Distinct Nuclear Proteins Competing for an Overlapping Sequence of Cyclic Adenosine Monophosphate and Negative Regulatory Elements Regulate Tissue-specific Mouse Renin Gene Expression

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Abstract

The mouse renin locus (Ren-1^d) exhibits specific patterns of tissue expression. It is expressed in kidney but not submandibular gland (SMG). This locus contains a negative regulatory element (NRE) and a cAMP responsive element (CRE) that share an overlapping sequence. In the kidney, CRE binding proteins (CREB) and NRE binding proteins (NREB) compete for binding to this sequence, with the CREB having a greater affinity. In the SMG, CREB is inactivated by an inhibitory protein, permitting NREB to bind to the sequence, thus inhibiting Ren-1^d expression. We hypothesize that the competition between NREB and CREB for this sequence governs tissue-specific expression of mouse renin. We speculate that this may be a general paradigm that determines tissue-specific gene expression. (*J. Clin. Invest.* 1993. 92:1805–1811.) Key words: renin • gene expression • transcriptional control • nuclear proteins • cyclic adenosine monophosphate

Introduction

Circulating renin has a well established role in blood pressure control and water-electrolyte homeostasis, and its synthesis and release by the juxtaglomerular (JG)[1] cells are regulated in accordance with this role (1). In the mouse, renin, which is encoded by two loci, is expressed in a variety of extrarenal tissues, such as the submandibular gland (SMG), adrenal, heart, testis, and ovary. The expression of the renin gene is regulated in a tissue-specific manner (2–4). To investigate the mechanism of this tissue-specific renin gene expression, we studied the renin locus, Ren-1^d which is expressed in the kidney, adrenal, and testis but not the SMG (4–5). In this study, we focused on the molecular mechanisms determining the expression of Ren-1^d in the kidney vs. the SMG.

Recently we reported that an XbaI fragment of Ren-1^d (−707 to −367) could suppress basal expression from a heterologous promoter in transient transfection experiments. Moreover, this fragment contains a sequence that is partially homologous to the chicken lysozyme silencer (6, 7). Subsequent experiments using a deletional mutant of the XbaI fragment of Ren-1^d, which lacks this negative regulatory element (NRE), confirmed that this putative NRE in fact confers negative regulation (8). However, the presence of the NRE binding protein in the mouse kidney has not previously been examined.

We have reported that the NRE in the mouse renin gene overlaps the sequence for a cAMP responsive element (CRE) (6), and that the CRE binding protein in the SMG nucleus cannot bind to the CRE due to the presence of an excess amount of an inhibitory protein (I-CRE) that inactivates this CRE binding protein. This inhibitory protein was not detected in kidney extracts (9). We hypothesize that the interaction of the NRE and CRE binding proteins at this overlapping sequence can explain, in part, the tissue-specific expression (SMG vs. kidney) of the Ren-1^d gene. If, for example, the CRE binding protein has a greater affinity than that of the NRE binding proteins, the NRE binding protein would be excluded from exerting its action in the kidney since CRE binding activity is present. However, in the SMG, an inhibiting protein forms a transcriptionally inactive complex with the CRE binding protein. This would allow the NRE binding protein to bind to the sequence, thus inhibiting transcription. Therefore, our aim in this study was to determine if the NRE binding protein was expressed in the mouse kidney and SMG, and to determine the relative binding affinities of the NRE and CRE binding proteins.

Methods

Preparation of nuclear extract. Nuclear extracts were prepared according to published methods (10). Kidneys and SMG were quickly removed from 15 male 40-d-old mice (strain DBA/2J; Charles River Laboratories, Boston, MA) and homogenized with a Potter-Elvehjem homogenizer in 4 vol of ice-cold homogenization buffer containing 10 mM Hepes, pH 7.5, 0.5 mM spermidine, 0.15 mM spermine, 5 mM EDTA, 0.25 mM EGTA, 7 mM β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride. After centrifugation at 12,000 *g* for 30 min at 4°C, the pellet was resuspended with a Dounce homogenizer (Kontes Glass Co., Vineland, NJ) in 1 vol of ice-cold homogenization buffer containing 0.5 M sucrose and 0.1% NP-40 and then centrifuged at 12,000 *g* for 30 min at 4°C. Pelleted nuclei were washed twice with ice-cold homogenization buffer containing 0.35 M sucrose. After washing, nuclei were preextracted with 1 vol of homogenization buffer containing 0.05 M NaCl and 10% glycerol for 15 min at 4°C, and again centrifuged at 12,000 *g* for 30 min at 4°C. The nuclear pellets were then extracted with homogenization buffer containing 0.3 M NaCl and 10% glycerol for 1 h at 4°C, and the concentration of DNA was adjusted to 1

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Received for publication 4 November 1992 and in revised form 13 May 1993.

