Retinoic Acid-mediated Activation of the Mouse Renin Enhancer*

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Previous studies demonstrate that the mouse renin gene is regulated by a complex enhancer of transcription located 2.6 kilobases upstream of the transcription start site which is under both positive and negative influence. We demonstrate herein that a positive regulatory element (Eb) is repeated 10 bp upstream (Ec), and both are required for baseline activity of the enhancer. The Eb and Ec core sequences are identical to the consensus sequence for the nuclear hormone receptor superfamily of transcription factors, and transcriptional activity of constructs containing the enhancer is increased after treatment with retinoic acid. Maximal induction requires both Eb and Ec. Expression of endogenous renin and a renin-promoter controlled transgene in As4.1 cells, and kidney renin mRNA in C57BL/6J mice was induced after retinoid treatment. Gel mobility supershift analysis revealed the binding of RARα and RXRα to oligonucleotides containing both Eb and Ec. Reverse transcriptase-polymerase chain reaction analysis revealed that As4.1 cells express both receptor isoforms, along with RARγ, but do not express RARβ, RXRδ, or RXRγ. Co-transfection of an expression vector encoding wild-type RXRα increased enhancer activity, whereas a dominant negative mutant of RARα significantly attenuated retinoic acid-induced activity of the enhancer. These results demonstrate the importance of the Eb and Ec motifs in controlling baseline activity of the renin enhancer, and suggest the potential importance of retinoids in regulating renin expression.

The renin-angiotensin system is a critical regulator of arterial pressure and electrolyte homeostasis and is required for continued development of the kidney after birth. The cleavage of angiotensinogen by renin is thought to be the rate-limiting step in the biosynthesis of angiotensin II and is tightly regulated. Transcription of the renin gene, storage and processing of renin in juxtaglomerular cell secretory granules, and secretion of renin into the systemic circulation, each dictate the level of renin expression. The m40 segment contained two regulatory elements. The first sequence, element a (Ea), bound the factor NF-Y and acted as a transcriptional repressor because mutations abolishing binding of NF-Y significantly stimulated enhancer-mediated transcription. The second sequence, element b (Eb), was required for maximal activity of the enhancer, and its mutagenesis essentially abolished enhancer activity. Given that Ea and Eb overlapped, we hypothesized that NF-Y blocks enhancer activity by preventing the binding of transcription factors to Eb. This is supported by experiments in which the spacing between Ea and Eb is altered.2

Based on the observation that the m40 sequence is insufficient to stimulate transcription on its own, we speculated that additional sequences further upstream of m40, but within the 242-bp enhancer are required for maximal induction. Herein we demonstrate that a third element, a direct repeat of Eb, termed Ec, lying upstream of m40 is also required for baseline enhancer activity. This sequence when multimerized can strongly stimulate renin promoter activity on its own. Moreover, the Ec-10 bp-Eb sequence matches the consensus binding site for members of the nuclear hormone receptor superfamily. This sequence can bind the RXRα and RXRγ transcription factors and is required for induction of the renin promoter by retinoic acid. That retinoic acid can stimulate endogenous renin mRNA in As4.1 cells and mouse kidney suggests they may play a potentially important role in regulating renin expression.

MATERIALS AND METHODS

Plasmids—The luciferase (LUC) reporter vectors m4.1kLUC, mE2.6kLUC, mEμ2.6kLUC, mEβ2.6kLUC, mEα2.6kLUC, and

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¶ The abbreviations used are: kb, kilobase(s); bp, base pair(s); Ea, element a; Eb, element b; LUC, luciferase; RAR, retinoic acid receptor; RXR, retinoic X receptor; FBS, fetal bovine serum; tRA, all-trans-retinoic acid; EMSA, electrophoretic mobility shift assay; RT-PCR, reverse transcriptase-polymerase chain reaction; RARE, retinoic acid receptor element.

1 Q. Shi and C. D. Sigmund, submitted for publication.
mE117LUC were described previously (4) (Fig. 1A). mE represents the 242-bp mouse renin enhancer sequence. mE.1k represents a 4.1-kb 5′-flanking sequence of mouse renin (−4.1 kb to +6) containing mE in its native position. mE.2k represents a 2.6-kb 5′-flanking sequence of mouse renin lacking mE. mE.17 is a minimal mouse renin promoter spanning −176 to +6. mE.17m lambs were fed the ADF for 6 h after subcutaneous injection. The kidney was collected and frozen on dry ice immediately. Care and use of the mice met the standard procedures approved by the University Animal Care and Use Committee at the University of Iowa.

