Regulated Expression of Human Angiotensinogen Gene by Hepatocyte Nuclear Factor 4 and Chicken Ovalbumin Upstream Promoter-Transcription Factor*


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We previously identified various upstream and downstream regulatory elements and factors important for hepatic expression of the human angiotensinogen (ANG) gene, the precursor of vasoactive octapeptide angiotensin II. In the present study, to further investigate the molecular mechanism of human ANG transcriptional regulation, we generated transgenic mice carrying the fusion gene composed of the 1.3-kilobase promoter of the human ANG gene, its downstream enhancer, and the chloramphenicol acetyltransferase reporter gene. Because expression of the chloramphenicol acetyltransferase gene was observed strongly in the liver and weakly in the kidney, we suspected that hepatocyte nuclear factor (HNF) 4 with a tissue expression pattern similar to that of the reporter gene would regulate ANG transcription. In vitro assays indicated that HNF4 bound to the promoter elements and strongly activated the ANG transcription, but that chicken ovalbumin upstream promoter transcription factor (COUP-TF), a transcriptional repressor, dramatically repressed human ANG transcription through the promoter elements and the downstream enhancer core elements. Furthermore, COUP-TF dramatically decreased the human ANG transcription in the mouse liver by the Helios Gene Gun system in vivo. These results suggest that an interplay between HNF4 and COUP-TF could be important in hepatic human ANG transcription.

Hypertension is one of the most important risk factors for cardiovascular disease, including myocardial infarction, stroke, heart failure, and renal failure. The renin-angiotensin system plays a key role in the regulation of blood pressure and electrolyte homeostasis as well as being a growth regulator of cardiac myocytes. The reaction between renin and angiotensinogen (ANG) is the initial and rate-limiting step of this enzymatic cascade that generates the decapeptide angiotensin I, which is further processed to the functional octapeptide angiotensin II by angiotensin-converting enzyme (1–3). Because plasma ANG concentration is close to the Km of the renin reaction, variation of ANG transcription is thought to influence blood pressure (4). This notion is supported by elevation of blood pressure in transgenic animals that overexpress the ANG gene (5, 6) and genetic association between plasma ANG concentration and essential hypertension (7). In particular, we reported that naturally occurring molecular variants of AGCE1 (ANG core promoter element 1), located between the TATA box and transcription initiation site, alter the binding affinity of ubiquitous transcriptional mediator, AGCF1 (AGCE-binding factor 1), and affect human ANG transcriptional activity (8, 9). Indeed, Sato et al. (10) and Ishigami et al. (11) indicated that a genetic variant in AGCE1 is directly associated with increased risk of hypertension. Therefore, it is considered to be etiologically important to understand the molecular mechanisms of human ANG transcriptional regulation.

A variety of cis-acting transcription elements and trans-acting nuclear factors responsible for the preferential expression of various genes in the liver have been identified (12–15). Of these liver preference factors, hepatocyte nuclear factor 4 (HNF4) is a member of the nuclear receptor superfamily that interacts with an element containing the AGGTCA motif (16). HNF4 was originally identified as an orphan member of the superfamily, but fatty acyl-CoA thioesters, which modulated the onset and progression of various disease including insulin resistances and hypertension, have recently been demonstrated as ligands of HNF4 (17). Binding sites for HNF4 have often been found in the regulatory regions of many liver-enriched genes encoding apolipoproteins, coagulation factors, serum proteins, and cytochrome P450s and those involved in the metabolism of fatty acids, amino acids, and glucose (18). Chicken ovalbumin upstream promoter transcription factor (COUP-TF), an orphan member of the nuclear receptor superfamily, was initially characterized as a transcriptional stimulator of the chicken ovalbumin promoter (19), yet has recently

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1 The abbreviations used are: ANG, angiotensinogen; AGCF1, human ANG core promoter binding factor 1; AGCE1, human ANG core promoter element 1; CAT, chloramphenicol acetyltransferase; COUP-TF, chicken ovalbumin upstream promoter transcription factor; EMSA, electrophoretic mobility shift assays; HNF4, hepatocyte nuclear factor 4; kb, kilobase(s); bp, base pair(s).
been recognized to play a suppressive role in the transcriptional control of the expression of several genes (20–23). Most of the binding sites initially found to bind HNF4 are also recognized by COUP-TF, and overexpression of COUP-TF generally antagonizes HNF4-mediated transcriptional activation (24–28). Therefore, it is accepted that this antagonistic effect finely tunes HNF4-dependent gene expression by an intracellular balance of these positive and negative regulators.

