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C/EBPα Regulates Hepatic Transcription of Hepcidin, an Antimicrobial Peptide and Regulator of Iron Metabolism

CROSS-TALK BETWEEN C/EBP PATHWAY AND IRON METABOLISM*

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Originally identified as a gene up-regulated by iron overload in mouse liver, the *HEPC* gene encodes hepcidin, the first mammalian liver-specific antimicrobial peptide and potential key regulator of iron metabolism. Here we demonstrate that during rat liver development, amounts of HEPC transcripts were very low in fetal liver, strongly and transiently increased shortly after birth, and reappeared in adult liver. To gain insight into mechanisms that regulate hepatic expression of hepcidin, 5′-flanking regions of human and mouse *HEPC* genes were isolated and analyzed by functional and DNA binding assays. Human and mouse *HEPC* promoter-luciferase reporter vectors exhibited strong basal activity in hepatoma HuH-7 and mouse hepatocytes, respectively, but not in non-hepatic U-2OS cells. We found that CCAAT/enhancer-binding protein α (C/EBPα) and C/EBPβ were respectively very potent and weak activators of both human and mouse promoters. In contrast, co-expression of hepatocyte nuclear factor 4α (HNF4α) failed to induce *HEPC* promoter activity. By electrophoretic mobility shift assay we demonstrated that one putative C/EBP element found in the human *HEPC* promoter (−250/−230) predominantly bound C/EBPα from rat liver nuclear extracts. Hepatic deletion of the *C/EBPα* gene resulted in reduced expression of HEPC transcripts in mouse liver. In contrast, amounts of HEPC transcripts increased in liver-specific HNF4α-null mice. Decrease of hepcidin mRNA in mice lacking hepatic C/EBPα was accompanied by iron accumulation in periportal hepatocytes. Finally, iron overload led to a significant increase of C/EBPα protein and HEPC transcripts in mouse liver. Taken together, these data demonstrate that C/EBPα is likely to be a key regulator of *HEPC* gene transcription and provide a novel mechanism for cross-talk between the C/EBP pathway and iron metabolism.

To ward off infections by pathogenic microorganisms, mammals display two distinct but complementary lines of defense: the acquired immune system, characterized by highly specific but delayed onset of response to initial pathogen exposure and the non-targeted innate immunity acting as a first barrier host defense. The ability to avoid microbial infection relies for the major part on constitutive or inducible endogenous synthesis of a battery of microbicidal substances named antimicrobial peptides that behave as natural antibiotics of the organism (1, 2). These molecular effectors, universally found in procaryotes, plants, and animals ranging from insects to mammals, exhibit a broad spectra of activities on bacteria and fungi (3–6). Antimicrobial peptides, typically 20–40 amino acids in length, are rich in cationic residues and adopt an amphipathic structure.

Recently, we identified a novel mouse gene named *HEPC* (7) encoding a small 83-aa protein that shares significant homology in its C-terminal region with human peptide hepcidin (also termed LEAP-1) isolated in two parallel studies from human urine (8) and plasma ultrafiltrate (9). Hepcidin exhibits a broad range of activity against Gram-positive and Gram-negative bacteria and fungi (8, 9). However, in contrast to described mammalian antimicrobial peptides including α- and β-defensins (4, 5), which are expressed mainly in circulating neutrophil granulocytes and in various epithelia including gastrointestinal, urogenital, and respiratory tracts, hepcidin exhibits predominant liver expression. Because in *Drosophila* most antimicrobial peptides were produced in the fat body, a functional equivalent of the liver (10), hepcidin could represent the first mammalian hepatic homologue of insect cysteine-rich antimicrobial peptides.

Hepcidin was identified as a gene induced in mouse liver by iron excess (7). In addition, expression of hepcidin was enhanced in mouse hepatocytes both *in vivo* and *in vitro* after exposure to bacterial lipopolysaccharides (7) and was dramatically induced following bacterial challenge in bass liver (11). Induction of hepcidin in these two unrelated physiopathological situations could indicate that besides its antimicrobial functions this peptide exhibits other functions. Several antimicrobial peptides display functions not directly related to host defense including prevention from oxidative stress (12), antitumor activity (13), and regulation of angiogenesis (14).