Electrophoretic Mobility Shift (EMSA) and Supershift Assay—Preparations of the nuclear extract from As4.1 cells and probes for EMSA and supershift assay were described previously (4). The labeled probes, 5′-32P-end-labeled on both ends of the annealed double-stranded oligonucleotides were filled with [α-32P]dATP (PerkinElmer Life Sciences) and 3 other cold nucleotides using Klenow DNA polymerase. The probes were purified through Sephadex G-50. Each binding reaction contained 0.02 pmol of labeled probe (about 60,000 dpm), 1 μg of nuclear extract, 1 μg of poly(dI-dC) (Roche Molecular Biochemicals), and binding buffer with the final concentration of (in mmol/liter): Tris-HCl (pH 7.5), 10, EDTA 1, dithiothreitol 1, MgCl2, 1, and KCl 60, as well as 5% glycerol in a total volume of 20 μl. For competition assays, cold competitor oligos were preincubated with nuclear extract and binding buffer for 15 min on ice before the addition of the probes. The binding reactions were then incubated on ice for another 15 min, and the products were resolved on a 5% nondenaturing polyacrylamide gel.

Rabbit polyclonal antibodies against human RAR subtypes: RARα, RARβ, and RARγ, and RXX subtypes: RXXα, RXXβ, and RXXγ were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The human anti-erasera cross-reacts with the specific RAR and RXX subtypes in the mouse. Universal monoclonal antibody against the mouse RXR family (including all three subtypes of α, β, and γ) was the generous gift from Dr. Pierre Chambon (CNRS, INSERM, Universite Louis Pasteur, Strasbourg, France). To prepare affinity-purified polyclonal antibodies (Santa Cruz) and the indicated amount monoclonal antibodies (Chambon) were added following the initial incubation of probe, nuclear extract, and binding buffer, and were left on ice for 60 min before electrophoresis.

RNA Isolation, RNase Protection Assay, and RT-PCR—Total cellular RNA was isolated from mouse As4.1 cells using TRI-REAGENT (Molecular Research Center, Inc., Cincinnati, OH) using the manufacturer’s protocol. Total renal RNA was isolated from mouse kidneys using our standard procedure (6). T7 RNA polymerase was used to prepare antisense RNA transcripts as RNase protection assay probes. The full-length and protected probe for mouse Ren-1′′ mRNA was 235 and 175 nucleotides, respectively. The full-length and protected probe for mouse Ren-1′′ mRNA was 235 and 175 nucleotides, respectively. RNase protection was performed using the Hyb-speed kit (Ambion Inc., Austin, TX). The protected RNA probes were resolved on 6% polyacrylamide denaturing gel (containing 8 M urea) and quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

RT-PCR was described as performed previously (7). The following synthetic oligonucleotides were used: RARα, 5′-CATGGCATGCGAGCGCATGCTGAAGCTC-3′ (1774 bp); RARγ, 5′-CAGGCTTGATATTTGTGTGAT-3′ and 5′-CATGAGTGTTGGCAGCAGATGTC-3′ (405 bp); RXRα, 5′-TCCTGGCCCAAGCAGCCATATGAC-3′ and 5′-CCCGGCAAGACCAAGAC-3′ (420 bp); RXRβ, 5′-CATGGCCCAGAGTCATTGCTGAAACCCG-3′ (459 bp); RXRγ, 5′-CTGAGCCCAAGACTGACATCC-3′ and 5′-CTGCTTAGCGCCTCTGACCCG-3′ (459 bp); RXRβ, 5′-CACAGCCGCTCATGATGACCGCTTCTT-3′ (404 bp); RXRγ, 5′-GGTTGCCTGCGAGTGGG-3′ and 5′-CATGTTGACGAGGAGGAGTCCTT-3′ (420 bp).

