

Hepatocyte Nuclear Factor 4 α Contributes to Thyroid Hormone Homeostasis by Cooperatively Regulating the Type 1 Iodothyronine Deiodinase Gene with GATA4 and Krüppel-Like Transcription Factor 9[†]

Hiroto Ohguchi,^{1,2} Toshiya Tanaka,¹ Aoi Uchida,¹ Kenta Magoori,¹ Hiromi Kudo,¹ Insook Kim,³ Kenji Daigo,¹ Iori Sakakibara,¹ Masashi Okamura,¹ Hideo Harigae,² Takeshi Sasaki,² Timothy F. Osborne,⁴ Frank J. Gonzalez,³ Takao Hamakubo,¹ Tatsuhiko Kodama,¹ and Juro Sakai^{1*}

Laboratory of Systems Biology and Medicine, Research Center for Advanced Science and Technology, University of Tokyo, Tokyo 153-8904, Japan¹; Department of Hematology and Rheumatology, Tohoku University Graduate School of Medicine, 1-1 Seiryomachi, Aoba-ku, Sendai 980-8574, Japan²; Laboratory of Metabolism, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892³; and Department of Molecular Biology and Biochemistry, University of California, Irvine, California 92717-3900⁴

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Type 1 iodothyronine deiodinase (Dio1), a selenoenzyme catalyzing the bioactivation of thyroid hormone, is highly expressed in the liver. Dio1 mRNA and enzyme activity levels are markedly reduced in the livers of hepatocyte nuclear factor 4 α (HNF4 α)-null mice, thus accounting for its liver-specific expression. Consistent with this deficiency, serum T₄ and rT₃ concentrations are elevated in these mice compared with those in HNF4 α -floxed control littermates; however, serum T₃ levels are unchanged. Promoter analysis of the mouse *Dio1* gene demonstrated that HNF4 α plays a key role in the transactivation of the mouse *Dio1* gene. Deletion and substitution mutation analyses demonstrated that a proximal HNF4 α site (direct repeat 1 [TGGACAAA GGTGC]; HNF4 α -RE) is crucial for transactivation of the mouse *Dio1* gene by HNF4 α . Mouse Dio1 is also stimulated by thyroid hormone signaling, but a direct role for thyroid hormone receptor action has not been reported. We also showed that thyroid hormone-inducible Krüppel-like factor 9 (KLF9) stimulates the mouse *Dio1* promoter very efficiently through two CACCC sequences that are located on either side of HNF4 α -RE. Furthermore, KLF9 functions together with HNF4 α and GATA4 to synergistically activate the mouse *Dio1* promoter, suggesting that Dio1 is regulated by thyroid hormone in the mouse through an indirect mechanism requiring prior KLF9 induction. In addition, we showed that physical interactions between the C-terminal zinc finger domain (Cf) of GATA4 and activation function 2 of HNF4 α and between the basic domain adjacent to Cf of GATA4 and a C-terminal domain of KLF9 are both required for this synergistic response. Taken together, these results suggest that HNF4 α regulates thyroid hormone homeostasis through transcriptional regulation of the mouse *Dio1* gene with GATA4 and KLF9.

Thyroid hormone plays important roles in growth, development, differentiation, and the basal metabolic rate in vertebrates. Synthesis of thyroid hormone occurs exclusively in the thyroid gland, whose predominant secretory product is the prohormone thyroxin (T₄) and which produces only a small amount of the biologically active hormone 3,5,3'-triiodothyronine (T₃) (29). The majority of plasma T₃ derives from extrathyroid tissues via outer-ring deiodination of T₄ (9). This activation is catalyzed by two different deiodinases, type 1 iodothyronine deiodinase (Dio1) and type 2 iodothyronine deiodinase (Dio2). Dio1 is one of a family of selenoenzymes

extensively expressed in the liver, kidney, thyroid, and pituitary in mammals (5, 6). Unlike Dio2, Dio1 can catalyze both activation of T₄ by outer-ring deiodination (5'D) to generate T₃ and inactivation of T₄ by inner-ring deiodination to produce 3,3',5'-triiodothyronine (rT₃) (5D) (9). The expression and activity of Dio1 are modulated by a variety of hormonal, nutritional, and developmental factors, the most potent being thyroid hormone. Thyroid hormone-induced Dio1 expression contributes to the T₃ excess commonly found in hyperthyroidism. Propylthiouracil and amiodarone are the two commonly used drugs that inhibit Dio1, which can have substantial effects on circulating thyroid hormone levels. Previous studies reported that T₃ and retinoids induce the expression of the *Dio1* gene via two complex thyroid hormone response-retinoic acid response elements located in the promoter region of the human *Dio1* gene (66). Although both the rat and mouse liver Dio1 mRNAs are markedly increased by T₃, canonical thyroid hormone response elements (TREs) have not yet been identified in the available 5'-flanking regions (5'-FR) of these genes (9, 35, 36).

* Corresponding author. Mailing address: Laboratory of Systems Biology and Medicine, Research Center for Advanced Science and Technology, University of Tokyo, 4-6-1 Komaba, Meguro, Tokyo 153-8904, Japan. Phone: 81-3-5452-5472. Fax: 81-3-5452-5429. E-mail: jmsakai-ky@umin.ac.jp.

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Hepatocyte nuclear factor 4 α (HNF4 α ; NR2A1) is a highly conserved member of the nuclear receptor superfamily. It is highly expressed in the liver, kidney, intestine, and pancreas in mammals (54). The active form of HNF4 α is a homodimer which recognizes a direct repeat (DR) of the AGGTCA motif separated by 1 nucleotide (DR1) as a binding site. Binding sites for HNF4 α have been found in the regulatory regions of many genes encoding proteins preferentially expressed in the liver, such as apolipoproteins, coagulation factors, serum proteins, and cytochromes P450, and those genes involved in the metabolism of fatty acids, amino acids, and glucose. Mice lacking hepatic expression of HNF4 α have revealed that HNF4 α regulates the expression of these target genes (17, 21–24, 49). HNF4 α , as an orphan nuclear receptor, activates gene transcription in the absence of exogenous ligand (28, 55, 56); therefore, unlike classic nuclear receptors, the transcriptional activity of HNF4 α is largely dependent on the selective interaction of tissue-specific or independently regulated coregulators with its activation function 2 (AF-2) domain to stimulate target genes in a tissue-specific and metabolically regulated gene-specific manner (16). For example, GATA factors function together with HNF4 α to synergistically activate transcription through a direct protein-protein interaction (58).

The six GATA factors show homology in two zinc finger domains that mediate DNA binding and cofactor interactions. GATA1, -2, and -3 are expressed in developing bone marrow cells and are critical for hematopoiesis (14, 43), whereas GATA4, -5, and -6 have a more diverse pattern of expression that includes the liver, small intestine, heart, lungs, and gonads (3, 25, 31, 42, 60). Although the wide-ranging expression patterns of GATA4, -5, and -6 argue against the notion that these proteins are master regulators of tissue- or cell type-specific gene expression, there is increasing evidence that this subfamily might be critical in regulating cell-specific gene expression through unique interactions with other semirestricted transcription factors and cofactors (34, 41, 61, 64, 67). A recent study showed that GATA4 also forms a complex with Krüppel-like factor 13 (KLF13), a member of the KLF family of zinc finger proteins, and this interaction is important for activating genes involved in cardiac growth and differentiation (30).

To uncover possible additional roles for HNF4 α in gene expression in an intact-animal model, we compared the gene expression profiles of liver-specific HNF4 α -null mice and littermate HNF4 α -floxed control mice. Through the evaluation of transcriptional changes by microarray and quantitative real-time PCR, we revealed that expression of the *Dio1* gene is dependent on HNF4 α expression and further demonstrated that the mouse *Dio1* promoter is directly regulated by HNF4 α . In this study, we also describe a likely mechanism by which T₃ induces the expression of Dio1 in mouse liver and show that T₃-inducible Krüppel-like transcription factor 9 (KLF9) functions together with HNF4 α and GATA4 to synergistically activate the mouse *Dio1* promoter through direct interaction between these transcriptional factors. These data suggest that HNF4 α plays a key role in thyroid hormone homeostasis by cooperatively regulating the 5' deiodination of T₄ with GATA4 and KLF9 and also reveal that regulation of mouse Dio1 by T₃ is likely due to an indirect mechanism responding to the T₃-dependent stimulation of KLF9.

MATERIALS AND METHODS

Reagents. T₃, rT₃, and 6-propyl-2-thiouracil (PTU) were obtained from Sigma; ¹²⁵I-labeled rT₃ was from Perkin-Elmer Life Sciences (Boston, MA). Other reagents were obtained from sources described previously (18, 19, 58, 68).

Antibodies. Mouse monoclonal antibodies immunoglobulin G (IgG)-H1415 against human HNF4 α 2 (amino acids [aa] 394 to 461) (62) and IgG-H2429 against human GATA4 (aa 332 to 442) (58) were described in the indicated references. Other antibodies were obtained from the following sources. Goat polyclonal anti-HNF4 α (sc-6556), goat polyclonal anti-KLF9 (sc-12996), rabbit polyclonal anti-GATA4 (directed against aa 328 to 439 of human GATA4) (sc-9053), and control goat IgG (sc-2028) were from Santa Cruz Biotechnology. Goat polyclonal anti-human GATA4 antibody (directed against aa 27 to 211 of human GATA) (AF2606) was from R&D Systems. Monoclonal anti-FLAG M2 (F3165), peroxidase-conjugated anti-FLAG M2 (A8592), mouse monoclonal anti- β -actin (A1978), and affinity-purified goat anti-mouse IgG (A4416) were from Sigma. Monoclonal antinucleoporin antibody (610497) was from BD-Biosciences. Control mouse IgG (132-13723) was from Wako (Osaka, Japan). Affinity-purified donkey anti-goat IgG was from Jackson ImmunoResearch Laboratories.

Animals. Liver-specific HNF4 α -null mice were generated as described previously (17). All experiments were performed with 2-month-old male mice (HNF4 α ^{flox/flox} \times albumin-Cre^{+/-} [H4LivKO] and HNF4 α ^{flox/flox} \times albumin-Cre^{-/-} [FLOX]). Mice were housed under a standard 12-h light/12-h dark cycle with ad libitum water and chow.

Affymetrix microarray analysis and quantitative real-time PCR (QRT-PCR). Total RNA for microarray analyses was extracted from three independent H4LivKO and FLOX livers with ISOGEN (Wako Pure Chemical Industries, Japan). Equivalent amounts from each animal were pooled before the preparation of biotin-labeled cRNA according to the Affymetrix GeneChip Expression Analysis Technical Manual. Hybridizations were performed with 10 μ g of fragmented cRNA in triplicate samples, on GeneChip Mouse Genome 430 2.0 arrays (Affymetrix), three for H4LivKO samples and three for FLOX samples. After washing, arrays were stained with streptavidin-phycoerythrin and image data were collected and analyzed with an Affymetrix GeneChip Scanner 3000 (Affymetrix). The GeneChip Analysis Suite software version 5.0 was used to calculate the average difference for each gene probe on the array. The average differences were normalized for each array so as to have a mean value of 100. The average change (2ⁿ-fold) is expressed relative to FLOX mice. The methods for QRT-PCR have been described previously (33, 63, 68). The sequences of all of the primer used in this study are available on request.

