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Genetics

Mouse kidney and submaxillary gland renin genes differ in their 5′ putative regulatory sequences

(molecular cloning/DNA sequence/steroid hormones/transcriptional regulation/duplicated genes)

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ABSTRACT **The genomic DNAs that specify the renin mRNAs found in the submaxillary gland and in the kidney of Swiss mice have been isolated by molecular cloning in phage λ. The *Ren1* and *Ren2* genes, encoding the kidney and submaxillary renins, respectively, span about 9.5 kilobases each. Restriction maps of both renin genes and their flanking sequences are presented. To correlate the differential expression of the renin genes with their structures, a sequence analysis of the putative 5′ regulatory sequences of both genes was conducted. The results show that the leader sequences and promoter regions of both genes are highly homologous to a point located 179 nucleotides upstream from the transcription start point; the sequences diverge beyond this position.**

In mammals, kidney juxtaglomerular cells constitute the main source of renin. However, in some strains of mice, the granular convulated tubules of the submaxillary gland (SMG) also synthesize large amounts of renin. This SMG renin production is under genetic and hormonal controls. Inbred strains of mice can be divided into two distinct phenotypic groups having different levels of SMG renin: renin activity is >100-fold greater in the SMG of Swiss and AKR mice than in the SMG of BALB/c, C3H, and C57Bl/6 mice (1). Furthermore, SMG renin synthesis is regulated by androgenic steroids. After treatment with androgen hormones, renin activity increases in the SMG, while remaining unchanged in the kidney (2).

The difference in the SMG renin activity between inbred strains of mice has been assessed at the RNA level (3) and has been associated with a single duplication of the renin structural gene (4–6). Strains of mice with low levels of SMG renin possess one renin gene per haploid genome, whereas strains of mice with high levels of SMG renin have two renin genes, named *Ren1* and *Ren2*. In mice having only one renin gene, such as BALB/c mice, *Ren1* is expressed mainly in the kidney, but also at a low level in the SMG. In Swiss mice, *Ren1* is expressed mainly in the kidney, whereas *Ren2* is highly expressed in the SMG, but not in the kidney (7). Following treatment with testosterone the SMG renin activities in both groups of mice increase proportionally to the basal activity (1). In other words, both renin genes, whose expression is tissue-specific, are inducible by testosterone in the SMG, but the rate of transcription of *Ren2* is enhanced in the SMG of either untreated or testosterone-treated mice compared to the rate of transcription of *Ren1* in the SMG.

Recently, we have reported the isolation and the characterization of the entire *Ren1* from BALB/c mice (8). Comparison of the nucleotide sequence of kidney renin mRNA (encoded by *Ren1*) from BALB/c mice with the previously reported nucleotide sequence of SMG renin mRNA (encoded by *Ren2*) from Swiss mice (9) revealed that *Ren1* and *Ren2* nucleotide sequences are 96% homologous in the protein coding regions, with the most important difference between kidney and SMG renins being the presence of three potential glycosylation sites in the kidney enzyme (8).

We report here the isolation and the characterization of the renin genes, together with their flanking sequences, from Swiss mice. The nucleotide sequences of the 5′ ends of both genes are presented and comparisons are made between the two putative regulatory regions.

MATERIALS AND METHODS

Construction and Screening of a Swiss Mouse Library. Genomic DNA was prepared from the livers of Swiss mice as described (10) and partially digested with *Sau*3A restriction endonuclease according to Maniatis *et al.* (11). DNA fragments of 10–20 kilobases (kb) were extracted from 0.5% low-melting agarose gel as described by McMaster *et al.* (12). λ EMBL3 vector DNA (unpublished data; described in ref. 13) was prepared according to Maniatis *et al.* (11).

Two micrograms of the genomic DNA fragments were coprecipitated with 4 μg of vector DNA. Ligation was carried out at 14°C for 16 hr at a final concentration of 200 μg of total DNA per ml. Recombinant molecules were packaged *in vitro* into infectious phage particles according to the method of Hohn and Murray (14). The library was screened by using the *in situ* plaque hybridization technique of Benton and Davies (15). Hybridization was carried out by using renin pRn1-4 cDNA as a probe (5). The probe was ligated to itself and labeled by nick-translation to high specific activity (10^8 cpm/μg) according to Rigby *et al.* (16).

