Chicken Ovalbumin Upstream Promoter Transcription Factor II Regulates Renin Gene Expression*

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Background: The production of the hormone renin is transcriptionally regulated.

Results: The nuclear receptor COUP-TFII binds to the renin gene promoter and is necessary for the cAMP-induced renin gene expression.

Conclusion: COUP-TFII stimulates renin gene transcription.

Significance: The molecular mechanisms controlling the expression of renin are crucial for the understanding of its role in blood pressure regulation and nephrogenesis.

This study aimed to investigate the possible involvement of the orphan nuclear receptor chicken ovalbumin upstream promoter transcription factor II (COUP-TFII) in the regulation of renin gene expression. COUP-TFII colocalized with renin in the juxtaglomerular cells of the kidney, which are the main source of renin in vivo. Protein-DNA binding studies demonstrated that COUP-TFII binds to an imperfect direct repeat of the cAMP response element (CRE) in the proximal renin promoter. Because cAMP signaling plays a central role in the control of the renin gene expression, we suggested that COUP-TFII may modulate this cAMP effect. Accordingly, knockdown of COUP-TFII in the clonal renin-producing cell lines As4.1 and Calu-6 diminished the stimulation of the renin mRNA expression by cAMP agonists. In addition, the mutation of the proximal promoter element in renin promoter reporter gene constructs abrogated the inducibility by cAMP. The proximal promoter sequence was identified in the proximal promoter of the human renin gene. However, the deficiency of COUP-TFII did not further diminish the renin expression when CREB was knocked down. In agreement with the cell culture studies, mutant mice deficient in COUP-TFII have lower renin expression than their control strain. Altogether our data show that COUP-TFII is involved in the control of renin gene expression.

The renin-angiotensin system is one of the key regulatory systems that control blood pressure. The renin-angiotensin system plays a central role in the regulation of arterial tone and NaCl excretion (1). The kidney hormone renin determines the activity of the renin-angiotensin system. Renin is produced in the juxtaglomerular (JG) cells, which represent a small cellular population in the afferent arteriole. The renin production is precisely regulated at the transcriptional level (1, 2). The renin gene is controlled by a variety of transcription factors that interact to produce a distinctive developmental and cell-specific expression pattern. Therefore, the proper expression of renin during the embryogenesis is decisive for the kidney development (nephrogenesis). COUP-TFII is an orphan nuclear receptor transcription factor (3, 4). It belongs to a nuclear receptor subfamily that includes also the closely related COUP-TFI. In addition, it is also expressed in organs and tissues (11, 12). In addition, it is also expressed throughout the kidney, starting in the early embryonic stages (13). However, nothing is known about the possible role of COUP-TFII in the regulation of the kidney functions in the adult. Interestingly, a putative COUP-TFII recognition sequence was identified in the proximal promoter of the human renin gene (14). DNase I footprint assays demonstrated protein binding to this motif, but it remained elusive whether COUP-TFII is part of the bound complex (15). Because the proximal promoter is decisive for the control of renin transcription, we examined whether COUP-TFII is involved in the regulation of renin gene expression. Such a function of COUP-TFII is not unlikely, because a row of nuclear receptors is already known to regulate renin gene transcription (1, 2). We used for the experi-

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Supplemental Figs. S1 and S2.

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iments As4.1 and Calu-6 cells, which are clonal cell lines with endogenous renin production, as well as mice deficient in COUP-TFII (11, 16, 17). We found that COUP-TFII is expressed in the renin-producing cells and that it binds to the proximal renin promoter. We also show that COUP-TFII is involved in the regulation of the renin gene by cAMP signaling, which is considered to be the principal intracellular cascade regulating the synthesis of renin.

EXPERIMENTAL PROCEDURES

Cell Culture—The clonal renin-producing cell lines As4.1 and Calu-6 were already described by our group (18–24). Briefly, the As4.1 mouse cells (ATCC-CRL-2193) were propagated in Dulbecco’s modified essential medium supplemented with 10% fetal calf serum, 1-glutamine and sodium pyruvate, 100 units/ml penicillin, and 100 μg/ml streptomycin and incubated at 37 °C in a humidified atmosphere containing 10% CO2. The human Calu-6 cells (ATCC-HTB-56) were cultured in Eagle’s minimal essential medium supplemented with 5% fetal bovine serum, sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin, and 1% nonessential amino acids at 37 °C in a humidified atmosphere containing 5% CO2. The cells were treated with cAMP agonists overnight (16–20 h) where indicated. Forskolin was applied at 5 μM, the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine at 100 μM, and pituitary adenylate cyclase-activating polypeptide at 30 nM.

