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A combination of upstream and proximal elements is required for efficient expression of the mouse renin promoter in cultured cells

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ABSTRACT

Renin, a key enzyme controlling blood pressure, is produced mainly in the kidney. To identify the transcriptional regulatory elements of the mouse *Ren-1*^{c} gene, the promoter regions were fused to the CAT reporter gene and transfected into embryonic kidney-derived 293 cells and four extrarenal cell lines, HeLa, HepG2, HT1080 and NIH3T3 cells. Transient transfection assay showed that sequences from −365 to +16 of the renin gene could direct transcription of the CAT hybrid gene only in 293 cells. Deletion analysis identified two transcriptionally active regions; the renin upstream-promoter element (RU-1 element; position −224 to −138) and the renin proximal-promoter element (RP-2 element; position −75 to −47). Although the RU-1 element functioned as an activator, depending on its orientation, it failed to *trans*-activate the renin promoter when the RP-2 element was deleted. By contrast, the proximal element alone exhibited a weak *trans*-activator property. Gel shift assay identified RU-1 element-binding factors in both 293 and HeLa cells, whereas 293 cell-dominant factors were shown to bind only to RP-2 element. Therefore, both RU-1 and RP-2 elements were found to be necessary for efficient CAT expression from the renin promoter in 293 cells, suggesting that activation of the *Ren-1*^{c} promoter requires combined action between cell type-dominant and ubiquitous nuclear factors.

INTRODUCTION

Renin, an aspartyl proteinase, is primarily synthesized in the kidney and secreted into the bloodstream and it is the rate-limiting factor for generation of angiotensin II, which is a potent vasoconstrictor and stimulator of aldosterone release (1,2). The renin-angiotensin system plays an important role in the regulation of blood pressure, fluid volume balance and other biological responses (3), and particularly its participation in several disease states including hypertension has been proposed. Previous studies have reported that genetic susceptibility forms a significant basis in the pathogenesis of hypertension, and it has been estimated that approximately one-third of human blood pressure variance is genetically determined (4). Among several proposed candidate genes in the pathogenesis of hypertension, renin gene expression appears particularly relevant. Clinical observation has revealed that overproduction of renin due to a renin-secreting tumor or renal arterial stenosis, elevates blood pressure, and that the blockade of the renin-angiotensin system by angiotensin-converting enzyme inhibitors is highly effective in controlling the blood pressure of the patients with essential hypertension.

In some strains of mice, two closely linked loci are located on the same chromosome as duplicated renin genes. *Ren-1* designates the renin gene present in all strains and encodes the circulating renin from kidney, and *Ren-2* is the duplicated renin gene which specifically exhibits abundant expression in the submandibular gland. *Ren-1* and *Ren-2* genes are highly homologous, including their 5′-flanking regions to a point located 179 nucleotides upstream from the transcription start site; the sequences diverge beyond this position (5,6). Two common alleles of the *Ren-1* locus are designated by their origin; *Ren-1*^{c} is the renin gene in strains which contain a single renin locus and *Ren-1*^{d} in strains with two renin loci. All three renin genes are expressed equivalently in the kidney (7,8), and thus it is supposed that the highly homologous 5′-flanking regions of the *Ren-1* and *Ren-2* genes are responsible for basal expression of these renin genes in the kidney.

Previous studies have suggested that renin biosynthesis is influenced by various signalling factors such as cAMP, calcium ions, phorbol esters and steroid hormones (9−12). These effects are probably mediated at the stages of transcription, processing and secretion (13,14). For instance, the far upstream 5′-flanking region of the mouse *Ren-1* and *Ren-2* genes contains putative cAMP-responsive elements (CRE), and cAMP treatment increases transcription from a chimeric herpes simplex virus (HSV)-thymidine kinase (TK) promoter linked downstream to the putative renin CRE in transient transfection assays (8,15,16). However, there have not been any detailed reports that investigate basal transcriptional regulatory elements of the mouse renin gene, due to the lack of suitable renal cell lines capable of driving the native promoter by transient transfection assay.

As a first study to establish a functional assay, 293 cells derived

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from embryonic kidney (17) were used to investigate the control of transfected genes containing the mouse renin (*Ren-1*^{c}) promoter region isolated from C57BL/6. We identified two *cis*-acting elements responsible for 293-dominant transcription of the renin promoter-bacterial chloramphenicol acetyltransferase (CAT) hybrid gene, which are termed the renin upstream-promoter (RU-1) element and the renin proximal-promoter (RP-2) element. Functional characterization of the two elements suggests that combined action of these elements is involved in 293 cell-dominant renin promoter activity.

MATERIALS AND METHODS

Plasmid constructions

Genomic clones were isolated from a library made with DNA obtained from C57BL/6 mouse liver, using a *Ren-2* cDNA probe (18). The partial DNA sequences were determined using the dideoxynucleotide chain termination procedure. Results of DNA sequence analysis of specific genomic fragments including 5'-flanking region were then compared with the published sequence (5,6,19). There were no differences between the sequenced fragments of our genomic clones and the previous published sequence (data not shown).

