Transcriptional Networks in the Liver: Hepatocyte Nuclear Factor 6 Function Is Largely Independent of Foxa2

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A complex network of hepatocyte nuclear transcription factors, including HNF6 and Foxa2, regulates the expression of liver-specific genes. The current model, based on in vitro studies, suggests that HNF6 and Foxa2 interact physically. This interaction is thought to synergistically stimulate Foxa2-dependent transcription through the recruitment of p300/CBP by HNF6 and to inhibit HNF6-mediated transcription due to the interference of Foxa2 with DNA binding by HNF6. To test this model in vivo, we utilized hepatocyte-specific gene ablation to study the binding of HNF6 to its targets in the absence of Foxa2. Chromatin immunoprecipitation using anti-HNF6 antibodies was performed on chromatin isolated from *Foxa2*^{loxP/loxP} *Alfp.Cre* and control mouse livers, and HNF6 binding to its target, *Glut2*, was determined by quantitative PCR. In contrast to the current model, we found no significant difference in HNF6 occupancy at the *Glut2* promoter between Foxa2-deficient and control livers. In order to evaluate the Foxa2/HNF6 interaction model on a global scale, we performed a location analysis using a microarray with 7,000 mouse promoter fragments. Again, we found no evidence that HNF6 binding to its targets in chromatin is reduced in the presence of Foxa2. We also examined the mRNA levels of HNF6 targets in the liver using a cDNA array and found that their expression was similar in Foxa2-deficient and control mice. Overall, our studies demonstrate that HNF6 binds to and regulates its target promoters in vivo in the presence and absence of Foxa2.

The liver performs essential functions by expressing hepatocyte-specific genes which encode plasma proteins, enzymes involved in metabolism, and proteins involved in the detoxification of exogenous chemicals (13). Many of these hepatocyte-specific genes are regulated primarily at the transcriptional level. This is achieved through the interaction of *cis*-acting DNA sequences with liver-enriched transcription factors called hepatocyte nuclear factors (HNFs). The HNFs are grouped into distinct families based on their structural properties. The families include the Pou homeodomain proteins HNF1 α and - β , the winged helix factors Foxa1, -2, and -3 (formerly HNF3 α , -3 β , and -3 γ), the orphan nuclear receptor HNF4 α , the basic leucine zipper CCAAT/enhancer binding protein (C/EBP) family, and the onecut homeodomain protein HNF6.

HNF6 binding sites are present in the enhancers and/or promoters of different hepatocyte-specific genes, and several of them have been shown to be transcriptionally activated by HNF6 in cotransfection assays (5, 12). Among its potential target genes are glucokinase (15), tyrosine aminotransferase (TAT), transthyretin, 6-phosphofructo-2-kinase, α -fetoprotein (Afp), glucose transporter 2 (*Glut2*) (24), and the transcription factor Foxa2 (19). During liver development, HNF6 is expressed in hepatocytes and in the epithelial cells of the intrahepatic and extrahepatic bile ducts and gall bladder (4). Consistent with this expression pattern, HNF6^{-/-} mouse embryos

fail to develop a gallbladder and exhibit severe abnormalities in both intrahepatic and extrahepatic bile ducts (4, 12).

The hepatocyte nuclear factors function in regulatory networks rather than in simple transcriptional hierarchies (2, 11). In these networks, several HNFs can bind simultaneously to the regulatory regions of a given gene in multi-input regulatory schemes. In addition, there are regulatory chains, in which one HNF activates a second HNF, and regulatory loops, in which two HNFs occupy each other's promoters (10, 18). For instance, HNF6 binds to the HNF4 α promoter, and HNF4 α and HNF1 α regulate each other (1, 6, 22). An even more complex relationship has been proposed for HNF6 and Foxa2. In vitro studies have shown that these two transcription factors can physically interact and that this contact can either stimulate or repress transcription, depending on the target sequence (20). On a Foxa-dependent promoter, the interaction between HNF6 and Foxa2 synergistically stimulates transcription by HNF6-mediated recruitment of the histone acetyltransferase p300/CBP proteins. In contrast, on an HNF6-specific site, the association between HNF6 and Foxa2 results in an inhibition of HNF6 DNA binding activity by Foxa2, thereby causing reduced transcription of HNF6-dependent target genes (see Fig. 1a). In contrast to the interaction between Foxa2 and HNF6, no interaction was observed between HNF6 and the two closely related proteins Foxa1 and Foxa3, and HNF6 was not able to stimulate the transcription of their targets (20).

