Rat renin gene transcription is initiated at a single start site

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Abstract The promoter region of the renin gene in man, mouse and rat contains several putative transcription start sites, which in mouse have been shown to be tissue specific and differently regulated. To investigate which of these start sites are used during stimulation of renin gene transcription by the major physiological control factors, we determined the trancription start sites of rat renin in the kidney and adrenal glands by RNase protection using a cRNA probe spanning 387 bases upstream and 121 bases downstream from the canonical transcription initiation site. To stimulate renin gene expression, we used renal artery stenosis, angiotensin II antagonists, furosemide and isoprenaline infusions and low sodium diet. Our results suggest that only one TATA box is functional in rat kidney and adrenal.

Key words: Renin promoter; TATA box; mRNA level

1. Introduction

The systemic renin angiotensin system (RAS) plays an important role in the control of blood pressure and of the extracellular volume. The activity of the RAS is primarily determined by the rate of renin secretion from the kidneys, where renin is synthetized, stored and secreted by the juxtaglomerular epitheloid (JGE) cells. A variety of physiological relevant factors control concordantly renin secretion and renin gene expression in these cells. These factors comprise the intrarenal perfusion pressure [1–3], a signal generated by the adjacent tubular macula densa cells [4–6], the activity of renal nerves and circulating catecholamines [7,8], angiotensin II [9] and salt intake [10–16].

With the exception of the renal nerve activity, considered to stimulate renin gene expression by increasing cAMP levels in the juxtaglomerular cells [17], the intracellular pathways mediating the effects of these rather heterogeneous factors on renin gene expression in the JGE cells are largely unknown. Findings obtained in vitro suggest that renin gene expression in JG cells is stimulated by cAMP and nitric oxide and inhibited by increased intracellular calcium concentration [18–19]. Sequence analysis of the 5′-flanking region of the renin genes from human, mouse and rat revealed the presence of several putative promoter regions, so-called TATA boxes [17,20–23]. The functionality of these promoters, termed P_{1a}, P_1 and P_2 from 5′ to 3′ and present in a 150-bp segment preceding exon 1, could in part explain the existence of multiple forms of renin in different tissues [17,24,25]. Whether factors influencing renin gene expression trigger transcription by the use of different start sites is at present not understood. In mouse, the preferential use of a particular transcription start site appears to be tissue specific and in part influenced by the administration of cAMP [20–22]. To detect a possible common denominator by which the above-mentioned factors could influence renin gene expression, we sought to obtain more information about the possible role of these additional start sites in the transcription of the renin gene, in particular in states of stimulated renin gene expression.

To localize the transcription start site(s), we constructed a cRNA probe covering 387 bases of the promoter and 121 bases 3′ from the putative transcription start site. With this cRNA, we performed RNase protection assay with total RNA isolated from the kidneys of the animals subjected to different treatment regimen. Stimulation of renin gene expression was achieved by renal artery stenosis, by inhibition of the macula densa function by the loop diuretic furosemide, by inhibition of angiotensin II AT_1 receptors by losartan, by the β-adrenergic agonist isoprenaline, and by low sodium diet.

To test for a role of the different putative start sites in the organotypic expression of the renin gene, we also used total RNA from adrenal glands, because these organs are also considered as relevant sites of renin expression.

Our results show that under all the conditions examined, transcription of the renin gene in kidneys and adrenal glands started from the same site, which was allocated to the canonical TATA box located 27 bp upstream from the transcription start site determined by Fukamizu et al. [23].

2. Materials and methods

2.1. Animal experiments

Male Sprague–Dawley rats (180–220 g) were used for the experiments: (1) Control rats. (2) Rats with losartan feeding; losartan (40 mg/kg) was administered by gavage in the morning of each experimental day for 2 days. (3) Rats with unilateral renal artery clip; rats were anesthetized with methohexital (75 mg/kg i.p.) and the left kidney was exposed by an abdominal incision. Sterile silver clips (Degussa, Frankfurt, Germany) with an i.d. of 0.2 mm were then placed on the left renal arteries. (4) Rats with furosemide infusion; osmotic minipumps (2ML1; Alzet, Palo Alto, CA) filled with 100 mg furosemide in 2 ml saline were implanted s.c. under the skin of the neck. The daily dosage of furosemide was 12 mg/animal. To avoid a negative salt and water balance, the animals had free access to an additional drinking bottle filled with 0.8% NaCl/0.1% KCl. (5) Rats with isoprenaline infusion; 40 μg/kg isoprenaline were supplied for 36 h with an osmotic minipump. (6) Rats with low sodium diet; rats were fed with a low salt diet (Na^+ 0.14 mg/g, Cl^- 0.18 mg/g) for 10 days. The salt diet and distilled water were available ad libitum.

