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DNA insertions distinguish the duplicated renin genes of DBA/2 and *M. hortulanus* mice

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Abstract. In a survey of inbred and wild mouse DNAs for genetic variation at the duplicate renin loci, *Ren-1* and *Ren-2,* a variant *Not* I hybridization pattern was observed in the wild mouse *M. hortulanus*. To determine the basis for this variation, the structure of the *M. hortulanus* renin loci has been examined in detail and compared to that of the inbred strain DBA/2. Overall, the gross features of structure in this chromosomal region are conserved in both *Mus* species. In particular, the sequence at the recombination site between the linked *Ren-1* and *Ren-2* loci was found to be identical in both DBA/2 and *M. hortulanus,* indicating that the renin gene duplication occurred prior to the divergence of ancestors of these mice. Renin flanking sequences in *M. hortulanus,* however, were found to lack four DNA insertions totaling approximately 10.5 kb which reside near the DBA/2 loci. The post-duplication evolution of the mouse renin genes is thus characterized by a number of insertion and/or deletion events within nearby flanking sequences. Analysis of renin expression showed little or no difference between these mice in steady state renin RNA levels in most tissues examined, suggesting that these insertions do not influence expression at those sites. A notable exception is the adrenal gland, in which DBA/2 and *M. hortulanus* mice exhibit different patterns of developmentally regulated renin expression.

Introduction

In vertebrates, blood pressure and electrolyte levels are in part regulated by the enzyme renin. The genes encoding renin in mice have experienced a number of genetic rearrangements which have influenced not only the organization of flanking sequences, but also gene copy number. Inbred mouse strains such as C57BL/6 have a single renin locus and have fixed the allele, *Ren-1*^{c}. Other strains, such as DBA/2, carry the *Ren-1*^{d} allele plus a second locus, *Ren-2*^{d}, believed to be the result of a relatively recent gene duplication (Piccini et al., 1982; Mullins et al., 1982; Panthier et al., 1982b). The physical arrangement of the duplicate loci in DBA/2 has been characterized, and the precise site of the recombination event which resulted in the duplication has been identified (Abel and Gross 1988, 1990). The *Ren-1*^{c} and *Ren-1*^{d}/*Ren-2*^{d} genotypes are approximately equally represented among inbred mouse strains. Among wild-derived mice, however, two renin loci have been observed in most individuals representing commensal and aboriginal species, but only a single renin locus has been observed in mice distantly related to the inbreds (Dickinson et al. 1984).

Structural comparisons between inbred mouse, rat, and human renin genes have also identified DNA insertions within flanking sequences at the DBA/2 loci. Some of these insertions distinguish between the renin genes of mice and other species, and others distinguish between the renin genes in inbred mice. A 3 kb partial intracisternal A particle (IAP) genome lies just downstream of *Ren-2*^{d} (Burt et al. 1984) and a 7 kb insertion termed M1 lies upstream of *Ren-1*^{d} (Abel and Gross 1990). A number of much smaller insertions have also been identified upstream of the inbred renin genes including type-1 and type-2 *Alu* equivalent repeats (Field et al. 1984b; Soubrier et al. 1986) and others of unknown identities (Tronik et al. 1988; Nakamura et al. 1989). Including the duplication, rearrangements of renin flanking sequences have thus been identified which distinguish between the *Ren-1* and *Ren-2* loci, and between alleles at the *Ren-1* locus.

In addition to the structural variation, each inbred renin gene exhibits a unique pattern of tissue specific- and developmentally-regulated expression. This raises the question of the relationship between the structural

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variation and expression differences. These differences have been shown to be mediated by closely linked sequences in *cis* with the structural genes (Wilson et al. 1978; Fabian et al. 1989; Mullins et al. 1989), although the precise regulatory sequences have not been identified as yet. Direct tests of recombinant DNA constructs have been used to identify specific transcriptional regulatory elements. This approach is presently limited by the paucity of suitable cell culture assay systems. Nevertheless, recent reports have proposed that one or more of the insertions, and/or the duplication event, may be responsible for several of the gene-specific expression patterns noted (Burt et al. 1984; Tronik et al. 1988; Nakamura et al. 1989; Abel and Gross 1990).

Naturally occurring genetic variants provide an alternative opportunity to correlate specific structural features with gene expression patterns. A detailed comparison of renin gene structure in the inbred strain DBA/2 and the wild mouse *M. hortulanus* has provided new insights into the evolution of the mouse renin genes subsequent to the duplication. Correlating gene structure and expression has enabled us to assess the putative involvement of several of these rearrangements in renin expression, and ultimately may help to focus upon the DNA sequences which regulate mouse renin expression.

Material and methods

Mice

Inbred mouse strains DBA/2JRos, C57BL/6JRos, SWR/J, and O20/A, and wild mouse individuals were obtained from stocks maintained at the Roswell Park Cancer Institute. *M. hortulanus* (Pancevo) mice are currently maintained as an outbred stock derived from animals trapped by R. Sage (Museum of Vertebrate Zoology, Berkeley, Calif.). Inbred strains AKR/FuRdA and STS/A were obtained from J. Hilgers (The Netherlands Cancer Institute, Amsterdam).

Conventional Southern blot analysis

Restriction endonuclease digests of genomic DNAs were separated on 0.8% agarose gels, transferred to nitrocellulose (Schleicher and Schuell), and hybridized as described (Abel and Gross 1988) except that filters were washed to a final stringency of 1× SSC, 0.1% SDS at 65°C. DNA probes were : *pDD-1D2*—a full-length renin cDNA isolated from a DBA/2 submandibular gland (SMG) cDNA library (Field et al. 1984a); *pSM479*—an incomplete DBA/2 SMG renin cDNA, containing only a portion of exon 8 plus exon 9 (Piccini et al. 1982); *probe A* (Fig. 4A)—a 0.5 kb *Eco*R I–*Kpn* I fragment isolated from a genomic subclone containing *Ren-1^d* 5′ flanking sequences (pRn34, kindly provided by D. Burt and W. Brammar); and *probe B* (Fig. 4B)—a 0.15 kb *Alu* I–*Rsa* I fragment containing exon I, also isolated from pRn34. DNA probes were prepared by the oligonucleotide random priming method (Feinberg and Vogelstein 1983) to a specific activity of 10^9 dpm/μg.