Western Blotting—Protein extracts were isolated from As4.1 and Calu-6 cells, and Western blotting was performed as described (20, 22, 24). Confluent cells grown in 25-cm² flasks were lysed in protein lysis buffer (10 mm Tris, 1% SDS, 1% Nonidet P-40, 5 mM Pefabloc), and the protein concentration was measured. The samples were boiled in Laemmlni sample buffer for 5 min and applied onto 10% SDS-polyacrylamide gels. The proteins were separated by electrophoresis and then transferred onto nitrocellulose membranes (Bio-Rad) in transfer buffer (48 mM Tris, 39 mM glycine, 0.037% SDS, 20% methanol). After blocking, anti-COUP-TFII, CREB, or anti-β-actin primary antibody (Perseus Proteomics, Cell Signaling, and Sigma, respectively) was applied. Bound antibody was detected with horseradish peroxidase-conjugated secondary antibody (DiaNova) and visualized by enhanced chemiluminescence.

Renal tissues were homogenized and lysed in radioimmune precipitation assay buffer (150 mm NaCl, 10 mm Tris-Cl (pH 7.5), 0.1% SDS, 0.1% Triton X-100, 1% deoxycholate, and 5 mm EDTA). Protein content of the lysates was determined by the BCA protein assay system (Pierce). The lysates were subjected to SDS-PAGE and blotted onto nitrocellulose membranes. Immunoblotting was performed using goat anti-mouse renin-1 antibody (R&D Systems) or mouse anti-β-actin antibody (Sigma). The signals were visualized by using the ECL Plus Western blotting detection system (Amersham Biosciences). Densitometry was done using ImageJ (National Institutes of Health).

Electrophoretic Mobility Shift Assay (EMSA)—Nuclear proteins were isolated from As4.1 and Calu-6 cells following the standard protocol of our laboratory (20, 21, 23). The nuclear protein extracts were probed with a mouse or human renin COUP-TFII recognition sequence (designated proxDR) in EMSAs. To this end, we used the GelShift™ chemiluminescent assay kit from Active Motif, following the instructions of the manufacturer. Double-stranded 3’-biotinylated oligonucleotides were used as probes (sense strand (human), 5’-ccagggctcacagggccaagc-3’; sense strand (mouse), 5’-ctagagtttgcgggccaggccagc-3’ (the proxDR sequences are underlined)). The COUP-TFII antibody used for the supershifts was from Perseus Proteomics.

Chromatin Immunoprecipitation (ChiP)—DNA-protein complexes isolated from As4.1 or Calu-6 cells were analyzed in ChiP assays using the ChIP-IT kit from Active Motif (19). In brief, DNA-protein cross-linked complexes were preclared with Protein G-agarose beads (input samples) or were further precipitated with anti-COUP-TFII antibody (Perseus Proteomics). After reversal of the cross-linking and digestion of RNA and proteins, DNA was eluted and PCR-amplified for 36 cycles. The primers used for the amplification of the mouse and the human renin promoter fragment containing the COUP-TFII-binding site proxDR were as follows: 5’-ggtggtctgtagtagag-3’ (sense) and 5’-caggctgtagtagtagag-3’ (antisense) (amplicon length 176 bp) and 5’-ggtggtgctgtagtagg-3’ (sense) and 5’-gagaagcacagctgtgg-3’ (antisense) (amplicon length 183 bp), respectively. Mouse peroxisome proliferator-activated receptor γ (PPARγ) exon 2 fragment (166 bp) and human renin exon 10 fragment (116 bp) were amplified as negative controls in As4.1 and in Calu-6 cells with the following primers: 5’-ggtgtctctctgtgagtaa-3’ (sense) and 5’-ggtgtctctctgtgagtaa-3’ (antisense) and 5’-ggtgtctctctgtgagtaa-3’ (antisense) (the proxDR sequences are underlined).

