Species-Specific Differences in Positive and Negative Regulatory Elements in the Renin Gene Enhancer

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Abstract—A distal transcriptional enhancer has been previously reported upstream of the mouse renin gene. A homologous sequence is also present upstream of the human renin gene, but the mouse and human renin enhancers differ markedly in their ability to activate transcription of a renin promoter. Although the 2 enhancers share high homology in their 202-bp promoter distal portions, their 40-bp proximal portions are heterogeneous. Chimeric enhancers were used to test the role of the 40-bp segment (m40) of the enhancer by using transient transfection analysis in mouse kidney renin-expressing As4.1 cells. Deletion of m40 from the mouse renin enhancer or its addition to the human renin enhancer did not significantly change transcriptional activity when placed close to a mouse or human renin promoter. However, when placed further upstream of a renin promoter, the same deletion and substitution markedly altered enhancer activity. Electrophoretic gel mobility shift analysis identified 2 elements, a and b, in m40 that specifically bound nuclear proteins from As4.1 cells. Mutagenesis and transient transfection analysis revealed that element b accounts for the function of m40 and that element a antagonizes the positive influence of element b. Gel competition and supershift analysis revealed that nuclear factor-Y, a ubiquitous CAAT-box binding protein, binds to element a. Sequence analysis revealed that the human renin enhancer has a natural loss-of-function mutation in element b that affects its ability to transactivate when placed far upstream of a promoter. Reversion of the human renin element b to match the mouse sequence restored transactivation of the enhancer in mouse As4.1 cells. These data suggest that element b cooperates with the rest of the enhancer to maintain full enhancer activity, whereas element a may regulate enhancer activity. Sequence differences in these elements may explain the functional differences in the mouse and human renin enhancer sequences. (Circ Res. 1999;85:479-488.)

Key Words: transfection | transcription factor | kidney | juxtaglomerular cell | electrophoretic mobility shift assay

Renin (REN) is thought to be the rate-limiting enzyme controlling the generation of angiotensin II, the effector hormone of the REN-angiotensin system. The main source of circulating REN is juxtaglomerular cells of the kidney, although REN expression has been reported in a number of extrarenal tissues. Expression of REN mRNA and its release from the kidney is thought to be controlled at the transcriptional, posttranslational, and posttranslational level and is regulated by physiological cues such as arterial pressure, plasma sodium, and sympathetic nerve activity.1 Although the REN-angiotensin system has been studied for decades, the molecular mechanisms controlling the regulation of REN gene expression and REN release remain unclear (reviewed in Reference 2).

Initial clues for the existence of a major regulatory element(s) controlling REN gene expression came from a number of transgenic studies that implicated a region between −2.5 kb and −4.6 kb as controlling the spatial and temporal expression of the mouse REN (mREN) gene.3–7 An enhancer of transcription was later found by deletion mutagenesis and transient transfection analysis to lie from −2866 to −2625 bp relative to the initiation of transcription of the mREN gene.8 The studies were performed using a REN-expressing mouse kidney cell line (As4.1) sharing many properties in common with bona fide juxtaglomerular cells.9 The enhancer can stimulate an 80- to 100-fold increase in the activity of a basal mREN promoter in these cells in an orientation- and position-independent manner consistent with its definition as a classical enhancer of transcription. Moreover, reporter constructs containing the enhancer respond similarly to physiological cues controlling expression of the endogenous REN gene.10

In contrast, studies of the human REN (hREN) gene 5′-flanking sequence extending to −5 kb failed to identify sequences with enhancer-like activity. Indeed, most regulatory studies initially focused on determining the importance of the relatively small transcriptional effects imparted by 2 elements close to the promoter, a cAMP-responsive element (CRE) and a POU-domain factor binding site with homology to Pit-1.11–14 Although genomic transgenes containing the entire hREN coding region and relatively small amounts of 5′-flanking sequence are cell-specifically expressed in renal juxtaglomerular cells, they also exhibit ectopic expression suggesting the absence of potentially important regulatory sequences.15,16 Moreover, reporter genes containing up to 5 kb of 5′-flanking sequence either
are not expressed or are inappropriately expressed in transgenic mice (C.D. Sigmund, unpublished observations, 1998). Recent studies suggest the presence of a sequence homologous to the mREN enhancer ∼12 kb upstream of the hREN promoter.\textsuperscript{17} However, despite having substantial homology, transcriptional activity of DNA fragments containing the hREN enhancer-like sequence are markedly lower than corresponding fragments containing the mREN enhancer.