PFGE-Southern blot analysis

Preparation and digestion of high molecular weight spleen DNAs, transfer to nitrocellulose, and hybridizations were performed as described (Abel and Gross 1988). Pulsed field gel electrophoresis (PFGE) was performed using a contour-clamped homogeneous electric fields (CHEF) apparatus (Chu et al. 1986) built by Owl Scientific Plastics (Cambridge, Mass.). CHEF gels were 1% agarose in 0.5 × TBE running buffer cooled to 13°C (0.5 × TBE = 45 mM Tris base pH 8.2, 45 mM boric acid, 1 mM EDTA). The gel in Fig. 2A was run for 24 h at 170 V, using a switching interval of 3.5 seconds to separate DNA fragments within a relatively low molecular weight range. Oligomers of intact λ DNA (unit length 48.5 kb) were included as size standards.

Genomic clones

Clones were isolated from a *M. hortulanus* genomic library prepared using the λ vector EMBL-3B and a 10–25 kb fraction of *Sau3* A partially digested DNA from a single adult male (Howles 1986). Screening of libraries was done according to Benton and Davis (1977). DNA probes containing renin exon sequences were nick-translated (Rigby et al. 1977) to a specific activity of ~10^9 dpm/μg and included in the hybridization mix at a concentration of 10^6 dpm/ml. Screening of approximately 2.5 × 10^6 recombinant phage yielded a total of 14 clones encompassing both renin loci plus 10–20 kb of sequences flanking each locus. The *M. hortulanus* sequence shown in Fig. 3 was derived from the clone λHRenII-3 which physically links *Ren-2^h* and *Ren-1^h*. *M. hortulanus* exon 7 and 8 sequences were derived from genomic clones λHRenII-16 (*Ren-1^h*) and λHRenII-10 (*Ren-2^h*).

Subcloning/sequencing

Restriction fragments from genomic clones were subcloned into the plasmid vector pTZ19R (Pharmacia) which yielded single-stranded sequencing templates upon infection of host cells with the helper phage M13K07 (Vieira and Messing 1987). Dideoxynucleotide sequencing was performed with Sequenase (United States Biochemical) using the M13 reverse sequencing primer. To complete internal sequences, custom oligonucleotide primers were synthesized on an Applied Biosystems 380A DNA synthesizer. The 2.5 kb *Xho* I–*Eco*R I fragment of λHRenII-3 (Region D in Figs. 1, 3) was subcloned into *Sal* I + *Eco*R I-cut pTZ19R; sequencing proceeded into the insert from the 5′ *Xho* I end. The 1.4 kb (exon 7) and 2.5 kb (exon 8) *Eco*R I–*Hind* III fragments from λHRenII-16 and λHRenII-10 were subcloned into *Eco*R I + *Hind* III-cut pTZ19R; sequencing proceeded into exon sequences from *Hind* III sites in the intron between exons 7 and 8. All sequences were determined in duplicate, once using dGTP in the labeling reaction and again using dITP, which eliminates compression artifacts observed with dGTP (Tabor and Richardson 1987). The labeling reactions included [α-^{35}S]dATP, and the ddNTP-terminated products were fractionated on 6% acrylamide/7 M urea sequencing gels using 0.4–1 mm wedge spacers.

Expression analyses

Total RNAs were isolated by homogenizing tissues in guanidine isothiocyanate (Kingston 1987). RNAs were purified by centrifugation over a 5.7 M CsCl cushion at 35,000 rpm for 15 h in a Beckman SW-41 rotor. The pelleted RNAs were recovered from the bottom of the centrifuge tube, and trace CsCl was removed by two ethanol precipitations.

Primer extension analysis was performed as described (Field and Gross 1985), except using a 30-mer oligonucleotide primer complementary to exon 8 sequences in renin mRNA (K. Lilleycrop and W.J. Brammar, personal communication). Briefly, 50,000 dpm of end-labeled oligonucleotide was hybridized with 0.8 or 80 μg of total organ RNAs. The primer was extended using avian myeloblastosis virus reverse transcriptase (Bethesda Research Laboratories) in the presence of dATP, dCTP, dTTP, and ddGTP. Following RNA hy-