Plasmids—Plasmid constructs used in the cell culture experiments are shown in Table 1. Construct proxDR represents pGL3 vector (Promega) encoding firefly luciferase under the control of the human renin promoter (bases −258 to +23 relative to the transcription start site). Construct proxDRdel con-

| Table 1
| Plasmids used for the experiments
| The cis-regulatory elements are illustrated with ellipses. A filled ellipse indicates a mutation. |
| Human constructs |
| proxDR | CRE | CNR | Luciferase |
| proxDRmut | Luciferase |
| proxDR-CREmut | Luciferase |
| proxDR-CNREmut | Luciferase |
| proxDR-del | Luciferase |
| Mouse constructs |
| proxDR | +23 |
| Luciferase |
| 4.2kb proxDRmut | Luciferase |
| 4.2kb | Luciferase |
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**TABLE 2**

| Gene            | Sense primer | Antisense primer | Amplicon bp |
|-----------------|--------------|------------------|-------------|
| Human renin     | atgagggggtgctgtggggtc | ggaatccatggcctgtggcgc | 304         |
| Human B-actin   | ttctttcagaacctccggtgggt | ggaagccccagctcgtag | 313         |
| Human COUP-TFI  | ttcgctgtggggttaggtaaaaa | tcctttctactcccccccccttcatttt | 120         |
| Human COUP-TFI  | ttcgctgtggggttaggtaaaaa | tcctttctactcccccccccttcatttt | 131         |
| Human CREB      | atgagggggtgctgtggggtc | ggaatccatggcctgtggcgc | 193         |
| Mouse renin     | atgagggggtgctgtggggtc | ggaatccatggcctgtggcgc | 193         |
| Mouse L32       | ttaaccgaaacctcgccggaacc | tcctttctactcccccccccttcatttt | 100         |

**RESULTS**

**COUP-TFII Knock-out Mice**—Mice were maintained in accordance with the National Institutes of Health Standards for the care and use of experimental animals. All procedures for animal studies were approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine. *ROSA26CREERT2; COUP-TFII*FOx/FOx mice (CreERT2CIIFF) were described previously (11). Control mice (wtCIIFF) were Cre-negative and homozygous for the floxed COUP-TFII allele. The mice were back-crossed to C57BL/6 background for at least four generations, and the comparisons were made between littermates. To induce COUP-TFII gene deletion, 2-month-old mice were intraperitoneally injected with 1 mg of tamoxifen (Sigma) for 3 consecutive days. The mice were subjected to experiments 1 month after injection.

**Statistics**—The results from at least two independent cell culture experiments were taken for the statistical analysis of the data. In each experiment, at least 3 samples/condition (control, treatment with different chemicals or siRNAs, or combinations of them) were assigned. Thus, the number of cell culture samples in the groups subjected to comparison was at least 6 (n ≥ 6). Levels of significance were determined by analysis of variance and Student’s unpaired t test. p < 0.05 relative to the corresponding comparison group was considered significant.
proxDR. The mouse proxDR sequence binds a single specific protein complex as detected by EMSA (Fig. 1, top, lane 2). This protein complex contains COUP-TFII because it disappears when a COUP-TFII-specific antibody is added (Fig. 1, top, lane 7). Interestingly, the human proxDR sequence binds two specific protein complexes as detected by EMSA (Fig. 1, bottom lane 7). Both complexes appeared to contain COUP-TFII, because the addition of COUP-TFII antibody strongly interfered with their binding to the human proxDR probe (Fig. 1, bottom, lane 7). The interaction of the COUP-TFII antibody with the proxDR-bound complexes was specific in both cell types, because the addition of an unrelated antibody (negative control; Fig. 1, lane 8) did not affect the protein-DNA interaction. The binding of COUP-TFII to an ~200-bp native DNA region containing proxDR in either cell type was confirmed by ChIP (Fig. 2). COUP-TFII did not bind to an unrelated exon sequence that served as negative control (Fig. 2). This confirmed the specificity of the COUP-TFII interaction with the proximal renin promoter.

