

Threshold Levels of Hepatocyte Nuclear Factor 6 (HNF-6) Acting in Synergy with HNF-4 and PGC-1 α Are Required for Time-Specific Gene Expression during Liver Development

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During liver development, hepatocytes undergo a maturation process that leads to the fully differentiated state. This relies at least in part on the coordinated action of liver-enriched transcription factors (LETFs), but little is known about the dynamics of this coordination. In this context we investigate here the role of the LETF hepatocyte nuclear factor 6 (HNF-6; also called Onecut-1) during hepatocyte differentiation. We show that HNF-6 knockout mouse fetuses have delayed expression of glucose-6-phosphatase (*g6pc*), which catalyzes the final step of gluconeogenesis and is a late marker of hepatocyte maturation. Using a combination of in vivo and in vitro gain- and loss-of-function approaches, we demonstrate that HNF-6 stimulates endogenous *g6pc* gene expression directly via a synergistic and interdependent action with HNF-4 and that it involves coordinate recruitment of the coactivator PGC-1 α . The expression of HNF-6, HNF-4, and PGC-1 α rises steadily during liver development and precedes that of *g6pc*. We provide evidence that threshold levels of HNF-6 are required to allow synergism between HNF-6, HNF-4, and PGC-1 α to induce time-specific expression of *g6pc*. Our observations on the regulation of *g6pc* by HNF-6 provide a model whereby synergism, interdependency, and threshold concentrations of LETFs and coactivators determine time-specific expression of genes during liver development.

Liver development is regulated by a dynamic network of liver-enriched transcription factors (LETFs) (5, 8, 34, 35, 51). During this process the two major cell types in the liver, hepatocytes and cholangiocytes, derive from common precursor cells called hepatoblasts (9, 37). Cholangiocytes delineate bile ducts (19, 38), and hepatocytes perform the liver metabolic functions. Differentiating hepatocytes organize into cords and undergo a maturation process during which they progressively acquire their functions. This maturation process relies on the coordinated action of the LETFs, which interact by mutually controlling their expression and by coordinately regulating target genes (1, 10, 15). Transcriptional regulators, including the LETFs, recruit coactivators harboring chromatin-modifying activities to their target promoters. In addition, the activity of LETFs and hepatocyte maturation are dependent on extracellular signals (14). Time- and tissue-specific gene expression is thus achieved through the concerted action of tissue-specific factors and coactivators on their target genes, under the control of extracellular cues. Genome-wide analyses identified target promoters of several LETFs in normal or regenerating adult liver, and mechanisms for coordinated action of LETFs have been proposed with regard to their respective target promoter occupancies (22, 48). In contrast, the molecular dynamics of LETF action in the developing embryo, and how a given transcription factor can exert different functions at different developmental stages, are not well known.

Hepatocyte nuclear factor 6 (HNF-6, also called Onecut-1),

a member of the Onecut family of transcriptional activators, belongs to the LETF network (4, 22, 27, 33). It is expressed in the hepatic epithelial cells, i.e., in the hepatoblasts and then in the cholangiocytes and hepatocytes, where it persists in adults. HNF-6 regulates the decision of hepatoblasts to differentiate toward the biliary or hepatocyte lineage, by controlling genes that modulate the response to transforming growth factor β (7). It is also required for bile duct morphogenesis and indirectly regulates B lymphopoiesis (3, 6). In hepatocytes HNF-6 controls the expression of other LETFs (4, 22, 27, 33), and in vivo data indicate that it regulates genes involved in detoxification and glucose metabolism (17, 42, 46). Finally, it has been shown to stimulate transcription by sequence-specific recruitment of the coactivator CBP or p300/CBP-associated factor (pCAF) to its target genes (16).

In the present work we investigated how HNF-6 controls gene expression in differentiating hepatocytes during development. We found that HNF-6 synergistically and interdependently cooperates with HNF-4 α 1 (referred to below as HNF-4), a LETF critical for hepatocyte differentiation (11, 20, 23, 39, 47), and with the coactivator PGC-1 α to induce time-specific expression of the catalytic subunit of glucose-6-phosphatase (*g6pc*). We propose a model whereby synergism, interdependency, and threshold concentrations of LETFs and coactivators control time-specific expression of genes during hepatocyte differentiation.

MATERIALS AND METHODS

Animals. All mice, raised in our animal facilities, were treated according to the principles of laboratory animal care of the local Animal Welfare Committee.

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hnf6 knockout mice and mice homozygous for allele *hnf4*^{tm1.1Gonz} (*hnf4*^{fl/fl}) were obtained as described elsewhere (11, 12).

Plasmids. pCMV-HNF6, pCMV-Flag.HNF6, pCMV-Myc.HNF6, pRL-138, and pEF-GFP have been described elsewhere (16, 24, 25). pCMV-HNF4 and pCMV-Flag.HNF4 were gifts from S. A. Duncan, and pSVSPORT-PGC1 α and pSVSPORT-HA.PGC1 α were gifts from B. M. Spiegelman. To construct pG6PC(-207/+46)luc, the mouse *g6pc* promoter was PCR amplified (with primers 5'-GCTCTAGAAATAATGGCTCTGCCAATG-3' and 5'-CGGGATCCA GCCCGTGCAGTGAGTCCAG-3') and subcloned into pBluescript. The fragment was excised by BamHI/XbaI restriction and inserted at the corresponding sites in pGL3-MCS (Promega).

Reverse transcription-PCR (RT-PCR) and real-time quantitative RT-PCR (qRT-PCR). Total RNA was extracted from embryonic liver, neonate liver, bipotential murine embryonic liver (BMEL) cells, and NIH 3T3 cells with TriPure reagent (Roche). RNA (1 μ g) was reverse transcribed as described previously (27). For semiquantitative PCR, the number of cycles corresponded to the mid-logarithmic phase. Primer sequences were 5'-ACCTTCACCAATGACT CCTATG-3' and 5'-ATGATGACTGCAGCAAATCGC-3' for the mRNA coding for TATA-binding protein (*tbp*), 5'-TGTCTGTGATTGCTGAC-3' and 5'-GTAGAAGTGACCATAACATAG-3' for *g6pc*, 5'-CTCAGCTGGCAGCA TGGGGTG-3' and 5'-AACAGCTCTCCACGTTGACG-3' for the mRNA coding for phosphoenolpyruvate carboxylase (*pepck*), and 5'-GAATTCAGA GCCTCTCGCCCTCTC-3' and 5'-CAGGAGCTGTCCGTGGC-3' for *hnf6*. Real-time quantitative PCR was performed with the SYBR Green PCR Core kit (Eurogentec) on a MyIQ thermal cycler (Bio-Rad). Threshold cycles were transformed into copy numbers according to the standard calibration curve. The absolute copy number for each mRNA was normalized to the absolute copy number for the mRNA coding for β -actin (*β -act*). Primer sequences were 5'-T CCTGAGCGCAAGTACTCTGT-3' and 5'-CTGATCCACATCTGCTGGAA G-3' for *β -act*, 5'-TTCCAGCGCATGTCCGGCGCTC-3' and 5'-GGTACTAGT CCGTGGTCTCTC-3' for *hnf6*, 5'-TGCAGCCAAAGACTCTGTATG-3' and 5'-CATCAAGTTCAGAAAGGTCAAG-3' for *pgc1 α* ; 5'-GAAAATGTGCAGGT GTTGACCA-3' and 5'-AGCTCGAGGCTCCGTAGTGT-3' for *hnf4*, and 5'-TACATCATCGCCAGTGTGTG-3' and 5'-AGCTGCAGAGTGCCAAT GATC-3' for the mRNA coding for aquaporin-1 (*aqp1*). *g6pc* mRNA was quantified using the Master Mix for Probe Assays (Eurogentec). Primer sequences were generated using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Primer sequences were 5'-GCCAGAGGGACTTCTGGT-3' and 5'-TCGGAGACTGGTTCAACCTC-3'; the Oligold (Eurogentec) 6-carboxy-fluorescein (FAM)/6-carboxytetramethylrhodamine (TAMRA) probe sequence was 5'-GCCCGTATTGGTGGTCTCGG-3'.

