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Forkhead Box A1 (FOXA1) and A2 (FOXA2) Oppositely Regulate Human Type 1 Iodothyronine Deiodinase Gene in Liver

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Type 1 iodothyronine deiodinase (D1), a selenoenzyme that catalyzes the bioactivation of thyroid hormone, is expressed mainly in the liver. Its expression and activity are modulated by several factors, but the precise mechanism of its transcriptional regulation remains unclear. In the present study, we have analyzed the promoter of human D1 gene (hDIO1) to identify factors that prevalently increase D1 activity in the human liver. Deletion and mutation analyses demonstrated that a forkhead box (FOX)A binding site and an E-box site within the region between nucleotides -187 and -132 are important for hDIO1 promoter activity in the liver. EMSA demonstrated that FOXA1 and FOXA2 specifically bind to the FOXA binding site and that upstream stimulatory factor (USF) specifically binds to the E-box element. Overexpression of FOXA2 decreased hDIO1 promoter activity, and short interfering RNA-mediated knockdown of FOXA2 increased the expression of hDIO1 mRNA. In contrast, overexpression of USF1/2 increased hDIO1 promoter activity. Short interfering RNA-mediated knockdown of FOXA1 decreased the expression of hDIO1 mRNA, but knockdown of both FOXA1 and FOXA2 restored it. The response of the hDIO1 promoter to USF was greatly attenuated in the absence of FOXA1. Taken together, these results indicate that a balance of FOXA1 and FOXA2 expression modulates hDIO1 expression in the liver. (Endocrinology 153: 492–500, 2012)

Thyroid hormone activation and inactivation are mediated by three selenoenzymes, type 1 iodothyronine deiodinase (D1), D2, and D3. D1 and D2 catalyze the conversion of T_4 to T_3 via removal of outerring iodine (1). The human D1 gene (hDIO1) is expressed in the liver, kidney, thyroid, and pituitary (2). The D2 gene is expressed in the central nervous system, pituitary, heart, and skeletal muscle, but it is absent in the liver (1). The D3 gene is expressed in the central nervous system and placenta, and it is involved in thyroid hormone inactivation by mediating the removal of innerring iodine. Unlike D2, D1 activity is considered to be regulated predominantly at the pretranslational level. The expression and activity of D1

are modulated by a variety of factors. T_3 induces the expression of hDIO1 via two thyroid hormone responsive elements within its promoter (3), and nuclear factor κB induced by TNFα inhibits the T_3-dependent induction of D1 (4). However, the precise mechanism of the transcriptional regulation of hDIO1 expression remains unclear.

In this study, we sought to identify factors that increased D1 activity in the liver, a main organ that expresses hDIO1. We assessed the promoter activity of the 5-kb 5'-flanking region of hDIO1 and characterized regulatory element-binding proteins within this region. In this study, we identify responsive elements for the forkhead box (FOX) transcription factors FOXA1/FOXA2 and the ba-

Abbreviations: D1, Type 1 iodothyronine deiodinase; FOX, forkhead box; hDIO1, human D1 gene; HNF, hepatocyte nuclear factor; MUT, mutated oligonucleotide; siRNA, short interfering RNA; USF, upstream stimulatory factor; WT, wild type.

sic/helix-loop-helix-leucine zipper transcription factor upstream stimulatory factor (USF), and we show that FOXA1, FOXA2, and USF all participate in the regulation of *hDIO1*. We also show that FOXA1 is required for the activation of the *hDIO1* promoter by USF and that FOXA2 represses the transcription of *hDIO1* and disrupts the interaction of USF with FOXA1 by occupying the FOXA binding site. Collectively, these results demonstrate that FOXA1 and FOXA2 display opposing activity in the regulation of *hDIO1* expression in the liver.

Materials and Methods

Cell culture

The human liver carcinoma cell line HepG2 was obtained from American Type Culture Collection (Manassas, VA) and cultured in MEM (Life Technologies, Carlsbad, CA) with 0.1 mM nonessential amino acid solution (Life Technologies), 1 mM sodium pyruvate (Life Technologies), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO) at 37 C in a humidified atmosphere containing 5% CO_2. TSA 201 cells, a clone of human embryonic kidney 293 cells (5), were cultured in DMEM (Life Technologies) with 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B supplemented with 10% fetal bovine serum at 37 C in a humidified atmosphere containing 5% CO_2.

Plasmid construction

Deletion mutants of the 5′-flanking regions of *hDIO1* (-4949, -2023, -343, -187, -150, -131, and $-103/-4$, the translational start site was set at $+1$) were prepared by PCR using human genomic DNA from leukocytes as a template. The resulting PCR products were subcloned into *Eco*RV or *Kpn*I/*Hin*dIII-digested pGL4.10 (Promega, Madison, WI) to create a fusion with the luciferase gene (-4949, -2023, -343, -187, -150, -131, and $-103/-4$ hDIO1-Luc). The PCR primers, containing *Eco*RV, *Kpn*I, or *Hin*dIII linker, are listed in Table 1. The correct orientation of these deletion mutant constructs was confirmed by sequencing.

Mutations were created using the QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA), according to the manufacturer's instruction; $-187/-4$ and $-150/-4$ hDIO1-

Luc were used as templates. For mutagenesis, the sequences of the FOXA binding element and E-box were specified in figure 3 below. Mutated constructs were isolated from each reaction and verified by sequencing.

