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Biochimica et Biophysica Acta 1625 (2003) 280–290
BIOCHIMICA ET BIOPHYSICA ACTA
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Regulation of renin enhancer activity by nuclear factor I and Sp1/Sp3

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Received 17 June 2002; received in revised form 11 November 2002; accepted 23 December 2002

Abstract

Transcription of the mouse *Ren-1*^{c} gene in kidney tumor-derived As4.1 cells, which express high levels of renin mRNA, is dependent on a proximal promoter element and a 242-bp enhancer region located 2.6 kb upstream of the transcription start site. We showed previously that the enhancer contains a cAMP responsive element (CRE) and an E-box. Mutation of either element resulted in almost complete loss of the *Ren-1*^{c} expression. In this report we show that there are additional transcription factor-binding sites within the *Ren-1*^{c} enhancer contributing to the enhancer activity. Electrophoretic mobility shift and supershift assays have identified four nuclear factor I (NFI)-binding sites, an Sp1/Sp3 site and an unidentified transcription factor-binding site (Ei) located upstream of the CRE and E-box. Mutation of the Sp1/Sp3 site or Ei reduced *Ren-1*^{c} expression by 40% or 30%, respectively, while mutations of four NFI-binding sites resulted in an 89% decrease in expression. Thus, these protein–DNA interaction sites are essential for transcription of mouse renin genes. There are four homologous NFI genes (NFI-A, -B, -C and -X) in vertebrates and multiple alternatively spliced isoforms from each gene. Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) assays have demonstrated that NFI-X is the predominant NFI mRNA expressed in As4.1 cells. Direct study of involvement of NFI-X in regulation of renin genes is underway.

Keywords: Renin gene; Transcription; Enhancer; As4.1 cell; NFI; Sp1/Sp3

1. Introduction

Renin is an aspartyl protease which catalyzes the first step in the formation of angiotensin II, a potent vasoactive hormone. The expression of renin genes is developmentally and tissue-specifically regulated (for reviews, see Refs. [1,2]). Renin expression can be detected at 14.5 and 15.5 days of gestation in mice and rats, respectively, in the earliest developing intrarenal arteries [3,4]. As the renal arterial tree develops, renin is expressed in the newly forming arterial branches. Subsequently, renin expression is progressively restricted to smaller arteries and arterioles until in the adult it is normally found in a small population of modified smooth muscle cells of the afferent arteriole called juxtaglomerular cells. A number of other tissues also express renin genes in mice, including submandibular gland, adrenal gland, testes, ovary, anterior prostate, placenta and fetal subcutaneous tissue [5–11].

Some strains of mice have only a single renin gene (*Ren-1*^{c}), whereas others contain a duplicated renin locus designated as *Ren-2*, which is closely linked to the *Ren-1* locus [12,13]. In the mouse kidney, these renin genes are approximately equivalently expressed [7]. However, they are differentially expressed in some extrarenal tissues.

Progress has been made recently in elucidating the mechanisms involved in the transcriptional regulation of mouse renin genes. Two important regulatory regions have been identified in *Ren-1*^{c} using a kidney tumor-derived As4.1 cell line, which was developed from transgenic mice containing the *Ren-2* 5′-flanking sequence fused to SV40 T antigen [14]. A proximal promoter element located at − 60 base pairs (bp) is essential for *Ren-1*^{c} expression since its mutation almost completely abolishes the transcriptional activity of 4.1 kilo-base pairs (kb) of *Ren-1*^{c} 5′-flanking sequence [15]. The proximal promoter element has recently been found to be a HOX·PBX binding site [16]. Homeodomain proteins PBX1b and HOX9/10 members

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doi:10.1016/S0167-4781(03)00016-2

can bind to this element with high affinities. A 242-bp enhancer from −2866 to −2625 is also essential for *Ren-1*^{c} expression [15]. A number of transcription factor-binding sites have been identified within the enhancer, including a cyclic AMP responsive element (CRE) (Ed), an E-box (Ee), two retinoic acid receptor/retinoic X receptor-binding sites (Eb and Ec) and a nuclear factor-Y binding site (Ea) [17–19]. Mutation of either the CRE or E-box results in almost complete loss of enhancer activity, suggesting the critical roles these two elements in regulating renin gene expression [17]. Meanwhile, Eb and Ec have been shown to be essential not only for basal but also for retinoic acid-activated renin gene expression [18]. Besides the above elements, the renin gene enhancer also contains other important and unidentified *cis*-acting elements located 5′ to the CRE [17].

Here we report the identification of transcription factor-binding sites within the distal portion of the renin gene enhancer and transcription factors binding to these elements. We have identified four nuclear factor I (NFI)-binding sites, an Sp1/Sp3 site and an unidentified transcription factor-binding site within the enhancer region from −2866 to −2699. Results from mutational analysis suggest that these *cis*-acting elements are essential for high-level expression of mouse *Ren-1*^{c} gene.

2. Materials and methods

2.1. Plasmid construction

Plasmid 2866, which is same as 2625enh, and 2625 are described in Pan et al. [17]. Plasmids 2777, 2738, 2713 and 2699 were constructed by inserting the polymerase chain reaction (PCR)-synthesized *Ren-1*^{c} enhancer sequences from −2777 to −2625, −2738 to −2625, −2713 to −2625 and −2699 to −2625 into the *Xho*I/*Bgl*II sites in 2625, respectively. All the site-specific mutants in Fig. 8 were constructed by inserting the PCR-synthesized mutant *Ren-1*^{c} enhancers into the *Xho*I/*Bgl*II sites in 2625.

2.2. Cell culture and transient transfections

As4.1 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and transfected using FuGENE 6 (Roche Applied Science). For each transfection in a 35-mm culture dish, 2.2-μg DNA including 0.5 μg of reporter plasmid, 1.5 μg of non-specific plasmid and 0.2 μg of plasmid containing Rous sarcoma virus promoter driving β-galactosidase (RSV-βgal) were mixed with 4.4 μl of FuGENE reagent. Twenty-four hours after transfection, cells were harvested and measured for luciferase (Luc) and β-galactosidase (β-gal) activities using Luciferase Assay System (Promega) and Galacto-Light Plus™ chemiluminescent reporter assay (Tropix), respectively. The Luc activity is normalized with β-gal activity to correct differences in transfection efficiency between experiments. All transfection results represent the mean ± S.E. of at least three separate experiments.

2.3. Electrophoretic mobility shift assay (EMSA)

For each reaction (10 μl), about 0.2 ng of labeled DNA probe (20,000 cpm) was mixed with As4.1 cell nuclear extract (3–6 μg) and 1 μg of polydI–dC or polydG·polydC in 10 mM Hepes pH 7.9, 10 mM KCl, 50 mM NaCl, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 10% glycerol. Reaction mixture was incubated on ice for 15 min and then run on 5% polyacrylamide gel in 0.5× Tris–borate–EDTA buffer. In a competition or supershift assay, excess amount of unlabeled DNA or 1 μl of antibody was added to reaction mixture 15 min or 1 h prior to the addition of labeled DNA probe, respectively. Antibodies against Sp1 and Sp3 were purchased from Santa Cruz Biotechnology, Inc. The NFI antiserum and preimmune serum (8199) [20] were kindly provided by Dr. N. Tanese (New York University Medical Center).

2.4. RNA isolation and cDNA synthesis

Total RNA from As4.1 cells was isolated by Trizol reagent (Invitrogen), treated with RNase-free DNase I (0.125U/μg RNA) (Promega), and then subject to reverse transcription using oligo dT primer and MultiScribe™ reverse transcriptase in TaqMan (Applied Biosystems).

