NF-Y Antagonizes Renin Enhancer Function by Blocking Stimulatory Transcription Factors

Qi Shi, Kenneth W. Gross, Curt D. Sigmund

Abstract—We previously reported that the promoter proximal portion of the mouse renin enhancer contains a binding site for NF-Y (Ea) that overlaps with a positive regulatory element (Eb). In the context of the renin enhancer, NF-Y acts to oppose enhancer activity. We tested the hypothesis that NF-Y acts as a negative regulator by physically blocking the binding of transcription factors to element-b (Eb). Increasing the spacing between the NF-Y binding site (Ea) and Eb by 2, 5, or 10 nucleotides increased activity of the enhancer to the same extent as mutations abolishing NF-Y binding. The increase in transcription caused by increasing the spacing between Ea and Eb was not due to a shift of NF-Y from a negative regulator to a positive regulator because there was no loss of activity when Ea was also mutated. Oligonucleotides containing the normal or increased spacing mutants still allowed the binding of both NF-Y to Ea and transcription factors to Eb. In fact, we present evidence that both NF-Y and the Eb-binding factor(s) can each bind together on the same oligonucleotide containing either a 5- or 10-bp spacing between Ea and Eb.

Key Words: transcription ■ renin-angiotensin system ■ activator ■ repressor

The renin-angiotensin system is an important regulator of arterial pressure and electrolyte homeostasis. The level of renin transcription, processing, and secretion dictates the eventual level of angiotensin II, because the angiotensinogen cleavage step is thought to be rate limiting. Knowledge of the molecular mechanisms defining renin gene regulation remain incomplete but has been aided recently by the identification of an enhancer of transcription located upstream of the mouse renin gene, although its position is much further from 5' than the promoter.2 mE consists of a 242-bp sequence isolated from the kidney.2 mE consists of a 242-bp sequence isolated from the kidney.2 mE consists of a 242-bp sequence isolated from the kidney.2

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Received January 29, 2001; first decision February 21, 2001; revision accepted February 23, 2001.

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332
Methods

Plasmids
The luciferase (LUC) reporter vectors 2.6 kLUC, mE2.6 kLUC, and mEμa2.6 kLUC were described previously.3 mE represents the 242-bp mouse Ren enhancer; 2.6 k represents a 2.6-kb 5′-flanking sequence of mouse Ren lacking mE; and mE2.6 k is a 2866-bp sequence with an SpI I site separating mE from mE2.6 k. Site-directed mutagenesis was performed by use of the GeneEditor in vitro Site-Directed Mutagenesis System (Promega). The sequence of all mutants was confirmed by DNA sequencing and restriction digestion analysis. The 2-base insertion mutant mE(b2a)2.6 kLUC, 5-base insertion mutant mE(b5a)2.6 kLUC, and 10-base insertion mutant mE(b10a)2.6 kLUC were generated with the following oligonucleotides: GTAACCTGACCTCCTGAGTGCTGG, GGCTG-TACTGACCTCAGGCTTGAGTGCTGG, and CTCTGAC-CTAGGGCATGCTTGAGTGCTGG, where the 5′-GATC-dinucleotide sequence is underlined. Spacing mutations were indicated in lowercase; μ mutations indicated by italics.

Cell Culture and Transient Transfection
Cell culture and transient transfection of As4.1 cells (ATCC CRL2193) were previously described.3 In brief, As4.1 cells were cultured in reduced-serum OptiMEM supplemented with 2% fetal bovine serum, 1 mg/mL Albumax-II (GIBCO-BRL), penicillin (100 U/mL), and streptomycin (100 mg/mL) for 2 days. The cells (2.5×10⁷) were transfected with equal-molar amounts of plasmid DNA by electroporation balancing with pUC19. RSV-LUC was used as an internal control. Cells were harvested and assayed for luciferase activity and then calculated as a percentage of RSV promoter activity. All activity assays were performed in duplicate, the average of 2 readings being 1 data point.

Electrophoretic Mobility Shift and Supershift Assay
Preparations of the nuclear extract from As4.1 cells and probes for electrophoretic mobility shift assay (EMSA) were previously described.3 The parent probe sequence was gatcGTAACCTGACCTCCTGAGTGCTGGTTG (top strand), where the 5′-GATC overhangs at each end of the annealed double-stranded oligonucleotides were filled with [α-32P]dATP (NEN) and 3 other cold nucleotides using Klenow DNA polymerase. The mutant probes were generated by inserting CT (b2a), AGGCT (b5a), or AGGC- GATGCT (b10a) between the 5′-GATC-dinucleotide sequence with an underlined. Spacing mutations are indicated in lowercase; μ mutations indicated by italics.

