Identification of a Nuclear Orphan Receptor (Ear2) as a Negative Regulator of Renin Gene Transcription

Xuebo Liu, Xiaodong Huang, Curt D. Sigmund

Abstract—A potent transcriptional enhancer was previously identified upstream of the mouse renin gene. Within the enhancer is a TGACCT direct-repeat motif, required for enhancer activity, that is the consensus sequence recognized by members of the thyroid hormone subfamily of steroid hormone receptors. We previously reported that RAR/RXR bind to this sequence and mediate the induction of renin promoter activity by retinoids. However, gel mobility shift assays clearly show that other as yet unidentified factors also bind to this motif. In order to identify some of these TGACCT binding factors, we screened a yeast one-hybrid cDNA library derived from mouse As4.1 cells. One of these encoded the orphan nuclear receptor Ear2. Recombinant Ear2 was purified from Escherichia coli and an antipeptide antiserum was generated. EMSA showed that purified recombinant Ear2 specifically binds the TGACCT direct-repeat motif. Transfection assays showed that Ear2 potently decreases both baseline and retinoid-induced mouse renin promoter activity in a dose-dependent, enhancer-dependent, and sequence-specific manner. Mutations in Ear2, which abolish its binding to the TGACCT motif, also abolish transcriptional repression. Ear2 was identified as a nuclear protein in As4.1 cells, is one of the proteins binding to the TGACCT repeat motif, and its overexpression can repress transcription of the endogenous renin gene in As4.1 cells. These data suggest that Ear2 is a negative modulator of renin gene transcription in As4.1 cells, and that the renin enhancer may actually encode a complex positive and negative regulator of transcription. (Circ Res. 2003;92:1033-1040.)

Key Words: transcription ♦ transfection ♦ gel shift ♦ repression ♦ nuclear receptor

Renin is synthesized in the kidney and is known to play a pivotal role in blood pressure and extracellular fluid regulation. As the rate-limiting enzyme in the renin-angiotensin cascade leading to production of angiotensin II, understanding its regulation of expression and secretion has been the focus of many investigators. Renin is regulated at the transcriptional level, although posttranscriptional regulation has also been reported. Among the transcriptional elements proposed to regulate renin expression are promoter proximal response elements for cAMP and Pit-1 and the Abd-class of Hox genes. Further upstream is a nonclassical binding site for LXR/sterol regulatory element (RARE). In addition, this sequence may also bind members,13,14 little is known regarding the importance of transcription of the endogenous renin gene and at −12 kb upstream of the human renin gene.6–8 This transcriptional enhancer contains at least 5 different transcription factor binding sites that have both stimulatory and inhibitory activities.9–11 Two of these binding sites match the consensus sequence for members of the thyroid hormone family of transcription factors (TGACCT) but contain a spacer sequence (DR10), which does not conform to any previously identified steroid hormone receptor response element. This sequence is necessary for enhancer activity and can substitute for the other transcriptional elements when concatemerized. We previously reported that sequence binds RARα/RXRα, mediates the induction of renin promoter activity by retinoic acid, and may therefore act as a RAR response element (RARE).9 In addition, this sequence may also be responsible for the downregulation of renin promoter activity mediated by vitamin D3, observed by us and others.9,12

Despite these observations, gel shift studies reveal that at least 3 other proteins may form a complex with the RARE. To identify these proteins and determine their role in the regulation of renin expression, we used the yeast one-hybrid system to isolate RARE binding proteins. One orphan nuclear receptor, Ear2, a member of the COUP-TF group belonging to the thyroid receptor subfamily was identified and cloned. Although much progress has been made in understanding the biochemical characterization and function of other COUP-TF members,13,14 little is known regarding the importance of Ear2. In the present study, we show that Ear2 may be an endogenous negative regulator of renin promoter activity through its interaction with the renin enhancer.

Materials and Methods

Yeast One-Hybrid Analysis
The yeast one-hybrid system was used to isolate cDNAs encoding proteins binding to the RARE (MATCHMAKER, Clontech). Two
bait sequences were generated in pHISi, pHISi-1, and pLacZi, consisting of (1) 4-tandem copies of element-B, and (2) 2-tandem copies of element-B and -C separated by the native DR10 sequence found in the enhancer. The sequence of the top-strand oligonucleotides were CTCTGACCTCTCTCTGACCTCTCTCTGACCTCTCTCTGACCT and CAGATGGTGACCTGGCTGTACTCTGACCTCTGACCT (TGACCT motifs underlined). Upstream EcoRI and downstream MluI or SalI sites were added for cloning. A custom cDNA library was generated from As4.1 cells15 consisting of 2.0 × 10^6 independent clones (insert size ranging from 0.4 to 4.0 kb and averaging 2.0 kb) in pGAD10. One million clones were screened, and cDNA from positive colonies was sequenced, reconfirmed by one-hybrid analysis in yeast cells, and identified by BLAST (Table).

