

The Role of HNF-1 α in Controlling Hepatic Catalase Activity

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Received June 17, 1999; Accepted September 30, 1999

This paper is available online at <http://www.molpharm.org>

ABSTRACT

Mice deficient in hepatocyte nuclear factor 1 α (HNF-1 α) develop Laron dwarfism and non-insulin-dependent diabetes mellitus (Lee et al., 1998). Oxidative stress was present in the diabetic HNF-1 α -null mice. To understand the mechanism underlying the oxidative stress in HNF-1 α -null mice, we examined whether HNF-1 α deficiency affects the integrity of the cellular defense system against oxidative stress. The glutathione level and activities of superoxide dismutase and glutathione reductase in liver and other tissues examined were not affected by HNF-1 α deficiency. However, activities of cytosolic glutathione peroxidase and catalase, two enzymes responsible for detoxification of hydrogen peroxide within cells, were reduced spe-

cifically in liver of HNF-1 α -null mice. The mRNA and protein levels of hepatic catalase in HNF-1 α -null mice did not differ from those in normal mice. The loss of hepatic catalase activity in HNF-1 α -null mice is probably caused by an insufficient heme pool in liver cells, because the mRNA level of ferrochelatase, the enzyme that catalyzes the last step of heme biosynthesis, was significantly reduced in liver, and the daily hemin treatment restored partial catalase activity in liver of HNF-1 α -null mice. Furthermore, our results of cell transfection and luciferase reporter assay indicated that the mouse ferrochelatase promoter could be *trans*-activated directly by HNF-1 α .

Hepatocyte nuclear factor 1 α (HNF-1 α) is a liver-enriched homeodomain-containing transcription factor (Baumhueter et al., 1990; Blumenfeld et al., 1991; De Simone et al., 1991). It has an important role in regulating genes that are preferentially expressed in liver, such as those that encode for phenylalanine hydroxylase and albumin (Tronche et al., 1989; Pontoglio et al., 1996; Lei and Kaufman, 1998).

A role for HNF-1 α in controlling development, as well as glucose homeostasis, was established in our previous study by using an HNF-1 α -null mouse line (Lee et al., 1998). Although HNF-1 α is dispensable in embryonic development, it is essential in postnatal development and growth in mice. HNF-1 α plays an important role in regulating the hepatic *Igf-I* gene expression to maintain the circulating insulin-like growth factor I (IGF-I) level (Lee et al., 1998). The circulating IGF-I, produced mainly in the liver, mediates many of the growth factor functions in growth (Lowe, 1991). In addition, HNF-1 α is involved in controlling pancreatic insulin production to maintain a normal glucose level in blood (Dukes et al., 1998; Lee et al., 1998).

HNF-1 α deficiency elicits hyperglycemia in mice 2 weeks after birth and a phenotype reminiscent of non-insulin-dependent diabetes mellitus (Lee et al., 1998). Diabetes status in HNF-1 α -null mice, once developed, persists thereafter

(Lee et al., 1998). In animal models of diabetes or diabetic patients, long-term hyperglycemia causes vascular dysfunction and pathologies involving retina, glomeruli, peripheral nerves, and cardiovascular tissues, etc. (Giugliano et al., 1996; Zhang et al., 1997; Koya and King, 1998). It is documented that oxidative stress is present in the diabetes state and is caused by glucose autooxidation that generates reactive oxygen species (Freitas et al., 1997). If the elevated oxidative stress persists, it might lead to long-term vascular complications of diabetes (Low et al., 1997; Koya and King, 1998).

In this study, we have found that oxidative stress was increased with age in HNF-1 α -null mice. In HNF-1 α -null mice, oxidative stress might arise in a manner similar to that found in other diabetic animals, or it might have resulted from a defect in other metabolic systems. To understand the mechanism underlying the oxidative stress in HNF-1 α -null mice, we compared the ability of antioxidation of normal and HNF-1 α -null mice by carrying out activity assays for several antioxidant enzymes. Our studies indicate that activities of glutathione peroxidase (GPx) and catalase, two antioxidant enzymes involved in the cellular defense system against hydrogen peroxide (H₂O₂), were significantly reduced in liver but not in other tissues of HNF-1 α -null mice. In addition, we explore the possible molecular mechanism by which HNF-1 α deficiency decreases catalase activity specifically in liver. Our results suggest that the reduced catalase activity might

This work was supported in part by Research Grant NSC-88-2311-B-001-106 and NSC-89-2311-001-078 from the National Science Council of Taiwan.

ABBREVIATIONS: HNF-1 α , hepatocyte nuclear factor 1 α ; IGF-I, insulin-like growth factor I; GPx, glutathione peroxidase; FC, ferrochelatase; MDA, malonaldehyde; SOD, superoxide dismutase; C/EBP α , CCAAT enhancer binding protein; HNF4, hepatocyte nuclear factor 4; GR, glutathione reductase.

result from an insufficient heme pool in liver cells caused by the reduced gene expression for ferrochelatase (FC), the enzyme that catalyzes the last step of heme synthesis. Furthermore, analysis of the mouse FC promoter activity demonstrates that HNF-1 α is able to *trans*-activate the FC promoter in a cell-type-specific manner.

Materials and Methods

Mice. The HNF-1 α -null mice (Lee et al., 1998) and their heterozygous littermates, HNF-1 α (+/-), were kept in a sterile microisolator and maintained in a Specific Pathogen-Free animal facility at the Institute of Molecular Biology, Academia Sinica, Taiwan. For analyzing serum chemistry and collecting tissues, mice at indicated ages (see below) were euthanized by CO₂ asphyxiation. Blood was collected to obtain serum samples. Mice were then perfused with PBS through the heart, and the selected tissues were snap frozen in liquid nitrogen for use in later studies.

Serum Chemistry Analysis. Mouse serum taken from different developmental stages was analyzed in an auto-dry chemical analyzer (SP4410; Spotchem, Kyoto, Japan) to monitor hepatocyte function and serum glucose levels. Serum samples were also analyzed for the combined levels of malonaldehyde (MDA) and 4-hydroxyalkenals according to the method of Esterbauer and Cheeseman (1990).

