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Regulated expression of the Ren-2 gene in transgenic mice derived from parental strains carrying only the Ren-1 gene

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The Ren-2 gene encoding the mouse submaxillary gland (SMG) renin was microinjected into the pronuclei of fertilized eggs from mice carrying only the Ren-1 gene. In addition to the whole transcription unit, the injected DNA contained 2.5 and 3 kb of upstream and downstream flanking sequences, respectively. Three independent transgenic mice lines were obtained; two of them had integrated one copy of the Ren-2 gene, the last one had integrated five and eleven copies at two independent sites. Independently of the number of Ren-2 copies integrated, the pattern of Ren-2 gene expression in all the transgenic mice was identical to that observed in wild-type animals in which Ren-1 and Ren-2 are closely linked on chromosome 1. In particular, the exogenous Ren-2 gene was only transcribed in the kidney and in the SMG. In the kidney, Ren-1 and Ren-2 mRNAs were present at a comparable level, whereas in the SMG Ren-2 mRNA was at least 100-fold more abundant than Ren-1 mRNA. Moreover, Ren-2 expression in the SMG was positively regulated by androgens. Only one difference between transgenic mice and wild-type mice carrying the Ren-2 gene has been observed: the basal level of Ren-2 transcription in the SMG of transgenic females was lower than in two-gene strain females. Androgen treatment of transgenic females induced SMG renin mRNA to a level identical to that of transgenic males. This suggests that the basal level of SMG renin mRNA is dependent upon *cis*-acting elements which are not present in the microinjected fragment.

Key words: juxtaglomerular cells/renin gene/submaxillary gland/transgenic mice

Introduction

Genes expressed in a particular tissue contain *cis*-acting elements which are required for their tissue-specific transcription. Three classes of such elements have been distinguished. Firstly, upstream promoter elements, which are located 5′ of the TATA box, are the targets for the promoter-specific transcription factors (for review see Breathnach and Chambon, 1981; Dynan and Tjian, 1985). Secondly, cellular enhancers increase transcription rate independently of their orientation and distance from the promoter. Unlike certain viral enhancers which are active in many different cell types, most cellular enhancers so far characterized appear to be tissue specific (Dynan and Tjian, 1985; Voss *et al.*, 1986). Finally, the steroid response elements (SRE), which bind steroid hormone receptors, have enhancer-like characteristics (Yamamoto, 1985). At present, it is unclear whether the upstream promoter elements are fundamentally different from the two classes of enhancers and whether different enhancer elements can act independently from each other on the same promoter in different cell types.

The renin system in the mouse provides a very attractive model to analyze how these three classes of *cis*-acting elements control gene transcription in different cell types. In most wild-type and domestic mice there are two renin genes, named Ren-1 and Ren-2 (Wilson *et al.*, 1978; Piccini *et al.* 1982; Panthier *et al.*, 1982; Mullins *et al.*, 1982; Dickinson *et al.*, 1984). The two genes have originated from a relatively recent duplication event as shown by their high degree of sequence homology (Holm *et al.*, 1984) and their close linkage on the chromosome 1. The Ren-1 gene encodes the plasma renin. This thermostable glycoprotein is synthesized in the juxtaglomerular cells of the kidney and plays a key role in blood pressure homeostasis (Wilson and Taylor, 1982; Ondetti and Cushman, 1982). The level of expression of Ren-1 is nearly the same in the kidney of males and females. The Ren-2 gene encodes the submaxillary gland (SMG) renin which is a salivary secreted thermolabile protease. This unglycosylated form of renin does not participate in the regulation of blood pressure (Bing *et al.*, 1980). Although no protein encoded by the Ren-2 gene is found in the kidney, Ren-2 mRNA is accumualted at nearly the same level as the Ren-1 mRNA in this tissue (Field and Gross, 1985). In contrast, the level of Ren-2 mRNA in the SMG is at least two orders of magnitude higher than that of Ren-1 mRNA. The expression of both Ren-1 and Ren-2 in the SMG is positively controlled by androgens (Wilson *et al.*, 1981). Moreover, these authors have suggested that the level of SMG renin activity is also controlled by a complex synergistic action of gonadal, thyroid, and adrenal hormones. Thus, hypophysectomy decreases basal renin levels in females but full expression can be restored by androgen treatment; conversely, the absence of the androgen receptor does not affect the basal level but prevents induction by testosterone.

