Functional Characterization of Evolutionarily Conserved DNA Regions in Forkhead Box F1 Gene Locus*

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The Forkhead Box f1 (Foxf1) transcription factor (previously known as HFH-8 or Freac-1) is expressed in the septum transversum and splanchnic (visceral) mesoderm and is required for proper development of gut-derived organs. Sequence comparisons of mouse and human Foxf1 genes have revealed highly conserved DNA sequences located within the −5.3-kb Foxf1 promoter region and the 400-nucleotide regulatory element located 1 kb 3′ to the Foxf1 gene (3′ RE). To examine their transcriptional activity during mouse embryonic development, we generated transgenic mice in which the expression of the β-galactosidase transgene was controlled by the −2.7-kb Foxf1 promoter region, the −5.3-kb Foxf1 promoter region, or the −5.3-kb Foxf1 promoter region fused to the 3′ RE. The −5.3-kb Foxf1 promoter sequences induced appropriate transgene expression in the midgut and developing intestine, whereas the −2.7-kb Foxf1 promoter region was transcriptionally inactive. Addition of 3′ RE to the −5.3-kb Foxf1 promoter restored proper transgene expression in the foregut, liver, and lung mesenchyme and prevented ectopic transgene expression in the developing nervous system. Cotransfection studies demonstrated that FoxA2 protein bound to the 3′ RE region (+43506/+4529 bp) and was sufficient to inhibit expression of the −5.3-kb Foxf1 promoter. Furthermore, C/EBPβ and HNF-6 proteins bound to the 3′ RE region (+4647/+4694 bp) and provided synergistic transcriptional activation of the −5.3-kb Foxf1 promoter in cotransfection assays. These studies demonstrated that the conserved Foxf1 3′ RE region is essential for proper tissue-specific regulation of the Foxf1 promoter region during mouse embryogenesis.

Embryonic development of gut-derived organs is regulated by distinct signaling pathways, which stimulate expression of mesenchymal or endoderm-specific transcription factors. These in turn bind cooperatively to distinct promoter regions and activate target gene expression (1–3). Dynamic changes in gene expression during embryonic development are critical to mediate proper organ morphogenesis, which involves extensive cellular proliferation, migration, and establishment of appropriate positioning of epithelial cells with developing mesenchyme. Understanding the regulation of tissue-specific transcription factors will provide insight regarding organ morphogenesis during embryonic development.

The Forkhead Box (Fox) proteins belong to an extensive family of transcription factors, which share homology in the winged helix/Forkhead DNA binding domain (4, 5). Fox proteins play important roles in regulating the transcription of genes involved in cellular proliferation (6–10), differentiation (1, 2, 11–13), metabolic homeostasis (14, 15), and development of cancer (16–18). The expression of the mesenchymal-specific Fox1 (HFH-8 or Freac-1) gene initiates during gastrulation at 6.5 days post-coitum (dpc)§ in extraembryonic mesoderm, allantois, and lateral mesoderm (19, 20). Foxf1 expression continues in splanchnic (visceral) mesoderm and septum transversum mesenchyme, which are critical for the mesenchymal-epithelial induction of gut-derived organs such as the liver, gallbladder, lung, stomach, and intestine (19, 21, 22). Foxf1 is also expressed in hepatic stellate cells, capillary endothelial and peribronchial smooth muscle cells of the lung, and mesenchymal cells of the gallbladder and intestine (21, 23, 24).

Genetic studies demonstrated that Foxf1−/− embryos die by 8.5 dpc due to defects in extraembryonic mesoderm development (20, 24). Foxf1 haploinsufficiency is associated with a variety of developmental abnormalities in the lung, gallbladder, esophagus, and trachea, suggesting that Foxf1 regulates mesenchymal-epithelial signaling during organ morphogenesis (21, 24–26). Approximately 55% of the Foxf1+/− newborn mice, which have only 20% of the pulmonary wild type levels of Foxf1 mRNA (low Foxf1+/−), exhibited defects in the development of peripheral lung sacs and microvasculature, severe fusions of the right lung lobes, and pulmonary hemorrhage causing perinatal lethality (24–26). These lung defects were associated with diminished expression of Flik-1, Pecam-1, Bmp4, Notch-2, and lung Kruppel-like factor, as well as delayed expression of Fgfl0, implicating the Foxf1 gene in regulation of branching lung morphogenesis and vasculogenesis (24, 25). Most interestingly, 40% of the newborn Foxf1+/− mice displayed compensatory increases in pulmonary levels of Foxf1 mRNA (high Foxf1+/− mice). High Foxf1+/− mice survived and exhibited normal development of the peripheral lung microvasculature (24) but died from pulmonary hemorrhage in response to lung injury, suggesting that Foxf1 is essential for lung repair (27). Furthermore, Foxf1 is expressed in hepatic stellate cells, and haploinsufficiency of the Foxf1 gene caused defective stellate cell activation during liver regeneration (23).