Cell culture and transfection. NIH 3T3 cells were grown in Dulbecco's modified Eagle medium supplemented with 10% bovine serum (Gibco), 5 mM sodium pyruvate, and antibiotics. BMEL cells were cultured in monolayers or as aggregates as described previously (27, 41). For endogenous *g6pc* transcriptional stimulation assays (see Fig. 1C, 2B and C, 4A and C, and 7B), 10⁵ BMEL cells or 7 \times 10⁴ 3T3 cells were seeded on 12-well plates (TPP) 18 h prior to transfection. Cells were transfected with 4 μ l of Lipofectamine 2000 (Invitrogen) and the following amounts of expression vectors: 750 ng of pCMV-Flag.HNF6, 750 ng of pCMV-Flag.HNF4, and 1.5 μ g of pSVSPORT-PGC1 α . The total amount of plasmid DNA was adjusted to 3 μ g by adding pEF-GFP. For dose-dependent stimulation of *g6pc* expression (see Fig. 8A), we used the indicated amounts of pCMV-Myc.HNF6 and pCMV-Flag.HNF4, and the total DNA amount was adjusted to 1 μ g using pEF-GFP. In all transcriptional stimulation assays, RNA was extracted 24 h after transfection. For luciferase assays, 5.5 \times 10⁴ BMEL cells were seeded on 24-well plates (TPP) and transfected with 2 μ l of Lipofectamine 2000, 400 ng of pG6PC(-207/+46)luc, 15 ng of pRL-138 as an internal control, and 10 ng of pCMV-Flag.HNF6 and/or pCMV-Flag.HNF4 α , and the total amount of plasmid DNA was adjusted to 465 ng by adding pEF-GFP. Luciferase activity was measured 24 h after transfection using the Dual-Luciferase reporter assay system (Promega) and a DLR-ready TD-20/20 luminometer (Turner Design). For small interfering RNA (siRNA) experiments, BMEL cells were transfected with 4 μ l of Lipofectamine 2000 in 12-well plates with 750 ng of pCMV-Flag.HNF6, 750 ng of pCMV-Flag.HNF4, and 1.5 μ g of pEF-GFP in the presence of 100 nM of a pool of control siRNAs or a pool of siRNAs directed against mouse *pgc1 α* (Dharmacon).

Isolation of *hnf4*^{-/-} BMEL cells from livers of *hnf4*^{fl/fl} mice. Mice homozygous for allele *hnf4*^{tm1.1Gonz} (*hnf4*^{fl/fl}), in which exons 4 and 5 are flanked by loxP sites (11), were crossed with heterozygous *hnf4*^{fl/+} animals. At embryonic day 14.5 (e14.5), embryos were removed and livers dissected. Clonal cell lines from both *hnf4*^{fl/fl} and *hnf4*^{fl/+} livers were isolated as described previously (41). To obtain *hnf4* gene disruption, the cells were electroporated with expression vectors coding for an eGFPnlsCre-fusion protein and containing a neomycin resistance

cassette. The cells were plated at clonal density. Following selection in G418, isolated colonies were picked, expanded, and subjected to Southern blotting to confirm deletion efficiency. Full details will be given in a future article by G. P. Hayhurst et al.

CoIP. Wild-type BMEL cells (1.25 \times 10⁶) were seeded on 10-cm plates (TPP), grown for 16 h, and transfected with 60 μ l of Lipofectamine 2000, 10 μ g of pCMV-Flag.HNF6, and/or 10 μ g of pSVSPORT-HA.PGC1 α , and the total amount of DNA was adjusted to 20 μ g by adding pEF-GFP. On the same day, 50 μ l of protein G-Sepharose beads per sample was washed four times with ice-cold coimmunoprecipitation assay (CoIP) binding buffer (20 mM Tris [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton X-100, and protease inhibitors) and resuspended in 300 μ l CoIP binding buffer. A rabbit polyclonal anti-hemagglutinin (anti-HA) antibody (3 μ l per sample; Sigma) was added to the mixture, which was incubated overnight at 4°C with constant rotation. Beads were then washed in CoIP binding buffer. Twenty-four hours after transfection, cells were washed four times in ice-cold phosphate-buffered saline supplemented with protease inhibitors. Cells were then scraped in 300 μ l of ice-cold CoIP binding buffer. Following a 10-s sonication, lysates were centrifuged at 4°C and 14,000 \times g for 5 min to remove insoluble cell debris. Inputs (50 μ l of the supernatant) were stored at -80°C. One-half of the rest of the supernatant was frozen for other applications, and the other half was immediately brought to a volume of 400 μ l by adding CoIP binding buffer. The bead-antibody solution (50 μ l) was added to the lysates, and the mixture was incubated for 2 h at 4°C with constant rotation. Beads were then washed three times in CoIP wash buffer (20 mM Tris [pH 7.4], 500 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton X-100, and protease inhibitors) and three times in CoIP binding buffer. Beads were boiled for 5 min, and extracts were immediately loaded onto sodium dodecyl sulfate-polyacrylamide gels for Western blotting with a mouse monoclonal anti-Flag M2 antibody (Sigma). The secondary antibody was horseradish peroxidase-conjugated mouse IgG True Blot (eBioscience). The secondary antibody was detected by chemiluminescence (Amersham Pharmacia Biotech).

ChIP. Chromatin immunoprecipitation (ChIP) was performed essentially as described previously (10, 25). For HNF-6 and HNF-4 binding assays (see Fig. 3), 1.5 \times 10⁶ BMEL cells were transfected with 60 μ l of Lipofectamine 2000, 7 μ g of pCMV-Flag.HNF6, and/or 7 μ g of pCMV-HNF4 or 7 μ g of pCMV-Flag.HNF4 and/or 7 μ g of pCMV-HNF6. The total amount of plasmid DNA was adjusted to 28 μ g by adding pEF-GFP. Binding on the *g6pc* promoter was measured by qPCR. Primer sequences were 5'-CTGGGTATAGGGCGGAAA GAC-3' and 5'-GGCCCCAAGACCTCTAATCAT-3' for 28S genomic DNA and 5'-GTTTTTGTGTGCTGTTTGTG-3' and 5'-GCTATCAGTCTGCCTTG C-3' for the *g6pc* promoter. The Oligold (Eurogentec) FAM/TAMRA probe sequence for the *g6pc* promoter was 5'-TTGAGTCCAAAGATCAGGGC-3'. Enrichments were calculated as 2^{exp} - [(G6PC_{ChIP} - 28S_{ChIP}) - (G6PC_{Input} - 28S_{Input})]. For the PGC-1 α recruitment assay (see Fig. 5), BMEL cells were transfected as described above. One or more of the following plasmids were used: pCMV-HNF6 (7 μ g), pCMV-HNF4 (7 μ g), and pSVSPORT-PGC1 α (14 μ g). Primer sequences were as indicated above, and binding of PGC-1 α was here visualized by an agarose gel. The antibody was monoclonal anti-Flag M2 (10 μ g; Sigma), anti-PGC-1 (10 μ g; sc-13067; Santa Cruz Biotechnologies), or anti-tubulin (2 μ g; Sigma). One-fifth of each ChIP eluate was used in each PCR.