The strikingly higher levels of Ren-2 transcription in the SMG, as compared to Ren-1 might reflect differences in the chromatin environment of the two genes in this particular tissue; alternatively, it is possible that differences in primary structure between the two genes modulate their expression in the SMG but not in the kidney. In fact, while these two genes are highly homologous up to 175 nucleotides upstream from the major transcription start point (Panthier *et al.*, 1984), two major differences exist beyond that point: Ren-2 possesses a mouse type-2 Alu equivalent insertion and Ren-1 has two polypurine tracts (Field *et al.*, 1984). Further upstream, the homology is restored for at least 2.5 kb (unpublished results).

Transfection analysis with mutated renin genes cannot be used to identify the putative regulatory sequences responsible for their differential transcription since cell lines derived from renin-synthesizing tissues are not yet available. However, we took advantage of the existence of some inbred strains of mice which possess only the Ren-1 gene (Piccini *et al.*, 1982; Panthier *et al.*, 1982; Mullins *et al.*, 1982; Holm *et al.*, 1984), to produce transgenic mice (Palmiter and Brinster, 1986) carrying one or

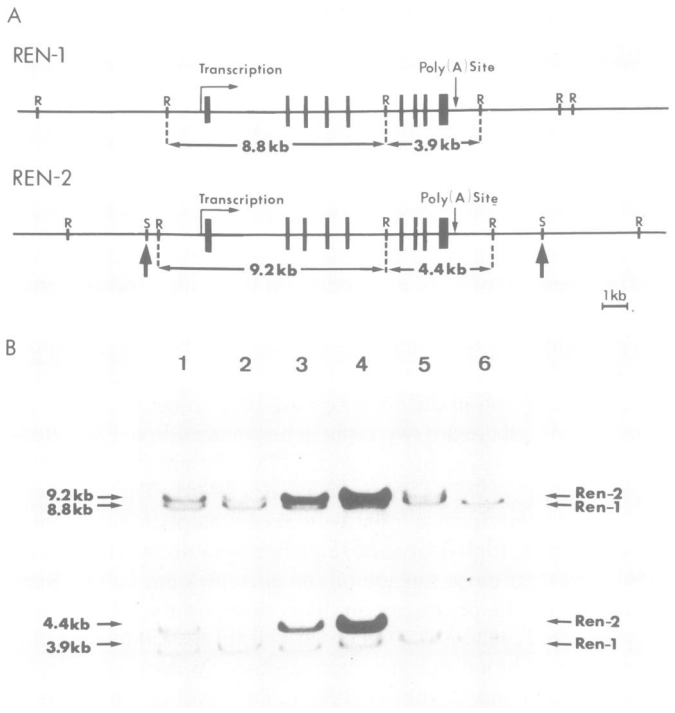

Fig. 1. (**A**) Organization of the mouse renin genes. The figure shows the protein coding segment (dark blocks) and *Eco*RI restriction sites (R). The *Eco*RI fragments of 9.2, 8.8, 4.4 and 3.9 kb revealed by the renin cDNA probe are also shown. The *Sau*3A (S) fragment inserted in λ phage is indicated by dark arrows. (**B**) Southern blot analysis of transgenic mice. Ten micrograms of tail DNA, digested with *Eco*RI, were subjected to electrophoresis on a 0.7% agarose gel and transferred to Zetabind nylon membranes. The filter was hybridized to the cDNA renin probe, which consists of the *PstI*–*PstI* fragment of plasmid pRn1-4 (Panthier *et al.*, 1982). As a control, DNA of one- and two-gene mice was digested, blotted and hybridized under the same conditions. **Lane 1**: Swiss mice (two-renin gene strain); **lane 2**: Rn2-1 (one-renin gene insertion); **lane 3**: Rn2-6(a) (five renin genes inserted in one chromosomal site); **lane 4**: Rn2-6(b) (16 renin genes inserted at two different chromosomal sites); **lane 5**: Rn2-9 (one renin gene insertion); **lane 6**: Balb/c mice (one-renin gene strain).