Previous studies revealed that the Foxf1 promoter is highly GC-rich and is regulated by DNA methylation (28). Cotransfection assays demonstrated that the −1-kb Foxf1 upstream sequences are sufficient to drive the luciferase reporter construct in distinct mouse cell lines (28). Despite growing interest in the Foxf1 gene as a very important transcriptional regulator in developing mesoderm, the in vivo regulation of the

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2 The abbreviations used are: dpc, days post-coitum; Foxf1, Forkhead Box f1; β-gal, β-galactosidase gene; Foxf1 3′ RE, 3′ Foxf1 regulatory element; LUC, luciferase; Foxf1−/−, Foxf1 heterozygous null allele; EMSA, electromophoretic mobility shift assay; HNF6, hepatocyte nuclear factor 6; bZIP, basic leucine zipper; C/EBP; CCATF enhancer binding protein J1; FoxA, Forkhead box A; CMV, cytomegalovirus; TTR, transthyretin.
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*Foxf1* promoter remains to be characterized. To investigate *Foxf1* regulatory regions, we have generated transgenic mice that use β-galactosidase (β-gal) reporter transgene driven by either the −2.7-kb *Foxf1* promoter, the −5.3-kb *Foxf1* promoter, or the −5.3-kb Foxf1 region fused to 3′ *Foxf1* conserved regulatory element (3′RE), which is located approximately 1 kb 3′ to the mouse *Foxf1* gene. Although the −2.7-kb Foxf1 region was insufficient to drive β-gal expression in vivo, the −5.3-kb *Foxf1* promoter induced transgenic β-gal expression in the mesenchyme of midgut and developing intestine. Addition of the 3′RE to the −5.3-kb *Foxf1* promoter restored proper β-gal transgene expression in the foregut mesoderm and in the mesenchyme of liver and lung, and inhibited ectopic expression of the −5.3-kb *Foxf1* transgene in a developing brain and spinal cord. Our results suggest that the Foxf1 3′RE is essential for mesenchyme-specific *Foxf1* promoter activity in developing embryos.

**MATERIALS AND METHODS**

*Generation of Foxf1 Transgenic Mice*—The −2.7-kb *Foxf1* or −5.3-kb *Foxf1* upstream promoter sequences were fused in-frame with a nuclear localizing β-galactosidase (β-gal) gene and then cloned into an Xhol site of the pBS plasmid (Stratagene). The 3′ Foxf1 regulatory element (+4400 to +4800 bp) was generated by PCR amplification from mouse genomic DNA and cloned into an Xhol site 5′ to the −5.3-kb Foxf1-β-gal construct. The −2.7-kb Foxf1-β-gal, −5.3-kb Foxf1-β-gal, and −5.3-kb Foxf1 + 3′RE-β-gal DNA constructs were injected into pronuclei of FVB/N mouse eggs. The fertilized mouse eggs were transferred to surrogate mothers by the University of Illinois at Chicago Transgenic Mouse Facility. Transgenic mice were identified by PCR analysis of the mouse tail DNA using primers specific to the β-gal gene as described previously (24). Four distinct −5.3-kb Foxf1-β-gal mouse lines, three −2.7-kb Foxf1-β-gal mouse lines, and two −5.3-kb Foxf1 + 3′RE-β-gal mouse lines were established and analyzed for β-gal transgene activity.

We described previously the generation of heterozygous *Foxf1* knock-in mice (*Foxf1*/+−), in which the *Foxf1* winged helix DNA binding domain was replaced by an in-frame insertion of a nuclear-localizing β-gal gene. These *Foxf1*/+− mice were bred for six generations into the Black Swiss mouse genetic background (24). Expression of the β-gal gene in *Foxf1*/+− mice was under the control of genomic *Foxf1* regulatory sequences, thus allowing the use of β-gal enzyme staining for visualizing *Foxf1*-expressing cells. *Foxf1*/+− or Foxf1-β-gal transgenic male mice were mated with wild type female mice to generate mouse embryos at various days of gestation. Embryonic tail DNA was used for genotyping by PCR analysis as described previously (24).

*Immunohistochemical and β-Galactosidase Staining*—To visualize β-gal-expressing cells, mouse embryos and adult tissue were stained for β-gal enzyme activity with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) substrate for 16 h at 30 °C and then paraffin-embedded, sectioned, and counterstained with nuclear fast red as described previously (24). For colocalization studies, the −5.3-kb Foxf1 + 3′RE 15.5 dpc transgenic livers were dissected, stained for β-gal enzyme activity, and then stained with desmin antibodies (clone DE-U-10; Sigma) as described (23). The cellular expression pattern of the desmin protein was visualized by using secondary antibody conjugated with biotin followed by avidin-horseradish peroxidase complex and 3′,3′-diaminobenzidine substrate (all from Vector Laboratories) as described (29). Colocalization studies were also performed using β-gal and biotinylated isoleucin B4 following by avidin-horseradish peroxidase complex and 3′,3′-diaminobenzidine substrate (Vector Laboratories) as described previously (24).

HNF-6 (30) or C/EBPβ antibody (C-19, Santa Cruz Biotechnology) was used at 1:100 dilution and visualized by using biotinylated anti-rabbit antibody, avidin-alkaline phosphatase complex, and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate (all from Vector Laboratories). Nuclear fast red (Vector Laboratories) was used as a counterstain.