Dio1 assay. Dio1 activity was determined by the release of radioiodide from ¹²⁵I-labeled rT₃ according to the method of Leonard and Rosenberg (32). Mouse liver or kidney was homogenized in ice-cold homogenization buffer (0.1 M potassium phosphate at pH 7.2, 1 mM dithiothreitol, 2 mM EDTA) with a Polytron (Kinematica AG, Lucerne, Switzerland) and clarified by centrifugation at 1,000 \times g for 10 min at 4°C, and the supernatants were used for the Dio1 assay. Prior to the assay, ¹²⁵I-labeled rT₃ was purified by column chromatography with Sephadex LH-20 (GE Healthcare) on the day of the experiment. Aliquots of the supernatants (20 μ g protein for liver and 75 μ g for kidney) were incubated with rT₃ (1.0 μ M for liver and 0.5 μ M for kidney) as a substrate and ¹²⁵I-labeled rT₃ (100,000 cpm/tube) as a tracer in deiodinase buffer (0.1 M potassium phosphate at pH 7.2, 10 mM dithiothreitol, 2 mM EDTA) at 37°C for 30 min. The reaction was stopped by the addition of 100 μ l of 5% (wt/vol) bovine serum albumin, and the protein-bound iodothyronines were precipitated by adding 800 μ l of ice-cold 10% trichloroacetic acid. After centrifugation, the supernatants were applied to minicolumns packed with AG 50W-X2 resin (Bio-Rad Laboratories) and eluted in 1 ml of 10% acetic acid. The released ¹²⁵I⁻ was counted in a gamma counter (1480 WIZARD Automatic Gamma Counter; Perkin-Elmer). Background levels of deiodination were assessed under the same conditions with the addition of 1 mM PTU. The difference between incubations with and without PTU represented Dio1 activity. Reaction rates were linear with time from 5 to 60 min and with protein content in the range used. The enzymatic activity was expressed as picomoles of I⁻ released per milligram of protein per minute.

Assays for serum T₄, T₃, rT₃, and TSH concentrations. Total serum T₄ and T₃ levels were determined with the competitive enzyme-linked immunosorbent assay kits from Alpha Diagnostic International (San Antonio, TX) and Diagnostic Systems Laboratories (Webster, TX), respectively. The reference ranges for T₄ and T₃ were 1.5 to 24 μ g/dl and 50 to 750 ng/dl, respectively. Total serum rT₃ was measured by radioimmunoassay with a kit from Adaltis Italia (Rino, Italy). The reference range was 2.5 to 200 ng/dl. Serum thyroid-stimulating hormone (TSH) was measured by radioimmunoassay with the rat TSH assay kit obtained from

GE Healthcare. The reference range for TSH was 1.0 to 64 ng/ml. All assays were performed according to the manufacturers' instructions.

Construction of the promoter reporter gene for the luciferase assay. pDio1(1998) is the mouse *Dio1* promoter-luciferase reporter gene that spans positions -1998 to -1 relative to the translation initiation site. pDio1(978), pDio1(500), pDio1(380), pDio1(190), pDio1(110), pDio1(75), and pDio1(65) each contain deletion-containing constructs of the 5'-FR with the 5' end of each noted in parentheses and the same 3' endpoint at -1. pDio1(1998) was constructed by the application of PCR to mouse genomic DNA with a forward primer starting at -1998 (5'-GCTGTGTTAGAAGGACTGGTGGTCTGTCTCGT TCTTG-3') and reverse primer Oli-1R (5'-CCCAAGCTTCTCAGCACGGGG CAGAAGTGGC-3') flanked by a HindIII site. The digested PCR product was cloned into the SmaI-HindIII sites of pGL3 basic. pDio1(978), pDio1(500), pDio1(380), pDio1(190), pDio1(110), pDio1(75), and pDio1(65) were constructed in a manner identical to that used for pDio1(1998), with forward primers starting at positions -978, -500, -380, -190, -110, -75, and -65, respectively, and coupled with a common reverse primer, Oli-1R. Base substitution mutants were generated in pDio1(500) with the QuikChange II site-directed mutagenesis kit according to the manufacturer's protocol. Oligonucleotides were designated to mutate each element as follows: DR1 motif at -400, from 5'-GGATCTTCT GACT-3' to 5'-GGATCC-3'; DR1 motif at -88, from 5'-GCACCTTTGTCC A-3' to 5'-GGATCC-3'; KLF motif at -165, from 5'-CCACCC-3' to 5'-GGA TCC-3'; KLF motif at -71, from 5'-CCACCC-3' to 5'-GGATCC-3'.

Expression plasmids. Cytomegalovirus promoter-driven mammalian expression vectors for full-length and deletion mutant HNF4 α 1 that encode aa 1 to 455 and an internal deletion, Δ 359-368 [pCMV-HNF4 α 1 and pCMV-HNF4 α 1(Δ 359-368)], were constructed in pcDNA3 (Invitrogen). Cytomegalovirus promoter-driven expression vectors for full-length and deletion mutant GATA4 that encode aa 1 to 442, 1 to 303, 1 to 332, and 202 to 442 and internal deletions Δ 202-303, Δ 304-332, and Δ 202-332 constructed in pcDNA3 were described previously (58). Cytomegalovirus promoter-driven expression vectors for mutant GATA4 that encode internal deletions Δ 202-244, Δ 265-303, and C274G were constructed in pcDNA3 in an identical manner. pCMV-KLF9, a cytomegalovirus promoter-driven expression vector encoding full-length mouse KLF9, pCMV-KLF15 encoding rat KLF15, and pCMV-KLF6 encoding mouse KLF6 were described previously (68). pCMV-FLAG-KLF13 encoding mouse KLF13 containing a FLAG epitope at the N terminus was constructed by reverse transcription-PCR with total RNA from mouse liver as a template and by insertion of the full-length coding sequence into pCMV-Tag2 (Stratagene). The mammalian expression plasmid for KLF2 (pBK-LKLF) (2) was generously provided by Jerry Lingrel (University of Cincinnati College of Medicine), and the mammalian expression plasmid for KLF4 (pcDNA3-GKLF) (69) was a kind gift from Shaw-Fang Yet (Harvard Medical School). The mammalian expression plasmid for KLF5 (45) was kindly provided by Ryozi Nagai (University of Tokyo Graduate School of Medicine). pCMV β , a plasmid encoding the *Escherichia coli* β -galactosidase reference gene, was obtained from Stratagene. All mutants were confirmed by DNA sequencing.

Cell culture. Monolayers of HepG2 cells, a human hepatoma cell line; HEK293 cells, a human embryonic kidney cell line; and NMuLi (ATCC CRL1638) (46) cells, a murine normal liver epithelial cell line, were maintained in Dulbecco's modified Eagle's medium containing 100 μ g/ml streptomycin sulfate and 100 U/ml penicillin supplemented with 10% (vol/vol) fetal bovine serum. Chinese hamster ovary (CHO) cells were maintained in Ham F-12 medium containing 5% (vol/vol) fetal bovine serum. All cells were cultured in 5% CO₂ at 37°C.

Luciferase reporter assay. HEK293 and HepG2 cells were plated at a density of 1×10^5 /24-well plate on the day prior to transfection. On day 1, cells were transfected with luciferase reporter plasmid (0.1 μ g), the indicated amount of expression plasmids, and pCMV β (0.05 μ g) with Lipofectamine 2000 reagent according to the manufacturer's instructions. The total amount of DNA per well was kept constant by adding the corresponding amount of expression vector without a cDNA insert. On day 2, the cells were harvested and assayed for firefly luciferase activity and normalized to β -galactosidase activity as described previously (18, 20, 58). All experiments were performed at least three times in duplicate or triplicate, and the most representative results are shown.

ChIP assay. The chromatin immunoprecipitation (ChIP) assay was performed as previously described (18, 19, 58). Frozen mouse liver (~0.2 g) was chopped into small pieces with a mortar and pestle and cross-linked for 15 min at room temperature with formaldehyde at a final concentration of 1% (wt/vol) in phosphate-buffered saline. The samples were subsequently washed twice with phosphate-buffered saline containing protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 2.8 μ g/ml aprotinin, 10 μ g/ml leupeptin, and 5 μ g/ml pepstatin A), and then tissue samples were disaggregated with a Dounce homogenizer. After centrifugation, the pellets were resuspended in sodium dodecyl sulfate (SDS)

TABLE 1. Double-stranded oligonucleotides used for gel shift analyses

Probe	Sequence ^a
Dio1 (-95/-66) wild	5'-GTAGCCTGCACCTTTGTCCAGC TCCACCCA-3' 5'-TGGGTGGAGCTGGACAAAGGT GCAGG-3'
Dio1 (-95/-66) mutant	5'-GTAGCCTGT <u>GT</u> TCCCACTCAGC TCCACCCA-3' 5'-TGGGTGGAGCT <u>GAGTGGGAACA</u> CAGG-3'
Dio1 (-407/-379) wild	5'-GCAGCTGGGATCTTCTGACTCC TTTTTGC-3' 5'-GCAAAAAGGAGTCAGAAGATC CCAG-3'
Human CYP8B1	5'-GGCCACAGGGCAAGTCCAGG TGCTCAGAC-3' 5'-GTCTGAGCACCTGGACCTTGC CCTGTGG-3'

^a Bold and underlined base pairs represent the specific mutations in each oligonucleotide.

lysis buffer (50 mM Tris-HCl at pH 8.1, 1% SDS, 10 mM EDTA) containing protease inhibitors and sonicated to generate a 200- to 1,000-bp DNA fragment. ChIP was performed with anti-HNF4 α (IgG-H1415), anti-GATA4 (AF2606) (R&D Systems), anti-KLF9 (sc-12996) (Santa Cruz Biotechnology), or control IgG according to the manufacturer's protocol (Upstate). The primers used to amplify the proximal mouse *Dio1* promoter sequences were 5'-AGAGAGAGC TCTGTGCCCTGG-3' and 5'-GAAGTGCTCTGAGCCTGCAG-3' (positions -15 to -199 with respect to the first ATG codon). The primers for the distal mouse *Dio1* promoter sequences were 5'-GCGACAATGCCCTGATCA AGA-3' and 5'-GATTCCACATCTGTTGCTTCA-3' (positions -1769 to -1948).

Immunoprecipitation and immunoblot analysis. For protein expression, HEK 293 cells were transfected with 1 μ g of the indicated constructs by FuGENE 6 (Roche Applied Science). Cell lysates were prepared with Nonidet P-40 lysis buffer (50 mM HEPES-HCl at pH 7.4, 100 mM NaCl, 1.5 mM MgCl₂, 1% [vol/vol] Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 5 μ g/ml pepstatin A, 25 μ g/ml *N*-acetyl-leucinal-leucinal-norleucinal, 1 mM dithiothreitol) as previously described (50) and immunoprecipitated with immune monoclonal or polyclonal antibodies. The eluate was separated by denaturing SDS-polyacrylamide gel electrophoresis (PAGE) transferred to Hybond-C extra nitrocellulose filters (GE Healthcare), and immunoblotted with one of the antibodies described above.