Construction of Wild-type and Dominant Negative RARα—The full-length cDNA encoding mouse RARα was PCR amplified using the primers 5′-GACTGTCTAGACTAC-3′ (Xhel site underlined) and 5′-CTGAATTCCTGGTGGTCGAGTTGCTG-3′ (EcoRI site underlined). This cDNA clone contains 1441 bp including the entire mRARα coding sequence. The PCR product was digested with Xhel and EcoRI to expose the restriction ends and was cloned into the mammalian expression vector, pCI (Promega, Madison, WI) under the control of the cytomegalovirus enhancer/promoter. To make the dominant negative mRARα403 expression vector, we first amplified a segment of cDNA, spanning the first 513 bp of the coding sequence corresponding to amino acids 1-170 using the primers 5′-CAGAGATACATCCTGGAAGATAAAC-3′ and 5′-GGTCTAGACCTGGGATCCCTAGGAAGC-3′ (EcoRI site underlined). This cDNA clone contains 1441 bp including the entire mRARα coding sequence. The PCR product was digested with Xhel and EcoRI to expose the restriction ends and was cloned into the mammalian expression vector, pCI (Promega, Madison, WI) under the control of the cytomegalovirus enhancer/promoter. To make the dominant negative mRARα403 expression vector, we first amplified a segment of cDNA, spanning the first 513 bp of the coding sequence corresponding to amino acids 1-170 using the primers 5′-CAGAGATACATCCTGGAAGATAAAC-3′ and 5′-GGTCTAGACCTGGGATCCCTAGGAAGC-3′ (EcoRI site underlined). This cDNA clone contains 1441 bp including the entire mRARα coding sequence. The PCR product was digested with Xhel and EcoRI to expose the restriction ends and was cloned into the mammalian expression vector, pCI (Promega, Madison, WI) under the control of the cytomegalovirus enhancer/promoter. To make the dominant negative mRARα403 expression vector, we first amplified a segment of cDNA, spanning the first 513 bp of the coding sequence corresponding to amino acids 1-170 using the primers 5′-CAGAGATACATCCTGGAAGATAAAC-3′ and 5′-GGTCTAGACCTGGGATCCCTAGGAAGC-3′ (EcoRI site underlined). This cDNA clone contains 1441 bp including the entire mRARα coding sequence. The PCR product was digested with Xhel and EcoRI to expose the restriction ends and was cloned into the mammalian expression vector, pCI (Promega, Madison, WI) under the control of the cytomegalovirus enhancer/promoter. To make the dominant negative mRARα403 expression vector, we first amplified a segment of cDNA, spanning the first 513 bp of the coding sequence corresponding to amino acids 1-170 using the primers 5′-CAGAGATACATCCTGGAAGATAAAC-3′ and 5′-GGTCTAGACCTGGGATCCCTAGGAAGC-3′ (EcoRI site underlined). This cDNA clone contains 1441 bp including the entire mRARα coding sequence. The PCR product was digested with Xhel and EcoRI to expose the restriction ends and was cloned into the mammalian expression vector, pCI (Promega, Madison, WI) under the control of the cytomegalovirus enhancer/promoter. To make the dominant negative mRARα403 expression vector, we first amplified a segment of cDNA, spanning the first 513 bp of the coding sequence corresponding to amino acids 1-170 using the primers 5′-CAGAGATACATCCTGGAAGATAAAC-3′ and 5′-GGTCTAGACCTGGGATCCCTAGGAAGC-3′ (EcoRI site underlined). This cDNA clone contains 1441 bp including the entire mRARα coding sequence. The PCR product was digested with Xhel and EcoRI to expose the restriction ends and was cloned into the mammalian expression vector, pCI (Promega, Madison, WI) under the control of the cytomegalovirus enhancer/promoter. To make the dominant negative mRARα403 expression vector, we first amplified a segment of cDNA, spanning the first 513 bp of the coding sequence corresponding to amino acids 1-170 using the primers 5′-CAGAGATACATCCTGGAAGATAAAC-3′ and 5′-GGTCTAGACCTGGGATCCCTAGGAAGC-3′ (EcoRI site underlined). This cDNA clone contains 1441 bp including the entire mRARα coding sequence. The PCR product was digested with Xhel and EcoRI to expose the restriction ends and was cloned into the mammalian expression vector, pCI (Promega, Madison, WI) under the control of the cytomegalovirus enhancer/promoter.
and Ea. The original m40 sequence containing Eb competitors used for EMSA. The stranded oligonucleotides probes and indicates the 5' head in the Ec sequence indicates the sequence employed are aligned. The sensus sequences for their cognate factors Eb, and Ea are underlined and the consensus sequences for their cognate factors are aligned. The dotted overlined sequence indicates the sequence employed in the Ec → Eb constructs and the double stranded oligonucleotides probes and competitors used for EMSA. The arrowhead indicates the 5' most nucleotide of the original m40 sequence containing Eb and Ea.