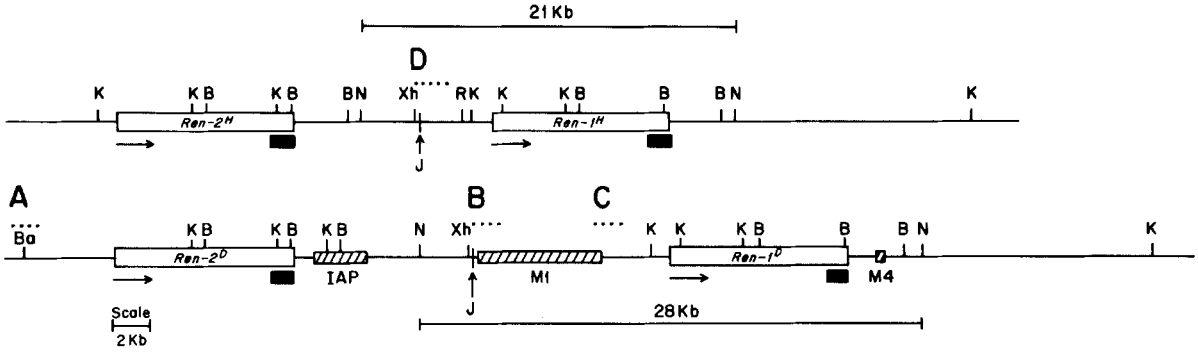

Fig. 1. Restriction maps of the renin loci in DBA/2 and *M. hortulanus* mice. The sequence organization of DBA/2 renin coding and flanking regions **(lower map)** has been previously characterized (Abel and Gross 1990). The sequence organization in *M. hortulanus* **(upper map)** was compiled from the data in Figs. 2–4, and from additional mapping of *M. hortulanus* genomic DNAs and clones (Dickinson et al. 1984; Howles 1986). Shown are several insertions near the DBA/2 loci which are absent in *M. hortulanus* (IAP, M1, M4; see text). The variant *Not* I fragments containing the *Ren-1*^{h} and *Ren-1*^{d} alleles (21 and 28 kb, respectively) are indicated. The duplication junction between *Ren-2* and *Ren-1* flanking sequences is indicated by "J." **Dotted lines** identify the sequenced regions A–D which are shown in Fig. 3. **Arrows** indicate the direction of transcription. **Solid boxes** represent the 3′-end renin cDNA probe, pSM479, which was used in Southern blot analyses in Fig. 2 (panels B and C). Restriction sites are represented by: B = *Bgl* I, Ba = *Bam*H I, K = *Kpn* I, N = *Not* I, R = *Eco*R I, Xh = *Xho* I.

drolysis, samples were neutralized, ethanol precipitated, and electrophoresed on an 8% acrylamide/7 M urea sequencing gel.

Results

In this report, the genomic structure at the closely linked *Ren-1* and *Ren-2* loci was compared between the inbred strain DBA/2 and the wild mouse, *M. hortulanus*. Figure 1 shows restriction maps of this chromosomal region in both mice and summarizes the following data.

The genomic organization of the renin genes in the inbred strain DBA/2 was previously characterized using PFGE and sequence analysis of cloned genomic segments (Abel and Gross 1988, 1990). *Ren-2*^{d} and *Ren-1*^{d} are separated by approximately 21 kb and lie within adjacent *Not* I fragments of 550 kb and 28 kb, respectively. In an effort to identify additional genetic variation at the renin loci, this analysis has been extended to other inbred and wild mice which carry both *Ren-1* and *Ren-2*.

High molecular weight DNAs from these mice were digested with *Not* I, size-fractionated by PFGE, and assayed by Southern blot hybridization using a mouse renin cDNA probe. *Not* I hybridization patterns similar to that of DBA/2 were observed in DNAs from inbred strains AKR, STS, SWR, and O20, and from individuals of wild-derived *M. domesticus* and *M. spretus* (not shown). A variant hybridization pattern, however, was observed in DNA of the wild mouse *M. hortulanus* (Fig. 2A). Whereas the *Ren-1* alleles in DBA/2 and *M. spretus* lie within *Not* I fragments of 28 kb, the *Ren-1* allele in *M. hortulanus* (*Ren-1*^{h}) was found to lie within a smaller fragment, estimated using conventional gels to be approximately 21 kb. *Not* I sites were mapped to homologous positions flanking the *Ren-1* alleles in both DBA/2 and *M. hortulanus* (Fig. 2B), indicating that the fragment length difference is due to the relative absence of approximately 7 kb of DNA near *Ren-1*^{h}.

Sequences flanking Ren-1^h *lack two insertions which reside near Ren-1*^{d}

In a previous report, no evidence for relative insertions or deletions was apparent within the 10 kb exon/intron structures of *Ren-1*^{d} and *Ren-1*^{h} (Dickinson et al. 1984). *Ren-1*^{h} flanking sequences were therefore examined for the presence of two insertions which have been identified flanking *Ren-1*^{d}. One is a 7 kb insertion beginning 3.8 kb upstream of *Ren-1*^{d} termed M1 (Abel and Gross 1990). The other, which we refer to as M4, is approximately 0.3 kb in length and lies several kb downstream (Dickinson et al. 1984). As shown in Fig. 2C, a *Bgl* I fragment in DBA/2 which contains M4 is slightly larger than its counterpart in *M. hortulanus*. The size difference between these fragments is consistent with the absence of M4, but not additional sequences, from this site in *M. hortulanus*. This is not simply a restriction site polymorphism, as several other restriction fragments overlapping this region showed similar length differences in these mice (not shown).

To test for the presence of the upstream insertion M1 in *M. hortulanus*, DNA sequence information was obtained for the corresponding region 5′ of *Ren-1*^{h} and was compared to that previously determined for DBA/2. Analysis of this region in *M. hortulanus* also enabled us to compare the sequences at the recombination site which resulted in the gene duplication, as this site lies only 0.25 kb further 5′ of M1. To obtain *Ren-1*^{h} and *Ren-2*^{h} genomic sequences, a *M. hortulanus* genomic library was prepared in the λ vector EMBL-3B (Howles 1986). A total of 14 clones were isolated, which encompass both renin loci plus 10–20 kb flanking each locus. One of these, λHRenII-3, was found to physically link *Ren-2*^{h} and *Ren-1*^{h}. An *Xho* I–*Eco*R I fragment, putatively containing the duplication junction and the M1 integration sequence, was isolated from this clone and sequenced. The compared DBA/2 and *M. hortulanus* sequences are indicated as A–D in