**TABLE 3**

| COUP-TFII binding sequences | Canonical | Human renin | Mouse renin |
|----------------------------|-----------|-------------|-------------|
| probe                     | GTGTGA    | aAGGTCA     | AGGTGACG    |
| nuclear protein            | + + + + + | - + + + + + | - + + + + + |
| competitor oligo           | _ NS     | WT          | CII Iso     |
| antibody                   | _         |             |             |

**FIGURE 1. COUP-TFII binds to the COUP-TFII recognition sequence proxDR in the renin promoter.** Nuclear extracts from As4.1 (top) or Calu-6 (bottom) cells were probed with mouse (top) or human (bottom) proxDR oligonucleotide in EMSAs. The samples in lanes 1 and 5 contained only the corresponding labeled probe. Nonspecific unlabeled oligonucleotide (NS) was added in 100-fold molar excess to the samples in lane 3 to demonstrate the specificity of the binding. Non-labeled probe (WT) was added in 100-fold molar excess to the samples in lane 4 for competition analysis. Anti-COUP-TFII (CII) antibody or isotype control (Iso) antibody (2 µl of each) was added to the samples in lanes 7 and 8, respectively. S, shifted protein complexes; NS bands, nonspecific bands; FP, free probe.

**FIGURE 2. COUP-TFII binds to the proximal renin promoter in its native context.** Cross-linked nuclear extracts isolated from As4.1 (top) or Calu-6 cells (bottom) were used in ChIP with COUP-TFII antibody (COUP-TFII Ab) followed by isolation of the precipitated DNA. DNA isolated from non-precipitated samples was used as positive control (input). The input and the COUP-TFII antibody-precipitated samples were subjected to PCR with a primer pair amplifying a proximal renin promoter fragment containing the COUP-TFII recognition sequence proxDR (promoter) or with a primer pair amplifying a non-related exon sequence that served as a negative control (exon).

**COUP-TFII and the proxDR Sequence Are Involved in the cAMP-dependent Stimulation of Renin Gene Expression**—The protein-DNA binding experiments provided evidence suggesting that COUP-TFII is involved in the transcriptional control of the renin gene. However, COUP-TFII is an orphan nuclear receptor, and therefore little is known about how its transcriptional activity is regulated. The signaling cascade downstream of the second messenger cAMP is regarded as the most important intracellular pathway that controls the renin synthesis (1). Therefore, we examined the possible role of COUP-TFII and the proxDR sequence in the cAMP-dependent stimulation of the renin gene expression. To this end, the adenylate cyclase activator forskolin, in combination with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine, was applied to Calu-6 cells. These maneuvers are well known to increase the intracellular cAMP levels and to stimulate the renin gene expression. To this end, the adenylate cyclase activator forskolin, in combination with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine, was applied to Calu-6 cells. These maneuvers are well known to increase the intracellular cAMP levels and to stimulate the renin gene expression. However, COUP-TFII is an orphan nuclear receptor, and therefore little is known about how its transcriptional activity is regulated. The signaling cascade downstream of the second messenger cAMP is regarded as the most important intracellular pathway that controls the renin synthesis (1). Therefore, we examined the possible role of COUP-TFII and the proxDR sequence in the cAMP-dependent stimulation of the renin gene expression. To this end, the adenylate cyclase activator forskolin, in combination with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine, was applied to Calu-6 cells. These maneuvers are well known to increase the intracellular cAMP levels and to stimulate the renin gene expression.