DNA Sequencing. The 2.0-kb *Sac* I/*Hin*dIII fragment and the 1.6-kb *Kpn* I fragment encoding the 5′ ends of SMG and kidney renin mRNAs, respectively, were inserted into the *Pst* I site of pBR322 by the dC/dG tailing method (17). The corresponding plasmids, ppRn1 and ppRn2, were used for sequencing studies according to Maxam and Gilbert (18). The sequence was checked independently by using the enzymatic method of Messing *et al.* (19) as modified by Biggin *et al.* (20), after subcloning of the *Hae* III fragments of the ppRn1 and ppRn2 inserts into M13 mp701. The sequence is numbered from the translational start of the renin gene (9). Downstream and upstream sequences are given "+" and "−" prefixes, respectively.

Mapping of the 5′ Ends of the Transcripts. Labeled, single-stranded DNA probes were synthesized according to Kelly *et al.* (21) and hybridized to poly(A)$^+$ RNA prepared according to Auffray and Rougeon (22). Hybridization was done in 50% formamide/500 mM NaCl/1 mM EDTA/40 mM Pipes, pH 6.4, for 18 hr at 42°C (S1 nuclease protection experiment) or in 0.1 M NaCl/0.1 mM EDTA/20 mM Tris·HCl, pH 8.0, for 3 hr at 60°C (primer extension experiment). After hybridization, samples were reacted for 1 hr at 37°C with 15 units of

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Abbreviations: SMG, submaxillary gland(s); kb, kilobase(s); bp, base pair(s).

S1 nuclease (Sigma) or 8 units of avian myeloblastosis virus reverse transcriptase. This treatment was followed by phenol extraction, ethanol precipitation, and electrophoresis on a 6% sequencing gel.

RESULTS

Construction of a Swiss Mouse Library and Isolation of *Ren1* and *Ren2*. A Swiss mouse DNA library was constructed and screened with the ^{32}P-labeled SMG renin cDNA probe (5, 9). Twelve plaques yielding strong hybridization signals were purified. Recombinant DNAs were isolated and restriction maps were constructed. All 12 recombinant clones are different and fall into two categories of overlapping sequences corresponding to the previously identified *Ren1* and *Ren2* present in Swiss mouse chromosomal DNA (5). In particular, *Eco*RI digestion of recombinant phage SW35 produces the two 8.8- and 3.9-kb *Eco*RI fragments distinctive for *Ren1*, whereas SW10 yields the two 9.2- and 4.4-kb *Eco*RI fragments distinctive for *Ren2*.

The restriction maps of the various overlapping insert DNAs allowed their unambiguous orientation with respect to each other. The overlapping clones were thus arranged into two regions of 19.6 and 23.1 kb length of continuous chromosomal DNA containing *Ren1* and *Ren2* sequences, respectively. The results presented in Fig. 1 indicate that the *Ren1* and *Ren2* cloned sequences are contained within two and three overlapping clones, respectively. Since the 3.0-, 3.4-, and 0.5-kb and the 3.5- and 5.8-kb *Eco*RI fragments, localized at the ends of the *Ren1* and *Ren2* cloned regions, respectively, do not hybridize with a cloned cDNA probe derived from an almost complete transcript of SMG renin gene, we consider that these clones contain the complete sequence of both renin genes of the Swiss mouse.

We have shown previously that a ^{32}P-labeled cDNA probe corresponding to the 3′ moiety of the mRNA hybridizes only with the 3.9- and 4.4-kb *Eco*RI fragments of *Ren1* and *Ren2*, respectively (5). This provides the orientation of the renin genes in the restriction maps as shown in Fig. 1.

To map the positions of the 5′ and 3′ ends of kidney and SMG renin mRNAs, the cloned DNAs were digested with restriction endonucleases, electrophoresed in agarose gels, and hybridized with total or 5′- or 3′-^{32}P-labeled cDNA probes. These experiments (data not shown) indicate that both *Ren1* and *Ren2* span about 9.5 kb of chromosomal DNA. Arrows in Fig. 1 indicate the orientation and give the approximate locations for transcription initiation (5′) and termination (3′) sites; comparison of the two restriction maps does not reveal any major DNA rearrangement inside the genes.