Gel mobility shift assay. The gel mobility shift assay was performed with double-stranded probes as previously described (58). The sequences of the oligonucleotides used are listed in Table 1.

Glutathione S-transferase (GST) pull-down and mammalian two-hybrid assay. GST fusion constructs containing the full length and aa 41 to 245 of KLF9 were created in bacterial expression vector pGEX4T-2 (GE Healthcare), expressed in BL21 bacteria, and purified with the MagneGST protein purification system (Promega) as described previously (58). Purified GST or GST fusion proteins were incubated with ³⁵S-labeled GATA4 synthesized with the TNT Quick Coupled Transcription/Translation system (Promega) for 2 h at 4°C and washed three times before SDS-PAGE was carried out. For the mammalian two-hybrid assay, we used the CheckMate Mammalian Two-Hybrid System (Promega) as described previously (58). The reporter plasmid pGAL4-luc, GAL4-HNF4 α (117-465), and the full length and deletion mutant (Δ 202-332) of VP16-GATA4 were previously described (58). The various VP16-GATA4 mutants (Δ 202-244, Δ 268-303, and C274G) were created in the full-length VP16-GATA4 fusion construct by site-directed mutagenesis.

Statistical analysis. Student's *t* test was used to compare data between the control and treated groups. A value of *P* < 0.05 was considered significant.

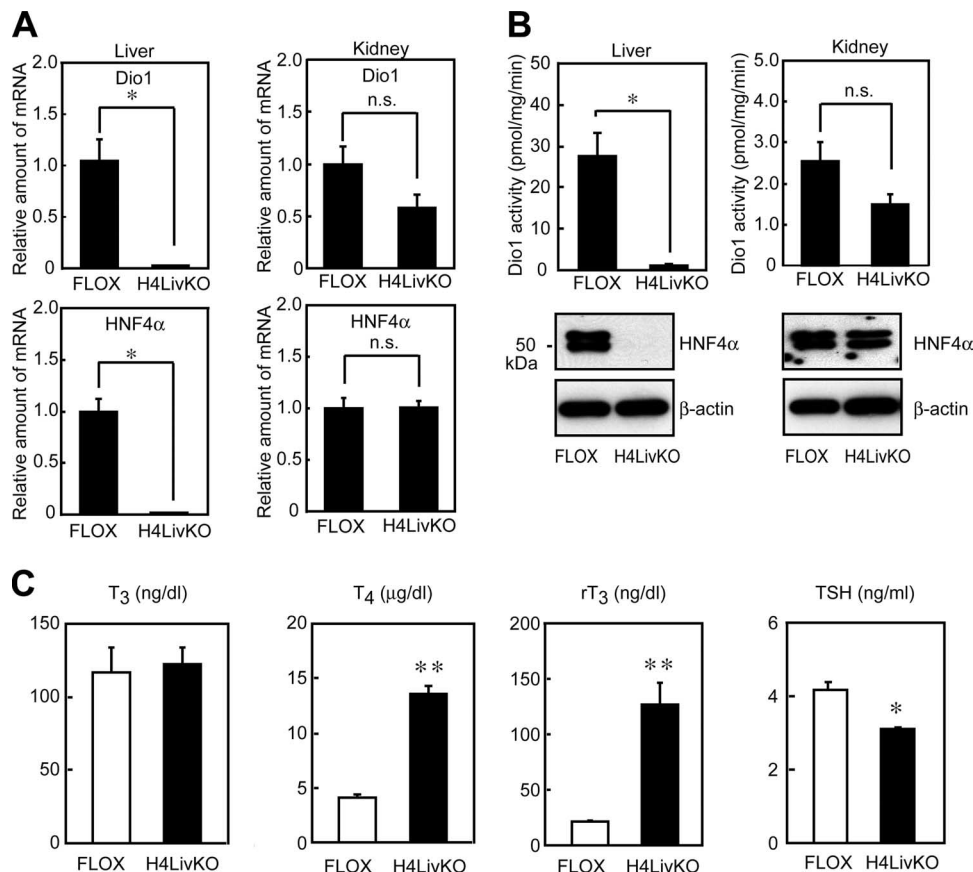


FIG. 1. *Dio1* gene expression is profoundly reduced in the livers of liver-specific HNF4 α -null mice. (A) *Dio1* mRNA levels in the livers and kidneys of control (FLOX) mice or mice lacking liver HNF4 α (H4LivKO). Total RNA from the livers and kidneys of FLOX or H4LivKO mice (17) (2-month-old male mice, $n = 4$ or 5 per group) was subjected to QRT-PCR quantification for *Dio1* and HNF4 α as a control. Cyclophilin was used as the invariant control. Values represent the amount of mRNA relative to that in the control group (FLOX), which is arbitrarily defined as 1. Each bar represents the mean \pm the standard error. *, $P < 0.01$ compared with FLOX; n.s., not significant. (B) *Dio1* activities in the livers and kidneys from FLOX and H4LivKO mice (2-month-old male mice, $n = 5$ per group) were prepared, and *Dio1* activities were determined with rT₃ as the substrate as described in Materials and Methods. Each bar represents the mean \pm the standard error. *, $P < 0.01$ (compared with FLOX); n.s., not significant (top). The tissue homogenates from each group were separately pooled, and 10- μ g aliquots of whole-cell lysate were subjected to SDS-PAGE and immunoblot analysis with anti-HNF4 α (IgG-H1415) and β -actin antibody (bottom). (C) Serum T₃, T₄, rT₃, and TSH levels in FLOX and H4LivKO mice. Serum samples from FLOX and H4LivKO mice (2-month-old male mice, $n = 6$ or 7 per group) were analyzed. Each bar represents the mean \pm the standard error. *, $P < 0.05$; **, $P < 0.01$ (compared with FLOX).

RESULTS

Expression of the *Dio1* gene is reduced in livers from liver-specific HNF4 α -null mice. To reveal an additional role for HNF4 α in the liver, the gene expression profile was examined in a liver-specific HNF4 α -null mouse. The expression of $\sim 39,000$ transcripts was measured by using Affymetrix Mouse Genome 430 2.0 arrays. To identify genes that are likely to be direct targets of HNF4 α , genes were filtered with a stringent cutoff. For a list of the genes whose expression was decreased to less than 0.05 ($2^{-4.4}$ -fold) in the livers of hepatic HNF4 α -null mice (designated H4LivKO) compared with their littermate control mice (designated FLOX), see Table S1 in the supplemental material. They include two genes encoding apolipoproteins, four genes encoding cytochromes P450, six genes encoding enzymes involved in lipid and steroid metabolism, one gene encoding an enzyme involved in amino acid metabolism, and one gene encoding an enzyme associated with glucose metabolism. Two genes had been characterized previously

as direct HNF4 α targets (23, 48) (see Table S1 in the supplemental material; denoted by the symbol *). Eight genes had been reported to be down-regulated in HNF4 α -null embryonic livers (4) (see Table S1 in the supplemental material; denoted by the symbol **). With our stringent cutoff, other potential HNF4 α target genes were identified (see Table S1 in the supplemental material; for the potential target genes with a low-stringency cutoff, see Table S2 in the supplemental material). One of these new putative HNF4 α target genes was *Dio1*, and it encodes a member of a small family of selenoenzymes and catalyzes the bioactivation of thyroid hormone.

To validate the *Dio1* array results, the levels of *Dio1* mRNA in the livers were determined by QRT-PCR. As shown in Fig. 1, the expression of *Dio1* was profoundly reduced in the livers of H4LivKO mice compared with the control group (FLOX, Fig. 1A). This reduction is due to the loss of HNF4 α expression, since the levels of *Dio1* mRNA in the kidney, where HNF4 α was still present, did not show any significant changes.

As expected, HNF4 α mRNA was present and absent in the livers of FLOX and H4LivKO mice, respectively (Fig. 1A).

Dio1 activity is reduced in the livers of liver-specific HNF4 α -null mice. To determine whether the reduced *Dio1* mRNA expression seen results in reduced enzymatic activity, *Dio1* activity was assessed in livers from FLOX and H4LivKO mice with ¹²⁵I-labeled rT₃, the preferred substrate for *Dio1* in the absence and presence of PTU. As a control, we also examined *Dio1* activity in the kidney. As shown in Fig. 1B, high levels of 5'D activity (outer-ring deiodination activity) were found in the livers of FLOX mice. In contrast, consistent with mRNA levels, 5'D activity in the livers of H4LivKO mice was markedly reduced, by more than 90%, while 5'D activities in the kidneys were comparable between FLOX and H4LivKO mice. In these assays, 5'D activity was reduced by more than 99% in the presence of PTU (data not shown), indicating that it was primarily a result of *Dio1* (27, 52). These data indicate that hepatic *Dio1* gene expression and *Dio1* activity are regulated by HNF4 α . To further confirm that *Dio1* activity was lost in the liver, serum T₄, T₃, rT₃, and TSH concentrations were assessed in FLOX and H4LivKO mice. Both T₄ and rT₃ concentrations in serum were approximately three- to fourfold higher in H4LivKO mice (Fig. 1C). In contrast, serum T₃ concentrations were comparable. The serum TSH concentration was slightly, yet significantly, reduced in H4LivKO mice (Fig. 1C). The above thyroid hormone profile was similar to that of *Dio1*KO mice (52), with the exception of the serum TSH concentration. In the *Dio1*KO mice, the serum TSH concentration was comparable to that of wild-type mice. This may be explained in part by the high serum T₄ concentration contributing to T₃ production in the pituitaries of the H4LivKO mice, where, unlike in the complete *Dio1*KO mice, expression of *Dio1* and *Dio2* is normal.

***Dio1* is a direct transcriptional target of HNF4 α .** To determine whether HNF4 α directly regulates *Dio1* expression, the *Dio1* gene promoter was examined for the presence of an HNF4 α binding site and transactivation studies were carried out. HNF4 α preferentially binds DNA as a homodimer to the HNF4 α -responsive element (HNF4 α -RE), which is composed of two nuclear receptor consensus half sites of AG(G/T)TCA organized as a direct repeat and separated by a single nucleotide (direct repeat 1, DR1). Scanning the 5'-flanking sequences of the mouse, rat, and human genes for the presence of putative DR1 motifs by a computer-assisted homology search (Genomatix) revealed a highly conserved DR1-like element (namely, DR1 motif at -88). This region is located 88 nucleotides upstream of the translation start site for *Dio1* in mice (Fig. 2A) and adjacent to a conserved GC/CA-rich sequence, a binding motif for the Sp1/KLF family transcription factor (59). In addition, another potential HNF4 α binding site was located at position -400 (DR1 motif at -400) in the mouse promoter (Fig. 2A and B).