Mouse fetal liver electroporation. The procedure used was an adaptation of the recently described whole-embryo electroporation (26). Livers from e12.0 embryos were dissected in Hanks balanced salt solution (HBSS) buffer (Gibco). Each liver was microinjected (20 pulses, 6 ms per pulse) with a mixture consisting of 20% carboxymethyl cellulose, 0.02% trypan blue stain (Gibco), and 0.5 μ g/ μ l plasmid DNA in HBSS buffer using a Picospritzer III (Parker Instrumentation) microinjector. Plasmid DNA mixtures consisted of 0.5 μ g/ μ l pEF-GFP alone (control explants) or a mix consisting of 0.1 μ g/ μ l pCMV-Flag.HNF6, 0.1 μ g/ μ l pCMV-Flag.HNF4, 0.2 μ g/ μ l pSVSPORT-PGC1 α , and 0.1 μ g/ μ l pEF-GFP. Livers in HBSS were then electroporated (3 pulses of 100 V, 50 ms per pulse, 1-s delay between each pulse) with an ECM 830 Electro Square Porator (BTX Genetronics) and filter cultured for 48 h at 37°C in BMEL cell culture medium prior to RNA extraction.

RESULTS

HNF-6 controls the expression of glucose-6-phosphatase during hepatocyte differentiation. HNF-6 is expressed in hepatocytes throughout liver development and after birth. It is known to control hepatocyte-specific expression of genes in the

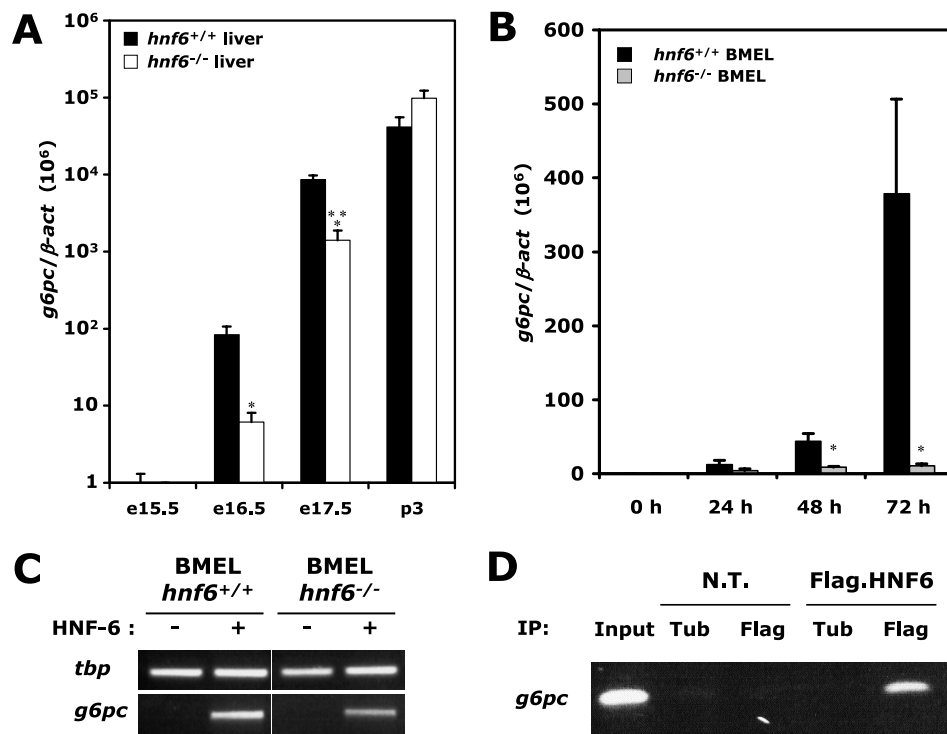


FIG. 1. HNF-6 controls expression of *g6pc* during hepatocyte differentiation. (A) Developmental regulation of *g6pc* expression is impaired in the *hnf6*^{-/-} liver. Total-liver RNA was extracted from wild-type (*hnf6*^{+/+}) and *hnf6*^{-/-} fetuses at different stages of development, and *g6pc* mRNA concentrations were measured by qRT-PCR. Data are expressed as the ratio of *g6pc* mRNA levels to β -act mRNA levels (means \pm standard errors of the means; $n \geq 4$ for each genotype). Significant differences are indicated (*, $P < 0.05$; ***, $P < 0.001$). Note the logarithmic scale. (B) Impaired *g6pc* expression in in vitro differentiating *hnf6*^{-/-} BMEL cells. Wild-type and *hnf6*^{-/-} BMEL cells were cultured in aggregates (38) to induce differentiation toward the hepatocyte lineage. RNA was extracted from those cells before culturing or after 24 h, 72 h, or 120 h of the aggregate culture. *g6pc* mRNA concentrations were measured as for panel A (means \pm standard errors of the means; $n \geq 3$ for each genotype). Significant differences between wild-type and *hnf6*^{-/-} values are indicated (*, $P < 0.05$). (C) HNF-6 induces *g6pc* mRNA expression in undifferentiated embryonic liver cells. Wild-type and *hnf6*^{-/-} BMEL cells were transfected with a vector coding for HNF-6, and *g6pc* mRNA was amplified by RT-PCR 24 h after transfection. We used the mRNA coding for TATA-binding protein (*tbp*) as a control. (D) HNF-6 binds endogenous *g6pc* promoter. BMEL cells were transfected with a vector encoding Flag-tagged HNF-6 and were submitted to a ChIP directed against the Flag epitope 24 h after transfection. A fragment spanning the -207-to-+46 region of *g6pc* was amplified by PCR. Nontransfected cells (N.T.) and a ChIP against tubulin (Tub) were used as negative controls. The input corresponds to a PCR product obtained from untreated chromatin representing 10% of the amount of chromatin used in each ChIP.

adult (17, 22, 33, 42, 46) and to control hepatoblast differentiation at early stages of liver development (7), but its function in differentiating hepatocytes, i.e., prior to birth, is not well characterized. Therefore, we compared the expression of genes in *hnf6*^{+/+} and *hnf6*^{-/-} livers at various stages of development and found that glucose-6-phosphatase (*g6pc*) expression was abnormal in HNF-6-deficient livers (Fig. 1A). Indeed, *g6pc* expression started around e16.5 in *hnf6*^{+/+} livers and increased sharply until postnatal day 3. In contrast, in *hnf6*^{-/-} livers at e16.5, *g6pc* expression was reduced about 10-fold, and it reached normal levels only at postnatal day 3. Therefore, HNF-6 controls the time-dependent stimulation of *g6pc* expression.

To rule out the possibility that the control of *g6pc* expression depends on nonhepatic cells, we used cell lines derived from e14.5 *hnf6*^{+/+} and *hnf6*^{-/-} livers (27). These BMEL cells behave like undifferentiated hepatoblasts in that they express the complete set of LETFs and can be induced to differentiate toward either the hepatocytic or the biliary lineage (41). Quantitative RT-PCR revealed that undifferentiated BMEL cells did not express detectable amounts of *g6pc* mRNA (Fig. 1B).

When induced to differentiate toward the hepatocytic lineage by culture in aggregates, *hnf6*^{+/+} and *hnf6*^{-/-} BMEL cells started to express *g6pc*, but this expression was reduced in *hnf6*^{-/-} BMEL cells, thereby mimicking in vivo liver development.