Recent reports argue in favor of a role of hepcidin in iron metabolism. Indeed, analysis of upstream stimulatory factor 2 (USF2)[1] knockout mice revealed a development of iron overload

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[1] The abbreviations used are: USF2, upstream stimulatory factor 2; aa, amino acid; AFP, α-fetoprotein; C/EBP, CCAAT/enhancer-binding protein; EMSA, electrophoretic mobility shift assay; FCS, fetal calf serum; GFP, green fluorescent protein; HNF4α, hepatocyte nuclear factor 4α; MEM, minimum essential medium; MOPS, 4-morpholinepropanesulfonic acid; STAT, signal transducers and activators of transcription.

in liver, pancreas, and heart. In these mice, it appeared that two very highly related mouse hepcidin genes, *HEPC1* and *HEPC2*, lying in close proximity to the *USF2* gene, were transcriptionally inactive, and livers of $USF2^{-/-}$ mice did not contain HEPC transcripts. It has been suggested that the lack of hepcidin expression was responsible for the liver iron-overload phenotype observed in $USF2^{-/-}$ (15, 16). Inversely, early liver expression of the *HEPC1* gene in transgenic mice resulted in phenotypic traits of iron deficiency (17). These results strongly suggest that hepcidin plays an important role in the regulation of iron homeostasis (15, 16).

In this study, to understand molecular mechanisms involved in the regulation of hepcidin in the liver, we have cloned and analyzed human and mouse *HEPC* promoters and investigated the transcriptional control of the *HEPC* gene *in vitro* and *in vivo*. We demonstrated that liver-enriched nuclear factor C/EBPα, a critical regulator of several hepatic metabolic processes (18), is likely to play an essential role in the control of *HEPC* gene expression.

MATERIALS AND METHODS

Animals—For fetal rat liver samples, pregnant female Sprague-Dawley rats were purchased from Charles River (St. Aubin-les-Elbeuf, France). Breeding was done by placing female rats with males of the same strain overnight, and the noon of the next day was considered as 0.5 day postcoitum. On the indicated days of gestation, rats were anesthetized, embryos were removed, livers were minced, and washed briefly with phosphate-buffered saline.

The mice with liver-specific deletion of the *C/EBPα* gene will be reported elsewhere. Briefly, C/EBPα-floxed allele described earlier (19) was bred with the rat albumin Cre transgene (20). The gene deletion commenced at about 2 weeks of age at the onset of expression of the albumin promoter and completely deleted at age 6 weeks when the promoter was maximally expressed (21). Liver-specific HNF4α-null mice were generated by Cre-loxP-mediated deletion of exons 4 and 5 of the *HNF4α* gene as previously described (21, 22).

For experimental iron overload, 5-week-old BALB/cJ male mice were obtained from CERJ (Le Genet St. Ile, France). Minced rat and mice liver samples were frozen in liquid nitrogen and kept at −80 °C until further processing.

Iron Overload—Carbonyl iron overload was performed as previously described (23). Briefly, a group of five mice was iron-overloaded by 3% carbonyl iron supplemented in the diet (AO3, UAR, France) over 8 months. Control mice had a carbonyl iron-free diet. Under these experimental conditions, liver iron concentration was on average 13.8-fold higher than the control value (158.2 μmol of iron/g of dry weight liver *versus* 11.5 μmol, respectively).

Hepatocyte Isolation and Culture—Hepatocytes were isolated by a two-step collagenase perfusion procedure from normal male Sprague-Dawley rats or C57BL6 mice. Hepatocytes were seeded in a mixture of 75% minimum essential medium (MEM) and 25% medium 199, supplemented with 10% FCS and per ml: 100 IU of penicillin, 50 μg of streptomycin sulfate, 1 mg of bovine serum albumin, and 5 μg of bovine insulin. Four hours later, the medium was renewed with the same medium deprived of FCS and supplemented with 1×10^{-6} M hydrocortisone hemisuccinate. It was changed every day thereafter.