To determine whether HNF4 α has the potential to activate the mouse *Dio1* promoter, a DNA fragment containing the 5'-FR of the mouse *Dio1* gene was subcloned into the promoterless luciferase reporter gene, pGL3 basic, and transiently transfected into HEK293 and HepG2 cells along with increasing amounts of HNF4 α expression plasmid. As shown in Fig. 2C, the expression levels of luciferase were increased in proportion to the amount of cotransfected HNF4 α expression

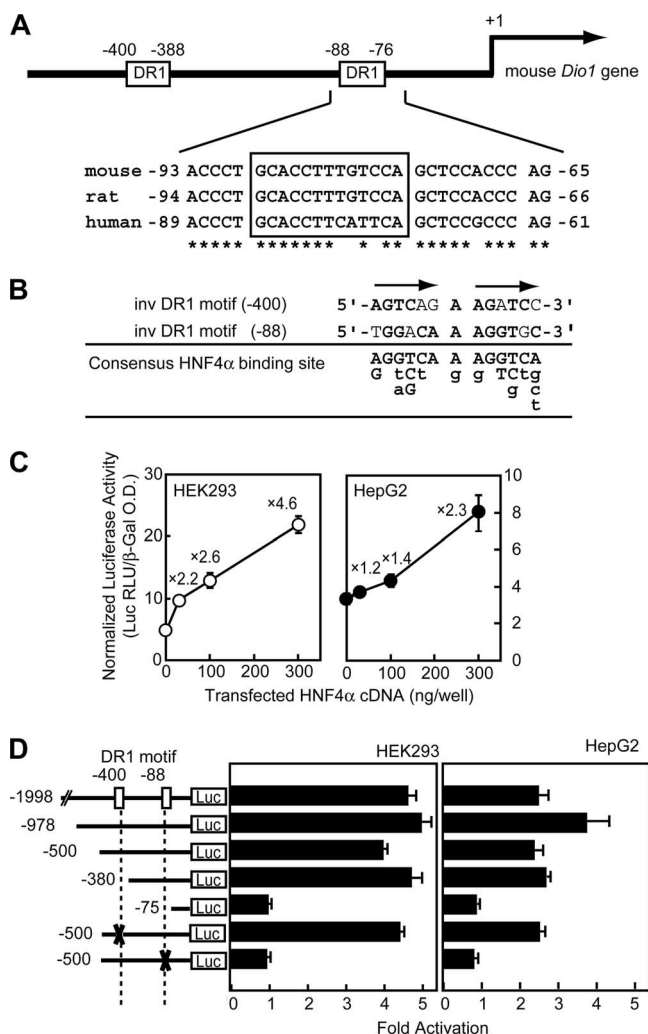


FIG. 2. *Dio1* is the direct transcriptional target of HNF4 α . (A) Schematic representation of the mouse *Dio1* promoter, illustrating two DR1 motifs (top). The DR1 motif at position -88 is conserved in the mouse, rat, and human promoters. Conserved nucleotides are indicated by asterisks (bottom). Nucleotide position +1 is assigned to the A of the ATG initiator codon. (B) Alignment of the two DR1 motifs in the mouse *Dio1* promoter. The sequences of DR1 motifs at -400, -88, and the consensus nucleotide(s) found in the HNF4 α binding sequences are aligned. Since the DR1 motifs at -400 and -88 are in the inverted orientation, the bottom strand of the DNA is presented. At the consensus site, the consensus nucleotide(s) found in the HNF4 α binding sequences is represented by capital letters; the lowercase letters point out divergences from the consensus that are represented as described in reference 54. (C) HNF4 α activates the mouse *Dio1* promoter. The indicated amounts of HNF4 α expression plasmids were transfected into either HEK293 or HepG2 cells together with pDio1(500) and incubated for 24 h, after which the cells were harvested and assayed for luciferase and β -galactosidase activities as described in Materials and Methods. The values above the open and closed circles refer to the induction levels relative to those measured in the absence of the HNF4 α expression plasmid. O.D., optical density. (D) Identification of an HNF4 α -RE in the mouse *Dio1* promoter by progressive deletion and mutation analyses. HEK293 or HepG2 cells were transfected with the indicated *Dio1*-luciferase reporter, in which each DR1 motif was deleted or mutated, together with 0.3 μ g of the HNF4 α expression plasmid and assayed for transcriptional activity. Transfection and luciferase assays were performed as described above. The relative activation level (normalized luciferase activity cotransfected with HNF4 α expression plasmid versus empty vector) is shown. (C and D) Each value represents the mean \pm the standard deviation of triplicate experiments.

plasmid (maximum, 4.6- and 2.3-fold, respectively), suggesting that *Dio1* is an HNF4 α target gene.

Identification of the HNF4 α binding site in the mouse *Dio1* promoter. To define the location of the control element that mediates the effects of HNF4 α , a series of 5' deletions were introduced into the promoter region. Deletion of the sequence from -1998 to -380 did not change the sensitivity to overexpressed HNF4 α (Fig. 2D). A further deletion to -75 resulted in complete loss of induction by HNF4 α in both HEK293 and HepG2 cells. Furthermore, in the context of pDio1(500), mutation of the DR1 motif at -88 reduced activity in HepG2 cells, indicating that this proximal HNF4 α site is crucial for activation of the *Dio1* gene, while mutation of the DR1 motif at -400 did not abolish the responsiveness to HNF4 α . It should be noted that the response to transfected HNF4 α expression plasmid was significantly higher in HEK293 than in HepG2 cells. This is likely due to the higher cellular levels of endogenous HNF4 α in HepG2 cells.

To determine whether HNF4 α is capable of binding to the DR1 motif at -88 of the mouse *Dio1* promoter, an electrophoretic mobility shift assay (EMSA) was performed with ³²P-labeled probes covering the HNF4 α binding site (double-stranded 30-mer corresponding to -95 to -66) with either nuclear extracts from HepG2 cells (Fig. 3A, lanes 1 to 8) or in vitro-translated HNF4 α (Fig. 3A, lanes 9 to 14). HepG2 nuclear extracts contain a protein that binds to the *Dio1* DR1 motif and reacts with the HNF4 α antibody (IgG-H1415; Fig. 3A, lanes 2, 6, and 8). Analyses with in vitro-translated HNF4 α produced similar results (Fig. 3A, lanes 10 and 14). In both cases, the protein-DNA complex was eliminated by the addition of an excess of unlabeled wild-type probe (Fig. 3A, lanes 3 and 11) but not by the addition of the corresponding mutant oligonucleotides (Fig. 3A, lanes 4 and 12). Also, no specific complex was detected with the labeled mutant probe and the HepG2 nuclear extract (Fig. 3A, lane 5). In contrast, ³²P-labeled probes covering the other putative DR1 motif at -400 (corresponding to -407 to -379) did not produce any specific bands under these conditions (data not shown).

To further evaluate the ability of the proximal and distal DR1 motifs (at -88 and -400, respectively) to compete for binding to a consensus HNF4 α -RE found in the human *CYP8B1* promoter (70), an EMSA was carried out. The *Dio1* probe carrying the DR1 motif at -88 was able to compete for binding to a radiolabeled band corresponding to the human *CYP8B1* promoter HNF4 α binding site (70) (Fig. 3B, lanes 7 to 9), while the *Dio1* probe carrying the DR1 motif at -400 was not (Fig. 3B, lanes 10 to 12), indicating that only the proximal DR1 motif at -88 is able to displace binding over the well-characterized HNF4 α -RE of the *CYP8B1* promoter with an efficiency similar to that of the unlabeled self probe. Taken together, these results indicate that the *Dio1* promoter has a single HNF4 α binding site that is critical for the activation of gene expression. Therefore, hereafter we refer to the DR1 motif at -88 as an HNF4 α -RE in the mouse *Dio1* promoter.

Finally, we assessed HNF4 α binding to the *Dio1* promoter in the mouse liver by a ChIP assay. After formaldehyde-based cross-linking of protein and DNA, chromatin fragmented by sonication was immunoprecipitated with anti-HNF4 α antibody or with IgG as a control, followed by PCR amplification with specific primers for HNF4 α -RE, as schematically depicted in

the upper part of Fig. 3C. As a control, a ChIP was carried out with liver extracts from H4LivKO mice. In FLOX mouse liver extracts, endogenous HNF4 α bound to the promoter of the *Dio1* gene (Fig. 3C, bottom left, compare lanes 2 and 3), while in H4LivKO liver extracts, no band was detected (Fig. 3C, bottom left, compare lanes 5 and 6). In addition, there was no enrichment of DNA corresponding to another unrelated distal region of the *Dio1* promoter (positions -1948 to -1769). The immunoblot in the bottom right part of Fig. 3C shows that HNF4 α protein levels were decreased in the H4LivKO mice (Fig. 3C, Pre IP) and that HNF4 α was efficiently captured by the immunoprecipitation procedure with anti-HNF4 α antibody (Fig. 3C, Post IP) in proportion to the overall levels (Fig. 3C, compare the gels labeled "Pre IP" for direct immunoblotting with those labeled "Post IP").

Consistent with the EMSA results showing that HNF4 α binds to HNF4 α -RE in vitro, the ChIP results revealed that HNF4 α associates with the HNF4 α -RE-containing region of the mouse *Dio1* promoter in vivo.

Regulation of the *Dio1* gene by T₃-regulated KLF9. Although the above data demonstrated that HNF4 α activates the mouse *Dio1* promoter through a single HNF4 α -RE, HNF4 α is a very weak activator of this promoter (Fig. 2; a maximum of 2.3-fold in HepG2 cells). Like most eukaryotic genes, *Dio1* is likely to be regulated by additional transcriptional mechanisms in addition to HNF4 α . In fact, *Dio1* is highly regulated by T₃ (8, 9). Studies with thyroid hormone receptor (TR) knockout mice indicate that TR β is primarily responsible for T₃-mediated *Dio1* stimulation (1). However, unlike the human promoter, canonical TREs or TRE-like elements have not been identified in the available 5'-FR of mouse and rat *Dio1* genes (9, 35, 36). We therefore tested whether its transcriptional potency might be enhanced in the presence of a thyroid hormone-regulated transcription factor, which may coactivate HNF4 α transcriptional activity of the *Dio1* promoter.

