Expression of the α7 Isoform of Hepatocyte Nuclear Factor (HNF) 4 Is Activated by HNF6/OC-2 and HNF1 and Repressed by HNF4α1 in the Liver*‡‡

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The hepatocyte nuclear factor (HNF) 4α gene possesses two promoters, proximal P1 and distal P2, whose use results in HNF4α1 and HNF4α7 transcripts, respectively. Both isoforms are expressed in the embryonic liver, whereas HNF4α1 is almost exclusively in the adult liver. A 516-bp fragment, encompassing a DNase I-hypersensitive site associated with P2 activity that is still retained in adult liver, contains functional HNF1 and HNF6 binding sites and confers full promoter activity in transient transfections. We demonstrate a critical role of the Onecut factors in P2 regulation using site-directed mutagenesis and embryos doubly deficient for HNF6 and OC-2 that show reduced hepatic HNF4α7 transcript levels. Transient transgenesis showed that a 4-kb promoter region is sufficient to drive expression of a reporter gene in the stomach, intestine, and pancreas, but not the liver, for which additional activating sequences may be required. Quantitative PCR analysis revealed that throughout liver development HNF4α7 transcripts are lower than those of HNF4α1. HNF4α1 represses P2 activity in transfection assays and as deduced from an increase in P2-derived transcript levels in recombinant mice in which HNF4α1 has been deleted and replaced by HNF4α7. We conclude that although HNF6/OC-2 and perhaps HNF1 activate the P2 promoter in the embryo, increasing HNF4α1 expression throughout development causes a switch to essentially exclusive P1 promoter activity in the adult liver.

Hepatocyte nuclear factor (HNF) 1α, an orphan member of the steroid/thyroid receptor superfamily, is highly expressed in the adult liver (1). During mouse development, it is one of the first liver-enriched transcription factors (LETF) to be expressed, HNF4α transcripts being detected at E (embryonic day) 4.5 in primitive endoderm, in the visceral endoderm (E 5.5), and in the liver bud (E 8.5) (2, 3). The crucial role of this factor in the embryo was demonstrated by the inactivation of the HNF4α gene, causing perigastrulation lethality (4) because of failure to activate visceral endoderm functions (5). When null embryos were transiently rescued by tetraploid complementation with wild-type morulae-derived visceral endoderm, HNF4α was dispensable for hepatic specification but not for activation of hepatic functions (6). In addition, liver-specific HNF4α deletion in the embryo (7) led to disorganization of liver architecture (8), and its forced expression in cultured hepatic cells resulted in the reexpression of some liver-specific genes (9) and restoration of epithelial morphology (10, 11). HNF4α plays a pivotal role, regulating expression of genes involved in nutrient metabolism and transport (for review, see Ref. 12), and its induced disruption in the adult liver provokes lethal defects in lipid and bile acid metabolism and ureagenesis (13, 14). Hence, HNF4α is essential for both the induction and the maintenance of hepatic functions. The HNF4α nuclear receptor contains six domains (A–F) and possesses two activation functions (AF): AF1 corresponds to the 24 N-terminal amino acids (15), and AF2 is located in the E domain. Different isoforms of HNF4α arise by alternative splicing. The most important for the adult liver appear to be α1, the prototype protein (1), and α2, which possesses a 10-amino acid insertion in the C-terminal F domain (16; for review, see Ref. 12).

The isoforms considered in this work result from alternative promoter usage. An N-terminal variant of HNF4α was cloned from the mouse (17). It arises from a distal upstream promoter, P2 (18), located about 40 kb upstream from the P1 promoter from which the α1 and α2 isoforms are transcribed (3, 19). The HNF4α7 protein derived from P2 usage differs from α1 (and its splicing variants) only by the first exon, which in the case of α1 but not α7 contains a functional AF1. The differences in activation functions of these isoforms result from differences in

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cofactor recruitment (20). In the liver, the α7 isoform is expressed mainly in the embryo and is nearly extinguished in the adult. In accordance with this expression profile, the transcription capacity of the HNF4α/7 isoform is efficient for fetal functions such as α-fetoprotein, whereas that of α1 is higher than α7 for most hepatocyte promoters (21). The HNF4α/7 isoform is also expressed in the adult stomach, intestine, and pancreas (17). In human, mutations in the HNF4α coding sequence that is common to P1 and P2-derived isoforms are associated with maturity-onset diabetes of the young (22, 23). Because predominantly P2 is active in the pancreas (17, 24, 25), mutant HNF4α/7 is probably incriminated in the insulin-secretion defects of diabetes, whereas the associated hepatic dysfunctions (26, 27) can likely be ascribed to mutant α1 (for review, see Ref. 12).

Because the HNF4α/7 and HNF4α1 isoforms show qualitative differences in their AF domains, it can be speculated that the regulation of expression of each is critical for ontogenesis of HNF4α-expressing tissues. In addition, because HNF4α/7 transcripts are present in embryonic liver but are mainly extinguished in the adult where HNF4α/7 transcripts are particularly high, it is possible that the products of one promoter influence activity of the other, providing another link in the transcription factor network regulating liver-specific gene expression. Although regulation of expression of the P1 promoter and its enhancer in liver cells has been analyzed (28, 29), the factors driving expression of the P2 promoter in embryonic liver and its down-regulation in the adult remain to be determined.

Here we demonstrate the existence of a single DNAse I-hypersensitive site specifically associated with P2 activity. This site is present in embryonic liver and is retained in the adult even though HNF4α/7 transcripts are barely detectable in adult liver, suggesting that chromatin at the promoter region remains in an open conformation after birth. Using a combination of embryonic liver-derived cultured cells and knock-out mice, we show that two Oncatin factors, HNF6 and OC-2, are derived transcripts are always less abundant than those from P1. Finally, using knock-in mice expressing only one of the two N-terminal HNF4α isoforms under control of both promoters, we describe a dramatic repressive effect of HNF4α1 on the P2 promoter, providing a novel mechanism to explain HNF4α/7 postnatal repression in the liver.