Rat hepatoma cell lines FAZA and HTC were grown in a mixture of 50% Ham's F-12 and 50% NCTC135 and in DMEM, respectively, supplemented with 10% FCS. Human osteosarcoma cells U-2OS were maintained in William's E medium containing 10% FCS. The human hepatoma cell lines HepG2, Hep3B, HuH-7, and PLC/PRF/5 were grown in a mixture of MEM (75%) and M199 (25%) supplemented with 10% FCS and 100 IU/ml penicillin, 50 μg/ml streptomycin sulfate, 1 mg/ml bovine serum albumin, 5 μg/ml bovine insulin, and 5×10^{-7} M hydrocortisone hemisuccinate.

RNA Isolation and Northern Blot Analysis—Total RNA was extracted by the guanidine thiocyanate method following sedimentation through a cesium chloride cushion or using the SV total RNA isolation system (Promega, Charbonnières, France). 10–20 μg of RNA was separated by electrophoresis through 1.2% agarose gel in 2.2 M formaldehyde, 20 mM MOPS (pH 7.0), 10 mM sodium acetate, 1 mM EDTA and transferred onto a nylon membrane (Hybond N+, Amersham Biosciences) by capillary blotting. Hybridization was carried out in the presence of ^{32}P-labeled cDNA probe. The equivalence of RNA loading was assessed by ethidium bromide staining and/or hybridization with a glyceraldehyde-3-phosphate dehydrogenase cDNA probe.

cDNA Probes—Mouse hepcidin cDNA probe was prepared as described previously (7). cDNAs encoding rat and human hepcidin were generated by polymerase chain reaction (PCR) from reverse-transcribed rat liver and human hepatoma HepG2 mRNAs, respectively. Obtained PCR products were cloned in pCRII-TOPO vector (InVitrogen, NV Leek, The Netherlands) and sequenced. The cloned fragments were then released by *Eco*RI digestion and used as probes for Northern blot analysis. Rat α-fetoprotein (AFP) probe was obtained from J. Kruh. The mouse C/EBPα and HNF4α cDNA probes were generated as described in Refs. 19 and 21, respectively. Mouse ApoC-III probe was amplified from mouse liver cDNAs using gene-specific primers and cloned in pCRII-TOPO vector and sequenced.

Real-time RT-PCR Analysis—Total RNA extraction was carried out from normal human liver and HuH-7, HepG2, and U-2OS cells using the SV total RNA isolation system (Promega), and cDNA first-strand synthesis was performed with the Advantage RT-for-PCR kit (Clontech, Palo Alto, CA). Primers and probes were designed with the assistance of Primer Express Software (PE Biosystems). The following probes were used to detect human HEPC and β-actin amplification products: 5′-FAM-CTGCGGCTGCTGTCATCGATCAA-TAMRA-3′ and 5′-FAM-ATGGGACGACATGGAGAAAATCTGGC-TAMRA-3′, respectively. The relative expression of HEPC in the different samples was determined by standardization to its expression in U-2OS cells (which exhibits the lowest level of HEPC expression) using a two-step calculation method. First, for each sample, Ct_{actin} value was subtracted from the corresponding Ct_{HEPC}. Then, these normalized values were used to calculate relative level of HEPC expression as follows: HEPC expression $\text{ratio}_{(\text{sample/U2OS})} = 2^{\Delta Ct}$ where $\Delta Ct = (Ct_{U2OS} - Ct_{sample})$. The HEPC expression level in U-2OS cells was used as a reference and was set as 1-fold expression. All quantitative real-time PCR assays were performed in triplicate in 96-well microplates using qPCR™ Core reagent kit (Eurogentec, Seraing, Belgium) and ABI PRISM 7700 Sequence Detection System (PE Biosystems). For each microplate the reactions contained target cDNAs, two standard curves (prepared from human liver), and two negative controls: no-template control (sterile water) and no-amplification control (omitting the RT step). The liver sample was obtained from a patient who underwent hepatic surgery for a liver metastasis of colonic adenocarcinoma. A sample of non-tumoral area of the liver was immediately frozen in liquid nitrogen and stored at −80° before use. Surgical procedures and collection of liver samples were performed under informed consent and in compliance with French law and regulations.