In this report, we examined the molecular mechanism regulating mouse and human REN enhancer activity by using chimeric mouse/human REN enhancers that were generated by taking advantage of natural sequence variation between them. Our results reveal that the promoter proximal 40 bp of the mREN enhancer is critical for its full transcription-inducing activity. Moreover, we demonstrate that this region contains both positive and negative regulatory elements and that the hREN enhancer sequence is deficient in its transcriptional activation because of a natural loss-of-function mutation in the positive element.

**Materials and Methods**

**Plasmid Construction**

Two hREN P1 artificial chromosome (PAC) clones were obtained from Genomes Systems (St. Louis, Mo) by screening a PAC library with previously described polymerase chain reaction (PCR)–amplified cDNA clone encompassing exons I through V.\textsuperscript{18} The presence of the entire coding region of hREN was established, and a 23-kb KpnI fragment was identified by Southern blot analysis as containing the mREN enhancer homology. The 23-kb KpnI subclone was subcloned into pBlueScript II SK– to form clone 67. PAC clones were handled and PAC DNA was purified according to directions provided by the manufacturer. Similar P1 clones containing hREN were previously described.\textsuperscript{17}

A 4.1-kb 5′-flanking DNA of the mouse Ren-\textsuperscript{1} gene was subcloned from RIC-1.1CAT\textsuperscript{a} first into the pGEM-2 vector to form pGEM-m4.1K and then into pGL2-basic to form the construct m4.1Kp-luc. This segment provided a positive control containing the mREN enhancer in its normal orientation and context upstream of the mREN promoter. The mREN test promoters, m2.6kP and m117P, each extending upstream to the coordinate indicated and downstream to +6, were derived from pGEM-m4.1K, and cloned into pGL2-basic to form the plasmids m2.6kP-luc and m117P-luc, respectively. The hREN test promoters, h896P and h149P, derived from a longer subclone containing 2.75 kb of hREN 5′-flanking DNA and extending upstream to the coordinate indicated and downstream to +13, were subcloned into pGL2-basic to form the plasmids h896P-luc and h149P-luc, respectively. All 4 promoters have their own endogenous mouse or human REN transcription start site. All 4 promoters were cloned upstream of the luciferase reporter gene in pGL2-basic in the forward orientation except those otherwise specified in text.

The 1018-bp PstI fragment containing the hREN enhancer homology was subcloned from clone 67 as a PstI fragment into pSL301 (Invitrogen) to form the plasmid pSL-h1018E. The hREN enhancer fragment consisting of the entire homologous region (h224E) was PCR amplified with the primers CGGGATCCACACTGGGAGAAGACCCTC and CGCGTCGACTCATGCTGTTGGAAATGGGGCCCC from pSL-h1018E and cloned into pBlueScript II SK– as a BclI fragment containing the sequence GATCCTGTACCTCTGACCGAGGTTGCGTGGTTGCAATGGTGAGACCCATG and GATCCCACTGGGCTATACGCACTCCAGAGGTCTAGCTACG with the hREN promoter. The minimal functional mREN enhancer fragment (m242E) was obtained from (−2625→−2866)m117P by previously described\textsuperscript{19} and subcloned into pSL301. The m40 sequence was obtained by directly cloning overlapping oligonucleotides containing the sequence GATCCTGTACCTCTGACCGAGGTTGCGTGGTTGCAATGGTGAGACCCATG and GATCCCACTGGGCTATACGCACTCCAGAGGTCTAGCTACG with the hREN promoter.

To generate chimeric enhancers (ie, the mREN enhancer lacking the m40 element [m242E–m40]), the hREN enhancer plus the m40 element [h202E–m40]) were exchanged between the plasmids m242E-h896P-luc and h202E-h896P-luc to form m242E–h896P-luc and [h202E–m40]–h896P-luc, respectively. The small to BstXI fragment contains the 202-bp segment of highest homology between the mouse and human minimal enhancers. The chimeric enhancers were then subcloned upstream of the other promoters described above. The m40 segment was removed from the positive control plasmid m4.1Kp-luc by cloning a small BstXI fragment from m4.1Kp-luc, containing 5′-flanking DNA from −4.1 to −2605 kb (including m242E without m40) upstream of m2.6kP-luc.

To generate mutations in transcription factor binding sites, binding sites a and b, site-directed mutagenesis was performed in the plasmid containing the entire minimal mREN enhancer (m242E) cloned into pGem-7Zf(−) (Promega). Oligonucleotides generated by Genosys Biotechnologies Inc were used for the mutagenesis after phosphorylation. Mutation in site a was generated with the mX30a oligonucleotide TGATCTCTGACCCCGCGTCCCCTGCTGTTGGTGTTGAGACCCATG and CGCATCTCCACACTGGGAGAAGACCCTC and the hREN enhancer plus the m40 element [h202E–m40] upstream of m2.6kP-luc. The m40 segment was removed from the positive control plasmid m4.1Kp-luc by cloning a small BstXI fragment from m4.1Kp-luc, containing 5′-flanking DNA from −4.1 to −2605 kb (including m242E without m40) upstream of m2.6kP-luc.