KLF9 (or BTEB1) is a member of the Sp/KLF family of transcription factors and is robustly activated by thyroid hormone T₃ treatment (15). Like *Dio1*, the regulation of this gene is specific to the TR β 1 isoform (15). KLF9 is one of 25 Sp/KLF family members that are characterized by the presence of a highly conserved carboxy (C)-terminal region containing three zinc finger domains that bind GC-rich sequence motifs in DNA and extremely divergent amino (N)-terminal regions that confer specificity and selectivity on their transcriptional activities and protein-protein interactions (26, 59). We evaluated thyroid hormone regulation of KLF9 and other KLF family members in mouse NMuLi hepatocytes. We chose the mouse line because of the differences in thyroid hormone regulation of mouse and human *Dio1* genes as mentioned in the introduction. As shown in Fig. 4A, consistent with other reports (12, 15, 39), the expression of the *KLF9* gene, but not that of other KLF family members, was increased by the treatment of normal mouse NMuLi hepatocytes with T₃. To examine whether KLF9 might act as a transcriptional activator of the *Dio1* promoter, transient cotransfection assays were carried out. Several other representative KLF proteins were chosen on the basis of structural similarity to KLF9 (30), and their effects on the *Dio1* promoter were tested. As shown in Fig. 4B, KLF9 dose dependently activated the *Dio1* promoter up to 20-fold in HepG2 cells. Closely related KLF13 also stimulated the *Dio1* pro-

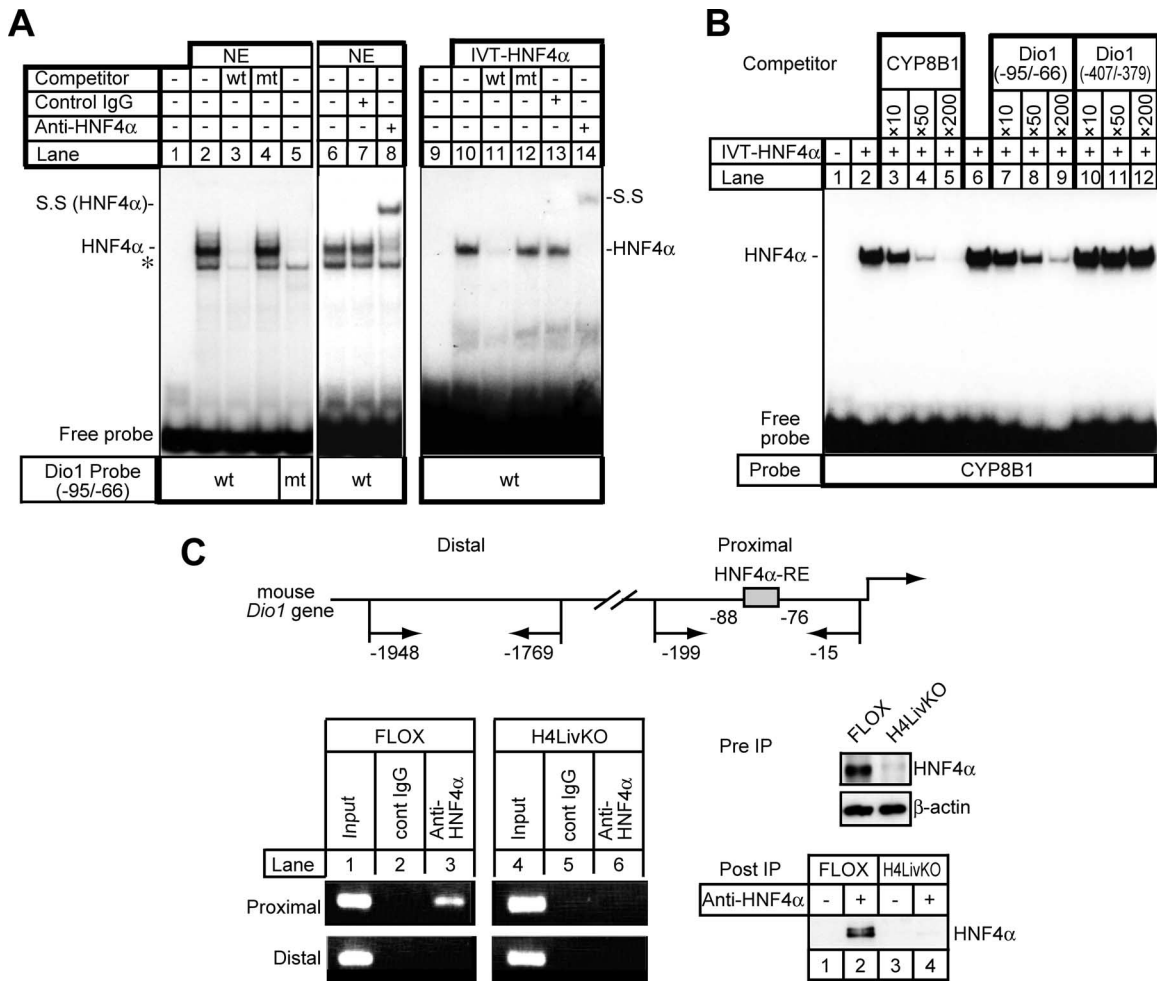


FIG. 3. Specific binding of HNF4 α to the mouse *Dio1* gene promoter. (A) EMSA. Nuclear extracts (NE) isolated from HepG2 cells (20 μ g) or in vitro-translated HNF4 α protein (IVT-HNF4 α) were incubated with a ³²P-labeled wild-type (wt) (lanes 1 to 4 and 6 to 8) or mutant (mt) (lane 5) oligonucleotide probe carrying HNF4 α -RE in the absence or presence of a 200-fold excess of the unlabeled wild-type (lanes 3 and 11) or mutant oligonucleotide (lanes 4 and 12). For the supershift assay, an anti-HNF4 α antibody, IgG-H1415 (lanes 8 and 14), was added to the binding reaction mixture. Each anti-HNF4 α antibody (2 μ g) was added to the reaction mixture 30 min after the addition of the probe and incubated for an additional 10 min. The HNF4 α -DNA complex and its supershifted complex (S.S) is indicated. Nonspecific bands are denoted by an asterisk. (B) IVT-HNF4 α was incubated with a double-stranded, radiolabeled oligonucleotide probe containing the sequence of the high-affinity HNF4 α binding site of the human *CYP8B1* promoter (70), followed by EMSA. Competition experiments were performed in the presence of a 10- to 200-fold molar excess of the unlabeled oligonucleotides for the HNF4 α binding site of the human *CYP8B1* promoter (lanes 3 to 5), the DR1 motif at -88 (Dio1 -95/-66) (lanes 7 to 9), or the DR1 motif at -400 (Dio1 -407/-379) (lanes 10 to 12) of the mouse *Dio1* promoter. (C) HNF4 α -RE of the mouse *Dio1* promoter binds to HNF4 α in the context of an intact chromatin structure as demonstrated by ChIP. Schematic representation of the 5'-FR of the mouse *Dio1* gene. Positions of primer sets (arrow) relative to the translation start site are denoted (top). Cross-linked DNA-protein complexes from either FLOX or H4LivKO mouse liver extracts were immunoprecipitated with anti-HNF4 α and control IgG, followed by PCR amplification with specific primers as schematically depicted in the diagram. Genomic DNA in the input cell lysates was used as a positive control. As a negative control, the distal region, which has no HNF4 α binding sites, was used for PCR amplification. Amplified PCR products were separated on an ethidium bromide-stained 2% agarose gel (bottom left). The presence of HNF4 α in cell lysate before immunoprecipitation (Pre IP) and the samples immunoprecipitated with anti-HNF4 α antibody (Post IP), detected by immunoblot analysis with anti-HNF4 α antibody, is shown (bottom right).

moter, albeit less efficiently than KLF9, whereas other, more distantly related, KLF proteins were ineffective (Fig. 4C). The KLF expression plasmids used in this study were characterized by the original investigators, from whom we obtained the constructs (2, 45, 69). For the expression of KLF6 and KLF13, since they contain a FLAG epitope tag, immunoblot analyses were performed with an anti-FLAG antibody, confirming that the proteins were expressed properly (data not shown).

There are two potential KLF9 binding sites within the 0.5-kb

region of the mouse *Dio1* promoter that was activated by KLF9 in the transfection assay (Fig. 4D). Both of the sequences contain the same CACCC core sequence that is specifically bound by the KLF family of transcription factors (44, 47, 68). To define the location of the control sequence that mediates the effects of KLF9, a series of 5' deletions was introduced into the promoter region. Deletion of the sequence from -500 to -190 did not change the sensitivity to overexpressed KLF9 (Fig. 4D). A deletion to -110 resulted in profound loss of

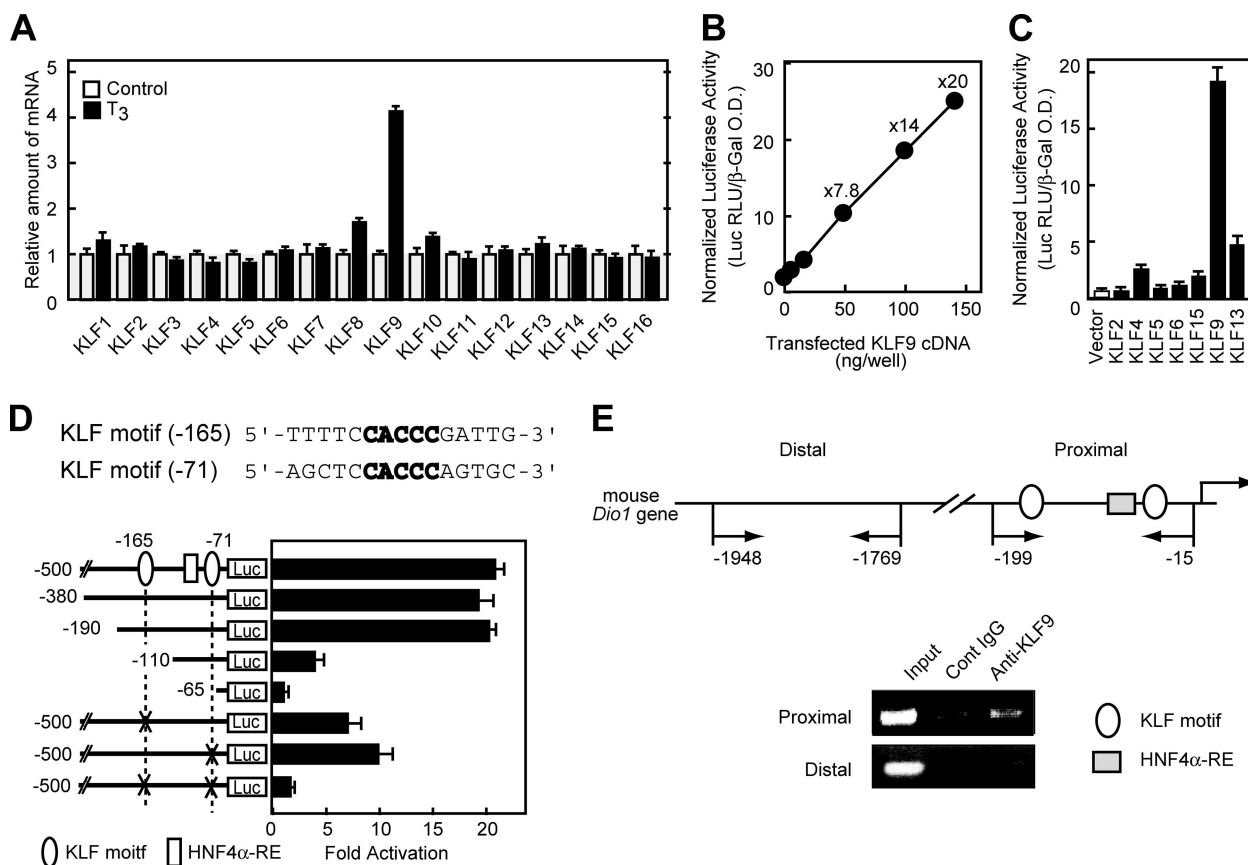


FIG. 4. T_3 -regulated KLF9 activates the mouse *Dio1* promoter. (A) Effects of T_3 treatment on various KLF mRNA levels in the normal mouse liver epithelial NMuLi cell line. On day 0, cells were set up at 5×10^5 /six-well plate in 2 ml of Dulbecco modified Eagle medium supplemented with 10% charcoal-dextran-treated fetal bovine serum (HyClone). On day 2, the cells were switched to the medium with or without 30 nM T_3 . On day 3, cells were harvested for RNA analysis by QRT-PCR. (B) KLF9 activates the mouse *Dio1* promoter. The indicated amounts of KLF9 expression plasmids were transfected into HepG2 cells together with pDio1(500) and incubated for 24 h, after which the cells were harvested and assayed for luciferase and β -galactosidase activities. The values above the closed circles refer to the level of induction relative to that found in the absence of the KLF9 expression plasmid. O.D., optical density. (C) Transactivation of the mouse *Dio1* promoter by various KLF proteins in HepG2 cells. HepG2 cells were transfected with pDio1(500) and 0.1 μ g of KLF expression plasmids. Luciferase assays were performed as described above. (D) Localization of KLF9-responsive element in the mouse *Dio1* promoter by progressive deletion and mutation analyses. HepG2 cells were transfected with the indicated *Dio1*-luciferase reporter, in which each KLF motif was deleted or mutated, together with 0.1 μ g of KLF9 expression plasmid, and assayed for transcriptional activity. The relative activation level (normalized luciferase activity cotransfected with KLF9 expression plasmid versus empty vector) is shown. (B to D) Each value represents the mean of duplicate experiments. Error bars indicate the range of the duplicates. (E) ChIP assays for KLF9 association with the *Dio1* gene promoter. Cross-linked DNA-protein complexes from mouse liver extracts were immunoprecipitated with anti-KLF9 or control IgG, followed by PCR amplification with specific primers as schematically depicted in the diagram. Genomic DNA in the input cell lysates was used as a positive control.