We next verified if HNF-6 could directly stimulate *g6pc* expression in embryonic cells. Indeed, previous studies have shown that HNF-6 binds in vivo to the *g6pc* gene promoter in the adult human liver (22) and activates a *g6pc* promoter-luciferase reporter construct in transiently transfected HepG2 cells (40). Transient transfection of undifferentiated *hnf6*^{+/+} and *hnf6*^{-/-} BMEL cells with a HNF-6 expression vector stimulated endogenous *g6pc* expression (Fig. 1C), and ChIP experiments showed that this was associated with binding of HNF-6 to the *g6pc* promoter (Fig. 1D). These experiments validated the BMEL cells as a model with which to study the control of *g6pc* expression by HNF-6.

From this set of data we concluded that HNF-6 is required to allow *g6pc* to become expressed at normal levels at the appropriate stage of hepatocyte differentiation. This control

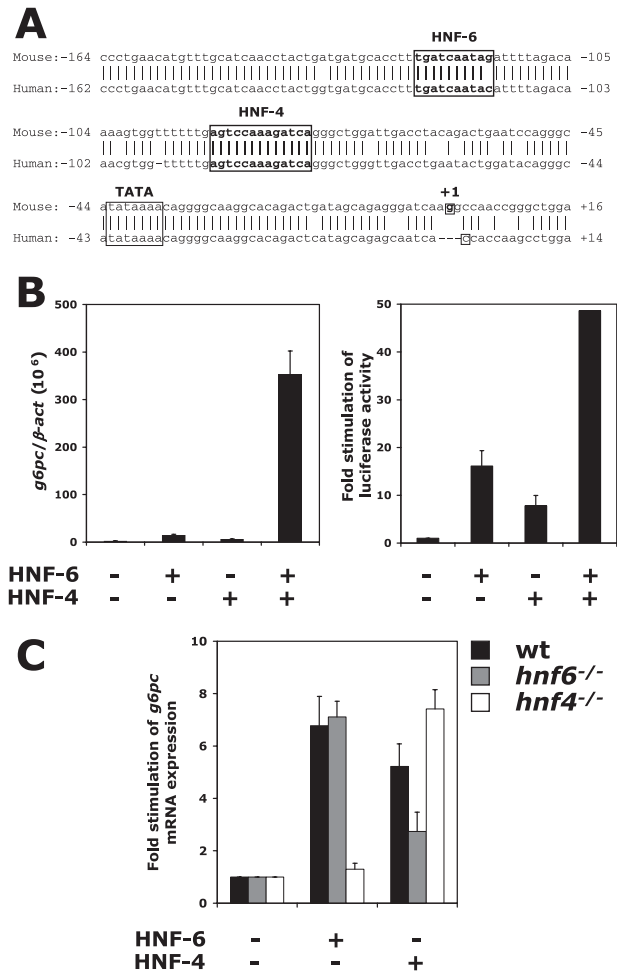


FIG. 2. HNF-6 and HNF-4 regulate *g6pc* expression in a synergistic and interdependent way. (A) Alignment of the human and murine *g6pc* gene promoters. Binding sites for HNF-6 and HNF-4, as well as the TATA box and the transcription initiation site, are indicated. (B) HNF-6 and HNF-4 synergistically stimulate *g6pc* expression. (Left) BMEL cells were transfected with combinations of vectors encoding HNF-6 and HNF-4, and *g6pc* mRNA was quantified by qRT-PCR 24 h after transfection. Data are expressed as the ratio of *g6pc* mRNA levels to β -act mRNA levels (means \pm standard errors of the means for at least four independent transfections performed in duplicate). (Right) HNF-6 and HNF-4 can synergistically stimulate the *g6pc* promoter. A reporter plasmid containing the *g6pc* promoter (-207 to $+46$) upstream of the firefly luciferase gene was transfected into BMEL cells together with combinations of vectors coding for HNF-6 and HNF-4, and luciferase activity was measured 24 h after transfection. Firefly luciferase activity was normalized to *Renilla* luciferase activity (internal control) and was expressed as *n*-fold induction of basal promoter activity in control, GFP-transfected cells (means \pm standard errors of the means; $n \geq 3$). (C) Mutual requirement for HNF-6 and HNF-4 in transcriptional control of *g6pc* expression. Wild-type, *hnf6*^{-/-}, and *hnf4*^{-/-} BMEL cells were transfected with vectors coding either for HNF-6 or for HNF-4, and *g6pc* mRNA concentrations were measured 24 h after transfection. Values are expressed as relative stimulation of *g6pc* mRNA expression (means \pm standard errors of the means; $n \geq 4$).

mechanism is independent of nonhepatic cells and is associated with binding of HNF-6 to the *g6pc* promoter.

HNF-6 and HNF-4 stimulate *g6pc* expression in a synergistic and interdependent way. HNF-4, a LETF that is critical for hepatocyte differentiation, regulates *g6pc* expression in vitro by

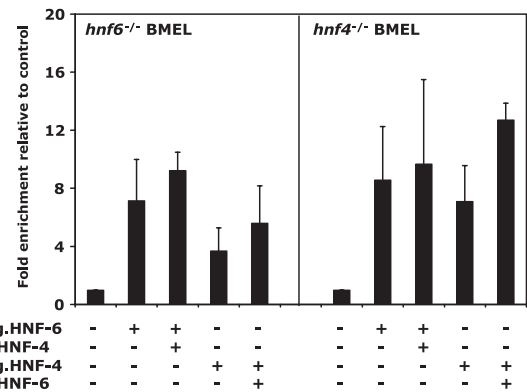


FIG. 3. Independent binding of HNF-6 and HNF-4 to the *g6pc* promoter. *hnf6*^{-/-} (left) and *hnf4*^{-/-} (right) BMEL cells were transfected with the indicated combinations of expression vectors and were subjected to ChIP directed against the Flag epitope 24 h after transfection. Binding of Flag-tagged proteins was visualized by qPCR amplification of the *g6pc* promoter. Enrichments relative to the control were calculated as indicated in Materials and Methods. Data are means \pm standard errors of the means from at least three independent experiments.

binding to several sites on the *g6pc* promoter (30). It also binds to the *g6pc* promoter in the adult human liver (22). The conserved HNF-4 binding site at -90 relative to the transcription initiation site is of particular importance, since its mutation causes a dramatic decrease in *g6pc* promoter activity (2). Those observations and the fact that the HNF-4 binding site at -90 is located close to the HNF-6 binding site at -124 (Fig. 2A) led us to hypothesize that HNF-4 and HNF-6 cooperate to regulate *g6pc* expression. To investigate this possibility, we transfected undifferentiated BMEL cells with HNF-4 and HNF-6 expression vectors and measured endogenous *g6pc* mRNA by qRT-PCR. The results showed that HNF-6 or HNF-4 alone stimulated endogenous *g6pc* gene expression but that the two factors together exerted a strong synergistic effect (Fig. 2B, left panel). These data were extended by transfecting BMEL cells with a luciferase reporter construct containing the -207 -to- $+46$ *g6pc* promoter region, which harbors the HNF-4 and HNF-6 binding sites. HNF-4 and HNF-6 synergistically stimulated this construct in transient transfection experiments (Fig. 2B, right panel), indicating that the -207 -to- $+46$ region of the *g6pc* promoter can mediate a synergistic effect of the two factors.