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To generate A-to-G mutation in m242E, overlapping double-stranded oligonucleotides with the sequence GATCCTGTACCTCTGACCGAGGTTGCGTGGTTGCAATGGTGAGACCCATG and GATCCTGTACCTCTGACCGAGGTTGCGTGGTTGCAATGGTGAGACCCATG and the hREN enhancer plus the m40 element [h202E–m40]] upstream of m2.6kP-luc. The m40 segment was removed from the positive control plasmid m4.1Kp-luc by cloning a small BstXI fragment from m4.1Kp-luc, containing 5′-flanking DNA from −4.1 to −2605 kb (including m242E without m40) upstream of m2.6kP-luc.

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To generate the A-to-G mutation in m242E, overlapping double-stranded oligonucleotides with the sequence GATCCTGTACCTCTGACCGAGGTTGCGTGGTTGCAATGGTGAGACCCATG and GATCCTGTACCTCTGACCGAGGTTGCGTGGTTGCAATGGTGAGACCCATG and the hREN enhancer plus the m40 element [h202E–m40], the upstream segment of the mouse minimal enhancer (m202) was expressed with h202 by Smal-BstXI. The mutated enhancer sequences were then cloned upstream of the m2.6kP promoter in m2.6kP-luc.

The generation and characterization of the mouse kidney REN-expressing As4.1 cell line (American Type Culture Collection, CRL2193) was previously described.\textsuperscript{20} As4.1 cells were propagated in DMEM containing 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL). Seventy-two hours before transfection, As4.1 cells were split and cultured in 75-cm\textsuperscript{2} flasks. Forty-eight hours before transfection, the cell culture medium was switched to Opti-MEM reduced-serum medium supplemented with 2% FBS, 1 mg/mL Albumax-II (Gibco-BRL), penicillin (100 U/mL), and streptomycin (100 μg/mL). The conditioned As4.1 cells were transfected with plasmid DNA by electroporation. In brief, the cells were lifted with trypsin-EDTA solution, washed, and suspended in Dulbecco’s PBS (5.0×10\textsuperscript{5}/mL). Plasmid DNA was mixed with 0.5 mL of cells (2.5×10\textsuperscript{6}/10\textsuperscript{5}) and DNA cell mixture was transferred into an electroporation chamber (0.4-cm electrode spacing) and electrically shocked using a Cell Porator (Life Technologies, Inc). A single electric impulse was used at 260 V and 1180 μF. After electroporation, the transfected cells were grown in a 60-mm dish containing 5 mL of the supplemented Opti-MEM reduced-serum medium. After 4 hours of incubation, culture media were changed with fresh supplemented Opti-MEM reduced-serum medium. Cells
were harvested and assayed for luciferase and β-galactosidase activities 48 hours after initial transfection.

The Rous sarcoma virus (RSV) promoter–LUC vector was always used as a standard positive control in each experiment. Test constructs were transfected in equal molar ratios using 25 μg of m4.1K-P-LUC as the starting point. In addition, the amount of nucleic acid in each transfection was brought to a total of 27 μg using PUC19 vector DNA as a carrier. A cytomegalovirus-promoter–β-galactosidase reporter vector (0.1 μg) (a gift from Dr Andrew F. Russo, University of Iowa) was cotransfected as an internal control to monitor transfection efficiency. Plasmid DNA for transfection was prepared by using the DNA Purification System (Promega) and purified by 2 ultracentrifugation steps through cesium chloride/ethidium bromide density gradients.

Luciferase and β-galactosidase activity assays were performed using the Luciferase Assay System (Promega) and the Galacto-Light Plus chemiluminescent reporter assay system (Tropix Inc) according to the instructions provided by the manufacturers and as previously reported.14 Luminescence was determined as light units on a Monolight 2010 luminometer (Analytical Luminescence Laboratory). All assays were performed in duplicate, and the average of 2 readings was used as 1 data point. Luciferase activity was normalized to β-galactosidase activity from the same cytoplasmic extract and expressed as a percentage of relative transcriptional activity of the RSV promoter.

Statistical Analysis

All data are presented as mean±SEM. Data were analyzed by 1-way ANOVA with correction for multiple analyses using the Bonferroni t test. When the test for normalization failed, the analysis was performed by Kruskal-Wallace ANOVA on ranks. All analysis was performed using the SigmaStat for Windows software package (SPSS Scientific).