**EXPERIMENTAL PROCEDURES**

**Library Screening for Mouse Genomic Sequence Upstream from Exon 1D—**Genomic clones were obtained by screening a mouse 129/Sv BAC library (Incyte, Inc.) for exon 1D sequences. One clone called m21 was used as a template for most experiments. This clone contained at least 12 kb upstream from the P2 promoter, the P1 promoter and its enhancer region, and exon 2.

**Cell Culture—**MMH (Met Murine Hepatocyte) palmate and epithelial cells (MMH-pal and MMH-ep)-immortalized cell lines derived from mouse embryonic liver at E 14.5 representing, respectively, bipotential and committed hepatocytic liver cells (30). They were grown in RPMI 1640 supplemented with 10% fetal calf serum (Invitrogen), 50 ng/ml epidermal growth factor, 30 ng/ml insulin-like growth factor II, and 10 μg/ml insulin on collagen I-coated (Sigma) dishes (Falcon Plastics, Inc.). FG4C cells are differentiated rat hepatoma cells of adult phenotype (31) which express mainly HNF4α1. However, these cells probably are not a faithful model for HNF4α/7 down-regulation in the adult liver because they derive from H4II hepatoma cells, which express both isoforms.4 They were maintained in Ham’s F-12 medium containing 5% fetal calf serum, Mouse NIH/3T3 fibroblasts, 3TDM-1 transformed fibroblasts (33), and βTC1 insulinoma cells (34) were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. βTC1 cells were kindly provided by P. K. Wellauer (ISREC, Epalinges, Switzerland). Cells were cultured at 37 °C under humidified 5% CO2.

**Analysis of DNAse I-hypersensitive Sites—**Nuclei were prepared from freshly excised organs of adult mice and liver from E 18.5 fetuses as described previously (35) or from cultured cells scraped into phosphate-buffered saline on ice. Purified nuclei were resuspended in 5 vol Tris-Cl, pH 7.5, 15 mM NaCl, 60 mM KCl, 5 mM MgCl2, 2 mM EDTA, 0.15 mM spermine, 0.5 mM spermidine, treated for 10 min at 0 °C with 0–10 units of DNAse I (Roche Applied Science)/μg of DNA. Nuclei were lysed, and DNA was purified and cleaved with the appropriate restriction enzyme prior to Southern blot analysis (36). Probes abutting the restriction cleavage sites (a 644-bp EcoRI-BamHI fragment for EcoRI or BamHI and a 566-bp AlwNI-HindIII fragment for HindIII digestions) were [α-32P]dCTP labeled by random priming (Amersham Biosciences kit).

**RNA Isolation—**Total RNA was isolated from cells or tissues either by lysis in guanidine thiocyanate followed by centrifugation through a CsCl cushion (37) or by the method of Chomczynski and Sacchi (38) using TRIzol reagent (Invitrogen). Poly(A)+ RNA was selected by use of PolyATtract® poly(DT) columns according to the manufacturer’s instructions (Promega).

**RT-PCR and Quantitative Real-time PCR—**Reverse transcription (RT) was performed using SuperScript II™ Moloney murine leukemia virus reverse transcriptase and random hexamers on 5 μg of DNAse I-treated RNA following the manufacturer’s instructions (Invitrogen). Semiquantitative PCR (Bioline Tng) was performed with 1 μl of cDNA and 0.8 μl of each primer and quantified with ImageQuant software. Forward primers for mouse HNF4α/7 and HNF4α1 were 5'-cctgagtctggaagcgcggccg-3' and 5'-cattgctgtggagcgcagcgc-3', and a common reverse primer was used (21). Reverse and α7 forward primers matched rat cdna perfectly, whereas the α1 forward primer contained two mismatches with the rat sequence, leading to poor amplification of HNF4α1. For FG4C rat cells, the primer (5'-cattgctgtggagcgcagcgc-3') was used with the same reverse primer for as many as 36 cycles because it was difficult to detect traces of α7 transcripts in the wild-type adult liver. Primers for 28 S rRNA (21) and HNF6 (39) have been published, and OC-2 primers are as follows: 5'-ggcagccgctgccacacggtgccgag-3' and 5'-cagctgctgcaagctggtgccgag-3'. Real-time PCR was performed with SYBR Green Master Mix (Applied Biosystems) using HNF4α1 (5'-ggaattgataggggtgaagagc-3' and 5'-gtggattggtgataggggtgaagagc-3'), HNF4α7 (5'-gtttggaattggcttgattgtttct-3' and 5'-gctttggaattggcttgattgtttct-3'), or β-actin primers (5'-gacatgctttgcaagtttcagc-3' and 5'-gactttgcaagtttcagc-3'), on a PerkinElmer Life Sciences ABI PRISM 7700 Sequence Detection system. To take into account the amplification efficiency of primers, we used the absolute standard curve method. Partial mouse HNF4α1 and HNF4α7 cDNA were amplified by PCR, cloned in pCR2.1-TOPO® (Invitrogen) and subcloned. These plasmids were used in series to serve as standard dilutions on each 96-well plate. For β-actin, gel-purified PCR products were used as well. Data were analyzed by using Sequence Detector Systems software (Applied Biosystems). The numbers of target molecules in cDNA samples were estimated using the linear regression curve equation (R2 > 0.99) of the logarithm of the target molecules number plotted versus Ct values (threshold) in the corresponding standard.

**Reporter Constructs—**The 516-bp and 4.1-kb luc constructs were obtained by subcloning of a 516–13 bp and a 4096–13 bp restriction fragment (relative to the ATG) into XhoI-HindIII (blunted) and Nhel-HindIII (blunted) sites upstream from the pGL2-Basic (Promega) promoterless luciferase gene. Mutations in HNF1/J6 sites were performed using a QuikChange site-directed mutagenesis kit (Stratagene). All promoter constructs were verified by sequencing. The apoB-luc reporter has been described previously (40).

