SUMMARY  The Ren-1 locus of mice encodes the protease renin, which with converting enzyme processes angiotensinogen to the potent vasoressor angiotensin II. Some strains of mice appear to carry a duplication of the renin structural gene (Ren-2) near the Ren-1 locus. Strains with the gene duplication can exhibit as much as 100-fold higher levels of submaxillary gland renin compared to strains with a single gene copy. In contrast, kidney renin levels appear to be unaffected by the gene duplication. Sequence analysis of a 319 bp renin cDNA recombinant isolated from a kidney library from the two-gene strain DBA/2Ha corresponds to a transcript of the Ren-1 gene. Moreover, a single base substitution of A for G at residue #996 in the kidney renin mRNA creates a potential glycosylation recognition site that may, in part, account for the differential glycosylation of kidney and submaxillary gland renins. In addition, our tissue surveys indicate that mature mRNAs from the Ren loci are detectable in adrenal gland and testes, as well as sublingual and parotid salivary glands, and reveal length variation for the renin transcripts in at least the submaxillary gland.

Tissue and Gene Specificity of Mouse Renin Expression

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RECENTLY, the use of recombinant techniques has resulted in the molecular characterization and isolation of portions of this locus. The available data suggest that strains with low levels of SMG renin have a single structural gene, while strains with high levels of SMG renin have two. The gene presumed to be held in common has been designated Ren-1, and the duplicated gene designated Ren-2. The increase in gene copy number is in itself insufficient to account for the increased SMG renin activity (as well as the increased mRNA level) observed in two-gene strains. Enzymatic activity assays and immunohistochemical assays indicate that kidney renin levels are unaffected by the gene duplication. Interestingly, a recent report by Holm et al., which compares the Ren-1 coding sequence (obtained from Ren-1 genomic clones) to a previously identified Ren-2 cDNA sequence, has shown that Ren-1 and Ren-2 share 96% homology in their coding regions.

Several groups have presented models to account for the varying levels of tissue-specific renin expression observed between one- and two-gene strains of mice. An appealing interpretation of the available data is that the duplicated gene is responsible for the increased...
levels of SMG renin in two-gene strains and that this gene is not appreciably expressed in the kidney, with the result that kidney and thus circulatory renin levels are equivalent in one- and two-gene strains. This model can be directly tested by analysis of kidney renin transcripts from two-gene strains. Here we report our analysis of a kidney renin cDNA clone from the two-gene strain DBA/2Ha and show by sequence analysis that it corresponds to a transcript of the Ren-I gene (the gene held in common in one- and two-gene strains). We have also determined other sites of primary renin synthesis by conducting a comprehensive tissue survey for the expression of renin mRNA and have found that at least two mRNA size classes are evident in the SMG.

Materials and Methods

RNA Extraction

Total RNA was extracted by the guanidine HCl method of Cox,16 with some modifications. Tissues were homogenized in 8 M guanidine HCl, 10 mM EDTA, 10 mM Tris base, pH 7.5. The homogenate was centrifuged at 10,000 g for 10 minutes and the supernatant was precipitated by the addition of 0.5 vol ethanol. The ribonucleic acid (RNA) was dissolved and reprecipitated from 6 M guanidine HCl three times and resuspended in nuclelease free H2O. The quantity and purity of the RNA was determined spectrophotometrically. Unless stated otherwise, RNA was isolated from male mice. To minimize the risk of cross-contaminating tissues during the dissection of the salivary glands, the lobes of the SMG and the highly adhering sublingual glands were carefully separated from the surrounding parotid tissue. The SMGs and sublinguals were then separated from one another in situ and removed. Finally, the parotid tissue was excised. RNAs were then separated from one another in situ and recovered from 6 M guanidine HCl three times and resuspended in nuclelease free H2O. The quantity and purity of the RNA was determined spectrophotometrically. Unless stated otherwise, RNA was isolated from male mice. To minimize the risk of cross-contaminating tissues during the dissection of the salivary glands, the lobes of the SMG and the highly adhering sublingual glands were carefully separated from the surrounding parotid tissue. The SMGs and sublinguals were then separated from one another in situ and removed. Finally, the parotid tissue was excised. RNAs were then separated from one another in situ and removed.

Northern Blot Hybridizations

Two methods were used to denature RNA before electrophoresis. The first was the method of Lehrach et al.17 as modified by Shaw et al.18 Total RNA (20 μg) was heated to 50° C for 10 minutes in 50% formamide, 6.6% formaldehyde, 10 mM Na-phosphate, pH 6.5. Samples underwent electrophoresis on 1.5% agarose gels that contained 6.6% formaldehyde and 10 mM Na-phosphate, pH 6.5.