Functional synergy between HNF-4 and HNF-6 may reflect their mutual requirement to stimulate the *g6pc* gene. To test this hypothesis, we used wild-type, *hnf6*^{-/-} (27), and *hnf4*^{-/-} (this paper; see Materials and Methods) BMEL cells. We transfected the three cell lines with HNF-6- or HNF-4-expressing vectors and measured endogenous *g6pc* mRNA levels. As in Fig. 2B, each of these two factors stimulated *g6pc* expression in wild-type cells (Fig. 2C). However, HNF-6 failed to stimulate *g6pc* expression in the absence of HNF-4 (*hnf4*^{-/-} cells), and HNF-4 did not significantly stimulate *g6pc* in the absence of HNF-6 (*hnf6*^{-/-} cells). Taken together, our data demonstrate that HNF-6 and HNF-4 synergistically and interdependently stimulate *g6pc* promoter activity.

Independent binding of HNF-6 and HNF-4 to the *g6pc* promoter. We next investigated the molecular mechanism underlying the interdependence between HNF-4 and HNF-6 and

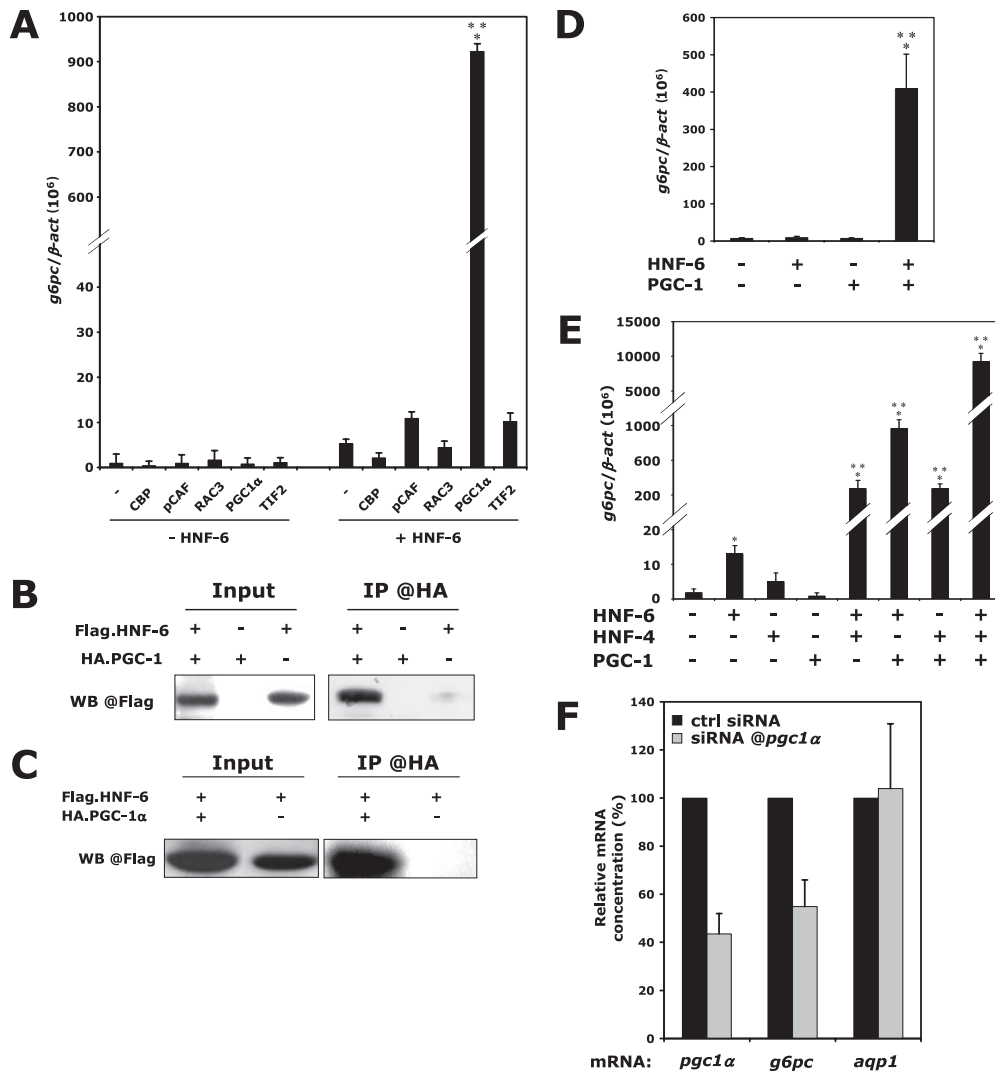


FIG. 4. HNF-6 and HNF-4 synergistically stimulate *g6pc* expression through recruitment of the coactivator PGC-1 α . (A) PGC-1 α is a coactivator of HNF-6 in stimulation of the *g6pc* gene. *hnf6*^{-/-} BMEL cells were transfected with vectors coding for the indicated coactivators, in the absence or the presence of a HNF-6-coding vector, and *g6pc* mRNA levels, expressed relative to β -act mRNA levels, were quantified by qRT-PCR 24 h after transfection (means \pm standard errors of the means; *n* = 3). Significant differences relative to HNF-6-mediated stimulation of *g6pc* are indicated (***, *P* < 0.001). (B) HNF-6 physically interacts with PGC-1 α . Protein extracts of BMEL cells expressing Flag.HNF-6 and/or HA.PGC-1 α were subjected to an anti-HA coimmunoprecipitation. The presence of Flag.HNF-6 in the eluate was revealed by anti-Flag Western blotting (WB). (C) HNF-6/PGC-1 α interaction does not require HNF-4. *hnf4*^{-/-} BMEL cells were transfected and treated as for panel B. (D) Functional interaction between HNF-6 and PGC-1 α is HNF-4 independent. *hnf4*^{-/-} BMEL cells were transfected with combinations of expression vectors encoding HNF-6 and PGC-1 α . *g6pc* mRNA concentrations were measured by qRT-PCR 24 h after transfection. Results are means \pm standard errors of the means from at least four independent transfections. Significant differences are indicated (***, *P* < 0.001). (E) PGC-1 α amplifies HNF6- and HNF-4-mediated stimulation of *g6pc* mRNA expression. BMEL cells were transfected with all possible combinations of vectors coding for HNF-6, HNF-4, and PGC-1 α , and *g6pc* mRNA levels, expressed relative to β -act mRNA levels, were quantified by qRT-PCR 24 h after transfection (means \pm standard errors of the means; *n* \geq 4). Significant differences relative to control, GFP-transfected cells are indicated (*, *P* < 0.05; ***, *P* < 0.001). (F) PGC-1 α is involved in the synergy between HNF-6 and HNF-4. BMEL cells were transfected with vectors coding for HNF-6 and HNF-4, together with control siRNAs or siRNAs directed against *pgc1 α* . Relative mRNA concentrations were measured 24 h after transfection. Aquaporin-1 (*aqp1*) mRNA was used as a negative control. Values are expressed relative to those for control siRNA-transfected cells, which were set at 100% (means \pm standard errors of the means; *n* = 5).

asked if it is associated with cooperative binding of HNF-6 and HNF-4 to their cognate sites on the *g6pc* promoter. To test this hypothesis, we performed ChIP experiments on *hnf6*^{-/-} and *hnf4*^{-/-} BMEL cells transfected with combinations of vectors encoding either Flag-tagged HNF-6 and untagged HNF-4 or Flag-tagged HNF-4 and untagged HNF-6. We immunoprecipitated the chromatin of those cells with an antibody directed

against the Flag epitope. Figure 3 shows that HNF-6 and HNF-4 bound the *g6pc* promoter in an independent manner. Indeed, Flag.HNF-6 was detected on the *g6pc* promoter in *hnf4*^{-/-} BMEL cells, and the addition of excess HNF-4 did not improve HNF-6 binding. We obtained similar results for HNF-4: it bound the *g6pc* promoter in *hnf6*^{-/-} BMEL cells, and excess HNF-6 did not significantly enhance HNF-4 bind-

ing. These results indicate that the two proteins bind the *g6pc* promoter independently of each other. Hence, the functional synergy and interdependence between HNF-6 and HNF-4 do not rely on interdependent binding.