**Ear2 Constructs**

Ear2 cDNA was PCR cloned into pcDNA3.1 (Invitrogen) and pQE30 (Qiagen). To prepare mutants, DNA fragments were obtained with splicing overlap extension by PCR and similarly subcloned. The primers were designed to change the amino acid sequence from 74-CEGCKS-79 (wild type) to 74-CESCIV-79 (mutant) and from 93-CRSNRDCQ-100 (wild type) to 93-CGVNRLCQ-100 (mutant). These mutations were in the zinc finger DNA binding domain.16

**Purification, Identification, and Expression of Ear2 From Escherichia coli**

*E. coli* M15 containing pQE-Ear2 plasmid was cultivated, induced with IPTG (1.0 mmol/L) for 4 hours, pelleted, lysed in 50 mmol/L NaPO4 (pH 8.0), 300 mmol/L NaCl, and 10 mmol/L imidazole, and Ear2 was purified by Ni-agarose column. The lysate were separated by electrophoresis on 10% SDS polyacrylamide gel, transferred to nitrocellulose, and proteins were detected with His-tag antisera or Ear2 antisera. Ear2 antisera was generated in rabbits against the peptide TSDAEPGDEERP based on previous generation of a homologous human Ear2 antiserum (Biosynthesis).

Whole cell proteins were prepared by sonicating cells in lysis buffer [10 mmol/L HEPES (pH 7.8), 10 mmol/L KCl, 2 mmol/L MgCl2, 0.1 mmol/L EDTA, 1% NP40]. For nuclear proteins, cells were resuspended in hypotonic buffer on ice, detergent was added to 0.5%, nuclei were pelleted, then suspended in nuclear protein extract buffer [50 mmol/L HEPES (pH 7.8), 300 mmol/L NaCl, 50 mmol/L KCl, 0.1 mmol/L EDTA, 10% (v/v) glycerol] on ice for 30 minutes, and spun with the supernatant saved. Cytoplasmic and membrane proteins were obtained by suspending cells in lysis buffer without detergent, sonication on ice, and spun at 120,000 g for 30 minutes. The supernatant (cytoplasmic proteins) was saved. The pellet was resuspended in lysis buffer with 1% detergent, sonicated on ice, and pelleted; the supernatant is membrane proteins.

**Immunohistochemistry of As4.1 Cells**

As4.1 cells were plated at 2 × 10^5 cells/well in a 6-well plate with 22 × 22 mm cover glass one day before the experiment. Cells were washed 3 × with PBS to remove growth media, fixed with 4% paraformaldehyde with triton X-100 for 15 minutes, rinsed, and then blocked with 5% Donkey Serum/1% BSA in PBS overnight at 4C. Ear2 antisera at 1:100 dilution in block solution was added for 2 hours. Cells were washed with PBS, and the secondary antisera was added for 1 hour. Cells were rinsed and stained with DAPI and mounted with Vectashield.

**Electrophoretic Mobility Shift Assays**

Nuclear proteins (5 μg) from As4.1 cells or 0.5 μg purified recombinant Ear2, 10 fmol/L oligonucleotide probe containing elements b and c of the renin enhancer (5'-GAT CCCAGATGGTGACCTGGCTGTACTCTGACCTCTGACCTCTGACCT-3') end labeled with [32P]-ATP (NEN Life Science Products) were incubated in 20 μL binding

### Yeast One-Hybrid System Hits

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*No sequence matches in nucleotide database (nr); homology found in EST database.
†Similar to the indicated gene based on search of EST database and UniGene assignment.
USF-1, and USF-2 (Figure 1A). RNase protection. Quantification was performed using the 30 hours after transfection and renin mRNA was quantified by pcDNA3.1 or Ear2 expression vector. RNA was harvested from cells endogenous renin expression, As4.1 cells were transfected with /H11005