Western Blot Analysis—Liver tissue lysates were prepared as follows: minced liver samples were lysed in a buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 5 mM EGTA, 0,1% Tween 20, 10% glycerol, 0.1 mM phenylmethylsulfonyl fluoride, and 1× protease inhibitor mixture (Roche Diagnostics, Meylan, France). Protein concentrations were determined by Bio-Rad protein assay. Total cellular proteins (100 μg/lane) were resolved by SDS-PAGE, transferred to nitrocellulose membrane (Amersham Biosciences), incubated with blocking buffer (3% bovine serum albumin in Tris-buffered saline). Rabbit polyclonal anti-C/EBPα antibody was a gift from G. Darlington (Baylor College of Medicine, Houston). Following incubation with peroxidase-conjugated goat anti-rabbit immunoglobulins (DAKO, Denmark) as second antibodies, peroxidase activity was detected by enhanced chemiluminescence according to the manufacturer's instructions (Amersham Biosciences).

Cloning of Human and Mouse HEPC Promoters and Construction of Reporter Vectors—950- and 624-bp nucleotide fragments of the 5′-flanking genomic region of the human *HEPC* gene were obtained from human genomic DNA (Clontech) by PCR amplification using the following forward primers: 5′-GGCTCGAGGTACTCATCGGACTGTAGATGTTAGC-3′ and 5′-GGCTCGAGCTGTGCTGGGCCATATTACTGCTGTC-3′, respectively, and the reverse primer: 5′-GGAAGCTTGTGACAGTCGCTTTTATGGGGCCTGC-3′ (the incorporated *Xho*I and *Hin*dIII sites are underlined). PCR fragments were digested with *Xho*I-*Hin*dIII and were inserted into the same sites of the promoter-less luciferase reporter vector pGL3-Basic (Promega). These were designated pHEPC−960/−9 and pHEPC−633/−9. Constructs containing 5′-deletions of the human *HEPC* promoter (pHEPC−327/−9, −275/−9, −223/−9, and −108/−9) were generated by PCR using pHEPC−633/−9 as template and specific primers containing *Xho*I and *Hin*dIII restriction sites. After digestion with *Xho*I-*Hin*dIII, PCR fragments were cloned in the pGL3-Basic vector. A 789-bp nucleotide fragment of the 5′-flanking genomic region of the mouse *HEPC1* gene were obtained from mouse genomic DNA (Clontech) by PCR amplification using the forward primers: 5′-GCGCTCGAGGAATACATCGTCAAGCCAGAC-3′ and the re-

verse primer: 5′-GCGAAGCTTGTGTGGTGGCTGTCTAGGAGC-3′ (the incorporated *Xho*I and *Hin*dIII sites are underlined). The PCR fragment was digested with *Xho*I-*Hin*dIII and was inserted into the pGL3-Basic vector. The obtained construct was designated pmHEPC−783/+5. Similar to human *HEPC* promoter constructs, 5′-deletions of mouse *HEPC1* promoter (pmHEPC−554/+5, −338/+5, and −267/+5) were generated by PCR. Details of constructs are available upon request. All constructs were verified by DNA sequencing. Searches for transcription factor binding sites was performed using MatInspector (24).