The renin promoter-CAT chimeric constructs were made as follows: mRn365CAT, mRn224CAT, mRn183CAT and mRn164CAT contain 381-bp (−365 to +16) *Xba*I/*Eco*T14I, 240-bp (−224 to +16) *Alu*I/*Eco*T14I, 199-bp (−183 to +16) *Mva*I/*Eco*T14I and 180-bp (−164 to +16) *Hin*fI/*Eco*T14I fragments, respectively, and these DNA fragments were subcloned into the *Bgl*II/*Hin*dIII sites of pUCSV0CAT (20); mRn114CAT and mRn75CAT consist of 130-bp (−114 to +16) *Eco*T14I and 91-bp (−75 to +16) *Hae*III/*Eco*T14I DNA fragments, respectively, cloned in the *Hin*dIII site of pUCSV0CAT; mRn47CAT consists of a 29-bp synthetic oligonucleotide (corresponding to the region from −47 to −19) and a 34-bp (−18 to +16) *Dde*I/*Eco*T14I fragment ligated into the *Bgl*II/*Hin*dIII sites of pUCSV0CAT; mRn365(Δ1)CAT is composed of a 182-bp (−365 to −184) *Xba*I/*Mva*I DNA fragment derived from mRn365CAT inserted into the *Bgl*II site of mRn164CAT; mRn365(Δ2)CAT and mRn365(Δ3)CAT contain the 182-bp (−365 to −184) *Xba*I/*Mva*I and 228-bp (−365 to −138) *Xba*I/*Hae*III DNA fragments derived from mRn365CAT, respectively, inserted into the *Bgl*II site of mRn114CAT.

Cell culture and transient transfection assay

Embryonic kidney-derived 293 cells were maintained in minimum essential medium (MEM) containing 10% heat-denatured horse serum. HeLa, HepG2 and HT1080 cells were cultured in MEM supplemented with 10% fetal bovine serum (FBS). NIH3T3 cells were in Dulbecco's modified Eagle's medium supplemented with 10% FBS. These cell lines were kept in 5% CO_2 and plated approximately 24 h before transfection at a density of 5×10^5 cells in 60-mm-diameter plastic dishes. Transient transfection assay was performed essentially as described previously (21), using 3 μg of plasmid DNA as a $CaPO_4$ coprecipitate. Cells were harvested 48 h after transfection and assayed for CAT activity.

Electrophoretic mobility shift assay (EMSA)

Crude nuclear extracts from 293 and HeLa cells were prepared using the methods of Dignam *et al.* (22). Nuclear extracts were preincubated for 15 min on ice in a 20-μl reaction mixture containing 12 mM HEPES (pH 7.9), 60 mM KCl, 4 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 12% glycerol, 0.5 mM phenylmethylsulfonylfluoride and 1 μg of poly (dI-dC). Non-labeled competitor was included in some of the binding reactions as indicated. Radio-labeled double-stranded renin promoter sequences (−224 to −138, −114 to −19 or −75 to −47) were added and the incubation continued for 30 min at 4°C. The incubation mixture was loaded on a 4% polyacrylamide gel in a buffer containing 50 mM Tris-HCl (pH 8.3), 192 mM glycine and 1 mM EDTA, then electrophoresed at 120 V for 3 h followed by autoradiography. Double-stranded oligonucleotides containing the consensus binding site for AP-1, AP-2 and Sp-1 were purchased from Stratagene (GELSHIFT™ KIT, La Jolla, CA), and oligonucleotide for CRE (corresponding to human chorionic gonadotropin α-subunit gene) was synthesized on an Applied Biosystems model 392 oligonucleotide synthesizer (23).

RESULTS

The renin promoter directs transcription in 293 cells but not in HeLa cells

The 293 cell line was established from embryonic kidney (17). We first examined, therefore, whether basal-level transcription from the renin promoter is efficient in transient transfection assay using this cell line. While 293 cells do not express renin mRNA (24), we nevertheless decided to use this cell line for analysis of transcriptional regulation of the renin promoter for the following reason. Recent experiments have demonstrated that the renin-angiotensin system is a local tissue system with autocrine, paracrine and intracrine functions and a role for this system during development and in disease has been indicated. Furthermore, renin expression in the embryonic kidney exhibits an interesting developmental pattern as observed by immunocytochemistry (25) and *in situ* hybridization (26). During fetal development in the kidney, foci of cells containing renin mRNA are detectable from the early stages of gestation and are observed in more extensive regions than in the adult kidney. After birth, the renin-producing cells are progressively restricted to more localized areas. Therefore, it is probable that the embryonic kidney-derived 293 cells possess trans-acting factors required to elicit renin promoter-CAT hybrid gene transcription. In fact, our previous work has demonstrated that 293 cells can direct human renin promoter-CAT hybrid gene expression (24).