**Statistical Analysis**—All data are presented as mean ± S.E. Multiple comparison of data was analyzed by one-way ANOVA using SigmaStat (SPSS Scientific). When the test for normalization failed, the analysis was performed nonparametrically. Single comparisons are performed using Student's t test.

**RESULTS**

Using chimeric enhancers derived from the divergent regions of the mouse and human renin enhancer we previously identified two regulatory elements in the promoter proximal portion of the mouse renin enhancer. These studies were performed using As4.1 cells, which express renin and are likely derived from juxtaglomerular cells. Ea acted as a negative regulatory element and bound the factor NF-Y, and Eb acted as a positive regulatory element (4). Mutation of Eb revealed that it is required for maximal enhancer activity, but that Eb and Ea alone were insufficient to stimulate renin promoter activity, suggesting the presence of other transcription factor-binding sites in the enhancer. The purpose of the current study is to further examine the requirements for enhancer-mediated transcriptional activation and identify the stimulatory factors.

Mutational analysis of Eb revealed that it has the core sequence TGACCT. Sequence analysis of the 242-bp mE revealed two other TGACCT motifs which lie upstream (more distal) of Eb. The first motif, termed Es is present at the 5' terminus of the enhancer at coordinates -2847 to -2852. The other TGACCT motif, termed Ec, is located 10 bp upstream of Eb from -2668 to -2673, and thus lies upstream of the original m40 sequence containing Eb and Ea (Fig. 1B). Since our previous study demonstrated that enhancer function requires Eb, we performed transient transfection analysis in mouse renin expressing As4.1 cells to test whether Es or Ec were also required for enhancer activity. Site-directed mutagenesis was performed to convert the GA in TGACCT to TT, which in our previous study caused a loss of function of Eb. Both mutations were individually generated in mE2.6kLUC, which contains the mouse enhancer fused upstream of a 2624-bp mouse renin promoter (Fig. 1A). Mutation of Ec significantly reduced transcriptional activity (Fig. 2), whereas mutation of Es had no effect (data not shown). Interestingly, mutation of Ec caused a significantly greater drop in enhancer activity than did mutation of Eb. Moreover, the increase in enhancer activity caused by mutation of the negative regulatory Ea required both Eb and Ec (Fig. 2). The importance of Ec and Eb was confirmed by transfection into As4.1 cells. The motifs (a, b, or c) after the μ indicate which sequences were mutated. For example, mEμcb lacks both Eb and Ec. Fold induction over enhancerless control vector are indicted in parentheses. *, p < 0.05 versus no mE; †, p < 0.05 mEμc and mEμcb versus mEμb (n = 6).

The mouse renin enhancer acts in a position-independent manner and can strongly stimulate (>100-fold) a minimal mouse renin promoter when placed directly upstream. To characterize whether Ec + Eb has intrinsic enhancer activity on its own, we placed one or three copies of the Ec + Eb sequence directly upstream of the 117-bp promoter. Although one copy of the Ec + Eb sequence only slightly increased promoter activity (5.2-fold), three tandem copies of Ec + Eb markedly increased promoter activity (110-fold) to nearly the same level as mE (Fig. 3). Mutation of Eb and Ec in the 3XEc + Eb) construct abolished this induction.

We previously reported that Eb specifically interacted with unidentified nuclear proteins from As4.1 cells by EMSA. Since
both Ec and Eb consisted of a TGACCT stretch we hypothesized that they possessed the same protein binding activity. We identified two major DNA-protein complexes (L and S) formed on Ec + Eb (Fig. 4A). The two complexes were efficiently competed by Ec + Eb as well as mutants lacking either Ec or Eb. On the contrary, a mutant lacking both Eb and Ec was not able to compete. The results suggest that both Ec and Eb have the ability to form both complexes. This was confirmed by demonstrating that mutants lacking either site, but not both, could still form complexes L and S when used as probes in EMSA (Fig. 4B). The two complexes formed on both Ecμb and μE were efficiently competed by competitor DNAs containing either one or two TGACCT stretches (data not shown).