Plasmids expressing cDNA for FOXA2 and USF1, pF1KB7038 and pF1KB8339, respectively, were generated by Kazusa DNA Research Institute (Chiba, Japan) and purchased from Promega. These plasmids were digested with *Sgf*I and *Pme*I, and cDNA for FOXA2 and USF1 were ligated into the *Sgf*I/*Pme*I-digested pF4A CMV Flexi vector (Promega), which uses the human cytomegalovirus intermediate-early enhancer/promoter to allow constitutive protein expression at native levels in mammalian cells. The open reading frame of human USF2 was generated by PCR using HeLa cell cDNA as a template. The PCR primers containing *Sgf*I or *Pme*I linker are listed in Table 1. The PCR product was digested with *Sgf*I and *Pme*I, cloned into the *Sgf*I/*Pme*I-digested pF4A CMV Flexi vector, and verified by sequencing.

Transient transfection and luciferase assay

HepG2 and TSA 201 cells were plated at 1.5–2×10^5 and 0.5–1×10^5 cells/well in 24-well tissue culture plates, respectively. Cells were maintained in 0.5 ml of antibiotic-free medium for 1 d before transfection. Transient transfections were performed using the Lipofectamine LTX reagent (Life Technologies) for HepG2 cells and the Lipofectamine 2000 reagent (Life Technologies) for TSA 201 cells according to the manufacturer's instruction. In HepG2 cells, transfections included 500 ng of experimental reporter constructs and 25 ng of pGL4.74, which contained the cDNA encoding *Renilla* luciferase (Promega) as an internal control for transfection efficiency. In TSA 201 cells, transfections included 100 ng of experimental reporter constructs and 5 ng of pGL4.74. In the experiments with plasmids expressing FOXA2 and/or USF, total amount of plasmid DNA was kept constant by adding the corresponding amount of pF4A without a cDNA insert. After transfection, cells were grown in antibiotic-free medium and harvested after 48 h. Luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega), and luminescence was measured by a 2030 ARVOX multilabel reader (PerkinElmer, Waltham, MA). Firefly luciferase activity was normalized to *Renilla* luciferase activity in each well to control for transfection efficiency.

Computational analysis of the putative transcription factor binding sites

The putative transcription factor binding sites on the 5′-flanking region of *hDIO1* were identified by computational

TABLE 1. Oligonucleotides used in plasmid construction and RT-PCR

	Forward primer (5′-3′)	Reverse primer (5′-3′)	Accession no.
Plusmid construction			
$-4949/-4$ hdio1-Luc	GGGGATATCGCAGGTGCAGCCTAGAGATGTAACG	CCCAAGCTTGGCAAAGCCAGAGTAAGCTC	AL031427
$-2023/-4$ hdio1-Luc	CGGGGTACCACACTTCCATTCCAGTTACAG	CCCAAGCTTGGCAAAGCCAGAGTAAGCTC	AL031427
$-343/-4$ hdio1-Luc	CGGGGTACCGAGAGAGCATCTAACAGGTTC	CCCAAGCTTGGCAAAGCCAGAGTAAGCTC	AL031427
$-187/-4$ hdio1-Luc	CGGGGTACCGACCTTTGTGCACCTGGTTAG	CCCAAGCTTGGCAAAGCCAGAGTAAGCTC	AL031427
$-150/-4$ hdio1-Luc	CGGGGTACCGACAGAAAGGCAAACATCTTC	CCCAAGCTTGGCAAAGCCAGAGTAAGCTC	AL031427
$-131/-4$ hdio1-Luc	CGGGGTACCTCTGACCTGACTCCTTCCCCTG	CCCAAGCTTGGCAAAGCCAGAGTAAGCTC	AL031427
$-103/-4$ hdio1-Luc	CGGGGTACCGGTTGGCTGCTCCTACCCTGC	CCCAAGCTTGGCAAAGCCAGAGTAAGCTC	AL031427
pF4A-USF2	AGCAGCGATCGCCATGGACATGCTGGACCCGGGTCTGGA	CGAGGTTTAAACCTGCCGGGTGCCCTCGCCCA	NM_003367
RT-PCR			
hDIO1	CAGAGTCAAGCGGAACATCC	CCGTTGGTCACCTAGAATTG	NM_000792
Cyclophilin A	GCACTGGAGAGAAAGGATTTGG	CAGCAATGGTGATCTTCTTGC	NM_021130

analysis using TFSEARCH databases (http://www.cbrc.jp/research/db/TFSEARCHJ.html), based on the TRANSFAC databases (6).

RNA isolation, RT-PCR, and quantitative PCR

Total RNA was extracted from HepG2 cells using the RNeasy Plus Mini kit (QIAGEN, Valencia, CA) according to the manufacturer's instruction. One microgram of total RNA was reverse transcribed with random hexamers using a First-strand cDNA Synthesis kit (GE Healthcare UK Ltd., Buckinghamshire, UK) according to the manufacturer's instruction. The resulting cDNA were diluted 1:10 and subjected to PCR amplification with 0.5 mM each of the sense and antisense primers and 0.5 U of AmpliTaq Gold DNA polymerase (Life Technologies). The PCR primers used for *hDIO1* and human cyclophilin A gene are indicated in Table 1. The PCR conditions were 40 cycles of denaturation for 1 min at 95 C, annealing for 1 min at 52 C, and extension for 1 min at 72 C. The PCR products were electrophoresed in 2% agarose gels.