The chimeric construct used for these experiments, mRn365CAT, is shown in Fig. 1A. It has a 381-bp fragment containing the mouse *Ren-1*^{c} promoter region (nucleotide positions −365 to +16) in the sense orientation with respect to the CAT gene. The promoterless plasmid pUCSV0CAT was used as a background reference and pUCSV3CAT was used as a positive control including the SV40 enhancer-promoter region (20). All results were corrected for variations in transfection efficiency by reference to pUCSV3CAT. The results shown in Fig. 1B demonstrate that mRn365CAT directs transcription in 293 cells. In HeLa cells, CAT activities were less than 0.1% relative to pUCSV3CAT. This complete inactivity in HeLa cells was not due to a lower transfection efficiency, since expression directed by pUCSV3CAT was almost at the same level in these cells as in 293 cells. Three other extra-renal cell lines, NIH3T3, HepG2 and HT1080, were also used to evaluate expression of mRn365CAT. In these cell lines, however, CAT activities were less than the background references (data not shown).

To define the DNA sequence responsible for dominant transcription from the renin promoter in 293 cells, a series of 5′-deletion mutants of mRn365CAT were tested for their ability to direct transcription (Fig. 1B). In 293 cells, deletion of the promoter to position −224 resulted in a two-fold increase in CAT expression relative to the undeleted construct, mRn365CAT. It is uncertain whether this small increase in CAT activity is significant enough to suggest the presence of any negative regulatory elements in the 142-bp region upstream of −224, because deletion of the same region does not result in any detectable increase in CAT expression in HeLa cells. At present, we consider that the sequences within −365 to −225 DNA region contribute very little to the cell-type dominancy of the renin promoter activity. Deletion to position −164 reduces the promoter-CAT hybrid gene expression to 20% of mRn365CAT. Further deletion up to position −47 decreased CAT activity below the background level demonstrated in the negative control, pUCSV0CAT. None of the deletion mutants were expressed upon transfection into HeLa cells. These results suggest that the regions from −224 to −165 and from −75 to −48 are important for 293 cell-dominant CAT expression.

Nuclear factors from 293 and HeLa cells bind to the upstream-promoter (RU-1) element of the renin gene

The 5′-deletion analysis suggested that the sequences from −224 to −165 were important for high-level CAT expression in 293 cells. In order to identify protein-factors binding to this region, we performed electrophoretic mobility shift assay (EMSA) with nuclear extracts from 293 and HeLa cells (Fig. 2). A double-stranded DNA fragment covering the region −224 to −138 (RU-1 element) of the renin promoter was used as a probe in EMSA. Two protein-DNA complexes were observed with extract from 293 cells (Fig. 2B, lanes 2 and 3). These retarded bands represent sequence-specific interactions between RU-1 element and proteins in the extract since these can be competed out with an excess of the same unlabeled RU-1 element (Fig. 2B, lanes 4 and 5). To further define the DNA sequences involved in these bindings, competition experiments were performed with fragments designated A (−224 to −165), B (−224 to −184), C (−183 to −165), D (−164 to −138) and E (−183 to −138), spanning different regions of RU-1 element. Fragments A, B, C, D and oligonucleotides containing the consensus binding sequence for either AP-1 and Sp-1 failed to compete with RU-1 element binding (Fig. 2B, lanes 6−9, 11 and 12). Fragment E, however, competed with its binding effectively (Fig. 4B, lane 10), implying that the binding region of these factors overlies the boundary between fragments C and D. Interestingly, similar band shift patterns and binding competitions were obtained in extracts from HeLa cells (Fig. 2B, lanes 15−24) despite the decreased intensity of protein-DNA complexes. This indicates that nuclear proteins present in both 293 and HeLa cells bind to the sequences from −183 to −138 of RU-1 element.

RU-1 element *trans*-activates the renin promoter in an orientation-dependent manner

To establish the functional importance of the RU-1 element (position −224 to −138) in renin promoter activity, we first constructed a series of internal-deletion mutants from mRn365CAT. Deletion of parts of the protein-binding region, mRn365(Δ1)CAT lacking from −183 to −165 and mRn365(Δ2)CAT lacking from −183 to −115, resulted in a significant decrease in CAT activity (Fig. 3). However, deletion of the region outside of the RU-1 element, mRn365(Δ3)CAT, did not reduce CAT expression, implying that disruption of nuclear factors binding to RU-1 element leads to a large reduction in the renin promoter activity in 293 cells.

To examine the effect of orientation of the RU-1 element on renin promoter activity, this element was ligated in 5′ to 3′ and 3′ to 5′ orientation into the renin promoter (Fig. 4). In 293 cells, RU-1 element activated CAT expression by 7.3-fold when this element was placed in its forward orientation (5′ to 3′) upstream of mRn76CAT, but this was reduced to 80% of mRn76CAT when this element was placed in the reverse orientation (3′ to 5′). No CAT expression was detected in transfected HeLa cells.