*Cotransfection Studies and Electrophoretic Mobility Shift Assays*—For transient transfection experiments, U2OS, HepG2, or HMEC cells were plated in 6-well plates and transfected with 1.6 μg of the −5.3-kb Foxf1-luciferase (LUC), −5.3-kb Foxf1 + 3′RE-LUC, or −5.3-kb Foxf1 + 3′RE Δ = +4641/+4752 bp mutant-LUC reporter constructs using FuGENE 6 reagent (Roche Applied Science) according to the manufacturer’s protocol. The following primers were used for PCR amplification of the 3′RE and 3′RE Δ = +4641/+4752 bp mutant: forward primer, 5′-ccactgagaaacaaataataag, and reverse primers, 5′-ctc-cccgcctccgcttccg and 5′-aagaatatccctcgccagca.

In separate experiments, these LUC reporter plasmids were cotransfected with 250 ng of CMV expression vectors containing the following transcription factors: FoxA2, HNF-6, C/EBPβ, Nkx2.5, GATA-4, or a combination of them. We also included 30 ng of CMV-Runx1 luciferase reporter plasmid as an internal control to normalize transfection efficiency. Thirty six hours post-transfection, cells were prepared for dual luciferase assays (Promega). Luciferase activity was normalized to a CMV-empty vector and calculated as a fold induction compared with pGL3Basic-LUC activity. Experiments were performed at least three times in triplicate, and the mean ± S.D. was determined.

For electrophoretic mobility shift assays (EMSA), U2OS cells (100-mm plate) were either left untransfected or transiently transfected with 15 μg of CMV-FoxA1, CMV-FoxA2, CMV-HNF6, or CMV-C/EBPβ expression vectors using FuGENE 6. Nuclear protein extracts were prepared 24 h after transfection as described previously (19). EMSAs were performed with 5′ end radioactively labeled double-stranded oligonucleotides and 5 μg of nuclear extracts that were prepared from either untransfected or transfected U2OS cells using binding reaction conditions as described previously (19, 31). We used the following double-stranded oligonucleotides made to the Foxf1 3′RE: +4506/+4529 bp 5′-tagggataacaaataactacg and +4647/+4694 bp 5′-tcgctttcatcataaatcttactacagttcgccagcagaatg. For DNA competitions in EMSA, we added 500-fold molar excess of cold competitor double-stranded oligonucleotide or nonspecific SP1 oligonucleotide to the binding reaction as described previously (6, 23, 30).

*Infection of Hepa1-6 Cells with Recombinant Adenoviruses and Chromatin Immunoprecipitation (ChIP) Assays*—Hepa1-6 cells (1 × 10⁷ cells per 150-mm dish), which do not express endogenous FoxA2, were infected at a multiplicity of infection of 100 infectious units/cell with adenovirus containing FoxA2 expression vector (AdFoxA2) as described (31). AdLacZ was used as a control. Infected Hepa1-6 cells were cross-linked in situ by the addition of 37% formaldehyde and were used to prepare protein extract as described (32). The resulting extract was subsequently sonicated and used for the immunoprecipitation (IP) with FoxA2 rabbit antisera as described previously (33). Immunoprecipitation with P-selectin rabbit antisera (BD Biosciences) was used as a control. Cross-links were reversed on all samples by the addition of TE buffer containing 10 μg of RNase A (15 min at 25 °C). Proteinase K (10 μg) was then added, and samples were digested for 16 h at 65 °C. DNA was extracted from the digested samples using PCR purification columns according to the manufacturer’s instructions (Qiagen). We then used these ChIP DNA samples for PCR by using primers specific to Foxf1 3′RE region +4504/+4680 bp (sense 5′-actctagagaacacaaataataag and antisense 5′-acttagattgataattttgag).
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**Statistical Analysis** — Student’s t test was used to determine statistical significance. p values < 0.05 were considered significant. Values for all measurements were expressed as the mean ± S.D.

**RESULTS**

Homology Searches between Mouse and Human Foxf1 Genes Identify Highly Conserved Regions Containing Potential Transcription Factor Binding Sites — In order to identify conserved DNA regions outside of the Foxf1 exons, which are the most likely locations of regulatory sequences (34), we performed homology searches between the 11-kb Foxf1 human and the mouse gene (−5.5 to +5.5 kb). This analysis identified two regions of homology within the −5-kb Foxf1 promoter region (I and II; Fig. 1, A and B) and a homologous 241-nucleotide region (III; +4511 to +4752 bp) located approximately 1-kb 3′ to the mouse Foxf1 gene (3′ RE; Fig. 1, A and B). We used the TFSearch, TRANSFAC, and MacVector Subsequence Transcription factor binding site search programs to identify potential binding sites in the Foxf1 conserved regions. The binding sites that are conserved in both the mouse and human Foxf1 regulatory sequences have been listed in Fig. 1B. The homologous Foxf1 sequences contain potential binding sites for the following families of transcription factors: basic leucine zipper (bZIP) CCAAT/enhancer binding protein β (C/EBPβ), winged helix/Forkhead Box A (FoxA), zinc finger GATA, homeodomain Nkx2.5, winged helix FoxA, and cut-homeodomain HNF-6.