Quantitative PCR reactions were performed, recorded, and analyzed using TaqMan Gene Expression Assays with StepOnePlus real-time PCR system (Life Technologies). The probe and primers were Hs00270129_m1 (human *FOXA1*), Hs00232764_m1 (human *FOXA2*), and Hs00174944_m1 (*hDIO1*) and purchased from Life Technologies. Diluted cDNA were amplified using the following conditions: 50 C for 2 min, 95 C for 10 min, and 40 cycles of 95 C for 15 sec and 60 C for 1 min, followed by continuous incubation at 25 C. Expression levels of FOXA1, FOXA2, and hDIO1 were normalized to cyclophilin A to compensate for variations in input RNA.

Preparation of cell extracts and EMSA

Nuclear extracts were prepared from HepG2 cells using the Nuclear Extract kit (Active Motif, Carlsbad, CA), according to the manufacturer's instruction. EMSA were conducted using a LightShift chemiluminescent EMSA kit (Thermo Fisher Scientific, Rockford, IL) with slight modifications of the original manufacturer's instruction. Oligonucleotides 3′-end labeled with biotin were synthesized (Life Technologies) and annealed to generate double-stranded oligonucleotide probes. Two hundred femtomoles of oligonucleotide probe were incubated with 10–15 μg of nuclear protein and 0.5 μg of poly (dI-dC) in the presence or absence of competing oligonucleotide in 10× binding buffer [containing 100 mM Tris, 500 mM KCl, and 10 mM dithiothreitol (pH 7.5)] and 75 mM KCl, and 5% glycerol was added to solutions containing probes with an E-box element. After a 30-min incubation at room temperature, DNA-protein complexes were separated by electrophoresis on a 6% DNA retardation gel (Life Technologies) at 4 C in 0.5× Tris-borate, EDTA buffer [containing 89 mM Tris-borate and 2 mM EDTA (pH 8.0)]. For supershift assays, binding reactions were incubated for 45 min at room temperature with antibodies before the addition of labeled probes. The antibodies used in the supershift assays were as follows: 1 μl (200 μg/0.1 ml) of USF1 (sc-8983X), USF2 (sc-861X), E47 (sc-763X), FOXA1 (sc-6553X), FOXA2 (sc-6554X), and FOXA3 (sc-5361X) and 5 μl (200 μg/0.5 ml) of normal goat and normal rabbit IgG, and all were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). After electrophoresis, samples were transferred onto nylon membranes and fixed by UV irradiation. Biotinylated DNA was detected using a Fujix Lumino-image analyzer (LAS-1000; Fuji Photo Film Co., Ltd., Tokyo, Japan).

Transfection of short interfering RNA (siRNA)

An aliquot of 6 pmol siRNA specific for FOXA1 and/or FOXA2 (Stealth Select RNAi, Life Technologies) or a negative control siRNA (Stealth RNAi Negative Control, Life Technologies) was transfected into HepG2 cells using the Lipofectamine RNAiMax reagent (Life Technologies) by reverse transfection according to the manufacturer's instruction. After transfection, HepG2 cells were plated at 1.5–2 × 10^5 cells/well in 24-well tissue culture plates and maintained in 0.5 ml of antibiotic-free medium for 24–48 h. mRNA extraction and analysis were performed as described above.

Statistics

The data represent the mean ± SEM and were obtained from at least three separate experiments, each performed in triplicate. Statistical analyses were performed to examine the significance of differences among the results using unpaired *t* test or ANOVA followed by Student-Newman-Keuls test or Dunnett's test.