Because ANG is mainly synthesized in the liver, we have studied the mechanisms of hepatic human ANG transcription using human hepatoma cell line (HepG2) as a model and identified various regulatory elements and factors that regulate the human ANG gene transcription including the upstream elements (29–31), core promoter elements (8, 9), and downstream enhancer elements (32, 33). In the present study, to further investigate the mechanisms of human ANG transcription, we generated transgenic mice carrying the reporter gene which include all components of previously identified regulatory elements of the human ANG gene. Expression patterns of the reporter gene in two independent transgenic lines brought us the notion that HNF4 participated in the regulation of human ANG transcription. In vitro transfection analyses and electrophoretic mobility shift assays (EMSA) showed that HNF4 regulated human ANG transcription. Furthermore, COUP-TF acted as a strong repressor of the human ANG transcription through multiple mechanisms in vitro and in vivo.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructions**—The human ANG promoter-chloramphenicol acetyltransferase (CAT) chimeric constructs were made as follows: 13cat, DM4cat, DM7cat, DM5.8cat, DM5cat, DM10cat, DM12cat contained 1268-bp (–1222 to +44), 791-bp (–747 to +44), 569-bp (–516 to +44), 388-bp (–344 to +44), 356-bp (–312 to +44), 340-bp (–296 to +44), 287-bp (–243 to +44), 150-bp (–106 to +44), and 76-bp (–32 to +44) fragments, respectively, and these DNA fragments were subcloned into the BglII/HindIII sites of SVOcat. DM12CEBPcat was constructed by inserting eight copies of C/EBP-binding sequence (5′-AATGGGCAATCAG-3′) at the upstream of the human ANG core promoter. G-SVPluc and J-SVPluc were constructed by inserting six copies of G and J fragments at the upstream of the SV40 promoter of SVPluc (Wako, Osaka, Japan), respectively. 13B2cat, DM12B2cat, DM12B2–5cat, DM12GMcat, DM12MEcat, DM12EBcat, DM12CB2–1cat, DM12E3cat, DM12d61–2cat, and SV3cat were constructed as described previously (32, 33). Expression vectors for HNF4 (pEF-BOS/HNF4) and for COUP-TF (pEF-BOS/COUP) were constructed as described previously (26).

**Generation of Transgenic Mice and Measurement of CAT Activity**—The 3.8-kb fragment containing human ANG 1.3-kb promoter, its downstream enhancer, and CAT reporter gene was isolated (Fig. 1A). The DNA was used directly for microinjection in a concentration of 4 μg/ml and about 1,000 copies/embryo. One-cell zygotes fertilized in vitro were obtained from C57BL/6 mice, and outbred ICR females were used as the pseudopregnant recipients. The transgenic procedure used was essentially as described previously (34). Heterozygous transgenic progeny were obtained by mating males and females with the transgene. Introns and flanking regions are shown by thin lines. The positions of the promoter and the downstream enhancer are indicated below. B, the CAT activities were determined in tissue extracts from a transgenic mouse bearing 1.3-kb promoter and 0.8-kb enhancer of the human ANG gene as described in A. Various tissue extracts of two independent transgenic mice lines were prepared from two to four progeny. Tissues were isolated and homogenized. The suspension was freeze-thawed three times and centrifuged. Extracts containing equal amounts of 5 μg (line 1) or 50 μg (line 2) of protein were used in CAT assays. The CAT activity of each liver extract is designated as 100, and each value of CAT activity represents the mean ± S.E. for at least four independent experiments. N.D., not detected.