Cell Transfection and Luciferase Assay—U-2OS or HuH-7 cells were seeded in 35-mm-diameter dishes and grown to 50–70% confluency. Cells were transiently transfected with 1.5 μg of promoter-less pGL3-Basic vector or positive control pGL3-Promoter vector or constructs containing 5′-flanking regions of the *HEPC* gene and 0.5 μg of C/EBPα, C/EBPβ, HNF4α expression plasmids, or empty vector. The pEGFP-C3 vector encoding green fluorescent protein (GFP) used as a co-transfectant (0.5 μg per dish) was served as the control for transfection efficiency. Transfections were performed using liposome-based transfection reagent Lipofectin (Invitrogen). Cells were maintained in the presence of transfection reagent in Opti-MEM (Invitrogen) for 16 h and then switched to growth medium. In all cases, the transfection efficiency was at least 10–15%. Primary mouse hepatocytes were transfected 24 h after seeding using GB12 reagent as previously described (25). The following expression plasmids were used for co-transfection: pCMV-C/EBPα and pCMV-NF-IL6 encoding C/EBPα and C/EBPβ, respectively (kindly provided by G. Darlington, Baylor College of Medicine, Houston) and a HNF4α expression vector pSG5/rHNF4. 42 or 30 h after transfection of cell lines or primary mouse hepatocytes, respectively, cells were lysed in luciferase cell culture lysis reagent (Promega), and cellular extracts were analyzed for luciferase activity by liquid scintillation counting using the Luciferase assay system from Promega. The luciferase activity was expressed as cpm/μg of cellular protein.

Electrophoretic Mobility Shift Assay (EMSA)—Rat liver nuclear extracts were prepared according to the procedure of Gorski *et al.* (26). To prepare double-stranded oligonucleotide probes, equal amounts of complementary single-stranded DNA were heated to 85 °C for 5 min in a buffer containing 50 mM Tris, pH 7.5, 1 mM spermidine, 10 mM $MgCl_2$, 5 mM dithiothreitol solution and then allowed to cool to room temperature. The following double-stranded oligonucleotides were used as probes in EMSA: the wild type C/EBP consensus sequence element as described in Ryden and Beemon (27) was used as a reference probe: 5′-CTAGGGCTTGCGCAATCTATATTCG-3′ and 3′-GATCCCGAACGCGTTAGATATAAGC-5′. The mutant form of the C/EBP consensus sequence element: 5′-CTAGGGCTTGCTACCCCTATATTCG-3′ and 3′-GATCCCGAACGATGGGGATATAAGC-5′. The oligonucleotide probe encompassing first putative C/EBP binding site in 5′-flanking region of the human *HEPC* gene, HEPC(−300/−280): 5′-CTTAACCGCTGAAGCAAAAGGGGGA-3′ and 3′-TGGCGACTTCGTTTTCCCCCTTGAA-5′. Second putative C/EBP binding site, HEPC(−250/−230): 5′-CATCGTGATGGGGAAAGGGCTCCCC-3′ and 3′-CACTACCCCTTTCCCGAGGGGTCTA-5′, and third putative C/EBP binding site, HEPC(−92/−72): 5′-CACCACCTTCTTGGAAATGAGACAG-3′ and 3′-TGGAAGAACCTTTACTCTGTCTCGT-5′.

Double-stranded C/EBP wild type and mutant oligonucleotides were purchased from Geneka Biotechnology (Montréal, Canada). The single-stranded oligonucleotides, which contain the putative C/EBP binding site in the 5′-flanking region of the *HEPC* gene, were from InVitrogen.

Oligonucleotide probes were labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (Amersham Biosciences). Binding reactions were performed in 24 μl of binding buffer containing 6 μg of rat liver nuclear extract and 100×10^3 cpm of labeled oligonucleotide probe. The reaction mixture was incubated at 4 °C for 20 min and then was loaded onto a 6% nondenaturating polyacrylamide gel. The electrophoresis was run in 0.5× Tris-Boric acid electrophoresis buffer.

For competition experiments, cold competitors were included in the binding mixture before adding the nuclear extract. For supershift experiments, nuclear extracts were preincubated with the antibody to C/EBPα or C/EBPβ (Geneka Biotechnology) for 20 min at 4 °C.

Preparation of Liver Tissues and Iron Staining—Livers from 2-month-old liver-specific C/EBPα-null and control mice were fixed in 10% neutral buffered formalin and embedded in paraffin. Then, sections cut at a thickness of 5 μm were mounted on the slides. Iron staining was performed using Accustain iron stain kit (Sigma).

Statistical Analysis—Results were expressed as mean ± S.D. Student's *t* test was used for estimation of statistical significance ($p < 0.01$ was considered as significant).