The −5.3-kb Foxf1 Promoter Drives Embryonic β-Gal Transgene Expression in Mesenchyme of Midgut and Developing Intestine — To determine the role of conserved Foxf1 DNA regions in vivo, we generated transgenic mice in which the β-gal reporter transgene was expressed under the control of either −2.7-kb Foxf1 promoter (contains region I), the −5.3-kb Foxf1 promoter (contains regions I and II), or the −5.3-kb Foxf1 promoter fused to the 3′ RE (−5.3-kb Foxf1 + 3′ RE; contains regions I, II, and III; Fig. 2A). Three distinct transgenic −2.7-kb Foxf1 mouse lines, four −5.3-kb Foxf1 lines, and two −5.3-kb Foxf1 + 3′ RE mouse lines were established and analyzed for β-gal transgene expression. Similar results were obtained among the lines in each group, suggesting that the observed β-gal expression patterns were not because of the precise sites of transgene integration. The −2.7-kb Foxf1, −5.3-kb Foxf1, or −5.3-kb Foxf1 + 3′ RE transgenic embryos were dissected at different stages of mouse development, stained for β-gal enzyme activity, and then compared with β-gal staining of the Foxf1+/− embryos, which contain a β-gal reporter gene knocked into endogenous Foxf1 gene locus (24). The −2.7-kb Foxf1 promoter region was insufficient to drive β-gal transgene in vivo, as demonstrated by the absence of β-gal staining in −2.7-kb Foxf1 9.5–18.5 dpc embryos (data not shown). Both −5.3-kb Foxf1 and −5.3-kb Foxf1 + 3′ RE transgenic embryos displayed transgenic β-gal staining in the mesoderm of midgut (Fig. 2, F, G, and J) and mesenchymal cells of developing 12.5 dpc intestine (Fig. 2, K and L), which was similar to the results from the Foxf1+/− mouse embryos (Fig. 2, E, H, and J). Transgenic β-gal expression was also detected in muscle layers and lamina propria of the adult mouse intestine in both −5.3-kb Foxf1 and −5.3-kb Foxf1 + 3′ RE transgenic mice (Fig. 2, M–O), suggesting that the −5.3-kb Foxf1 promoter contains regulatory sequences that are sufficient for proper β-gal transgene expression in mesenchyme-derived intestinal tissues.

Foxf1 3′ RE Is Essential for the Foxf1 Promoter to Drive β-Gal Transgene Expression in the Foregut Mesoderm — Although β-gal staining was not detected in septum transversum mesoderm of either −5.3-kb Foxf1 or −5.3-kb Foxf1 + 3′ RE 9.5 dpc embryos (Fig. 2, C and D), only the
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FIGURE 2. The 3′ Foxf1 regulatory element is essential for the −5.3 kb Foxf1 promoter to drive proper β-gal expression in foregut mesoderm. A schematic drawing of β-gal transgenic constructs. Schematically shown are transgenic constructs that use either −2.7 kb Foxf1, −5.3 kb Foxf1, or the −5.3 kb Foxf1 + 3′ RE promoter to drive expression of the β-gal reporter gene (A–O, β-gal expression pattern in Foxf1+/−, −5.3 kb Foxf1, and −5.3 kb Foxf1 + 3′ RE embryos. Transgenic or Foxf1+/− 9.5 dpc (B–G), 10.5 dpc (H and I), 12.5 dpc embryos (J–L), or adult intestine (M–O) were dissected and stained for β-gal enzyme activity (blue). The β-gal-stained embryos were photographed (H and I) and paraffin-embedded, sectioned, and counterstained with nuclear fast red (red). The −5.3 kb Foxf1 promoter is insufficient to drive β-gal expression in mesoderm of the foregut and septum transversum (C). The −5.3 kb Foxf1 + 3′ RE 9.5 dpc embryos display β-gal staining in foregut mesenchyme (D and F) similar to β-gal staining in Foxf1+/− embryos (B and H). The −5.3 kb Foxf1, −5.3 kb Foxf1 + 3′ RE, and Foxf1+/− embryos exhibit similar β-gal staining in the mesenchyme of midgut (E–G), and mesenchymal cells of 12.5 dpc (J–L) and adult intestine (M–O). Abbreviations used are as follows: Br, brain; M, mouth; Fg, foregut; Mm, midgut; NT, notochord; ST, splanchic mesoderm; ST, septum transversum; In, intestine; Li, liver; He, heart. Magnification: B–G, ×50; H and I, ×15; J–L, ×100; M–O, ×200.

−5.3 kb Foxf1 + 3′ RE transgenic embryos displayed strong β-gal staining in the splanchic mesenchyme of the foregut (Fig. 2D). This β-gal expression in the foregut was similar to the staining pattern from endog-

FIGURE 3. The −5.3 kb Foxf1 + 3′ RE embryos display a proper β-gal staining in peribronchial mesenchyme and hepatic stellate and endothelial cells. A–F, mouse 12.5 dpc embryos or 15.5 dpc lung and liver tissues of Foxf1+/− (A and D), −5.3 kb Foxf1 (B and E), and −5.3 kb Foxf1 + 3′ RE embryos (C and F) were stained for β-gal enzyme activity, paraffin-embedded, sectioned, and counterstained with nuclear fast red. Foxf1+/− mice exhibit abundant β-gal staining in lung mesenchyme (A). The −5.3 kb Foxf1 promoter is insufficient to drive β-gal reporter in mesenchyme of lung (B) and liver (E). The −5.3 kb Foxf1 + 3′ RE transgenic lungs display β-gal staining in peribronchial mesenchyme of 12.5 dpc embryos (C) as well as in embryonic liver (F), gallbladder (J), and smooth muscle (Sm) cells surrounding pulmonary airways (Br) of 15.5 dpc lungs (L), G, H, J, and K. The localization experiments to determine β-gal expression pattern in embryonic liver. β-gal stained −5.3 kb Foxf1 + 3′ RE 15.5 dpc liver sections were stained with mouse monoclonal desmin antibody (H), isotype control antibody (G), or isoelectric B4 and K. Nuclear β-gal staining (blue, shown with arrows) was colocalized with cytoplasmic desmin staining (brown in H) or with isoelectric B4 staining (brown in J and K). Abbreviations used are as follows: CV, central vein; Br, bronchi. Magnification: A–F and J, ×100; G, H, and K, ×630; J and L, ×400.