**FIG. 1.** Tissue distribution of CAT reporter activities in transgenic mice. A, the structures of the human ANG gene and the reconstructed transgenic construct are shown. Black boxes represent exons. Introns and flanking regions are shown by thin lines. The positions of the promoter and the downstream enhancer are indicated below. B, the CAT activities were determined in tissue extracts from a transgenic mouse bearing 1.3-kb promoter and 0.8-kb enhancer of the human ANG gene as described in A. Various tissue extracts of two independent transgenic mice lines were prepared from two to four progeny. Tissues were isolated and homogenized. The suspension was freeze-thawed three times and centrifuged. Extracts containing equal amounts of 5 μg (line 1) or 50 μg (line 2) of protein were used in CAT assays. The CAT activity of each liver extract is designated as 100, and each value of CAT activity represents the mean ± S.E. for at least four independent experiments. N.D., not detected.

pCMV-β-gal. Total amounts of DNA were adjusted to 4 μg by pEF-BOS. After 48 h of culture, β-galactosidase activities were measured and cell extracts containing equivalent amounts of β-galactosidase activity were used for CAT assay (35). In the case of luciferase assay, the cells were plated at a density of 3 × 10⁴ cells/24-well dish and transfected 16 h later by FuGENE™6 (Roche Molecular Biochemicals) with reporter plasmids (50 ng), pCMV-β-gal (25 ng), and EF-BOS/HNF4 (10 to 100 ng). Total amounts of DNA were adjusted to 275 ng by pEF-BOS. After 24 h of culture, β-galactosidase and luciferase activities were measured according to the manufacturer’s protocol (Wako, Osaka, Japan).

EMSAs—Nuclear extracts from HepG2 cells and the liver of C57BL/6 mice were prepared using the protocols of Dignam et al. (36) and Gorski et al. (37), respectively. Nuclear extracts of COS7 cells transfected with pEF-BOS/HNF4 were prepared by the mini-scale detergent treatment procedure (38). The maltose-binding protein fusion and purification system from New England Biolabs was used to prepare the recombinant COUP-TF protein. Double-stranded DNA probes were end-labeled using [γ-32P]ATP and T4 polynucleotide kinase. Ten micrograms of nuclear extracts or bacterially expressed proteins were incubated with 1 μg of polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and added to the reaction mixture. The reaction mixtures were directly loaded onto a 4.5% nondenaturing polyacrylamide gels containing 4% glycerol made in 1 × TBE (90 mM Tris-HCl, pH 8.0, 89 mM boric acid, and 2 mM EDTA). After electrophoresis was
Constructs used are listed to the right. Right, HepG2 cells were transfected with 2 μg of the CAT reporter plasmids, 0.5 μg of pEF-BOS or pEF-BOS/HNF4, and 1 μg of pCMV-β-gal. Transfection experiments and CAT assays were performed as described in A. CAT activities of the reporter genes in the presence of pEF-BOS/HNF4 are expressed as fold activation relative to the activity obtained with each reporter plasmid in the presence of pEF-BOS. Each value of CAT activity represents the mean ± S.E. for at least four independent experiments.

**Fig. 3.** Deletion analysis of HNF4-activating elements. Left, 5′-deletion constructs of the human ANG promoter-CAT hybrid genes. Thick horizontal lines and open boxes marked by CAT represent the promoter and CAT vector sequences, respectively. Names of constructs used are listed to the right. Right, HepG2 cells were transfected with 2 μg of the CAT reporter plasmids, 0.5 μg of pEF-BOS or pEF-BOS/HNF4, and 1 μg of pCMV-β-gal. Transfection experiments and CAT assays were performed as described in Fig. 2A. CAT activities of the reporter genes in the presence of pEF-BOS/HNF4 are expressed as fold activation relative to the activity obtained with each reporter plasmid in the presence of pEF-BOS vector. Each value of CAT activity represents the mean ± S.E. for at least four independent experiments.