**Transient Transfections—**MMH, 3TDM-1, and NIH3T3 cells (1.7 × 106 cells/dish) were transfected using 3.3 μg of reporter construct and 1.7 μg of internal control RSV-lacZ (pMMJ20) by the calcium phosphate procedure as described previously (41) unless specified. For MMH cells, DNA precipitates were added in Ham’s F-12 medium containing 10% fetal calf serum, epidermal growth factor, insulin-like growth factor II, and insulin. Cells were harvested about 64 h later. FG4C cells were transfected by electroporation and βTC1 cells by the calcium phosphate procedure or electroporation using 10 μg of reporter construct and 5 μg of internal control RSV-lacZ as described previously (28). Cotransfection experiments of NIH3T3 and MMH cells were per-
formd using pCB6tagHNF6a.2 pXJ42-OC-2 (49), RSV-αHNF1 (43), RSV-HNF1 (44), and pMT7-HNF4a(7) (45) as indicated in the figures, appropriately completed as needed with the corresponding empty vectors. Luciferase activity was determined as described previously (46) and β-galactosidase activity by the standard colorimetric method. Luciferase activity was normalized to β-galactosidase activity for transfection efficiency and calculated relative to that of pGL2-Basic unless specified otherwise. Values are the means of three to ten independent experiments (±S.D.).

Electrophoretic Mobility Shift Assays (EMSA)—Nuclear extract preparations and EMSA were performed as detailed previously (28). Sequences of the α7H1 and α6H6 oligonucleotides were 5′-gtgactgtttcctgtaatc-3′ and 5′-ccaatcttgggtataggatgataagaaagagactgaggggtcaatgggccgga-3′. Bases in bold were replaced in α7H1mut with GT and GG (47) and T and G in α6H6mut oligonucleotide. For competitions, a 50× molar excess of unlabeled oligonucleotides was used. The antibody against the HNF6 peptide GTAREPNSVTG in rabbit (UC-6), the antibody raised against the OC-2 amino acids 36–311 in rat (UC-1), and the HNF3a antibody (Santa Cruz) were used at dilutions of 1/10 and 1/3.

Transient Transgenesis—The 4.096-kb sequence upstream from the HNF4a7 ATG was subcloned in front of an nls-lacZ cassette (pSKT-nls-lacZ plasmid, a kind gift from S. Tajbakhsh, Institut Pasteur, Paris) taking care to keep the entire α7 5′-UTR intact. The HindIII-Nol restriction fragment (374 kb) containing the construct was microinjected into zygote pronuclei (Centre d’Ingénierie Génétique Murine, Institut Pasteur). Embryos were harvested at E 17.5, fixed for 1 h in 4% paraformaldehyde following a sagittal section, and X-gal stained at 30 °C overnight. Genotyping was performed by duplex PCR with IacZ-specific primers and primers specific for an endogenous gene (a kind gift from J. Barra, Institut Pasteur). Results are representative of three independently obtained transgenic embryos, all sharing identical staining profiles.

Recombinant Mice—OC-2 knock-out mice were obtained by deleting the OC-2 first exon by homologous recombination in embryonic stem cells. Double knock-out HNF6F−/− OC-2−/− E 12.5 embryos were harvested at the expected proportions by crossing double heterozygous mice. The HNF4a7 knock-in mice were obtained by replacement of the α1 first exon coding sequence (1A) by exon 1D coding sequence for “α7-only” mice and vice versa for “α1-only mice” using homologous recombination in embryonic stem cells. In all cases, the entire 5′-UTR and Kozak consensus were conserved. Both types of homozygous mice are viable and fertile.5

Terminology—Because up to nine isoforms of HNF4a have been described or proposed (for review, see Ref. 12), each time that HNF4a7 is mentioned, this includes not only α7 but also the constitutively produced splicing-derived isoforms, α8 and α9. Likewise, HNF4a1 is meant to encompass at least the α2–α4 isoforms.

RESULTS

A Single Hypersensitive Site Is Associated with P2 Activity and Retained in the Adult Liver—We began by defining the transcription initiation site of the murine HNF4a7 gene because the 5′-end of the cDNA published sequence (17) did not match either the sequence of our BAC clone or that of a mouse chromosome 2 genomic contig. To verify in addition that the first exon of the HNF4a7 gene, exon 1D, is not preceded by an additional exon in hepatic cells, we performed both primer extension and RNase protection assays (see figures in the supplemental material; Refs. 83, 84). The major start site was situated at −103 bp relative to the ATG.

DNase I-hypersensitive site analysis was undertaken to identify genomic regions implicated in hepatic regulation of the HNF4a7 gene in the vicinity of exon 1D. The experiments were performed with fetal liver and cultured cells expressing α7 (Fig. 1A) and with tissues that have been described (19, 21, 29) to express only traces of α7 (adult liver) or none at all (kidney, spleen). Using the E-B probe (Fig. 1B), which hybridized to an 8.7-kb EcoRI genomic fragment spanning exon 1D, we identified at least seven sites. However, only one was present exclusively in α7-expressing samples, including the βTC1 insulinoma cell line (Fig. 1A), and absent from kidney and spleen. This site was positioned −300 bp upstream from the ATG, within the promoter region. Strikingly, this site was also strongly digested in adult liver chromatin, suggesting that the reduction in HNF4a7 transcript amounts compared with fetal liver is not the consequence of a dramatic change in chromatin at the promoter. The signal corresponding to this site was less pronounced in fetal liver than in MMH-pal cells or adult liver, probably because of dilution by hematopoietic cells that do not express the HNF4a gene (48).