Cellular Glutathione and Antioxidant Enzyme Assays. Frozen tissues were homogenized in various buffers with respect to the proceeding assays. For measuring cellular glutathione level (the reduced form), liver homogenate was extracted with 6% metaphosphoric acid and level of the reduced glutathione was measured using a glutathione assay kit (Calbiochem, San Diego, CA). For assaying the catalase activity, tissues were homogenized in 50 mM Tris · HCl, pH 7.0, 1% Triton X-100, and 250 μ g of total protein were assayed accordingly (Aebi, 1984). For assaying the activities of cellular GPx and superoxide dismutase (SOD), tissues were homogenized in 100 mM Tris · HCl, pH 7.5, at 4°C, centrifuged at 5000g, and the supernatant was assayed for both GPx and SOD activities using the respective assay kit (Oxis, Portland, OR). For measuring activities of glutathione reductase (GR), tissues were homogenized in 50 mM Tris · HCl, pH 7.0, 5 mM EDTA and were assayed accordingly (Carlberg and Mannervik, 1985).

Measurement of Tissue Protoporphyrin. Liver protoporphyrin was extracted and measured, based on the method of Grandchamp et al. (1980). Briefly, liver was homogenized in 10 mM Tris · HCl, pH 7.0, and 150 μ l of homogenate was extracted with 3 ml of 1 M perchloric acid/methanol (1:1, v/v). After centrifugation to remove precipitate, the extracts were measured in a spectrofluorometer (SLM8000C; SLM Instruments Inc., Urbana, IL) at various excitation wavelengths with emission wavelength set at 595 or 605 nm.

Hemin Treatment. Hemin stock solution was prepared by dissolving 36 mg of hemin chloride (Sigma, St. Louis, MO) in 0.4 ml of 0.5N NaOH and buffering with 0.5 ml of 1 M Tris · HCl, pH 8.0. Dilution was made in PBS just before injection. Three to four 6-week-old mice of either genotype were injected i.p. with hemin at 15 mg/kg of body weight/day for 4 consecutive days. The control littermates of the same genotype received only PBS. Twelve hours after the last injection, mice were perfused with PBS, and their livers were removed and snap frozen in liquid nitrogen.

Western Blot Analysis. Livers were homogenized in 50 mM Tris · HCl, pH 7.0, 1% Triton X-100. Fifty micrograms of liver protein were electrophoresed in a 10% denaturing polyacrylamide-bis gel and transferred to a nitrocellulose membrane. The membrane was blocked in PBS containing 5% (w/v) nonfat dry milk and 0.2% Tween 20, incubated with the primary goat IgG against catalase (The Binding Site, Ltd., Birmingham, UK) and developed by using peroxidase-labeled donkey anti-goat IgG (The Binding Site, Ltd.).

RNA Extraction and Northern Blot Analysis. Frozen mouse tissues were homogenized in TRIzol RNA reagent (Life Technologies-BRL), and total RNA was isolated according to the manufacturer's protocol. Total RNA (15 μ g) was denatured, electrophoresed, transferred to a nylon membrane, and probed with cDNA probes as described previously (Lee et al., 1998). Mouse cDNA fragments for cellular GPx, catalase, and FC were obtained by reverse transcription-polymerase chain reaction of total liver RNA. Briefly, 2 μ g of total RNA was reverse-transcribed at 42°C in 20 μ l of reaction mixture containing 0.2 μ g of oligo-dT primer and 5 U of avian myeloblastosis virus reverse transcriptase. Three microliters of the cDNA product was used in the subsequent polymerase chain reaction amplification with primer sets designed to amplify the GPx, catalase, and FC coding regions. The primer sets used to amplify were as follows: GPx forward primer, 5'-CCCTAGGAGAATGGCAAG; GPx backward primer, 5'-CAGAGTGCAGCCAGTAATCACCAG; catalase forward primer, 5'-GCTGAAGTTGAACAGATG; catalase backward primer, 5'-GTCATCAGCGTGAGTCTG; FC forward primer, 5'-GACCGAGACCTCATGACACTTC; FC backward primer, 5'-GACAGTTCAGACTCAACTGCGTGGAGC.

Promoter-Luciferase Reporter Constructs and Expression Vectors. Mouse FC genomic Bac clones containing the entire coding and 5' upstream region were obtained by screening a mouse genomic Bac library (Genome Systems, St. Louis, MO) with the FC cDNA fragment mentioned above. A 3-kilobase *Hind*III to *Sma*I genomic fragment containing the upstream promoter and 47 base pairs of the 5' untranslated regions was subcloned into pGEM7Z vector (Promega, Madison, WI) to generate pG7Z.FC. The region of -279 to +47 and -918 to +47 were then excised out by *Xba*I/*Xho*I and *Sac*I/*Xho*I digestion of pG7Z.FC, respectively, and subcloned into the promoterless luciferase reporter pGL2.basic vector (Promega). The resulting plasmids were named pFC(-279/+47) and pFC(-918/+47). pFC(-2920 to +47) was then generated by inserting the 2-kilobase *Sac*I fragment isolated from pG7Z.FC into pFC(-918/+47) at the *Sac*I site. The plasmid pCH110 was purchased from Pharmacia Biotech (Piscataway, NJ). pCH110 contains a functional *LacZ* gene encoding β -galactosidase under the control of the simian virus 40 early promoter for expression and was therefore used as an internal marker for normalizing luciferase activity between different transfection experiments described below. The plasmid pMex contains the murine osteosarcoma virus early promoter for expression in mammal cells. pMex and the CCAAT enhancer binding protein α (C/EBP α) expression vector, pMex.C/EBP α , were obtained from Dr. Peter Johnson at Frederick Cancer Research and Development Center-National Cancer Institute (FCRDC-NCI, Frederick, MD). The hepatocyte nuclear factor 4 (HNF-4) expression plasmid pMex.HNF-4 was generated by inserting the HNF-4 coding region, isolated from the plasmid pSG5.HNF-4, into pMex.