In Vivo Transient Expression Assay—The Helios Gene Gun system (Nippon Bio-Rad Laboratories, Tokyo, Japan) was used essentially as described (39). Fifty milligrams of Au particles (radius 1.0 μm, Bio-Rad) were washed with 70% ethanol, and 100 μl of 0.05 M spermidine (Sigma) were added to the washed Au particles. Then, 50 μg of reporter plasmid (13B2cat) and 200 μg of effector plasmid (pEF-BOS/COUP) or empty vector (pEF-BOS) were added to the mixture. This suspension was mixed with 100 μl of 1 M CaCl₂ and vortexed. The suspension was centrifuged after 10 min, and the resulting DNA-coated Au particles were washed with 70% ethanol and suspended in 200 μl of PVP solution (polyvinyl pyrrolidone (molecular weight 360,000), 0.02 mg/ml 99.5% ethanol). The suspension was used to prepare the cartridge as described in the manual. ICR mice (12 weeks old) were anesthetized by intraperitoneal injection of pentobarbital. The abdomen was shaved, and the abdomen was closed by a running suture. Control experiments were performed with a Helios Gene Gun at 180 p.s.i., as an internal control for transfection efficiency. Total amounts of DNA were adjusted to 4 μg of pCMV-gal and empty vector showed that about 10% of the -galactosidase activities were measured, and extracts containing equivalent amounts of β-galactosidase activities were used for CAT assays. A value of CAT activity represents the mean ± S.E. for at least four independent experiments.

The results from transgenic mice brought us the notion that HNF4 with a tissue expression pattern similar to that of the reporter gene would participate in the regulation of human ANG transcription. Indeed, HNF4 activated CAT activity of 13B2cat and 13cat, but could not do that of DM12B2cat (Fig. 2B). To localize HNF4-responsive elements within the promoter, we examined the 5′-deletion mutants fused to the reporter gene (Fig. 3). Deletion of the sequences from −1222 bp to −516 bp did not essentially influence the HNF4-responsive activity, but there was a significant reduction in CAT activity when the upstream sequence was eliminated up to −344 bp. Further deletion of the sequences to

**RESULTS**

To investigate the mechanisms of hepatic human ANG transcriptional regulation, we generated transgenic mice carrying the fusion gene that comprises the human ANG 1.3-kb promoter, its downstream enhancer, and the CAT reporter gene (Fig. 1A). The extracts from major tissues of the two independent transgenic lines were analyzed by CAT assays. Expression of the reporter gene construct was observed strongly in the liver and weakly in the kidney, although the other tissues exhibited no activity (Fig. 1B). These results indicated that the reconstituted gene construct composed of the human ANG 1.3-kb promoter and its downstream enhancer are sufficient for the liver-preferential expression in vivo. The results from transgenic mice brought us the notion that HNF4 with a tissue expression pattern similar to that of the reporter gene would participate in the regulation of human ANG transcription. Indeed, HNF4 activated CAT activity of 13B2cat and 13cat, but could not do that of DM12B2cat (Fig. 2B). To localize HNF4-responsive elements within the promoter, we examined the 5′-deletion mutants fused to the reporter gene (Fig. 3). Deletion of the sequences from −1222 bp to −516 bp did not essentially influence the HNF4-responsive activity, but there was a significant reduction in CAT activity when the upstream sequence was eliminated up to −344 bp. Further deletion of the sequences to
−296 bp did not essentially affect it; however, HNF4 hardly activated CAT activity when the region between −296 bp and −243 bp was removed. These results suggest that the regions from −516 bp to −344 bp and from −296 to −243 bp are important for the HNF4-dependent human ANG transcriptional activation.