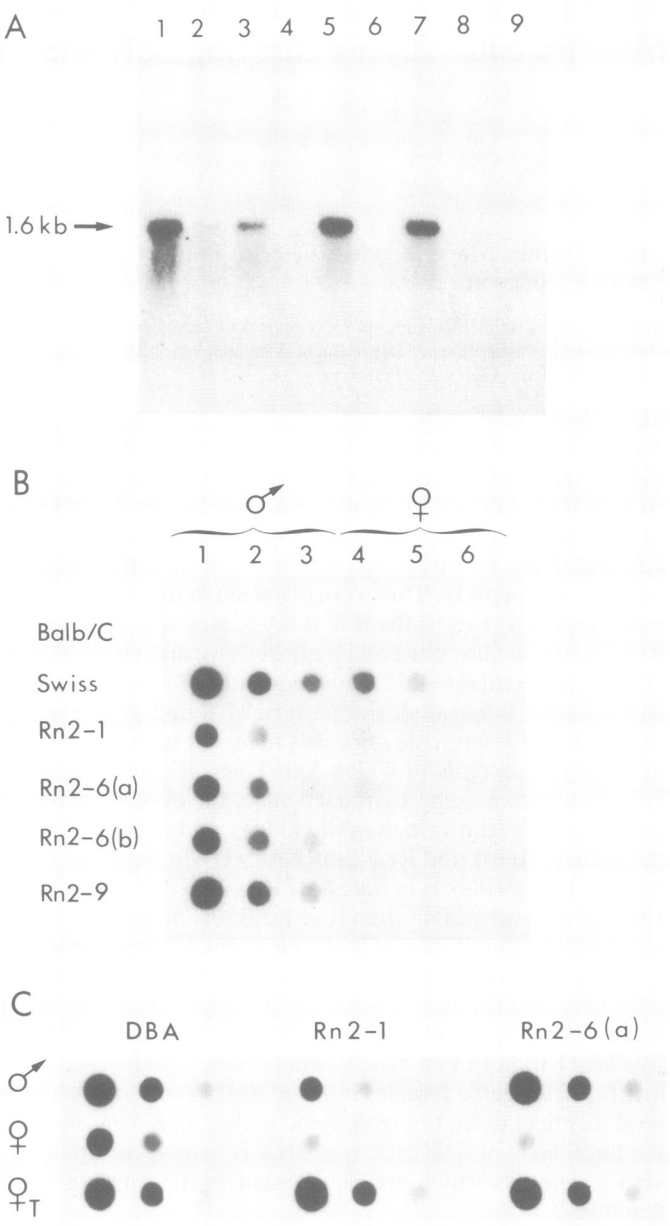

Fig. 2. Analysis of SMG renin mRNA of male and female transgenic mice. (**A**) Northern blot of total SMG RNA (1 μg). **Lane 1**, Swiss male mice; **lane 2**, Swiss female mice; **lane 3**, male Rn2-1; **lane 4**, female Rn2-1; **lane 5**, male Rn2-6(b); **lane 6**, female Rn2-6(b); **lane 7**, male Rn2-6(a); **lane 8**, female Rn2-6(a); **lane 9**, male Balb/c. (**B**) Dot-blot analysis of SMG RNA of male and female transgenic mice compared to Swiss mice (two-renin gene strain) and Balb/c (one-renin gene strain). The amounts of RNA applied to the filter were: lanes 1 and 4, 0.5 μg; lanes 2 and 5, 0.125 μg; lanes 3 and 6, 0.031 μg. (**C**) Dot-blot analysis of androgen-treated transgenic females. Total SMG RNA of males (♂), females (♀), and testosterone-treated females ($♀_T$) were spotted into nitrocellulose and hybridized to a renin probe. DBA/2 mice (two-renin gene strain) were used as control. The amount of RNA spotted was 0.5 μg and subsequent serial dilutions 1:4.

several Ren-2 gene copies. Here we show that the Ren-2 gene is expressed in the SMG and kidney in an identical manner in all transgenic mice and in wild-type two-gene mice strains.