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extract was prepared from A4.1 cells as previously described.19 The concentration of the nuclear protein was determined by using the Bio-Rad Protein Assay (Bio-Rad Laboratories). Aliquots of the dialyzed nuclear extract were stored at −80°C. Double-stranded oligonucleotides were made by heating equal amounts of 2 complementary single-stranded oligonucleotides (Genosys Biotechnologies Inc) at 94°C for 5 minutes, followed by slow cooling to room temperature. The probes were made from double-stranded oligonucleotides (10 pmol) containing a 5′-GATC overhang at both ends using Klenow DNA polymerase incorporating [α-32P]dATP (NEN Life Science Products) and 3 other cold nucleotides into full-length double-stranded oligonucleotides. The binding reactions contained 0.02 pmol of labeled probe (~60 000 cpm), 3 μg of nuclear extract, 1 μg poly[dI-dC] (Boehringer Mannheim), and binding buffer (containing, in mmol/L, Tris-HCl [pH 7.5] 10, EDTA 1, DTT 1, MgCl2 1, and KCl 300, as well as 5% glycerol). Competitor DNA was preincubated with the nuclear extract for 15 minutes on ice before the addition of probes. After the addition of probes, the binding reactions were incubated on ice for 15 minutes. For supershift, 1, 2, and 3 μg of antibody to nuclear factor-Y (NF-Y) subunit A or B (BioDesign International [catalog Nos. K59100R and K50101R, respectively]) were incubated with nuclear proteins at 4°C for 15 minutes either before or after complex formation. Both antibodies were generated to N-terminal peptides. Loading dye was added to the reactions, and the binding products were resolved on a 6% nondenaturing polyacrylamide gel (40% stock; acrylamide:bisacrylamide, 19:1) in 0.25× Tris borate–EDTA (pH 8.5) running buffer.14 After electrophoresis at 4°C, the gels were dried for autoradiography.

Southern Blot Analysis

m4.1K-P-LUC (100 ng) and clone 67 plasmid DNA (200 ng) was restriction digested and then run on a 0.7% agarose gel. The gel was first stained with ethidium bromide, photographed, and then blotted to a nylon-supported nitrocellulose membrane using standard procedures. The blot was probed with a single-stranded oligonucleotide matching the antisense of m40 that was labeled with [32P] by T4 polynucleotide kinase.

Results

The absence of a functional enhancer element or a sequence homologous to the mREN enhancer within the proximal 5000 bp of known 5′-flanking DNA sequence of the hREN gene led us to consider the possibility that an enhancer element might be present in the distal flanking regions. To test this possibility, 2 PAC clones containing the entire hREN gene were obtained from Genome Systems. Southern blot analysis using a 242-bp fragment encoding the minimal functional mREN enhancer (m242E)b revealed the presence of a sequence homologous to the enhancer within a 23-kb KpnI, a 3733-bp HindIII, and a 1018-bp PstI fragment (Figure 1B). Sequence analysis of the ends of the KpnI fragment revealed the upstream end to be new, unique sequence and the downstream anchor end to be contiguous with another KpnI fragment that spans most of the hREN coding region. Interestingly, the hREN enhancer homology is located ~12 to 13 kb upstream from the hREN promoter, 9 kb farther upstream than in the mREN gene.17 Sequence analysis of 5943 bp of hREN 5′-flanking DNA centering on the enhancer homology (~9 to ~15 kb) revealed a number of blocks of segmental homology with the mREN 5′-flanking sequence between −4.1 and +1 (Figure 1A). The blocks ranged in homology from >50% to >80% with the enhancer lying in a region of highest homology (block 7).

We first tested whether the hREN enhancer homology was functional by cloning a 1018-bp PstI fragment (h1018E) containing the homology directly upstream of 2 hREN promoters differing in length of 5′-flanking DNA (149 and 896 bp) fused to luciferase. As a control the minimal mREN enhancer (m242E) was also fused to the same promoters. Transient transfection analysis showed that m242E strongly enhanced hREN promoter activity (25-fold for h149P and 10-fold for h896P), whereas h1018E was much less effective in inducing the activity of either promoter (2-fold for h149P and 3-fold for h896P, Figure 2). This initial result suggested that the hREN enhancer homology may not function as a classical enhancer element. This may be due to the presence of a negative regulatory element in the h1018E sequence or the absence of critical elements required for full enhancer activity.

DNA sequence analysis of the minimal mREN enhancer and the 1018-bp PstI fragment revealed that the enhancer sequences shares 71% DNA-sequence identity. With respect to the promoters, the distal 202-bp portions (h202 and m202) share higher homology (80% similarity), whereas their proximal 40-bp sequences (h40 and m40) are quite different (45% similarity) (Figure 1C). To rule out the presence of negative regulatory elements within h1018E, we subcloned the region of highest homology between the mREN and hREN enhancers.