Positions of the hypersensitive sites were verified on HindIII-digested DNA, using the probe abutting the 3′-HindIII site (Fig. 1B), permitting analysis of the same genomic region but from the other direction. The specific site was observed again in fetal and adult liver (data not shown). We also searched for hypersensitive sites upstream from the 5′-EcoRI

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3 P. F. Lemaigre, unpublished data.
4 P. Jacquemin, F. Clotman, G. G. Rousseau, and F. P. Lemaigre, unpublished data.
5 N. Briançon and M. C. Weiss, manuscript in preparation.
site using BamHII and the E-B probe, but only ubiquitous sites were found (data not shown and Fig. 1B).

As a complement to this analysis, we performed a Blast search against the human genome (chromosome 20) of the sequence upstream from the murine HNF4α7 ATG to pinpoint conserved regions that could represent regulatory elements. In the 4.1-kb upstream region, two homology stretches were indeed found, at −520/+54 bp (containing exon 1D and the specific hypersensitive site shown previously) and a smaller stretch at −3523/−3388 bp relative to the ATG (Fig. 1C). The same alignment was performed against the rat genome, and the two conserved elements were found within longer homology stretches (not shown).

516 Base Pairs Confer P2 Promoter Activity in HNF4α-expressing Cells—To characterize cis-elements necessary for HNF4α7 hepatic expression, we prepared luciferase constructs containing the 4.1-kb and a smaller 516-bp proximal conserved fragment (Fig. 1C) for transfection analysis. We selected cell lines that expressed no HNF4α (MMH3T3), mainly HNF4α1 (FGC4 hepatoma cells (21)), both types of transcripts (embryonic liver-derived MMH-ep), and mainly or only HNF4α7 (MMH-pal, 3TDM-1 trophoblastoma cells, and βTC1 insulinoma cells). These expression patterns are illustrated by the RT-PCR gel pattern of Fig. 2A.

In all cell lines expressing the endogenous HNF4α7 gene, activity levels of the 4.1-kb and 516-bp constructs were close to (MMH-ep) or superior to that of the ubiquitous SV40 promoter used as a control (Fig. 2B). This was in contrast to NIH3T3 fibroblasts for which reporter activity levels represented only about 5% of the SV40 promoter. This suggests that cell type-specific transcription factors are necessary for reporter activity of the HNF4α7 constructs.

We expected to observe only weak promoter activity of the HNF4α7 constructs in FGC4 cells because these cells mimic an adult hepatocyte phenotype, including high expression of HNF4α1 and only very low HNF4α7 transcript amounts. We initially anticipated that transfection tests would reproduce the extinction of HNF4α7 expression characteristic of adult liver, but this turned out not to be the case because these constructs showed high reporter activity levels in FGC4 cells (see “Experimental Procedures”).

The activity levels of both HNF4α7 constructs were similar in all cells, suggesting that the distal homology stretch (Fig. 1C) is not implicated in HNF4α7 expression in the transfected cells and that the 516-bp element is sufficient to drive consistent cell type-specific expression. When isolated and tested by transfection (Fig. S2), this distal homology stretch showed at best only a weak enhancer effect.

Functional HNF1 and HNF6 Sites Activate the P2 Promoter—The 516-bp fragment was analyzed for consensus binding sites. Others have localized Pdx1 and HNF1 sites implicated in HNF4α7 expression in pancreatic cells (18, 24). As shown in Fig. 3A, there is also a perfect HNF6 consensus that we found to be strictly conserved in the human sequence (GenBank accession number AF509467).

EMSA was employed to verify that the relevant transcription factors bind to the HNF1 and HNF6 sites in hepatic cells. Fig. 3B shows that both HNF1α and HNF1β bind to the HNF1 site. In adult liver, adult-type FGC4 hepatoma cells and in the differentiated embryoid-derived MMH-ep cells, HNF1α is the major form, whereas the undifferentiated MMH-pal cells express mainly HNF1β (Fig. 3B). In line with these observations, HNF1β is the first isoform to be expressed at the onset of liver development, whereas HNF1α rapidly becomes predominant in hepatocytes (49, 50). The specific complexes were abolished by competition with a well known HNF1-binding oligonucleotide but not a mutant oligonucleotide.

Fig. 3C provides a similar analysis of the HNF6 site. MMH-pal, FGC4 cells, and adult liver nuclear extracts all showed binding to the putative HNF6 site (α’βH6 oligonucleotide), and this was effectively competed with an excess of the α’βH6 but not the mutant oligonucleotide (not shown), which does not bind any complex. It was observed consistently that the MMH-pal band migrated slightly more slowly than that of FGC4 or adult liver extract. This could indicate that a different but related protein is bound to the HNF6 site in MMH-pal extracts. There are three different members of the Onecut family: HNF6, present as two splicing-derived isoforms, α and β (51, 52), as well as OC-2 (42) and OC-3 (53). In the mouse, the OC-2 protein is larger than HNF6α (39) and it is known that both OC-2 and HNF6 are expressed very early in the developing liver (51, 54, 55), a stage that is mimicked by MMH-pal cells. Fig. 3D shows that MMH-pal cells do not harbor detectable HNF6 transcripts, but they do express OC-2. In addition, Fig. 3E reveals that the protein bound to the α’βH6 oligonucleotide in MMH-pal cells is displaced by anti-OC-2, whereas that in adult liver reacts mainly with anti-HNF6. In MMH-pal cells, attenuation of the complex was also observed with anti-HNF6, probably because of antibody cross-reaction. These results demonstrate that the HNF6 site is bound by HNF6 and by OC-2.