2.5. Analysis of NFI gene expression using reverse transcriptase-PCR (RT-PCR) with degenerate primers

The conserved DNA-binding regions of NFI genes were amplified from the reverse transcription product by PCR. The degenerate PCR primers are 5′-tgacaagcttGGATGA(A/G)TT(T/C)CA(T/C)CCITT(T/C)AT(T/C)GA(A/G)GC-3′ and 5′-tgactctagaAT(A/G)TG(A/G)TG(C/G/T) GGC-TGIA(T/C)(A/G)CAIAG-3′ [21,32]. The 490-bp fragments resulting from RT-PCR were isolated and cloned. Sequences of individual clones were then determined.

2.6. Analysis of NFI gene expression using real-time RT-PCR

PCR was performed with 1.5 μl of reverse transcription reaction, 12.5 μl of SYBR Green Master Mix (Applied Biosystems) and 0.1 μg of each primer in a total volume of 25 μl. The PCR program was 10 min at 95 °C, and then 30 cycles of 15 s at 95 °C and 1 min at 60 °C. The reaction was run on an ABI Prism 7700 Sequence Detection System (Applied Biosystems). A threshold cycle (Ct) value, which is the cycle number at which an increase in signal associated with an exponential growth of PCR product is detected, was determined by the Sequence Detection System. The relative

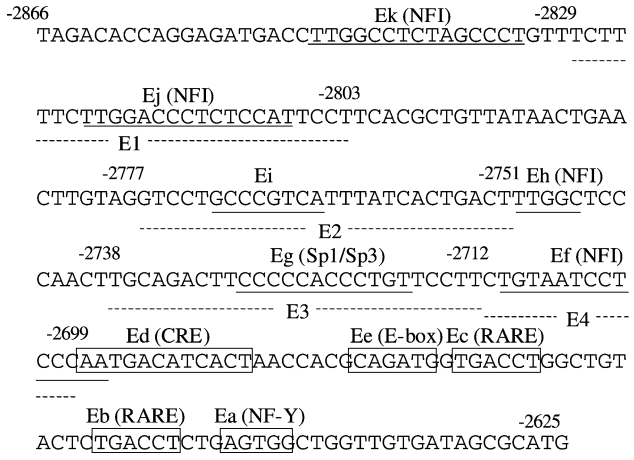


Fig. 1. Sequence of the *Ren-1*^{c} enhancer. The sequence of the *Ren-1*^{c} enhancer (-2866 to -2625) is shown. Both previously (boxed) and presently (underlined) identified transcription factor-binding sites, including the NF-Y binding site (NF-Y), retinoic acid responsive element (RARE), E-box, CRE, the NFI binding site (NFI) and the Sp1/Sp3 binding site (Sp1/Sp3), are labeled (Ea–Ek). The regions (E1–E4) shown to contribute to the enhancer activity when the enhancer was placed immediately upstream of the *Ren-1*^{c} promoter (-117 to $+6$) [17] are indicated by the dotted lines.

expression level of a specific gene is expressed as ΔCt, which equals the Ct for this gene minus the Ct for β_2-microglobulin. All primer sets were validated by showing that the slopes of the standard curves of dilutions of RT products were between -3.4 and -3.7. The NFI-A, NFI-B, NFI-C and NFI-X primers were also validated using standard curves of plasmid DNAs and show an absolute Ct difference of less than 1 at the target levels used here. Primer sequences used in SYBR RT-PCR are as follows: NFI-A forward 5′-GGCATACTTTGTACATG-CAGC-3′, reverse 5′-CCTGATGTGACAAAGCTGTCC-3′; NFI-B forward 5′-TTTTTGGCATACTACGTGCAGG-3′, reverse 5′-TCTGATACATTGAAGACTCCG-3′; NFI-C forward 5′-ACCTGTACCTGGCCTACTTTG-3′, reverse 5′-ACACCTGACGTGACAAAGCTC-3′; NFI-X forward 5′-TGGCTTACTTTGTCCACACTC-3′, reverse 5′-CAGC-TCTGTCACATTCCAGAC-3′; β_2-microglobulin forward 5′-GACTGATACATACGCCTGCAG-3′, reverse 5′-CAG-GTTCAAATGAATCTTCAG-3′. ΔCt values for mNFI mRNAs obtained from As4.1 cells represent the mean $\pm$ S.E. of four separate experiments. The mean Ct value for control transcript β_2-microglobulin in As4.1 cells was 17.6 ± 0.2. High ΔCt's reflect lower levels while low ΔCt's indicate higher levels of transcripts.

3. Results

3.1. Distal portion of the *Ren-1*^{c} enhancer is essential for the mouse renin expression in As4.1 cells

Our previous results showed that four elements (designated E1–E4) within the distal portion of *Ren-1*^{c} enhancer (-2866 to -2699) (see Fig. 1 for sequence of the *Ren-1*^{c} enhancer and positions of E1–E4) contributed to the full enhancer activity when the enhancer was placed immediately upstream of the *Ren-1*^{c} promoter (-117 to $+6$) [17]. To test whether these elements are necessary for the enhancer activity when the enhancer is placed in its natural position at -2625, a series of deletion mutants were made based on construct 2866 and transfected into As4.1 cells

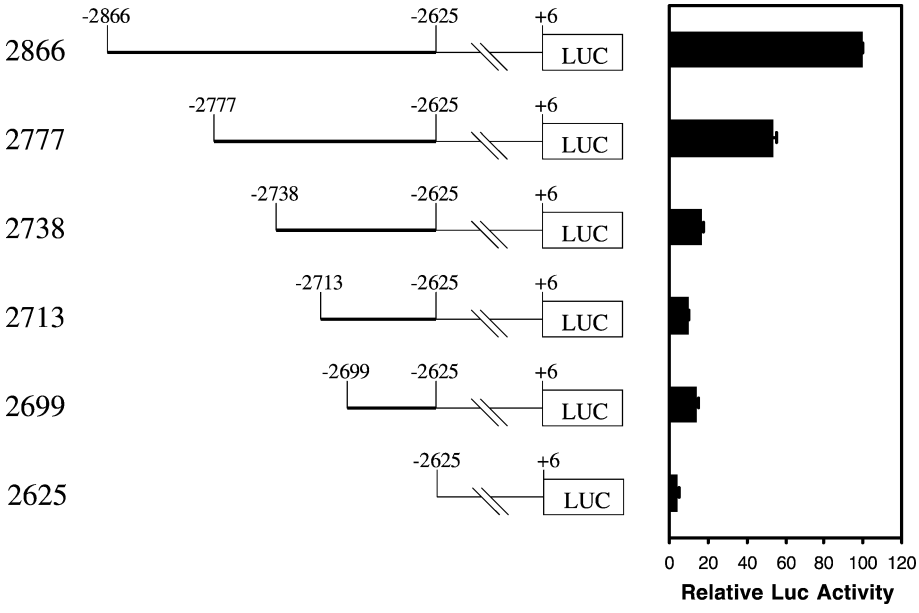


Fig. 2. Distal portion of the *Ren-1*^{c} enhancer (-2866 to -2699) contains regulatory regions essential for *Ren-1*^{c} expression in As4.1 cells. As4.1 cells were transfected with constructs shown in the left panel. The luciferase (Luc) activity is expressed relative to that of plasmid 2866 (arbitrarily set to 100).