induction by KLF9, and further deletion to -65 resulted in almost complete loss of induction. Furthermore, in the context of pDio1(500), mutation of either the CACCC site at -165 (KLF motif at -165) or the CACCC site at -71 (KLF motif at -71) decreased the response of the promoter to overexpressed KLF9 by 40 to 60%. Furthermore, when both sites were mutated simultaneously, activation of the *Dio1* promoter was almost negligible, indicating that both KLF sites are crucial for the transactivation of the mouse *Dio1* gene by KLF9 (see also Fig. 5B below).

To determine whether endogenous KLF9 protein associates with the *Dio1* promoter in the liver, ChIP assays were performed with mouse liver extracts and an anti-KLF9 antibody. As shown in Fig. 4E, the mouse *Dio1* promoter fragment encompassing the putative KLF9 site was enriched by the anti-KLF9 antibody relative to the signal derived from control IgG.

A promoter distal fragment lacking an identifiable KLF site (positions -1948 to -1769) did not show specific enrichment. These data demonstrated that endogenous KLF9 occupies the KLF site in the chromatin-associated *Dio1* promoter in vivo and strongly suggest that KLF9 is critical for the activation of hepatic *Dio1* gene expression.

Synergistic activation of the mouse *Dio1* promoter by HNF4 α , GATA4, and KLF9. Next we examined whether KLF9 can act as a coactivator with HNF4 α . We previously reported that GATA4 is capable of binding directly to HNF4 α and coexpression of GATA4 with HNF4 α resulted in the synergistic activation of the intergenic promoter of the HNF4 α target genes for ABCG5 and ABCG8 (58). In addition, previous studies demonstrated that KLF and GATA transcription factors interact to stimulate gene expression. For example, KLF1 (erythroid KLF), the founding member of a KLF family, was

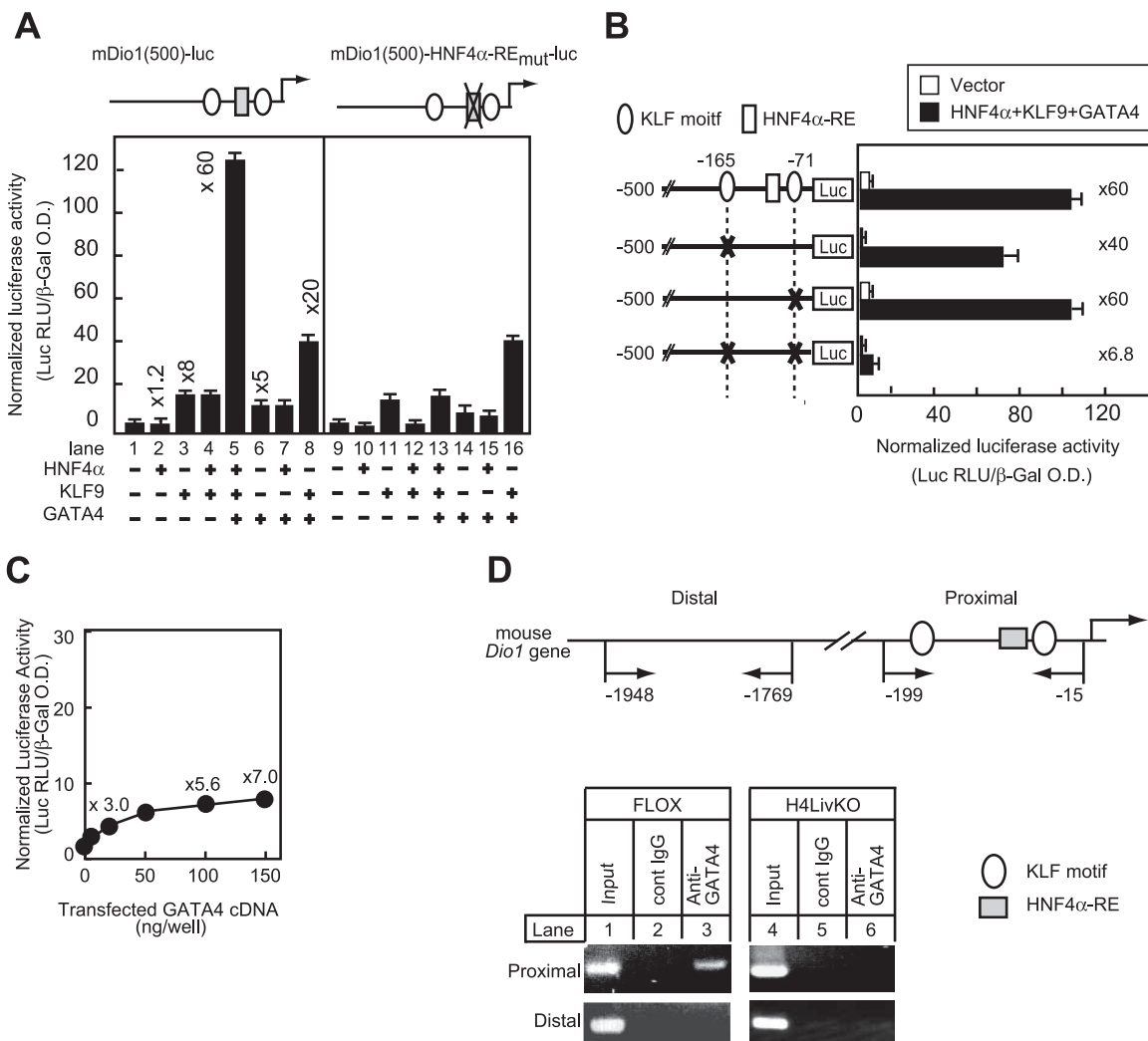


FIG. 5. KLF9 and GATA4 synergistically activate the mouse *Dio1* promoter with HNF4 α and HNF4 α -RE, and KLF motifs are required for the synergy. (A) HepG2 cells were transfected with either pDio1(500) or its mutant in which HNF4 α -RE is mutated, together with HNF4 α , KLF9, and GATA4 expression plasmids (0.05 μ g each), as indicated. The values above the bars refer to the level of induction relative to that found in the absence of the expression plasmid. O.D., optical density. (B) HepG2 cells were transfected with the indicated Dio1-luciferase reporter, in which either each KLF motif or both were mutated, together with HNF4 α , KLF9, and GATA4 expression plasmids (0.05 μ g each). The values on the right of the bars are the levels of induction relative to that found in the absence of the expression plasmid. (C) HepG2 cells were transfected with the indicated amounts of GATA4 expression plasmids together with pDio1(500). (A to C) Luciferase activities were measured and normalized to β -galactosidase activity as described in the legend to Fig. 2. Each value represents the mean of duplicate experiments. Error bars indicate the range of the duplicates. (D) ChIP analysis of the *Dio1* promoter in either FLOX or H4LivKO mouse liver with anti-GATA4 (Af2606) or control IgG, followed by PCR amplification with specific primers as schematically depicted in the diagram. Genomic DNA in the input cell lysates was used as a positive control. The results are representative of multiple different ChIP measurements.

initially isolated as a protein that functions in concert with GATA to stimulate genes required for erythropoiesis (11). KLF13, the family member most closely related to KLF9 (59), was identified as a novel GATA4-interacting protein critical for cardiac gene transcription and heart development (30). These reports suggest that KLF9 may also interact with GATA proteins and may contribute to the transcriptional regulation of KLF9 by T₃ in the promoter of the *Dio1* gene, a target gene for HNF4 α . We thus tested this hypothesis by using cotransfections and immunoprecipitation assays.

To examine synergy, we transfected a relatively small amount (50 ng) of each plasmid based on the dose dependency curve (Fig. 2C, 4B, and 5C). When the *Dio1*-luc promoter

reporter construct was cotransfected with this amount of either HNF4 α or KLF9 expression plasmid alone into HepG2 cells, luciferase activity was minimally affected or relatively modestly affected (~1.2- and 8-fold, respectively) (Fig. 5A, lanes 2 and 3), and cotransfection of the HNF4 α and KLF9 expression constructs together did not result in any significant further stimulation (Fig. 5A, lane 4). However, when both expression constructs were cotransfected with GATA4 expression constructs, there was a dramatic (60-fold) stimulation of promoter activity (Fig. 5A, lane 5). Interestingly, the coexpression of GATA4 and KLF9 resulted in a lower level of synergy: expression of either the GATA4 or the KLF9 plasmid activated the *Dio1* promoter 5-fold and 8-fold, respectively, and coexpress-

sion of GATA4 and KLF9 resulted in 20-fold activation (Fig. 5A, lanes 3, 6, and 8). The strong synergy of the three activators was dependent on the proximal HNF4 α DNA-binding site, since the mutation of HNF4 α -RE significantly reduced the level of stimulation (Fig. 5A, compare lanes 5 and 13). In this transfection assay, endogenous HNF4 α may not be sufficient to form an optimal transcription complex with transfected GATA4 and KLF9 to synergize with KLF9 and GATA4, since mutation of HNF4 α -RE did not change the activation by KLF9 and GATA4 (Fig. 5A, compare lanes 8 and 16). This strong synergy of three activators was also dependent on the KLF sites. When both motifs were mutated simultaneously, activation of the *Dio1* promoter was markedly reduced (Fig. 5B).

Further supporting a role for GATA4 in the activation of *Dio1*, titration of increasing amounts of the expression plasmid for GATA4 stimulated the *Dio1* promoter up to sevenfold in proportion to the amount of DNA transfected (Fig. 5C). ChIP assays demonstrated that endogenous GATA4 bound to the chromatin-associated *Dio1* promoter in the wild-type mouse liver, which was profoundly reduced in the livers of H4LivKO mice, indicating that endogenous GATA4 associates with the *Dio1* promoter and HNF4 α is required for the efficient recruitment of GATA4 to the *Dio1* gene (Fig. 5D).