—Foxf1 gene locus in Foxf1+/− mouse embryos (Fig. 2B), suggesting that Foxf1 3′ RE enhances the −5.3 kb Foxf1 promoter activity in foregut mesoderm.

The Foxf1 3′ RE Is Essential for β-Gal Transgene Expression in Mesenchymal Cells of the Embryonic Liver and Lung—We have previously used Foxf1+/− mice to demonstrate that β-gal is abundantly expressed in the mesenchyme of the embryonic lung (24) (Fig. 3A). Consistent with the absence of β-gal staining in the foregut of −5.3 kb Foxf1 9.5 dpc embryos (Fig. 2C), transgenic β-gal expression was not observed in the −5.3 kb Foxf1 12.5 dpc lungs (Fig. 3B). In contrast, −5.3 kb Foxf1 + 3′ RE 12.5 dpc lungs displayed mesenchymal β-gal staining, which was increased in peribronchial regions (Fig. 3C). However, these β-gal levels were significantly diminished when compared with age-matched lungs from Foxf1+/− embryos containing a knock in of the β-galactosidase gene (Fig. 3A). Transgenic β-gal expression was also observed in smooth muscle cells surrounding pulmonary airways and trachea of 15.5 dpc lungs from −5.3 kb Foxf1 + 3′ RE transgenic embryos (Fig. 3L and data not shown).

We demonstrated previously that Foxf1 is expressed in the mesenchyme of the gallbladder and embryonic and adult liver (21, 23) (Fig. 3D). Although β-gal staining was not observed in −5.3 kb Foxf1 transgenic livers at all developmental time points (Fig. 3E and data not shown), we detected abundant hepatic β-gal expression in the mesen-
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induced LUC activity in both epithelial (HepG2) and mesenchymal (U2OS and HMEC) cell lines (Fig. 5A), a result consistent with previous Foxf1 promoter studies (28). Addition of the 3′RE to the −5.3-kb Foxf1 promoter construct caused a significant decrease in Foxf1 promoter-driven LUC activity in all three transfected cell lines (Fig. 5A), suggesting that the 3′RE functions as a repressor when linked to the −5.3-kb Foxf1 promoter region.

To investigate the effect of 3′RE on the activity of a heterologous minimal promoter, we generated LUC reporter constructs, which contained the 3′RE fused with either TATA box sequences (21, 23, 30) or the minimal hepatocyte-specific −202-bp transferrin (TTR) promoter region (1, 2, 11–13) driving expression of the LUC reporter gene. Transient transfection experiments demonstrated that the 3′RE does not influence expression of the TATA-LUC basal promoter construct in either human osteosarcoma U2OS or human hepatoma HepG2 cells (Fig. 5B). Consistent with the hepatocyte-specific activity of the TTR promoter region, the 3′RE sequence induced TTR promoter activity only in HepG2 cells and not in U2OS cells (Fig. 5B). These cotransfection experiments demonstrated that the 3′RE functions as an enhancer in HepG2 cells when linked to the −202-bp TTR promoter region.

FoxA2 Transcription Factor Binds the 3′RE Region and Represses the Activity of −5.3-kb Foxf1 Promoter—Our transgenic studies demonstrated that 3′RE functions as a repressor to −5.3-kb Foxf1 promoter in the developing midbrain and spinal cord, both of which exhibit strong expression for FoxA proteins (35–37). Because a conservative FoxA-binding site is present in the 3′RE region (Fig. 1B), we wanted to determine whether FoxA proteins are able to mediate the repression function of the 3′RE. We generated the LUC reporter construct driven by the −5.3-kb Foxf1 promoter fused to 3′RE ∆+4641/+4752-bp deletion mutant, which contains only the FoxA-binding site. Cotransfection studies demonstrated that either the 3′RE region or the 3′RE ∆+4641/+4752-bp deletion mutant diminished the activity of the −5.3-kb Foxf1 promoter (Fig. 5C), suggesting that +4511/+4640-bp nucleotide region of the 3′RE was sufficient to repress the −5.3-kb Foxf1 promoter activity.

To determine the role of the FoxA2 protein in 3′RE function, we performed cotransfection experiments using the −5.3-kb Foxf1 or −5.3-kb Foxf1 + 3′RE luciferase constructs and CMV-FoxA2 expression vector. Although FoxA2 transfection did not influence the −5.3-kb Foxf1 promoter activity compared with transfection with the empty CMV plasmid, transfected FoxA2 protein significantly diminished LUC activity driven by the −5.3-kb Foxf1 + 3′RE promoter (Fig. 5D). These results suggest that FoxA2 protein represses the Foxf1 promoter through the 3′RE conserved region.