(Fig. 2). Deletion of region from −2866 to −2777, which includes the element E1, caused a 46 ± 1.0% decrease in *Ren-1*^{c} expression. Further deletion to −2738 reduced the expression by 83 ± 0.9%, which is about equal to the percentage drop (86 ± 1.0%) when the distal portion of the enhancer is deleted. These results demonstrate that the distal portion of the *Ren-1*^{c} enhancer contains binding sites for transcription factors which are critical for the *Ren-1*^{c} expression.

3.2. Sequence from −2866 to −2803 contains two NFI binding sites

To determine whether there is a DNA–protein interaction site within E1, EMSA was performed using double-stranded oligonucleotide E1 (Fig. 3A) and nuclear extracts prepared from As4.1 cells. A DNA·protein complex was observed (Fig. 3B, lane 1) which was competed out by 100-fold molar excess of unlabelled E1 (Fig. 3B, lane 2). To better define

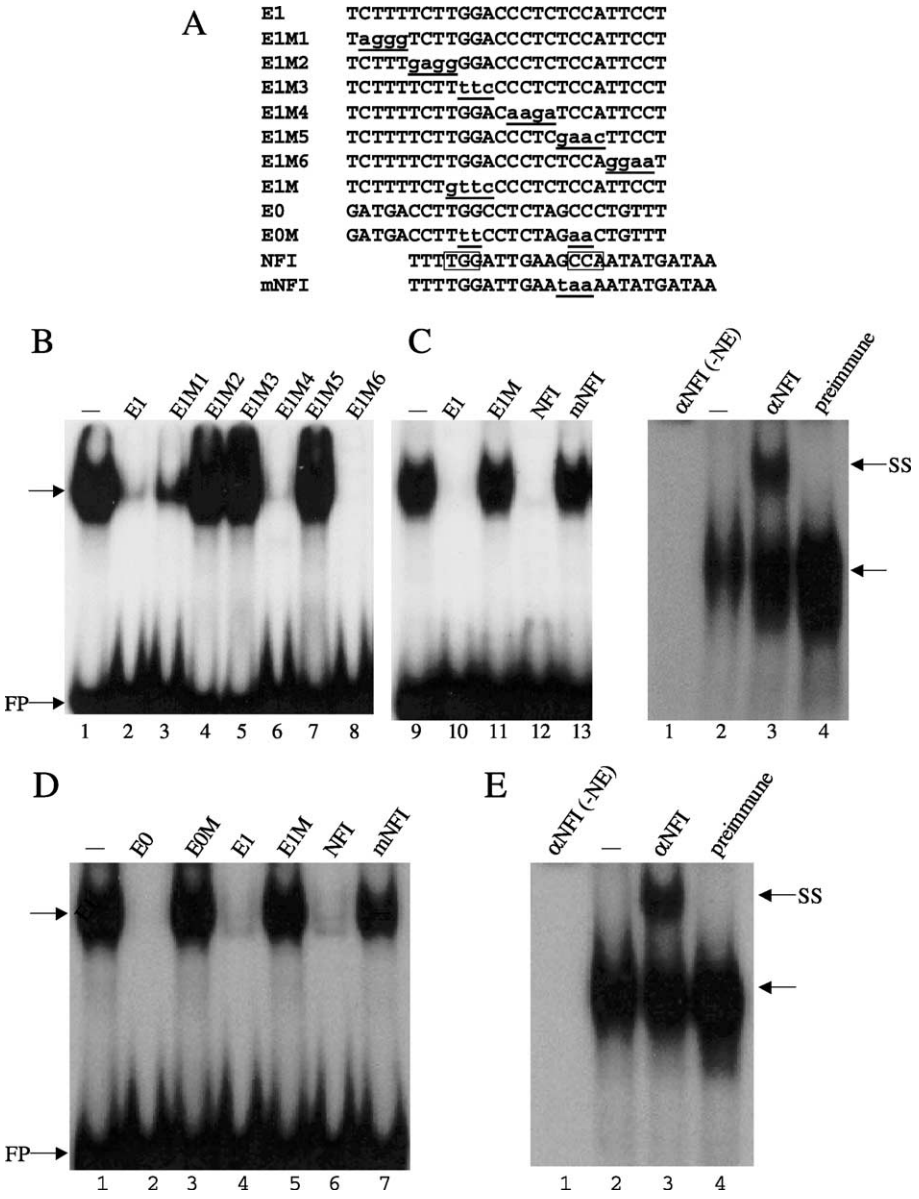


Fig. 3. Identification of two NFI-binding sites within the region from −2854 to −2803 (E1). (A) Shown are the sequences of the double-stranded oligonucleotides that were used in EMSAs. E1 and E0 represent wild-type mouse *Ren-1*^{c} sequences from −2829 to −2803 and −2854 to −2829, respectively. E1M1–E1M6, E1M and E0M contain selectively altered bases (indicated by underlined lower case letters). The critical nucleotides in oligonucleotides NFI involved in the NFI binding are boxed. E1 (B) or E0 (D) was used as the probe in EMSAs without competitor (− lane), or with specific oligonucleotides indicated as competitors. A 100-fold molar excess of competitor over the probe was used. Free probe is indicated by FP. Supershift EMSAs were performed using E1 (C) or E0 (E) as probe. As4.1 cell nuclear extract was added in each lane except lane 1, in which only antibody against NFI (αNFI) was added. αNFI and preimmune serum were added in lanes 3 and 4, respectively. In lane 3, a supershifted complex (SS) was observed. The specific DNA·protein complexes are indicated by arrows.

the specific base pairs within E1 involved in the complex formation, competition assays were performed using six double-stranded oligonucleotides containing 3- or 4-bp mutations (Fig. 3A). The results showed that 100-fold molar excess of mutant E1 oligonucleotides E1M1, E1M4 and E1M6 competed for complex formation whereas E1M2, E1M3, E1M5 and E1M failed to compete (Fig. 3B, lanes 3–8). Thus, the protein–DNA interaction site was confined to sequence motif TCTTGGACN$_4$TCCA, which is similar to the consensus binding site for NFI (TGGN$_7$CCA) [22]. Further competition assays showed that the As4.1·E1 complex was competed out by 100-fold molar excess of unlabelled NFI, an oligonucleotide containing a consensus NFI binding site (Fig. 3B, lane 12) but not by an oligonucleotide containing a mutant NFI site (mNFI) (Fig. 3B, lane 13). The presence of NFI binding site (Ej) within E1 was also demonstrated by the supershift EMSAs. The As4.1·E1 complex was supershifted by an antibody against NFI (Fig. 3C, lane 3) but not by the preimmune serum (Fig. 3C, lane 4). Addition of either the anti-NFI or preimmune serum increased the intensity of retarded bands (Fig. 3C, lane 3C, lane 2 versus 3 or 4), suggesting that the added serum proteins stabilized the formation of DNA·protein complex.

A sequence homologous to an NFI recognition sequence (Ek) was also identified within the region from −2866 to −2829 by sequence comparison (Fig. 1). EMSA analysis showed that double-stranded oligonucleotide E0, which contains Ek sequence, was able to bind As4.1 cell nuclear proteins (Fig. 3D, lane 1). The E0·As4.1 cell nuclear protein complex was competed out by 100-fold molar excess of unlabelled E0 itself (Fig. 3D, lane 2), E1 (Fig. 3D, lane 4) and NFI (Fig. 3D, lane 6) but not by a mutated E0 (E0M) containing mutations in nucleotides critical for the NFI binding (Fig. 3D, lane 3), E1M (Fig. 3D, lane 5) and mNFI (Fig. 3D, lane 7). Results from supershift EMSAs showed that the E0·As4.1 nuclear protein complex was supershifted by the anti-NFI antibody (Fig. 3E, lane 3) but not by the preimmune serum (Fig. 3E, lane 4). These results suggest that Ek element is an NFI-binding site.