For this study, we used promoter and cDNA microarrays to investigate HNF6 binding to its target promoters in vivo in the presence or absence of Foxa2. We found that Foxa2 did not reduce HNF6's occupancy of its target promoters, nor did it alter the expression levels of HNF6-regulated genes. Rather,

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FIG. 1. HNF6 binding to the Glut2 promoter in hepatic chromatin in the presence and absence of Foxa2. (a) Schematic representation of the model suggesting that the interaction between Foxa2 and HNF6 can synergistically stimulate or repress transcription. On a Foxa2 target promoter, HNF6 recruitment of p300/CBP activates transcription, whereas on an HNF6 target promoter, an interaction with Foxa2 inhibits HNF6 binding (modified with permission from reference 20). Liver chromatin isolated from wild-type and HNF6^{-/-} mice (b and c) or from wild-type and Foxa2^{loxP/loxP} Alfp.Cre mice (d and e) was immunoprecipitated with an anti-HNF6 antibody or control IgG. Input chromatin and precipitated DNA were amplified with primers surrounding the HNF6 binding site in the Glut2 promoter. Occupancy of the HNF6 site in the Glut2 promoter is detectable by this qualitative assay in wild-type and Foxa2-deficient liver chromatin (d), but not in HNF6^{-/-} chromatin (b), confirming the specificity of the anti-HNF6 antibody. In addition, immunoprecipitation with control IgG demonstrates the specificity of the assay. (c and e) Quantitative real-time PCR analysis of ChIP assays. Enrichment of the target gene Glut2 was calculated by using the 28S rRNA locus as a control for nonspecific DNA and is shown relative to the input chromatin. (c) HNF6 binding to the Glut2 promoter is 13-fold higher in wild-type than in HNF6^{-/-} chromatin, confirming the specificity of the anti-HNF6 antibody. (e) HNF6 binds to the Glut2 promoter in both wild-type and Foxa2-deficient liver chromatin, with a trend towards less binding in the absence of Foxa2. Black bars, control mice; white bars, HNF6^{-/-} mice (c) or Foxa2^{loxP/loxP} Alfp.Cre mice (e). Values are represented as means plus standard errors ($n = 6$ for each group [c] and $n = 3$ for each group [e]). P values were determined by Student's t test. ***, $P < 0.0005$. N.S., not statistically significant. (f) Glut2 mRNA levels are similar in Foxa2-deficient and control livers. Quantitative real-time PCR was performed on RNAs isolated from livers of wild-type and Foxa2^{loxP/loxP} Alfp.Cre mice with primers specific for Glut2, TBP, and HPRT mRNA sequences. Glut2 mRNA levels were normalized to either TBP or HPRT. Black bars, control mice; white bars, Foxa2^{loxP/loxP} Alfp.Cre mice. Values are represented as means plus standard errors ($n = 6$ for each group) N.S., not statistically significant.

we demonstrate that for certain promoters, HNF6 binding was higher in the presence of Foxa2, suggesting that Foxa2 is either neutral to or synergistic with HNF6 binding.

MATERIALS AND METHODS

Animals. The derivation of *Foxa2^{loxP/loxP} Alfp.Cre* mice was described previously (23). Mice were genotyped by PCR using tail-tip DNA. Two- to three-month-old mice were used for these studies. Venous blood was collected from the tail, and serum chemistry was analyzed by Ani Lytics Incorporated (Gaithersburg, MD). The HNF6^{-/-} liver samples were a kind gift from Frederic Lemaigre (12).