Results

Functional analysis of the 5′-flanking region of the *hDIO1* gene

To identify regions within the promoter region of *hDIO1* important for regulating its expression, a series of 5′-deletion constructs was subcloned into the pGL4.10 vector and transiently transfected into HepG2 and TSA 201 cells (Fig. 1A). In both HepG2 and TSA 201 cells, luciferase activity increased by deletion of nucleotides −150 to −131 and decreased after deletion of −131 to −103. Among the tested constructs, the luciferase activity produced by transfection of −150/−4 hDIO1-Luc was specifically and markedly decreased in HepG2 cells. Additionally, more pronounced differences were seen between the activity of −150/−4 hDIO1-Luc and −131/−4 hDIO1-Luc in HepG2 cells compared with TSA 201 cells. In addition, luciferase activity was markedly increased by deleting the region from −343 to −187 and decreased after deletion of −187 to −150 only in HepG2 cells. Taken together, these results indicate that the region between nucleotides −187 and −132 is important for *hDIO1* promoter function in HepG2 cells. To confirm the expression of *hDIO1*, we performed RT-PCR using total RNA isolated from HepG2 and TSA 201 cells, a liver and kidney cell line, respectively. As shown in Fig. 1B, although there was a difference in the degree of gene expression, *hDIO1* was expressed in both cell lines; this was consistent with a previous report examining *hDIO1* tissue distribution (2). Additionally, multiple PCR products were detected, because there are several alternative splice variants of *hDIO1* (7). These results indicate that there may exist a sequence
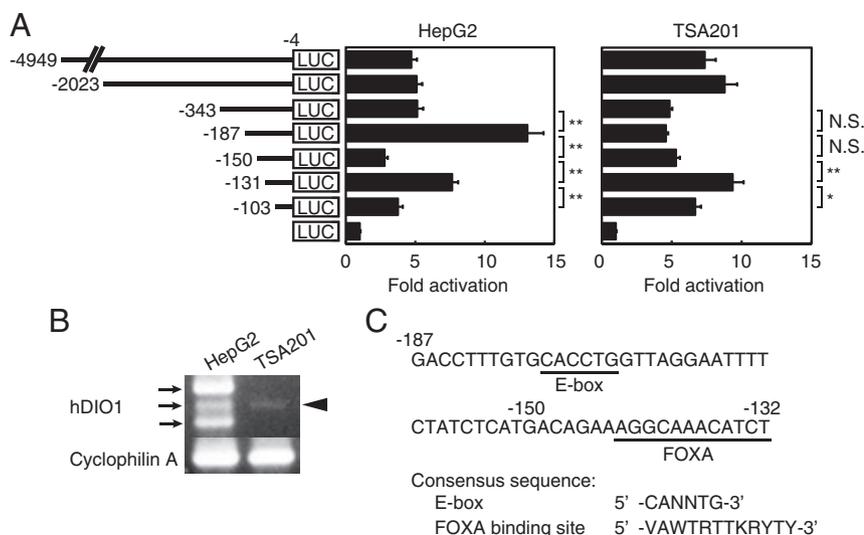
FIG. 1. Liver-specific changes in *hDIO1* promoter activity. A, A series of 5′-deletion constructs of the *hDIO1* promoter were transiently transfected into HepG2 or TSA 201 cells. Promoter activity was normalized to *Renilla* luciferase activity and expressed as the relative activity to promoterless pGL4.10. Statistical significance was determined by ANOVA followed by Student-Newman-Keuls test. *, $P < 0.05$; **, $P < 0.01$. N.S., Not significant. B, RT-PCR analysis of *hDIO1* expression. Electrophoretic analysis of RT-PCR products using total RNA from HepG2 and TSA 201 cells was performed. The *arrows* and *arrowhead* correspond to the RT-PCR products using total RNA from HepG2 and TSA 201 cells, respectively. Cyclophilin A was used as a positive control. C, The nucleotide sequences of the 5′-flanking region of *hDIO1* are shown. The translational start site was set at +1. *Underlined sequences* indicate putative binding sites for transcription factors. Consensus sequences of E-box site and FOXA binding site are shown at the *bottom*. Abbreviations for nucleotides: W (A or T), K (G or T), Y (C or T), R (A or G), V (A, C, or G), and N (A, C, G, or T). LUC, Luciferase.

essential for liver-specific expression of *hDIO1* within the −187 to −132 region of its promoter. A computational analysis of this region revealed the presence of a consensus E-box site between nucleotides −187 and −151 and a FOXA binding site between nucleotides −150 and −132 (Fig. 1C).

Promoter activity associated with the FOXA binding site and the E-box

To better understand the contribution of the FOXA binding site and the E-box on the expression of *hDIO1* in liver-derived HepG2 cells, we examined luciferase activity in cells transfected with wild-type (WT) or mutated *hDIO1* promoter constructs. In HepG2 cells, luciferase activity was increased 2-fold by mutating the FOXA binding site when cells were transfected with a −150/−4 hDIO1-Luc construct (Fig. 2A). In addition, when cells were transfected with a −187/−4 hDIO1-Luc construct, luciferase activity was nearly completely lost by destruction of the E-box, and mutation of the FOXA binding site caused a decrease in luciferase activity by 50% (Fig. 2B). In TSA 201 cells transfected with −187/−4 hDIO1-Luc, luciferase activity was almost completely abolished by mutation of the E-box, but mutation of the FOXA binding site in both −187/−4 hDIO1-Luc and −150/−4 hDIO1-Luc did not significantly affect luciferase activity (Fig. 2). Thus, the E-box present

within the *hDIO1* promoter is required for the enhancer activity in both liver- and kidney-derived cells, but the FOXA binding site exhibits liver-specific enhancer and repressor activity.

Binding of FOXA1/FOXA2 to the FOXA binding site and USF to the E-box

To determine the transcription factors that bind to these elements in the promoter of *hDIO1* in HepG2 cells, we performed EMSA using oligonucleotides with the FOXA binding site and the E-box. Incubation of HepG2 cell extracts with oligonucleotides containing the FOXA binding site (Fig. 3A, WT-F) led to the formation of several DNA/protein complexes (Fig. 3B, lane 2). Formation of one of these complexes was inhibited by incubation with excess WT-F, but not mutated oligonucleotide (MUT)-F, demonstrating the specificity of this complex (Fig. 3B, lanes 3–6). Additionally, the complex was supershifted by addition of anti-FOXA1 and anti-FOXA2 antibodies (Fig. 3B, lanes 7 and 8). However, an antibody specific for FOXA3, which binds an identical sequence, or normal goat IgG did not disrupt complex formation (Fig. 3B, lanes 9 and 10). These results suggest that the putative FOXA binding site is specifically bound by FOXA1 or FOXA2. We next examined binding to the E-box sequence, and several complexes were formed by
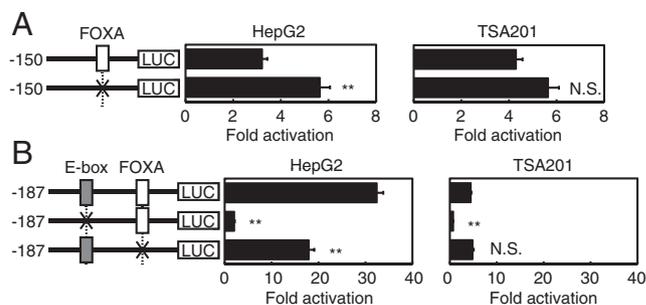
FIG. 2. Changes in *hDIO1* promoter activity by FOXA binding site and E-box. Schematic diagram in the *left* of each figure representing WT and site-specific mutations of the *hDIO1* promoter, introduced into the upstream region of the luciferase gene. A *cross* represents the site-specific mutation of the putative FOXA binding site or E-box. Each construct was transiently transfected into HepG2 or TSA 201 cells. Promoter activity was normalized to *Renilla* luciferase activity and expressed as the relative activity to promoterless pGL4.10. Statistical significance was determined by unpaired *t* test (A) or ANOVA followed by Dunnett's test (B). **, $P < 0.01$. N.S., Not significant.