To determine whether FoxA proteins directly bind the 3′RE region, we synthesized a double-stranded oligonucleotide corresponding to the mouse 3′RE region +4506/+4529 bp, which contains a potential FoxA-binding site. EMSAs were performed with this oligonucleotide and nuclear protein extract from CMV-FoxA1- or CMV-FoxA2-transfected U2OS cells. Both FoxA1 and FoxA2 proteins formed specific DNA-protein complexes, which were inhibited by the addition of cold competitor oligonucleotide interfering with formation of the protein-DNA complexes (Fig. 5E). In contrast, the formation of FoxA-DNA complexes was not inhibited by a nonspecific oligonucleotide containing the Sp1-binding site. These results show that FoxA1 and FoxA2 proteins directly bind to the +4506/+4529-bp 3′RE region. Furthermore, we used ChIP assays to determine whether FoxA2 protein binds to the 3′RE Foxf1 region in the context of endogenous mouse DNA. These ChIP assays demonstrated that adenovirally expressed FoxA2

FIGURE 4. The Foxf1 3′RE is essential for inhibiting ectopic β-gal transgene activity in the developing brain and spinal cord. Foxf1 +/– (A and D), −5.3-kb Foxf1 (B and E), and −5.3-kb Foxf1 + 3′RE 12.5-dpc embryos (C and F) were dissected, stained for β-gal enzyme activity, and paraffin-embedded. Transverse sections were dewaxed, rehydrated, and then counterstained with nuclear fast red. Ectopic β-gal staining is observed in diencephalon (Di), midbrain (Mi), and tegmentum of pons (te, basal plate), and ventral part of epiphymal (ep) layer of neurotube (NT) in the −5.3-kb Foxf1 mice (B and E). Expression of β-gal was diminished in these regions in the −5.3-kb Foxf1 + 3′RE embryos (C and F). Abbreviations used are as follows: FV, fourth ventricle; TV, third ventricle; fl, floor plate; ma, mantel layer. Magnification: A–C, ×50; D–F, ×100.
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FIGURE 5. FoxA2 protein binds to the 3′RE region and retention of FoxA2-binding sequences is sufficient to repress the −5.3-kb Foxf1 promoter. A, the Foxf1 3′RE inhibits the −5.3-kb Foxf1 promoter activity in cotransfection assays. We transiently transfected U2OS, HepG2, or HMEC cells with the −5.3-kb Foxf1 or −5.3-kb Foxf1 + 3′RE LUC reporter plasmids. Cells were harvested at 36 h after transfection and processed for dual luciferase assays to determine luciferase activity. Transfections were performed twice in triplicate and used to calculate the fold transcriptional induction relative to pGL3 basic luciferase plasmid (□). B, the Foxf1 3′RE enhances TTR promoter activity in HepG2 cells. Transient transfection experiments were performed in HepG2 and U2OS cells using one of the following luciferase reporter constructs: TATA-LUC, 3′RE-TATA-LUC, TTR-LUC, and 3′RE-TTR-LUC. C, the +4511/+4641-bp nucleotide region of the 3′RE was sufficient to repress the −5.3-kb Foxf1 promoter activity. We transiently transfected U2OS cells with LUC reporter plasmid driven by the −5.3-kb Foxf1 promoter alone, −5.3-kb Foxf1 + 3′RE, or −5.3-kb Foxf1 + 3′RE 4641–4752-bp deletion mutant. Transfections were performed in triplicate and used to calculate the fold transcriptional induction relative to pGL3 basic luciferase plasmid (□). D, a p value of 0.05 is shown with an asterisk. E, FoxA2 protein represses the activity of −5.3-kb Foxf1 promoter through 3′RE. U2OS cells were transiently transfected with −5.3-kb Foxf1 or −5.3-kb Foxf1 + 3′RE luciferase reporter plasmids as well as CMV-empty and CMV-FoxA2. The double asterisk indicates statistical significance in FoxA2 transfected cells compared with CMV-empty plasmid. E, EMSAs show that FoxA1 and FoxA2 transcription factors bind to their potential binding sites in the Foxf1 3′RE. Nuclear protein extract was prepared from untransfected (UN) U2OS cells or cells transfected with CMV-FoxA1 or CMV-FoxA2. EMSA was performed with the +4506/+4529-bp oligonucleotide containing a potential FoxA-binding site. Specificity of these protein-DNA complexes was demonstrated by the ability of the cold competitor DNA (C, 500-fold molar excess) and the inability of the nonspecific Sp1 oligonucleotide to interfere with formation of protein-DNA complexes (shown with arrows). F, ChIP assays show that FoxA2 protein binds to the 3′RE Foxf1 region in context of endogenous DNA. Cross-linked chromatin was prepared from Hepa1-6 cells at 24 h after infection with AdFoxA2 or AdLacZ. The cross-linked and sonicated chromatin was then immunoprecipitated (IP) with antibodies specific to FoxA2 or P-selectin (control). Immunoprecipitated genomic DNA was analyzed for the amount of mouse 3′RE DNA using PCR analysis with primers specific to the mouse Fox1 3′RE region (+4504 to +4680 bp).

protein specifically binds to chromatin-associated Foxf1 3′RE region in the context of endogenous DNA (Fig. 5F).