**Role of COUP-TFII and the proxDR Sequence in cAMP-dependent Control of Renin Gene Transcription**—We were further interested in studying the molecular mechanisms of the interaction of COUP-TFII with cAMP signaling to the renin gene promoter. Using the Calu-6 cells as a model, we found that...
The knockdown of COUP-TFII abolished the stimulatory effect of cAMP on the transcriptional activity of the proximal human renin promoter containing the proxDR sequence (Fig. 5A). Because we had already seen that the mutation of the COUP-TFII-binding proxDR site abrogated the stimulation of the renin transcription by cAMP (Fig. 4), we assumed that the proxDR sequence is necessary for the function of the cAMP target sequences within the renin gene. There are two cAMP target sites in the proximal renin promoter construct proxDR: CRE at \( /H11002 \) to \( /H11002 \) bp and cAMP and CNRE at \( /H11002 \) to \( /H11002 \) bp (26, 27). Mutation of CRE eliminated, whereas mutation of CNRE did not affect, the stimulation of the renin promoter activity by cAMP. Thus, the proximal CRE sequence seems to mediate the stimulation of the renin promoter activity by cAMP. Importantly, the deletion of proxDR to yield a shorter renin promoter reporter construct (proxDRdel) resulted in a loss of the cAMP-induced stimulation, demonstrating that the proximal CRE alone is not sufficient to mediate the effect of cAMP on renin gene transcription (Fig. 5B). These results indicated that the COUP-TFII-binding site proxDR cooperates with the proximal promoter CRE site in the cAMP-dependent transactivation of the renin gene. CREB is the archetypal transcription factor targeted by cAMP. Earlier experiments with Calu-6 cells have demonstrated that CREB binds to the renin proximal promoter CRE and is necessary for the renin gene expression (27). In agreement with these previous data, we found that selective deficiency of either COUP-TFII or CREB decreased the renin gene expression (Fig. 6, B and C). The combined deficiency of COUP-TFII and CREB did not further decrease the renin gene expression as compared with the situation when a single transcription factor was knocked down (Fig. 6B). Moreover, the knockdown rate of COUP-TFII as well as of CREB was comparable when only one of them or when simultaneously both were targeted by sequence-specific siRNAs (Fig. 6D). Thus, COUP-TFII and CREB appear to share a common mechanism to regulate renin gene expression. Altogether, the last data strongly suggest that COUP-TFII bound to proxDR synergizes with CREB bound to CRE in the cAMP-dependent control of the renin gene.

**COUP-TFII Is Involved in the Control of Renin Production in Vivo**—To study the relevance of COUP-TFII for the control of the renin gene expression in vivo, we generated inducible COUP-TFII knock-out mice. The deletion of COUP-TFII was proven to be efficient in the kidneys of the induced adult animals (Fig. 7A). The COUP-TFII deficiency was accompanied by
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FIGURE 5. COPU-TFII and the proxDR sequence are necessary for the cAMP-induced expression of the renin gene mediated by a CRE site in the proximal promoter. A. COPU-TFII is necessary for the cAMP-induced renin gene transcription in Calu-6 cells. COPU-TFII was knocked down with sequence-specific siRNA (siCOPU-TFII). Control cells were transfected with nontargeting siRNA (siControl). Calu-6 cells were transfected with the 258-bp human proximal promoter construct proxDR containing the COPU-TFII binding site, and the effect of cAMP agonists on its transcriptional activity was tested. B. Calu-6 cells were transfected with the proxDR construct, with construct proxDR-CREmut carrying the mutated cAMP target site CRE, with construct proxDR-CNREmut carrying the mutated cAMP target site CNRE, or with construct proxDRdel in which proxDR was deleted and the effect of cAMP agonists on the transcriptional activity was tested. The data shown are means ± S.E. (error bars), *p < 0.05.

decreased renal expression of renin (Fig. 7, B and C). This finding fits to the paradigm that cAMP is decisive for renin production in vivo (1, 28). Furthermore, it is in agreement with our cell culture data, which have shown a synergism between COPU-TFII and the cAMP signaling in the control of renin gene expression. Thus, the in vivo data confirmed that COPU-TFII is involved in the control of renin expression.