FIG. 3. Specific binding of the transcription factors within the −187 and −132 region in the *hDIO1* promoter using nuclear proteins from HepG2 cells. Panel A, Sequences of the double-stranded oligonucleotides used in EMSA. WT-F, WT *hDIO1* sequence identical to the −153 to −125 region, containing the FOXA binding site at −143 to −132. MUT-F contains a mutated FOXA binding site. WT-E, WT *hDIO1* sequence identical to the −187 to −159 region, containing the E-box at −177 to −172. MUT-E contains a mutated E-box. Each putative binding site is *underlined*; the mutated base pairs are indicated by *italic letters*. Panel B, Oligonucleotide WT-F was used as the probe for EMSA either without competitor (lane 2) or in the presence of 50- and 100-fold molar excesses of unlabeled WT-F (lanes 3 and 4, respectively) or MUT-F (lanes 5 and 6, respectively). The specific complex formed from HepG2 cell nuclear extract and WT-F is indicated by an *arrowhead*. Supershift assay experiments were performed using antibody against FOXA1 (F1; lane 7), FOXA2 (F2; lane 8), or FOXA3 (F3; lane 9) and normal goat IgG (C; lane 10). Panel C, Oligonucleotide WT-E was used as the probe for EMSA either without competitor (lane 2) or in the presence of 50- and 100-fold molar excesses of unlabeled WT-E (lanes 3 and 4, respectively), MUT-E (lanes 5 and 6, respectively), or 100-fold molar excesses of unlabeled E-box from the human ghrelin gene [E2 and E3 (in Ref. 8); lanes 7 and 8, respectively]. The specific complex formed from HepG2 cell nuclear extract and WT-E is indicated by an *arrowhead*. D, Supershift assay experiments were performed using antibody against USF1 (U1; lane 7), USF2 (U2; lane 8), or E47 (E47; lane 9) and normal rabbit IgG (C; lane 10). C, Negative control (normal goat and normal rabbit IgG).

incubation of HepG2 cell extracts with an appropriate oligonucleotide (Fig. 3A, WT-E, and C, lane 2). Formation of one of these complexes was inhibited by incubating with an excess of WT-E and the E-box from human ghrelin gene (8) but not by a mutant oligonucleotide MUT-E (Fig. 3C, lanes 3–8). Computational analysis predicted that the putative E-box site binds the basic/helix-loop-helix-leucine zipper transcription factor USF, and to investigate this hypothesis, a supershift assay was performed using antibodies specific for USF1 and USF2. Addition of antibodies specific for USF1 and USF2 completely inhibited complex formation (Fig. 3D, lanes 7 and 8). However, incubation with an antibody against E47, a protein that binds a similar E-box sequence, or normal rabbit IgG did not disrupt complex formation (Fig. 3D, lanes 9 and 10). These results suggest that USF1/USF2 bind the putative E-box, and this complex likely contains a USF1/USF2 heterodimer. Collectively, FOXA1, FOXA2, and USF likely participate in the regulation of *hDIO1* expression.

Effect of overexpression of FOXA2 or USF on *hDIO1* promoter activity

To determine whether FOXA and USF have the potential to affect the activity of the *hDIO1* promoter in liver

cells, a −187/−4 hDIO1-Luc construct was transiently transfected into HepG2 cells along with increasing amounts of either a FOXA2 or USF expression plasmid. The luciferase activity decreased in a dose-dependent manner by cotransfection of the FOXA2 expression plasmid with −187/−4 hDIO1-Luc (Fig. 4A). In contrast, luciferase activity increased dose dependently by the cotransfection of the USF1 or USF2 expression plasmid, and overexpression of USF2 consistently led to greater *hDIO1* promoter activity than expression of USF1 alone (Fig. 4B). Thus, transcription of *hDIO1* is negatively regulated by FOXA2 and positively regulated by USF. Although we transiently transfected a −187/−4 hDIO1-Luc construct into HepG2 cells along with increasing amounts of a FOXA1 expression plasmid, we could not obtain appropriate data, indicating that the FOXA1 expression plasmid we used did not function in our experimental system for unknown reason.