We tried to identify HNF4-binding elements by EMSA using the dissected DNA fragments that covered with the HNF4-responsive elements (A–J; Fig. 4A). Incubation of the well known HNF4-binding element, C3P, located at −90 to −66 upstream from the apolipoprotein CIH gene transcriptional start site (28), with the mouse liver nuclear extracts produced retarded complexes (Fig. 4B, lane 2), but no shifted band was observed without the nuclear extract (Fig. 4B, lane 1). The complexes represented a sequence-specific interaction between C3P fragment and a nuclear factor, since the formation of this complex was specifically reduced with molar excess of unlabeled competitors (Fig. 4, lane 3). Supershift experiments using an anti-HNF4 antibody showed that this factor was HNF4 (data not shown). The DNA-protein complex formed by HNF4 binding to C3P was inhibited by molar excess of non-labeled C and J fragments, although the other fragments could not compete for this binding (Fig. 4, lanes 4–13). Although incubation of C3P, C, and J fragments with COS7 nuclear extracts produced no retarded complexes (Fig. 5, lanes 1, 5, and 7), nuclear extracts of COS7 cells transfected with the HNF4 expression plasmid produced the complexes between HNF-4 and C3P, C, or J fragments (Fig. 5, lanes 2, 3, 4, 6, and 8). To further characterize C and J region binding activities, mouse liver nuclear extracts were incubated with C and J fragments as a probe (Fig. 6). The complexes formed by mouse nuclear extracts with C or J fragments had biochemical properties similar to the ones by the nuclear extracts with C3P, since the formation of this complex was specifically reduced with molar excess of unlabeled C3P competitor (Fig. 6, lanes 4 and 10). Further-

**FIG. 4.** Binding of HNF4 to different portions of the human ANG promoter. A, top, the promoter region of human ANG gene is represented. The oligonucleotides used to detect specific interactions between the promoter sequences and HNF4 were indicated below. B, EMSA. The C3P double-stranded oligonucleotides were labeled with T4 polynucleotide kinase using [γ-32P]ATP. Five µg of mouse liver nuclear extract (N.E.) were incubated with 0.3 ng of the 32P-labeled probe. In a competition assay, 200-fold molar excess of the unlabeled oligonucleotides, as indicated for each lane, were added to the reaction mixture. Binding reactions were resolved by 4.5% acrylamide, 1× TBE electrophoresis. HNF4 is indicated.

**FIG. 5.** C and J fragments as binding sites for HNF4. A, nucleotide sequences of C and J fragments. Imperfect direct repeats, HNF4 motif, are indicated by arrows below the sequences. B, the indicated double-stranded oligonucleotides were labeled with T4 polynucleotide kinase using [γ-32P]ATP. Five µg of nuclear extracts of the COS7 cells transfected with HNF4 expression vector (HNF4 +) or empty vector (HNF4 −) were incubated with 0.3 ng of 32P-labeled probes. In a competition assay, 200-fold molar excess of the unlabeled oligonucleotides, as indicated for each lane, were added to the reaction mixture. Binding reactions were resolved by 4.5% acrylamide, 1× TBE electrophoresis. HNF4 is indicated.

**FIG. 6.** C and J regions as binding sites for HNF4. Five µg of mice liver nuclear extract (N.E.) were incubated with 0.3 ng of 32P-labeled C and J probes in the presence or absence of 200-fold molar excess of the unlabeled oligonucleotides. In supershift assays, HNF4 antibodies were added to the reaction mixture. Binding reactions were resolved by 4.5% acrylamide, 1× TBE electrophoresis. HNF4 and supershifted complexes are indicated.
more, supershift experiments using anti-HNF4 antibody showed that these sequence-specific binding factors were HNF4 (Fig. 6, lanes 6 and 12). To confirm the role of C and J regions as HNF4-responsive elements, we inserted these fragments in front of the heterologous SV40 promoter (Fig. 7A). C-SVPLuc and J-SVPLuc were activated by HNF4 as a dose-dependent manner, although SVPLuc was little affected (Fig. 7B). These results suggested that HNF4 binds to C region (−429 to −386) and J region (−281 to −252) of the human ANG gene and activates its transcription through these elements.