To better understand the mechanisms and protein motifs involved in KLF9-GATA4-HNF4 α synergy, we carried out a structure-function analyses of GATA4. A series of GATA4 mutants were cotransfected with KLF9 and HNF4 α expression constructs, and *Dio1* promoter activity was measured. The GATA proteins contain two transcriptional activation domains, one at the N terminus and one at the C terminus flanking the two zinc finger DNA-binding domains (Fig. 6A). Removal of either the N-terminal activation domain by deletion of the first 201 aa (mutant 1-201) or deletion of the C-terminal activation domain by eliminating aa 333 to 442 at the C terminus (mutant 1-332) reduced but did not abrogate synergy; deletion (Δ 202-303) or mutation (C274G) of the C-terminal zinc finger domains (Cf) and deletion of the adjacent basic domain (Δ 304-332) profoundly decreased GATA transcriptional activity and abolished KLF9-GATA-HNF4 α -mediated transcription over the mouse *Dio1* promoter (Fig. 6A). These results are unlikely to be due to inefficient protein expression by the various mutants, as the only one that may be expressed at reduced levels upon transfection is the GATA4 variant with the Δ 202-332 mutation (Fig. 6D).

KLF9 interacts functionally and physically with GATA4. To determine whether the modulation of KLF9 activity by GATA4 requires direct interaction between the proteins, coimmunoprecipitation assays were performed. HEK293 cells were transfected with FLAG-tagged KLF9 and GATA4 expression vectors, immunoprecipitated with either anti-GATA4 or anti-Flag (anti-KLF9) antibody, and subjected to SDS-PAGE and immunoblotting with either anti-GATA4 or anti-Flag antibody. As shown in Fig. 6B, Flag-KLF9 and GATA4 were coprecipitated with anti-GATA4 and anti-Flag antibodies, respectively, indicating that the two proteins are present as a complex in vivo.

To determine the region of GATA4 required for its interaction with KLF9, we tested the abilities of various GATA4 mutants to interact with KLF9. These experiments demonstrated that the N-terminal region of GATA4 (aa 1 to 201) was

not required for interaction with KLF9 proteins since even deletion of the entire N-terminal transactivation domain (deletion of aa 1 to 201 [Δ 1-201]) did not hinder protein interaction with KLF9 (Fig. 6C). GATA4(Δ 1-201) appeared to be recognized by the anti-GATA4 antibody less efficiently than the full-length GATA protein in the immunoprecipitation (Fig. 6C). Nonetheless, KLF9 was associated with both the wild type and GATA4(Δ 1-201) at proportional levels (compare lanes 2 and 3). Moreover, as shown in Fig. 6D, deletion of either or both zinc fingers did not compromise the ability to interact with KLF9 (Δ 202-303, Δ 202-244, and Δ 268-303; lanes 4, 7, and 8, respectively). In contrast, deletions of the basic region sequence following the Cf of GATA4(Δ 304-332) resulted in a loss of interaction with KLF9 (lane 5). Consistently, deletion mutants that lack this domain, i.e., mutants 1-303 (lane 2) and Δ 202-332 (lane 6), hindered the protein-protein interaction. These results are unlikely to be due to a failure to accumulate in the nucleus, as the nuclear fraction showed that the Δ 304-332 mutant was present in the nucleus of transfected cells. Here, the integrity of the subcellular fractionation was confirmed by detection of the nucleoporin protein in the nuclear extract fraction (Fig. 6E).

With a GST pull-down assay, we next determined whether KLF9 directly binds to GATA4. Full-length KLF9 and a deletion mutant that lacks 40 aa at the N terminus of KLF9 were expressed in *Escherichia coli* as fusion proteins with GST, a series of ³⁵S-labeled GATA4 constructs were synthesized in vitro, and GST pull-down assays were performed. The data in Fig. 6F show that deletion of aa 304 to 332 of GATA4 abolished the interaction with full-length KLF9 and the first 40 aa of KLF9 are not required for binding to GATA4.

Functional interaction between the Cf of GATA4 and AF-2 of HNF4 α . By using two-hybrid and GST pull-down assays, we previously demonstrated a physical interaction between GATA4 and HNF4 α through two zinc fingers in GATA4 (58). By making smaller deletions in the present study, we further deduced that the Cf is required for the interaction in a mammalian two-hybrid assay (Fig. 7A). The deletion of aa 268 to 303 or the replacement of cysteine 274 with glycine in the Cf with an intact N-terminal zinc finger (Nf) resulted in the loss of stimulation of Gal4 reporter gene expression. The loss of stimulation was not observed with the deletion of aa 202 to 244 in the Nf. We also previously demonstrated that HNF4 α interacts with GATA4 through its AF-2 motif by mammalian two-hybrid and GST pull-down assays (58). The crucial role of AF-2 for HNF4 α in the physical interaction was further supported by coimmunoprecipitation assays in this study (Fig. 7B). It should be noted that deletion of aa 268 to 303 and the C274-to-G mutation of GATA4 also abrogated synergistic activation with KLF9 and HNF4 α (Fig. 6A) and the deletion of the AF-2 domain of HNF4 α abolished the synergistic activation (Fig. 7C, compare lanes 6, 7, and 8). Thus, the domains critical for protein-protein interaction were required for synergistic activation of the *Dio1* promoter in the transfection assays.

DISCUSSION

HNF4 α plays a central role in the regulation of the genes preferentially expressed in the liver (54). In the present study,

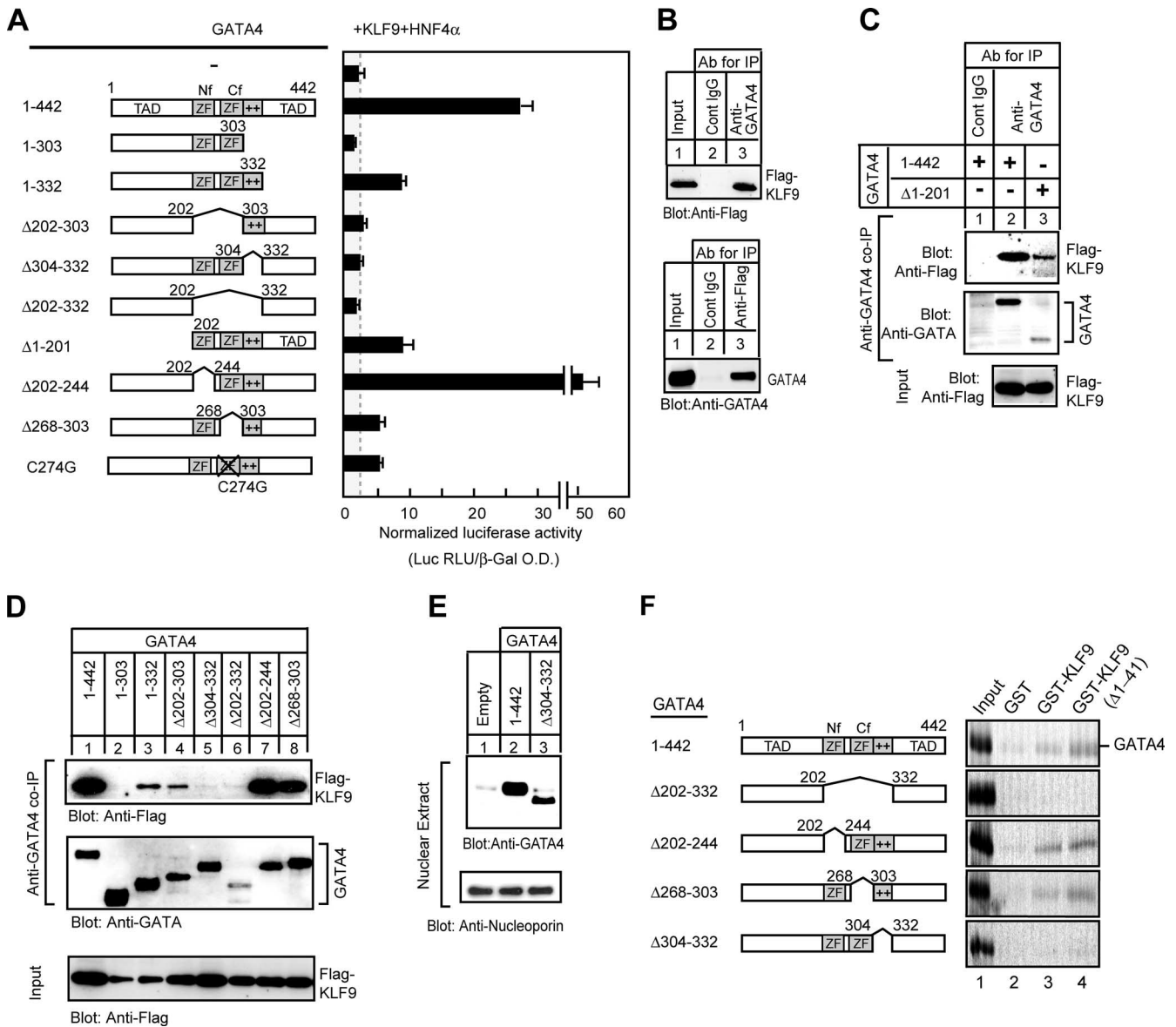


FIG. 6. KLF9 interacts functionally and physically with the basic domain adjacent to the Cf of GATA4. (A) Schematic diagram of various GATA deletion mutants and the ability to stimulate the mouse *Dio1* promoter in association with KLF9 and HNF4 α as evaluated by luciferase reporter assay. TAD, transcriptional activation domain. The data shown are the mean of duplicate experiments. Error bars indicate the range of the duplicates. (B) Interaction of GATA4 and KLF9 in transfected HEK293 cells. HEK293 cells were transfected with expression plasmids for GATA4 along with FLAG-tagged KLF9. Cells were harvested, and whole-cell lysate were immunoprecipitated with polyclonal anti-GATA4 (AF2606; R&D Systems) or control IgG (top) or monoclonal anti-FLAG M2 or control IgG (bottom) as described in Materials and Methods. The pellets from immunoprecipitation were subjected to SDS-PAGE and immunoblotted with anti-FLAG (top) or anti-GATA antibody (bottom). The expression of GATA4 or KLF9 was confirmed by immunoblot analysis of 10 μ g of whole-cell lysate (Input) with anti-FLAG or anti-GATA4 antibody (AF2606), respectively. Ab, antibody; IP, immunoprecipitation; Cont, control. (C and D) Mapping of the region of GATA4 required for the interaction with KLF9 by coimmunoprecipitation analyses. HEK293 cells expressing FLAG-KLF9 and various GATA4 deletion mutants were immunoprecipitated with polyclonal antibody directed against the C-terminal part of GATA4 (aa 328 to 439) (sc-9053; Santa Cruz Biotechnology) (C) or the N-terminal part of GATA4 (aa 27 to 211) (AF2606) (D) as described for panel B. To detect KLF9 in the protein complex, peroxidase-conjugated anti-FLAG M2 antibody was used, and to detect GATA4, monoclonal anti-GATA4 (IgG-H2429) (for panel C) and polyclonal GATA4 (AF2606) antibodies (for panel D) were used for the immunoblot analyses. (E) Nuclear fractions were prepared from lysates of HEK293 cells expressing full-length (aa 1 to 442) or mutant (Δ 304-332) GATA4. The presence of GATA4 in these subcellular fractions was detected by immunoblotting with an anti-GATA4 antibody. The nuclear fraction was confirmed by detection of the nuclear protein nucleoporin. (F) GST pull-down assays were performed with various ³⁵S-labeled GATA4 mutants in the presence of GST or GST fusions containing either the full length (aa 1 to 244) or the deletion mutant form of KLF9 [KLF9(Δ 1-41)] immobilized on glutathione beads. After washing, specifically bound proteins were eluted, separated by SDS-PAGE, and detected by autoradiography. The input samples contain 1% of the material added to each GST assay. ZF, zinc finger.