The 202-bp distal portion of the hREN enhancer (h202E) was cloned upstream of the h149P and h896P promoters. In contrast to the h1018E sequence, h202E homology induced transcriptional activity of h149P by 19-fold in comparison with 25-fold caused by m242E, a difference of only 1.3-fold that was not statistically significant, suggesting that the h202E sequence does have intrinsic enhancer-like activity (Figure 2A). In contrast, however, the same sequence only marginally stimulated transcription from the longer h896P promoter, suggesting that h202E does not contain all the essential cis-acting elements needed to act as a classical enhancer when placed a greater distance upstream from the promoter (Figure 2B).

m242E and h202E differ in that m242E contains the promoter-proximal 40-bp sequence (termed m40), whereas this
sequence is lacking from h202E. Recall that m40 and h40 shared 50% identity. We next tested the hypothesis that m40 may be required for full enhancer activity when it is located at a distance from the promoter. To accomplish this, we designed a set of chimeric enhancers in which m40 is removed from m242E (m242E–m40) and placed behind h202E (h202E + m40). We speculated that if m40 is required for full enhancer function when located a distance from the promoter, then deletion of m40 from m242E would diminish its activity, whereas addition of m40 to h202E would confer full enhancer activity when located a distance from a REN promoter. All 4 enhancers (m242E, m242E–m40, h202E, and h202E + m40) increased transcriptional activity of the short h149P promoter from 16- to 25-fold (Figure 3B). However, removal of m40 from m242E significantly lowered transcriptional induction of the longer h896P promoter from 10-fold to 3-fold (Figure 3D). Consistent with the hypothesis, addition of m40 to the promoter-proximal end of h202E increased promoter activity from 2-fold to 8-fold (Figure 3D).

Although the difference in enhancer-driven activity was significantly lower for h202E than for m242E, both enhancers still markedly stimulated the activity of the basal m117 promoter. However, as with the h896P promoter, elimination of m40 from m242E or its addition to h202E caused a significant change in the activity of the longer 2.6-kb mREN promoter (m2.6kP, Figure 3C). The 3-fold change in enhancer-driven activity in response to elimination of m40 from m242E or its addition to h202E was similar on both h896P and m2.6kP. The function of m40 was further confirmed by examining the consequences of its removal from its native position within element b in the 4.1-kb segment of mREN promoter (m4.1kP, Figure 4). However, removal of m40 from m242E significantly lowered transcriptional induction of the longer h896P promoter from 10-fold to 3-fold (Figure 3D). Consistent with the hypothesis, addition of m40 to the promoter-proximal end of h202E increased promoter activity from 2-fold to 8-fold (Figure 3D). Similarly, only modest changes in enhancer activity were obtained when the 4 enhancer combinations were placed upstream of a short 117-bp mREN promoter (m117P, Figure 3A).

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We next used EMSAs to identify DNA-protein interactions that may regulate the function of m40. EMSAs were performed using double-stranded DNA oligonucleotides encoding the m40 sequence and nuclear extracts prepared from As4.1 cells. These studies revealed the formation of 2 major complexes on m40, labeled a and b, which were efficiently competed by increasing molar excess of unlabeled m40 oligonucleotide (Figure 5). The specificity of binding was confirmed by the inability of 3 different unrelated oligonucleotides (encoding the hREN CRE and activator protein 1 and 2 consensus sequences) to compete for m40 binding (data not shown). To begin to narrow down the location of the 2 complexes on m40, competition was performed using two 30-bp overlapping double-stranded oligonucleotides, mX30 and mY30. Complexes a and b were both easily competed with mX30, but not with mY30 (Figure 5), suggesting that the 2 specific DNA-nuclear protein complexes require sequence elements downstream of m40.

We next assessed which bases are important for the DNA-nuclear protein interactions by EMSA and competition using mutant oligonucleotides. In the first experiment, we introduced 9 sequential 3-bp deletion mutations in mX30, generating 9 different double-stranded mutant oligonucleotides (1 to 9 in Figure 6A). Deletion mutants 1, 2, 8, and 9 were able to efficiently compete for both complexes a and b (Figure 6B). Mutants 3 to 7 were unable to effectively compete for complex a, whereas mutants 3 and 4 were unable to compete for complex b. These data suggest that 2 sequence motifs, GACCTCCT-AGGTGGCT and GACCTC, form complexes a and b, respectively, with nuclear extracts from As4.1 cells. Interestingly, sites a and b appear to overlap (see below).