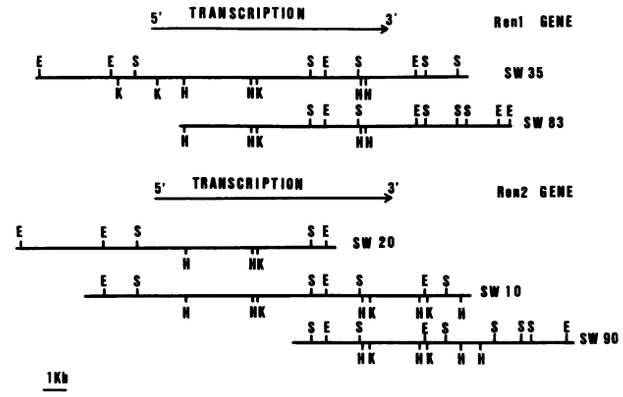

FIG. 1. Endonuclease cleavage maps of *Ren1* and *Ren2*. Recombinant phage DNAs were digested to completion either with *Eco*RI (E), *Kpn* I (K), *Sac* I (S), or *Hin*dIII (H) restriction endonuclease. DNA fragments were separated on neutral 0.9% agarose gels and transferred to nitrocellulose as described by Southern (23). Hybridization of DNA immobilized on nitrocellulose with cDNA probe was done according to Groner and Hynes (24). For ordering the restriction fragments, λ DNAs were labeled at the right cohesive end with [α-^{32}P]dGTP and DNA polymerase Klenow fragment. Partial digestions of labeled λ DNAs were made and DNA fragments were separated by electrophoresis on 0.4% neutral agarose gels, transferred to DE-81 filters (Whatman). The overlapping series of restriction fragments, all with a common labeled terminus, were obtained after autoradiography.

Mapping of the 5′ Ends of the Renin Genes. Although the regions involved in the control of eukaryotic gene transcription are not well defined, the accumulated evidence suggests that the control region for RNA polymerase II is located upstream from the transcription initiation site. Only the 1.6-kb *Kpn* I fragment of SW35 phage and the 2.0-kb *Sac* I/*Hin*dIII fragment of SW10 phage hybridize with a 104-base-pair (bp) *Pst* I/*Hin*fI probe corresponding to the 5′ end of SMG renin cDNA. These fragments, named ppRn1 and ppRn2, were subcloned into vector pBR322. The DNA sequence of the 500 bp preceding the ATG start codon and the sequence corresponding to the first exon of both genes were determined by the methods of Maxam and Gilbert (18) and of Messing *et al.* (19) according to the strategies shown in Fig. 2.

Comparison of both sequences, numbered from the ATG start codon, is shown in Fig. 3. We searched for canonical RNA polymerase II promoter sequences upstream from the 5′ end of the cloned cDNA (position −40). Two putative "TATA" sequences (25), T-A-T-A-A-A-A and T-A-A-T-A-A-A, are found at positions −61 and −97, respectively. That

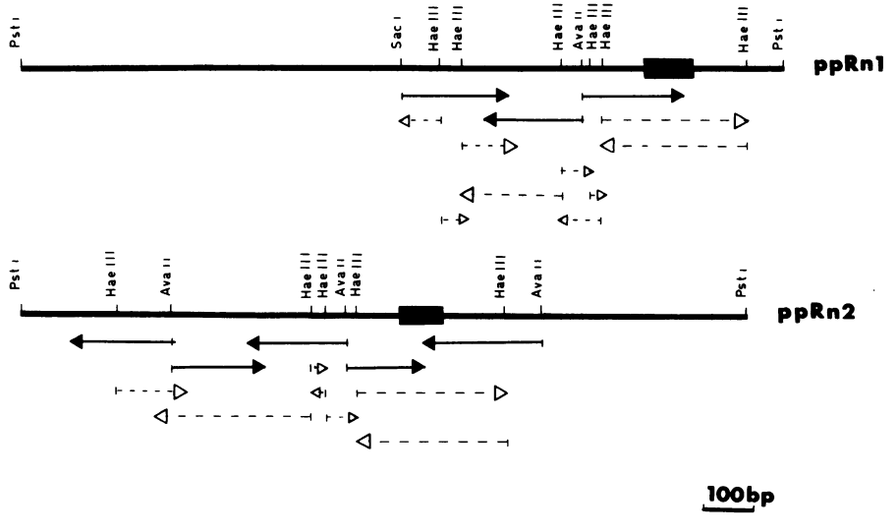

FIG. 2. Strategies for nucleotide sequencing. The black boxes represent the first exon of both genes. Nucleotide sequences were determined by the methods of Maxam and Gilbert (18) (—) and of Messing *et al.* (19) (– – –) by using the restriction sites indicated. The direction and extent of nucleotide reading are indicated by horizontal arrows below the restriction maps. A 400-bp *Pst* I fragment localized at the right end of plasmid ppRn2 has been omitted for clarity.