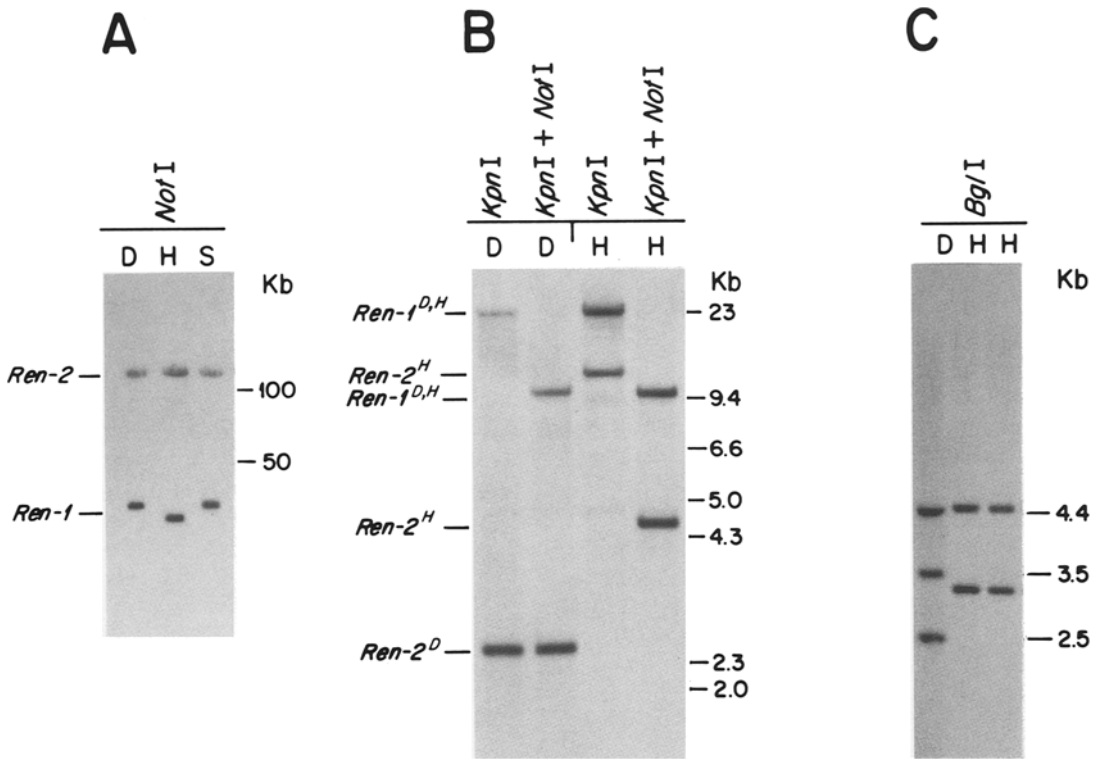

Fig. 2. Southern blot analysis reveals a variant *Not* I fragment containing the *Ren-1* locus in *M. hortulanus*. (**A**) *Not* I digests of embedded spleen DNAs were separated by CHEF using a switching interval of 3.5 seconds to resolve within a relatively low molecular weight range. The *Ren-2* hybridizing fragments lie within a region of compressed mobility above the range of resolvable sizes. The DNAs were transferred to nitrocellulose and hybridized with a full-length renin cDNA probe, pDD-1D2. Approximate lengths in kb of included size markers (oligomers of intact λ DNA) are indicated. D = DBA/2, H = *M. hortulanus*, S = *M. spretus*. (**B**) Spleen DNAs digested with *Kpn* I or *Kpn* I + *Not* I were separated by conventional electrophoresis, transferred, and hybridized with a 3′-end renin cDNA probe, pSM479. This probe detects *Kpn* I fragments extending into 3′ flanking regions (refer to Fig. 1). Combined *Hind* III and *Hind* III + *Eco*R I digests of λ DNA were included as size markers. (**C**) DNAs digested with *Bgl* I were separated by conventional electrophoresis, transferred, and hybridized with the 3′-end cDNA probe, pSM479. The lengths of hybridizing fragments are indicated.

Fig. 1, and the relevant sequence information is shown in Fig. 3.

Beginning at the *Xho* I site and extending for 875 base pairs (bp), the *M. hortulanus* sequence (region D) displayed nearly 99% similarity to sequences in DBA/2 which flank the M1 insertion (regions B,C). The recombination site, or duplication junction between the *Ren-2* and *Ren-1* loci, is indicated by "J" in Figs. 1 and 3. The sequence of the junction region in *M. hortulanus* was identical to that previously determined for DBA/2, including 3 bp of uncertain origin (AGG) at the precise junction. The M1 insertion in DBA/2 begins 251 bp further downstream of the junction. The homologous sequence in *M. hortulanus*, however, was not