Because COUP-TF was known to act as a transcriptional repressor by antagonizing the function of HNF4 (24–28), we suspected that COUP-TF would also regulate ANG transcription. As expected, COUP-TF dramatically repressed the reporter gene, 13B2cat, in a dose-dependent manner in HepG2 cells (Fig. 8A). Next, we examined whether COUP-TF-responsive region localized in the promoter including HNF4-binding sites (Fig. 8B). Interestingly, COUP-TF repressed CAT activities of not only 13cat but also DM12B2cat, which does not contain HNF4-responsive elements, although COUP-TF did not significantly affect SV3cat and DM12C/EBPcat including the eight copies of C/EBP-binding site fused in front of the human ANG core promoter. These results suggest that COUP-TF influences human ANG transcriptional activity by other mechanisms than by antagonizing the function of HNF4.

To localize COUP-TF-responsive elements within the promoter, we examined the 5′-deletion mutants linked to the reporter gene (Fig. 9A). Deletion of the sequences from −1222 bp to −516 bp did not essentially affect the COUP-TF-responsive activity, but there was a significant reduction in repression activity when C element was eliminated (Fig. 9A, DM7.8cat). Furthermore, COUP-TF hardly repressed CAT activity when J element was removed (Fig. 9A, DM9cat). These results suggested that COUP-TF repressed the human ANG promoter activity through the regions including HNF4-responsive elements (C and J). To identify the COUP-TF-responsive elements within the downstream enhancer, we divided the fragment B2 into three DNA segments, B2–5′ (+1399 to +1510 bp), ΔB2–1 (+1510 to +1811 bp), and ΔE3 (+1807 to +2230 bp), and examined the effects of COUP-TF (Fig. 9B). COUP-TF repressed the transcriptional activities of DM12B2–5′ cat and DM12E3cat, but did not significantly repress the CAT activity of DM12ΔB2–1cat. Since we previously reported that the enhancer core elements, GM (+1399 to +1438 bp), ME (+1435 to +1478 bp), and EB (+1472 to +1510 bp), exist within B2–5′ fragment and the other core element, d61–2 (+2191 to +2214), exists within E3 fragments, these core elements were next explored for COUP-TF-responsive activity. DM12GMcat, DM12Mcat, and DM12d61–2cat were repressed by COUP-TF, although DM12E8cat was not affected (Fig. 9B). Taken together, these results suggested that GM, ME, and d61–2 fragments in addition to C and J fragments were important for the COUP-TF-dependent repression.

We tried to detect COUP-TF-complex by EMSA. Incubation of C3P fragment also known as a COUP-TF-binding element (28) with the bacterially expressed-COUP-TF produced retarded complexes (Fig. 10, lane 2). The complexes represented a sequence-specific interaction between C3P fragment and COUP-TF, since the formation of this complex was specifically reduced with molar excess of unlabeled C3P competitors (Fig. 10).
Regulation of ANG Gene Expression by HNF4 and COUP-TF

In the present study, to further investigate the molecular mechanism of human ANG transcriptional regulation, we generated the transgenic mice carrying the reconstituted human ANG gene, and indicated that the reporter gene contains the regulatory elements sufficient for liver preferential expression (Fig. 1). Because of expression patterns of the reporter gene similar to that of HNF4, we suspected that HNF4 would participate in the human ANG transcriptional regulation and found that HNF4 dramatically activated the reporter gene in HepG2 cells (Fig. 2). Deletion analyses and EMSAs showed that HNF4 bound to C and J region (Figs. 3–6) and activated the transcription through these regions. Moreover, COUP-TF was shown to dramatically repress human ANG transcription through the HNF4-binding sites (C and J region) and the downstream enhancer core elements (GM, ME, and d61–2) (Figs. 7–9). The EMSAs showed that COUP-TF bound to C, J, and GM fragment and ME fragment, to antagonize activators including HNF4, and COUP-TF simultaneously binds to ME fragment with HNF3, by interacting with distinct trans-factors. Nevertheless, the reason why such multiple factors are required for the enhancer activ-

![Diagram](http://www.jbc.org/)