3.3. Sequence from −2777 to −2738 contains an unidentified transcription factor binding site and a half site for NFI

EMSA was used to investigate whether there are transcription factors binding to E2 (Fig. 4A). Results showed the formation of the E2/As4.1 cell nuclear protein complex (Fig. 4B, lane 1), which was competed out by 100-fold molar excess of unlabelled E2 itself (Fig. 4B, lane 2). To more precisely define the DNA–protein interaction site, a series of E2 mutants containing sequential 4-bp mutations were used in competition EMSAs (Fig. 4A). E2M1, E2M4, E2M5 and E2M6 were able to compete for the complex formation (Fig. 4B, lanes 3, 6–8) whereas E2M2 and E2M3 were

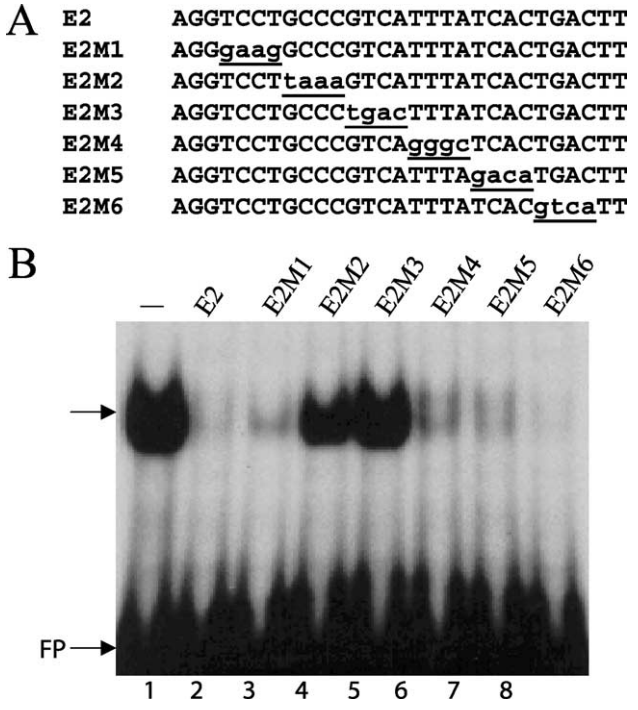


Fig. 4. Sequence from −2777 to −2738 (E2) contains a transcription factor-binding site. (A) Shown are the sequences of the double stranded oligonucleotides that were used in EMSAs. E2 represents wild-type mouse *Ren-1*^{c} sequence (−2777 to −2738). E2M1–E2M6 contain selectively altered bases (indicated by underlined lower case letters). (B) E2 was used as the probe in EMSAs without competitor (− lane), or with specific oligonucleotides (100X) indicated as competitors. The specific complex formed with As4.1 cell nuclear extract and E2 is indicated by an arrow. Free probe is indicated by FP.

unable to compete. These results suggest that sequence motif GCCCGTCA (Ei) is a DNA–protein interaction site. Search of the transcription factor database suggested that this motif is similar to the *sis*-inducible factor-binding element, cyclic AMP responsive element and AP-1 binding site [23]. However, competition EMSAs indicated that none of these elements could compete for E2 complex formation (data not shown). Thus, the transcription factors binding to Ei remains to be determined.

A survey of nucleotide sequences immediately downstream of the Ei element revealed a half site for NFI (Eh: TTGGC), which interacts with NFI with lower affinity compared to a strong NFI binding site [24]. EMSA showed formation of two complexes (L and S) between E5 (Fig. 5A) containing the NFI half site and As4.1 cell nuclear proteins only with polydG·polydC as nonspecific competitor but not with poly(dI–dC) (Fig. 5B, lane 1). E5 itself but not E5M (Fig. 5A), which contains a mutated NFI half site, could compete for the major complex (L) formation (Fig. 5B, lanes 2 and 3). Moreover, the major complex was competed out by E1 (Fig. 5B, lane 4) and NFI (Fig. 5B, lane 6), both of which contain a strong NFI binding site, but not by the mutated E1 (E1M) (Fig. 5B, lane 5). However, the mutated consensus NFI binding site (mNFI) was able to compete the

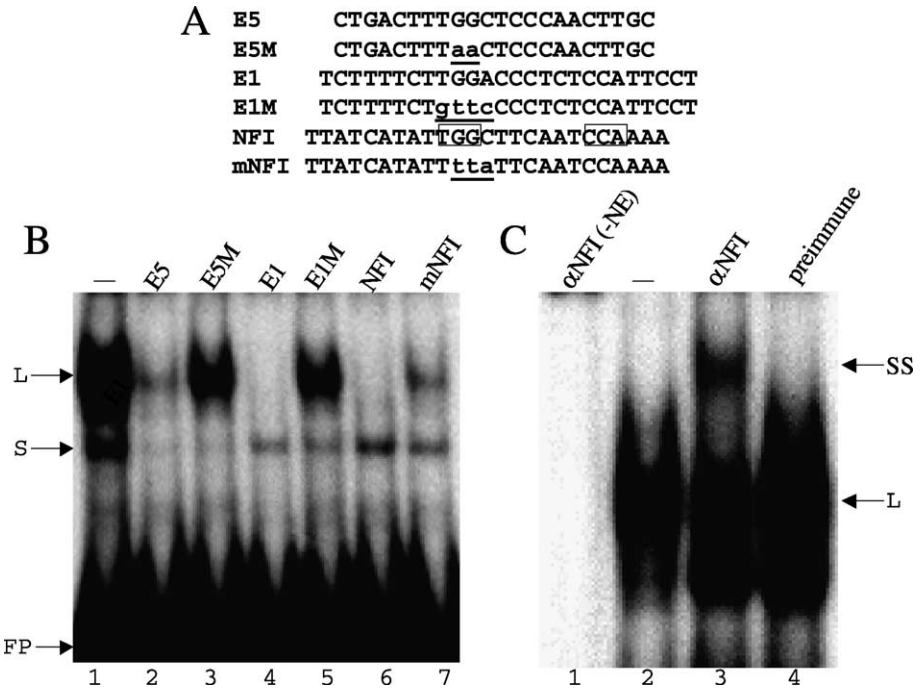


Fig. 5. Identification of a half binding-site for NFI. (A) Shown are the sequences of the double stranded oligonucleotides that were used in EMSAs. E5 represents wild-type mouse *Ren-1*^{c} sequence (− 2757 to − 2736). E5M contains selectively altered bases (indicated by underlined lower case letters). (B) E5 was used as the probe in EMSA without competitor (− lane), or with specific oligonucleotides (100 ×) indicated as competitors. Free probe is indicated by FP. (C) E5 was used as probe in supershift EMSAs, which were performed as described in Fig. 3C. The specific complexes formed with As4.1 cell nuclear extract and E5 are indicated by L and S.

major complex formation to some extent (Fig. 5B, lane 7) since there is still a weak half NFI binding site (TTGGA) present in mNFI. Furthermore, supershift EMSAs demonstrated that the major complex was supershifted by the anti-NFI antibody (Fig. 5C, lane 3) but not by the preimmune serum (Fig. 5C, lane 4). These results suggest that the major complex formed contains NFI binding to the Eh element. The minor complex (S) formed between E5 and As4.1 cell nuclear proteins could be competed out by both E5 and E5M but not by either E1 or NFI, suggesting that the minor complex does not contain NFI. Mutated oligonucleotide E5 containing mutations in both the third and forth nucleotides (CC) downstream of Eh failed to compete for the minor complex formation (data not shown), suggesting that these two nucleotides are involved in the formation of the minor complex. However, results from transfection assays showed that mutation of these two nucleotides did not affect the *Ren-1*^{c} expression in As4.1 cells, indicating that proteins involved in the formation of minor complex might not be important for the renin gene expression in As4.1 cells (data not shown).