To facilitate our identification of the Ec + Eb-binding factors, we determined which bases in the TGACCT stretch were essential to its binding activity. To accomplish this we first demonstrated that complexes L and S could be efficiently competed by DNAs containing a single TGACCT motif from either Eb or Ec (Fig. 4C). We then examined the requirement of each base in the binding activity by using double stranded oligonucleotides mutated one base at a time as competitors in EMSA (Fig. 4C). The two complexes were efficiently competed by Ec and Eb as probes. The indicated competitors were added in 100-fold molar excess. Eb was derived from the 5′-half of EcEb. Both DNAs only contained one TGACCT motif. The mutant competitors contain the indicated single nucleotide mutation in the TGACCT motif of Ec. The last mutation (G to C) lies outside the TGACCT motif and does not prevent competition.

A direct repeat of the TGACCT motif separated by a spacer of variable length is the consensus recognition sequence for transcription factors in the thyroid/retinoid superfamily of nuclear hormone receptors. As a candidate approach to identify which nuclear receptor could bind to Ec + Eb, we transfected As4.1 cells with the 3X(Ec + Eb)117LUC reporter vector, and then treated the cells with four different common nuclear receptor cognate ligands. To eliminate potential effect of li-

Based on these results we hypothesized that RAR bind to Ec

**Fig. 3. Intrinsic enhancer activity of Eb + Ec.** Transient transfection analysis of LUC vector carrying a minimal mouse renin promoter (m117) with or without inserts were transiently transfected into As4.1 cells. No mE, no enhancer; mE, m117 plus the mE sequence; Ec + Eb, m117 plus a single copy of Eb + Ec (includes the 10-bp spacer); 3X, three tandem copies of Ec + Eb or mutations in Eb + Ec (Ecμ + Ebμ). Values for fold induction over m117 promoter are shown in parentheses. *p < 0.05 versus no insert (n = 6–9).

**Fig. 4. Nuclear protein binding to Ec + Eb.** EMSA of nuclear protein binding activity of Ec + Eb is shown. A. Ec + Eb is used as a probe. Competitor DNAs are indicated and present in 20- and 100-fold molar excess (indicated by ramps). Position of the large (L) and small (S) complex are indicated. B, EMSA was performed using the indicated double stranded oligos as probes. C, EMSA was performed using Ec + Eb as probe. The indicated competitors were added in 100-fold molar excess. Eb was derived from the 3′-half of EcEb. Both DNAs only contained one TGACCT motif. The mutant competitors contain the indicated single nucleotide mutation in the TGACCT motif of Ec. The last mutation (G to C) lies outside the TGACCT motif and does not prevent competition.

**Fig. 5. Evaluation of candidate nuclear hormone receptor ligands.** The 3X(Ec+Eb)117LUC construct was transiently transfected into As4.1 cells, and the transfected cells were treated with 3,3′,5-triiodo-l-thyronine (T3), α1,25-dihydroxyvitamin D3 (D3), 9-cis-retinoic acid (cRA), and tRA. Open bar, vehicle; filled bar, ligand treated. *p < 0.001 versus vehicle (n = 6–7).
mice received subcutaneous injection of either tRA (5 nM, 5) or vehicle (n = 5) as described under "Materials and Methods." Mouse kidneys were collected 54 h after the initial tRA treatment. RNA (10 μg) containing mutations in Eb (mEmE), containing the wild-type enhancer (mE), or containing mutations in Eb (mEmEg), or both (mEmEc). Cells were either treated with vehicle (open) or tRA (1.0 μM, closed). *, p < 0.05 versus untreated (n = 5).

FIG. 7. Role of Eb and Ec in mediating the response to retinoids. As4.1 cells were transiently transfected with 2.6kLuc either caused the appearance of a supershifted complex. Subtype-specific antisera for RXR revealed that RXRα bound to Ec + Eb (data not shown). That both RXRα and RXRα antisera only partially supershifted the complex suggests that other proteins, perhaps other members of the nuclear hormone superfamily may also bind to mE. RT-PCR and DNA sequencing verified the expression of RARα, RARβ, and -γ, and 0.025 μM of anti-RXR monoclonal antibody indicated by the Xi. L and S indicate the protein-DNA complexes and the supershift complexes are indicated by the open arrow.