Results

Characterization of transgenic mice carrying the Ren-2 gene

Approximately 500 copies of a 16-kb *Sal*I DNA fragment containing the renin gene with its 5′ and 3′ flanking region (2.5 and 3 kb respectively) (Figure 1) were microinjected into (C57BL/6 × CBA) F_2 or (C57BL/6 × Balb/C) F_1 eggs as described by Brinster *et al.* (1981). These parental strains have only one renin gene (Ren-1) per haploid genome. Altogether 144 eggs were microinjected, 94 (65%) of which survived and were implanted into seven foster mothers. Five became pregnant and gave birth to 10 mice.

The integration of the Ren-2 gene in those mice was analyzed by Southern blot hybridization since animals having one or two renin genes can be distinguished on the basis of the *Eco*RI hybridization pattern (Panthier *et al.*, 1982). Thus, DNA of one-gene mice strains gives rise to two bands of 8.8 and 3.9 kb (Ren-1) whereas DNA of two-gene strains gives two additional hybridization bands of 9.2 and 4.4 kb corresponding to the Ren-2 copy (Figure 1A). Three out of 10 mice tested had the pattern expected for Ren-2 transgenic mice. The results presented in Figure 1B show the *Eco*RI hybridization pattern of the two transgenic males, Rn2-1 (lane 2) and Rn2-6 (lane 4), and of the trans-

genic female Rn2-9 (lane 5). The hybridization patterns of one-gene or two-gene mice strains are included for comparison (lane 6 and lane 1 respectively). The number of Ren-2 copies integrated in each mice was determined by comparing the intensities of the bands due to the Ren-2 gene with that of the endogenous Ren-1 gene. Thus, Rn2-1 and Rn2-9 mice have integrated a single copy and Rn2-6 16 copies per diploid genome.

Upon breeding with C57BL/6 animals, Rn2-1 and Rn2-9 transmitted the Ren-2 gene to ~50% of their offspring (11 out of 18 in the case of Rn2-1 and 14 out of 25 in the case of Rn2-9). In contrast, when Rn2-6 progeny was analyzed, 27 out of 33 offspring were found to have the Ren-2 gene integrated into their DNA. This high transmission rate (82%) suggests the presence in F_0 Rn2-6 of more than one site of integration. This was confirmed by the appearance of a heterogeneous F_1 progeny: some mice carry about five copies of the transgene [hereafter referred to as Rn2-6 (a)], and some carry a larger number of copies. Since we cannot distinguish easily between F_1 mice bearing the two insertions (i.e. 16 copies) and those having integrated 11 copies in one chromosomal site, they will both be referred to as Rn2-6 (b) in the text (Figure 1B).

Ren-2 is transcribed in the submaxillary gland and in the kidney of transgenic animals

Total RNA was extracted from the submaxillary gland of individual F_1 males from the independent transgenic lines, and analyzed by dot-blot hybridization. Since the amount of renin mRNA found in the SMG of two-gene strains (like Swiss mice) is two orders of magnitude higher than in one-gene strains (like Balb/c), we used RNA amounts that allow discrimination between the two categories of mice. The results of dot-blot experiments showed that these transgenic mice express Ren-2 in the SMG, at a level nearly identical to that of the two-gene Swiss male used as a control, irrespective of the number of Ren-2 copies integrated (Figure 2B). Northern blot analysis also demonstrated that the size of this Ren-2 mRNA is identical to that of Ren-2 mRNA from the SMG of Swiss males (Figure 2A) (Rougeon *et al.*, 1981). Moreover, *in vitro* translation of SMG mRNA in a rabbit reticulocyte lysate showed that the Ren-2 mRNA from the SMG of transgenic mice is functional: a polyclonal antibody raised against SMG renin recognized a 45 000-dalton polypeptide present in translation products of transgenic or two-gene males, but not of one-gene mice (results not shown).