RNA interference

Next, we determined the effects of FOXA on the native *hDIO1* promoter using siRNA-mediated knockdown of FOXA in HepG2 cells. As shown in Fig. 5, A and B, knock-

FIG. 4. Effect of overexpression of FOXA2 or USF on *hDIO1* promoter activity. −187/−4 hDIO1-Luc was transiently transfected into HepG2 cells in the presence of increasing amounts of vectors expressing FOXA2 (F2) (A) or USF1 or USF2 (U1 and U2, respectively) (B). Promoter activity was normalized to *Renilla* luciferase activity, then expressed as the relative activity to −187/−4 hDIO1-Luc cotransfected with pF4A without a cDNA insert. Statistical significance was determined by ANOVA followed by Dunnett's test. **, $P < 0.01$.

down of FOXA1 decreased the expression of *hDIO1* mRNA, and knockdown of FOXA2 increased the expression level of *hDIO1* mRNA. FOXA1 and FOXA2 did not affect each other's expression by knockdown of them (Fig. 5, A and B). In addition, when both FOXA1 and FOXA2 were knocked down simultaneously, no change in the expression of *hDIO1* mRNA was seen (Fig. 5C). Thus, *hDIO1* expression is positively regulated by FOXA1 and negatively regulated by FOXA2, and FOXA1 and FOXA2 interact with each other to regulate *hDIO1* expression.

Interaction between FOXA and USF in the activation of the *hDIO1* promoter

Transcription factors frequently interact to coordinately regulate gene expression, and we first wished to determine whether the FOXA binding site and the E-box present in the *hDIO1* promoter interact. We cotransfected a WT or mutated −187/−4 hDIO1-Luc construct and USF expression plasmids into HepG2 cells. When the FOXA binding site was mutated, the transcription activity of the *hDIO1* promoter in the presence of transfected USF was attenuated (Fig. 6A). Thus, activation of the *hDIO1* promoter by USF depends on the presence of a functional FOXA binding site. Next, we investigated the effects of FOXA on the response of the *hDIO1* promoter to USF.
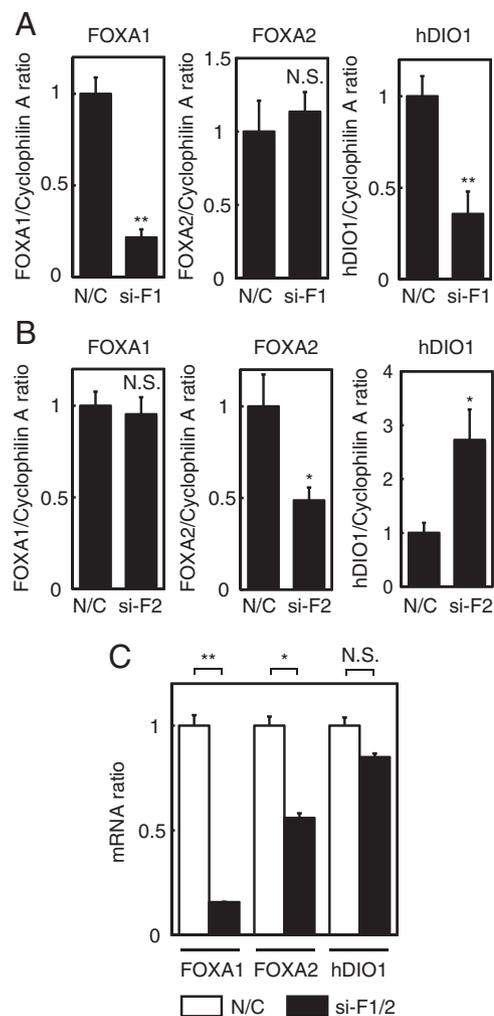
FIG. 5. Knockdown of FOXA1 and/or FOXA2. Effect of transfection of FOXA1 (A) and FOXA2 (B) siRNA on the mRNA expression level of FOXA1, FOXA2, and D1. Simultaneous knockdown of FOXA1 and FOXA2 (C) was performed using siRNA that has identical sequences in both FOXA1 and FOXA2. N/C, Negative control siRNA; si-F1, siRNA specific for FOXA1; si-F2, siRNA specific for FOXA2; si-F1/2, siRNA specific for both FOXA1 and FOXA2. mRNA expression level was normalized to that of cyclophilin A in each sample and expressed as the relative activity to the basal expression (N/C). Statistical significance was determined by unpaired *t* test. *, $P < 0.05$; **, $P < 0.01$. N.S., Not significant.