To test the transcriptional properties of RU-1 element on a heterologous promoter, this element was fused to the TK promoter (Fig. 4). Interestingly, RU-1 element activated TK promoter in both 5′ to 3′ and 3′ to 5′ orientations in contrast with the renin promoter. In HeLa cells, the RU-1 element did not show any apparent transcriptional activity able to drive the heterologous TK promoter. Furthermore, when RU-1 element was linked to the enhancerless SV40 early promoter, activation of CAT expression was not observed in transfected HeLa cells (data not shown). From these results, RU-1 element was shown to be able to act as an activator element only in 293 cells.

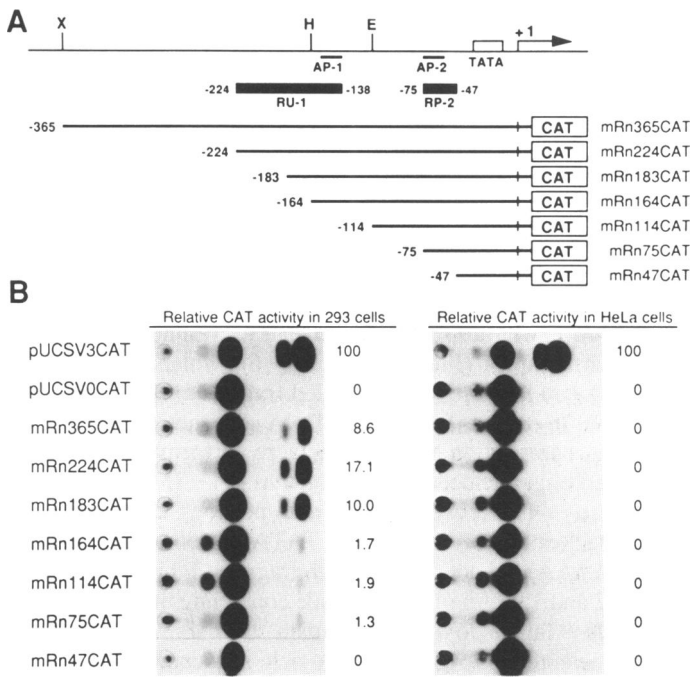

Figure 1. 5′-Deletion analysis of the mouse renin promoter. (A) Schematic representation of the mouse renin promoter and construction of the mouse renin promoter-CAT hybrid genes. Restriction endonuclease cleavage sites are indicated as follows: X, *Xba*I; H, *Hin*fI; E, *Eco*T14I. AP-1 and AP-2 consensus-like sequences are underlined . The TATA box is located at nucleotide positions −31 to −25 and the transcription start site is indicated by +1. RU-1 and RP-2 elements are shown by the open boxes. (B) Basal expression of the mouse renin promoter-CAT hybrid genes in transfected 293 and HeLa cells. Transfections were performed using 3 μg of plasmid DNA as a $CaPO_4$ coprecipitate. Cells were harvested 48 h after transfection and aliquots of cell extract containing equal amounts of total protein (40 μg) were used in the CAT assay. pUCSV3CAT and pUCSV0CAT were used as positive and negative controls, respectively. The CAT activity of each construct was calculated by comparing the percentage conversion of [^{14}C] chloramphenicol to its acetylated forms relative to the activity achieved with pUCSV3CAT in each cell line. These relative CAT activities are averages of five independent experiments.