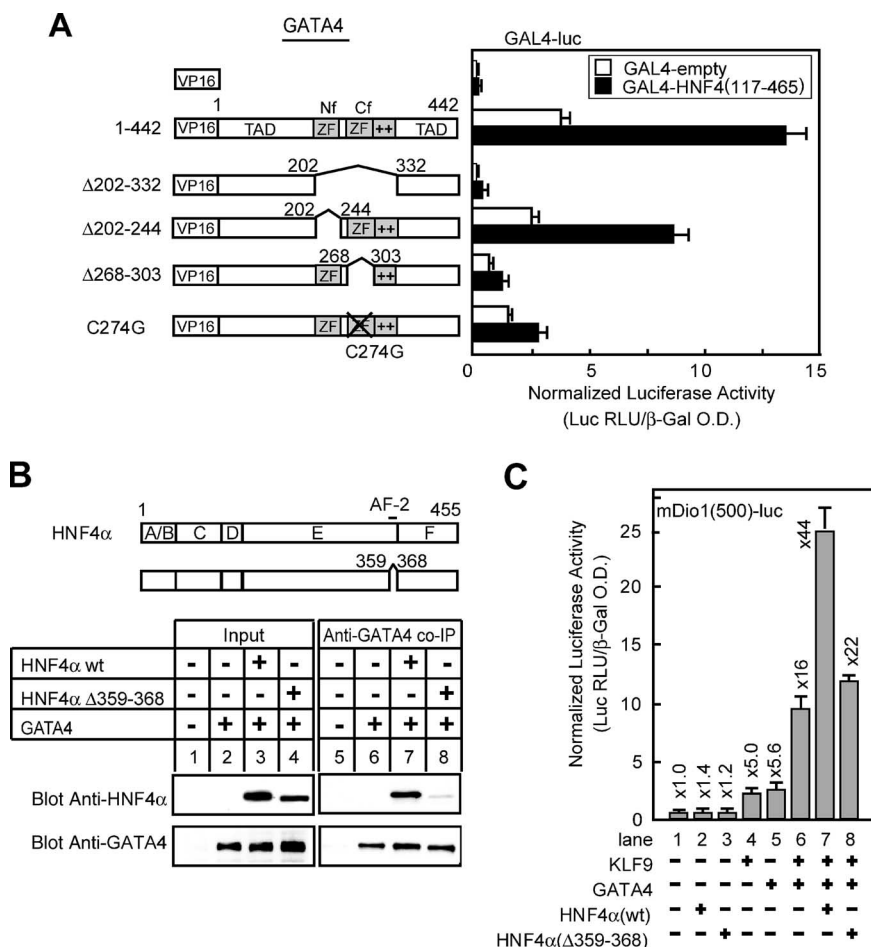


FIG. 7. The AF-2 domain of HNF4α and the Cf of GATA4 are required for both their association and the synergistic activation of the mouse *Dio1* promoter with KLF9. (A) The Cf of GATA4 is required for its association with HNF4α, as demonstrated by mammalian two-hybrid assay. CHO cells were transfected with the reporter vector pGAL4-luc (0.15 μg) and hybrid constructs or empty plasmids (0.015 μg each) indicated. The values represent the mean luciferase activity of duplicate experiments. Error bars indicate the range of the duplicates. β-Galactosidase activity was used as an internal control. ZF, zinc finger; O.D., optical density. (B) Schematic diagram of the AF-2 deletion mutant [HNF4α(Δ359-368)] and the ability to bind to GATA4, as evaluated by coimmunoprecipitation analyses (bottom). Letters A to F in the diagram indicate conventional nuclear receptor domains. AF-2, activation function 2. Anti-HNF4α antibody (IgG-H1415) was used to detect HNF4α in protein complexes immunoprecipitated (IP) with an anti-GATA4 antibody (AF2606) from lysates of HEK293 cells expressing wild-type (wt) or AF-2 deletion mutant HNF4α along with GATA4. (C) Cotransfections were carried out with HepG2 cells with pDio1(500) and 0.05 μg of the wild type (wt) or the AF-2 deletion mutant (Δ359-368) of HNF4α together with either 0.05 μg of KLF9 or GATA4 expression plasmid or both together, as indicated. The values above the bars refer to the level of induction relative that found in the absence of the expression plasmid. The values represent the mean luciferase activity of duplicate experiments. Error bars indicate the range of the duplicates.

by using liver-specific HNF4α-null mice made by using the Cre-loxP system, we found that HNF4α is essential for thyroid hormone metabolism in the liver. Expression of the *Dio1* gene and the 5'-deiodination activity of Dio1 were markedly reduced in the livers of liver-specific HNF4α-null mice. The 5'-FR of the mouse *Dio1* gene contains sufficient elements to control Dio1 expression in HepG2 hepatoblastoma cells. Indeed, there are two potential HNF4α-binding sites in the mouse promoter, and results obtained with mutated constructs revealed the proximal HNF4α-binding site be the crucial site. We previously found that *Dio1* gene expression is reduced in HepG2 cells when HNF4α expression is knocked down by RNA interference (Table 2 in reference 58). The Dio1 activity in HepG2 cells was also reduced by knockdown of HNF4α, and HNF4α binds to the endogenous human *Dio1* promoter, indi-

cating that HNF4α is also crucial for human *Dio1* promoter activity (see Fig. S1 in the supplemental material).

Currently, four mouse models have been reported with various deficiencies in the deiodinases: mice with a targeted disruption of the *Dio1* gene (Dio1KO) (52); mice with a targeted disruption of the *Dio2* gene (Dio2KO mice) (51); C3H mice, which have genetically low levels of Dio1 (7, 53); and Dio2KO mice backcrossed into a C3H background (13). Each of these animals has a normal serum T₃ concentration and an increased serum T₄ concentration (8). Consistent with these findings, H4LivKO mice also exhibit a normal serum T₃ concentration and an increased serum T₄ concentration. Clearly, the potent compensatory mechanisms for maintaining optimum levels of serum T₃ function efficiently. One difference between H4LivKO and Dio1KO mice was in the serum TSH concen-

tration, i.e., reduced in H4LivKO mice and unchanged in Dio1KO mice. One explanation for this finding is that much (threefold) higher levels of T₄ in H4LivKO mice may lead to the suppression of TSH gene expression in the pituitary. Expression of the TSH gene in the pituitary is negatively regulated by local T₃ that is generated from T₄ by Dio2 in the pituitary, which is critically important in the feedback regulation of TSH secretion (9, 51). Because H4LivKO mice only have reduced Dio1 in the liver, the pituitary expression which is important for TSH regulation is unaffected, resulting in local T₃ for suppression of TSH.

Physiological regulation of Dio1 expression likely requires additional regulatory proteins in addition to HNF4 α . In the process of searching for proteins that act with HNF4 α to coregulate *Dio1*, we found that KLF9 could activate the *Dio1* promoter very efficiently through a CACCC sequence that lies both distal and proximal to the HNF4 α -RE identified here. Activation of the mouse *Dio1* promoter by overexpressed KLF9 does not absolutely require HNF4 α since KLF9 itself could efficiently activate the mouse *Dio1* promoter harboring the mutated HNF4 α -RE. Thus, it is possible that KLF9 may play a role in the expression of the *Dio1* gene in the pituitary and the thyroid gland, where KLF9 is expressed but HNF4 α is absent (see the gene expression array database of our laboratory at http://www.lsbm.org/site_e/).

However, interestingly, HNF4 α and KLF9 dramatically activate the mouse *Dio1* promoter, which is even more active when GATA4 is also expressed. We propose that GATA4 serves as a coactivator of HNF4 α and KLF9 and that all three proteins are required for maximal activation of hepatic *Dio1* gene expression. This is due to the formation of protein interactions among KLF9, GATA4, and HNF4 α . The mutation of their interaction domain or mutation of the DNA-binding site(s) of HNF4 α or KLF9 diminished the synergy of the three proteins. KLF9 contacts the basic region of GATA4 (aa residues 304 to 332), which follows the Cf. This domain is critical for KLF9 interaction but not for nuclear localization. With the exception of CEBP α (65), which also contacts the basic region following the Cf of GATA proteins, most protein-protein interactions by the GATA factors involve either N-terminal or C-terminal activation domains (reviewed in reference 40). Moreover, KLF proteins, including KLF9, associate with coactivators/corepressors such as CBP/p300, PCAF, and ctBP2 (10, 57). As GATA factors also interact physically with CBP/p300, corecruitment of these coactivators by the GATA and KLF proteins may be the mechanism underlying transcriptional cooperativity. Together with the reported interaction of KLF1 and GATA1 in erythroid cells (11) and KLF13 and GATA4 in the heart (30), our findings suggest that GATA-KLF interaction may be relevant to transcriptional regulation in a broader array of other cell types and tissues.

We previously found, by using transfection and GST pull-down assays, that GATA4 and GATA6 can directly bind to HNF4 α , leading to synergistic activation by HNF4 α and GATA4 (58). We have recently succeeded in isolating the endogenous GATA4 and HNF4 α protein complex in HepG2 cells by means of targeted proteomic analysis by mass spectrometry (K. Daigo et al., submitted for publication). In addition, ChIP assays revealed that the coexistence of native

GATA4 protein and HNF4 α on the chromatin-associated *Dio1* promoter in the mouse liver.

Recent papers reported that the carboxy zinc finger of GATA2 directly binds to the DNA-binding domain of TR β . This GATA2-TR β protein complex is essential for T₃-regulated expression of the *TSH* gene (38). In this case, TR β does not bind the DNA sequence directly but interacts with GATA2 via its DNA-binding domain. A number of studies have shown that Dio1 mRNA and activity are regulated by thyroid hormone (reviewed in references 8 and 9). Additionally, the human *Dio1* promoter contains thyroid hormone response-retinoic acid response elements that mediate TR activation of the human *Dio1* gene (66). In rodents, a TRE has not been identified in the 5'-FR of the *Dio1* promoter and the precise mechanism of T₃-mediated induction of *Dio1* gene expression is not clearly understood. Maia et al. reported that the response of the *Dio1* gene to T₃ in FRTL5 cells and in a rat pituitary cell line is due to transcriptional activation and is not blocked by cycloheximide, indicating that this is a direct effect of T₃ not requiring the synthesis of an intermediate protein (37). If this is the case, it is tempting to speculate that TR β is recruited to the GATA4-HNF4 α complex on the *Dio1* promoter, and upon T₃ binding, it may regulate *Dio1* gene transcription through a non-DNA-binding mechanism similar to GATA2-TR β in *TSH* gene regulation. On the basis of our results, it is likely that at least part of the T₃ regulation may be indirect through that T₃-regulated KLF9 expression which would contribute to the KLF9, HNF4 α , and GATA4 synergy revealed by the present studies.

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