Next, we used the approach developed by Field and Gross (1985) to assess whether the exogenous Ren-2 gene is transcribed in the kidney of transgenic mice. These authors took advantage of one of the differences between the Ren-1 and Ren-2 mRNA sequences to quantify the two transcripts by a primer extension technique. As see in Figure 3, female mice from Rn2-1 and Rn2-6 lines expressed the exogenous Ren-2 gene in the kidney. In both lines, the level of expression of Ren-2 was somewhat smaller (2- to 3-fold) than in *bona fide* two-gene mice. In addition, these experiments confirmed that the high level of renin expression in the SMG of transgenic mice was due to the exogeneous Ren-2 gene and not to some deregulation of Ren-1 expression following transgenesis (Figure 3).

No Ren-2 expression was detected in the liver of transgenic mice using this assay (Figure 3). In addition dot-blot analysis failed to reveal renin mRNA in other tissues tested: heart, muscle, brain, lung and spleen (results not shown). Identical experiments made with F_2 progeny of the different transgenic lines showed no significant differences between individuals of the same family. Moreover, the Ren-2 gene was transmitted and expressed faithfully in successive generations.

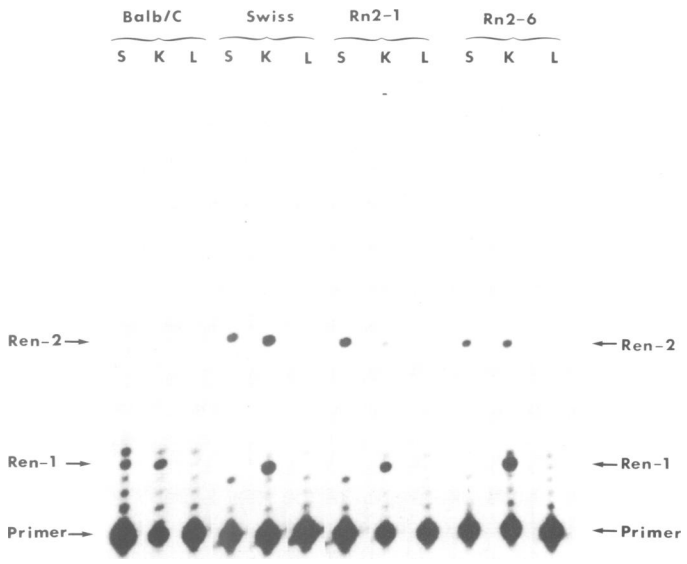

Fig. 3. Primer extension analysis of renin transcripts according to Field and Gross (1985). Briefly, a 38-mer oligonucleotide complementary to nucleotides 1039–1075 of the renin cDNA was end-labelled with [^{32}P]ATP and hybridized with total RNA from kidney (K), SMG (S) or liver (L) of Balb/c (one-gene strain), Swiss (two-gene strain) and transgenic (Rn2-1 and Rn2-6) mice. This probe corresponds to a region immediately adjacent to a sequence divergence between the two genes, with a G being found five nucleotides upsteram from the probe in Ren-1 mRNA, while in Ren-2 mRNA the first G is encountered 17 nucleotides upstream from the probe. Therefore, if the hybrids are elongated with reverse transcriptase in the presence of dATP, dTTP, dGTP and ddCTP, the hybrid from Ren-1 mRNA will lead to a transcript shorter than the one from Ren2 mRNA. In each case, 10 000 c.p.m. of the probe were hybridized with 80 μg of total RNA, except for the SMG RNA from Swiss or transgenic mice where 0.8 μg were used. All samples were taken from male mice, except for the kidney RNAs from transgenic lines Rn2-1 and Rn2-6, which were prepared from females.