1. *Abbreviations used in this paper:* CN, combined CRE and NRE sequences; CRE, cAMP responsive element; CREB, cAMP responsive element binding protein; DOC, sodium deoxycholate; hCG, human chorionic gonadotropin; JG, juxtaglomerular; MR-CRE, mouse renin cAMP responsive element; NRE, negative regulatory element; NREB, negative regulatory element binding protein.

J. Clin. Invest.

0021-9738/93/10/1805/07 $2.00
Volume 92, October 1993, 1805–1811

mg/ml. After pelleting the extracted nuclei at 12,000 *g* for 30 min at 4°C, the supernatant fraction was brought to 45% $(NH_4)_2SO_4$ and stirred for 30 min at 4°C. The precipitated proteins were collected at 10,000 *g* for 30 min, resuspended in homogenization buffer containing 0.35 M sucrose, and stored in aliquots at −70°C.

The JG cells, which are the main renin-producing cells in the kidney, constitute only a small fraction of the total cells in the kidney. To examine for the presence of NREB and CREB in these cells, we prepared nuclear extracts from enriched JG cells isolated from 50 male DBA/2J mouse kidneys according to the method previously described (9). Protein concentration was determined (11) with BSA as a standard.

Preparation of oligonucleotides. The sequence of the NRE and CRE found in the mouse Ren-1^d 5′ flanking region is described in Fig. 1. The oligonucleotide containing NRE sequence was synthesized as complementary pairs of single-strand deoxyoligonucleotides with cohesive 5′ ends.

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5′gatcc C T A A C T T G G T C T C A C A G G C T A G A A T T T A t3′
3′     g G A T T G A A C C A G A G T G T C C G A T C T T A A A T agatc5′
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The left-hand end of each pair is the gatc-cohesive end and the c-g base, which is essential to reconstitute a BamHI site. The a-t base pair at the right-hand end allows the right-hand ctag-cohesive end to reconstitute XbaI site. After NAP 10 column (Pharmacia LKB Biotechnology, Piscataway, NJ) purification, complementary oligonucleotides were annealed in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA for 2 h, while the temperature descended from 80 to 25°C. Using the same strategy, oligonucleotides containing CRE were also synthesized.

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5′gatcc T A T A C C T A C C T A A C T T G G T C T C A C A G G t 3′
        gA T A T G G A T G G A T T G A A C C A G A G T G T C C agatc
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We also made a synthetic DNA probe spanning both the CRE and NRE (5′ TATACCTACCTAACTTGGTCT CACAGGCTAGAATTTA3′) oligonucleotide and human chorionic gonadotropin (hCG) (AAATTGACGTCATCGTAA) oligonucleotide (12) which contains the typical 8-bp palindromic CRE (TGACGTCA).

End-labeling of oligonucleotides. Oligonucleotides were labeled at the 3′ end with the Klenow fragment of *Escherichia coli* DNA polymerase I (Bethesda Research Laboratories, Gaithersburg, MD) using [^{32}P]-dCTP (300 Ci/mmol, DuPont/New England Nuclear, Boston, MA). After end-labeling, ^{32}P-labeled oligonucleotides were purified by 10% PAGE, eluted with 500 mM ammonium acetate, 10 mM $MgCl_2$, 1 mM EDTA, and 0.1% SDS at 37°C overnight, and concentrated by ethanol precipitation. For the DNase I footprinting assay, we used an oligonucleotide with 5′ cohesive end of aag (*left hand*) and ttc (*right hand*) to label only cohesive G residue by [^{32}P]dCTP.

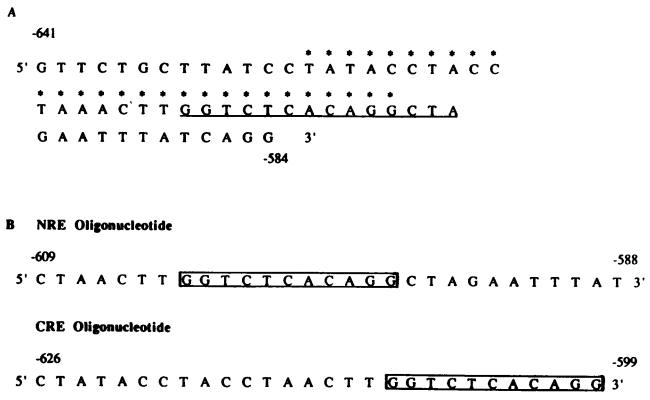

Figure 1. Sequence of 5′-flanking region of mouse Ren-1^d gene. (*A*) Asterisks show functional CRE and underlining shows negative regulatory element as previously reported (8). (*B*) Sequences of synthetic NRE oligonucleotide and CRE oligonucleotide are shown. Boxed areas indicate the overlapping sequence of NRE and CRE.