Transient Transfection and Luciferase Activity Assay. HepG2 and CV-1 cells were transfected essentially as described elsewhere (Lee et al., 1994). Cells were grown to 50% confluence in 60-mm cell culture dishes. One microgram of promoter-luciferase plasmid DNA, 0.5 μ g of internal standard, the β -galactosidase reporter pCH110, and 4 μ g of expression vector DNA for HNF-1 α (or others where indicated) were mixed with 125 μ l of 0.25 M CaCl₂, and 125 μ l of 50 mM HEPES, 280 mM NaCl, 1.75 mM NaH₂PO₄, pH 7.1, was added drop-wise. The mixture was added to the cells after 15 min of incubation. Cells were harvested 40 to 48 h after transfection. Cells were lysed and the cell lysates were then used directly for both luciferase and β -galactosidase activity measurements by using the Dual-Light system (Tropix, Inc., Bedford, MA). The luciferase- or β -galactosidase-initiated light signals were measured in a microplate luminometer (Model TR717; PE Biosystems, Foster City, CA). The relative luciferase activity was calculated based on the activity of β -galactosidase internal standard.

Statistical Analysis. Student's *t* test was performed with unpaired data for the control and HNF-1-null mouse samples of the same age. The data presented in the figures are the means \pm S.E. of

at least three samples. *P* values indicate the significance of HNF-1 α -Null mice with respect to control littermates.

Results

Oxidative Stress Is Present in HNF-1 α -Null Mice.

HNF-1 α -null mice develop hyperglycemia around 2 weeks after birth, and their diabetic status persists thereafter (Fig. 1; Lee et al., 1998). In humans, it is documented that, because of glucose autooxidation, which generates reactive oxygen species, oxidative stress is present in the diabetes state (Brownlee et al., 1988; Freitas et al., 1997; Koya and King, 1998). To determine whether oxidative stress also exists in HNF-1 α -null mice, lipid peroxidation, the marker of oxidative stress, was monitored in the HNF-1 α -null mice at different ages. Serum samples were analyzed for the levels of MDA and 4-hydroxyalkenals, which are end products derived from the breakdown of polyunsaturated fatty acids and related esters and are the commonly used markers of lipid peroxidation (Esterbauer and Cheeseman, 1990). As shown in Fig. 1, the combined level of MDA and 4-hydroxyalkenals in the serum of 3-month-old HNF-1 α -null mice was significantly

higher than that in the heterozygous littermates, indicating that a high degree of oxidative stress exists in HNF-1 α -null mice. In addition, oxidative stress was increased with age in HNF-1 α -null mice, despite the similar degree of hyperglycemia present in every stage (except for the age of 2 weeks) examined (Fig. 1). At the age of 6 months, the HNF-1 α -null mice had levels of MDA and 4-hydroxyalkenals 10 times higher than the heterozygous littermates (Fig. 1). Mice carrying the heterozygous mutant *Hnf-1 α* allele do not differ from their wild-type littermates in serum glucose levels (Lee et al., 1998) or the degree of lipid peroxidation at the age of 6 months and earlier, as indicated in Fig. 1 (serum glucose and lipid peroxidation levels of wild-type littermates were not shown).

HNF-1 α Deficiency Reduces Activities of Glutathione Peroxidase and Catalase in Livers. With hyperglycemia, autooxidation of glucose, as seen in diabetic patients, might lead to the oxidative stress found in HNF-1 α -null mice. However, oxidative stress could also arise from other mechanisms, such as a defect in the antioxidation defense system. HNF-1 α is a transcription factor and is important in controlling expression of many essential genes, such as IGF-I and albumin (Tronche et al., 1989; Lee et al., 1998). A role for HNF-1 α in regulating expression of genes involving in anti-oxidation has not yet been reported. To examine whether a cellular defense system against antioxidative stress is impaired because of HNF-1 α deficiency, the integrity of antioxidant and antioxidant enzymes in tissues of HNF-1 α -null mice were analyzed. As shown in Fig. 2A, the activities of both hepatic SOD and GR in HNF-1 α -null mice did not differ from those in control heterozygous littermates at all stages examined. Activities of these enzymes in kidney and erythrocytes were also analyzed, and no difference was found between the control and null mice (data not shown). In addition, the level of antioxidant, the reduced form of glutathione, was not reduced in tissues of HNF-1 α -null mice (Fig. 2A).

On the other hand, there was a significant decrease in the activities of cellular GPx and catalase in liver of HNF-1 α -null mice at all stages examined (Fig. 2B). The activity of cellular GPx in liver of the HNF-1 α -null mice was 50 to 60% of the control level at every stage, whereas the activity of catalase was decreased to a greater degree in mice of older ages (Fig. 2B). These results suggest that the protection against cellular H₂O₂ is thwarted in HNF-1 α -null mice, because GPx and catalase are two types of enzymes that exist to remove H₂O₂ within cells (Halliwell and Gutteridge, 1989). However, the reduction in both enzyme activities were not found in kidneys (Fig. 2B) or in erythrocytes (data not shown), indicating that the decrease of cellular GPx and catalase activities is liver-specific in HNF-1 α -null mice.