Analysis of 5′-deletion mutants of the 516-bp fragment transfected in hepatic MMH-pal, MMH-ep, FGC4 cells, and fibroblastic NIH3T3 cells (Figs. S3 and 4A) revealed that when the region upstream from 221 bp was deleted, all cell types...
FIG. 3. HNF1β/OC-2 in MMH-pal cells and HNF1α/HNF6 in FGC4 and adult liver bind the corresponding consensus sites within the P2 promoter. A, putative binding sites for ubiquitous (SP1, Oct1, Smad4) and tissue-restricted factors expressed in endoderm-derived tissues (CHOP/C/EBP, Cdx2, GATA4/6, HNF1α/β, Pdx1, HNF6/OC-2) were determined using the MatInspector program (81) on the P2 promoter. Arrow, major transcription start site. Bold, translation start codon. * denotes the upstream limit of sequence conservation compared with that of Ref. 17 (GenBank accession number AF015275). B, EMSA: [32P]labeled α7H1 oligonucleotide containing the HNF1 site was incubated with 20 μg of cell nuclear extract and 5 μg of adult rat liver nuclear extract. PE56, a well known HNF1-binding oligonucleotide, and mutations introduced into α7H1 (giving α7H1mut oligonucleotide) to suppress binding are from Ref. 47. n.s., nonspecific complex. C, EMSA: [32P]labeled α7H6 or α7H6mut (mutated in the HNF6 binding site) oligonucleotides were incubated with 10 μg of cell nuclear extract and 7.1 μg of adult liver nuclear extract (left panel) or 10.8 μg of cell and 6.2 μg of liver extract (right panel). Comp., competition. D, RT-PCR products showing the presence of OC-2 and the absence of HNF6 transcripts in MMH-pal cells compared with Onecut-expressing fetal liver (E 17.5) and adult kidney as a negative control. E, EMSA: [32P]labeled α7H6 oligonucleotide was incubated with 10.8 μg of cell and 6.2 μg (left) or 2.2 μg (right) of liver nuclear extract. Experiments were performed in the presence or absence of increasing amounts of antisera directed against HNF6, OC-2, or HNF3α as a control.
showed a similar reduction in reporter activity, implying that ubiquitous factors are involved in transcriptional activity of this region. However, when a 56-bp fragment containing the HNF1 site was deleted, the majority of tissue-specific promoter activity was lost. This is in agreement with previous studies in INS-1 insulinoma cells and FTO-2B hepatoma cells (18). Indeed, the 165-bp construct retained very low reporter activity, indicating that the HNF6 site, when separated from upstream sequences, is not sufficient to confer significant promoter activity. This suggests that Onecut factors interact with yet to be identified factors binding within these upstream sequences. No hepato-specific complex in addition to HNF1 and Onecut (see above) could be detected by EMSA using oligonucleotides spanning the −450/−122 bp region (Fig. S4).

Site-directed mutagenesis in the context of the −516 and −221 promoter fragments was used to test the functionality of the HNF1 and HNF6 sites in hepatic cells. In MMH-pal and MMH-ep cells, mutation of the HNF6 site resulted in a more dramatic loss of activity than did that of the HNF1 site, indicating that in models of embryonic hepatic cells the HNF6 site plays a predominant role (Fig. 4A). In these cells, disruption of the HNF1 site showed a very weak effect, which was surprising compared with results of 5′-deletions. In contrast, in the adult hepatocyte model (FGC4), mutation of the HNF1 site destroyed the majority of activity, whereas that of the HNF6 site had a smaller effect. In all hepatic lines, the simultaneous mutation of both sites compromised promoter activity more severely than single mutations. These mutations had no effect upon promoter activity in NIH3T3 cells, but they abolished transactivation by HNF1β and HNF6 (data not shown).

The effects of combinations of transactivators upon promoter activity in fibroblasts are depicted in Fig. 4B. It can be seen that HNF1α is a more potent transactivator than HNF1β and that HNF6 is more robust than OC-2. When used in combination, HNF1α with HNF6 produced an additive enhancement, whereas HNF1β with HNF6 gave activity similar to that of HNF6 alone. The effect of OC-2 was not additive with that of HNF1 family members, although together with HNF1α the effect was higher than with either alone. We conclude that both sites are indeed functional in the context of the HNF4α7 promoter and that members of the HNF1 and HNF6 families are active, with the strongest contribution being observed for HNF6 and HNF1α.

In Vivo Role of HNF6/OC-2 in HNF4α7 Expression in the Developing Liver—Targeted mutation of the HNF6 gene has been described (56), and that of the OC-2 gene has been obtained recently. E 12.5 livers of both types of mutants as well as of the double mutant HNF6−/− OC-2−/− (Fig. 5) were examined by quantitative real-time RT-PCR for HNF4α transcripts originating from the P1 or P2 promoter. In no case were the transcript levels of HNF4α1 affected, but the HNF4α7 transcripts were reduced by two-thirds in the double mutant, without being affected in the single mutants. This reduction in HNF4α7 transcripts amount was also observed at E 13.5 and 15.5 (not shown). This attests that the Onecut family indeed plays a central role in P2 promoter regulation in vivo and demonstrates redundancy between HNF6 and OC-2 in activation of the HNF4α7 gene.

Four Kilobase Pairs of the HNF4α7 Promoter Region Is Sufficient to Drive Expression of a Reporter Gene in the Stomach, Intestine, and Pancreas, but Not the Liver, of Transgenic Embryos—To test whether the region containing the two conserved elements (Fig. 1C) is sufficient to ensure faithful expres-

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6 F. Clotman, manuscript in preparation.
sion of HNF4α7 in vivo, a fragment containing the 4.1-kb upstream from the ATG was fused to an nls-lacZ reporter, injected into mouse zygotes, and embryos harvested at E 17.5 were stained for β-galactosidase activity. Fig. 6A shows the stained viscera of a transgenic (left) and wild-type (right) embryo. No staining was obtained in tissues outside of the abdominal cavity (data not shown). Intense staining of the intestinal loops and pancreas of the transgenic embryo is clearly visible, as is the total absence of staining in the liver, whereas in the nontransgenic embryonic background staining is present in the intestine. However, because the bacterial β-galactosidase activity is targeted to the nucleus, the transgene-directed staining has a punctate rather than homogeneous appearance, making it possible to distinguish transgenic staining from endogenous background (Fig. 6B, b). Fig. 6B shows higher magnifications of the stained tissues (above) compared with the wild-type control (below). In addition to intestine (b), the stomach (c) shows punctate staining throughout the glandular region, and the pancreas (c) presents intensely stained clusters of cells that correspond to the ducts (acini were not stained on cryosections; data not shown).