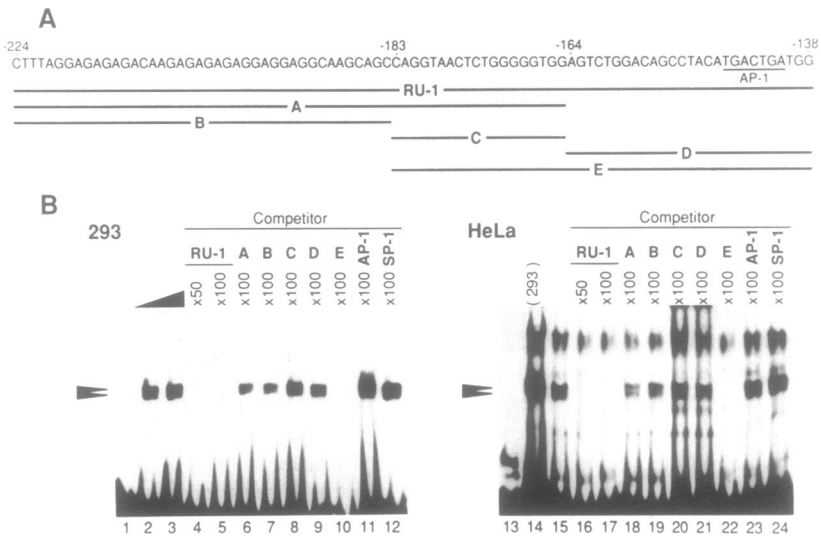

Figure 2. Identification of nuclear proteins that bind to the RU-1 element in 293 and HeLa cells. (A) Probe DNA sequences and competitors for the electrophoretic mobility shift assay. The *AluI-Hae*III (−224 to −138) fragment of the renin promoter was gel purified and end labeled with T4 polynucleotide kinase using [γ-^{32}P] ATP. The AP-1 consensus-like sequence is underlined with a thin horizontal line. The restriction fragments (RU-1; −224 to −138, A; −224 to −165, B; −224 to −184, C; −183 to −165, D; −164 to −138 and E; −183 to −138) for the competition assay are indicated by thick horizontal lines. (B) Electrophoretic mobility shift and competition analyses of complexes formed by factors in 293 and HeLa nuclear extracts with the RU-1 element (−224 to −138). 293 cell nuclear extract (lane 1, negative control; lane 2, 10 μg; lanes 3 to 12, 20 μg; lane 14, 20 μg) and HeLa cell nuclear extracts (lane 13, negative control; lanes 15 to 24, 20 μg) were incubated with 0.25 ng of ^{32}P-labelled probe. In a competition assay, 50-fold or 100-fold molar excess of unlabeled DNA restriction fragments and oligonucleotides (AP-1 and Sp-1), as indicated for each lane, were added to the reaction mixture. Arrows point to specific DNA-protein complexes.

The renin proximal-promoter (RP-2) element is relevant to 293-dominant transcription from the renin promoter

To further elucidate the mechanism of 293 cell-dominant expression of CAT activity, EMSA was carried out using a DNA fragment extending from nucleotide positions −114 to −19 (Fig. 5A, fragment F) as a probe. Incubation of end-labeled fragment F with 293 cell nuclear extract resulted in a specific retarded complex (Fig. 5B, lanes 4−6), which was competed by a 50×molar excess of unlabeled fragment F (Fig. 5C, lanes 8 and 9). In order to identify the DNA sequence involved in this binding, competition experiments were performed with double-stranded oligonucleotides designated as G (−75 to −47) and H (−47 to −19), covering different portions of fragment F. Oligonucleotide H containing the TATA box consensus sequence (TATAAAA) did not compete with fragment F binding (Fig. 5C, lane 12 and 13). An oligonucleotide containing the AP-2 binding site also failed to compete (data not shown). However, oligonucleotide G with the TA-rich sequence (TAATAAA) competed effectively (Fig. 5C, lanes 10 and 11). An additional upward shifted band occurs on competition with fragment G, the cause of which is unclear. No apparent protein-DNA complex was observed with HeLa cell nuclear extract (Fig. 5B, lanes 2 and 3).

Similar results were obtained by using a 30-bp double-stranded DNA probe spanning −75 to −47 bp (oligonucleotide G) of the renin promoter, which included the TA-rich sequence (Fig. 6A, lanes 1−6). The single shifted band observed with this probe after incubation with 293 cell nuclear extract was specifically competed by unlabeled oligonucleotide G (Fig. 6A, lanes 7 and 8). Oligonucleotides containing the binding sites for either AP-1, AP-2 and CRE did not compete with oligonucleotide G binding (Fig. 6A, lanes 10−12). These results demonstrated that 293 cell-dominant nuclear proteins bound to RP-2 element from −75 to −47. To investigate the possible relationship between RP-2 element-binding proteins and the TATA box binding protein TFIID, several core promoter fragments (TK promoter; −109 to +19, SV40 promoter; −43 to +64 and mouse angiotensinogen promoter; −52 to +23 (27)) containing the TATA box elements were used for competition assay (Fig. 6B). All of the promoter fragments tested failed to compete effectively for binding to RP-2 element (Fig. 6B, lanes 16−18).

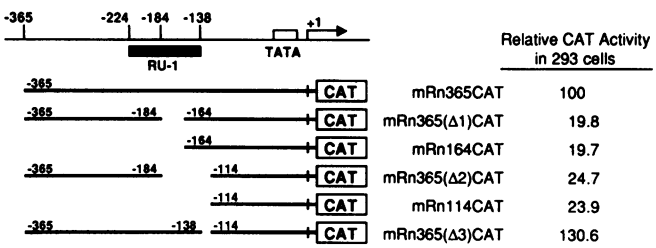

Figure 3. Internal-deletion analysis of the mouse renin promoter in 293 cells. The restriction fragment spanning −365 to +16 was digested by restriction enzymes and the resultant fragments were ligated to the CAT gene to make internal-deletion mutants. Internal-deletion regions are as follows: mRn365(Δ1)CAT, nucleotide positions −183 to −165; mRn365(Δ2)CAT, −183 to −115; and mRn365(Δ3)CAT, −137 to −115. The RU-1 element is denoted by the solid box. CAT activities are expressed relative to those achieved with mRn365CAT, and these values are averages of three independent experiments.