**FIG. 9.** Deletion analyses of the COUP-repressing elements. **A,** Deletion analysis of the promoter. Left, 5'-deletion constructs of the human ANG promoter-CAT hybrid genes. Thick horizontal lines, open boxes marked by B2, and open boxes marked by CAT represent the promoter, the downstream enhancer, and CAT vector sequences, respectively. Names of constructs used are listed to the right. Right, HepG2 cells were transfected with 2 µg of the CAT reporter plasmids, 0.5 µg of pEF-BOS or pEF-BOS/CAY, and 1 µg of pCMV-bgal. Transfection experiments and CAT assays were performed as described in Fig. 2A. A value of CAT activity represents the mean ± S.E. for at least four independent experiments. **B,** deletion analysis of the downstream enhancer. Transfection experiments were performed as described in A. A value of CAT activity represents the mean ± S.E. for at least four independent experiments.

10, lane 3), but not with that of HNF3 (lane 4). Although d61–2 fragment could not produce the retarded complex (Fig. 10, lane 9), the others produced COUP-TF complexes. These results indicate that COUP-TF binds to C, J, GM, and ME fragments, but not to d61–2. Next, we performed another EMSA assay to analyze the effects of COUP-TF on the mouse liver nuclear factors binding to each element (Fig. 11). The complexes formed by HNF4 and C or J fragments were replaced by the COUP-TF complexes in a dose-dependent manner (Fig. 11A). The complexes formed by mouse liver nuclear factors and GM fragment were also replaced by the addition of COUP-TF (Fig. 11B). In the case of ME fragment (Fig. 11C), the bands formed by mouse liver nuclear factors were gradually reduced by the addition of COUP-TF. The observation of lower mobility complexes than the complex produced by COUP-TF (Fig. 11C, lane 6) suggested that COUP-TF simultaneously binds to ME fragment with mouse liver nuclear factors. Interestingly, COUP-TF inhibited the formation of the low mobility complex produced by d61–2 fragment but did not affect the high mobility complex, although COUP-TF itself could not bind to this fragment (Fig. 11D). These results suggest that COUP-TF would repress the transcriptional activation property of d61–2 fragment through the interference with the binding activity of mouse liver nuclear factors.

Finally, we examined the effect of COUP-TF on the human ANG transcription in vivo, because the repression of human ANG transcription is important for the potential target of medical treatment of hypertension. The reconstituted human ANG gene, 13B2cat, was co-transfected with an empty vector or COUP-TF-expression vector in the mouse liver by the Helios Gene Gun system (39). The liver extracts prepared from treated mice were used for CAT assays. As shown in Fig. 12, COUP-TF repressed the human ANG transcription below 30%. These results indicate that COUP-TF dramatically repress the human ANG transcription in vivo as well as in vitro.

**DISCUSSION**

In the present study, to further investigate the molecular mechanism of human ANG transcriptional regulation, we generated the transgenic mice carrying the reconstituted human ANG gene, and indicated that the reporter gene contains the regulatory elements sufficient for liver preferential expression (Fig. 1). Because of expression patterns of the reporter gene similar to that of HNF4, we suspected that HNF4 would participate in the human ANG transcriptional regulation and found that HNF4 dramatically activated the reporter gene in HepG2 cells (Fig. 2). Deletion analyses and EMSAs showed that HNF4 bound to C and J region (Figs. 3–6) and activated the transcription through these regions. Moreover, COUP-TF was shown to dramatically repress human ANG transcription through the HNF4-binding sites (C and J region) and the downstream enhancer core elements (GM, ME, and d61–2) (Figs. 7–9). The EMSAs showed that COUP-TF bound to C, J, and GM, to antagonize activators including HNF4, and COUP-TF simultaneously binds to ME fragment with mouse liver nuclear factors. Furthermore, COUP-TF inhibited a mouse liver nuclear factor to bind to d61–2, although COUP-TF itself could not bind to d61–2 (Fig. 11). Finally, COUP-TF decreased the human ANG transcription in the mouse liver (Fig. 12). These results suggest that HNF4 plays an important role in hepatic human ANG transcription, and COUP-TF acts as a strong repressor of the human ANG transcription through multiple mechanisms in vitro and in vivo.