To understand the mechanism underlying the decreased activities of GPx and catalase in liver of HNF-1 α -null mice, hepatic mRNA levels for GPx and catalase, respectively, were analyzed. As shown in Fig. 3, neither GPx nor catalase mRNA levels were reduced in livers of HNF-1 α -null mice. On the contrary, the level of hepatic catalase mRNA was increased in HNF-1 α -null mice (Fig. 3 and 4A), and this increase of catalase mRNA level persisted in livers at all ages analyzed (Fig. 4A). These results suggest that the decreased activities of both GPx and catalase in liver of HNF-1 α -null mice were not caused by reduction of their transcription

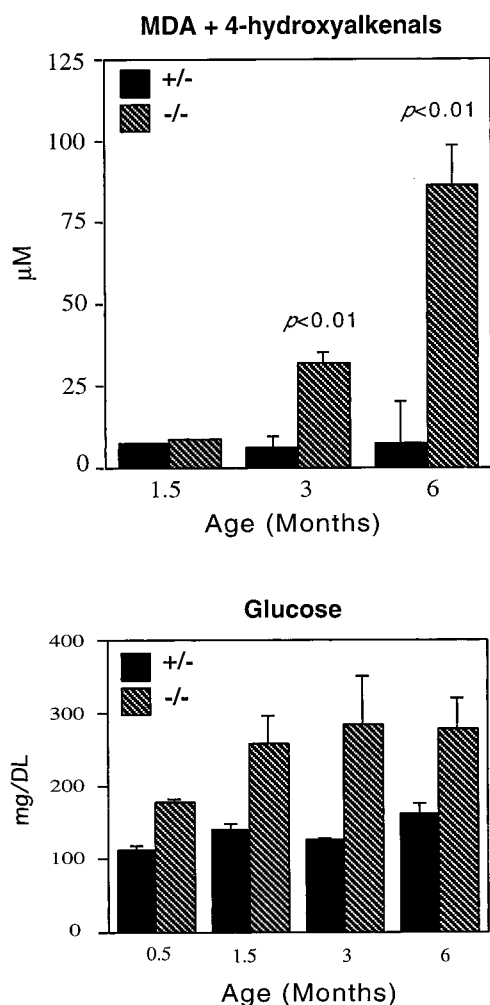


Fig. 1. Serum MDA and 4-hydroxyalkenal (top) and glucose (bottom) levels in HNF-1 α -null mice during development. The value for each group is the mean of at least three serum samples and is presented as a vertical column in the figure. The standard error for each group is shown as a vertical line. The *P* value indicates the level of significance for differences between knockout (-/-) and heterozygous (+/-) mice.

levels. Although GPx and catalase are both involved in detoxification of H_2O_2 , they are modulated by different mechanisms and by distinct factors in translational and post-translational levels. For example, GPx is a selenoprotein; its synthesis is controlled by the selenium status (Bermano et al., 1996). Catalase is a heme-containing protein; its activity is controlled by ions including heme (Eventoff et al., 1976; Hortner et al., 1982; Kirkman et al., 1987). The following experiments of this study were focused to explore the mechanism by which HNF-1 α deficiency affects catalase activity.

Hemin Treatment Increases Activities of Catalase in Livers of HNF-1 α -Null Mice. SDS-polyacrylamide gel electrophoresis and immunoblotting analyses of liver catalase showed that catalase protein was present in liver of HNF-1 α -null mice and that the amount of liver catalase protein in

HNF-1 α -null mice seemed to be comparable, if not higher, to that of control heterozygous littermates (Fig. 4B). This confirms that the decrease of catalase activity in livers of HNF-1 α -null mice was not caused by an insufficient protein level but rather by the level of active form. Catalase is a hemoprotein that consists of four protein subunits, each of which contains a heme group bound to its active site. Heme is required for the functional activity of catalase (Eventoff and Gurskaya, 1975; Eventoff et al., 1976). In addition to heme, other factors, such as NADPH, can also modulate catalase activity (Kirkman et al., 1987). To determine whether an altered heme pool caused the loss of catalase activity in liver of HNF-1 α -null mice, hemin was administered to the HNF-1 α -null mice. Hemin, an iron-containing protoporphyrin and an oxidized derivative of heme, has been widely used to treat