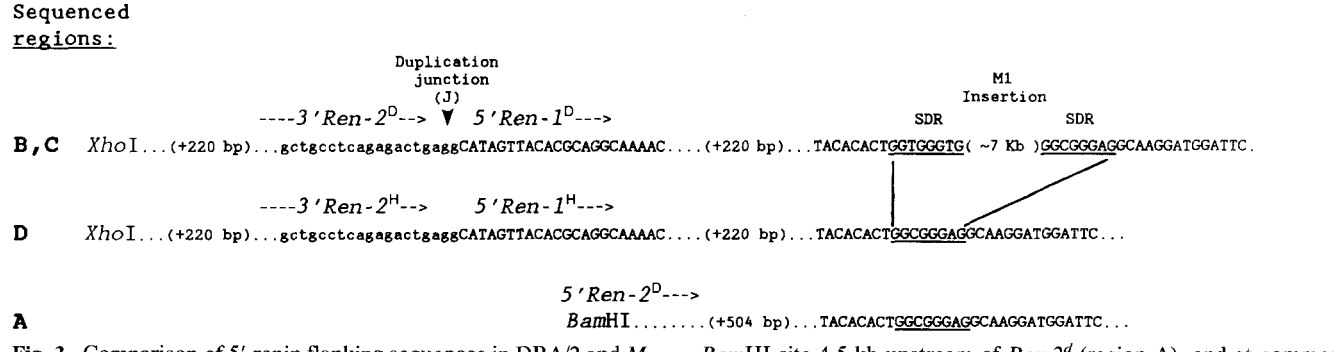

Fig. 3. Comparison of 5′ renin flanking sequences in DBA/2 and *M. hortulanus*. To the left of each sequence is the letter of the corresponding region in Fig. 1. A = 5′ *Ren-2*^{d}; B, C = sequences between *Ren-2*^{d} and *Ren-1*^{d} which include the duplication junction and which flank the M1 insertion; D = *M. hortulanus* sequences homologous to sequenced DBA/2 regions B, C. The sequences begin at a *Bam*HI site 4.5 kb upstream of *Ren-2*^{d} (region A), and at common *Xho* I sites 10 kb downstream of *Ren-2*^{d} and 7 kb downstream of *Ren-2*^{h} (regions B and D, respectively). The duplication junction (J) between 3′ *Ren-2* and 5′ *Ren-1* flanking sequences is indicated. "SDR" indicates imperfect short direct repeats of 8 bp bordering the M1 insertion.

interrupted by the M1 element and was instead identical to sequences 5′ of *Ren-2*^{d} which do not contain M1. Despite the absence of M1 at this site in *M. hortulanus*, Southern blot analysis has shown that sequences homologous to M1 are repetitive in the *M. hortulanus* genome, and in the genomes of most other murine species examined (K. Abel and K. Gross, unpublished data). Interestingly, the *Not* I hybridization results in Fig. 2A suggest that M1 lies between the renin loci in another aboriginal mouse, *M. spretus*, indicating that the presence of M1 at this location is not unique to commensal mice.

M. hortulanus mice also lack two insertions which flank Ren-2^d

M. hortulanus DNAs were examined for four additional small insertions which have been identified 5′ of the DBA/2 loci. Figure 4 illustrates the locations of these insertions relative to the inbred renin genes. B1 and B2 are, respectively, mouse type-1 and type-2 equivalents of *Alu* repetitive elements (Field et al. 1984b; Soubrier et al. 1986). M2 refers to a small insertion upstream of *Ren-2*^{d} described as either 143 or 160 bp (Tronik et al. 1988; Burt et al. 1989), and M3 refers to a 0.5 kb transposon upstream of both loci (Tronik et al. 1988).

In Fig. 4A, *M. hortulanus* DNAs were examined simultaneously for the B1 and M2 insertions. A *Hinc* II site within the B1 sequence is a useful diagnostic site for the presence of this insertion, while *Xba* I and *Sst* I sites are useful in the identification of M2, which lies between these two sites. The double intensity pattern in DBA/2 mice (lane 2) resolves into two equal intensity bands when a digest that discriminates between the presence or absence of M2 is performed (lane 4). However, this does not occur in *M. hortulanus*, leaving one double intensity band similar in size to those associated with *Ren-1*^{c} and *Ren-1*^{d} (lanes 5 and 6). Thus, B1 elements also lie at equivalent positions upstream of each *M. hortulanus* locus, but unlike *Ren-2*^{d}, the M2 insertion does not lie upstream of *Ren-2*^{h}. The results in Fig. 4B showed that the M3 insertion is present in the immediately upstream regions of both *M. hortulanus* loci, and that the B2 element is present upstream of *Ren-2*^{h}.

It was previously shown (Dickinson et al. 1984)