HNF-6 and HNF-4 recruit and synergize with PGC-1 α . To investigate if HNF-6 and HNF-4 could synergize by recruiting a common coactivator, we first looked for factors that can coactivate *g6pc* expression with HNF-6. We expressed various coactivators, namely, CBP, pCAF, RAC-3, PGC-1 α , or TIF-2, in *hnf6*^{-/-} BMEL cells, either in the presence or in the absence of exogenous HNF-6 (Fig. 4A). In the absence of HNF-6, none of these coactivators stimulated *g6pc* expression. In contrast, in the presence of HNF-6, PGC-1 α dramatically stimulated endogenous *g6pc* gene expression, thereby demonstrating a functional interaction between the two proteins. Such an interaction was supported by coimmunoprecipitation experiments. Indeed, protein extracts from *hnf6*^{-/-} BMEL cells transfected with vectors coding for Flag.HNF-6 and for HA.PGC-1 α were immunoprecipitated using an anti-HA antibody, and the immunoprecipitated proteins were detected by Western blot analysis with an anti-Flag antibody. The results (Fig. 4B) showed that Flag.HNF-6 was coprecipitated with HA.PGC-1 α , demonstrating a physical interaction between HNF-6 and PGC-1 α . These data identify PGC-1 α as a new coactivator of HNF-6.

Since PGC-1 α is a well-known coactivator of HNF-4, including in the regulation of *g6pc* expression (21, 29, 49), we tested whether the physical and functional interactions between HNF-6 and PGC-1 α depend on the presence of HNF-4. We performed CoIP experiments on *hnf4*^{-/-} BMEL cells transfected with Flag.HNF-6 and/or HA.PGC-1 α . The results (Fig. 4C) show that HNF-6 and PGC-1 α were able to physically interact in a cellular context devoid of HNF-4. In addition, functional interaction between the two proteins did not require HNF-4, since PGC-1 α was a strong coactivator of HNF-6 for the stimulation of *g6pc* expression in *hnf4*^{-/-} cells (Fig. 4D). Finally, we were unable to coprecipitate HNF-6 and HNF-4 in living cells, suggesting that those two factors do not interact directly (data not shown). These results demonstrate that HNF-6 and PGC-1 α are able to interact in the absence of HNF-4.

Given the ability of both HNF-6 and HNF-4 to interact with PGC-1 α , we hypothesized that this coactivator is involved in the synergy between HNF-6 and HNF-4 on the *g6pc* promoter. To test this hypothesis, we transfected BMEL cells with combinations of vectors encoding HNF-6, HNF-4, and PGC-1 α and measured *g6pc* mRNA concentrations by qRT-PCR. Figure 4E shows that the three proteins synergistically stimulated *g6pc* expression: whereas PGC-1 α had no effect on *g6pc*, it enhanced the effects of HNF-6 and HNF-4, and a very strong effect was observed when the expression of HNF-6, HNF-4, and PGC-1 α was combined.

To investigate if endogenous PGC-1 α is required for the synergy between HNF-6 and HNF-4, we transfected BMEL cells with HNF-6 and HNF-4 expression vectors in the presence of control or anti-*pgc1 α* siRNAs. As shown in Fig. 4F, anti-*pgc1 α* siRNA caused a 55% decrease in the *pgc1 α* mRNA concentration, and this was associated with a 50% decrease in the HNF-6/HNF-4 synergy for *g6pc* expression. This effect was specific, since an unrelated mRNA coding for aquaporin-1 was

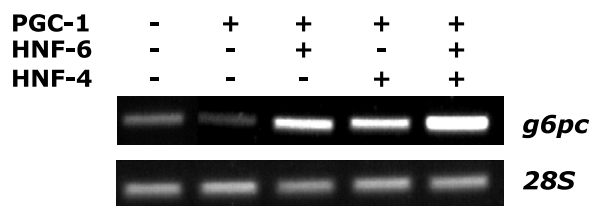


FIG. 5. HNF-6 and HNF-4 recruit PGC-1 α to the *g6pc* gene promoter. BMEL cells were transfected with a PGC-1 α -expressing vector, either in the presence or in the absence of vectors coding for HNF-6 or HNF-4, and were subjected to ChIP using an antibody against PGC-1 α . Binding of PGC-1 α to the *g6pc* promoter was detected by PCR amplification of the coimmunoprecipitated -207-to-+46 *g6pc* promoter fragment.

unaffected by anti-*pgc1 α* siRNA. These data indicate that PGC-1 α is required for the transcriptional synergy between HNF-6 and HNF-4.

To gain further insight into the molecular mechanism by which PGC-1 α participates in the synergism between HNF-6 and HNF-4, we verified if the latter factors corecruit PGC-1 α to the *g6pc* promoter. We transfected BMEL cells with combinations of vectors encoding HNF-6, HNF-4, and PGC-1 α and immunoprecipitated their chromatin with an anti-PGC-1 α antibody. Figure 5 shows that PGC-1 α alone was not associated with the *g6pc* promoter, consistent with the lack of transcriptional effect induced by overexpression of PGC-1 α alone (see Fig. 4A, D, and E). When HNF-6, HNF-4, or both were cotransfected with PGC-1 α , the *g6pc* promoter was readily immunoprecipitated by the anti-PGC-1 α antibody. This indicates that HNF-6 and HNF-4 promote the recruitment of PGC-1 α to the *g6pc* promoter.

Developmental regulation of *g6pc* expression in the liver. In the fetal liver, *g6pc* expression starts late in development, i.e., around e16.5 (Fig. 1). In contrast, the expression of HNF-6, HNF-4, and PGC-1 α , which we measured by qRT-PCR, starts earlier (Fig. 6). Thus, despite the fact that these three factors are present and play a role at early stages of liver development, they cannot induce *g6pc* expression at these stages. Since expression of HNF-6, HNF-4, and PGC-1 α increases progressively during development (Fig. 6), we hypothesized that sufficient amounts of the three factors have to be present to induce expression of *g6pc*. To address this hypothesis, we reasoned that increasing the expression of HNF-6, HNF-4, and PGC-1 α in early liver would result in precocious expression of *g6pc*. To test this, we electroporated liver explants from e12.0 embryos, either with a green fluorescent protein (GFP)-coding vector or with a combination of vectors encoding HNF-6, HNF-4, and PGC-1 α . The electroporated explants were cultured for 2 days before extraction of RNA. qRT-PCR measurement of the *g6pc* mRNA concentration revealed that livers electroporated with HNF-6, HNF-4, and PGC-1 α expressed *g6pc*, whereas GFP-electroporated livers did not (Fig. 7A). This indicates that increased expression of HNF-6, HNF-4, and PGC-1 α in the embryonic liver can induce precocious expression of *g6pc*.