In no case was staining of the liver observed, either in toto or on liver cryosections, and gallbladder, kidney, and spleen were also negative (not shown). At first sight these results imply that the 4.1-kb fragment contains the elements sufficient to direct expression to the stomach, the intestine, and the pancreatic ducts, but not to the liver. Alternatively, because quantitative analysis of HNF4α7 expression levels in these tissues has never been reported, it could be imagined that expression levels in the liver are below the sensitivity level of the staining. To address this question, real-time RT-PCR was employed to determine transcript levels of the two HNF4α isoforms in embryonic and adult mice.

Expression Levels of P2 and P1 Promoter-directed Transcripts during Liver Development and in Different Tissues—To determine levels of hepatic HNF4α1 and HNF4α7 transcripts during liver development, quantitative real-time RT-PCR was employed. In addition, in E 17.5 embryos, transcripts corresponding to the two isoforms were also evaluated for each of the X-gal-stained tissues of Fig. 6.

Fig. 7A shows the results obtained for hepatic transcripts corresponding to the two isoforms, normalized to β-actin. HNF4α1 transcripts are present at E 12.5 and increase thereafter in an exponential fashion nearly until birth, reaching a maximum in adult liver at a level that is 40-fold greater than that at E 12.5. Hepatic HNF4α7 transcripts increase with time during development (3-fold between E 12.5 and E 17.5) followed by a decline to very low values in the adult. HNF4α7 transcript levels are always lower than those of HNF4α1. This contradicts previous RT-PCR results, but the latter had been obtained with rat primers for which the α1 sequence contains mismatches with the mouse sequence leading to underestima-

**Fig. 5.** Oncut factors are essential for HNF4α7 expression in vivo in embryonic liver. Shown are HNF4α1 and α7 transcript amounts in HNF6−/−, OC-2−/−, and HNF6−/− OC-2−/− E 12.5 livers relative to wild-type (wt) after normalization to β-actin, as determined by quantitative real-time PCR analysis (means of three independent experiments, each using two independent samples for each genotype).

**Fig. 6.** The 4.1-kb sequence upstream from the ATG is sufficient to drive reporter expression of a transgene in vivo in pancreas, stomach, and intestine but not in the liver. A, abdominal cavity of in toto X-gal-stained E 17.5 embryos cut down the middle. A, heart; b, liver; c, lung; d, pancreas. Bar, 1 mm. B, higher magnification of dissected organs. Punctate nuclear transgene activity can be detected in the pyloric region of the stomach (a), the intestine (b), and in pancreatic ducts (c) on in toto stained transgenic versus wild-type embryos (d–f). The whole intestine, from duodenum to colon, expressed the transgene (not shown). Abbreviations: d, part of duodenum; p, pancreas; py, pyloric region of the stomach. Bar in a, c, d, and f is 0.5 mm; in b and e, 0.2 mm.
developmental evolution of the ratio of HNF4$\alpha$/H9251 transcripts in the liver, intestine, stomach, and pancreas, relative to the liver. Among tissue-expressing tissues, liver is the lowest, but only by a factor of 3 compared with the highest (pancreas). In addition, the ratio of HNF4$\alpha$ to $\alpha7$ transcripts was calculated, revealing that liver and intestine show similar ratios corresponding to similar abundance of total HNF4$\alpha$ transcripts, whereas stomach contains low transcript levels, all derived from P2. The pancreatic levels are intermediate. Among all of the HNF4$\alpha$-expressing tissues, only stomach contains exclusively $\alpha7$ transcripts, and only kidney exclusively presents $\alpha1$ transcripts.

The expression patterns of the reporter gene in the transgenic embryos indicate that only a small fraction of the cells in stomach, pancreas, and intestine, which show moderate to strong expression of the reporter gene, are positive for $\beta$-galactosidase staining. In contrast, it would be expected that in the liver the transcripts would be expressed by all hepatocytes, which occupy at this stage about 60% of the tissue (57). Taking together this anticipated widespread expression in the liver and the fact that the expression levels are always weak, it is possible that the expression level per cell would not be within the limits of detection, although we do not think that this is the case. However, taking together the promoter analysis, the results from HNF6/OC-2 double knock-out embryos, and quantitation of P2 expression levels, we favor the interpretation that the tissue-specific factors important for expression in the liver have indeed been identified and that additional elements necessary for maximal expression remain to be identified. In conclusion, with the exception of the liver, the staining of the tissues is a faithful reflection of the HNF4$\alpha$7 expression pattern in E17.5 embryos.

HNF4$\alpha$ Represses P2 Promoter Activity by a Mechanism That Is Not Dependent upon HNF4$\alpha$ Binding Sites—Given the high levels of HNF4$\alpha$ expression in postnatal liver, we investigated whether this isoform could participate in the postnatal repression of HNF4$\alpha$7. We show here that HNF4$\alpha$1 inhibits P2 promoter activity in transfection assays. Indeed, using concentrations of HNF4$\alpha$1 expression vector demonstrated to activate the apoB reporter (Fig. 8B), we found a 2.2–3.7-fold repression of P2 promoter activity, using either the 516- or 221-bp promoter in MMH-ep cells (Fig. 8A). Under these conditions, the effect should not be a reflection of squelching (58, 59). ChIP assays have shown binding of HNF4$\alpha$ protein at the P2 promoter in adult hepatocytes (60), but no consensus binding site was detected in this region (Fig. 3A). Thus, to define the site through which the repression operates, two approaches were used. First, the −516 bp–122 bp region was scanned for HNF4$\alpha$ binding activity, using a series of 44–47-bp-long oligonucleotides overlapping by 22–35 bp, in the presence of liver nuclear extracts with or without anti-HNF4$\alpha$ antiserum, as well as with extracts from transfected COS-7 cells expressing an HNF4$\alpha$1 expression vector. No bands were observed that could be displaced with the antibody (Fig. S4), implying that the region is devoid of sites that bind HNF4$\alpha$ in our EMSA conditions, even with weak affinity.

