The duplication model suggests that the ancestral gene encoded a polypeptide having a fold similar to that of one lobe of pepsin (Tang, 1979). This polypeptide was probably active as a dimer in which the two identical subunits were related by a 2-fold symmetry axis. After gene duplication and fusion, the two subunits are connected by the short peptide encoded in exon 6, while retaining the same 2-fold relationship. This gene fusion event could have been selectively important by permitting divergent evolution between the two lobes and by permitting the correct association between the two more or less divergent subunits.

Recently, Blundell *et al.* (1983) used interactive computer graphics to build a three-dimensional model of renin. They have shown that the structure of renin is probably similar to that of other aspartyl proteases. This suggests that every aspartyl proteinase gene may have the 1-4-4 basic organization of the renin gene. The human pepsinogen gene spans 9.6 kb and is divided into nine exons separated by eight intervening sequences (Sogawa *et al.*, 1983). The second exon, corresponding to the activation segment, is located near the first exon. Consequently the 1-4-4 organization does not appear clearly in that case (Figure 6). There are possible explanations: firstly, the first intervening sequence might have contained originally several exons, and different exons may have been selected for different proteins. A second explanation, although unlikely, is that some exon transposition could have occurred in the pepsinogen gene. A third explanation is that the second exon of the pepsinogen gene has not been correctly localized as a consequence of gene rearrangement during the cloning procedure. Thus, if we compare the DNA sequences at the exon-intron junctions (Figure 7) of pepsinogen and renin, it appears that these sequences are relatively well conserved, especially in the case of the second exon. The same splice junctions are used in both cases. This strongly suggests that the two first exons are localized at homologous positions on both genes. In conclusion, the analysis of the renin gene illustrates the essential role of gene duplication in the emergence of new proteins and new biological functions.

Materials and methods

Restriction enzymes were purchased from New England Biolabs, Boehringer Mannheim and Genofit. T4 DNA ligase was obtained from NEN, New England Nuclear. *Escherichia coli* DNA polymerase I was obtained from Boehringer Mannheim. *E. coli* DNA polymerase I large fragment was obtained from New England Biolabs. Terminal deoxynucleotidyl transferase and T4 polynucleotide kinase were obtained from P.L. Biochemicals. [γ-^{32}P]ATP, [α-^{32}P]dATP and [3′-α-^{32}P]dATP (cordycepin triphosphate) were obtained from Amersham.

Screening of BALB/c mouse gene library

1.6 x 10^6 phage from a λ Charon 4A library (Ollo *et al.*, 1981) were screened as described by Benton and Davis (1977) with ^{32}P-labeled renin cDNA plasmid pRn 1-4 (Panthier *et al.*, 1982b). Four clones were obtained, three of them give the same *Eco*RI restriction fragments.

Subcloning of renin gene fragments in pBR325 and pBR322

After cleavage with *Eco*RI or *Eco*RI and *Bam*HI, the renin gene fragments were subcloned in pBR322 and pBR325. The subclones were digested with restriction endonucleases and analyzed by agarose gel electrophoresis to generate a restriction endonuclease map of the renin gene.

DNA sequencing analysis

DNA fragments were labeled at the 5′ end using [γ-^{32}P]ATP and polynucleotide kinase in the exchange reaction, at the 3′ end using [3′-α-^{32}P]dATP (cordycepin triphosphate) or by filling in protruding restriction sites with *E. coli* DNA polymerase I large fragment. The nucleotide sequence was then determined by the partial chemical degradation method of Maxam and Gilbert (1980).

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