We knocked down the expression of FOXA and cotransfected a −187/−4 hDIO1-Luc construct along with USF expression plasmids into HepG2 cells. As shown in Fig. 6B, the transcription activity of the *hDIO1* promoter was attenuated by knockdown of FOXA1 and enhanced by knockdown of FOXA2. The transcription activity of the *hDIO1* promoter was also attenuated by simultaneous knockdown of FOXA1 and FOXA2 to an extent similar to that seen for the knockdown of FOXA1. The suppressed activity by knockdown of FOXA1 was not restored by overexpression of USF, and the enhanced activity by knockdown of FOXA2 was further enhanced by overexpression of USF. Thus, the response of the *hDIO1* pro-
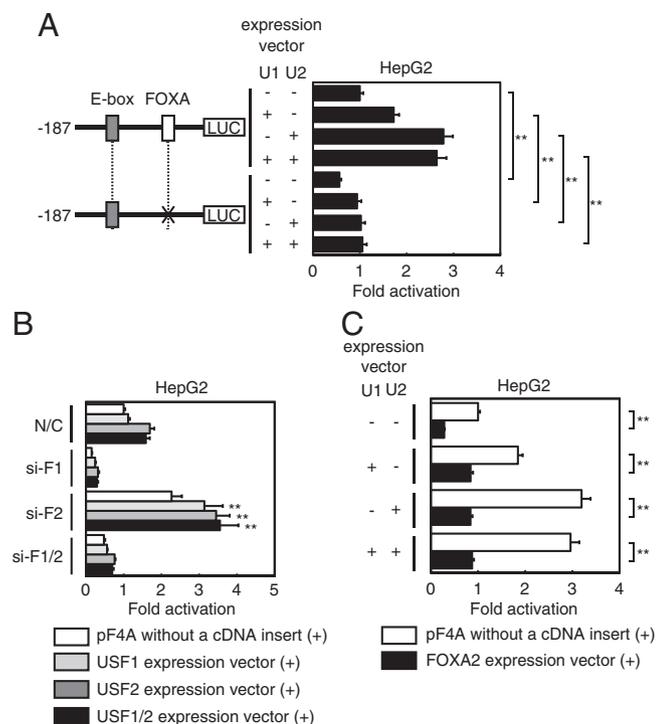
FIG. 6. Interaction between FOXA and USF in the activation of the *hDIO1* promoter. A, Dependence of the activation of *hDIO1* promoter by USF on the FOXA binding site. Schematic diagram in the *left* of figure representing WT and site-specific mutations of the *hDIO1* promoter, introduced into the upstream region of the luciferase gene. A *cross* represents the site-specific mutation of the putative FOXA binding site. Each construct was transiently cotransfected into HepG2 cells in the presence of 5 ng of vectors expressing USF1 (U1) and/or USF2 (U2). Promoter activity was normalized to *Renilla* luciferase activity, then expressed as the relative activity to −187/−4 hDIO1-Luc cotransfected with pF4A without a cDNA insert. Statistical significance was determined by ANOVA followed by Student-Newman-Keuls test. **, $P < 0.01$. B, Effect of knockdown of FOXA on the activation of the *hDIO1* promoter by USF. Short interfering RNA specific for FOXA1 and/or FOXA2 or a negative control siRNA were transfected into HepG2 cells as described in *Materials and Methods*. One day after siRNA transfection, −187/−4 hDIO1-Luc was transiently cotransfected in the presence of 5 ng of vectors expressing USF1 and/or USF2. N/C, Negative control siRNA; si-F1, siRNA specific for FOXA1; si-F2, siRNA specific for FOXA2; si-F1/2, siRNA specific for both FOXA1 and FOXA2. Promoter activity was normalized to *Renilla* luciferase activity, then expressed as the relative activity to −187/−4 hDIO1-Luc cotransfected with pF4A without a cDNA insert after knockdown by negative control siRNA. Statistical significance was determined by ANOVA followed by Student-Newman-Keuls test. **, $P < 0.01$ relative to −187/−4 hDIO1-Luc cotransfected with pF4A without a cDNA insert after knockdown by siRNA specific for FOXA2. C, Effect of overexpression of FOXA2 on the activation of the *hDIO1* promoter by USF. −187/−4 hDIO1-Luc was transiently transfected into HepG2 cells in the presence of 5 ng of vectors expressing USF1 (U1), USF2 (U2), and/or FOXA2. Promoter activity was normalized to *Renilla* luciferase activity, then expressed as the relative activity to −187/−4 hDIO1-Luc cotransfected with pF4A without a cDNA insert. Statistical significance was determined by ANOVA followed by Student-Newman-Keuls test. **, $P < 0.01$. LUC, Luciferase.

moter to USF was greatly attenuated by knockdown of FOXA1 and enhanced by knockdown of FOXA2. Furthermore, we cotransfected a −187/−4 hDIO1-Luc con-

struct and USF expression plasmids with or without a FOXA2 expression plasmid into HepG2 cells. The transcription activity of the *hDIO1* promoter was enhanced in the presence of transfected USF (Fig. 6C, *white bar*), but the activity was greatly attenuated by cotransfection of the FOXA2 expression plasmid (Fig. 6C, *black bar*). Thus, the response of the *hDIO1* promoter to USF was attenuated by the coexpression of FOXA2. Collectively, these results indicate that FOXA1 is required for the activation of the *hDIO1* promoter by USF and that FOXA2 represses the transcription of *hDIO1* and disrupts the interaction of USF with FOXA1 by occupying the FOXA binding site.

Discussion

In this study, we analyzed the 5′-upstream region of *hDIO1* to identify protein-DNA interactions within the *hDIO1* promoter. Our experiments demonstrated that the region between nucleotides −187 and −132 is important for *hDIO1* promoter activity in HepG2 cells. We identified functional elements for FOXA and USF within this region, and we showed that these sites are important for the transcriptional regulation of *hDIO1*. Recently, Ohguchi *et al.* (9) identified a proximal hepatocyte nuclear factor (HNF)4α binding site in mice, and they demonstrated that the HNF4α binding site is essential for the activation of the mouse D1 gene by HNF4α. Deletion analyses of the 5′-flanking region of *hDIO1* were performed by Jakobs *et al.* (10) by transfecting 1.5- and 0.1-kb constructs into HepG2 cells, and they found that both constructs substantially increased luciferase activity compared with a promoterless vector. However, they did not perform a higher resolution promoter analysis, and we are the first to identify functional elements other than thyroid hormone responsive element in the *hDIO1* promoter.