It is generally accepted that promoters and enhancers of eukaryotic genes are composed of multiple cis-elements that interact with distinct trans-factors. Nevertheless, the reason why such multiple factors are required for the enhancer activ-
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Fig. 11. Effects of COUP-TF on binding activities of mouse liver nuclear factors. Five μg of mouse liver nuclear extracts were incubated with 0.3 ng of 32P-labeled probes (A, C and J; B, GM; C, ME; D, d61–2) in the presence or absence of bacterially expressed COUP-TF. In a competition assay, 200-fold molar excess of the unlabeled oligonucleotides, as indicated for each lane, were added to the reaction mixture. Binding reactions were resolved by 4.5% acrylamide, 1 TBE electrophoresis. The positions of complex formed by COUP-TF and each probe in the absence of the mouse liver nuclear extract are indicated. Sequence-specific binding factors including HNF4 are also indicated.

Fig. 12. In vivo repression of human ANG transcription by COUP-TF. ICR mice (12 weeks old) were anesthetized by intraperitoneal injection of pentobarbital. The abdomen was shaved, and the liver surface was exposed by a transverse skin incision. The liver was bombarded with a Helios Gene Gun to transfect 13B2cat with pEF-BOS or pEF-BOS/COUP, and the abdomen was closed by running suture. The liver extracts were prepared 6 days after bombardment, and the extracts containing equal amounts of 100 μg of protein were used in CAT assays. The CAT activity of 13B2cat in the presence of pEF-BOS is designated as 100, and each value of CAT activity represents the mean ± S.E. for at least four independent experiments.

True QTLs are those identified in human pedigrees. Some transcriptional activators are down-regulated by inhibitory proteins with which they form protein complexes with altered or reduced DNA binding activities. This is demonstrated by the negative regulation of helix-loop-helix transcription factors and C/EBP by Id and CHOP proteins, respectively (45, 46). Glucocorticoid receptor also interferes with AP1 in the collagenase gene. The basis of this repression appears to be direct protein-protein interaction involving the DNA-binding domain of AP1, and resulting protein complexes lack DNA binding activity (47). Therefore, COUP-TF may directly interact with the low mobility-shifted nuclear factor and interfere with its binding activity. Further study will be necessary to define, by means of purifying the low mobility nuclear factor, the molecular mechanism of its repression by COUP-TF.

Most pathological lesions underlying human genetic disease lie within gene coding regions. A different class of molecular lesion, however, is represented by regulatory mutations that disrupt the normal processes of transcriptional initiation and control and serve either to increase or decrease the level of mRNA/gene product synthesized rather than altering its nature (48, 49). We recently demonstrated that naturally occurring molecular variants of AGCE1, located between the TATA box and transcription initiation site, alter the binding affinity of USF1/AGCF1 and the human ANG transcriptional activity, and act as a genetic risk factor for essential hypertension (8, 9). Moreover, genetically chronic overactivity of the renin-angiotensin system could favor renal sodium reabsorption, vascular hypertrophy, and/or an increase in sympathetic nervous sys-
tem activity, and predisposition to the development of common cardiovascular diseases. In this point, interestingly, associations between molecular variation of the ANG gene and diseases including pre-eclampsia, coronary atherosclerosis, myocardial infarction, and nephropathy in insulin-dependent diabetes have been reported (50–56). Therefore, the repression of human ANG transcription is thought to be important for the potential target of medical treatment of cardiovascular diseases including hypertension. In fact, a recent study showed that the inhibition of ANG transcription resulted in a reduction of plasma ANG levels associated with a decrease in blood pressure of spontaneously hypertensive rats, by using synthetic double-stranded oligonucleotides as “decoy” cis-elements to block the binding of nuclear factors to the targeted promoter regions (56). In the present study, we demonstrated that COUP-TF dramatically repress the human ANG transcription not only in vitro but also in vivo (Fig. 12). Repression of human ANG transcription by COUP-TF may be possibly available for medical applications.

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Regulated Expression of Human Angiotensinogen Gene by Hepatocyte Nuclear Factor 4 and Chicken Ovalbumin Upstream Promoter-Transcription Factor

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