Gel mobility shift assay. Binding reactions (10 μl) included ^{32}P-labeled double-stranded oligonucleotide (0.5–1 ng, 10,000–15,000 pm), 1 μg of poly (dI:dC/dI:dC), and 5–30 μg of nuclear proteins in a buffer containing 10 mM Tris-HCl, pH 7.5, 4% glycerol, 1 mM EDTA, 1 mM β-mercaptoethanol, and 40 mM NaCl. The reaction mixture was incubated for 30 min at room temperature and then loaded onto a 5% polyacrylamide gel (acrylamide/bisacrylamide, 79:1, 17 × 15 × 0.8 cm) containing 0.2 mM DTT. The gels were subjected to electrophoresis in a buffer containing 50 mM Trizma base, 380 mM glycine, 2 mM EDTA, and 0.5 mM β-mercaptoethanol. Gels were run at 17 mA for 2.5–3 h at 4°C. After electrophoresis, gels were soaked in 5% glycerol for 20 min, followed by drying and autoradiographing using X-Omat AR film (Eastman Kodak Co., Rochester, NY). In some experiments (as indicated), 0.8% sodium deoxycholate, 1.2% NP-40, and 1 μg of BSA were included. For the competition assay, unlabeled competitor DNA fragments were preincubated with the parallel samples 10 min before the addition of labeled probe.

Southwestern blotting analysis. Southwestern blotting analyses were performed essentially as described (13). Nuclear extracts prepared from kidney and SMG were mixed with 1:1 (vol/vol) with sample buffer (5 mM Tris-HCl, pH 6.8, 5% SDS, 200 mM DTT, 20% glycerol, 0.1% bromophenol blue) and resolved by electrophoresis on 10% SDS PAGE. The proteins were transferred to nitrocellulose electrophoretically in 25 mM Trizma base/190 mM glycine/20% methanol. After soaking for 30 min at 4°C in binding buffer (25 mM Hepes, pH 7.9, 50 mM NaCl, 4 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT), the nitrocellulose filter was incubated for 30 min at 4°C in binding buffer containing 5% (wt/vol) nonfat dry milk and then washed in several changes of binding buffer. The filters were incubated overnight at 4°C in binding buffer containing 10^6 cpm of ^{32}P-labeled oligonucleotides per milliliter with 100-fold excess of salmon sperm DNA. After hybridization, the filters were washed with several changes of binding buffer containing 0.25% nonfat dry milk and then exposed to Kodak X-Omat film.

DNase I footprinting analysis. Nuclear extracts were incubated in ^{32}P-labeled DNA (15,000 cpm), which contains the entire sequences of both CRE and NRE in the buffer containing 10 mM Tris-HCl, pH 7.5, 4% glycerol, 1 mM EDTA, 1 mM β-mercaptoethanol, 40 mM NaCl, and 1 μg of poly (dI:dC/dI:dC). After incubation at room temperature for 30 min, the concentrations of $MgCl_2$ and $CaCl_2$ were adjusted to 5 mM and 3 mM, respectively, and the DNA was digested with DNase I (Bethesda Research Laboratories), using 92 ng/ml for the blank and kidney extract and 23 ng/ml for the SMG extract, at 20°C for 1 min. The DNase digestion was terminated by addition of 0.1% SDS and extraction in phenol/chloroform/isoamyl alcohol. The deproteinized digestions were ethanol-precipitated, resuspended, and separated on 8% urea-polyacrylamide gels.

Results

Gel mobility shift assays were performed to examine whether nuclear extracts prepared from mouse kidney and SMG contain specific nuclear binding proteins, which recognize the NRE sequence. As shown in Fig. 2, we observed ^{32}P-labeled NRE oligonucleotide–protein complex formation (band *I*) in both kidney and SMG nuclear extracts. This binding was not inhibited by the preincubation of nuclear extracts with a 100-fold molar excess of unlabeled oligonucleotides containing the mouse renin CRE or the hCG CRE, but was competitively inhibited by preincubation with the oligonucleotide containing the NRE, suggesting that both kidney and SMG nuclear extracts contain a specific nuclear binding protein that recognizes the mouse renin NRE. Interestingly, we observed an additional shifted band Fig. 2 *A*, band *II*). This band was attenuated by