3.4. Sequence from −2738 to −2712 contains an Sp1/Sp3 binding site

To define the transcription factor binding site within E3 (− 2738 to − 2712) (Fig. 6A) EMSAs were performed using As4.1 cell nuclear extracts. Four E3/As4.1 cell nuclear protein complexes (I–IV) were detected, which could be competed out by E3 itself (Fig. 6B, lanes 1 and 2). Six double-stranded oligonucleotides containing sequential 4-bp mutations were used as competitors to better locate the DNA–protein interaction site (Fig. 6A). Oligonucleotides E3M1, E3M2 and E3M6 competed efficiently for the formation of Complexes I to IV (Fig. 6B, lanes 3, 4 and 8) whereas E3M3 and E3M4 failed to compete for any of four complexes (Fig. 6B, lanes 5 and 6). E3M5 failed to compete for two major complexes I and II while it competed complexes III and IV (Fig. 6B, lane 7). Thus, the sequence motif important for the transcription factor binding within E3 is CCCCCACCCTGT, which contains a CACCC-box, a binding site for Sp and Krüppel-like factor family of transcription factors (for review, see Ref. [25]).

Competition EMSAs were then performed using oligonucleotides containing the β-globin CACCC-box (CACCC) [31] or consensus Sp1-binding site (Sp1) as competitors (Fig. 6A and B). The Sp1 site competed for the formation of all four complexes (Fig. 6B, lane 14) whereas CACCC competed for only complexes I and II (Fig. 6B, lane 12). As expected, mutated CACCC and Sp1 oligonucleotides (Fig. 6A) failed to compete (Fig. 6B, lanes 13 and 15). Supershift EMSAs using antibodies against Sp1 (αSp1) and Sp3 (αSp3) demonstrated that

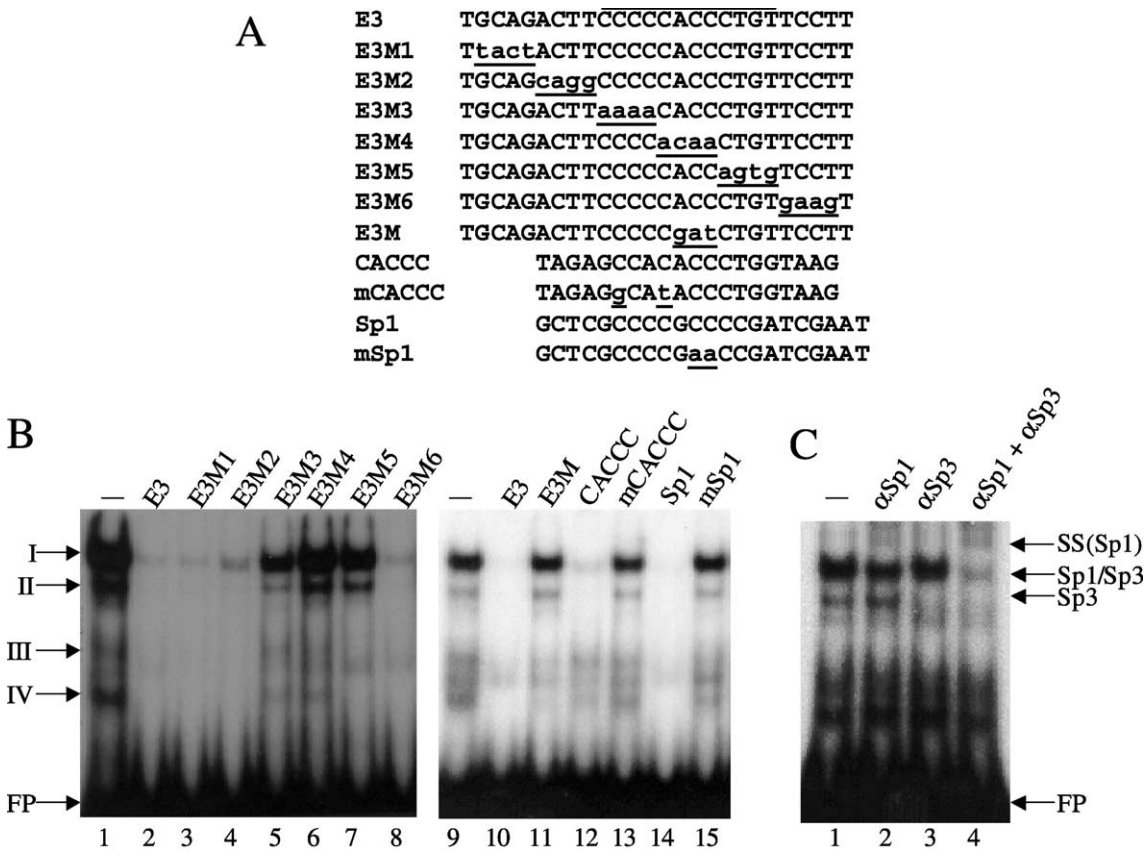


Fig. 6. Identification of an Sp1/Sp3 binding site within the region from −2738 to −2712 (E3). (A) Shown are the sequences of the double-stranded oligonucleotides that were used in EMSAs. E3 represents wild-type mouse *Ren-1*^{c} sequence (−2738 to −2712). E1M3–E3M6 and E3M contain selectively altered bases (indicated by underlined lower case letters). (B) E3 was used as the probe in EMSA without competitor (− lane), or with specific oligonucleotides (100×) indicated as competitors. Nuclear extracts were prepared from As4.1 cells. The specific complexes (I, II, III and IV) were formed with As4.1 cell nuclear extract and E3. Free probe is indicated by FP. (C) EMSAs were performed with probe E3 and As4.1 cell nuclear extracts. No antibody was added in − lane. Antibodies against Sp1 (αSp1) or/and Sp3 (αSp3) were added in lanes 2–4 as indicated. The αSp1-supershifted complex is indicated by SS (Sp1).

complex I contains Sp1 and Sp3 while complex II contains a truncated Sp3 (Fig. 6C, lanes 2–4), which might arise by the utilization of a different translational initiation site [26]. Since intensities of complexes III and IV were not affected by the addition of Sp antibodies, whether these two minor complexes are truncated Sp family members remains to be determined.

3.5. Sequence from −2712 to −2698 contains a weak NFI binding site

Like Eh, the half NFI binding site, oligonucleotide E4-2 containing E4 (Fig. 1) only forms complex with As4.1 nuclear proteins in the presence of nonspecific competitor polydG·polydC but not poly(dI–dC) (Fig. 7B, lane 1). Survey of nucleotide sequences of E4 revealed an NFI recognition sequence-like sequence (Ef: TGTN$_7$CCA). Competition EMSAs showed that the complex formed with E4-2 and As4.1 cell nuclear proteins could be competed out by E4-2 itself, E1 and NFI (Fig. 7B, lanes 2, 3 and 5) but not by E1 mutant (E1M) (Fig. 7B, lane 4). The complex could also be partially competed out by mNFI (Fig. 7B, lane 6), suggesting that Ef is a weak NFI-binding site similar to Eh. Binding of NFI to Ef element was further demonstrated by the supershift EMSAs. The Ef·As4.1 cell nuclear protein complex was supershifted by the anti-NFI antibody but not by the preimmune serum (Fig. 7C).