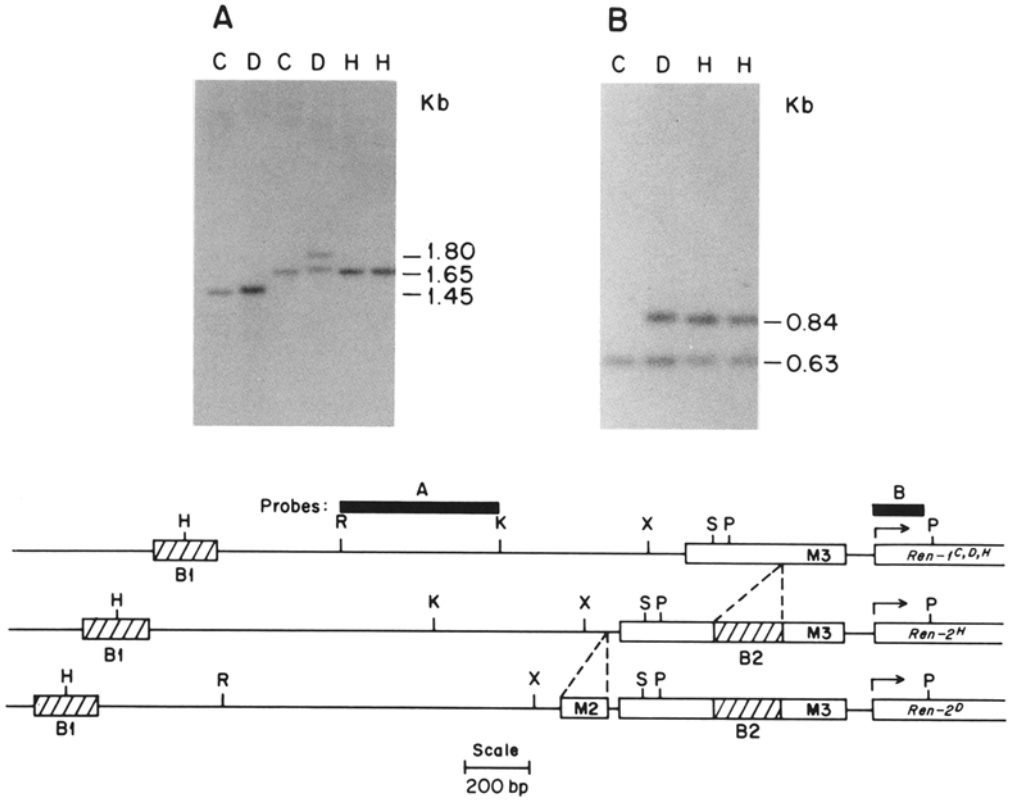

Fig. 4. Identification of insertions upstream of the *M. hortulanus* renin loci. **(A)** Genomic DNAs were digested with *Hinc* II + *Xba* I (lanes 1 and 2) or *Hinc* II + *Sst* I (lanes 3–6), size-fractionated, and transferred to nitrocellulose. The DNAs were then hybridized with the 5′ flanking probe A (see restriction maps, below). C = C57BL/6, D = DBA/2, H = *M. hortulanus*. DNAs from two *M. hortulanus* individuals were included. Approximate lengths of detected fragments in kb are indicated. **(B)** *Pvu* II-digested DNAs were size-fractionated, transferred, and hybridized with a 0.15 kb renin exon I probe (probe B). The restriction maps indicate the insertions (B1, B2, M2, M3) identified upstream of the inbred and *M. hortulanus* renin loci. **Heavy bars** represent the probes used for panels A and B. Restriction sites for *Ren-1*^{c}, *Ren-1*^{d}, and *Ren-2*^{d} were compiled from several reports (Field et al. 1984b; Panthier et al. 1984; Soubrier et al. 1986; Tronik et al. 1988). Sites upstreams of the *M. hortulanus* loci were determined from the present data by comparing restriction fragment lengths to corresponding fragments in the inbred strains, and from additional mapping of genomic clones (Howles 1986). H = *Hinc* II, K = *Kpn* I, P = *Pvu* II, R = *Eco*R I, S = *Sst* I, X = *Xba* I.

that *M. hortulanus* DNAs also lack the partial intracisternal A particle genome which begins 1 kb downstream of *Ren-2*^{d} (Burt et al. 1984). Altogether, four insertions near the DBA/2 renin loci (M1, M2, M4, and IAP) were found to be absent from sequences flanking *Ren-1* and *Ren-2* in *M. hortulanus*. Although Southern data for only two individuals were shown, no differences in the structural organization of 5′ or 3′ renin flanking sequences were observed in the DNAs of nine individuals from our outbred *M. hortulanus* stock.

Analysis of renin expression in DBA/2 and M. hortulanus

With regard to gene expression, the four additional insertions at the DBA/2 loci represent potentially important flanking sequence rearrangements. Steady state levels of renin RNA were therefore examined in a number of DBA/2 and *M. hortulanus* tissues to determine whether the additional insertions in DBA/2 correlated with differences in gene expression. RNAs isolated from the same *M. hortulanus* individuals examined by Southern hybridization (Figs. 2 and 4) were used in the expression analyses.

To identify sequence differences which could be utilized to distinguish between transcripts derived from either *M. hortulanus* locus, partial sequence information was determined for *Ren-1*^{h}- and *Ren-2*^{h}-encoded mRNAs. Adjacent *Eco*R I–*Hind* III fragments containing exons 7 and 8 were isolated from *Ren-1*^{h} and *Ren-2*^{h} genomic clones and subcloned for sequencing. The exon sequences within these genomic fragments were deduced by comparison to the reported renin coding sequences in inbred mice (Panthier et al. 1982a; Holm et al., 1984; Burt et al. 1989). The sequencing enabled us to identify a region that is conserved between the homologous loci in DBA/2 and *M. hortulanus*. This permitted quantitation of the *Ren-1*^{h} and *Ren-2*^{h} transcripts by a primer extension assay developed by K. Lilleycrop and W.J. Brammar for *Ren-1*^{d} and *Ren-2*^{d} (personal communication). Shown diagrammatically in Fig. 5A, a 30-mer oligonucleotide complementary to a sequence in exon 8 was hybridized to total organ RNAs and served as a primer for reverse transcriptase. The reverse transcripts were extended using deoxynucleotides until the incorporation of a chain-terminating dideoxynucleotide (ddGTP) at positions within exon 7 which distinguish between *Ren-1* and *Ren-2* mRNAs. The results in Fig. 5B showed little difference between DBA/2 and *M. hortulanus* in the relative accumulations of *Ren-1* and *Ren-2* transcripts in kidney, submandibular gland (SMG), and testis. Liver expression of *Ren-1*^{h} apparently exceeds that of *Ren-1*^{d}.

Northern hybridization analysis (not shown) of total RNAs isolated from a number of DBA/2 and *M. hortulanus* tissues indicated renin mRNA was detectable in kidney RNAs isolated from both types of mice, supporting the primer extension results. Neither species, however, expressed detectable levels of renin mRNA in adult male coagulating gland, a source of abundant renin mRNA in inbred mice carrying *Ren-1*^{c} (Fabian et al. 1989; unpublished results).

A significant difference observed between these mice was in the developmental pattern of adrenal renin expression. As reported previously (Field et al. 1984a; Mullins et al. 1989), renin mRNA was easily detectable in RNAs isolated from adult male and female DBA/2 adrenal glands (Fabian et al. 1989). In contrast, no renin mRNA was detectable in the adrenal glands of adult male or female *M. hortulanus* mice, an observation supported by in situ RNA hybridization of sectioned adult adrenal glands (C.M. Kane and K.W. Gross, unpublished data). The in situ hybridization also showed, however, that *M. hortulanus* mice were similar to the inbred strains in that they do express abundant levels of renin RNA in the fetal adrenal. Renin expression in the *M. hortulanus* adrenal thus more closely resembled that of the inbred strain C57BL/6 (*Ren-1*^{c} only), in which abundant fetal expression diminishes to undetectable levels by birth and remains undetectable in the adult adrenal (Jones et al. 1990; Fabian et al. 1989).