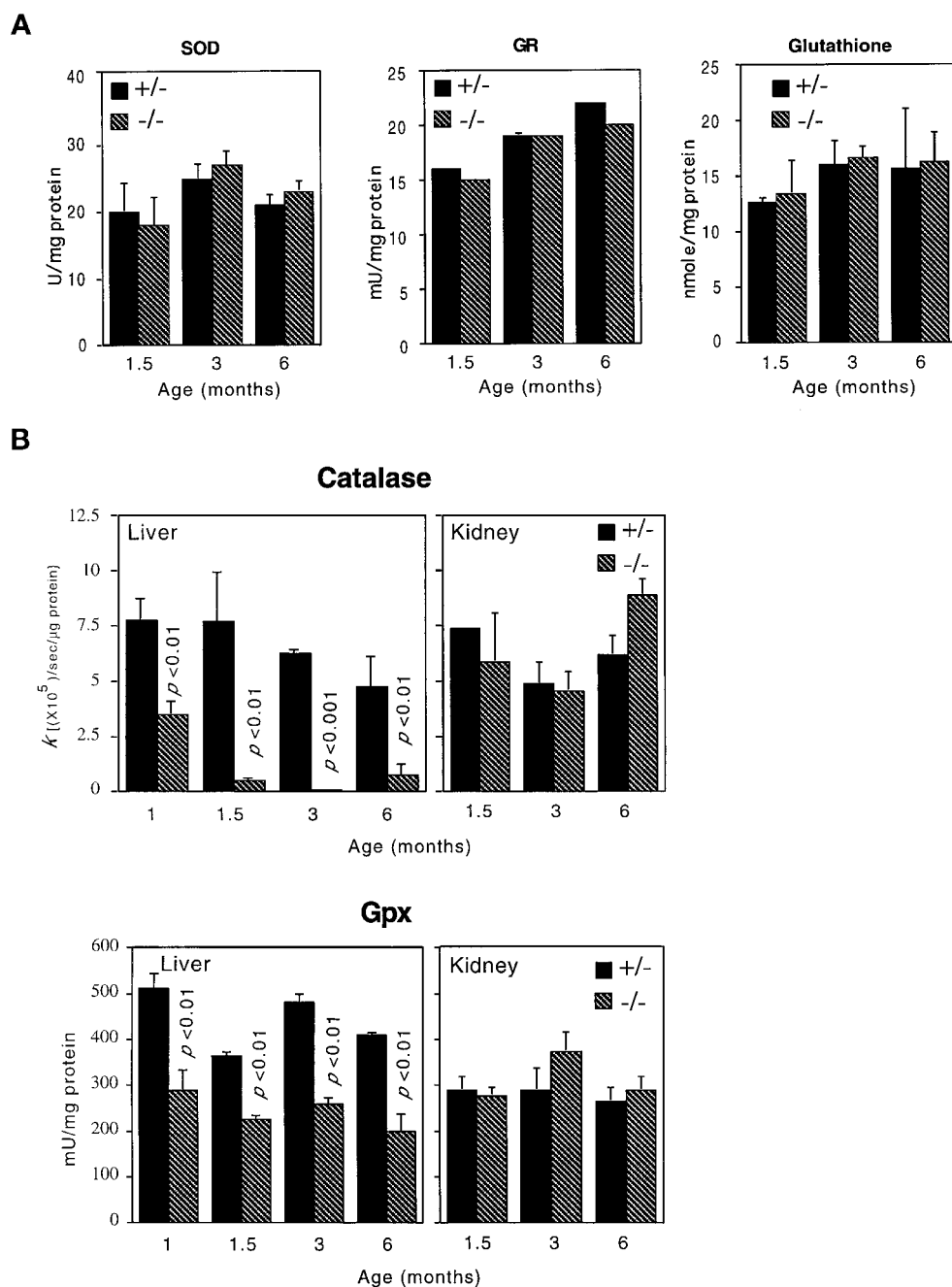


Fig. 2. Activities of catalase and glutathione peroxidase were reduced specifically in liver of HNF-1 α -null mice during development. A, activities of SOD and GR and levels of glutathione (reduced-form) in liver. B, activities of catalase and GPx in liver and kidney of HNF-1 α -null mice during development. k is the rate constant of a first-order reaction for the decomposition of H_2O_2 catalyzed by catalase. The value for each group is the mean of at least three liver samples and is presented as a vertical column in the figure. The standard error for each group is shown as a vertical line. The P value indicates the level of significance for differences between knockout (−/−) and heterozygous (+/−) mice.

porphyrias, which are characterized by an inherited defect in heme biosynthesis (Herbert et al., 1991; Tenhunen and Mustajoki, 1998). As shown in Fig. 5, daily administration of hemin for 4 days significantly increased the catalase activity in liver of HNF-1 α -null mice, but the same treatment did not affect the catalase activity in liver of control heterozygous littermates. These results suggest that the decrease of hepatic catalase activity in HNF-1 α -null mice might be attributable in part to an insufficient heme pool in livers.

HNF-1 α Deficiency Reduces FC mRNA Levels and Increases Protoporphyrin Levels in Livers. Heme is the final product of a biosynthesis pathway in cells that involves several enzymatic steps (Gidari and Levere, 1977). To examine the heme biosynthesis status in tissues of HNF-1 α -null mice, mRNA levels of enzymes involved in heme biosynthesis pathway were first analyzed. Aminolevulinic acid synthase is the first and normally rate-limiting enzyme of the heme synthesis pathway, whereas FC catalyzes the last step of heme biosynthesis, in which ferrous iron is inserted into protoporphyrin to form heme (Gidari and Levere, 1977; Harbin and Dailey, 1985; Bloomer, 1998). As shown in Fig. 6, no significant difference between HNF-1 α -null mice and control heterozygotes in the mRNA levels of liver and kidney aminolevulinic acid synthase was detected. On the other hand, the FC mRNA levels (existing as 2.9- and 2.2-kilobase species) in liver of HNF-1 α -null mice were significantly reduced to only 20 to 40% of control levels (Fig. 6). However, the levels of FC mRNA in kidney of HNF-1 α -null mice did not differ from those of control heterozygous littermates (Fig. 6), indicating that HNF-1 α deficiency decreases the levels of FC mRNA specifically in liver. Excessive accumulation of protoporphyrin occurs when FC is defective or deficient (Harbin and Dailey, 1985; Smith et al., 1997). Indeed, as shown in Fig. 7, the protoporphyrin levels were substantially higher in livers of HNF-1 α -null mice compared with those in control livers. In agreement with the analysis of FC mRNA, the protoporphyrin

levels in kidney of HNF-1 α -null mice did not differ from those of control mice (Fig. 7).

HNF-1 α *trans*-Activates the Promoter of FC Gene in HepG2 but Not CV-1 Cells. The role of HNF-1 α in regulating expression of the human and mouse FC genes has not yet been studied (Taketani et al., 1992; Taketani et al., 1999). To determine whether HNF-1 α can directly *trans*-activate the FC promoter, a series of FC promoter-luciferase reporters were constructed as illustrated in Fig. 8. In transient transfection studies carried out in the human hepatoblastoma cell line HepG2 and in monkey kidney cells CV-1, all three FC promoter-luciferase reporters were found to have substantial expression activity compared with the promoterless luciferase reporter (Fig. 8). In addition, the FC promoter-luciferase reporters had higher expression activity in HepG2 than in CV-1 cells. In the cotransfection studies, these FC promoter-luciferase reporters were cotransfected with an expression plasmid encoding HNF-1 α . As shown in Fig. 8, cotransfection of HNF-1 α increased significantly the expression activities of both pFC(-918/+47) and pFC(-2920/+47) but not that of pFC(-279/+47) in HepG2 cells, indicating that HNF-1 α can