3.6. Roles of identified transcription factor-binding sites in regulating *Ren-1*^{c} expression

To test the amount that each newly identified transcription factor-binding site contributes to the *Ren-1*^{c} enhancer activity, mutations were introduced into the enhancer region of construct 2866. Mutation of Ek alone did not significantly affect the *Ren-1*^{c} expression in As4.1 cells (Fig. 8A). Mutation of Ej, Ei, Eh, Eg or Ef resulted in

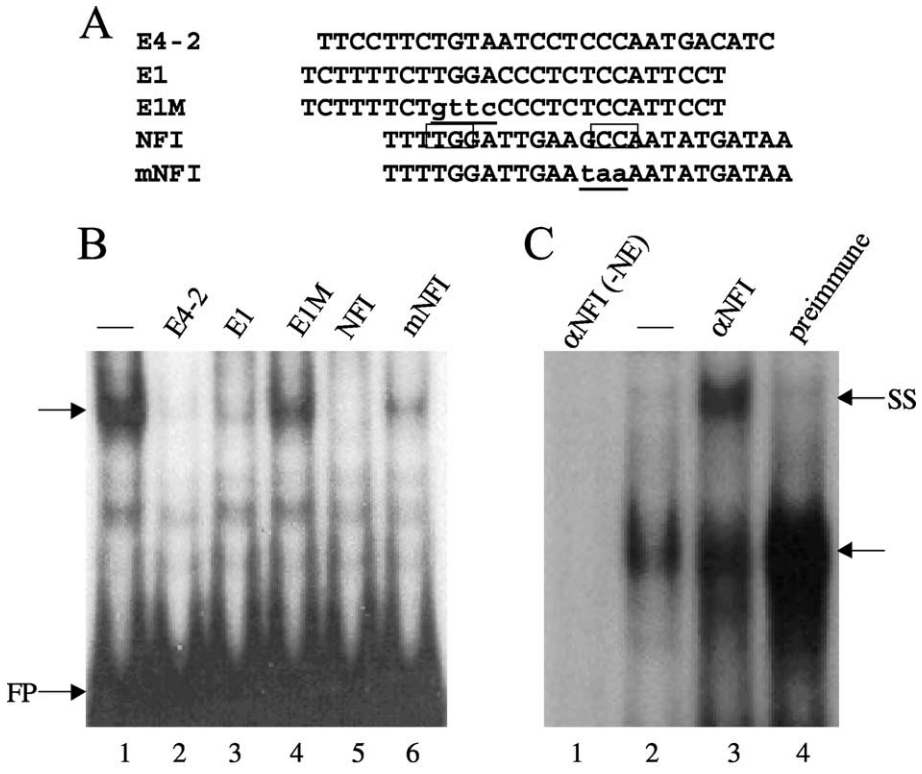
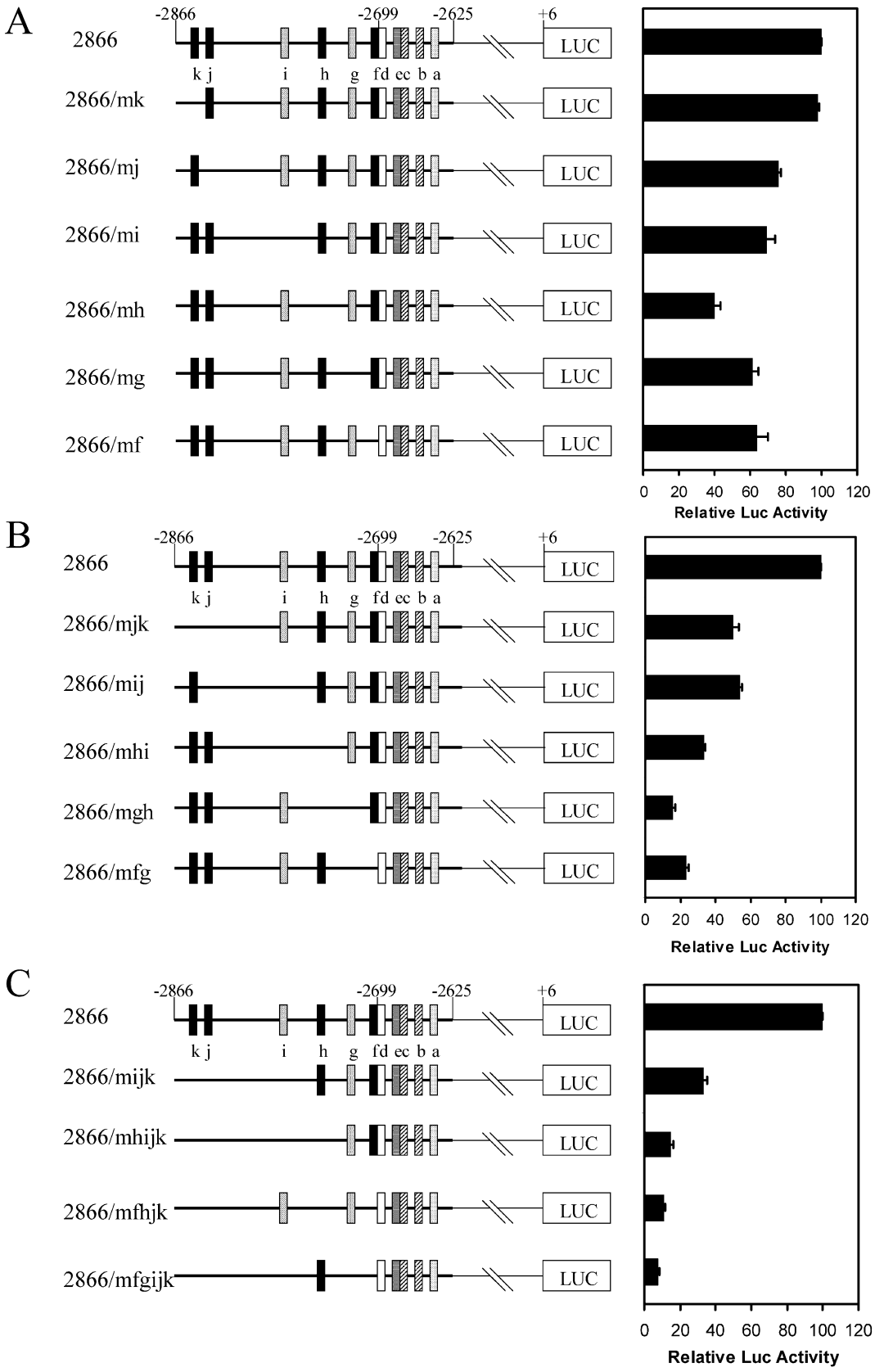


Fig. 7. Identification of an NFI-binding site within the region from −2712 to −2699 (E4). (A) Shown are the sequences of the double-stranded oligonucleotides that were used in EMSAs. E4-2 represents wild-type mouse *Ren-1*^{c} sequence (−2718 to −2691). (B) E4-2 was used as the probe in EMSA without competitor (− lane), or with specific oligonucleotides (100×) indicated as competitors. Free probe is indicated by FP. (C) E4-2 was used as probe in supershift EMSAs, which were performed as described in Fig. 3C. The specific complexes formed with As4.1 cell nuclear extract and E4-2 are indicated by arrows.