Discussion

Renin gene structure and expression have been well-characterized in only a few inbred mouse strains, including C57BL/6 and BALB/c (*Ren-1*^{c}), and DBA/2 and Swiss mice (*Ren-1*^{d}, *Ren-2*^{d}). A chromosomal region of approximately 580 kb, defined by adjacent *Not* I fragments containing either the *Ren-2* or *Ren-1* locus, was examined in a number of different mice using PFGE. This survey has indicated that the long-range structure in most inbred and wild mice examined was similar to that which is evident in DBA/2. However, a variant *Not* I fragment containing the *Ren-1* locus was observed in the aboriginal mouse *M. hortulanus*. Subsequent detailed analysis of *M. hortulanus* DNAs, together with previously reported findings (Dickinson et al. 1984), showed that renin flanking sequences in *M. hortulanus* lack four DNA insertions which reside near the DBA/2 loci. These include two insertions flanking the *Ren-2* locus (M2 and IAP), and two flanking the *Ren-1* locus (M1 and M4).

Correlation between renin gene structure and expression

Several reports have described causal relationships between the presence of DNA insertions and modified gene expression patterns, including the mouse dilute (Jenkins et al. 1981), hairless (Stoye et al. 1988), and *Slp* loci (Stavenhagen and Robins 1988), and the human amylase genes (Samuelson et al. 1990). Renin RNA levels in various DBA/2 and *M. hortulanus* tissues were therefore compared to determine whether the additional insertions in DBA/2 correlate with differences in gene expression. It appears unlikely that these insertions significantly influence renin expres-

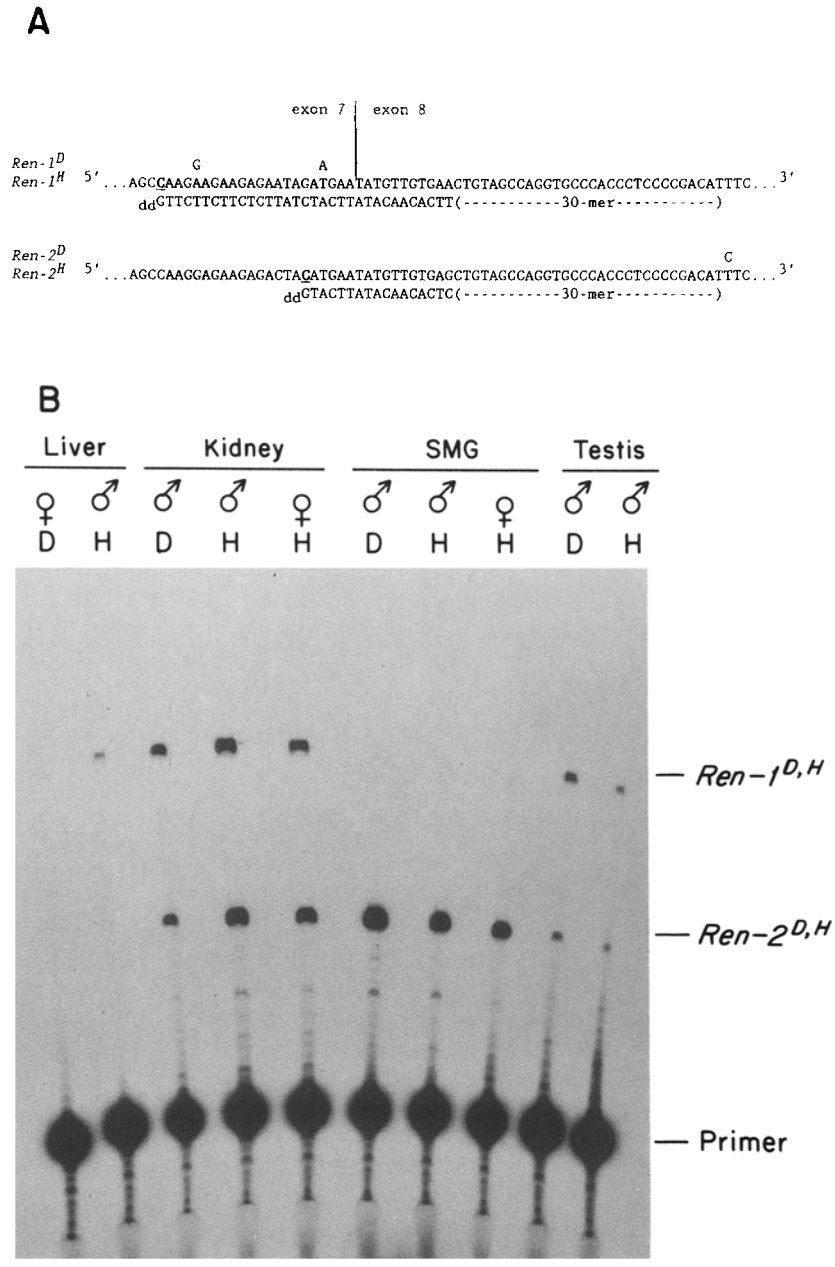

Fig. 5. Locus-specific expression of the renin genes in DBA/2 and *M. hortulanus* mice. **(A)** Schematic diagram of the expected locus-specific primer extension products generated by reverse transcription of 30-mer oligonucleotide/renin RNA hybrids in the presence of dATP, dCTP, dTTP, and ddGTP. The primer hybridizes to a region within exon 8 of identical sequence in DBA/2 and *M. hortulanus* renin RNAs (*Ren-2* bases 997–1026; Panthier et al. 1982a). The 5′–3′ orientation of the mRNA is indicated. The incorporation of ddGTP at different positions (*underlined* C residues in the mRNA sequence) generates truncated transcripts of 63 bases ($Ren\text{-}1^{d,h}$) or 47 bases ($Ren\text{-}2^{d,h}$). **(B)** Primer extension results, using the end-labeled 30-mer oligonucleotide primer, for various DBA/2 and *M. hortulanus* RNAs. The primer extension reactions included 80 μg of total RNA isolated from liver, kidney, or testis, and 0.8 μg of total submandibular gland (SMG) RNA. The truncated extension products were resolved on an 8% acrylamide/7 M urea sequencing gel. The mRNAs from which the extension products were generated ($Ren\text{-}1^{d,h}$ or $Ren\text{-}2^{d,h}$) are indicated.

sion in liver, kidney, testis, or coagulating gland, since little or no difference in expression at these sites was observed between the two mice.