Androgen treatment induces Ren-2 mRNA accumulation in the SMG of transgenic females

The SMG renin activity in two-gene strains has been shown to be about five times lower in females than in males. Androgen treatment of females between 6 and 10 weeks of age induces SMG renin activity to the same level as that of males (Wilson *et al.*, 1981).

To examine whether Ren-2 gene transcription was also regulated by androgens in transgenic mice, Ren-2 mRNA levels in the SMG of 8-week-old male and female mice were compared. The results of Northern blot experiments (Figure 2A) showed an evident sex-related difference between individual F_1 males and females of the same age and related genetic background. Quantification of the mRNA by dot-blot hybridization revealed that the basal level of renin mRNA in the SMG of transgenic females was 6- to 8-fold lower than in two-gene females (Swiss or DBA/2) whereas this level was nearly the same in transgenic males and Swiss males (Figure 2B). Testosterone treatment of transgenic females increased SMG renin mRNA to a level equivalent to that of transgenic males (Figure 2C). Thus, in transgenic mice, renin mRNA levels increased 20- to 25-fold by testosterone, versus ~5-fold in normal two-gene strains.

Discussion

Because of the lack of cell lines in which the mouse renin genes can be expressed, we have undertaken experiments based on transgenic mice methodology to characterize DNA sequences

responsible for tissue-specific expression and hormonal control of the renin genes. We show that the Ren-2 gene, when introduced into the germ line of mice carrying only the Ren-1 gene, has the same pattern of expression as in mice in which the two renin genes are closely linked on chromosome 1. In particular, we have demonstrated the following: (i) the exogenous Ren-2 gene is only transcribed in the SMG and in the kidney of transgenic mice; (ii) in the kidney, the level of Ren-2 mRNA is comparable to that of Ren-1 mRNA; (iii) in the SMG, the expression of Ren-2 is positively controlled by androgens; the level of Ren-2 mRNA in transgenic males is comparable to that of two-gene male mice and is two orders of magnitude greater than that of Ren-1 in the SMG of one-gene male mice. Therefore, all the elements required for the tissue-specific expression of the Ren-2 gene, for its higher expression in the SMG as compared to Ren-1, and for its androgen responsiveness, are included within the 16-kb fragment which has been injected. Our findings clearly demonstrate that the chromosomal localization of the Ren-2 gene does not play any important role in these properties.

Nevertheless, there are certain interesting differences between the Ren-2 mRNA levels in transgenic animals when compared to the reference strains. While the amount of SMG renin mRNA in transgenic males is similar to that of Swiss males, transgenic females have a very low level of renin mRNA when compared to Swiss females (6–8 times smaller). Since it has been suggested that the basal level of SMG renin is controlled by a complex synergistic action of gonadal, thyroid and adrenal hormones, but not by androgens (Wilson *et al.*, 1981), it is possible that target sequences for one of these hormones are not present in the injected fragment. However, we cannot exclude the possibility that the low basal mRNA level is in part due to the particular strains used for transgenesis, since basal SMG renin activity shows great variations between inbred strains (Wilson *et al.*, 1981). Experiments are in progress to settle this point.

No correlation was found between the number of Ren-2 copies integrated and the level of transcription. The amount of Ren-2 transcripts in the SMG of all transgenic males is comparable to that of two-gene animals. Moreover, we found more Ren-2 mRNA in the kidney of a transgenic mouse carrying one Ren-2 copy than in one which received 16 copies (Figure 3). The absence of gene dosage effects in transgenic mice has often been observed (Palmiter *et al.*, 1982; McKnight *et al.*, 1983; Overbeek *et al.*, 1986; Morello *et al.*, 1986) and may be due to limiting amounts of *trans*-acting factors. The small variations which exist between transgenic mice may reflect the chromosomal localization of the transgene: other authors have reported that the site of integration can modulate the transcription level, even though the general pattern of expression is not modified (Palmiter *et al.*, 1982; Stewart *et al.*, 1982; Krumlauf *et al.*, 1985).