C/EBPβ Protein Synergizes with HNF-6 to Activate the −5.3-kb Foxf1 + 3′RE Promoter—Because our transgenic studies demonstrated that Foxf1 3′RE stimulates the Foxf1 promoter in the mesenchyme of the developing lung and liver (Fig. 3), we wanted to determine whether the −5.3-kb Foxf1 promoter can be activated through the +4641/+4752-bp 3′RE region, which contains several overlapping binding sites for known transcriptional activators (Fig. 1B). We performed cotransfection experiments in mesenchymal U2OS cells using −5.3-kb Foxf1 or −5.3-kb Foxf1 + 3′RE LUC constructs and CMV expression vectors containing HNF-6, C/EBPβ, Nkx2.5, and GATA-4, or combinations of these proteins. Although Foxf1 3′RE maintained its inhibitory activity for the Foxf1 promoter after transfection with one of the CMV expression vectors (Fig. 6A), combinations of GATA-4 protein with C/EBPβ or HNF-6 as well as C/EBPβ protein with Nkx2.5 completely blocked the 3′RE repressor activity (Fig. 6A). In contrast, cotransfection of HNF-6 and C/EBPβ proteins caused a synergistic increase in LUC activity of the −5.3-kb Foxf1 + 3′RE reporter compared with either HNF-6 or C/EBPβ alone (Fig. 6A), suggesting that
HNF-6 and C/EBPβ synergize to convert 3’RE from transcriptional repressor into activator. Consistent with this hypothesis, both HNF-6 and C/EBPβ displayed specific bindings to their potential binding sites in the 3’RE region as demonstrated by electrophoretic mobility shift assays with the +4647/+4694-bp oligonucleotide (Fig. 6B). Furthermore, transcriptional synergy between C/EBPβ and HNF-6 proteins was significantly reduced after deletion of the +4641/+4752-bp 3’RE region, which contains HNF-6 and C/EBPβ-binding sites (Fig. 6C). These results suggest that the Foxf1 3’RE can function as an enhancer to the -5.3-kb Foxf1 promoter in the presence of both HNF-6 and C/EBPβ transcription factors. Most interestingly, immunostaining with antibodies specific to HNF-6 or C/EBPβ proteins demonstrated that these transcriptional regulators are coexpressed in the mesenchyme of trachea and proximal airways of 15.5 and 18.5 dpc mouse embryos (Fig. 6, D–F, and data not shown). These embryonic tissues displayed abundant β-gal staining in Foxf1+/- and -5.3-kb Foxf1 + 3’RE transgenic embryos but not in -5.3-kb Foxf1 embryos (Fig. 6G and Fig. 3L, and data not shown). These results suggest that HNF-6 and C/EBPβ proteins stimulate the β-gal transgene reporter through the 3’RE Foxf1 region in vivo.

**DISCUSSION**

Mouse genetic studies have demonstrated that the Foxf1 protein (previously known as HFH-8 or Freac-1) is an important developmental regulator of vasculogenesis and mesenchymal-epithelial cell signaling during mouse embryonic development. Foxf1+/- embryos die by 8 dpc due to extra-embryonic mesoderm defects (20, 24). Foxf1 haploinsufficiency is associated with severe defects in development of the lung, esophagus, trachea, and gallbladder (24–26). To gain insight into in vivo regulation of the Foxf1 gene, we performed a homology search between
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mouse and human Foxf1 DNA sequences. This analysis enabled us to identify two regions of homology within the −5.3-kb Foxf1 promoter region and a homologous 241-nucleotide region located approximately 1-kb 3′ to the mouse Foxf1 gene (3′ RE). To determine the role of conserved Foxf1 DNA regions in vivo, we generated transgenic mice in which the β-gal reporter transgene was expressed under the control of either the −2.7-kb Foxf1 promoter, the −5.3-kb Foxf1 promoter, or the −5.3-kb Foxf1 promoter fused to the 3′RE (−5.3-kb Foxf1 + 3′RE). In this study, we compared β-gal staining in these new transgenic mouse lines with β-gal expression in Foxf1+/− mice, in which the winged helix DNA binding domain was replaced by an in-frame insertion of a nuclear localizing β-gal gene to provide the true Foxf1 expression pattern from endogenous Foxf1 gene locus (24). The use of this β-gal gene knocked into the Foxf1 locus allowed us to determine that Foxf1 is expressed in the splanchic mesoderm of embryonic gut and septum transversum mesenchyme (21). In this study we demonstrated that the −2.7-kb Foxf1 promoter was insufficient to drive β-gal transgene expression in mouse embryos. In contrast, the −2-kb Foxf1 promoter exhibited correct temporal and spatial expression of a GFP reporter in transgenic Xenopus tadpoles (38), illustrating evolutionary complexity in the regulation of the Foxf1 gene among different species. Although the −5.3-kb Foxf1 promoter alone was sufficient to drive β-gal transgene expression in the mesenchyme of the midgut in transgenic mouse embryos, addition of the 3′RE was required for the Foxf1 promoter to elicit proper β-gal expression in the foregut mesoderm and mesenchymal cells of the developing liver and lung, suggesting that the Foxf1 3′RE functions as an enhancer to the −5.3-kb Foxf1 promoter in these mesenchymal tissues.