these sequences do correspond to functional renin promoters was demonstrated by mapping the 5′ end of the message. Both primer extension and S1 nuclease protection experiments showed the existence of two main transcripts of the *Ren2* gene (Fig. 4 *B* and *C*). The 5′ ends of both transcripts are somewhat heterogeneous and are found around positions −35 and −69—i.e., 27 and 29 nucleotides downstream from the two TATA boxes, respectively. Moreover, the 3′-proximal start point is used predominantly. The 5′ end of the previously described SMG renin cDNA, pRn1-4 (5), maps between the two transcription starts at position −41. Therefore, the corresponding mRNA must have arisen from the upstream minor transcription start point. The major transcription start point of the *Ren1* gene in BALB/c mouse kidney maps also around position −35 (Fig. 4*D*).

Comparison of the 5′-Flanking Regions of *Ren1* and *Ren2*. An extensive homology is observed extending from a point 179 bp upstream from the main transcription start point to at least the first intervening sequence including the first exon of both genes. Dot matrix analysis shows immediately that no significant homology can be detected further upstream with the exception of two G-A-G-A motifs. One of 25 bp, at position −226 of *Ren1* (where −226 denotes the center of the motif), has a single base-pair substitution when compared to its analogue, which is located 72 bp further upstream in *Ren2*. The second, a 20-bp G-A-G-A motif, is located at position −345 of *Ren1* and differs in 3 bp when compared to the corresponding G-A-G-A sequence, which is located at position −307 of *Ren2* (Fig. 5).

Computer analysis using the procedures described by Smith *et al.* (26) confirmed that no significant alignment of nucleotides can be found upstream from position −214 of both sequences. One interesting feature in the 5′-flanking sequence of *Ren2* is noted at position −199, around which the DNA sequence has a region of almost perfect dyad symmetry. This hairpin loop is located at the end of the homologous region and is not found in *Ren1*. Also striking is a 57-bp A+T-rich sequence that occurs within the nonhomologous part of *Ren2* around position −343.

DISCUSSION

The molecular study of the two renin genes seemed especially attractive for three reasons: (*i*) because both are testosterone-regulated in the SMG of mice, (*ii*) the transcription of *Ren2* is enhanced (compared to that of *Ren1*) in the SMG of mice either treated or untreated with testosterone, and (*iii*) *Ren2* is not expressed in the kidney. Here we describe the molecular cloning and the characterization of *Ren1* and *Ren2*. The restriction maps show that, although both genes are homologous in the coding region, differences exist either in the 5′- or 3′-flanking regions. Since current evidence suggests that the transcriptional control elements are located at the 5′ ends of the genes, close to the mRNA initiation site,