Oligonucleotides with specific base-substitution mutations were then used as competitors to confirm these findings (Figure 6A). We made 3 mutant oligonucleotides in mX30. The mX30ua contains a 4-base change of the central GAGTGG motif to TCACGT. These residues were selected to avoid the region overlapped by element b. The mX30ub oligonucleotide contains a 2-nucleotide change from GACCTC to TTCCCT, and mX30ua contains both mutations. mX30ua lost its ability to compete for complex a but not b, mX30ub lost its ability to compete for complex b.

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significant induction of promoter activity that was significantly attenuated by the removal of the m40 sequence (Figure 8A). Mutation of site b (or of both sites a and b together) attenuated enhancer-driven activity similar to that caused by the loss of the entire m40 segment, suggesting that the activity of m40 is dependent on complex b. Indeed, there was no statistically significant difference in the transcriptional induction caused by m242E-m40 and m242Eμb. Interestingly, mutation of the NF-Y binding site (m242Eμa) resulted in increased enhancer activity, suggesting that complex a may play a role in regulating the activity of the mREN enhancer. That complex b is necessary for this induction is evidenced by the loss of enhancer activity in the double mutant (m242Eμab).

We showed above that the m40 sequence can confer increased enhancer-driven activity to h202E when placed a distance upstream from a REN promoter (Figure 3). We therefore asked whether this property requires protein binding to element b. To accomplish this, we replaced the m40 element in the chimeric enhancer, h202E+ m40, with the m40μa, m40μb, and m40μab mutants and then inserted them upstream of the m2.6kP-luc reporter construct. As above, addition of m40 to H202E significantly increased enhancer activity, and this was further increased by the mutation of the NF-Y binding site (Figure 8B). However, removal of complex b (or a and b) results in an attenuation of the enhancer activity stimulated by addition of m40. Again, there was
no significant difference in enhancer activity between h202E and h202E+m40μb, both of which lack element b.

The data presented above indicate that site b and the nuclear proteins binding to it can account for the stimulating function of m40. Removal of site b attenuates (1) the activity of the mREN minimal enhancer and (2) the increase in transcriptional activity caused by the addition of m40 to the hREN enhancer homology. We therefore asked whether the 40-bp segment immediately downstream of the h202E functions similarly to m40. Recall that h40 and m40 share <50% overall sequence identity and that a 1018-bp PstI fragment containing the entire hREN enhancer homology (including h40) failed to stimulate transcription (Figure 2). Moreover, Southern blot analysis revealed the absence of a sequence homologous to m40 within a 23-kb KpnI restriction fragment containing hREN 5’-flanking DNA from approximately −23 kb to −149, whereas it was easily detected in the control plasmid containing 4.1 kb of mREN 5’-flanking DNA (Figure 9). Sequence analysis of h40 revealed that it contains a complex b binding site similarly positioned but with a single nucleotide mismatch (GGCCTC) compared with complex b in m40 (GACCCTC). The data in Figure 6C indicate this to be 1 of 2 sites that when mutated (GACCCTC to TTCCCT) result in loss of complex b formation.

Unless an oligonucleotide containing a reversion mutation generating a G-to-A transition within the complex b binding site is used (Figure 10). Similarly, mutation of GACCTC to GGCCTC in m40 causes a loss of competition for complex b and significantly attenuates the activity of the m242E enhancer in As4.1 cells (Figure 11A). The loss of activity in the A-to-G mutant is similar to that caused by the m40μb mutation tested above.

Finally, we made a set of chimeric enhancers to examine whether h40 has any m40-like function. As above, the chimeric enhancers were placed at the native position of the mREN enhancer upstream of the m2.6kP promoter. The results indicate that addition of h40 to the h202E sequence (to form h242E) does not induce an increase in promoter activity (Figure 11B). However, a G-to-A mutation restoring the GACCTC motif in h40 results in a marked increase in enhancer activity when placed upstream of the m2.6kP promoter (Figure 11B) and when introduced in the h1018-bp PstI fragment (Figure 11C). It is interesting to note that enhancer activity in the G-to-A mutants is greater than the activity of the minimal mREN enhancer (m242E).

It is therefore not surprising to note that (1) the positionally equivalent site a in h40 differs from site a in m40 at 3 of 9 bases, including 3 of the 4 positions that are mutated in m40μA; (2) the hX30 oligonucleotide cannot effectively compete for complex a formation (Figure 10); and (3) the increase in transcription in the G-to-A mutants is similar to that induced by mutation of site a in m40 (Figure 11B).
Discussion

Classical transcriptional enhancers are position- and orientation-independent cis-acting DNA elements, and the mREN gene enhancer functions as a classical enhancer. A homologous enhancer-like sequence was found 12 to 13 kb upstream of the human REN promoter. Although an hREN sequence containing the minimal homologous region could stimulate the activity of short REN promoters, it failed to induce transcriptional activity when placed farther upstream from the promoter. The data imply that sequences present in the mREN enhancer but lacking in the homologous hREN sequence are required for enhancer activity over a distance. Using a set of chimeric enhancers and REN promoter/5'-flanking DNA, even on very long exposures.