DISCUSSION

The main purpose of this study was to examine the role of the orphan nuclear receptor COPU-TFII in the regulation of the renin gene expression. We used two different renin-producing cell types and a mutant mouse line with deletion of COPU-TFII in the adult. The combined data clearly demonstrated that COPU-TFII is involved in the transcriptional control of the renin gene. We first detected COPU-TFII in the renin-producing JG cells of the mouse kidney. COPU-TFII was also expressed in the mouse As4.1 and the human Calu-6 cell lines. Thus, COPU-TFII colocalizes with renin species-independently, and in such a way, it could be essential for the regulation of the renin production in humans as well. We next provided evidence that COPU-TFII binds to the mouse and the human proximal promoter region containing a sequence (termed proxDR) that is homologous to the consensus COPU-TFII-binding site. Notably, COPU-TFII bound to the proxDR element as a part of a single protein complex in EMSA with nuclear extracts isolated from As4.1 cells and as a part of two protein complexes with nuclear extracts isolated from Calu-6 cells. It remains to be clarified whether this discrepant binding pattern is species-, sequence-, or cell-specific. In any case, the further data (Figs. 3 and 4) imply that this discrepancy has no significant functional impact on the role of COPU-TFII in the control of renin gene transcription. Similarly to many other nuclear receptors, COPU-TFII binds to a DR of the canonical HRE (5′-(A/G)GGTCA-3′) (29, 30). The originally identified COPU-TF-binding site in the chicken ovalbumin promoter is an imperfect DR with a 1-bp spacer (31). Mouse and human renin proxDRs are also imperfect repeats, but they are separated by 2 bp (Table 3). This is in agreement with earlier reports demonstrating that COPU-TFs bind to DRs with diverse spacings (30). Mouse and human renin proxDRs are over 70% homologous with highest degree of discrepancy in the 3′-flanking region of the first repeat and completely conserved second repeat (Table 3). Whereas the human proxDR element is located at −255 to −242 bp, the mouse proxDR is located at −717 to −704 bp relative to the transcription start site of the renin gene. This positional discrepancy is due to a 476-bp insertion at −68 bp in the mouse renin gene (32). This mouse-specific fragment is flanked by 14-bp imperfect repeats. Therefore, it was suggested that the 476-bp sequence represents a mobile element that has arisen since the divergence of mouse and rat renin genes (32). A conserved DR of the HRE sequence binding Ear2/retinoic acid receptor/retinoid X receptor/PPARγ exists also in the distal (kidney) enhancer of the renin gene (20, 21, 33, 34). Interestingly, COPU-TFII was identified as a yeast one-hybrid screen hit with the enhancer HRE and As4.1 cell nuclear extracts (33). Recently, the interaction of COPU-TFII with the renin enhancer HRE repeat was confirmed (35). COPU-TFII is an orphan nuclear receptor that could regulate the expression of its target genes either positively or negatively (29). In addition, it modulates the transactivating capacity of other transcription factors. Although nuclear receptors, such as retinoic acid receptors, retinoid X receptor, vitamin D receptor, liver X receptor α, or PPARγ, are known to be involved in the transcriptional control of the renin gene (20, 34, 36, 37), we did not look for their possible interactions with COPU-TFII. Instead, we investigated the role of COPU-TFII in the cAMP-dependent regulation of renin expression. In vitro and in vivo studies consistently demonstrated that cAMP signaling is one of the most important intracellular cascades regulating the renin gene expression (25, 28, 38 – 40). The combined data from As4.1 and Calu-6 cells showed that COPU-TFII and its binding sequence proxDR are necessary for the cAMP-dependent increase of the renin mRNA levels and promoter activity. Although the COPU-TFII deficiency only attenuated the mRNA increase, the mutation of proxDR completely abrogated the transactivation of the promoter. These findings are in agreement with the knowledge that cAMP induces the renin gene transcription and
at the same time stabilizes the renin mRNA (25, 41, 42). Therefore, the residual cAMP-dependent increase of the renin mRNA levels after COUP-TFII knockdown is most probably due to a prolonged half-life of the mRNA.

We used the Calu-6 cells and the proximal human renin promoter to examine the molecular mechanisms of the involvement of COUP-TFII in the cAMP-mediated transactivation of the renin gene. The renin production is better inducible by cAMP in the Calu-6 cells than in the As4.1 cells (25, 41). Furthermore, the proximal human renin promoter containing the proxDR site is shorter than the orthologous mouse sequence because of the 476-bp insertion (see above), thus allowing us to precisely map the cis-acting elements. We found that COUP-TFII and proxDR play a role in the cAMP-induced regulation of the renin gene. proxDR is necessary for the cAMP-dependent control of a proximal CRE at −225 to −219 which is known to bind CREB (27). Moreover, COUP-TFII and CREB seem to regulate the renin gene expression through a common transcriptional mechanism. In accordance with our data, it has already been reported that the HRE repeat in the distal renin enhancer, which is another COUP-TFII binding site, cooperates with a distal renin enhancer CRE site in the cAMP-dependent stimulation of the renin promoter activity (25). An activating effect of COUP-TF in the context of several transcription factor binding sites, including CRE, is also known for the phosphoenolpyruvate carboxykinase gene (43). Based on our data, a model of the cAMP-dependent regulation of the renin gene transcription could be proposed in which the transcription factors bound to proxDR and proximal CRE (COUP-TFII and CREB, respectively) cooperate within one transcription initiation protein complex (enhanceosome) at the proximal renin promoter. Interestingly, the proximal renin CRE was not active in a shorter promoter fragment lacking proxDR, whereas a consensus CRE (which differs from the proximal renin CRE by only two