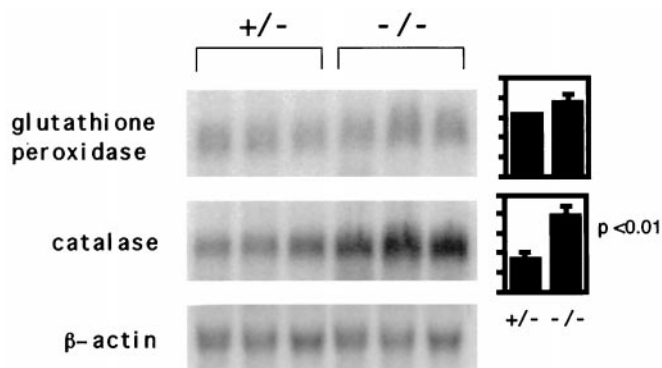


Fig. 3. mRNA levels of hepatic catalase and glutathione peroxidase are not reduced in HNF-1 α -null mice. Northern blotting analysis of representative liver biopsies of *Hnf-1 α ^{-/-}* mice. Liver total RNA (15 μ g) from 3-month-old *Hnf-1 α ^{-/-}* mice were denatured and electrophoresed on a formaldehyde-containing 1% agarose gel, blotted to nylon membranes and probed with the indicated cDNA probes. Each lane contains RNA from an individual animal. The signal of mRNA from each sample was quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and normalized with its respective actin mRNA signal. The value for each group shown on the right is the mean of three different samples shown on the left and is presented as a vertical column in the figure. The standard error for each group is shown as a vertical line. The *P* value indicates the level of significance for differences between knockout (-/-) and heterozygous (+/+) mice.

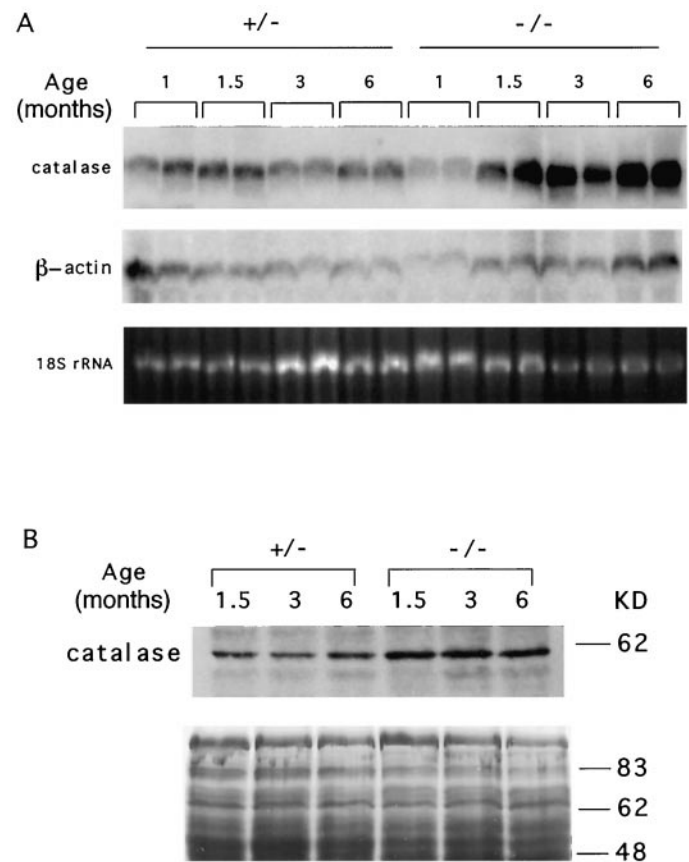


Fig. 4. Liver catalase mRNA levels increase with age in HNF-1 α -null mice. **A,** Northern blotting analyses of representative liver biopsies of *Hnf-1 α ^{-/-}* mice. Liver total RNA (15 μ g) from different ages of *Hnf-1 α ^{-/-}* mice were denatured and electrophoresed on a formaldehyde-containing 1% agarose gel, blotted to nylon membranes, and probed with the indicated cDNA probes. Each lane contains RNA from an individual animal. **B,** Western blotting analysis of liver protein of *Hnf-1 α ^{-/-}* mice. Liver protein (50 μ g) were denatured and electrophoresed on a 10% SDS-polyacrylamide gel, blotted onto a nitrocellulose membrane, and probed with goat anti-IgG against mouse catalase (top). A parallel electrophoresis was performed at the same time and the gel was stained with Coomassie Blue to confirm the equal sample loading (bottom).

directly *trans*-activate the FC promoter and that the HNF-1 α responsive element is located between -279 and -918. By contrast, no increase in reporter gene activity was found in HepG2 cells cotransfected with HNF-4, another liver-enriched transcription factor (Fig. 8). Interestingly, in CV-1 cells, HNF-1 α did not increase the reporter gene activity, suggesting that the transactivation of the FC promoter by HNF-1 α is cell-type specific. The FC promoter-luciferase reporters were also significantly activated in both cell lines by

another liver-enrich transcription factor, C/EBP α . However, this activation was also found in the promoterless reporter, suggesting that the activation by C/EBP α is not specific to the FC promoter.

Discussion

We report here that the activities of both cellular GPx and catalase, two antioxidant enzymes involved in disposal of H₂O₂ within cells (Tenhunen and Mustajoki, 1998), were reduced specifically in liver of HNF-1 α -null mice. The loss of catalase activity might involve defective heme biosynthesis in liver of HNF-1 α -null mice, whereas the mechanism by which HNF-1 α regulates GPx activity in liver remains to be elucidated.

Oxidative stress is present in the diabetic HNF-1 α -null mice, and its severity progresses with age. The oxidative stress in HNF-1 α -null mice might arise from hyperglycemia autooxidation of glucose, as seen in humans with diabetes (Brownlee et al., 1988). On the other hand, our finding that activities of both catalase and GPx were impaired in liver of HNF-1 α -null mice indicates that the oxidative stress in HNF-1 α -null mice might involve the defected cellular anti-oxidation system against H₂O₂ in liver. It remains to be studied, however, whether the defected antioxidation is one of the causes that leads to the high degree of oxidative stress in HNF-1 α -null mice.