Figure 9. Search for an m40 homology in hREN 5'-flanking DNA. m4.1K-LUC (m4.1K, lanes 1 through 3) and clone 67 (h23.0K, lanes A through E) plasmid DNA was digested with Smal (lane 1), Smal + HindIII (lane 2), Smal + HindIII + SpI (lane 3), BamHI (lane A), BamHI + XbaI (lane B), XbaI (lane C), XbaI + KpnI (lane D), and KpnI (lane E). Size markers are 1-kb ladder (M) and phage λ DNA cut with HindIII (M'), and selected sizes are indicated in kb. Left, Representative ethidium bromide staining pattern. Right, Southern blot of an identical gel run with only 100 ng of m4.1K and 200 ng of h23.0K probed with an end-label antisense m40 probe. Hybridization was observed to 10-, 4.1-, and 1.5-kb bands from m4.1K demonstrating the presence of m40 in mREN 5'-flanking DNA and the selectivity of the probe. No hybridization was detected in hREN plasmid DNA containing 23 kb of 5'-flanking DNA, even on very long exposures.

The fact that element b is not required when the enhancer is close to the promoter perhaps suggests that direct contacts compete for complex b formation, suggesting that neither CREB nor other members of the activating transcription factor family contribute to complex b.

The mREN enhancer functions as a classical enhancer of transcription when intact, but is highly sensitive to distance effects when element b is mutated. Numerous examples of functional synergism between transcription factors in enhancer-mediated transcriptional activation have been reported. That m40 does not itself have intrinsic enhancer activity makes it tempting to speculate that complex b may cooperate with other transcription factors that bind to the enhancer to activate transcription from a REN promoter. Among these may be additional sites for the formation of complex b, as sequence analysis identified 2 other stretches containing the GACCT heptanucleotide identified within element b (GACCTC). Interestingly, one of these motifs (termed element c) is present 16 bp upstream from element b. With a spacing of 1 to 2 turns of the helix it is possible that GACCT-bound nuclear factors may interact with each other as a multimer. If this is proven to be the case, the interaction of the GACCT nuclear protein(s) could be the molecular foundation for the functional synergism between the m40 and m202. Preliminary results suggest that mutation of element c has the same effect as mutation of element b, suggesting that both sites are required for enhancer activity (Q. Shi and C.D. Sigmund, unpublished observation, 1999). Nevertheless, it is also possible that the GACCT-bound nuclear protein(s) might cooperate with other nuclear protein(s) that bind elsewhere in the enhancer. Indeed, preliminary results suggest the formation of at least 5 different DNA:protein complexes on the mREN enhancer (T.A. Black and K.W. Gross, unpublished observation, 1998).
between enhancer binding proteins and proximal promoter binding proteins could take place when they are physically close to each other. Given that element b is required for enhancer activity when placed at a distance from the promoter suggests, but does not prove, that it may be required for the formation of a higher-order transcription complex involving not only enhancer-binding proteins, but also proteins binding close to the promoter. It is tempting to speculate that complex b might be involved in protein-protein interactions between the enhancer and proximal promoter that would bring them in close proximity (ie, through the formation of a loop in genomic DNA). A transcription factor identified to bind to a conserved AT-rich sequence at −60 with respect to the transcription start site may be a potential candidate, as mutations in that sequence severely diminish enhancer activity.8

We also identified a second functional element in m40, element a, a mutation of which markedly enhanced transcriptional induction of the enhancer. These results suggest that element a may act as a negative regulator of the enhancer. Potential mechanisms include the following: (1) competition for sequences in the m40 segment by proteins binding to elements a and b, where formation of complex a precludes the binding and formation of complex b, and (2) formation of complex a having some direct negative effect on formation of the transcription complex or initiation of transcription independent of complex b. Experiments are currently in progress to distinguish these mechanisms. We currently favor the former mechanism, because the induction of transcription caused by mutation of element a required a functional element b. This mechanism is not unique, given that 2 partially overlapped binding sites (one for hepatocyte nuclear factor 4 and the other for Sp1) compete for binding in the promoter region of the blood coagulation factor X gene,26 and DNA-bound Oct-1 inhibits IL-8 promoter activity by competing against the positive nuclear factor C/EBP.27