To determine the effects of Ear2 on protein in the lysate. Each luciferase assay was performed in Luciferase activity was determined and normalized to total cellular reporter vector by Fugen-6 (Roche). Forty to 48 hours after transfection in DMEM containing 10% fetal bovine serum. Cells were cotransfected with Ear2 expression vector and luciferase transfection in DMEM (Life Technologies) supplemented with 10% maintained in DMEM (Life Technologies) supplemented with 10% fetal bovine serum. Cells were cotransfected with Ear2 expression vector and luciferase reporter vector by Fugen-6 (Roche). Forty to 48 hours after transfection, the cells were harvested and lysed using the luciferase kit. Luciferase activity was determined and normalized to total cellular protein in the lysate. Each luciferase assay was performed in duplicate to get an n=1. To determine the effects of Ear2 on endogenous renin expression, As4.1 cells were transfected with pCDNA3.1 or Ear2 expression vector. RNA was harvested from cells 30 hours after transfection and renin mRNA was quantified by RNase protection. Quantification was performed using the IMAGEQuant software on a STORM 820 PhosphorImager.

Cell Culture, Transient Transfection, and Luciferase Assay
As4.1 cells (American Type Culture Collection CRL2193) were maintained in DMEM (Life Technologies) supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 mg/mL). Cells (2×10⁵/well) were plated on 6-well culture plates 24 hours before transfection in DMEM containing 10% fetal bovine serum. Cells were cotransfected with Ear2 expression vector and luciferase reporter vector by Fugen-6 (Roche). Forty to 48 hours after transfection, the cells were harvested and lysed using the luciferase kit. Luciferase activity was determined and normalized to total cellular protein in the lysate. Each luciferase assay was performed in duplicate to get an n=1. To determine the effects of Ear2 on endogenous renin expression, As4.1 cells were transfected with pCDNA3.1 or Ear2 expression vector. RNA was harvested from cells 30 hours after transfection and renin mRNA was quantified by RNase protection. Quantification was performed using the IMAGEQuant software on a STORM 820 PhosphorImager.

Statistical Analysis
Transfection results were compared by ANOVA using SigmaStat. A value of P<0.05 was considered significant.

Results
The renin gene enhancer is a complex regulatory element consisting of the binding sites for a number of transcription factors including NF-Y, RARα/RXRα, CREB, CREM, USF-1, and USF-2 (Figure 1A). One of these binding sites consists of a direct repeat of the canonical TGACCT motif recognized by members of the steroid hormone nuclear receptor superfamily, but containing an atypical spacer (DR10). We previously showed that the TGACCT-DR10-TGACCT motif acts as a retinoic acid response element (RARE), and is both required for retinoic acid–mediated induction of the renin promoter, and for baseline (retinoic acid–independent) activity of the enhancer. Electrophoretic mobility shift assays suggest that at least 4 proteins complex with the RARE. We hypothesize that one of these interactions may account for baseline enhancer activity. Therefore, in order to identify other transcription factors that bind to this site, we performed a yeast one-hybrid screen of 10⁶ colonies from a mouse As4.1 cell cDNA library using two different TGACCT combinations (see Materials and Methods) as bait. Mouse As4.1 cells express renin and are thought to represent the best model of a renal juxtaglomerular cell in culture.5 Two rounds of screening yielded 21 positive clones that were subjected to BLAST search of nucleotide and EST databases (Table). Ear2 attracted immediate attention as it was found most frequently (4 of 21 clones), was replicated in two different screens, gave a positive signal when transfected on its own into yeast with two different bait plasmids, and is an orphan member of the same subfamily of nuclear hormone receptors as RAR.

In order to determine if Ear2 has a role in regulating renin expression, we first cloned its full-length cDNA into expression vectors for purification of a 6XHis-tagged protein in bacteria and for transfection analysis in As4.1 cells. We also generated Ear2 antisera using a homologous peptide to one used previously to make human Ear2 antisera. A 45-kDa protein purified from bacteria was recognized by both anti-His and anti-Ear2 antisera (Figure 2A). Two different molecular weight forms of Ear2 were identified in As4.1 cells (Figure 2B). Two forms of Ear2 were previously detected by Zhu et al.16 in transfected CV1 cells. The 45-kDa form comigrated with His-tagged Ear2 purified from bacteria. Immunofluorescence studies revealed primarily nuclear localization of Ear2 in As4.1 cells (Figures 2D through 2F). Interestingly, cell fractionation revealed that both forms are present in the nucleus, whereas the lower molecular weight

Figure 1. Schematic representation of the renin enhancer and Ear2 DNA binding domain. A, Sequence of the functional region of the renin enhancer is shown along with identified transcription factor binding sites. B, DNA binding domain of mouse Ear2 protein showing the two zinc fingers, and the sites altered to generate the M1 and M2 mutations.