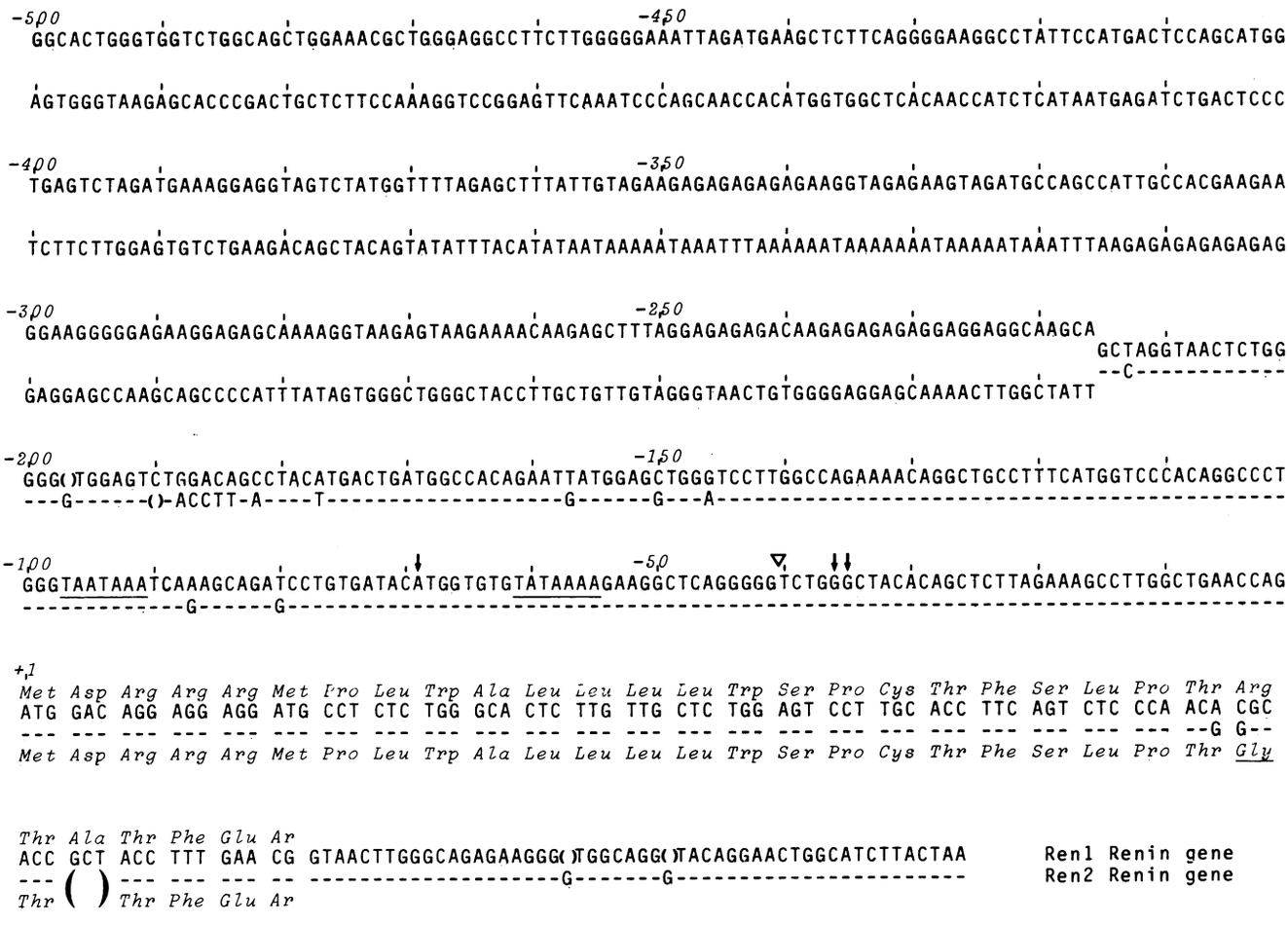

FIG. 3. Comparison of the DNA sequences at the 5′ ends of *Ren1* and *Ren2*. The two sequences are aligned at the ATG start codon (+1 position). In the homologous part of both sequences only nucleotides of *Ren2* that differ are shown; homologous positions are indicated by dashes. Deletions are indicated by parentheses. The deduced amino acid sequences of the first exons are shown above and below the nucleotide sequences of *Ren1* and *Ren2*, respectively. The nonhomologous amino acid is underlined in *Ren2* amino acid sequence. The TATA-box elements are underlined and the 5′ end of cloned mRNA is denoted by an open triangle. The main transcription start points are indicated by arrows.