Interestingly, a search of the transcription factor database revealed homology between sequences within element a and the binding site for NF-Y. Element a was homologous in 12 of 13 nucleotides with a NF-Y binding site but was different at one position (CTGA/GTGCTTG). That is invariant in other known NF-Y binding sites in the database. Despite this invariant base, both competition and supershift assays clearly identified NF-Y as the protein binding to element a. NF-Y is a ubiquitous transcription factor that acts as a CCAAT-box binding protein and is generally thought to impart a positive influence on transcription initiation by facilitating the binding of additional transcription factors to nearby promoter elements.28,29 CCAAT has also been reported to exert negative effects on transcription of the varicella-zoster virus immediate-early 62 promoter and the human CYP1A1 gene.30,31 NF-Y was also demonstrated to negatively regulate the transcriptional activity of the transforming growth factor-β receptor gene in embryonal carcinoma cells.32

Our data suggest that a complex regulatory circuit may be operating to regulate the mREN enhancer and therefore transcriptional activity of the mREN gene in As4.1 cells and perhaps in JG cells in vivo. Clearly, this leaves unresolved the function, if any, of the enhancer sequence upstream of the hREN gene. The hREN enhancer homology lies an additional 10 kb upstream compared with the mREN enhancer. This suggests that the hREN enhancer may function over a large distance. However, we demonstrated that the homologous element b upstream of hREN is nonfunctional because of a single nucleotide substitution and that reversion of that mutation in either a minimal enhancer (h242E) or a larger fragment (h1018E) caused marked transcriptional induction. No other regulatory elements closely linked to the enhancer homology could substitute for the loss of element b function, because a 3733-bp HindIII fragment containing the enhancer was also unable to stimulate transcription of a REN promoter (data not shown). It is possible that the lack of function of element b in hREN (GGCCCT) is caused by an experimental artifact of its analysis in a mouse cell line. In light of this possibility, it is important to point out that both h202E and h242E had minimal effects (either no induction or 1.2-fold induction) on the transcriptional activity of the h896 promoter in Calu-6 cells, the only permanent cell line expressing human REN mRNA endogenously (data not shown), indeed suggesting that our findings are not due to artifacts of the experimental system.34,35

It is further surprising that in addition to element b, there was no evidence for a functional element a in the hREN enhancer sequence. Of course, if element b has been rendered nonfunctional, there would be little need for another element to regulate its activity. This suggests the provocative hypothesis that the hREN enhancer is itself nonfunctional or does not act as a classical enhancer of hREN transcription. If so, there would be little selective pressure to maintain its position and sequence as suggested by mutational

### Figure 11. Transcriptional activity of the h40 element. A, Transcriptional activities ( % RSV-luc) of m242E, an A/G mutation in element b (m242E[A>G]), and m242E[b] placed upstream of the m2.6kP promoter. P indicates promoter alone. Numbers in parentheses indicate fold induction over promoter alone. Statistical comparison was between the enhancer-mediated effect of m242E vs mutant enhancers. *P < 0.05 vs m242E; n = 6 for all constructs. B, Transcriptional activities of h202E, h242E, m242E[μ], and a G-to-A reversion in element b placed upstream of the m2.6kP promoter (h242E[G>A]). Numbers in parentheses indicate fold induction over promoter alone. Statistical analysis was to compare enhancer-mediated induction of constructs with an m40 or h40 sequence to a basal construct lacking a functional m40 or h40 element (h202E). *P < 0.05 vs h202E; n = 6 for all constructs. C, Transcriptional activities of h1018E, m242E, and a G-to-A reversion within element b of h1018 (h1018[G>A]) placed upstream of the m2.6kP promoter are shown. Statistical analysis was to compare enhancer-mediated induction of constructs with a functional element b to a basal construct lacking a functional element b (h1018E). *P < 0.05 vs h1018E; n = 6 for all constructs.
analysis. Circulating levels of REN in humans are substantially lower than those in mice, suggesting less of a demand to maintain high-level transcription of the hREN gene. It is also possible that transcription of the hREN gene depends on additional sequences present between −13kb and the promoter. Our Southern blot analysis suggests the absence of an additional m40-like sequence in this region. However, Germain et al.86 recently identified an additional transcriptional enhancer in the hREN gene that is active in choriocarcinoma cells. In vivo, there may be cooperation between transcription factors binding to the “kidney” and “chorionic” enhancers to regulate hREN transcription, and experiments to directly test this possibility are in progress. Indeed, it is interesting to note that large hREN transgenes containing 5′-flanking DNA extending past the hREN enhancer (and containing both enhancer sequences) are expressed in a highly tissue-specific, cell-specific, and regulated manner.37,38

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