24 ± 1.2%, 31 ± 5.1%, 60 ± 3.9%, 39 ± 3.6% or 36 ± 6.0% reduction in *Ren-1*^{c} expression, respectively. When both Ek and Ej were mutated, transcriptional activity was reduced by 50 ± 3.1% (Fig. 8B), which is equal to the percentage drop when the region from −2866 to −2777 was deleted from construct 2866. The result indicates that Ek and Ej can cooperate with each other to contribute to the enhancer activity. Mutations of both Ej and Ei, Ei and Eh, Eh and Eg, or Eg and Ef reduced expression by 46 ± 1.0%, 66 ± 0.8%, 84 ± 1.6% or 77 ± 1.5%, respectively. Thus, mutations of both Eh and Eg resulted in a reduced expression level equal to that of construct 2699, in which the whole distal portion of enhancer is deleted (Fig. 2), suggesting the critical roles these two elements in regulating the enhancer activity. Constructs containing more than two *cis*-elements mutated were also tested in As4.1 cells (Fig. 8C). Mutations of distal four elements (2866/mhijk) or all four NFI binding sites (2866/mfhjk) completely abolished the activity of distal portion of the enhancer. These results demonstrated that all these newly identified transcription factor-binding sites contribute to the enhancer activity.

3.7. NFI-X is the predominant NFI mRNA expressed in As4.1 cells

There are four homologous NFI genes in vertebrates (NFI-A, -B, -C, and -X) and multiple alternatively spliced isoforms from each gene (for review, see Ref. [27]). To determine which NFI protein might be involved in regulation of mouse renin gene, real-time RT-PCR was performed using RNA from As4.1 cells. PCR primers for NFI were designed from the region of the 5′ DNA binding domains so that all the 3′ alternatively spliced NFI isoforms would be quantified in a single PCR. Results showed that the expression level of either NFI-A (ΔCt = 9.7 ± 0.5) or NFI-B (ΔCt = 12.4 ± 0.2) or NFI-C (ΔCt = 8.6 ± 0.4) is extremely low whereas the expression level for NFI-X (ΔCt = 1.3 ± 0.2) is very high. These results strongly suggest a critical role of NFI-X in regulating *Ren-1*^{c} expression in As4.1 cells. The relative abundance of NFI-X in As4.1 cells was also demonstrated by RT-PCR using degenerate primers. Fragments from the highly conserved NFI DNA binding domains were amplified and cloned into a vector. A total of 28 clones were sequenced and results showed that 27 of these are NFI-X.


A
-2866
-2699
-2625
+6
2866
k j
i
h
g
fd ec b a
LUC
2866/mk
2866/mj
2866/mi
2866/mh
2866/mg
2866/mf
0 20 40 60 80 100 120
Relative Luc Activity
B
2866
2866/mjk
2866/mij
2866/mhi
2866/mgh
2866/mfg
C
2866
2866/mijk
2866/mhijk
2866/mfhjk
2866/mfgijk

4. Discussion

In this study, six transcription factor-binding sites were identified within the distal portion of *Ren-1*^{c} enhancer (−2866 to −2699). These newly identified elements include four NFI-binding sites, an Sp1/Sp3-binding site and an unidentified transcription factor-binding site. Mutational analysis showed that all these elements contribute to high-level expression of the *Ren-1*^{c} gene. However, the half NFI binding site (Eh) appears to be the most critical element. Mutation of Eh caused a 60% reduction in *Ren-1*^{c} expression while mutations of both Eh and Eg resulted in almost complete loss of activity of the distal portion of the *Ren-1*^{c} enhancer. Results from mutational analysis also demonstrated the critical roles of NFI family members in regulating renin gene expression. Mutations of all four NFI-binding sites reduced the *Ren-1*^{c} expression by about 90%.

A sequence which is homologous to the *Ren-1*^{c} enhancer was also found in the human renin 5′ flanking sequence ~12 kb upstream of the promoter [19,30]. These two enhancers share a high degree of similarity, suggesting that the enhancer is important for both human and mouse renin gene expression. Analysis of their sequence alignment has revealed that the most critical *cis*-acting elements previously identified in the *Ren-1*^{c} enhancer are also present in the human enhancer, including the CRE (Ed), E-box (Ee) and the distal retinoic acid-responsive element (Ec). Moreover, results from EMSA analysis indicated that the human enhancer also contains two of the NFI-binding sites (Ej and Eh), the Sp1/Sp3-binding site (Eg) and element Ei identified in the distal portion of the *Ren-1*^{c} enhancer (L. Pan and K. W. Gross, unpublished data), suggesting the importance of these elements in regulating renin gene transcription. However, elements Eb, Ef and Ek, which contribute to the mouse *Ren-1*^{c} enhancer activity, are not present in the human enhancer, accounting for the lower activity of human enhancer in transcriptional activation of a renin promoter compared to the *Ren-1*^{c} enhancer in As4.1 cells [19].

NFI has been shown to be involved in tissue- and cell-specific regulation of gene expression [27]. It is believed that this is achieved by differential expression of many alternatively spliced isoforms of four NFI genes in different tissue and cell types. All four NFI proteins have very conserved N-terminal DNA-binding/dimerization domains but diverse C-terminal transactivation and repression domains. Thus, alternative splicing within the C-terminal domains may create NFI proteins with different transactivation or repression functions. NFI isoforms can form both homodimers and heterodimers on the NFI binding-site, providing more complexity in regulating gene expression [28]. Moreover, it has been shown that activation or repression of gene expression by NFI is cell- and promoter-specific. For example, NFI has been reported to induce the transcriptional activity of the α_{1B} adrenergic receptor gene middle promoter in Hep3B cells while inhibiting expression in DDT_1 MF-2 cells [29]. In JEG-3 cells, NFI-X is the strongest activator of a simple promoter containing a single NFI-binding site upstream of a TATA-box, whereas NFI-B is the strongest activator of the complex NFI-responsive mouse mammary tumor virus promoter [28]. Both real-time RT-PCR and RT-PCR with degenerate primers have shown that NFI-X is the only NFI mRNA abundantly expressed in As4.1 cells, suggesting that NFI-X binds to the NFI sites within the *Ren-1*^{c} enhancer and activates its expression. Whether other NFI isoforms are able to either induce or repress renin expression remains to be determined. The anti-NFI antibody used in this study was generated against a bacterial fusion protein containing human CTF2 (an isoform of NFI-C). The antibody reacts with the C-terminal half of NFI-C. However, whether it cross-reacts with other NFI proteins is not carefully studied. We have shown that this antibody could supershift the As4.1 cell nuclear extract·NFI-binding element complex, suggesting that it may weakly cross-react with NFI-X. Alternatively, the minimal level of NFI-C transcript detected here may be capable of yielding enough NFI-C proteins to form complexes detectable by EMSA.

Mutation of just the CACCC-box (Eg) within the *Ren-1*^{c} enhancer resulted in a 40% reduction in renin expression, indicating that it is an important regulatory element. The Sp1-4 and numerous Krüppel-like factors can bind to the CACCC-box to regulate gene expression [25]. We have shown that only Sp1/Sp3 factors bind to Eg element using As4.1 cell extracts. Also, the oligonucleotide containing consensus Sp1-binding site competed well for the binding activity of the Eg element, suggesting that Eg is an Sp1/Sp3 binding site. However, we cannot exclude the possibility that in some renin-expressing tissues or cells, the tissue-specifically expressed Krüppel-like factors bind to this CACCC-box to regulate renin expression.