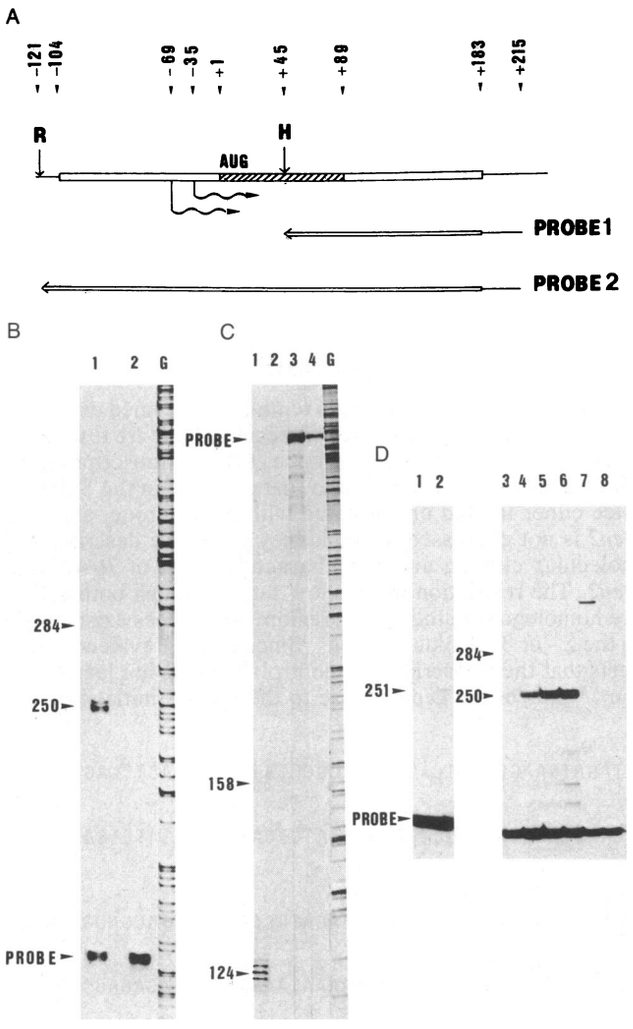

FIG. 4. Mapping of the 5′ end of renin mRNA. (*A*) Strategy for single-stranded DNA probe preparation. The *Hae* III fragments corresponding to nucleotides −104 to +184 (for *Ren1*) or −104 to +183 (for *Ren2*), with respect to the translational start point, were cloned into the *Hin*cII site of M13 mp701. ^{32}P-labeled single-stranded DNA probes complementary either to the 3′-proximal *Hin*fI–*Hae* III fragment of the insert (probe 1) or to the whole insert (probe 2) were synthesized. Sequences corresponding to the insert are boxed and the renin coding region is hatched. The wavy lines indicate the transcription start points. R and H stand for the *Eco*RI and *Hin*fI sites, respectively. Numbering above the scheme refers to *Ren2*. (*B* and *C*) Mapping of the 5′ end of SMG renin mRNA. Probe 1 (2×10^5 cpm) was hybridized either with 1 μg of poly(A)$^+$ RNA from male Swiss mouse SMG (*B*, lane 1) or with 1 μg of tRNA (*B*, lane 2) and elongated with reverse transcriptase. Probe 2 (2×10^5 cpm) was hybridized with 1 μg of poly(A)$^+$ RNA from male Swiss mouse SMG (*C*, lane 1) or with tRNA (*C*, lane 2) and digested with S1 nuclease. The undigested probe 2 is shown in lanes 3 and 4 (10^5 and 10^4 cpm, respectively). G, calibrating sequencing reaction (19). The position and length of the extended or protected probe is given. (*D*) Comparison of the 5′ ends of transcripts from *Ren1* and *Ren2*. Poly(A)$^+$ mRNA from male BALB/c mouse kidney (lane 1, 0.5 μg; lane 2, 3 μg) or poly(A)$^+$ mRNA from male Swiss mouse SMG (lane 3, 0.05 μg; lane 4, 0.1 μg; lane 5, 0.3 μg; lane 6, 1 μg) was hybridized with probe 1 (lanes 1 and 2, 40,000 cpm; lanes 3–6, 25,000 cpm) and the hybrid was elongated with reverse transcriptase. Lane 7, probe 1 (25,000 cpm) hybridized with 3 ng of the complementary M13 DNA, elongated with reverse transcriptase, and cut with *Eco*RI. Lane 8, same as lane 7, except that tRNA (0.5 μg) was used instead of M13 DNA. Other symbols as in *B* and *C*.

we mapped the transcription start point of both renin genes and compared the flanking nucleotide sequences.

The renin genes have two main transcription start points, separated by 36 nucleotides, the 3′-proximal one being the predominant. The functional significance of this dual promoter organization is unclear at present. However, it is rather intriguing to find that another testosterone-regulated gene, the *C3* gene of the rat prostatic steroid-binding protein, has a promoter region very similar to that of the renin, both with respect to the distance between the two promoters and their relative strengths (27).

The sequences of the 5′-flanking regions of the two genes are highly homologous up to a point located at position −179 with respect to the main transcription start point, while they differ beyond this point. Therefore, it seems likely that this nonhomologous region is responsible for the differences in the regulation properties of the two genes, which have been outlined above. This is consistent with a recent report showing that the sequences located 200–300 bp upstream from the transcription start site are necessary for cell-specific expression of insulin and chymotrypsin genes (28). Similarly, the higher rate of transcription of *Ren2* with respect to *Ren1* in the SMG may also be associated with this nonhomologous region. Alternatively, it might be due to an as-yet-undefined tissue-specific enhancer element located elsewhere in the gene (cf. ref. 29).