Finally, we constructed expression vectors encoding mouse RARα1, and a dominant negative mutant of mRARα (mRARα403) which lacked the C-terminal ligand-dependent AF-2 transactivation domain, to specifically test the role of RARα in the Ec + Eb-mediated retinoic acid-induced activation of the mouse renin promoter (8, 9). Both cDNAs were placed under the control of the cytomegalovirus promoter/enhancer and were transiently cotransfected into As4.1 cells along with the 4.1kLuc reporter vector. Co-transfection of the wild-type mRARα expression vector significantly increased promoter activity of m4.1kLuc whether induced with tRA or left untreated (Fig. 10). On the contrary, co-transfection of the mRARα403 expression vector significantly attenuated the tRA-induced promoter activity of m4.1kLuc vector, but did not alter baseline expression. The stimulatory effect of the wild-type mRARα was completely abolished when Ec and Eb were mutated (data not shown). Our results suggest an important role of the Ec and Eb sequence in controlling baseline activity of mE and demon
The spacing between Ea and Eb is altered strongly suggests blocks the binding of transcription factors to Eb. Studies where binding of NF-Y to Ea acts as a negative regulator because it binding sites which mechanistically oppose each other (4). The regulation. We previously identified two transcription factor-negative RAR

As4.1 cells were transiently co-transfected with empty ex-
teractive RAR. 

expression vector pCI (open), or wild-type RARα (crosshatch), or dominant negative RARα403 (closed), along with the 4.1kLUC reporter vector. Transfected cells were cultured in media supplemented with 2% charcoal-treated FBS and either treated with vehicle (V) or tRA 24 h after transfection. The cells were harvested for LUC and β-gal activity 24-h after treatment with tRA. *, p < 0.05 versus pCI (n = 5).

strate a potential role for retinoids in regulating expression of the renin gene.

**DISCUSSION**

The renin gene enhancer is a potent enhancer of transcription that works in a position- and orientation-independent manner in renin-expressing As4.1 cells (2). Its ability to stimulate up to a 100-fold increase in transcriptional activity of the renin promoter makes it an important candidate in renin gene regulation. We previously identified two transcription factor-binding sites which mechanistically oppose each other (4). The binding of NF-Y to Ea acts as a negative regulator because it blocks the binding of transcription factors to Eb. Studies where the spacing between Ea and Eb is altered strongly suggests that NF-Y sterically blocks the binding of factors to Eb.2 The importance of Eb was shown by a loss of enhancer activity after mutagenesis. In the present study, we demonstrate that a second TGACCT motif located 10 bp upstream of Eb termed Ec is also required for maximal enhancer activity. Mutation of either Ec or Eb in mE essentially eliminates enhancer function. Mutation of a third TGACCT motif (Es) did not effect enhancer function.

The Eb and Ec sequences are clearly required for baseline activity of the enhancer. In addition, their importance is supported by the observation that they fit the consensus sequence for a member of the nuclear hormone receptor superfamily, are required to mediate induction of the renin promoter by retinoid acid, and bind RARs and RXRs. There was no induction when cells were treated with thyroid hormone or vitamin D3. Within the superfamily, selective recognition of different ligand/receptors is determined by the number of intervening base pairs between the two TGACCT motifs. In general, heterodimers of TR/RXR selectively bind to DR4 and heterodimers of VDR/RXR selectively bind to DR3. Interestingly, heterodimers of RAR/RXR preferentially bind to DR2 or DR5 (10). There are 10 intervening base pairs between Ec and Eb in mE. Our EMSA and supershift studies clearly demonstrate the ability of the DR10 sequence to bind RARα and RXRα. Despite the preference for DR2 or DR5, the retinoic acid responsive γF-crystallin and medium chain acyl-coenzyme A dehydrogenase genes contain DR3, and the oxytocin and laminin B1 genes contain DR14 (11–14).

It is interesting to note that constructs containing mutations in either Eb or Ec, but not both, still responded to tRA stimulation, but with reduced responsiveness. This is consistent with our EMSA results showing that probes containing one intact TGACCT motif were still capable of forming two complexes that had the same mobility as those with probe Ec + Eb. Imperfect motifs for RAR/RXR exist in many genes, such as apolipoprotein A1, oxytocin, γF-crystallin, medium chain acyl-coenzyme A dehydrogenase, phosphoenolpyruvate carboxyki-
nase, and β-crystallin (11, 13–17). Each can still bind heterodimers of RAR/RXR and mediate retinoic acid-induced gene transcription. This may be an important consideration because the human renin enhancer (hE) has one perfect TGACCT motif (Ec) and one variant motif (TGGCCT, Eb). Baseline transcriptional activity of hE was considerably lower than that of mE (4), but nevertheless retained modest retinoic acid-induced transcription (data not shown).