To test if this inductive effect of the HNF-6/HNF-4/PGC-1 α combination is liver specific, we tested NIH 3T3 fibroblasts and compared *g6pc* mRNA levels in cells transiently transfected with HNF-6-, HNF-4-, and PGC-1 α -coding plasmids with lev-

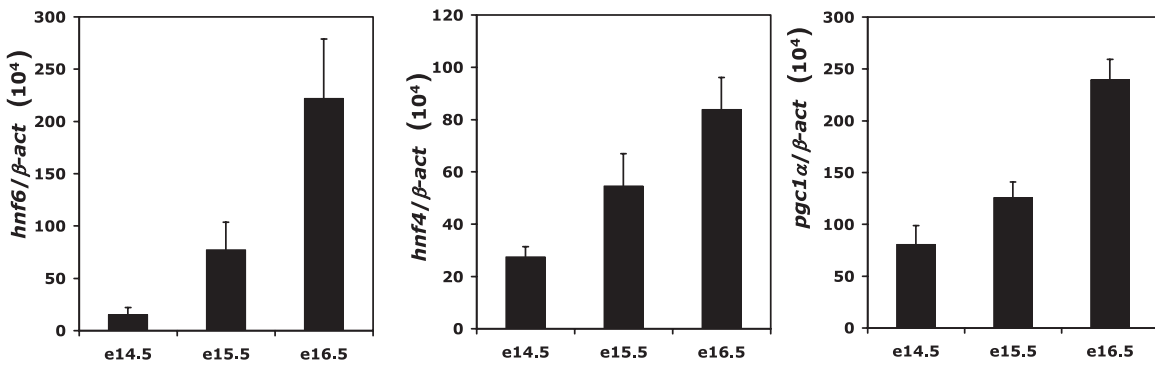


FIG. 6. Expression of *hnf6*, *hnf4*, and *pgc1 α* mRNAs increases in the liver between e14.5 and e16.5. Total RNA was extracted from wild-type embryonic livers at e14.5, e15.5, and e16.5, and *hnf6* (left), *hnf4* (center), and *pgc1 α* (right) mRNA levels were quantified by qRT-PCR and expressed relative to β -act mRNA levels (means \pm standard errors of the means; $n = 4$).

els in control cells transfected with a GFP-coding vector (Fig. 7B). *g6pc* mRNA levels were very low or undetectable in control NIH 3T3 cells, but high levels of *g6pc* mRNA were found after overexpression of HNF-6, HNF-4, and PGC-1 α . This effect did not result from induction of a hepatic differentiation program, since other markers of hepatic differentiation, namely, albumin, α -fetoprotein (*afp*), phosphoenolpyruvate carboxykinase (*pepck*), tyrosine aminotransferase (*tat*), and transthyretin (*ttr*), were not detected by RT-PCR (data not shown).

Taken together, our overexpression data in liver and NIH 3T3 cells suggested that threshold levels of HNF-6, HNF-4, and PGC-1 α are required to induce *g6pc* gene expression, regardless of the cellular context. This was tested by expressing increasing amounts of both HNF-6 and HNF-4 in NIH 3T3 fibroblasts (Fig. 8A), which express PGC-1 α but not HNF-6 or HNF-4 (data not shown). The results showed that low levels of HNF-6 (20 and 50 ng of expression vector) allow HNF-4 to

stimulate *g6pc* mRNA expression weakly, but this stimulation was not dependent on the dose of HNF-4. In contrast, at high HNF-6 concentrations (150 and 500 ng of expression vector), a strong and dose-dependent stimulation was obtained with HNF-4. These data indicate that threshold levels of HNF-6 are required to induce optimal stimulation of *g6pc* gene expression.

If this model is correct, one would expect that reduced expression of HNF-6 in the liver at the time when *g6pc* mRNA becomes detectable would result in impaired expression of *g6pc*. We compared *hnf6* and *g6pc* mRNA levels in e16.5 livers from wild-type, heterozygous *hnf6*^{+/-}, and homozygous *hnf6*^{-/-} embryos (Fig. 8B). We found that a 50% reduction of *hnf6* expression in *hnf6*^{+/-} livers suffices to reduce *g6pc* mRNA levels to those found in the absence of HNF-6. These data, combined with those above, led us to conclude that threshold levels of HNF-6 allow synergistic and time-specific expression of *g6pc* in the developing liver.

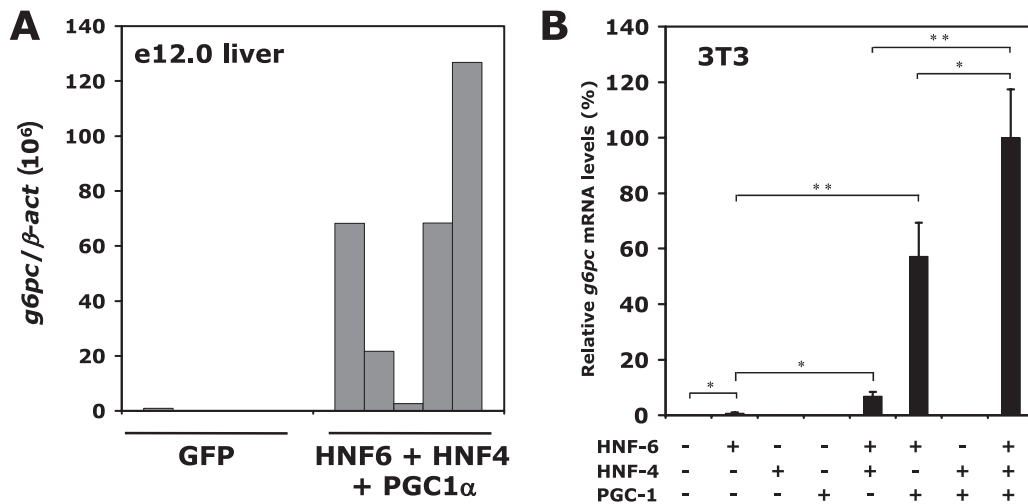


FIG. 7. HNF-6, HNF-4, and PGC-1 α can induce *g6pc* expression regardless of the cellular context. (A) Increased expression of HNF-6, HNF-4, and PGC-1 α in immature embryonic liver leads to precocious expression of *g6pc*. Livers from e12.0 mouse embryos were electroporated with the indicated vectors and filter cultured for 48 h. *g6pc* mRNA concentrations were measured by qRT-PCR. Results of five independent experiments are shown, and data are expressed as the ratio of *g6pc* mRNA levels to β -act mRNA levels. (B) The activity of the HNF-6/HNF-4/PGC-1 α complex is not tissue specific. NIH 3T3 fibroblasts were transfected with combinations of vectors coding for HNF-6, HNF-4, and PGC-1 α . *g6pc* mRNA concentrations were quantified by qRT-PCR 24 h after transfection. Results are expressed as percentages of maximal *g6pc* mRNA expression \pm standard errors of the means ($n = 3$). Significant differences are indicated (*, $P < 0.05$; **, $P < 0.01$).