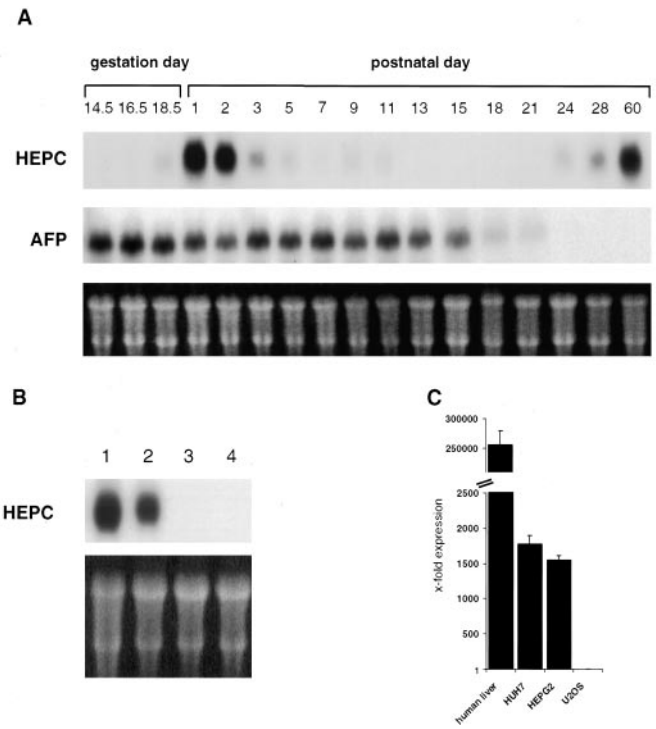

FIG. 1. **Expression of the *HEPC* gene is related to developmental and differentiation status of hepatocytes.** *A*, total RNA was extracted from rat livers at different stages of development and subjected to Northern blot analysis using rat hepcidin and AFP cDNA probes. Exposure time 3 h. *B*, total RNA was isolated from freshly isolated (*lane 1*) or maintained in culture for 2 days rat hepatocytes (*lane 2*), rat liver-derived cell lines HTC (*lane 3*), and FAZA (*lane 4*) and analyzed by hybridization for the presence of HEPC transcripts using rat hepcidin probe. Exposure time 4 h. *C*, expression of the *HEPC* gene by real-time quantitative RT-PCR. Total RNA was extracted from normal human liver or from HuH-7, HepG2, and U-2OS cells. The indicated x-fold expression of HEPC in human liver and in different cell lines was determined by standardization to the expression in U-2OS cells as described under "Materials and Methods." The lowest level of HEPC expression in U-2OS cells was set as 1-fold. For each sample, experiments were performed in triplicates.

RESULTS

HEPC Gene Expression Is Regulated during Rat Liver Development and Is Related to Hepatocyte Phenotype—Expression of the *HEPC* gene was analyzed during rat liver development from day 14.5 of embryonic age. HEPC transcripts were undetectable in 14.5- and 16.5-day fetal liver, appeared at 18.5 day of embryonic age, was very strongly induced shortly after birth, and then abruptly ceased from the third day of postnatal period. Then, amounts of HEPC mRNA began to accumulate from 28th day after the birth, reaching the highest levels in adult liver (Fig. 1*A*). In parallel, expression of AFP, a well known liver-specific developmentally regulated gene, was studied. AFP transcripts were highly expressed in fetal and perinatal liver and abruptly shut off after 15 days, in accordance to previous studies (28). These observations, along with our previous analysis of HEPC expression (7), suggested that this gene is liver-specific and its expression is related to the fully differentiated hepatocyte. To confirm this issue, we analyzed levels of HEPC transcripts by Northern blotting in normal hepatocytes and in a set of rat and human liver-derived cell lines. In accordance with our previous data on mice hepatocytes (7), freshly isolated rat hepatocytes contained higher amounts of HEPC transcripts in comparison with 48-h-old cultures (Fig. 1*B*). Furthermore, HEPC mRNAs could not be detected in rat hepatoma cell lines HTC and FAZA after a 2-week exposure.