The FOXA proteins were first identified as liver-enriched factors because of their ability to bind the transthyretin gene promoter, and they were originally termed HNF3 (11). There are three FOXA proteins, FOXA1 (HNF3α), FOXA2 (HNF3β), and FOXA3 (HNF3γ), which are encoded by different genes on different chromosomes (12). FOXA proteins play important roles in early embryonic development and organogenesis, and they are recognized as "pioneer factors" (13). In addition, the FOXA proteins control glucose metabolism through the regulation of multiple target genes in the liver, pancreas, and adipose tissue after birth (13). Our EMSA experiments demonstrated that FOXA1 and FOXA2 specifically bound the identical FOXA binding site of the *hDIO1* promoter. Although all three FOXA proteins exist relatively abundant in HepG2 cells (14) and recognize the same DNA sequences, slight differences in the binding affin-
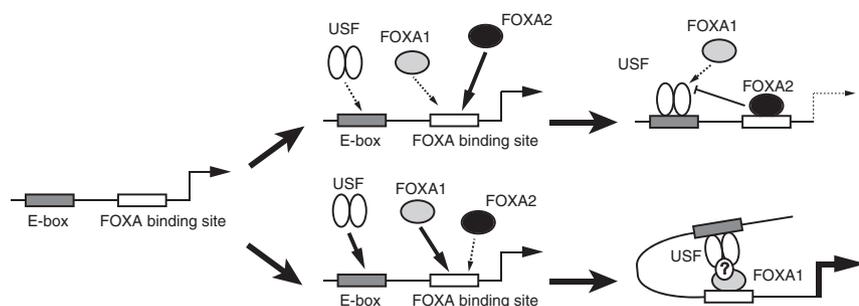
FIG. 7. A model for the regulation of liver-specific expression of *hDIO1* by FOXA1, FOXA2, and USF. FOXA1 and FOXA2 bind and share the identical FOXA binding site, and USF binds the E-box as a heterodimer. FOXA2 represses the transcription of *hDIO1* through the FOXA binding site (*upper panel*), and FOXA1 and USF work cooperatively to activate *hDIO1* transcription (*lower panel*).

ity and DNA binding capacity may account for their specificity (15). Indeed, there are very few reports that FOXA1 and FOXA2 share an identical binding site and coparticipate in the transcriptional regulation of a single gene (16). Our transfection assays and siRNA experiments demonstrated that *hDIO1* is positively regulated by FOXA1 and negatively regulated by FOXA2 and that FOXA1 and FOXA2 interact to coordinately regulate *hDIO1* expression. These results suggest that FOXA proteins are involved in thyroid hormone homeostasis.

USF proteins were first identified as regulators of adenovirus major late promoter transcription (17, 18). There are two USF proteins, 43 kDa (USF1) and 44 kDa (USF2), encoded by different genes on different chromosomes (19, 20). USF proteins primarily bind as dimers to consensus sequences containing the CACGTG motif termed an E-box (18, 19, 21). USF proteins are ubiquitously expressed, although different ratios of USF homo- and heterodimers are found in different cell types (22). The molecular details of USF binding and activity have been well characterized, but its biological role remains poorly understood. USF proteins regulate the expression of several genes related to glucose and lipid metabolism and peptide hormone synthesis, including liver-type pyruvate kinase (23) and glucokinase (24), fatty acid synthase (25), apolipoprotein A-II (26), calcitonin/calcitonin gene-related peptide (27), and ghrelin (8). In our study, we demonstrated that the putative E-box site in the *hDIO1* promoter specifically bound the USF1/USF2 heterodimer and that promoter activity increased in a dose-dependent manner with the cotransfection of the USF1/2 expression plasmid. These results suggest that USF positively regulate *hDIO1* expression. Additionally, promoter activity was almost completely abolished by mutation of the E-box motif, indicating that USF proteins are critical for the transcriptional regulation of *hDIO1* and thyroid hormone homeostasis in the liver and possibly kidney.

The response of the *hDIO1* promoter to USF was greatly attenuated by mutation of the FOXA binding site or knock-

down of FOXA1, indicating that FOXA1 is necessary for the expression of *hDIO1* by USF. FOXA1 plays an essential role in the "pioneering" of gene regulatory elements, allowing for the recruitment of additional factors required for gene regulation (28), and our data suggest that USF cooperates with FOXA1 to regulate *hDIO1* promoter activity. Although we could not confirm the interaction between FOXA1 with USF by coimmunoprecipitation experiments in our experimental condition (data not shown), a direct physical interaction between FOXA1 and USF has been reported through the use of immunoprecipitation and glutathione *S*-transferase pulldown assays (29). Furthermore, the cooperation between FOXA1 and USF likely contributes to the liver-specific activation of *hDIO1*; although FOXA1 was expressed in both HepG2 and TSA201 cells in our preliminary experiments (data not shown), only HepG2 cells demonstrated substantial differences in promoter activity by transfection of −187/−4 hDIO1-Luc and mutation of the FOXA binding site. Interactions between cell-specific factors and other regulators are thought to contribute to the tissue-specific control of gene expression by the ubiquitous USF proteins (26, 30–34), and Fig. 7 shows our working model for the regulation of liver-specific expression of *hDIO1* by transcription factor binding to the −187 to −132 region of the *hDIO1* promoter. In this model, *hDIO1* promoter activity is modulated by FOXA1, FOXA2, and USF proteins, and these transcription factors interact with each other to fine-tune the *hDIO1* promoter activity.

In conclusion, we have shown that FOXA1, FOXA2, and USF regulate *hDIO1* expression in the liver. FOXA1 and FOXA2 both participate in the liver-specific regulation of *hDIO1* expression, and FOXA1 and USF act together to promote the liver-specific activation of *hDIO1*. FOXA1 and FOXA2 are likely involved in thyroid hormone homeostasis in the liver.

Acknowledgments

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