RP-2 element is necessary for 293 cell-dominant transcription from the renin promoter

To define the functional role of RP-2 element in directing CAT expression in 293 cells, we performed deletion analysis as shown in Fig. 7. Transfection of the constructs with deletion of the RP-2

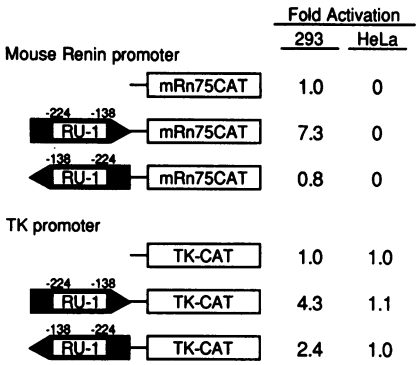

Figure 4. *Trans*-activation of the renin promoter by the RU-1 element in 293 cells. The RU-1 element (−224 to −138) was ligated in 5′ to 3′ and 3′ to 5′ orientations into the homologous (mouse renin; mRn75CAT) and the heterologous TK (nucleotide positions from −109 to +19) promoters. The CAT assay with these constructs was performed in three separate experiments in duplicate by transfection into 293 and HeLa cells. CAT activities are calculated relative to the level achieved with pUCSV3CAT included in each experiment, and fold activation for each construct is calculated by the CAT activity divided by the CAT activity of the mRn76 or TK promoters. Values are averages of at least three independent experiments.

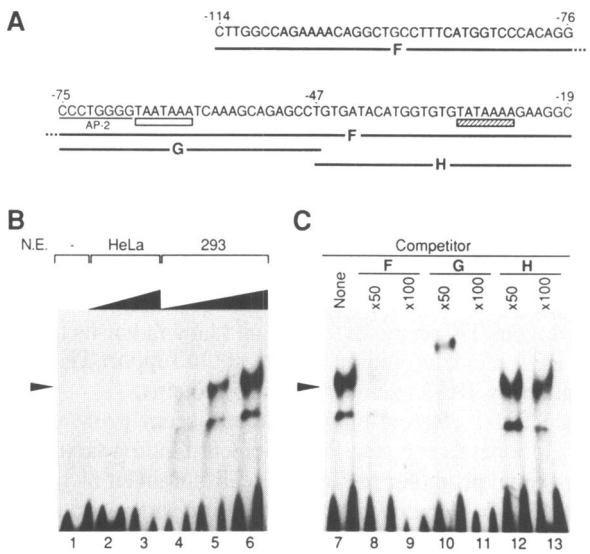

Figure 5. Electrophoretic mobility shift assay of 293-dominant nuclear proteins that bind to the proximal-promoter sequence between −114 and −19. (A) Probe DNA sequences and competitors for the electrophoretic mobility shift assay. The *Eco*T14I-*Dde*I (−114 to −19) fragment of the renin promoter was used as a probe. The AP-2 consensus-like sequence is underlined with a thin horizontal line. The TA-rich sequence (−67 to −61) is indicated by the open box and the TATA box (−31 to −25) is denoted by the hatched box. The restriction fragments (F; −114 to −19) and double-stranded oligonucleotides (G; −75 to −47 and H; −47 to −19) used in the competition assay are shown by thick horizontal lines. (B) Electrophoretic mobility shift analysis of complexes formed by factors in 293 and HeLa nuclear extracts with the proximal-promoter sequences between −114 and −19 (fragment F). HeLa nuclear extract (lane 2, 10 μg; lane 3, 20 μg) and 293 nuclear extract (lane 4, 5 μg; lane 5, 10 μg; lanes 6, 20 μg) were incubated with 0.2 ng of ^{32}P-labelled probe. Lane 1 contains no nuclear extract. Arrowheads point to DNA-protein complex formation. (C) Competition experiments with nuclear extract from 293 cells (lanes 7−13, 20 μg) using different portions of fragment F. Lane 1 contains no competitor. Lanes 8−13 show the effect of cold specific competitor (50× or 100× molar excess, as indicated above each lane) on the DNA-protein interaction.