Results
The purpose of this study was to determine the molecular mechanism by which NF-Y antagonizes mE. On the basis of previous studies of the mechanism of NF-Y–mediated transcriptional repression and the overlap between Ea and Eb in mE, we hypothesized that NF-Y binding to Ea may physically disrupt or prevent the binding of Eb-binding factor(s) to Eb. Our strategy was to alter the spacing between Ea and Eb to remove the overlap with the rationale that if transcriptional repression works via steric hindrance (NF-Y blocking Eb-binding protein to an overlapping site), then removing the overlap between Ea and Eb should relieve transcriptional repression even in the presence of NF-Y.

To test this hypothesis directly, we inserted 2, 5, or 10 nucleotides between Eb and Ea in mE by site-directed mutagenesis using the mE2.6 kLUC plasmid as the backbone (Figure 1). All 3 insertions contained the 3′ CT-dinucleotide to ensure that the binding sites for both Eb and Ea remained intact. We then performed transient transfection using renin expressing As4.1 cells. As previously reported, addition of mE to the enhancerless 2.6 k Ren promoter markedly induced transcriptional activity (Figure 2A).3 Consistent with our hypothesis, all 3 insertions increased promoter activity by 2-fold. The increase in transcription caused by the increased spacing between Ea and Eb was due to a shift in the mechanism of NF-Y action (Figure 2B). Mutation of Ea did not cause any significant loss of transcriptional activity in any construct tested and in fact caused a 2-fold increase in transcription caused by the increased spacing between Ea and Eb.

Because NF-Y is typically considered a transcriptional activator, it became important to determine whether the increase in transcriptional activity caused by increasing the spacing between Ea and Eb was due to a shift in the mechanism of NF-Y action from a repressor to an activator. To address this issue, we generated the same 3 insertion mutations (2, 5, or 10 nucleotides) in plasmids lacking a functional NF-Y binding site and compared transcriptional activity with plasmids with an intact Ea (Figure 2B). Mutation of Ea did not cause any significant loss of transcriptional activity in any construct tested and in fact caused
a significant increase in the b2a mutant. The increase in transcriptional activity caused by mutation of Ea in the b2a construct suggests that the 2-bp insertion may have only incompletely removed the overlap between the 2 sites. This finding is confirmed below by EMSA. However, the fact that mutation of the NF-Y binding site did not result in a loss of transcriptional activity suggests that the increased transcriptional activity observed in the b2a, b5a, and b10a mutants is not due to transcriptional activation by NF-Y.

We previously reported that a double-stranded oligonucleotide mX30 (Figure 3A) that contained Ea and Eb formed 2 major DNA-protein complexes (a and b) with nuclear extracts from As4.1 cells.3 To verify that both NF-Y and Eb-binding protein can still form a complex on the insertion mutants, we individually labeled double-stranded oligonucleotides containing the b2a, b5a, or b10a insertions and tested their binding activity using EMSA. Strong complex-a formation was evident on all 3 insertions (Figure 3B). Although strong complex-b formation was observed on the wild-type (WT) and b2a insertions, we noted a reproducible drop in the intensity of complex-b using b5a and b10a as a probe. Nevertheless, that b2a, b5a, and b10a still effectively bound to complex-a and complex-b is demonstrated by their effectiveness as competitors even when competing against the WT oligonucleotide. Moreover, competition analysis using varying levels of competitor DNAs revealed that there was no observable change in the affinity of NF-Y for Ea in the b2a, b5a, and b10a mutants, because each was equally effective in competing for binding to the WT probe (Figure 4). Significantly, the observation that antiserum against either the A or B subunit of NF-Y supershifted complex-a in all 3 mutants confirmed the interaction between NF-Y and Ea in b2a, b5a, and b10a (Figure 5).

The increase in transcriptional activity and the EMSA data above suggest that increasing the spacing between Ea and Eb binding activity using EMSA. Strong complex-a formation was evident on all 3 insertions (Figure 3B). Although strong complex-b formation was observed on the wild-type (WT) and b2a insertions, we noted a reproducible drop in the intensity of complex-b using b5a and b10a as a probe. Nevertheless, that b2a, b5a, and b10a still effectively bound to complex-a and complex-b is demonstrated by their effectiveness as competitors even when competing against the WT oligonucleotide. Moreover, competition analysis using varying levels of competitor DNAs revealed that there was no observable change in the affinity of NF-Y for Ea in the b2a, b5a, and b10a mutants, because each was equally effective in competing for binding to the WT probe (Figure 4). Significantly, the observation that antiserum against either the A or B subunit of NF-Y supershifted complex-a in all 3 mutants confirmed the interaction between NF-Y and Ea in b2a, b5a, and b10a (Figure 5).

The increase in transcriptional activity and the EMSA data above suggest that increasing the spacing between Ea and Eb may allow both factors to simultaneously bind to the en-
under other conditions, the antiserum causes a clear and abundant supershift to appear.16,17

Taken together, these data strongly suggest that NF-Y mediates its antagonistic activity by preventing the binding of Eb-binding protein(s) to Eb. Increasing the spacing did not cause NF-Y to act as a positive factor and did not affect the ability of NF-Y to bind to the modified enhancers. In fact, increasing the spacing by 5 and 10 nucleotides provided an opportunity for both NF-Y and Eb-binding protein to simultaneously bind to mX30.