When a heterodimer of RAR/RXR binds to a TGACCT direct repeat, RAR selectively binds to the upstream TGACCT while RXR selectively binds to the downstream TGACCT (18). Forcing RAR/RXR to bind RARE in the opposite direction abolished its transactivation activity. Our study, mutation of Ec always had a stronger effect than mutation of Eb, suggesting the possibility for asymmetric binding of RAR/RXR to the RARE. In a model described in Westin et al. (19), RAR plays a pivotal role to initiate the hierarchical assembly of transcriptional proteins on RARE. If this is true for RAR/RXR binding to Ec + Eb in mE, RAR will bind to Ec and RXR will bind to Eb. Since RAR/RXR-mediated transactivation depends on coactivators, and the interaction between RAR/RXR and coactivators initiates from RAR, disturbance of RAR binding to Ec should cause a marked loss of the functional RAR/RXR-ligand-coactivator complex.

Despite the retinoid-mediated induction of the renin promoter (and endogenous renin gene) mediated by RAR, it is likely that other transcription factors bind to the Ec + Eb sequence. EMSA revealed two major protein-DNA complexes, and supershift does not cause a reduction in either the L or S complex. Indeed, Eb and Ec are both required for the induction by retinoids and for baseline activity of the enhancer in the absence of ligand. These data suggest that other transcription factors, perhaps other members of the hormone receptor superfamily may also play a role in regulating renin gene expression. It is possible that the requirement for Ec + Eb in mediating baseline transcriptional activity of mE may occur independently of RAR/RXR, while the retinoic acid induced activity requires RAR/RXR. We are currently attempting to identify other Ec + Eb binding factors using yeast one-hybrid analysis and have preliminary data implicating an orphan nuclear receptor. Some orphan receptors have been reported to bind the TGACCT as a monomer to regulate RARE function (20).

In addition to Ec and Eb, Gross and colleagues3 have identified a fourth transcription factor-binding site located 16 bp upstream of Ec termed Ed. This sequence is similar to the consensus binding site for members of the CREB/ATF-1 family of transcription factors. Interestingly, the transactivation function of both RAR/RXR and CREB/ATF-1 requires an interaction with coactivators, and both are able to interact with p300/ CBP (21, 22). Therefore, it is possible that transcription factors binding to Ed and Ec may interact. Recall that the human renin enhancer retains an intact Ed and Ec, but lacks Eb. Mechanistically, the situation may be analogous to the pit-1 gene where RAR binds to a single core recognition motif to activate retinoic acid-dependent transcription (23). In the pit-1 gene, the RARE is immediately adjacent to a Pit-1-binding site. Retinoic acid-dependent transcription of pit-1 requires Pit-1, which like RAR and CREB/ATF-1 requires CBP as a coactivator (24).

In closing, our results pose the obvious question as to the relevance of renin gene regulation by retinoids in vivo. As this

3 T. A. Black and K. W. Gross, unpublished observation.
is the first study to implicate this pathway, its importance in adults remains unclear. However, it is now clearly recognized that retinoids are critical signaling molecules during development. Vitamin A deficiency during development leads to fetal vitamin A deficiency syndrome, which includes abnormalities in the urogenital tract including the kidney (25). Moreover, retinoic acid is thought to be the active metabolite of vitamin A during development. Knockout mice deficient in specific subtypes of both RARα and RARβ or RARα and RARγ develop severe renal malformations generally characterized by renal agenesis and aplasia (26). Histological analysis revealed the defect to be a failure of the Wolffian or mesonephric duct to contact the metanephric blastema. This interaction is critical for the differentiation of the tubular and eventual development of the vascular system in the kidney. Interestingly, mice lacking genes in the renin-angiotensin system also develop severe renal abnormalities although at a much later stage in development (27). Renin expression is first visible in the metanephric kidney at 15.5 days postcoitum and is localized in the developing arterial tree (28, 29). Expression of renin coincides with the growing branches of the arterial tree suggesting it may play an important developmental role. Therefore, it is possible that as retinoids are critical in very early events in renal development, so are they needed to induce renin expression developmentally. The expression of RAR and RXR in A54.1 cells which are believed to derive from juxtaglomerular cells is consistent with this notion. Clearly, additional studies examining the coexpression of RAR with renin during renal development would seem necessary.

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