Figure 2. Purification of recombinant Ear2 and expression in As4.1 cells. A, Western blot of purified recombinant Ear2 using Ear2 antipeptide antisera and anti-His tag antisera. B, Western blot of purified Ear2 and nuclear proteins from As4.1 cells using Ear2 antipeptide antisera. C, Western blot of subcellular fractionation of As4.1 cells. Lysates were prepared from whole cells (W), cytoplasm (C), membrane (M), and nucleus (N). D through F, Immunofluorescence image of As4.1 cultures examined with anti-Ear2 (D), DAPI stain (E), or merged (F).
RARE mutated in both half sites (H9262 with the b or c half sites mutated; and H9262). Ear2 erase activity was corrected with total protein and normalized to transfections lacking Ear2. *P<0.05 vs control (0 concentration) by ANOVA. B, Transfected cells were either left untreated or were treated with 1μmol/L all-trans retinoic acid (atRA). Total DNA concentration was held constant with carrier plasmid DNA. Relative luciferase activity was corrected with total protein and normalized to transfections lacking Ear2. *P<0.05 vs control (0 concentration) by ANOVA. C, Cells were cotransfected with either 4.1-kb luc, 3X-RARE-117P, or mE-117P along with either empty vector (pcDNA3.1, gray bars) or Ear2 expression vector (black bars). *P<0.05 vs 0; **P<0.05 vs retinoic acid–treated control by ANOVA.

Figure 4. Transcriptional repression by Ear2. As4.1 cells were co-transfected with 4.1-kb Luc and either empty pcDNA3.1 or with the indicated amount of Ear2 expression vector. Total DNA concentration was held constant with carrier plasmid DNA. A, Relative luciferase activity was corrected with total protein and normalized to transfections lacking Ear2. *P<0.05 vs control (0 concentration) by ANOVA. B, Transfected cells were either left untreated or were treated with 1μmol/L all-trans retinoic acid (atRA). Total DNA concentration was held constant with carrier plasmid DNA. Relative luciferase activity was corrected with total protein and normalized to transfections lacking Ear2. *P<0.05 vs control (0 concentration) by ANOVA. C, Cells were cotransfected with either 4.1-kb luc, 3X-RARE-117P, or mE-117P along with either empty vector (pcDNA3.1, gray bars) or Ear2 expression vector (black bars). *P<0.05 vs control by ANOVA.
all be equally competed with cold RARE or RARE half sites (Figure 6A). Recombinant Ear2 comigrates with either complex-b or complex-c. In order to formally identify which complex contains Ear2, we performed supershift analysis using Ear2 antisera. As anticipated, the His-antisera supershifted the complex formed with recombinant Ear2, but did not supershift any of the complexes formed with As4.1 nuclear extract (Figure 6B). On the contrary, in 3 independent experiments, Ear2 antisera caused a loss of complex-c (compare lane 2 or 3 to lane 5 in Figure 6B). There was also an enhancement of complex-a that is unlikely due to supershift of complex-c as the supershift position of recombinant Ear2 is at a higher molecular weight (lane 10). Ear2 antisera was unable to cause any mobility shift in the absence of nuclear extract.

Finally, we determined if Ear2 could regulate expression of the endogenous mouse renin gene expressed in As4.1 cells. We therefore transiently transfected As4.1 cells with either an empty expression vector (pcDNA3.1) or an expression vector containing Ear2. Cotransfection studies using an eGFP expression vector indicated that approximately 90% of the cells detected with DAPI stain also expressed eGFP (data not shown). RNase protection assay revealed that steady state renin mRNA levels were lower in cells transfected with the Ear2 expression vector (Figure 7A). Two independent experiments, each performed in duplicate, revealed a reduction of renin mRNA to 36% of control (Figure 7B).