Chromatin immunoprecipitation (ChIP). Mouse livers were minced in cold phosphate-buffered saline (PBS) and cross-linked in 1% formaldehyde-PBS for 10 min with constant shaking. Cross-linking was quenched by the addition of glycine to a final concentration of 0.125 M, with constant shaking, for an additional 5 min. The tissue was rinsed in cold PBS and homogenized with a Dounce homogenizer in 1 ml cold cell lysis buffer (10 mM Tris-Cl, pH 8.0, 10 mM NaCl, 3 mM MgCl₂, 0.5% NP-40) supplemented with protease inhibitors (Roche). Cells were incubated at 4°C for 5 min to allow the release of nuclei. Nuclei were sedimented by centrifugation at 13,000 × g for 5 min. The pellet was resuspended in nuclear lysis buffer (1% sodium dodecyl sulfate [SDS], 5 mM EDTA, 50 mM Tris-Cl, pH 8.1) supplemented with protease inhibitors and sonicated with a Sonic Dismembrator model 100 sonicator (Fisher Scientific) with a microtip probe set to a power output of 4 to 6 W for three cycles of 20 s each. Insoluble debris was removed by centrifugation at 13,000 × g for 10 min at 4°C, and the supernatant was collected and flash frozen in liquid nitrogen. Cross-linking of a 10 μM aliquot was reversed by the addition of NaCl to a final concentration of 192 mM, overnight incubation at 65°C, and purification using a Minelute PCR purification kit (QIAGEN). The chromatin concentration was determined by using a NanoDrop 3.1.0 nucleic acid assay (Agilent Technologies).

Three micrograms of chromatin was precleared by adding 125 μl of protein G-agarose in 1 ml of ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 167 mM NaCl, 16.7 mM Tris-Cl, pH 8.1) and rotating the sample for 1 h at 4°C. Protein G-agarose was sedimented by a 15-s pulse in a microcentrifuge. Two micrograms of anti-HNF6 antibody (Santa Cruz), rabbit anti-Foxa2 serum (a gift from J. A. Whitsett), or control preimmune immunoglobulin G (IgG; Upstate) was added to the supernatant and incubated overnight at 4°C. Protein G-agarose (100 μl) was blocked overnight at 4°C with 1 mg/ml bovine serum albumin and 0.1 mg/ml herring sperm DNA in ChIP dilution buffer, added to the chromatin, and rotated for 30 min at 4°C. Following three consecutive washes of 5 min each with TSE I (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-Cl, pH 8.1, 150 mM NaCl), TSE II (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-Cl, pH 8.1, 500 mM NaCl), and ChIP buffer III (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-Cl, pH 8.1), chromatin was eluted by adding 100 μl of freshly made ChIP elution buffer (1% SDS, 0.1 M NH₄CO₃) to the pellet and rotating the sample for 10 min. Elution was repeated with an additional 100 μl of ChIP elution buffer, and the eluates were combined. Cross-linking was reversed by the addition of NaCl to a final concentration of 192 mM and overnight incubation at 65°C.

Real-time PCRs were performed to quantify the relative enrichment of target DNA fragments in the immunoprecipitated DNA. PCRs were assembled using Brilliant SYBR green QPCR master mix (Stratagene), a 0.125 μM concentration of each specific primer, and the included reference dye at a 1:400 dilution. Reactions were performed in triplicate using the SYBR green program on the Mx4000 PCR System (Stratagene). The enrichment of target genes was calculated using the 28S rRNA locus as a reference for nonspecific DNA.

Mouse promoter microarray analysis. Amplification and labeling of immunoprecipitated DNA were performed as described previously (7). Labeled DNAs were hybridized to the Mouse PromoterChip BCBC 3.0, which contains 7,000 amplicons of mouse promoter sequences corresponding to over 3,300 genes with a known or suspected role in hepatocyte and/or pancreatic beta-cell function and metabolism (<http://www.betacell.org/microarrays>). Each gene in the array is represented by two tiles, with the first spanning 1 kb upstream from the transcriptional start site and the second spanning the sequence from -1 kb to -3 kb. Statistical analysis of the microarray data was performed using the significance analysis of microarrays (SAM) (25) and patterns from gene expression (PaGE) (16) packages with a false discovery rate (FDR) of 10 to 30%.

Microarray analysis of gene expression. Liver RNAs were isolated from three *Foxa2^{loxP/loxP} Alfp.Cre* and three control mice. RNAs were reverse transcribed and labeled as described previously (7). Fluorescently labeled cDNAs were hybridized to the Mouse PancChip 5.0 cDNA microarray (14, 21; <http://www>

[betacell.org/microarrays](http://www.betacell.org/microarrays)). This cDNA microarray contains over 13,000 mouse cDNAs, of which >90% are expressed in the liver.