Liver is an active site for heme biosynthesis, second only to bone marrow in terms of the quantity of heme produced (Bloomer, 1998). Heme is required for functional activity of cytochrome P-450 and other critical heme-containing proteins, such as catalase and tryptophan pyrrolase (Eventoff and Gurskaya, 1975; Badawy et al., 1986; Wong, 1998). Cytochrome P-450s use the majority of hepatic heme produced (Bonkovsky, 1991; Bloomer, 1998; Wong, 1998). Amounts of the different apoproteins and their affinity for heme may determine the distribution of hepatic heme (Bloomer, 1998). For example, tryptophan pyrrolase is the rate-limiting enzyme for tryptophan metabolism, and it binds heme with relatively low affinity. Accordingly, the saturation degree of

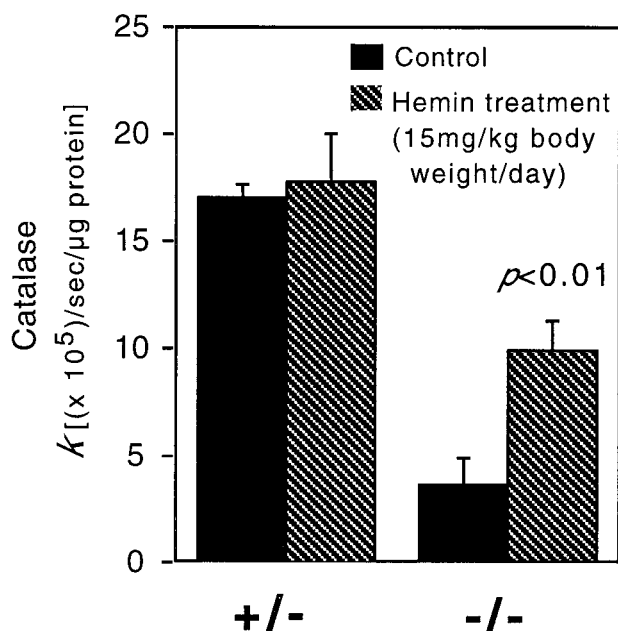


Fig. 5. Hemin restores partially the catalase activity in liver of HNF-1 α -null mice. One-month-old mice of either genotype were administered hemin solution i.p. at a dosage of 15 mg/kg of body weight/day for 4 consecutive days. Twelve hours after the last injection, livers were removed, homogenized, and assayed for catalase activity. The value for each group is the mean of at least three liver samples and is presented as a vertical column in the figure. The standard error for each group is shown as a vertical line. The *P* value indicates the level of significance for differences between knockout (-/-) and heterozygous (+/-) mice.

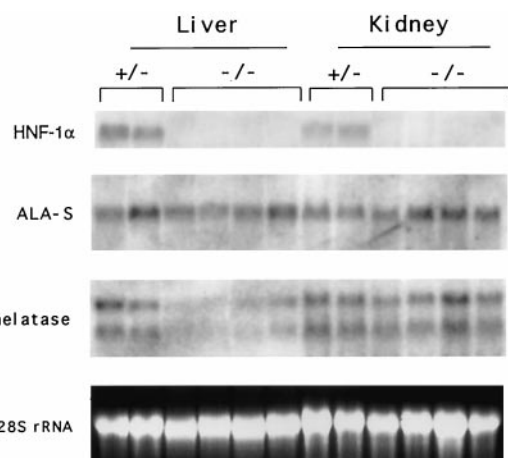


Fig. 6. Ferrochelatase mRNA levels are reduced specifically in liver of HNF-1 α -null mice. The figure depicts the results of Northern blotting analysis of representative liver and kidney biopsies of *Hnf-1 α* ^{-/-} mice. Liver and kidney total RNA (15 μ g) from 1-month-old *Hnf-1 α* ^{-/-} mice were denatured and electrophoresed on a formaldehyde-containing 1% agarose gel, blotted to nylon membranes, and probed with the indicated cDNA probes. Each lane contains RNA from an individual animal.

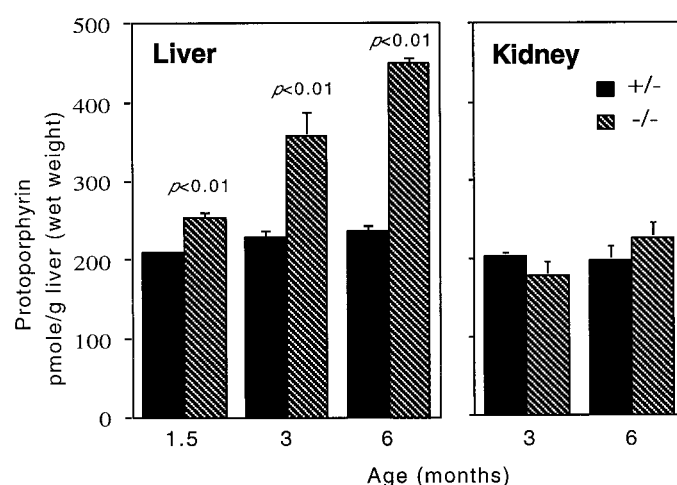


Fig. 7. Accumulation of protoporphyrin in liver of HNF-1 α -null mice. The value for each group is the mean of at least three liver samples and is presented as a vertical column in the figure. The standard error for each group is shown as a vertical line. The *P* value indicates the level of significance for differences between knockout (-/-) and heterozygous (+/-) mice.

tryptophan pyrrolase by heme has been used as an indicator for the relative size of the regulatory heme pool in hepatocytes (Badawy et al., 1986). Hepatic catalase activity is significantly reduced in HNF-1 α -null mice in which the gene expression for FC is defective in liver. Although other factors might be involved in the loss of liver catalase activity in HNF-1 α -null mice, the finding that hemin treatment partially restores the activity of hepatic catalase in HNF-1 α -null mice indicates that the degree of disrupted heme synthesis has an adverse effect in normal physiological function, at least in maintaining the catalase activity in liver.