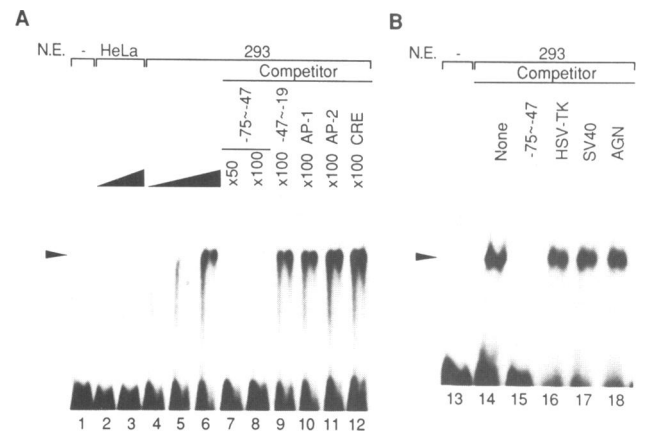

Figure 6. Identification of the target sequence of the proximal promoter-binding protein in 293 cells. (A) Electrophoretic mobility shift assay performed with fragment G (RP-2 element; −75 to −47). Double-stranded oligonucleotides containing the consensus binding site for AP-1, AP-2 and CRE (corresponding to human chorionic gonadotropin α subunit gene) were used as competitors. HeLa nuclear extracts (lane 2, 10 μg; lane 3, 20 μg) and 293 nuclear extracts (lane 4, 5 μg; lane 5, 10 μg; lanes 6 to 12, 20 μg) were incubated with 0.2 ng of ^{32}P-labelled probe G. Lane 1 contains no nuclear extract. The arrowhead points to the specific DNA-protein complex. (B) Competition experiments with DNA fragments spanning different core promoter regions. Binding reactions were performed as in (A). Several promoter fragments containing consensus TATA sequences were prepared and these were added at a 100-fold molar excess relative to the probe. DNA fragments used were: none (lane 14); RP-2 element (−75 to −47, lane 15); HSV-TK (−109 to +19, lane 16); SV40 (−43 to +64, lane 17); mouse angiotensinogen promoter (−52 to +23, lane 18). Lane 13 no added nuclear extract.

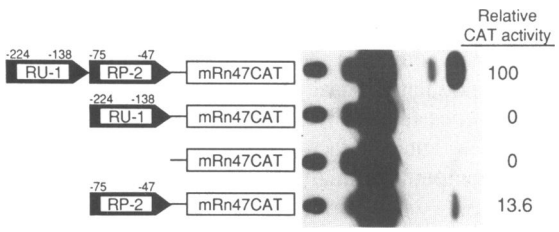

Figure 7. Effect of deletion of the RP-2 element on transcriptional activity in 293 cells. RU-1 and/or RP-2 elements were linked to the mRn47CAT-hybrid gene in a 5′ to 3′ orientation. CAT activity values are expressed relative to that obtained from the mRn48CAT construct containing the RU-1 and RP-2 elements, and these are averages of three independent experiments.

element (−75 to −47) resulted in a significant decrease of CAT expression in 293 cells. These reduced levels of expression were less than the background level for the negative control, pUCSV0CAT. This result is consistent with the proposal that the RU-1 element alone can not support renin promoter activity, and thereby suggests a critical contribution that the RP-2 element makes in 293 cell-dominant transcription from the renin promoter.

DISCUSSION

In the present study, we have identified two *cis*-acting elements in the native mouse *Ren-1*^{c} promoter that contribute to the basal expression of the CAT reporter gene in embryonic kidney-derived 293 cells. Expression of the renin promoter-CAT hybrid gene transfected into HeLa cells gave lower CAT activity

indistinguishable from that of the promoterless vector. Thus, we propose that DNA encoding information necessary to direct 293 cell-dominant expression of the CAT gene resides within 365 bp immediately upstream of the transcription start site of *Ren-1*^{c}, although 293 cells do not express renin mRNA (24). Several possibilities can be considered with respect to this discrepancy between CAT activity and mRNA levels in 293 cells. Firstly, the endogenous renin gene on the chromosome may be repressed by a negative *trans*-acting factor whose concentration is rate-limiting and therefore insufficient to repress the many copies of the transfected CAT hybrid gene. Alternatively, a negative regulatory element, which suppresses chromosomal renin gene expression might not reside on the renin promoter constructs used in the present transfection assays. This is consistent with recent studies suggesting that negative regulatory elements as well as positive regulatory elements are important for the tissue specific expression of many genes (28−30). Finally, the chromosomal renin gene might be inaccessible to positive *trans*-acting factors because of its chromatin structure or because of methylation at crucial residues. Recent findings concerning the functional role of histon H1 and methyl-CpG binding proteins in the repression of gene expression may support these possibilities (31−33).

One of the two *cis*-acting elements corresponds to a positive regulatory region located between −224 and −138, which has been identified by deletion analysis as the RU-1 element. This upstream element appears to be required for high-level renin promoter activity in transfected 293 cells. EMSA performed with nuclear extract from 293 cells revealed protein factors binding to the RU-1 element and a detailed competition analysis showed that the factors bound to sequences between −183 and −138. Interestingly, EMSA also showed that RU-1 element-binding proteins existed in HeLa cell nuclear extract. The TGACTGA sequence at positions −147 to −141 has partial homology to the consensus binding site for AP-1, a well-known transcription factor that modulates basal and induced expression of many eukaryotic genes (34−37). Therefore, we initially considered AP-1 to be a nuclear factor binding to the RU-1 element. However, competition analysis indicated that RU-1 element-binding proteins were distinct from the AP-1 family of nuclear proteins as an excess of AP-1 oligonucleotide was unable to inhibit the observed complex formation of the RU-1 element with nuclear proteins.