It now becomes possible to attempt to localize the various regulatory elements responsible for the hormonal regulation and basal expression of renin genes. Although there are numerous examples showing that the *cis*-regulatory elements are located in a short upstream region not exceeding 200 or 300 bp, some exceptions have been found such as the immunoglobulin (Gillies *et al.*, 1983; Banerji *et al.*, 1983) and lysozyme genes (Theisen *et al.*, 1986). It would be particularly interesting to determine whether the 175-bp region of homology between Ren-1 and Ren-2 is sufficient to control the tissue-specific expression and androgen regulation. If this is the case, the region which is not homologous in the two genes would act only as a modulator in the submaxillary gland. Experiments to answer these questions using shortened renin promoters associated with an indicator gene are in progress.

Materials and methods

Preparation of the Ren-2 gene for microinjection

The isolation of recombinant λ phages containing renin genomic sequences has been reported previously (Panthier *et al.*, 1984). The phage SW10, which carries the whole Ren-2 transcription unit and ~3 kb of both upstream and downstream flanking sequences, was selected for the present study. Pure SW10 DNA was restricted with *Sal*I which cuts it at the extremity of the insert, and after purification, the mixture was resuspended in 10 mM Tris, 0.25 mM EDTA, pH 7.5. In a typical experiment, 2 pl of this solution, corresponding to ~500 copies of the Ren-2 gene, were injected per fertilized egg.

Production of transgenic mice

Fertilized eggs were obtained from superovulated 3- to 4-week-old virgin females separately mated with males the day before. All these mice were of the Ren-1 genotype. Two types of crosses were used: (i) (C57BL/6 × CBA) F_1 females mated to males of the same genotype; (ii) (C57BL/6) females mated to Balb/c males. Pronuclei were microinjected with ~500 copies of the Ren-2 gene essentially as described (Brinster *et al.*, 1981) except that cytochalasin B was omitted from the medium. Eggs that survived microinjection were implanted into the oviducts of pseudopregnant (C57BL/6 × CBA) F_1 foster females on the first day of pseudopregnancy.

DNA analysis

DNA was prepared from biopsy samples from tails of 3- to 4-week old mice. The presence of the Ren-2 gene was checked by Southern blot analysis as previously described (Panthier *et al.*, 1982). Briefly, DNA of each animal was digested with the restriction enzyme *Eco*RI. The digests were separated by electrophoresis on 0.7% agarose gels and transferred to Zetabind nylon membranes. The renin probe used was obtained after *Pst*I digestion of the pRn1-4 plasmid (Panthier *et al.*, 1982). The probe was labeled by the multi-prime DNA labelling system method (Feinberg and Vogelstein, 1983) to a specific activity of 10^9 c.p.m./μg. The filters were hybridized to the probe and washed under stringent conditions.

RNA analysis

RNA was prepared from mouse tissues as previously described (Auffray and Rougeon, 1980). The only major modification was the utilization of 4 M guanidine thiocyanate instead of 6 M urea. For Northern blot experiments, RNA was electrophoresed through a 1.1% agarose/formaldehyde gel, transferred to nitrocellulose and hybridized as previously described (Thomas, 1980). The probe used is the same as for Southern blot analysis.

For RNA dot-blot experiments, RNA extracted from different tissues was spotted for dot-blot essentially as described (White and Bancroft, 1982) and hybridized under the same conditions as for Northern blot experiments.

Primer extension

The primer extension method described by Field and Gross (1985) to discriminate between Ren-1 and Ren-2 transcripts was used without modification except that the elongated products were separated on a 15% sequencing gel.

Hormone treatment

Hormone induction was carried out by injection by 12.5 mg of testosterone (Sterandryl Roussel) to transgenic and DBA/2 females. Animals were killed 1 week after hormone injection by severing the spinal cord.

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