The Foxf1 3′RE sequence contains high affinity binding sites for transcription factors FoxA, HNF-6, C/EBP, Nkx2.5, and GATA. Cotransfection studies demonstrated that Foxf1 3′RE exhibited repressor properties when cells were untransfected or transfected with the FoxA2 protein. EMSA and ChIP assays demonstrated that the FoxA2 protein binds to the +4506/+4529-bp 3′RE region, and retention of these FoxA2-binding sequences was sufficient to repress the −5.3-kb Foxf1 promoter. In contrast, the 3′RE sequence functioned as an enhancer when C/EBPβ expression vector was combined with HNF-6. Furthermore, deletion of +4641/+4752-bp 3′RE region, which includes C/EBPβ and HNF-6 DNA-binding sites, abolished transcriptional synergy between C/EBPβ and HNF-6 proteins in cotransfection experiments. This is the first demonstration that C/EBPβ and HNF-6 proteins can synergize to activate mesenchyme-specific genes. Although we did not observe transcriptional synergy between C/EBPβ and GATA4-1, other investigators have shown that C/EBPβ can directly interact with GATA-4 and GATA-1 proteins to provide transcriptional synergy for activation of the steroidogenic acute regulatory protein promoter and eosinophil granule major basic protein promoter, respectively (39, 40).

These results suggest that the Foxf1 3′RE plays an important role in the regulation of the Foxf1 promoter and that, depending on the combination of transcription factors, the 3′RE functions to inhibit or activate expression of the −5.3-kb Foxf1 promoter region.

Although the mechanism underlying a tissue-specific regulation of the Foxf1 3′RE in transgenic mice is not completely understood, several members of the Fox, zinc finger GATA, homeodomain Nkx, and bZIP C/EBP families of transcriptional regulators are expressed in foregut mesoderm as well as the mesenchyme of developing liver and lung (19, 41–44). We demonstrated that both HNF-6 and C/EBPβ proteins are coexpressed in the mesenchyme of trachea and proximal airways, and this was associated with abundant β-gal staining in Foxf1+/− and −5.3-kb Foxf1 + 3′RE transgenic embryos but not in −5.3-kb Foxf1 embryos. These results suggest that HNF-6 and C/EBPβ proteins may enhance the Foxf1 promoter activity through 3′RE Foxf1 region.

Foxf1 is expressed in the mesenchyme of the gallbladder (21) as well as in the hepatic stellate and endothelial cells of the embryonic and adult Foxf1+/− liver (see Ref. 23 and this study). Identical β-gal staining was observed in the liver of −5.3-kb Foxf1 + 3′RE embryos but not in the −5.3-kb Foxf1 transgenic livers, suggesting that the Foxf1 3′RE region functions as an enhancer in hepatic mesenchymal cells. The Foxf1 3′RE may be regulated by synergistic transcriptional interactions involving C/EBPβ transcription factor, a known coactivator of the mouse α1 collagen promoter in hepatic stellate cells (45). Because the β-gal transgene was not expressed in adult −5.3-kb Foxf1 + 3′RE liver, the 3′RE may enhance Foxf1 transcription during development or maturation of the hepatic mesenchymal cells. Alternatively, the β-gal transgene can be inactivated by DNA methylation through potential methylation sites in the −5.3-kb Foxf1 promoter region (28). However, in our hands, in vivo inhibition of histone deacetylases by trichostatin A treatment (46) did not reactivate β-gal transgene expression (data not shown), suggesting that DNA methylation does not play a role in β-gal transgene silencing.

Most interestingly, the β-gal transgene was not expressed in septum transversum mesoderm in both −5.3-kb Foxf1 and −5.3-kb Foxf1 + 3′RE transgenic embryos. These results indicate that the −5.3-kb Foxf1 promoter and 3′RE region are still missing important regulatory sequences required for proper transgene expression in the septum transversum.

Strong ectopic β-gal staining was observed in the developing brain and spinal cord of −5.3-kb Foxf1 transgenic embryos. We demonstrated that the addition of 3′RE to −5.3-kb Foxf1 promoter caused inhibition of this ectopic β-gal staining, a result consistent with the ability of the Foxf1 3′RE to repress the Foxf1 promoter in cotransfection studies. Most interestingly, the FoxA2 transcription factor is expressed in the developing midbrain and spinal cord (35–37), and FoxA2 expression partially overlaps with β-gal transgene expression in the −5.3-kb Foxf1 embryos. Because FoxA2 protein directly binds to the 3′RE region and retention of the FoxA2 binding site is sufficient to repress the −5.3-kb Foxf1 promoter activity, the FoxA2 protein may be involved in repression of Foxf1 promoter activity in the developing brain and spinal cord.

In summary, Foxf1 3′RE is required for the −5.3-kb Foxf1 promoter to drive β-gal transgene expression in foregut mesoderm and mesenchymal cells of the developing liver and lung. The Foxf1 3′RE prevents ectopic β-gal transgene expression in developing brain and spinal cord. Taken together, our data suggest that the Foxf1 3′RE is involved in tissue-specific regulation of the Foxf1 promoter during mouse embryonic development.

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