RNA reverse transcription and real-time PCR. Liver RNAs were isolated using an RNeasy kit (QIAGEN). RNAs were quantified using the RNA 6000 Nano Assay program of the Agilent 2100 bioanalyzer (Agilent Technologies). Each RNA (5 μg) was reverse transcribed using 1 μg oligo(dT) primer and Superscript II reverse transcriptase (Invitrogen) at 42°C for 1 h. Primers for real-time PCR were designed from National Center for Biotechnology Information mRNA sequences using Primer3 software with a 3' bias. PCRs were performed using SYBR green as described above. All reactions were performed with six biological replicates in triplicate, with reference dye normalization. The median cycle threshold (*C_T*) value of the technical replicates was used for analysis. *Glut2* and HNF6 mRNA expression levels were normalized to those of the TATA-box binding protein (TBP) and hypoxanthine guanine phosphoribosyl transferase (HPRT) mRNAs as internal controls. The specificity of the PCR amplification was confirmed by dissociation curve analysis and agarose gel electrophoresis.

Nuclear extracts and Western blots. Liver nuclear extracts were prepared as described previously (8). The protein concentration was measured using the Bradford assay, and an equal volume of 2× Laemmli buffer (100 mM Tris-Cl, pH 6.8, 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, 20% glycerol) was added. Samples were boiled for 5 min, sonicated for 20 s with a microsonicator, and stored at -80°C. For Western blots, 10 μg of each nuclear extract was resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. The primary antibodies used were rabbit anti-HNF6 (Santa Cruz) (1:200 dilution), goat anti-Foxa2 (Santa Cruz) (1:100), and mouse anti-TBP (Abcam) (1:2,000).

RESULTS

Foxa2 does not affect the binding of HNF6 to the *Glut2* promoter in the liver. The regulation of hepatic gene expression depends on complex interactions among liver-enriched transcription factors, including Foxa2 and HNF6. The current model, based on in vitro data, suggests that a physical interaction between Foxa2 and HNF6 inhibits HNF6 binding to its target promoters, as depicted in Fig. 1a (20). We set out to test this model by investigating HNF6 binding to its target promoters in vivo, in both the presence and absence of Foxa2, using ChIP. For this purpose, we employed the loxP-Cre system to specifically delete Foxa2 from hepatocytes. In this model, the *Foxa2^{loxP/loxP} Alfp.Cre* mouse, the Foxa2 gene is ablated in >99.9% of hepatocytes (Fig. 2c and data not shown). *Foxa2^{loxP/loxP} Alfp.Cre* mice display an apparently normal phenotype. They have comparable sizes and weights to those of control littermates. Their blood glucose levels, liver morphology, and functions are normal (data not shown).

We first examined whether HNF6 binding to the *Glut2* promoter, which has previously been shown to be transcriptionally activated by HNF6 (24), is dependent on Foxa2. In order for this assay to be informative, it is critical that the antibody against HNF6 used is specific for HNF6. Therefore, we first tested our anti-HNF6 antibody in ChIP assays of wild-type and HNF6^{-/-} livers. As shown in Fig. 1b and c, the antibody employed is able to immunoprecipitate the HNF6-bound *Glut2* promoter, and this binding is completely abrogated in HNF6^{-/-} liver chromatin. These data demonstrate that under the conditions used, the anti-HNF6 antibody does not recognize any other proteins that bind to the *Glut2* promoter.

Next, we investigated whether the binding of HNF6 to the *Glut2* promoter is inhibited by the presence of Foxa2, as suggested by the model described above. ChIP with the anti-HNF6 antibody was performed on chromatin that was isolated from livers of *Foxa2^{loxP/loxP} Alfp.Cre* mice and their control littermates. Surprisingly, HNF6 was bound to the *Glut2* pro-

FIG. 2. HNF6 mRNA and protein levels are similar in Foxa2-deficient and control livers. (a) RNAs were isolated from livers of wild-type or Foxa2^{loxP/loxP} Alfp.Cre mice and reverse transcribed. PCRs were performed with primers specific for HNF6, TBP, and HPRT mRNA sequences, and PCR products were resolved by agarose gel electrophoresis. (b) Quantitative real-time PCR analysis shows similar HNF6 mRNA levels in Foxa2^{loxP/loxP} Alfp.Cre and control mice. HNF6 mRNA levels were normalized as described in the legend to Fig. 1f. Black bars, control mice; white bars, Foxa2^{loxP/loxP} Alfp.Cre mice. Values are represented as means plus standard errors ($n = 6$ for each group). The y axis is in a logarithmic scale. (c) Western blot analysis of protein nuclear extracts (10 μ g) from two control (lanes 1 and 2) and two Foxa2^{loxP/loxP} Alfp.Cre (lanes 3 and 4) mice with antibodies against HNF6, Foxa2, and TBP (loading control). The faint bands in the two lanes for Foxa2^{loxP/loxP} Alfp.Cre mice detected with the anti-Foxa2 antibody are due to a partial cross-reactivity of this antibody with other Foxa family members.