The second method used to denature RNA was a modification of the method of Thomas19 that involved the use of glyoxal. Total RNA (20 μg) was heated to 50° C for 15 minutes in 1.0 M deionized glyoxal, 50% (vol/vol) Me3SO, 10 mM Na-phosphate and 0.1% SDS. Samples underwent electrophoresis on 2.0% agarose gels that contained 10 mM Na-phosphate. With both methods RNA was transferred to nitrocellulose by the method of Thomas.19 The transfer was hybridized to nick-translated insert from pDD-1D2, a full-length SMG renin clone constructed from poly A RNA from the two-gene strain DBA/2J (D.P. Dickinson, unpublished data). The cDNA clone was constructed by a modification of the method of Okayama and Berg20 in which oligo-dT was used to prime first-strand synthesis. The cDNA was then dC-tailed and ligated to dG-tailed pBR-322 (D.P. Dickinson, unpublished data). The nick-translation and hybridization conditions have been described.1 21

Isolation of a Kidney cDNA Clone

A 3500-member kidney cDNA library prepared from a size-enriched mRNA fraction from the strain DBA/2Ha (constructed by R.E. Ganschow)22 was screened by the method of Grunstein and Hogness.23 The probe used for hybridization consisted of a 300 bp SMG renin cDNA insert from pSM47917 subcloned into M13 mp-8 and labeled by DNA polymerase I-mediated elongation of the BRL mp-8 hybridization primer (catalog number 1544) in the presence of α-32-P dNTPs. The pSM479 was constructed with poly A SMG RNA from the two-gene strain DBA/2J.

Sequence Analysis

Plasmid to be sequenced was isolated from CsCl by the cleared lysate method of Clewell and Helinsky.24 DNA was digested with selected restriction endonucleases and end-labeled with the Klenow fragment of DNA polymerase I in the presence of appropriate α-32-P dNTPs. Specific fragments were recovered from agarose gels25 and sequenced by the chemical method of Maxam and Gilbert.26

Results

Gene Specificity of Kidney Renin Expression

To ascertain the gene specificity of kidney expression, a kidney cDNA library22 was screened with a SMG renin cDNA probe to recover homologous clones from this tissue. Previous hybridization has indicated that renin mRNA constitutes approximately 0.5% to 1% of induced submaxillary poly A mRNA in two-gene strains and that these levels are roughly 30- to 100-fold greater than those observed for kidney.1,27 Therefore, renin can be estimated to represent 0.005% to 0.01% of kidney poly A mRNA. The kidney library was constructed with size-fractionated poly A mRNA from the two-gene strain DBA/2Ha. The fractionation resulted in an approximately 6- to 10-fold enrichment of 20S message.

One clone of 3500 screened was detected by hybridization to our SMG renin cDNA probe (pSM479 insert, see Materials and Methods). The positive clone, designated pK-A1, produced the appropriate pattern of bands that corresponded to the renin genes when hybridized to genomic Southern blots (not shown). Sequence analysis of pK-A1 is illustrated in Figure 1. The schematic diagram shows the location of pK-A1 with respect to SMG renin mRNA28 and the location of a putative Ren-1 genomic exon analyzed by Brammar and co-workers.2 The sequence of pK-A1 was also compared to that of SMG renin as determined by Pantier et al.28 It is apparent that kidney and SMG
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A) Sequencing Strategy

B) Comparison of Overlapping Sequences

pK-A1

Ren 1 Exon

SMG Renin RNA

C) Sequence

FIGURE 1. Sequence analysis of pK-A1. A. Sequencing strategy of pK-A1. B. Region of sequence overlap of pK-A1 and the Ren-1 exon with respect to SMG renin mRNA. C. Sequence of pK-A1 (designated kidney) compared to that for SMG renin (designated SMG) as determined by Panthier et al. The pK-A1 was digested with selected restriction enzymes, end-labeled with DNA polymerase-I, and sequenced by the chemical method of Maxam and Gilbert. Divergent nucleotide and amino acid residues are indicated. Dashes indicate sequenced nucleotides (and the deduced amino acids) which agree with those for submaxillary mRNA as determined by Panthier et al. renin mRNA are quite homologous; only nine of the 319 residues sequenced varied. In addition, the sequence of pK-A1 agreed completely with the Ren-1 genomic sequence reported by Mullins et al. (see Figure 1) and with that recently reported by Holm and co-workers. This strongly suggests that pK-A1 originated from a transcript of the Ren-1 (common) gene. Interestingly, the substitution of A for G at residue #996 of kidney message resulted in a substitution of Asn for Ser in kidney renin; this substitution generated the glycosylation recognition sequence Asn/Cys/Ser which may, in part, account for the differential glycosylation observed between renal and submaxillary renin.