2.2. Isolation of RNA

Total RNA was extracted from the kidneys according to the protocol of Chomczynski and Sacchi [26]. Kidneys were homogenized in 10 ml of solution D (guanidinium-thiocyanate (4 M) containing 0.5% *N*-lauryl-sarcosinate, 10 mM EDTA, 25 mM sodium citrate and 700 mM β-mercaptoethanol) with a polytron homogenizer. Then, 1 ml of 2 M sodium acetate, pH 4, and 10 ml of phenol/chloroform (1 : 1) were added to the homogenate. Samples were mixed after addition of each reagent. After cooling on ice for 15 min, the samples were centrifuged at 10,000 × *g* for 15 min at 4°C. RNA was precipitated by adding an equal

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volume of isopropanol at −20°C for at least 1 h. After centrifugation, RNA pellets were suspended in 0.5 ml of solution D and again precipitated with an equal volume of isopropanol at −20°C. RNA pellets were finally dissolved in diethypyrocarbonate-treated water and stored at −80°C for further processing.

2.3. RNase protection for rat preprorenin

A preprorenin cRNA probe containing 296 bases from exons I to III (Fig. 1), generated from a pSP64 vector carrying a *Pst*I-*Kpn*I restriction fragment of a rat preprorenin cDNA was generated by transcription with SP6 RNA polymerase as described [8]. Transcripts were labelled with [α^{32}P]GTP (410 Ci/mmol).

cRNA probes (5×10^5 cpm) were hybridized at 60 °C overnight with 20 μg of total kidney RNA, respectively, with 100 μg total RNA from adrenals. RNA was dissolved in 50 μl buffer containing 80% formamide, 40 nmol/l 1,4-piperazine diethane sulphonic acid (PIPES), 400 mmol/l NaCl and 1 mmol/l EDTA, pH 8. RNase A and T1 digestion was carried out at 20°C for 30 min and terminated by incubation with proteinase K (0.1 mg/ml) and sodium dodecylsulphate (0.4%) at 37°C for 30 min. Protected mRNA fragments were purified by phenol-chloroform extraction, ethanol purification and subsequent electrophoresis on a denaturing 10% polyacrylamide gel. After autoradiography of the dried gel at −70°C, bands representing the protected renin mRNA fragment(s) were excised from the gel and radioactivity was counted with a liquid scintillation counter. The number of counts/min obtained from each sample of total kidney RNA was expressed relative to an external renin mRNA standard included in each hybridization, consisting of 20 μg pooled RNA extracted from the 12 kidneys of six normal Sprague–Dawley rats.

2.4. Determination of renin transcription start site(s)

The existence of multiple transcription start sites for rat renin was investigated by RNase protection assay using a 508-base-long cRNA probe covering 387 bases of the promoter and 121 bases from the putative transcription start site (Fig. 1). The plasmid for the generation of the cRNA probe was constructed by PCR-cloning of a fragment of the renin gene amplified by the primers Rat ren 1 (5′-CGG GAT CCA GAG CAA CAA GAG TGC-3′) and Rat ren 14 (5′-GGA ATT CCA AAG CTG GCT GTG TC-3′). Rat ren 1 and Rat ren 14 hybridize with nucleotides 832 to 850, respectively, 1323–1340 of the rat renin DNA sequence [23]. To facilitate cloning, the 5′-end of the primers contained *Bam*HI and *Eco*RI restriction sites, respectively. After amplification of the renin fragment from genomic DNA from the rat liver and *Bam*HI/*Eco*RI, the fragment was cloned into the respective polylinker site of a pGEM 4Z vector wich was then transformed into *E. coli* DH5a. Sequencing confirmed the identity of the insert with the renin DNA. For the generation of cRNA, the plasmid was linearized with *Hin*dIII. ^{32}P-labelled antisense probes were obtained by in vitro transcription with SP6 polymerase, according to the protocol of Promega Riboprobe System. Transcripts were purified on a Sephadex G50 spun column. RNase protection assay was performed as described for preprorenin.