**Discussion**

Renin gene transcription is regulated in response to a complex series of physiological cues. Some estimates suggest that the range of renin mRNA expression can be as high as 100-fold depending on the physiological status of the organism. Studies of the renin 5'-flanking sequence has identified a number of transcription factor binding sites both proximal to the promoter and further upstream. Transcription factor binding sites close to the promoter include CREB, Pit-1,2,3 and an atypical binding site for the transcription factor LXRα.5 Mutagenesis experiments suggest that all three of these sites are required for stimulation of renin expression by cAMP. The LXRα binding site, termed the CNRE (for cAMP responsive element–negative regulatory element), has also been implicated in the gene-specific regulation of mouse renin in submandibular gland.23 This notion has been chal-
enhancer including NF-Y, RAR, and RXR. The binding sites for numerous transcription factors in the renin enhancer are located 10 kb further upstream than in the mouse. The physiological significance of this observation remains unknown. Extensive mutagenesis, electrophoretic mobility shift, and supershift assays have revealed that at least four different proteins bind to the RARE, of which only a minor component is RARα and RXRα. We therefore performed a yeast one-hybrid screen to identify other RARE binding proteins.

Herein, we demonstrated that Ear2, an orphan member of the nuclear hormone superfamily, is present in As4.1 cells, serves to repress both endogenous renin expression and transfected renin promoter constructs, attenuates the transcriptional response to retinoic acid, binds to the same site as RAR/RXR with the same sequence specificity, and acts through the RARE. Given the robust transactivation function provided by the enhancer, we were initially surprised that Ear2 would act to oppose transcription. In agreement with this, however, are reports that Ear2 is a negative coregulator of thyroid hormone receptor (T3R) function46 and inhibits transcription of the luteinizing hormone receptor and a number of other genes.47,48 Studies of T3R function suggest that Ear2 binds to T3R, preventing its binding to the TRE.16 Ear2 is a member of COUP-TF family. In addition to retinoid receptors, a number of orphan receptors, including COUP-TF have been implicated in the regulation of the retinoid response.28–30 COUP-TF has been reported to repress transcription induced by RARs, as well as T3R and vitamin D receptor.31–34 In contrast, COUP-TF can also function as positive regulators for many different genes.35–38

Given the inhibitory effect of Ear2 on renin transcription, it is interesting to note that in the context of the renin enhancer, NF-Y, which normally is a transcriptional inducer, acts as a negative regulator. Mechanistically, NF-Y inhibits enhancer-mediated transactivation because its binding site overlaps with one of the RARE half sites and prevents the binding of transcription factors to that site.39 Increasing the spacing between the NF-Y binding site and the RARE eliminates the inhibitory influence of NF-Y. We propose that Ear2 acts by binding either directly to transcription factor(s), thus preventing their binding to the renin enhancer RARE, or by binding to the RARE and thus hindering the binding of other stimulatory factors. Functionally therefore, although they may act through different mechanisms, Ear2 and NF-Y may both prevent the binding of transcription factors to the RARE. Moreover, these two proteins may not be the only negative modulators of renin enhancer function. Recent studies suggest that vitamin D₃ is also a negative regulator of renin expression, and although the mechanism by which this occurs has yet to be established, vitamin D₃ receptor and RARα both share the same core binding site and heterodimerize with RXR.12,39 Clearly therefore, the enhancer is a complex regulatory element responding to both stimulatory and inhibitory stimuli.

Despite the effort aimed at characterizing the renin enhancer sequence, we have yet to determine its functional relevance in vivo. Transgenic mice containing the renin 5'-flanking region, including the enhancer fused to either SV40 T antigen or egFP, correctly express the gene in fetal development and in adults, and the transgene responds to physiological cues known to regulate the renin gene.40,41
Alternatively, transgenic mice containing a construct lacking the enhancer are inappropriately expressed. Similarly, fusions between the human renin promoter and several different reporter genes that lack the enhancer are poorly expressed and regulated, whereas large genomic transgenes derived from P1 artificial chromosomes containing the enhancer are tightly regulated. Despite this suggestive evidence, experiments designed specifically to test the in vivo significance of the enhancer or one of its transcription factor binding sites has yet to be reported. Using homologous recombination in bacteria, we have recently modified a PAC clone containing the human renin gene by substitution of the enhancer sequence with a loxP511 site. Transgenic mice have been generated and a comparison of renin expression will be performed between mice containing the wild-type and mutant constructs. These and additional experiments in which individual transcription factor binding sites are mutated should provide, for the first time, a model system to link physiological cues with specific transcriptional events.

Acknowledgments

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