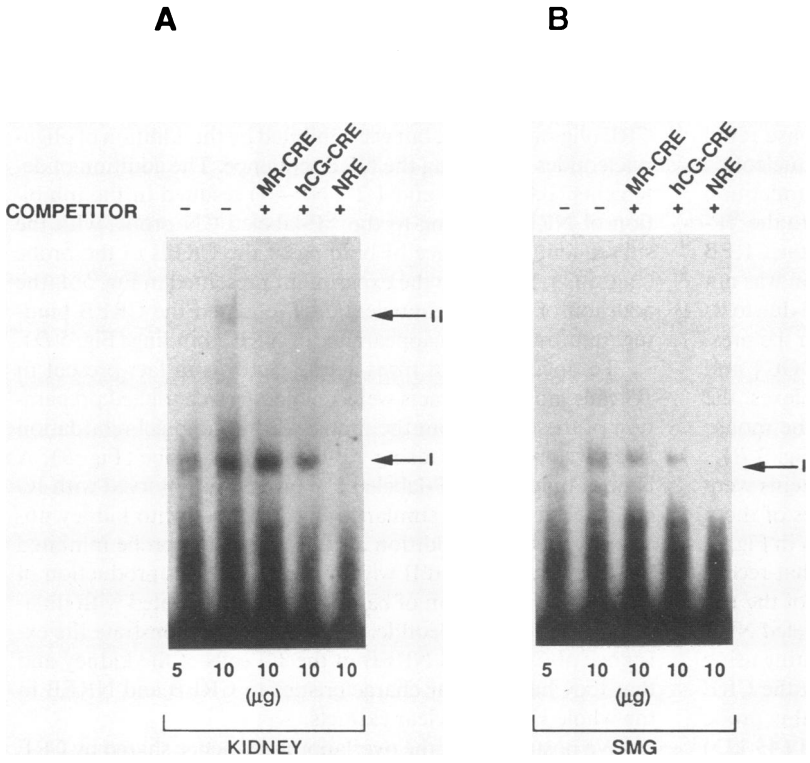

Figure 2. Detection of a specific NRE binding protein in mouse kidney (*A*) and SMG (*B*) nuclear extracts. Kidney and SMG nuclear extracts (5, 10 μg) were incubated with the ^{32}P end-labeled NRE oligonucleotide and analyzed by gel mobility shift assay. In some reactions, 100-fold excess of unlabeled oligonucleotides of mouse renin CRE (MR-CRE), hCG CRE, and NRE were preincubated with 10 μg of nuclear extracts before the addition of the radiolabeled probe. Arrows point to DNA–protein complex formation. Band I indicates the presence of the NREB–NRE complex (*lower band*) and band II indicates the presence of the CREB–CRE complex (*upper band*).

the addition of the mouse renin CRE, hCG CRE, or NRE oligonucleotides. We have reported previously (9) and confirmed in this study that hCG CRE completes effectively the binding of CREB to the mouse renin CRE. This is consistent with the presence in the kidney of a CRE binding protein that has affinity for both the CRE and NRE. We have previously reported (9) that mouse kidney nuclear extracts, but not those from the SMG, contain a specific CRE binding protein (CREB) that binds the mouse renin CRE. (Fig. 3). Since the mouse renin NRE and CRE share an overlapping sequence (Fig. 1), we hypothesize that the kidney CREB can bind to the NRE oligonucleotide at this overlapping site. To examine this

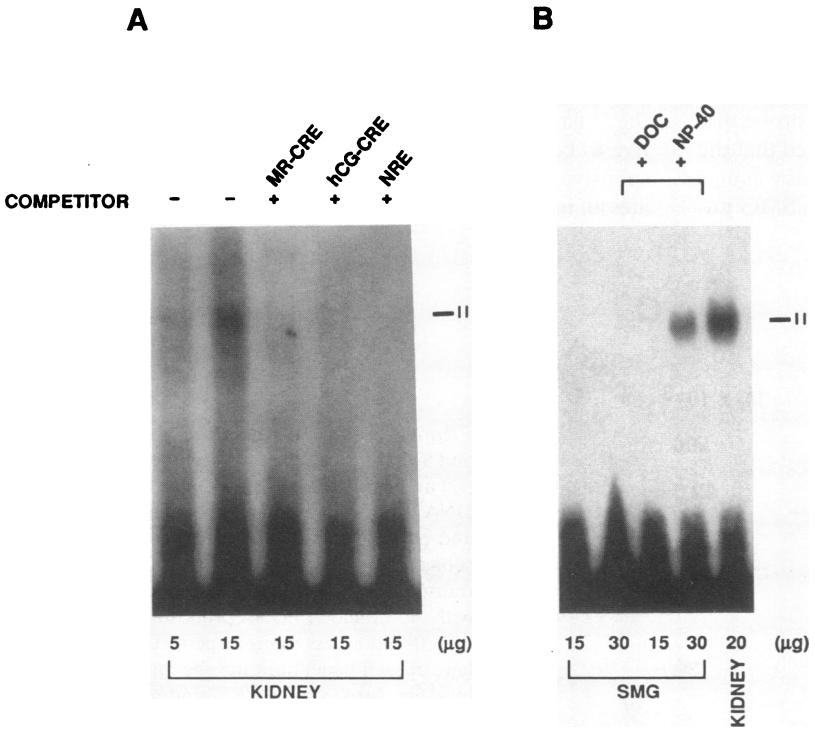

Figure 3. Detection of a CRE binding protein in kidney and SMG nuclear extracts. (*A*) Kidney nuclear extract (5, 15 μg) was incubated with ^{32}P-labeled MR-CRE oligonucleotide and the DNA-protein complex was analyzed by gel mobility shift assay. Competition analysis was performed with 100-fold excess of MR-CRE, hCG CRE, and NRE oligonucleotides. (*B*) SMG nuclear extracts (15, 30 μg) were incubated with ^{32}P-labeled MR-CRE oligonucleotide with or without 0.8% DOC and 1.2% NP-40. The right lane shows kidney nuclear protein (15 μg) binding to MR-CRE. As in Fig. 2, band II indicates the presence of the CREB–CRE complex.