Tissue Survey of Renin Expression

Recently, there have been indications that renin may be expressed in other tissues in addition to the SMG and kidney. Northern blot hybridizations were employed to screen various tissues of the two-gene strain DBA for the presence of renin mRNA. Total RNA from various tissues underwent electrophoresis in formaldehyde gels, transferred to nitrocellulose, and hybridized to our nick-translation SMG renin cDNA probe (pDD-1D2). Figure 2 shows autoradiographs of representative hybridizations from the survey. We have quantitated all positive signals by densitometry and normalized them to kidney levels. In addition to the SMG and kidney, we found renin mRNA in the sublingual, parotid, and adrenal glands, and in testes at levels of approximately 2.5, 0.5, 0.3, and 0.15 times that of kidney, respectively. Our Northern blots routinely detect a 1/625 dilution of two-gene SMG RNA in a 48-hour exposure (see Figure 2). This represents approximately 0.1 times the level of kidney renin mRNA. Because of potential difficulties in cleanly dissecting the three murine salivary glands, we are cautious about identifying the parotid and sublingual glands as sites of renin synthesis.
RNA are evident (see C3H/HeHa, Figure 3). These transcripts are approximately 1450 and 1525 bases long. Furthermore, these transcripts are also present in two-gene strain submaxillary RNA, but only the 1450 base transcripts are discernible by Northern analysis due to a marked difference in the relative abundance of the mRNAs. The heterogeneity of the SMG mRNA size is detectable in both one- and two-gene strains by S1 nuclease and primer extension analysis (Field, Philbrick, Howles, Dickinson, McGowan, and Gross, unpublished data).

Discussion
In this report we address two questions. First, what is the gene specificity of kidney renin expression? Second, what is the spectrum of tissues expressing renin? In addition, we have observed at least two discrete size classes of SMG renin mRNA in one-gene strains.

Sequence analysis of a kidney cDNA clone from the two-gene strain DBA/2Ha indicates that the proposed common gene (Ren-1) is expressed in this tissue. This result appears to be corroborated by Panthier and co-workers, who have recently reported a 76 bp kidney renin cDNA sequence from two-gene Swiss mice which agrees with a different portion of the Ren-1 exon. Of the 319 residues in our kidney cDNA clone, nine differ from the sequence reported for mouse SMG renin (see Results). Interestingly, the substitution of A for G at residue #996 in kidney message results in the substitution of Asn for Ser at residue #319 of the amino acid sequence. This, in turn, creates the glycosylation recognition sequence Asn/Cys/Ser at amino acid residues #319-#321. It has been noted that kidney renin is glycosylated while submaxillary renin is not. Examination of the primary amino acid sequence and the cDNA sequence of mouse SMG renin reveals no glycosylation recognition sequences. Thus, the substitution of A for G at residue #996 in kidney mRNA may, in part, account for the differential glycosylation of kidney and SMG renin. An additional glycosylation recognition sequence in Ren-1 has recently been identified by Holm and co-workers. Two arguments can be made that strongly suggest that Ren-1 is the predominantly expressed renin gene.
in the kidney of two-gene strains. The first argument addresses the apparent thermostable renin activity in the kidney of both one- and two-gene strains. Molecular analysis from three independent laboratories has shown that the presence of the gene duplication cosegregates with the high SMG renin phenotype. Wilson and Taylor have shown that this high SMG renin phenotype, in turn, cosegregates with a thermostable SMG renin activity, while the low SMG renin phenotype cosegregates with a thermostable SMG renin activity. In addition, they have shown that kidney renin activity in strains that carry either the high or low SMG renin activity phenotype is thermostable. These results suggest that kidney renin activity in both one- and two-gene strains is similar to one-gene strain SMG renin activity (presumed to be derived from the Ren-1 gene) and dissimilar with two-gene strain SMG renin activity (presumed to be derived from the Ren-2 gene). The identification of our kidney renin cDNA clone as having originated from a Ren-1 transcript provides direct evidence for the expression of Ren-1 mRNA in the kidney of two-gene strains. This fact, together with the thermostability studies of Wilson and Taylor, suggests that Ren-1 is the predominantly expressed renin gene in the kidney of two-gene strains.