**Discussion**

The ability of the renin gene enhancer to stimulate a 100-fold increase in transcriptional activity of the Ren promoter makes it an important candidate in Ren gene regulation.1 We previously identified 2 transcription factor binding sites that mechanistically oppose each other.2 We hypothesized that the binding of NF-Y to Ea acts as a negative regulator because it blocks the binding of transcription factors to Eb. Changing the spacing between Ea and Eb has the same effect as mutating Ea, suggesting that this hypothesis is correct. These data are supported by the observations that NF-Y can still bind to Ea even when the spacing between Ea and Eb is increased and that mutations of Ea in the b5a and b10a mutants does not inhibit transcription of the Ren promoter.

NF-Y is generally considered a ubiquitous transcriptional activator, although the exact mechanisms through which NF-Y regulates transcription is not totally clear. Sequence surveys have revealed that most stimulatory NF-Y binding sites are located from −80 through −100 in TATA-containing promoters or very close to the transcription start site in TATA-less promoters.8,18 Other studies of NF-Y containing promoters have demonstrated that the positive function of the NF-Y site also depends on other adjacent cis-acting elements.19,20 In mouse Ren, Ea is located far upstream of the promoter (about −2.6 kb) and does not have the previously reported adjacent cis-acting elements that cooperated with positive NF-Y sites in the other genes.

The negative regulatory activity of NF-Y has been reported in several genes.9–13 Mutation of an NF-Y binding site overlapping with a C/EBP site upstream of the human apolipoprotein A-I promoter increases transcription.9 Overlap of NF-Y and a CRE in the promoter proximal region of the rat insulin 1 gene attenuates transcriptional stimulation by cAMP.9,13 Although Ea does not match a perfect consensus NF-Y site (It has a single-base substitution in its core recognition motif), the same single-base substitution also exists in human apolipoprotein A-I gene, which is also repressed by NF-Y.9

Under normal conditions, activation of the enhancer may involve a competition between NF-Y and the factors binding to Eb. It is likely that both the amount of NF-Y and Eb-binding protein and their relative binding affinity may be determinants of this competition. EMSA experiments reproduced suggest that the level of NF-Y exceeds the level of Eb-binding protein under baseline conditions. It is possible that specific conditions exist that change the balance between these proteins and therefore favor binding to Eb. It is known that the level or affinity of NF-Y can be regulated by the
redox state of the cell, intracellular calcium concentration, cellular differentiation, and the presence of serum supplementation.\textsuperscript{16,21–23} Exactly what physiological cues regulate the level or affinity of the Eb-binding protein will have to wait for the unequivocal identification of that factor(s) (see below).

It is now becoming clear that the mechanism by which the renin enhancer regulates renin transcription will be quite complicated because, in addition to the elements described above, additional elements upstream of Eb have recently been shown to be required for maximal enhancer activity (Figure 7). One element, termed Ec, which located 10 bp upstream of Eb, is a direct repeat of the Eb TGACCT motif. TGACCT direct repeats have been reported to bind members of the nuclear hormone receptor superfamily of transcription factors.\textsuperscript{24} We have recently demonstrated that Eb is a half-site for retinoic acid receptor (RAR). Eb and Ec together can bind RAR\(\alpha\) and RVR\(\alpha\) and form a functional RAR element.\textsuperscript{14} In the studies described herein, Ec was intact and the distance between Ec and Eb remained unaltered. In addition, an element called Ed, which lies further upstream of Ec and Eb, has homology to a CRE and may thus bind members of the CREB/ATF-1 family of transcription factors (T.A. Black, et al., unpublished observation, 2000). It is likely that the interplay between transcription factors binding to these sites may be required because the CREB/ATF-1 and RAR/RXR pathways share a similar coactivator in p300/CPB.\textsuperscript{25,26} Other yet-unidentified regulatory elements may also be present in the \textit{Ren} enhancer, and other transcription factors may bind to Ed, Ec, Eb, and Ea. In the end, it is likely that ubiquitous positive and negative regulatory factors will be found to cooperate to regulate expression of renin through the enhancer.

\textbf{Acknowledgments}

Funds in support of this work were obtained from the NIH (HL-48058, HL-61446, and HL-55006 to C.D.S. and HL-48459 to K.W.G.). We acknowledge the outstanding technical assistance of Deborah Davis and Xiaoji Zhang. DNA sequencing was performed by guest on October 25, 2017 http://hyper.ahajournals.org/ Downloaded from

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_Hypertension_. 2001;38:332-336
doi: 10.1161/01.HYP.38.3.332

_Hypertension_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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