2.5. Determination of actin mRNA

The abundance of rat cytoplasmatic β-actin in total RNA isolated from the kidneys was determined by RNase protection assay exactly as described for renin. An actin cRNA probe containing the 76 nucleotide first exon and ~200 bases of surrounding sequence was generated by transcription with SP6 polymerase from a pAM19 vector carrying a *Aval*/*Hin*dIII restriction fragment of actin cDNA. For one assay, 2.5 μg RNA were hybridized under the conditions described for renin mRNA.

2.6. Determination of plasma renin activity

Plasma renin activity was determined using a commercial available RIA kit for angiotensin I (Sorin Biomedica, Düsseldorf, Germany).

3. Results

The existence of different renin transcripts and their eventual regulation was investigated in rats with stimulated renin gene expression. This was achieved by inducing renal hypoperfusion with a 0.2-mm renal artery clip, by inhibiting macula densa transport function with the loop diuretic furosemide, by inhibiting angiotensin II AT_1 receptors with losartan, by β-adrenergic stimulation with isoprenaline and, finally, by keeping the animals at low sodium diet for 10 days.

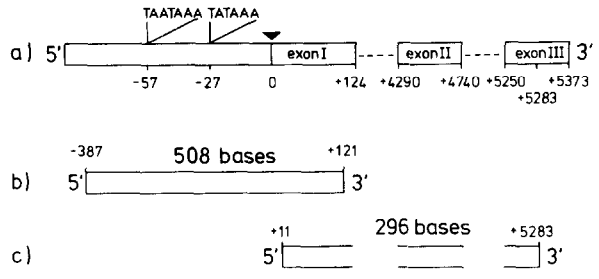

Fig. 1. Schematic presentation of the promoter region of the rat renin gene and the construction of the cRNA probes. (a) Structure of the rat renin gene (Fukamizu et al. [23]) from exons I to III, with location of TATA boxes and transcription start site (▽). (b) cRNA probe used in RNase protection assays for determination of transcription start sites, covering part of the renin promoter comprising the two TATA box and part of the first exon. (c) cRNA probe used for renin mRNA measurements covering part of exons I to III.

As shown in Fig. 2 (upper panel), all these maneuvers increased plasma renin activity.

Renin mRNA levels in the kidneys of the animals were first determined by RNase protection using a cRNA probe protecting a portion of the renin gene comprised between exons I and III (Fig. 1c). The hybridization signals obtained in the assays were set in proportion to the signal obtained with an external standard run in each experiment and generated by pooling total RNA from the kidneys of 6 untreated rats.

As shown in Fig. 2 (lower panel), renin mRNA levels increased at all the conditions investigated, with some differences in the magnitude of the stimulation.

Hybridization of 2.5 μg of the RNA samples with a cRNA probe for β-actin showed no significant variations among the different experimental conditions (data not shown).

To localize the transcription start site(s) of the renin gene in the animals, total renal RNA was also hybridized with a cRNA probe covering 121 bases of the first exon and 387 bases 5′ from the putative transcription initiation site (Fig. 1b). Fig. 3 shows a representative autoradiograph of such an assay with total renal RNA pooled from the animals of the different treatment groups. It is evident that this hybridization always yielded a single protected fragment with a size of 121 bp. The intensities of the bands corresponded very well with those obtained with the probe hybridizing with exons I to III.

Fig. 4 shows a representative autoradiograph of an RNase protection assay with the cRNA probe covering the promoter region using total RNA from the adrenal glands of animals treated with isoprenaline infusions or with low sodium diet. Adrenal RNA was pooled from 4 rats for each experimental condition. Both isoprenaline infusion and low sodium diet increased adrenal renin mRNA levels. As for kidney RNA, only a single protected fragment with a size of 121 bp was found in these organs.

4. Discussion

Renin gene expression is physiologically controlled by a group of well-characterized but strikingly different factors,