motor in both control and Foxa2-deficient hepatocytes (Fig. 1d and e), with a trend towards increased binding in the presence of Foxa2. In addition, quantitative reverse transcription-PCR performed on RNAs isolated from the livers of Foxa2^{loxP/loxP} Alfp.Cre mice and their control littermates revealed similar amounts of Glut2 mRNA in both groups (Fig. 1f). Thus, in the specific case of the Glut2 promoter, our data contradict the current model.

HNF6 mRNA and protein levels are not altered in Foxa2-deficient mouse livers. A trivial explanation for our findings would be that Foxa2 regulates the expression of HNF6 itself. In other words, if HNF6 were normally activated by Foxa2, then the deletion of Foxa2 would lead to reduced HNF6 levels and consequently decrease HNF6 binding to its targets. In order to address this possibility, we examined HNF6 mRNA levels in the livers of Foxa2^{loxP/loxP} Alfp.Cre mice and their control littermates by quantitative reverse transcription-PCR. Similar amounts of HNF6 mRNA were detected in both groups (Fig. 2a and b). To confirm this result, we performed an immunoblot analysis of nuclear extracts from Foxa2^{loxP/loxP} Alfp.Cre and control livers. HNF6 protein levels were also similar for the two groups (Fig. 2c). Thus, lower levels of HNF6 in Foxa2-deficient mouse livers do not account for the lower level of HNF6 binding to the Glut2 promoter.

Global target occupancy by HNF6 is not dependent on Foxa2. We expanded our analysis of HNF6 binding to its target promoters in the presence and absence of Foxa2. For this purpose, we performed a large-scale promoter microarray location analysis. ChIP samples were amplified by ligation-mediated PCR, fluorescently labeled, and hybridized to the Mouse PromoterChip BCBC 3.0 microarray (<http://www.betacell.org/microarrays>) (Fig. 3). Microarray data were analyzed using the SAM package (25). No significant differences were found between the promoters that were bound by HNF6 in Foxa2-deficient and control mouse chromatin, as shown in the SAM plot in Fig. 4a. In this plot, genes with significant changes in HNF6 binding will fall above or below the diagonal. No promoters were found to be differentially bound at an FDR of 10%, represented by the fact that no points are found outside of the two dashed lines located on either side of the diagonal (Fig. 4a), and only seven promoters were differentially bound when the FDR was increased to 28%. Overall, the SAM analysis detected very few significant differences between the promoters that were bound by HNF6 in Foxa2-deficient and control mouse chromatin. To confirm that our findings were not dependent on the particular analysis program employed, the same data were also analyzed using the PaGE (patterns from gene expression) package (16), which also did not find significant differences between the two data sets at a 10% FDR (data not shown). Thus, Foxa2 does not significantly alter HNF6 binding to the 7,000 promoter elements represented on the array.

Next, we used the promoter microarray results to examine the binding of HNF6 to over 100 promoters that we and others had shown previously to bind HNF6 in vivo (18; J. R. Friedman and K. H. Kaestner, unpublished data). Surprisingly, of the 97 known HNF6 promoter targets, none showed elevated HNF6 binding in the absence of Foxa2. Rather, we identified several promoters for which HNF6 binding was lower in the Foxa2-deficient liver than in control samples (Fig. 4b). Again, this

FIG. 3. Schematic representation of experimental approach combining promoter and expression microarray analysis. Chromatin and RNA were isolated from wild-type or *Foxa2*^{loxP/loxP} *Alfp*.Cre mice. Chromatin was cross-linked and immunoprecipitated with an anti-HNF6 antibody. The resulting material was amplified via ligation-mediated PCR, fluorescently labeled, and hybridized to the mouse promoter microarray. Isolated RNAs were reverse transcribed, and the cDNAs were fluorescently labeled and hybridized to the PancChip 5.0. Microarray data were analyzed using the SAM and PaGE software packages to determine whether there were significant differences between the expression levels or binding levels of promoters by HNF6 in the wild-type and *Foxa2*^{loxP/loxP} *Alfp*.Cre mice.