The second argument addresses the ratio of Ren-1 and Ren-2 transcripts. In the SMG, in which both Ren-1 and Ren-2 are expressed, the presence of the gene duplication results in 100-fold higher levels of renin message in two-gene strains relative to one-gene strains. Thus, we would anticipate Ren-1 (common) gene transcripts and Ren-2 (duplicated) gene transcripts to be present at a ratio of 1 to 100 in the SMG of two-gene strains. If kidney renin expression in two-gene strains is similar to SMG expression, the probability would be considerably in favor of isolating a cDNA clone derived from the Ren-2 gene. Yet, sequence analysis indicates that PK-AI originated from a Ren-1 transcript. Similarly, sequence analysis of an independently derived and nonoverlapping cDNA recombinant for kidney renin, recently reported by Rougeon and co-workers, indicates that this recombinant also derives from a Ren-1 transcript of the two-gene strain SWR. Clearly, sequence analysis of a limited sample of kidney cDNA recombinants cannot unequivocally address the issue of whether the Ren-2 gene is completely silent in the kidney. Nevertheless, the observed differences in physical and immunological properties of kidney and SMG renin and the above results are consistent with the notion that the Ren-1 gene is the predominantly expressed renin gene in the kidneys of two-gene strains. More precise quantitation of the relative levels of expression of the two Ren loci in different tissues will require molecular probes that can distinguish the two transcripts.

As part of our further investigation of the basis for the tissue-specific expression of the Ren-1 and Ren-2 loci, we have conducted an extensive tissue survey to identify additional primary sites of renin synthesis. A number of other potential sites of synthesis have been reported based on immunohistochemical, enzymatic activity assays, or Northern blot assays. These include human brain, rat pituitary, human chorion, as well as other tissues. Because renin is secreted into the circulatory system and shares homology in its reaction mechanism with other members of the family of acid proteases, verification of such tissues as sites of primary synthesis by methods that only detect enzymatic activity or protein has not been unequivocal. We have surveyed tissues by Northern analysis with the aim of identifying tissues containing significant concentrations of apparently mature renin mRNA species. In addition to the SMG and kidney, our hybridization analyses detected renin mRNA in the adrenal gland and testes, as well as in the sublingual and parotid salivary glands. We view the sublingual and parotid identifications as debatable, given technical difficulties with dissection. The adrenal and testes analyses, however, provide independent, direct support for the identification of these tissues as sites of primary synthesis. At present, the physiological significance of such synthesis is not clear. Nevertheless, confirmation of such sites may ultimately aid in the understanding of
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Acknowledgments

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more complex aspects of the involvement of the renin-angiotensin system in blood pressure regulation. It will be of interest in this regard to determine if these observations can be extended to other species and strains of mice harboring a single renin gene. It is also worth noting that portions of the kidney, adrenal glands, and testes derive from a limited region of the mouse embryo.44 Perhaps this relationship is manifested in a commitment event during embryogenesis that is necessary, but in itself not sufficient, for renin expression in adult tissue.

Although the pituitary gland was included in our brain preparations, we have been unable to detect renin mRNA in the brain; this may be due to the low percentage of cells that synthesize renin in this tissue.7 We cannot, of course, rule out the low levels of renin expression in other tissues, although our Northern blot hybridizations easily detected renin message in a 1/625-fold dilution of SMG RNA. Tissues that appeared positive for renin mRNA in our survey are currently being analyzed by in situ hybridization to ascertain the cell types exhibiting synthesis. In addition, tissues from one- and two-gene strains are being compared in an initial effort to discern which Ren loci are active in these tissues.

A particularly intriguing result obtained during the course of the tissue surveys was the finding that multiple transcript lengths for renin were discernible in the SMG of one-gene strains. In results to be presented elsewhere (Field, Philbrick, Howles, Dickinson, McGowan, and Gross, unpublished data) we have confirmed this finding by 5' and 3' S1 nuclease analysis and by primer extension analysis. We demonstrated that the longer transcripts harbor additional 5' sequences encoding a potentially open reading frame continuous with that previously identified.28 Such transcripts are also present in two-gene strain, but are not detected by Northern analysis due to their lower concentration relative to shorter renin mRNA species. They are, however, detectable by S1 nuclease and primer extension experiments.

The Ren structural genes are highly homologous and tightly linked genetically, yet they show markedly different levels of tissue specific expression. As such they provide an interesting model system for the study of tissue-specific transcription in higher eucaryotes. The availability of inbred strains harboring different Ren alleles has facilitated the genetic and molecular analysis of the locus. Molecular analysis of these alleles should help to elucidate the types of physical differences that alter their expression in different tissues and hormonal environments.
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