In contrast, correlations between the structure and expression of renin genes in different mice and in different species have led to recent proposals that one or more of these insertions influence expression in the submandibular gland (SMG). In particular, it has been suggested that the 0.5 kb transposon (M3) contains sequences necessary for SMG expression, and that either the M2 or B2 insertions might be responsible for the much higher SMG expression of $Ren\text{-}2^{d}$ (Tronik et al. 1988). It has also been proposed that M2 disrupts a negative regulatory element (Nakamura et al. 1989), leading to greater SMG expression of $Ren\text{-}2^{d}$ relative to the inbred *Ren-1* alleles.

Results reported here help clarify these issues. While it remains possible that SMG expression is enhanced as a result of the B2 insertion, the high level of $Ren\text{-}2^{h}$ expression in this gland (Fig. 5) argues against any straightforward role for the M2 insertion. Moreover, the M3 insertion is present upstream of both the $Ren\text{-}1^{d}$ and $Ren\text{-}1^{h}$ alleles, yet neither of these are expressed at detectable levels in SMG. This suggests that this insertion alone is not sufficient to direct significant SMG expression. Interestingly, a proportion of renin transcripts in the renin-expression epithelial cells of SMG and coagulating gland have been observed to initiate within the M3 insertion (Field et al. 1984b; J. Fabian and K. Gross, unpublished data). The sequences which promote transcription at these upstream sites, however, remain unclear. The profound difference in SMG expression characteristic of *Ren-1* and *Ren-2* in inbred mice was also observed in *M.*

hortulanus. Therefore, up-regulation of the *Ren-2* alleles in SMG or down-regulation of the *Ren-1* alleles may have been a very early post-duplication event, or a direct consequence of the duplication itself.

The primer extension results reported here have enabled us to resolve an outstanding question regarding the structure of the isozyme encoded by *Ren-2*^{h}. It was previously shown that SMG renin activities in DBA/2 and *M. hortulanus* differed with regard to thermostability and alloantibody inhibition (Dickinson et al. 1984). One explanation for these results was that *M. hortulanus* SMG renin was an equal mixture of isozymes encoded by *Ren-1*^{h} and *Ren-2*^{h}. The results in Fig. 5, however, demonstrating exclusive expression of *Ren-2*^{h} in SMG, support the alternative explanation that this allele encodes a structural variant of renin-2.

Unlike the adult DBA/2 adrenal gland, in which both renin loci are expressed at roughly equivalent levels (Fabian et al. 1989), no renin mRNA was detectable in adrenal gland RNAs from adult male or female *M. hortulanus* mice. One potential explanation is that one or more of the insertions which distinguish the DBA/2 loci contain sequences necessary for adrenal expression in adult mice. Particularly interesting candidates are M1 and IAP, as these large insertions lie between *Ren-2*^{d} and *Ren-1*^{d} and they are suitably situated to influence expression of both loci.

Post-duplication evolution of mouse renin genes

The identification of variant sequence organization provides insights into the history of rearrangements which have occurred at the mouse renin genes. Most interesting, the sequence of the duplication junction between the renin loci was identical in both DBA/2 and *M. hortulanus*. This indicates that the gene duplication event occurred prior to the divergence of commensal and aboriginal mice, estimated to be 2.75–5.5 million years ago (Ferris et al. 1983). Aboriginal mouse species, including *M. hortulanus*, were found to be the most distantly related to the inbreds which showed evidence of the duplication (Dickinson et al. 1984). Thus, a single duplication event apparently accounts for the presence of two renin loci in most, if not all, present day mice identified to carry two loci.

The B1 and M3 insertions (refer to Fig. 4) were found upstream of the renin loci not only of DBA/2 and *M. hortulanus*, but also of single renin locus mice, suggesting that these insertions may have been present near an ancestral, pre-duplication renin gene. The B2 element 5′ of both *Ren-2*^{d} and *Ren-2*^{h} may have inserted early after the duplication, or may have been present upstream of one of the ancestral alleles which participated in the duplication. The simplest explanation for the insertions which distinguish between the DBA/2 and *M. hortulanus* loci (M1, M2, M4, IAP) is that they inserted post-duplication, and subsequent to the divergence of ancestors of these mice. In turn, this would suggest that the structure at the *M. hortulanus* loci most closely resembles the earliest post-duplication structure.

Comparison of homologous 5′ flanking sequences (Fig. 3) provided no evidence to suggest the presence and subsequent deletion of the M1 element from this site in *M. hortulanus*. Sequences homologous to M1, however, are repetitive in the *M. hortulanus* genome, and also in the genomes of mice distantly related to the inbreds. This element thus may have been present in the genome early in mouse evolution, and at least some members of this class have apparently been mobile since the time of the renin gene duplication.

These results suggest relationships between certain flanking sequence rearrangements and gene-specific expression patterns. Proof of the relationships will require that the involved sequences be directly tested either by transfection assays in appropriate cell lines or by construction of transgenic animals.

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