FIG. 4. Global promoter occupancy by HNF6 is similar in *Foxa2*-deficient and control liver chromatin. (a) Plot generated by the SAM analysis package showing no differences in enrichment of HNF6-bound promoters between *Foxa2*^{loxP/loxP} *Alfp.Cre* and control liver chromatin. The solid line indicates $y = x$, where the observed differences are identical to the expected differences. The distance from the diagonal represents the likelihood of differential enrichment between the two groups. The two dashed lines on either side of the diagonal represent a false discovery rate of 10% (see the text for details). Notice that there are no points outside the area bound by the dashed lines. (b) Promoter microarray values of enrichment for 97 promoters, which were previously shown to be in vivo HNF6 targets, plotted for the *Foxa2*^{loxP/loxP} *Alfp.Cre* versus control mice. Enrichment was normalized relative to genomic DNA. Most promoters are enriched to a similar degree in *Foxa2*-deficient and control liver chromatin, and some are more highly enriched in the presence of *Foxa2* (circled dots). An arrow indicates *Glut2*, a well-established HNF6 target.

finding directly contradicts the idea that the presence of *Foxa2* inhibits HNF6 DNA binding.

In order to confirm the results from our promoter microarray by an independent method, we performed quantitative PCR on unamplified ChIP samples with primers specific for

several HNF6 target promoters. In most cases, *Foxa2* was either neutral to or increased the binding of HNF6 to its target sequences, while *Foxa2* significantly inhibited HNF6 binding only to the *Vdac2* promoter (Fig. 5a).

The promoter array results revealed that HNF6 binding to some of its targets is actually elevated in the presence of *Foxa2* (Fig. 4b, seven circled points to the right of the diagonal). This result indicates that *Foxa2* may cooperate with HNF6 on select promoters. To test this possibility, we performed ChIP on *Foxa2*^{loxP/loxP} *Alfp.Cre* and control liver chromatin with an anti-*Foxa2* antibody. Quantitative PCR was performed on the immunoprecipitated DNA with primers specific for the HNF6 binding site. We found that *Foxa2* binds to three of the seven HNF6 targets that were examined (Fig. 5b). These results suggest that HNF6 and *Foxa2* have mutual targets and that the binding of *Foxa2* to these targets enhances HNF6 binding. Thus, in the absence of *Foxa2*, as in *Foxa2*-deficient liver chromatin, HNF6 binding to these promoters is decreased.

Expression of HNF6 targets is not dependent on *Foxa2*. Our results suggest that *Foxa2* does not interfere with HNF6 binding to the vast majority of its targets. We next examined whether *Foxa2* influences the expression levels of HNF6 targets. If the model depicted in Fig. 1a were correct, we would expect that the expression of HNF6 targets would be altered in *Foxa2*-deficient mice. To test this prediction, RNAs were isolated from the livers of *Foxa2*^{loxP/loxP} *Alfp.Cre* and control mice, reverse transcribed, fluorescently labeled, and hybridized to the Mouse PancChip 5.0 cDNA microarray (<http://www.betacell.org/microarrays>). The expression profiles of several hundred HNF6 targets were analyzed and were found to be similar for *Foxa2*-deficient and control livers (Fig. 6). Two HNF6 targets exhibited decreased expression in the absence of *Foxa2* (Fig. 6, two dots to the right of the diagonal), although the promoter array results did not show reduced HNF6 binding to these targets in the absence of *Foxa2*. Thus, *Foxa2* has little effect on either DNA binding or transcriptional regulation by HNF6.

Taken together, our studies demonstrate that in the liver in vivo, HNF6 binds to its targets similarly in the presence or absence of *Foxa2*, and that in some cases the presence of *Foxa2* even increases HNF6 binding to its targets. Furthermore, the expression levels of >100 HNF6 target genes are not affected by the presence of *Foxa2*. Thus, *Foxa2* does not cause a reduced transcription of HNF6 target genes.