possibility, we studied the effect of incubation with excess NRE oligonucleotide on the binding of the mouse kidney CREB to ^{32}P-labeled mouse renin CRE. Indeed, the binding of ^{32}P-labeled CRE to the CREB was competed not only by the addition of oligonucleotides containing the sequences of mouse renin CRE, hCG, and CRE, but also by the NRE oligonucleotide (Fig. 3 *A*). These data, taken together with the competition analysis observed in kidney nuclear extract binding to the ^{32}P-labeled NRE probe (Fig. 2 *A*, band *II*), suggest that the CREB can bind to the NRE oligonucleotide. This interaction was not apparent in the SMG since the CREB was inactivated due to its interaction with an inhibitory protein (9). Indeed, in the presence of detergents (0.8% sodium deoxycholate [DOC] and 1.2% NP-40) that dissociated protein–protein complexes, the SMG nuclear extract exhibited specific binding to the mouse renin CRE, demonstrating the presence of CREB (Fig. 3 *B*).

To confirm that the NRE and CRE binding proteins were distinct proteins, we examined the molecular masses of these proteins by Southwestern blotting analysis. As shown in Fig. 4, the molecular mass of the kidney nuclear protein that recognized the CRE sequence, was ~ 43 kD, while that of the nuclear protein in kidney and SMG that bound ^{32}P-labeled NRE was ~ 72 kD. We then performed Southwestern blotting using a single ^{32}P-labeled DNA probe that contained both the CRE and NRE sequences (CN). As shown in Fig. 4 *C*, this probe recognizes both the NREB (72 kD) and the CREB (43 kD) proteins.

To examine further the hypothesis that the CREB and NREB competed for the overlapping sequence with differential affinities, we used the CN probe in gel mobility shift assays. As shown in Fig. 5 *A*, incubation of this probe with kidney nuclear extracts yielded a single DNA–protein complex (band *II*). This band was completed by the addition of 100-fold excess of either unlabeled CRE- or NRE-containing oligonucleotide. Interestingly, the addition of unlabeled CRE oligonucleotide not only attenuated this band but simultaneously produced a second shifted band (band *I*). This result would suggest that the displacement of radiolabeled CN probe from CREB by the addition of excess mouse renin CRE oligonucleotide enabled this probe to bind the NREB, presumably at the overlapping sequence. The fact that only the CREB bound to the probe that contained both the CRE and NRE sequence suggested that the CREB binds more strongly than does the NREB. This was also suggested by the results using SMG nuclear extracts. SMG nuclear extracts showed only one specific nuclear binding protein–DNA complex with the ^{32}P-labeled CN probe, which contains the entire sequence of CRE and NRE (Fig. 5 *B*). The formation of this band was not inhibited by the addition of CRE oligonucleotide, but was inhibited by the addition of oligonucleotides containing the NRE sequence. The addition of detergents (0.8% DOC and 1.2% NP-40) resulted in the inhibition of NREB binding to the ^{32}P-labeled CN probe, with the subsequent appearance of binding of the CREB to the probe (Fig. 5 *C*). Similar to the experiment presented in Fig. 5 *A*, the addition of CRE oligonucleotide attenuated the CREB binding, resulting in the reappearance of NREB binding (Fig. 5 *D*).

To document that *trans*-acting factor is in fact present in JG cells, nuclear extracts were isolated from enriched preparation of these cells from the mouse kidney, and gel retardation assay was performed using ^{32}P-labeled CN probe (Fig. 6). A band II binding to ^{32}P-labeled CN probe was observed with JG cell nuclear extracts, similar to that observed with kidney nuclear extracts. The addition of unlabeled CRE probe inhibited the formation of band II with the simultaneous production of band I. The formation of band I could be inhibited with unlabeled NRE oligonucleotide. These results demonstrate the existence of CREB and NREB in the JG cells of the kidney and that they have similar characteristics to CREB and NREB in the whole kidney nuclear extracts.

We postulate that the overlapping sequence shared by CRE and NRE is important for the binding of two distinct proteins, NREB and CREB, with differential affinities. Since kidney and SMG nuclear extracts each exhibited only a single complex formation (CREB and NREB, respectively) with the CN probe, these extracts were used for the footprinting assay. As shown in Fig. 7, an identical sequence (−607 to −600, TCTCACAG) was protected by both the NRE and CRE binding proteins.