such as the renal perfusion pressure [1–3], the function of the macula densa [4–6], renal nerve activity [7–8], catecholamines [17], angiotensin II [9] and the rate of salt intake [10–14]. With the exception of the renal nerve activity, considered to regulate renin gene expression by altering cAMP levels in the juxtaglomerular cells, it is not known how these very heterogeneous factors exert their effect at the cellular level, where renin gene expression has been reported to be stimulated by cAMP and nitric oxide levels and inhibited by increased intracellular calcium concentration [18,19]. Multiple transcription start sites exist for 5–10% of eukaryotic mRNAs [27]: since human, rat and mouse renin genes contain several potential transcription start sites [20–23], it would be conceivable that the different factors use different promoters and different start sites for renin gene transcription. Indeed, beside the predicted initiator termed P_2, which is active in the unstimulated state and in the kidney and in every mouse organ expressing renin mRNA, additional transcription start sites were detected in adrenal and testis (termed P_{1a}) and submaxillary glands (termed P_{1a} and P_1). 8-Bromoadenosine 3′,5′-cyclic monophosphate (cAMP) led to selective stimulation of P_{1a} in adrenal [21]. This particular transcription start site (P_{1a}), however, is located within a 476-bp insertion of the mouse renin promoter which is absent in man and rat [20]. We, therefore, attempted to localize the transcription start sites in conditions in which renin secretion and renin gene expression were stimulated. According to previous results, we found that renal artery clipping [1–3], inhibition of macula densa function [4–6] or angiotensin AT_1 receptors [28], activation of β-adrenergic receptors [7,8] or low sodium diet [10–14] caused a substantial activation of renin secretion and renin gene expression in the rat. The existence of different renin transcription start sites was analysed by RNase protection assay with a cRNA probe covering 121 bases of exon 1 and 387 bases of the renin promoter from the putative transcription start site described by Fukamizu et al. [23]. Within this region, additional TATA boxes are located in mouse, man and rat [17,20,23]. Only a single protected fragment of the apparent size of 121 bp was detected, suggesting that at all the conditions relevant for the physiological control of renin gene expression a single transcription start site is utilized in the kidney and that it corresponds to the one determined in primer extension experiments by Fukamizu et al. for rat renin [23]. Our results confirm previous findings, suggesting that only a single renin promoter is functional in normal mouse kidneys [21,29]. Since the use of multiple renin transcription start sites has been reported for various extrarenal tissues of one-gene and two-gene mouse strains [17,21,22], we investigated the possible use of additional promoters in rat adrenal, where the existence of multiple form of renin with an extended NH_2-terminus has been reported [24,25]. As observed with kidney RNA, in adrenal the same single transcription start site was used at all the conditions investigated. We could, therefore, not confirm the results obtained by others, showing the existence of three different renin transcripts in mouse adrenal, one (tsp_{1b}) being selectively stimulated by cAMP [21]. Apart from species differences, we have no satisfying explanation for these different findings. For these

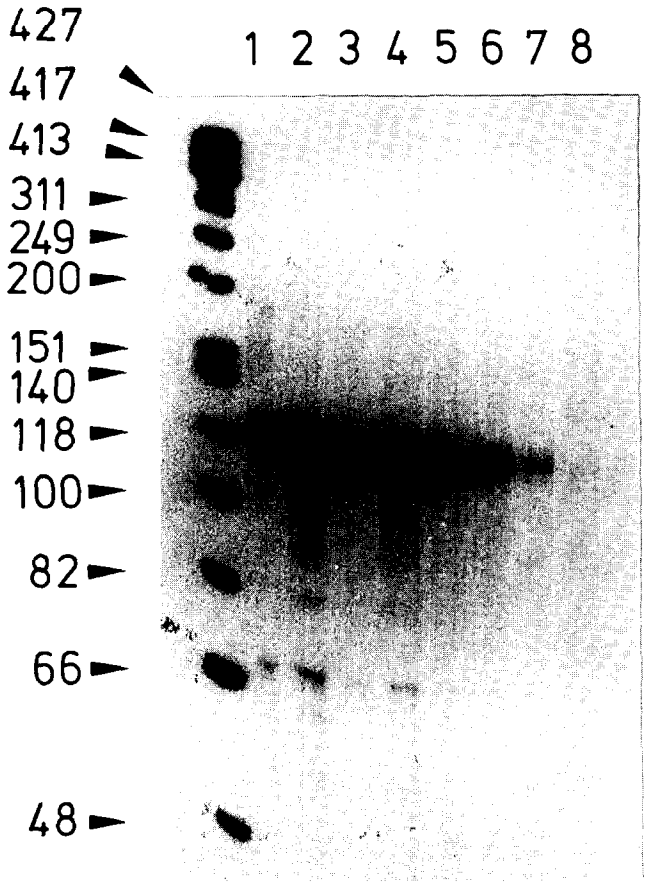

Fig. 3. Autoradiograph of an RNase protection assay for 20 μg of kidney total RNA using the cRNA probe covering the renin promoter/exon I region (Fig. 1b). A single protected fragment is visible in animals treated with furosemide (lane 1), clipped (lane 2) and contralateral (lane 3) kidney, losartan (lane 4), low sodium diet (lane 5), isoprenaline (lane 6) and control (lane 7). Lane 8 corresponds to 20 μg of tRNA. Left are Φ174 DNA/*Hin*fI markers.