We previously showed that the distal portion of *Ren-1*^{c} enhancer alone (−2829 to −2699) was incapable of activating transcription in As4.1 cells when it was placed immediately upstream of the renin promoter (−117 to +6) [17]. It only activates transcription when the proximal

Fig. 8. Mutational analyses of *cis*-acting elements within the distal portion of *Ren-1*^{c} enhancer. As4.1 cells were transfected with 2866 and 2866-based constructs containing single (A), double (B) and multiple (C) mutations in the identified transcription factor-binding sites within the distal portion of the enhancer. Ek, Ej, Ei, Eh, Eg, Ef, Ed, Ee, Ec, Eb and Ea are labeled as k, j, i, h, g, f, d, e, c, b and a, respectively. Mutations introduced into Ek, Ej, Ei, Eh and Eg in these constructs are same as in oligonucleotides E1M (Fig. 3A), E0M (Fig. 3A), E2M3 (Fig. 4A), E5M (Fig. 5A) and E3M (Fig. 6A), respectively, used in EMSAs. A 3-bp mutation was introduced into Ef (TGTAATCCTCCCAA→TGTAATCCgaaCAA) to create constructs containing mutated Ef. Oligonucleotide containing this mutated Ef was not able to compete for the formation of Ef·As4.1 cell nuclear protein complex (data not shown). The luciferase (Luc) activity is expressed relative to that of 2866 (arbitrarily set to 100).

portion of the enhancer (−2699 to −2625) is present. These results suggest that the transcription factors binding to the distal region of the enhancer, including NFI, may not interact with components of general transcription machinery directly. Rather, they may either directly or indirectly interact with factors binding to CRE, E-box or the retinoic acid responsive elements located within the proximal portion of enhancer to regulate renin gene expression.

Acknowledgements

We are grateful to Dr. Naoko Tanese for the gift of NFI antiserum. We also thank Colleen Kane for excellent technical assistance. This work was supported by NIH Grants HL48459 (to K.W.G.), DK58401 and HD34908 (to R.M.G.), and Bruce Cuvelier Family. L. Pan was supported by a postdoctoral fellowship from the National Institutes of Health. This research utilized core facilities supported in part by RPCI's NCI-funded Cancer Center Support Grant, CA16056.

References

[1] C.D. Sigmund, K.W. Gross, Hypertension 18 (1991) 446–457.
[2] C.A. Jones, J.R. Fabian, K.J. Abel, C.D. Sigmund, K.W. Gross, in: M.K. Raizada, M.I. Phillips, C. Sumners (Eds.), Cellular and Molecular Biology of the Renin–Angiotensin System, CRC Press, Boca Raton, USA, 1993, pp. 33–57.
[3] C.A. Jones, C.D. Sigmund, R.A. McGowan, C.M. Kane, K.W. Gross, Mol. Endocrinol. 4 (1990) 375–383.
[4] R.A. Gomez, K.R. Lynch, B.C. Strurgill, J.P. Elwood, R.L. Chevalier, R.M. Carey, M.J. Peach, Am. J. Physiol. 257 (1989) F850–F858.
[5] L.J. Field, K.W. Gross, Proc. Natl. Acad. Sci. U. S. A. 82 (1985) 6196–6200.
[6] C.C.J. Miller, A.T. Carter, J.I. Brooks, R.H.L. Badge, W.J. Brammar, Nucleic Acids Res. 17 (1989) 3117–3128.
[7] J.R. Fabian, L.J. Field, R.A. McGowan, J.J. Mullins, C.D. Sigmund, K.W. Gross, J. Biol. Chem. 264 (1989) 17589–17594.
[8] K.N. Pandey, M. Maki, T. Inagami, Biochem. Biophys. Res. Commun. 125 (1984) 662–667.
[9] M. Ekker, D. Tronik, F. Rougeon, Proc. Natl. Acad. Sci. U. S. A. 86 (1989) 5155–5158.
[10] C.D. Sigmund, C.A. Jones, U. Kim, J.J. Mullins, K.W. Gross, Proc. Natl. Acad. Sci. U. S. A. 87 (1990) 7993–7997.
[11] C.A. Jones, M.I. Hurley, T.A. Black, C.M. Kane, L. Pan, S.C. Pruitt, K.W. Gross, Physiol. Genomics 4 (2000) 75–81.
[12] K.J. Abel, K.W. Gross, Nucleic Acids Res. 16 (1988) 2111–2126.
[13] K.J. Abel, K.W. Gross, Genetics 124 (1988) 937–947.
[14] C.D. Sigmund, K. Okuyama, J. Ingelfinger, C.A. Jones, J.J. Mullins, C. Kane, U. Kim, C. Wu, L. Kenny, Y. Rustum, V.J. Dzau, K.W. Gross, J. Biol. Chem. 265 (1990) 19916–19922.
[15] N. Petrovic, T.A. Black, J.R. Fabian, C. Kane, C.A. Jones, J.A. Loudon, J.P. Abonia, C.D. Sigmund, K.W. Gross, J. Biol. Chem. 271 (1996) 22499–22505.
[16] L. Pan, Y. Xie, T.A. Black, C.A. Jones, S.C. Pruitt, K.W. Gross, J. Biol. Chem. 276 (2001) 32489–32494.
[17] L. Pan, T.A. Black, Q. Shi, C.A. Jones, N. Petrovic, J. Loudon, C. Kane, C.D. Sigmund, K.W. Gross, J. Biol. Chem. 276 (2001) 45530–45538.
[18] Q. Shi, K.W. Gross, C.D. Sigmund, J. Biol. Chem. 276 (2001) 3597–3603.
[19] Q. Shi, T.A. Black, K.W. Gross, C.D. Sigmund, Circ. Res. 85 (1999) 479–488.
[20] C. Santoro, N. Mermod, P.C. Andrews, R. Tjian, Nature 334 (1988) 218–224.
[21] A.Z. Chaudhry, G.E. Lyons, R.M. Gronostajski, Dev. Dyn. 208 (1997) 313–325.
[22] R.M. Gronostajski, S. Adhya, K. Nagata, R.A. Gruggenheimer, J. Hurwitz, Mol. Cell. Biol. 5 (1985) 964–971.
[23] S. Faisst, S. Meyer, Nucleic Acids Res. 20 (1992) 3–26.
[24] M. Meisterernst, I. Gander, L. Rogge, E.L. Winnacker, Nucleic Acids Res. 16 (1988) 4419–4435.
[25] A.R. Black, J.D. Black, J. Azizkhan-Clifford, J. Cell. Physiol. 188 (2001) 143–160.
[26] S.B. Kennett, A.J. Udvadia, J. Horowitz, Nucleic Acids Res. 25 (1997) 3110–3117.
[27] R.M. Gronostajski, Gene 249 (2000) 31–45.
[28] A.Z. Chaudhry, A.D. Vitullo, R.M. Gronostajski, J. Biol. Chem. 273 (1998) 18538–18546.
[29] B. Gao, G. Kunos, J. Biol. Chem. 273 (1998) 31784–31787.
[30] Y. Yan, C.A. Jones, C.D. Sigmund, K.W. Gross, D.F. Catanzaro, Circ. Res. 81 (1997) 558–566.
[31] J. van Vliet, J. Turner, M. Crossley, Nucleic Acids Res. 28 (2000) 1955–1962.
[32] H. Baumeister, R.M. Gronostajski, G.E. Lyons, F.L. Margolis, Mol. Brain Res. 72 (1999) 65–79.