The cell-type dominancy exhibited by the renin promoter may in part be a property of RP-2 element for the following reasons. Firstly, 293 cell nuclear extracts produced a specific retarded complex with the RP-2 element, whereas HeLa cell nuclear extracts lacked detectable binding activity. Secondly, deletion of this element abolished transcriptional activity of the renin promoter-CAT hybrid gene in transient transfection assays. Thus, RP-2 element-binding factors appear necessary in determining expression of the renin promoter in a cell type-dependent manner. Although inspection of the DNA sequence of this region revealed an element (CCCTGGGG at −75 to −68) that shares sequence identity with the consensus binding motif for AP-2 (38, 39), competition analysis by EMSA showed that RP-2 element-binding proteins were distinct from the AP-2 family of nuclear proteins.

Examination of the RP-2 sequences also revealed a TA-rich element from −67 to −61 (TAATAAA), which closely resembles the TATA box consensus sequence located at position −31 to −25 (TATAAAA). Therefore, the presence of a TA-rich sequence within RP-2 element raises an interesting question concerning the possible relationship between the RP-2 element-binding proteins and the TATA box-binding protein. Previous works have indicated that the TATA box is recognized by TFIID, a member of general transcription factors present in all cell types. Cloning of the TFIID gene of yeast (40,41) and human (42−44) has revealed that TATA box consensus sequences play an important role in the function of TFIID, and furthermore, has suggested a strong evolutionary conservation of this ubiquitous transcription factor. Promoter fragments of several genes containing the TATA box failed to compete RP-2 element protein binding, thereby consistent with RP-2 binding factors as being 293 cell-dominant and different from TFIID. In addition, TFIID binding to the TATA box is not usually detected in crude nuclear extracts (45), thus further supporting the argument that TFIID is not involved in RP-2 binding. Also, our previous studies have showed that the TATA box at −31 to −25 (TATAAAA), but not the TA-rich sequence at −67 to −61 (TAATAAA), is a functional promoter in kidney (46). Recently, functional heterogeneity of the TATA box-binding factor has been proposed (47), and the existence of tissue-specific TATA box-binding proteins has been suggested from studies with the GHF-1 promoter (48), the immunoglobulin heavy chain promoter (49) and the uteroglobin promoter (50). Tanese *et al.* (51) have suggested that TFIID is actually a large multi-component complex including the TATA-binding protein, coactivators and other associated factors. Therefore, further studies are needed to identify the nature of the factor interacting with the RP-2 element.

More interestingly, our results indicate that the 293 cell-dominant RP-2 element-binding factor is inefficient in activating transcription of the renin promoter by itself. Rather, it is dependent on the RU-1 element being possibly interacting with factors which are not restricted to 293 cells. RU-1 element-binding factors cannot activate transcription of the renin promoter by themselves. Therefore, we propose that the RP-2 element-binding protein is the cell type-dominant cofactor necessary for RU-1 element-binding proteins to activate the renin promoter. Interestingly, the RU-1 element has an ability to activate the heterologous TK promoter, implying that another factor distinct from RP-2 element-binding protein could support TK promoter activation by RU-1 element-binding proteins.

Since RU-1 element-binding factors seem to be ubiquitous transcriptional factors and RP-2 element-binding factor binds to the proximal promoter region, 293 cell-dominant factor binding to the RP-2 element may mediate an interaction between ubiquitous factors binding to the RU-1 element and other general transcriptional activators. Therefore, both RU-1 and RP-2 element-binding factors appear to activate the renin promoter efficiently only in combination with each other. It has been noted by other investigators that certain enhancer elements are inactive unless combined with other elements (52,53). In addition, several studies concerning the transcriptional regulation of tissue-specific genes suggest that an interaction between ubiquitous and tissue-specific factors, which either bind close to the cap site or in remote position, is important for tissue-specific gene expression, as seen with the aldolase B gene (54) and the rat insulin II gene (55). For example, a muscle-specific factor activates transcription of the myosin heavy-chain β gene in a tissue-specific manner only in concert with a ubiquitously expressed factor (56), and similar situations have been observed with the troponin I promoter (57) and the hepatitis B virus preS1 promoter (58).

Recent studies using further upstream regions of *Ren-1*^{d} (−707 to −367) and *Ren-2* (−1055 to −571) fused to the TK promoter have demonstrated a possible interaction between the

cAMP-responsive element (CRE) and a negative regulatory element (NRE) overlapping the CRE sequence (16,59). It is likely that the interplay between these further upstream elements such as CRE and NRE is responsible for tissue-specific and high-level renin gene expression, and that the interaction between RU-1 and RP-2 elements determines the level of basal transcriptional activity of the renin promoter. Furthermore, renin-expressing cell lines have been recently established from transgenic mice carrying a renin promoter-SV40 T-antigen fusion construct (60). Further study using these cell lines will greatly assest in addressing the nature of tissue-specific regulation of renin gene expression.

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