**FIGURE 6.** COUP-TFII cooperates with CREB in the control of renin gene expression. Calu-6 cells were transfected with nontargeting siRNA as control (siControl) or with COUP-TFII sequence-specific siRNA (siCOUP-TFII), CREB sequence-specific siRNA (siCREB), or a combination of COUP-TFII and CREB siRNAs (siCOUP-TFII + siCREB). A, efficacy of the CREB knockdown. Protein extracts were probed in Western blots with CREB and β-actin (internal control) antibodies. See Fig. 3B for the efficacy of the COUP-TFII knockdown. B, effect of the deficiency of COUP-TFII, CREB, or both on renin gene expression. Renin and human β-actin (internal control) mRNA levels were quantified by real-time PCR. C, specificity of the knockdown. COUP-TFI and human β-actin (internal control) mRNA levels were quantified by real-time PCR. The data shown are means ± S.E. (error bars). *, p < 0.05. NS, not significant.
swapped bases) was still functional in the same construct (18). These findings imply that the enhanceosome complex built at the proximal renin promoter upon cAMP-induced transactivation is highly specific. At last, we also provided evidence that the effect of COUP-TFII on the renin expression is relevant in vivo. Transgenic mouse strains with defects in cAMP signaling to the renin gene are, as a rule, renin-deficient (28, 40, 44). Because, on the other hand, we found that COUP-TFII is necessary for the cAMP-dependent control of the renin gene expression in vitro, it could be predicted that mice deficient in COUP-TFII should have reduced renin production. Consistent with this assumption, the knockout of COUP-TFII in mice resulted in decreased renin expression. In agreement with the cell culture data, the COUP-TFII knock-out mice were not completely devoid of renin, because the cAMP signaling also stabilizes the renin mRNA independently on its transcriptional effect (also see above for discussion). Surprisingly, there was no significant difference in the plasma renin concentration between control and COUP-TFII-deficient mice (data not shown). It is noteworthy that the renin expression and plasma renin concentration do not obligatorily change in parallel in genetically engineered mice (45, 46).3 One possible explanation for this phenomenon is that it is an artifact of the genomic manipulation. One further scenario is that the generalized COUP-TFII deficiency may modulate either the processing of the renin protein or the mechanisms of renin release. We are currently working on this issue by generating mice with inducible COUP-TFII deletion restricted to the renin-producing JG cells of the kidney.

The overall data presented here show that COUP-TFII is essentially involved in the molecular mechanisms of the stimulation of renin gene expression and that this effect is relevant in vivo. Because the COUP-TFII expression, similarly to the renin expression, is tightly regulated during embryogenesis, we provide the first evidence for a novel nuclear receptor transcription factor, which could be important for the developmental control of the renin gene.

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COUP-TFII and Renin

FIGURE 7. COUP-TFII knock-out mice have decreased renin expression. A, efficiency of the knockout. Shown are renin (green), COUP-TFII (red), and DAPI (nuclear marker) (blue) co-immunostaining in control (wtCIIFF) and COUP-TFII knock-out (CreERT2CIIFF) mouse kidneys. Arrows, renin immunoreactivity. B and C, renin expression is decreased in the COUP-TFII knock-out mice (CreERT2CIIFF, n = 3) compared with the control mice (wtCIIFF, n = 4). Renin and β-actin (internal control) in mouse kidneys are detected by immunoblotting (B) and quantified by densitometry (C). The data shown are means ± S.E. (error bars).

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