Second, the 516-bp promoter fragment harboring or not mutations of the HNF1 and/or HNF6 sites was transfected into MMH-ep cells in the presence or absence of HNF4$\alpha$1 expression vector, to test whether repression could act via indirect binding of HNF4$\alpha$1 at these sites, acting like a cofactor through
another LETF. The mutated constructs continued to be repressed by HNF4α1, suggesting that it acts via a yet to be identified factor bound to the P2 promoter (Fig. 8C).

The HNF4α1 P2 Promoter Is Repressed in Vivo by HNF4α1—To determine the physiological roles of the HNF4α isoforms originating from different promoters, we have prepared recombinant mice that express only one isoform, but under control of the two cognate promoters (α1-only and α7-only mice).5 To achieve this, it was necessary to replace the first exon coding sequence of one isoform with the first exon coding sequence of the other, without modifying the sequence transcribed from the latter promoter. To monitor transcriptional activity at the two promoters, a forward primer in each 5′-UTR sequence had to be used. Fig. 9A presents a scheme of these mutations.

RNA was prepared from adult liver of wild-type, homozygous α7-only, and homozygous α1-only mice. Semiquantitative radioactive RT-PCR that measures only transcription from P2 was carried out. Fig. 9B demonstrates that when HNF4α1 is replaced by HNF4α7, transcription from the P2 promoter is markedly higher than in livers that do express HNF4α1 (wild-type and α1-only). Although there is variability in P2 transcripts from one mouse to another, there is no overlap between α7-only and the two other genotypes. These results strongly suggest that the P2 promoter is actually repressed by HNF4α1 in the adult liver.

**DISCUSSION**

The use of alternative promoters is a prevalent mechanism (around 9% of genes in mice and up to 18% in humans (61)) employed for the differential regulation of genes with complex spatio-temporal expression patterns. In some cases, like that of HNF4α, the resulting protein is modified by alternative promoter usage, producing an additional level of complexity. Moreover, HNF4α is not an exception among nuclear receptors because alternative promoters have been reported for the peroxisome proliferator-activated receptor γ (62) and the retinoic acid receptor genes (63). Furthermore, cross- and autoregulatory loops are a commonly admitted feature among transcription factors implicated in ontogenesis and maintenance of differentiated cell status and in particular in the liver via the LETF (64–68). Here we have analyzed regulation of the distal promoter P2 of the HNF4α gene which gives rise to HNF4α7 transcripts. In the pancreas, the P2 promoter appears to be regulated by the homeoproteins Pdx1 and HNF1α (18, 24), and an important role for the HNF1 binding site within this promoter is suggested by the cosegregation of maturity-onset diabetes of the young in a Czech family with a mutation in this site (69). Here we describe that the Onecut factors HNF6/OC-2 and the HNF1 homeoproteins are involved in activation of the HNF4α7 gene in the developing liver and that the HNF4α1 isoform can mediate repression of P2, as depicted in the model for regulation of both the P1 and P2 promoters in Fig. 9C.

To detect regulatory cis-elements in the vicinity of exon 1D, we performed DNase I-hypersensitive site analysis. This led us to focus on the proximal promoter region because the only site found to be associated with P2 activity was revealed in this region. Ex vivo analysis of the P2 promoter required cell lines that could be used as faithful models of early liver development stages and expressing the transcription factors suspected to be important for P2 activity, which was the case for the nontransformed bipotential MMH-1 cells and their epithelial committed progeny MMH-ep. In transfection assays, a highly conserved 516-bp promoting element encompassing the hypersensitive site conferred high reporter activity. EMSA and site-directed mutagenesis analysis identified within this element two functional binding sites for LETF, HNF1 and HNF6.

We showed here that both HNF1α and HNF1β bind to the HNF1 site, yet only HNF1α was a strong transactivator of the P2 fragment, contrary to previous results (18). However, mutation of the site only weakly affected reporter activity in MMH cells expressing HNF1β exclusively or low levels of HNF1α. This argues against a critical role of the HNF1 binding site in these models. However, we do not exclude a role of HNF1 factors in vivo on P2 activity. Given that HNF1β in the embryo is expressed at the onset of liver ontogenesis and becomes almost exclusively restricted to biliary cells by E 14 (70, 71), we suggest that if this factor played a role upon regulation of the P2 promoter, it should be mainly restricted to early stages of development. In line with a lack of such a role, HNF4α7 expression is not affected either in HNF1β−/− embryonic stem cell-derived embryoid bodies (72) or in HNF6−/− embryonic liver in which HNF1β is significantly down-regulated (70).
contrast with HNF1β, the more potent transactivator HNF1α is highly expressed in hepatocytes from E 14.5 onward (49), and mutation of its binding site within the promoter construct provoked a strong decrease in reporter activity in FGCl4 cells, which express high levels of HNF1α. In addition, ChIP assays have shown that in adult hepatocytes HNF1α is bound to promoter P2 (60) but see also contradictory results in (24)) in accordance with our observation of retention of a prominent hypersensitive site in adult liver. Thus, we conclude that the HNF1 site may be functional in liver development via binding of HNF1α and probably not HNF1β, at least after E 14.5 when HNF1α is highly expressed in the liver. In the adult liver, the low HNF4α7 transcript levels could result from the stimulatory effect of HNF1α being strongly attenuated by the effect of repressor factors (see below).