In contrast, the inducibility of both genes by testosterone implies that they share a common regulatory element, presumably in the homologous region located immediately upstream from the 5′ end of the gene. It is well established that steroid hormones bind a specific class of proteins in target cells to form a steroid–receptor complex that accumulates in the nucleus. This complex would then bind to specific DNA site(s) located near the promoter of inducible genes. Supporting this view, it has been shown that purified glucocorticoid–receptor and progesterone–receptor complexes can bind to *in vitro* defined sequences near the promoters of mouse mammary tumor virus DNA (30, 31) and ovalbumin gene (32, 33), respectively, and that 5′-flanking regions of hormone-sensitive genes are necessary *in vivo* for inducibility (34–40). Hurst and Parker (27) have reported the DNA sequences of the 5′-flanking regions of two nonallelic genes encoding the *C3* subunit of the rat prostatic steroid-binding protein, whose expression is stimulated by testosterone. We have made a computer search for a consensus sequence for the binding site for testosterone receptor by comparing the 5′-flanking sequences of the four testosterone-regulated genes—i.e., the renin genes and the *C3*-encoding genes named *C3(1)* and *C3(2)*. No sequence exhibiting >65% homology over 20 bp or >85% homology over 9 bp can be found at similar locations in all four 5′-flanking sequences. However, the sequence A-G-G-C-C-C-T-G-G-G is found without substitution 63 and 392 nucleotides upstream from the 5′ end of renin and *C3*-encoding genes, respectively. This decanucleotide is absent from the 5′-flanking sequence of estrogen-regulated chicken genes (41, 42). It will be important to test experimentally the functional significance of these potential regulatory sequences.

The *Ren2* copy of renin genes has been located on mouse chromosome 1 near the *Pep-3* locus (43). *Ren1* has not been mapped yet because of the lack of known allelic form of this gene. Thus, it remains to be shown whether the renin genes are closely linked or not. The fact that both genes exhibit high similarity (8) has suggested that they may result from a recent duplication (5). Furthermore, the 3′- and the 5′-flanking sequences of *Ren1* and *Ren2* are nonhomologous (ref. 4; this work). This could be the result of either the duplication of *Ren2* followed by the transposition of a DNA fragment containing regulatory sequences or the transposition of *Ren2* itself in a different gene environment. This complex DNA rearrangement has changed the transcriptional control operating on *Ren2*.

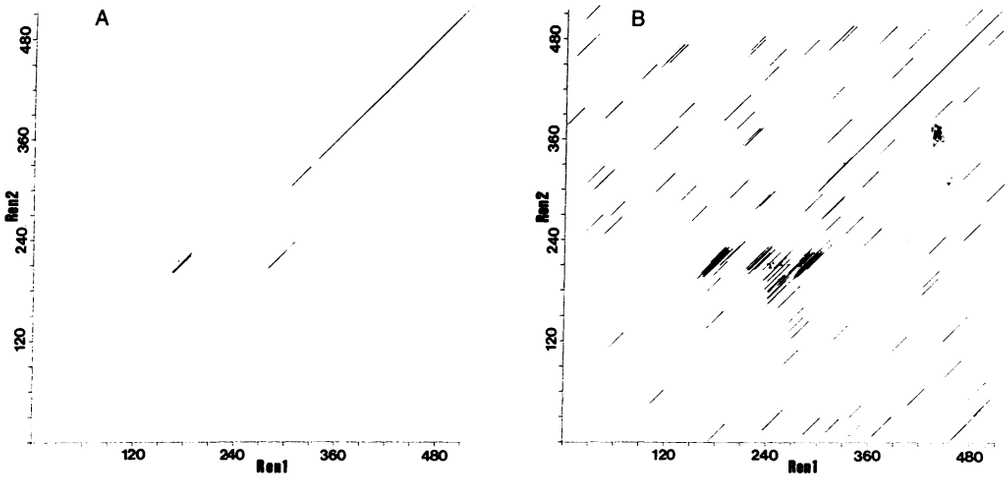

FIG. 5. Dot matrix comparison of the DNA sequences at the 5′ ends of *Ren1* and *Ren2*. The 500 bp preceding the ATG start codon of *Ren1* (abscissa) are compared with the 500 bp preceding the ATG start codon of *Ren2* (ordinate). The program compares each set of 20 bp of the ordinate with each set of 20 bp of the abscissa and places a dash when two sets display 80% (*A*) or 60% (*B*) homology or more.

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