The role of cytochrome P-450 in the liver function of metabolizing drugs and endogenous substances has been well established (Wong, 1998). Patients with severe liver disease frequently have reduced hepatic drug metabolism because of low levels of cytochrome P-450 in the liver, which is probably caused in part by a decrease in the hepatic heme pool (Howden et al., 1989; Guengerich and Turvy, 1991). In animals, depletion of intracellular heme can lead to limited synthesis of cytochrome P-450s in liver (Dwarki et al., 1987; Celier and Cresteil, 1991). Indeed, mRNA levels of several cytochrome P-450s, such as steroid 16 α -hydroxylase, were reduced in liver of HNF-1 α -null mice (VM and Y-HL, unpublished observations). However, whether the function of cytochrome P-450 is also affected in liver of HNF-1 α -null mice remains to be elucidated. Nevertheless, our finding that the expression of FC gene is reduced in liver, resulting in an insufficient hepatic heme pool, establishes HNF-1 α as an important regulator in detoxification as well as in metabolism, as described previously (Lee et al., 1998).

FC is the terminal enzyme of the heme biosynthesis pathway. It catalyzes the reaction in which a ferrous iron is inserted into protoporphyrin to form heme (Gidari and Levere, 1977; Bloomer, 1998). FC is a housekeeping enzyme; unlike albumin, which is expressed specifically in liver, it is present in all tissues and is particularly abundant in erythrocytes (Chan et al., 1993). The FC gene was isolated from both human and mouse, and its 5' flanking regulatory region has been analyzed (Taketani et al., 1992, 1999). The FC mRNA is transcribed from a single promoter in both ery-

throid and nonerythroid cells (Taketani et al., 1992). The FC promoter contains regulatory elements for the ubiquitous transcription factor-specific protein 1 as well as the erythroid-specific transcription factor GATA-1, which are responsible for the basal expression of FC in cells and the abundant expression of FC in erythrocytes, respectively (Taketani et al., 1992, 1999). FC is also abundantly expressed in liver (Chan et al., 1993). However, the possibility of a liver-specific transcription factor involved in directing the expression of FC in liver has not yet been explored. Our transient transfection assay demonstrates that the FC promoter can be *trans*-activated by HNF-1 α . Analysis of FC gene expression in liver of HNF-1 α -null mice reveals that HNF-1 α deficiency reduces the FC expression in liver. Thus, the regulatory role of HNF-1 α in controlling hepatic FC gene expression was established in this study.

HNF-1 α is enriched in liver but is also expressed in several other tissues, such as kidney (Blumenfeld et al., 1991; De Simone et al., 1991). Interestingly, HNF-1 α , despite its expression in kidney, seems to be dispensable in regulating the FC expression in kidney, as revealed in this study. Similarly, cotransfection studies indicate that HNF-1 α is able to *trans*-activate the FC promoter in a hepatoblastoma cell line (HepG2) but not in a kidney cell line (CV-1). The mechanism by which HNF-1 α controls hepatic FC expression remains to be elucidated. Nevertheless, our finding that the FC expression in liver is significantly affected by the HNF-1 α status provides an example that a tissue-enriched transcriptional regulator plays an important role in controlling expression of a ubiquitous factor in selected tissues.

Acknowledgments

We thank Dr. Peter Johnson at the Frederick Cancer Research and Development Center-National Cancer Institute for the expression plasmids pMex and pMex.C/EBP α , Dr. Gerald Crabtree at the Howard Hughes Medical Institute (Stanford University, Stanford, CA) for pBJ5.HNF-1 α , and Dr. Frances Sladek at the Environmental Toxicology Graduate Program, University of California (Riverside, CA) for pSG5.HNF-4.

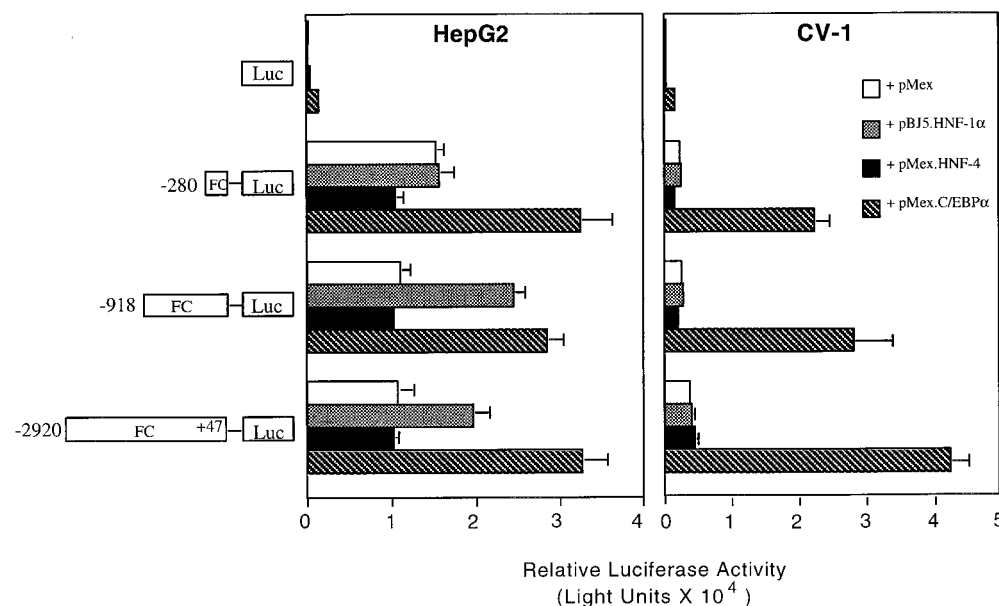


Fig. 8. Deletion analysis of the mouse FC promoter: transcriptional activation by HNF-1 α . A series of FC promoter-luciferase reporters (1 μ g) were cotransfected with 4 μ g of pMex, pMex.C/EBP α , pBJ5.HNF-1 α , or pMex.HNF-4 and 0.5 μ g of pCH110. The luciferase activity for each sample was normalized to β -galactosidase to control for transfection efficiency. The value for each group is the mean of at least three liver samples and is presented as a vertical column in the figure. The standard error for each group is shown as a vertical line.

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