The HNF6 consensus site in the P2 promoter can bind HNF6 and OC-2, both of which are expressed from the onset of liver ontogenesis (51, 54, 55). HNF6 is then retained in hepatocytes during development, although it becomes highly enriched in biliary cells (70). The HNF6 binding site is transactivated by both factors, and its mutation caused a decrease in P2 activity in hepatic cell lines expressing either OC-2 or HNF6. In addition to this ex vivo argument, the essential role of the Onecut factors was demonstrated in vivo by the use of HNF6−/− OC-2−/− double knock-out E 12.5 embryos where a drastic reduction in HNF4α7 transcripts in the liver was observed. Furthermore, because neither of the single deficiencies showed decreased α7 expression, we conclude that the Onecut factors act redundantly. The residual α7 expression may be attributed to HNF1 factors because the HNF1α transcript amount is not affected in double mutant livers.6

Although the model depicted in Fig. 9C shows that some of the same factors appear to regulate expression of P1 and P2, clear differences in promoter response have been observed. In contrast to HNF4α7, we observed no differences in the HNF4α1 transcript levels in any of the Onecut knock-out embryos, even though HNF6 has been shown to stimulate the P1 promoter in vitro (29, 51). In addition, the only HNF4α transcripts affected in HNF1β−/− embryonic stem cell-derived embryoid bodies are from P1 and not P2 (72). A synergistic induction of the P1 promoter has been reported in transactivation assays for HNF1α and HNF6 (29), whereas in the case of P2 the effect is additive. Taken together, these observations imply that the P1 and the P2 promoters of the HNF4α gene are regulated by distinct mechanisms during liver development, either by different combinations of factors within the LETF network and/or by the same factors using different activation modes.

We then asked whether the identified elements were sufficient to reproduce in vivo the expression pattern of HNF4α7. A 4.1-kb fragment upstream from the HNF4α7 ATG was shown to be sufficient to drive nls-lacZ expression in E 17.5 embryos in the intestine, pancreas, and stomach, tissues that express the endogenous α7 transcripts at this stage. With the exception of the liver, the transgene expression profile is consistent with the RT-PCR results. Expression of the transgene in the intestine could result from stimulation of the P2 promoter by Cdx2 (73), for which putative binding sites were found in silico, and by HNF1α, which have been described to act together in intestine-specific gene transcription (74, 75). In the pancreas, the candidate regulators HNF1β and HNF6 are both expressed in duct cells and/or their likely precursors, HNF6 acting upstream from HNF1β in this tissue (51, 55, 56, 71, 76). The lack of transgene expression in the fetal liver is likely the result of a lack of cis-elements that could be essential to visualize transgene activity in this tissue, where the HNF4α7 expression level is low. Nevertheless, the ensemble of data argues that a critical regulatory region and relevant binding sites have been identified for this tissue. Still, we cannot exclude the possibility that the α7 transcript levels per hepatocyte are below the detection threshold of the transgene product. The quantitative results obtained from real-time PCR showed in addition that, contrary to previous results (21), in the liver these transcripts are always less abundant than α1 transcripts from E 12.5 onward.

No information has been available on mechanisms involved in repression of α7 expression in the adult liver. Here we provide in vitro and in vivo evidence that HNF4α1 is implicated in repression of P2 activity. We showed that overexpression of HNF4α1 in α7-expressing hepatic cells leads to significant down-regulation of a reporter gene driven by the P2 promoter, at concentrations that activate a control reporter construct. This repression appears to be independent of a direct binding of HNF4α to DNA within the proximal promoter region because we were not able to identify a complex by EMSA, although we know from ChIP assays (60) that HNF4α is bound to the P2 promoter in adult hepatocytes. This suggests an indirect binding of HNF4α1 via interaction with another factor. We tested whether an interaction between HNF4α1 and HNF1 and/or Onecut factors could mediate the repression using P2 constructs mutated for these binding sites. Indeed, a functional interaction of HNF4α1 with HNF1α (77, 85) and other proteins such as ACBP, the repressor SHP, or transforming growth factor-β downstream effector Smad3 has been demonstrated (78–80). However, HNF1α and HNF6 do not seem to be involved because the repression was not substantially relieved by mutation of their binding sites. Hence, the sites and intervening protagonists implicated in the repression mechanism remain to be identified.

The repression by HNF4α1 was reproduced in recombinant mice engineered to express in all HNF4α-positive tissues only one kind of isoform under control of both the P1 and P2 promoters. Thus, in mice in which α1 is replaced by α7 (so that no α1 is produced at all), we found a large increase of transcript levels expressed from P2 in the adult liver, strongly suggesting that α1 protein is also a repressor of the P2 promoter in vivo. These results led us to propose that the presence of a functional AF1 in HNF4α1 compared with the AF1 lacking α7 isoform is implicated in the repressive effect. In transfection assays, HNF4α1 is able to mediate a stronger repression than α7, via recruitment of SMRT, which may be caused by an increased ability of the SMRT-HNF4α1 complex to recruit histone deacetylase activity (20). Taking together the results of P2 promoter activity in the α7-only mice and the quantitative PCR analyses of HNF4α1 and α7 expression in the liver at different stages of development, the HNF4α1-mediated repression of the P2 promoter may begin when the level of HNF4α1 transcripts exceeds a critical threshold and is essentially total in the adult liver where the HNF4α1 levels are very high. After birth, this repressor effect would chronically override the stimulatory effects mediated by the Onecut and HNF1 factors. This active mechanism, strictly HNF4α1 concentration-dependent, is entirely consistent with retention of the P2 hypersensitive site in adult liver and with results of ChIP analysis implicating binding of HNF4α1 to the P2 promoter in hepatocytes (60).

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Expression of the α7 Isoform of Hepatocyte Nuclear Factor (HNF) 4 Is Activated by HNF6/OC-2 and HNF1 and Repressed by HNF4 α1 in the Liver
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