DISCUSSION

Transcriptional regulation in the liver is mediated by a complex interplay of dozens of DNA binding proteins. The current model of this transcriptional regulatory network suggests that HNF6 and *Foxa2*, but not the related proteins *Foxa1* and *Foxa3*, interact physically (20). This interaction is thought to reciprocally stimulate *Foxa2* target gene transcription through the recruitment of p300/CBP by HNF6 and to inhibit HNF6 target gene transcription due to *Foxa2* interference with the DNA binding of HNF6. This model, based on in vitro studies, makes certain predictions that can be tested using genetic means. Here we utilized the *Foxa2*^{loxP/loxP} *Alfp.Cre* mouse model, in which *Foxa2* is ab-

FIG. 5. HNF6 binds its target promoters similarly in *Foxa2*-deficient and control mice. Liver chromatin isolated from wild-type or *Foxa2^{loxP/loxP}Alfp.Cre* mice was immunoprecipitated with an anti-HNF6 (a) or anti-Foxa2 (b) antibody. Input and precipitated DNAs were subjected to quantitative real-time PCR with primers specific for several putative HNF6 target promoters. Enrichment of the target promoters was calculated, using the 28S rRNA locus as a reference, and is shown relative to the input chromatin. Black bars, control mice; white bars, *Foxa2^{loxP/loxP}Alfp.Cre* mice. Values are represented as means plus standard errors ($n = 3$ for each group). P values were determined by Student's t test. *, $P < 0.05$; **, $P < 0.01$.

lated from hepatocytes, to assess whether *Foxa2* does indeed block HNF6 binding in the liver in vivo and whether the presence of *Foxa2* influences the expression of HNF6 targets. We performed chromatin immunoprecipitation using an anti-HNF6 antibody on chromatin isolated from livers of *Foxa2^{loxP/loxP}Alfp.Cre* mice and their control littermates, expecting that the occupancy of HNF6 on its target promoters would be enhanced in the absence of *Foxa2*. However, by investigating HNF6 binding to the promoter of the *Glut2* gene by quantitative real-time PCR, we found no increase in promoter occupancy in the absence of *Foxa2*.

In addition, when we examined HNF6 binding to thousands of promoters in the presence and absence of *Foxa2*, we found

none at which *Foxa2* inhibited HNF6 binding but several at which occupancy by HNF6 was increased in the presence of *Foxa2*. A global analysis of the expression profiles of HNF6 target genes revealed similar expression levels in *Foxa2*-deficient and control livers. Our results contradict the current model of HNF6 and *Foxa2* interaction, which suggests that *Foxa2* inhibits the binding of HNF6 to its targets. The current model is largely based on in vitro assays, in which cultured cell lines were transfected with different amounts of plasmids expressing HNF6 and *Foxa2*, usually under the control of a strong promoter such as the cytomegalovirus promoter. In these assays, the amounts of proteins found in the cells can be much higher than the physiological levels of these proteins and

FIG. 6. Expression levels of HNF6 targets in the liver are similar for Foxa2-deficient and control mice. Mouse PancChip 5.0 microarray fluorescence intensity values of enrichment for 97 HNF6 *in vivo* target genes were plotted for Foxa2-deficient versus control mice.

may not reflect the conditions and ratios in which the proteins interact *in vivo*. This effect is especially acute for assays that involve transcription factors, whose physiological expression levels are usually low, and may result in protein-protein and protein-DNA interactions that do not occur in the liver. In other experimental systems, mice were injected with recombinant adenoviruses expressing the two transcription factors, again under the control of the cytomegalovirus promoter (24). These experiments, although performed *in vivo*, still encountered the problems of nonphysiological high expression levels, which may alter interactions between proteins and between proteins and DNA. Thus, it is possible that in systems where Foxa2 is expressed at nonphysiological levels, it can inhibit HNF6 binding to its targets.

Our findings that Foxa2 may synergize with HNF6 binding to some of its targets are consistent with other studies which show that Foxa proteins are required for the activation of transcription by other DNA binding factors. The Foxa proteins were shown to function as chromatin-remodeling factors that decompact chromatin, thus facilitating the subsequent binding of other transcription factors to their targets (9, 17). Foxa proteins were found to bind the albumin gene enhancer before other transcription factors and to alter nucleosome positioning, thus allowing other factors to bind (3). Thus, it is possible that Foxa2 also facilitates HNF6 binding to some of its targets.

In summary, we have demonstrated that HNF6 binds its target promoters *in vivo* in the presence and absence of Foxa2 and that the expression levels of HNF6 targets are not influenced by Foxa2, thus revising the current model of transcriptional networks in the liver.

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