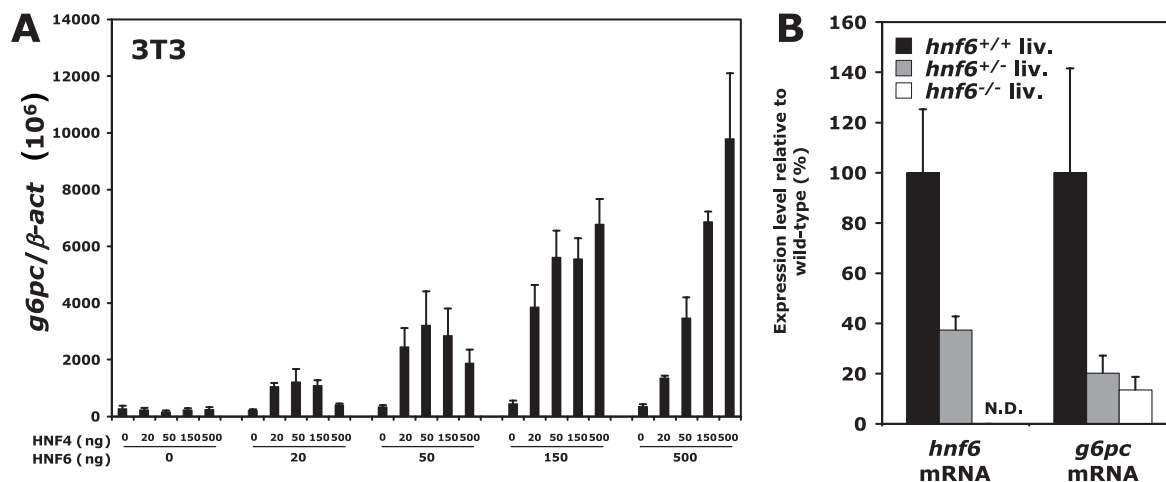


FIG. 8. Threshold levels of HNF-6 are required to induce stimulation of *g6pc* gene expression. (A) Dose-dependent stimulation of *g6pc* by HNF-4 requires threshold levels of HNF-6. NIH 3T3 fibroblasts were transfected with increasing amounts of HNF-6 and HNF-4 expression vectors as indicated, and *g6pc* mRNA levels, expressed relative to β -act levels, were measured 24 h after transfection. Data are means \pm standard errors of the means from three independent experiments. (B) Time-specific induction of *g6pc* in the developing liver requires an optimal concentration of HNF-6. *hnf6* and *g6pc* mRNA concentrations were quantified by qRT-PCR in e16.5 livers from *hnf6*^{+/+}, *hnf6*^{+/-}, and *hnf6*^{-/-} embryos (means \pm standard errors of the means; $n \geq 7$).

DISCUSSION

In the present study, we have shown that HNF-6 is required for proper time-specific regulation of *g6pc* in the liver. This occurs via a synergistic and interdependent action with HNF-4. The synergy and interdependence do not rely on synergistic or interdependent DNA binding but involve interaction with the coactivator PGC-1 α . Through a combination of in vitro and in vivo gain- and loss-of-function approaches, which focus on endogenous and not on transfected genes, we also provide arguments that support a model whereby time-specific expression of *g6pc* occurs only when a threshold level of HNF-6 in the presence of HNF-4 is reached.

The physical and functional interaction between PGC-1 α and HNF-6, which occurs on the *g6pc* gene, identifies PGC-1 α as a new coactivator of HNF-6. HNF-6 has been shown previously to interact with CBP and with pCAF (16, 31, 32, 36). Interestingly, CBP and pCAF had no or only a marginal effect on the HNF-6-mediated stimulation of *g6pc* mRNA expression. Also, PGC-1 α was unable to coactivate the *Foxa2* and *ttr* promoters with HNF-6 (data not shown). This highlights the promoter specificity of HNF-6-coactivator interactions. A similar promoter-specific HNF-4-coactivator interaction had been illustrated previously for HNF-4-SMRT interactions (44). On the other hand, PGC-1 α is a known coactivator of HNF-4 (49), and we now show that PGC-1 α is efficiently recruited to the *g6pc* promoter by HNF-6 and HNF-4, raising the possibility that a trimeric HNF-6/HNF-4/PGC-1 α complex is formed.

The mode of action of HNF-6 and HNF-4 on the *g6pc* promoter refines our knowledge of the coordinated regulation of common target genes by LETFs. Odom et al. (22) have proposed that such regulation would depend on coordinated binding of LETFs to a common target promoter. However, this model does not take into account the dynamics of such regulations. Our data add a new dimension to the model. Indeed, we show here that the mere presence of transcriptional activators is not sufficient to induce expression of a specific gene

but that this induction requires well-adjusted concentrations of the transcriptional activators. This observation is particularly relevant during development. Indeed, under normal conditions HNF-6 and HNF-4 control gene expression in the liver well before they induce *g6pc* gene expression (4, 6, 7, 20, 23). Our data show that the expression levels of HNF-6 and HNF-4 rise steadily during development and that up- or downregulation of HNF-6 or HNF-4 expression generates premature or delayed expression of the *g6pc* gene. Therefore, our data show that time- and tissue-specific expression of genes critically depends on the threshold level of transcriptional regulators that coordinately regulate common target genes.

The progressive rise in HNF-6 and HNF-4 concentrations during liver development (the HNF-4 α 1 isoform was studied throughout this work) has been documented earlier (6, 43), but to our knowledge, that of PGC-1 α before the perinatal period was not known (50). The coordinated regulation of *g6pc* expression during development by HNF-6, HNF-4, and PGC-1 α raises the question of their control. The known HNF-6 or HNF-4 regulatory elements are not sufficient to drive their expression during hepatocyte maturation (4, 28), and the developmental mechanisms governing PGC-1 α expression have not been analyzed. However, it was shown that signaling molecules originating from the hematopoietic cells in the embryonic liver promote hepatocyte maturation at the end of development. Indeed, oncostatin M-induced signaling in differentiating hepatocytes promotes morphological changes that resemble those associated with hepatocyte maturation; it also induces glycogen accumulation and the expression of several markers of hepatocyte maturation (13). Signaling molecules such as oncostatin M may thus contribute to allowing HNF-6, HNF-4, and PGC-1 α to reach threshold levels required to induce *g6pc* expression.

Our data also reveal that the transcription factor requirements for expression of *g6pc* differ before and after birth. In *hnf6*^{-/-} mice the expression of *g6pc* is perturbed during de-

velopment but reaches normal levels at birth, suggesting that HNF-6 is dispensable after birth. The difference between pre- and postnatal regulation of *g6pc* most likely reflects the critical metabolic role of *g6pc* in postnatal life, when it becomes essential for hepatic glucose production (45). In prenatal life, fetal blood glucose levels depend mainly on maternal supply, whereas after birth glycemia depends on a balance of food intake, glucose consumption, gluconeogenesis, and glycogenolysis. Hormones that control gluconeogenesis and glucose consumption exert a tight regulation of *g6pc* expression (18). Besides this essential hormonal regulation, postnatal liver-specific regulation must be maintained, and our data suggest that HNF-6 is not required in this process. HNF-4 is, together with PGC-1 α , a key mediator of hormonal control of *g6pc* expression (49). It has been shown to be essential for *g6pc* expression in the liver before birth (23), but to our knowledge, whether it is essential after birth has not yet been demonstrated through *in vivo* studies.

During fetal life, the hepatocytes progressively mature, and the expression of gluconeogenic genes at the end of gestation is a hallmark of this maturation process. The regulation of *g6pc* by HNF-6 positions this transcription factor as a hepatocyte maturation factor. HNF-6 is also required for glucokinase gene expression (17), indicating that HNF-6 is an important regulator of liver glucose metabolism. However, expression of other key enzymes of glucose metabolism, such as phosphoenolpyruvate carboxykinase and glycogen synthase 2, is not affected in the absence of HNF-6 (7) (data not shown). Therefore, HNF-6 participates in hepatocyte maturation and controls glucose metabolism at the transcriptional level but does not seem to drive metabolic pathways toward the anabolic or catabolic orientation.

In conclusion, our data suggest a model whereby the developmental regulation of hepatocyte differentiation relies, in part, on the synergistic action of LETFs and their coactivators on their target genes. Transcriptionally active complex formation requires threshold levels of transcription factors that allow cell- and time-specific regulation of gene expression.

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