Discussion

The regulation of renin Ren-1^d gene expression is accomplished via the interaction of both positive and negative factors. In this and previous studies we have focused on a fragment of the 5′ flanking region of the gene (−707 to −351) that is sensitive to both. We have described previously a novel cAMP responsive element that binds a CRE binding protein and mediates an increase in transcription in response to cAMP. Interest-

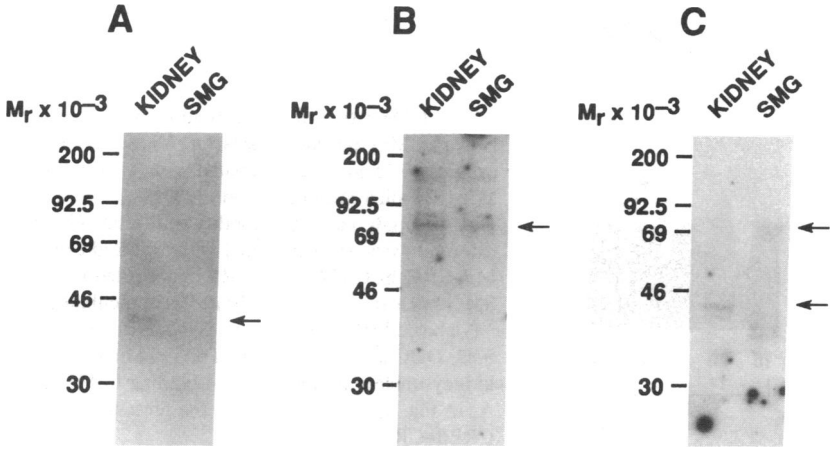

Figure 4. Southwestern blotting analysis of kidney and SMG nuclear extract with mouse renin CRE (*A*) and NRE (*B*) oligonucleotides, and with a DNA probe containing entire sequences of CRE and NRE (*C*). The nuclear extracts (60 μg) were subject to 10% SDS PAGE. After electrophoretic transfer to nitrocellulose, the filters were incubated with the ^{32}P-labeled DNA probe in the presence of 100-fold excess salmon sperm DNA. The right lane in each panel indicates the molecular mass markers. Arrows show CRE binding protein (*A* and *C*) and NRE binding protein (*B* and *C*).