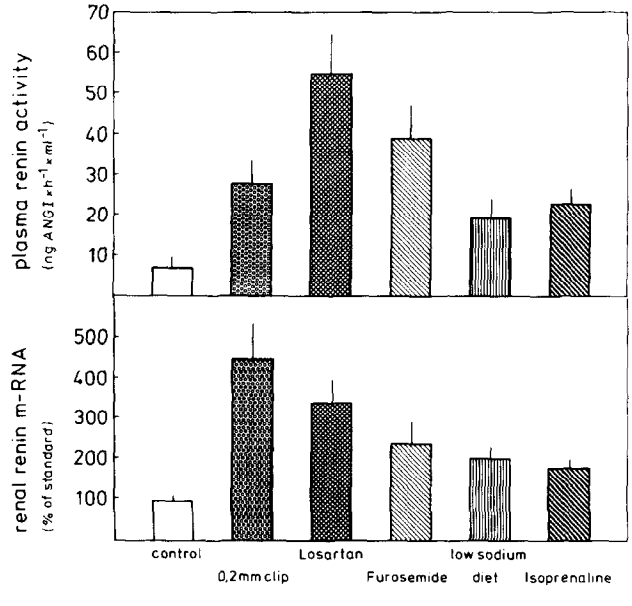

Fig. 2. Plasma renin activity (upper panel) and renin mRNA levels (lower panel) in the kidneys of rats treated with renal artery clips, losartan, furosemide, low sodium diet or isoprenaline. Renin mRNA levels are expressed as percentage of an external standard that was generated by pooling renal total RNA from 6 untreated rats. Data are mean ± S.E.M. of 5 animals in each group.

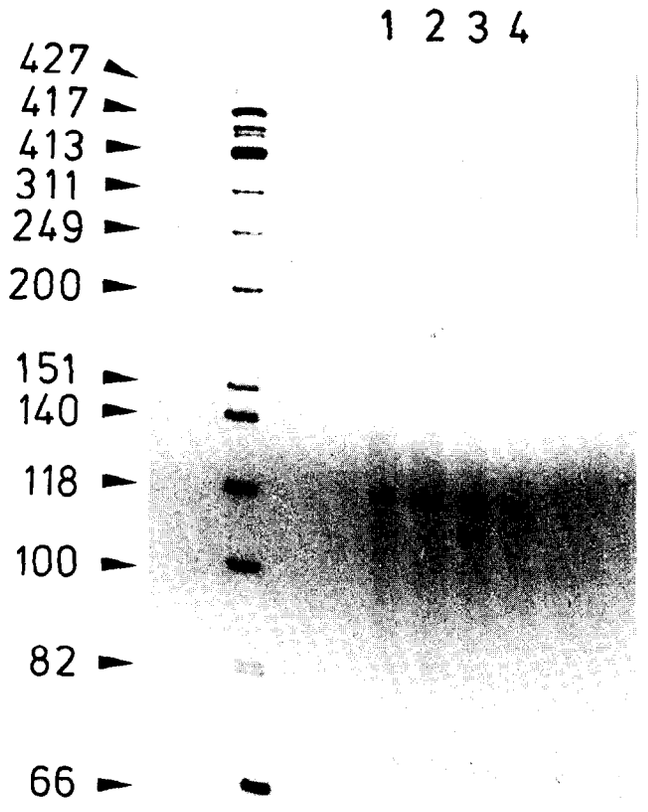

Fig. 4. Autoradiograph of an RNase protection assay for 100 μg adrenal total RNA using the cRNA probe covering the renin promoter/exon I region (Fig. 1b). A single protected fragment is visible in control animals (lane 1) and in animals treated with isoprenaline (lane 2) or low sodium diet (lane 3). Lane 4 corresponds to 100 μg of tRNA. Left are F174 DNA/*Hin*fI markers.

experiments, we used a RNase protection assay, considered to be more sensitive than primer extension technique. Our results suggest, therefore, that only a single TATA box is of importance for renin expression in the rat.

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