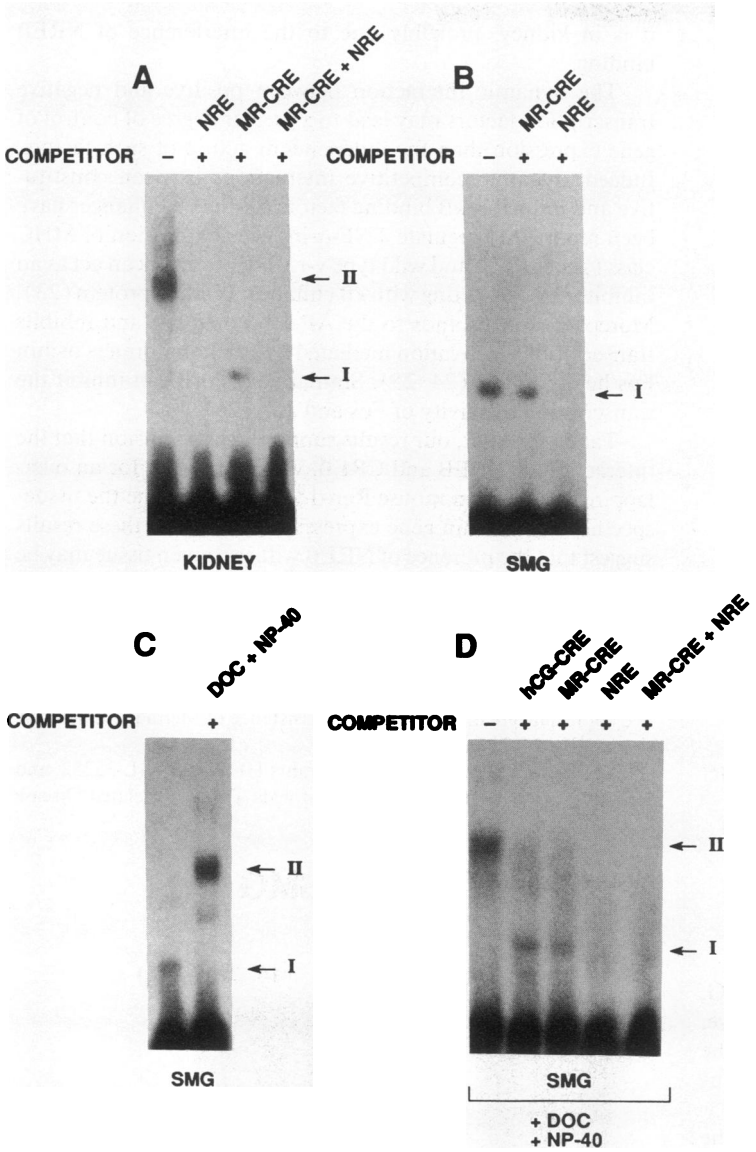

Figure 5. Gel mobility shift assay of nuclear protein binding in kidney (*A*) and SMG (*B–D*) to the oligonucleotide spanning the entire sequence of the CRE and NRE. Kidney and SMG nuclear extracts (10 μg) were incubated with the ^{32}P-labeled DNA probe containing entire sequences of CRE and NRE, and the DNA–protein complexes were analyzed by PAGE. Competition analysis was performed with 100-fold excess of NRE and/or MR-CRE. Band I indicates the presence of the NREB–NRE complex (*lower band*) and band II indicates the presence of the CREB–CRE complex (*upper band*). (*C*) SMG nuclear extract (30 μg) was incubated with the ^{32}P-labeled DNA probe containing entire sequences of CRE and NRE with or without 0.8% DOC and 1.2% NP-40. Band I indicates the presence of the NREB–NRE complex (*lower band*) and band II indicates the presence of the CREB–CRE complex (*upper band*). (*D*) SMG nuclear extract (30 μg), treated with 0.8% DOC and 1.2% NP-40, was incubated with the ^{32}P-labeled DNA probe containing entire sequences of CRE and NRE. Competition analysis was performed with 100-fold excess CRE and/or NRE. Band I indicates the presence of the NREB–NRE complex (*lower band*) and band II indicates the presence of the CREB–CRE complex (*upper band*).

ingly, this CRE overlaps a negative regulatory element. Additional analysis has confirmed that this NRE confers negative regulation in transient transfection experiments using JEG-3 cells. Sequences homologous to this NRE are found within the regulatory regions of the genes encoding human renin (14) rat collagen (15), T-cell receptor (16), chicken lysozyme (17), human c-*myc* (18), and human IL-2 (19). Previously, we have shown that a protein that binds this sequence is found in JEG-3 cell nuclei (8).

The Ren-1^d gene exhibits tissue specific expression. In addition to the kidney, it is expressed in multiple tissues. However, unlike the second renin loci, Ren-2^d, it is not expressed in the SMG. We postulated that this tissue-specific expression might be mediated by the NRE and its binding protein (8). Initially, we assumed that the binding protein would be absent or at low levels in the kidney. However, multiple lines of evidence suggested that it was in fact present at comparable levels. Both SMG and kidney nuclear extracts contained specific nuclear binding proteins that recognized the NRE sequence (Fig. 2). Moreover, the results from Southwestern blotting (Fig. 4) again demonstrated the presence of a protein in both organs. The molecular mass of this protein (72 kD) as determined by Southwestern blotting, and the fact that the binding to the NRE sequence was not competed by the mouse renin CRE or the hCG CRE, distinguished this protein from a CRE binding protein. Therefore, by itself, the interaction of the NRE and its binding protein would not explain fully the tissue-specific expression of Ren-1^d. However, we have previously demonstrated that the NRE and the CRE are overlapping sequences (8). Thus, a competition for this sequence by the NREB and CREB may play a role in the tissue-specific expression. Indeed, when an oligomer that contains the sequences of both the NRE and CRE is used in a gel shift assay, a single band, corresponding to the CREB binding to the CRE, is observed in kidney nuclear extracts (Fig. 5). Consistent with the results presented in Fig. 3, excess unlabeled NRE oligomer could inhibit formation of this complex. However, when excess unlabeled CRE oligomer was added, the complex formation was also inhibited, allowing the formation of a complex with characteristics similar to that of the NREB binding to the NRE. Thus, the results of these experiments suggest that the CREB has a higher affinity for this region than does the NREB. This is consistent with

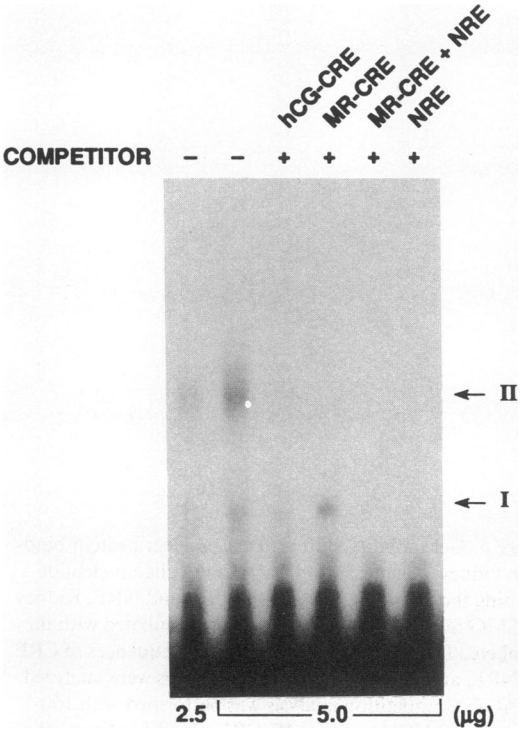

Figure 6. Gel mobility shift assay of nuclear extract prepared from JG cells to the oligonucleotide spanning entire sequence of CRE and NRE. Competition analysis was performed with 100-fold excess of unlabeled MR-CRE, hCG-CRE, and NRE. Band I indicates the NREB–NRE complex (*lower band*) and band II indicates the CREB–CRE complex (*upper band*).

the expression of the Ren-1^d gene in the kidney. In the SMG, however, we have shown previously that the CREB is inactive, due to the presence of an inhibitory protein that binds to the CREB (9). This allows the NREB to bind to the NRE, thus inhibiting expression in that tissue.

The above results may explain why renin is expressed in the kidney, but not the SMG, of the mouse strains (e.g., C57 B) that carry only one renin gene, i.e., Ren-1, and may also account for the same tissue-specific expression of renin in rat and human that also contain one renin locus. The DBA/2J strain of mouse contains two renin loci, i.e., Ren-1^d and Ren-2^d (20). Ren-2^d is thought to arise from a duplication of Ren-1^d (1). It is highly homologous to Ren-1^d in structure, including the 5′ flanking sequence, with the exception that a 150-bp insertional mutation is present immediately 5′ to the NRE (6). While the patterns of expression of Ren-1^d and Ren-2^d are similar in the kidney, adrenal, and testis, they differ strikingly in the SMG. Ren-2^d is expressed at a significant level in the SMG, whereas Ren-1^d is not expressed. We have previously demonstrated that the 150-bp insertional mutation interfered with the function of the NRE of the Ren-2^d, thereby permitting the expression of Ren-2^d in the SMG (8). These observations are in keeping with the results of this paper with specific regard to the SMG. Despite the inactivation of CREB and the presence of NREB, Ren-2^d cannot be silenced, since the NRE is nonfunctional. These data support the hypothesis of Field and Gross (21) that the high expression of Ren-2^d (compared to Ren-1^d) in SMG is due to nonresponsiveness of Ren-2^d to negative control. Thus, we suppose that Ren-2^d is expressed in SMG as it is in kidney, probably due to the interference of NREB binding.

The dynamic interaction between positive and negative transcription factors may lead to a greater degree of control of gene expression than the independent action of such factors. Indeed, dynamic competitive interactions between constitutive and inducible κB binding factors for the κB enhancer have been reported to regulate TNF-α-induced expression of MHC class I genes (22), and wild type v-*rel* oncoprotein can act as an inhibitor by competing with κB enhancer binding protein (23). Moreover, Jun-B binds to the AP-1 binding site and inhibits transcriptional activation mediated by Jun homodimers or Jun Fos heterodimers (24, 25). Similarly, Δ FosB can inhibit the transcriptional activity of Fos and Jun (26).

Taken together, our results support the conclusion that the interaction of NREB and CREB, which compete for an overlapping sequence in mouse Ren-1^d, may determine the tissue-specific mouse renin gene expression. Moreover, these results suggest that the presence of NREB within a given tissue may be necessary, but not always sufficient, to silence genes containing an NRE sequence.

Acknowledgments

We appreciate greatly the secretarial assistance of Melinda Hing in the preparation of this manuscript.

This work is supported by NIH grants HL-35610, HL-35252, and HL-42663, and the University of California Tobacco Related Disease

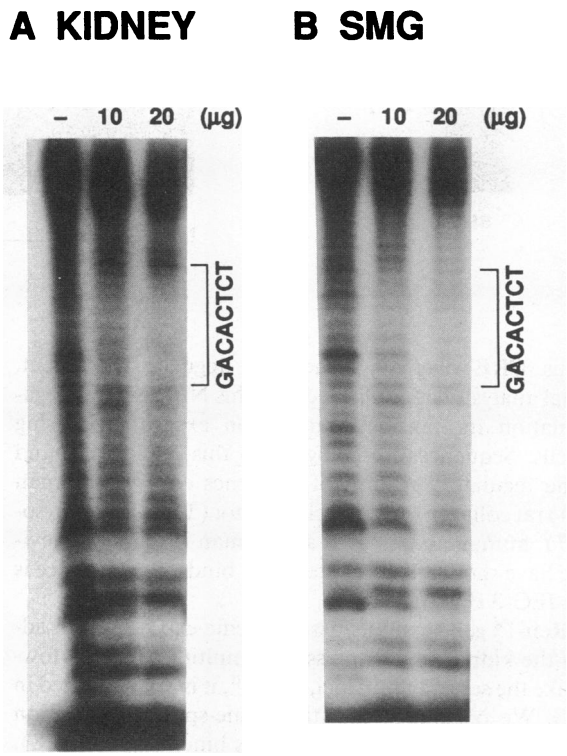

Figure 7. DNase I footprinting analysis of kidney (*A*) and SMG nuclear extracts with the ^{32}P end-labeled DNA probe (−625 to −586). Nuclear extracts (10, 20 μg) prepared from kidney and SMG were incubated with the ^{32}P end-labeled DNA probe (1.0 ng, 15,000 cpm), which contained the entire sequence of the NRE and CRE before addition of DNase. After digestion and deproteinization the samples were separated on 8% urea-polyacrylamide gels.

Program 1RT215, and an unrestricted gift from the Bristol-Myers Squibb Fund for Cardiovascular Research.

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