The survey of four human hepatoma cell lines (HepG2,

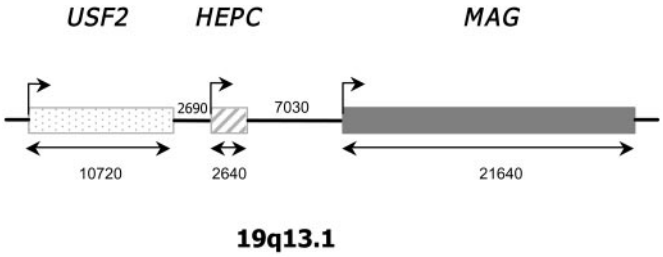

FIG. 2. **Genomic localization of the human *HEPC* gene.** The length of genes and intergenic regions are shown in base pairs. *Arrows* indicate the site of initiation and direction of transcription.

Hep3B, HuH-7, and PLC/PRF/5) for hepcidin expression by Northern blotting revealed a hybridization signal in HuH-7 and HepG2 cells following a 1-week exposure time. In contrast, HEPC transcripts from normal human liver were clearly detectable as early as 2 h after autoradiography (data not shown). In order to assess more accurately the relative level of hepcidin expression in adult liver in comparison to HuH-7 and HepG2 hepatoma cells and non-hepatic U-2OS cell line, we performed quantitative real-time RT-PCR assays. Expression of the *HEPC* gene in adult human liver was found to be 142 times stronger than in HuH-7 hepatoma cells. (Fig. 1*C*).

Localization of the Human HEPC Gene, and Sequence Analysis of Human and Mouse HEPC Promoters—To understand molecular mechanisms that confer liver specificity and are involved in transcriptional regulation of hepcidin during development and iron overload, we isolated and characterized the 5′-flanking region of the human *HEPC* gene and analyzed its promoter activity. Analysis of genomic sequence data of human clones R30879 and F24108 (GenBank™ accession numbers AD000684 and AC002132, respectively) allowed us to map the *HEPC* gene to chromosome 19, band q13.1 between the *USF2* gene and the *MAG* gene encoding myelin-associated glycoprotein precursor (Fig. 2). Interestingly, two liver-specific genes *LISCH7* and *C/EBPα* are also mapped to 19q13.1. In particular, the *LISCH7* gene is located immediately upstream of the *USF2* gene.

A 950-bp 5′-flanking genomic fragment of the human *HEPC* gene was cloned in the pGL3-Basic vector, sequenced, and analyzed using bioinformatic tools for the presence of potential transcription factor binding sites. This region contains a sequence with a TATA box homology. Among a number of putative response elements, we found binding sites for the liver-enriched transcription factor HNF-4 (−75 to −62 and −602 to −589 from the predicted translation start site) and for members of C/EBP family (−249 to −236, −298 to −285 and −90 to −77) (Fig. 3). The proximal putative C/EBP binding site TTCTTGGAAATGA overlaps with the STAT consensus binding site TTCNNNGAA.

Sequence analysis of a 789-bp nucleotide fragment of the 5′-flanking genomic region of the mouse *HEPC1* gene revealed that, similarly to human *HEPC* promoter, it contains at least three putative binding sites for the C/EBP family of transcription factors, including the proximal C/EBP binding site that overlaps with the STAT site and a sequence related to the HNF4 binding site (data not shown).

Hepatocyte-specific Promoter Activity of Hepcidin Gene—Two reporter vectors, pHEPC-960/−9 and pHEPC−633/−9, containing respectively 950-bp and 624-bp nucleotide fragments of the human HEPC 5′-flanking genomic region fused to a luciferase reporter gene were tested for transcriptional activity in human hepatoma cell line HuH-7 and non-hepatic osteosarcoma U-2OS cells. Promoter-less pGL3-Basic vector and pGL3-Promoter vector containing SV40 promoter upstream of the luciferase gene were used as negative and positive control,

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-958  GTACTCATCGGACTGTAATGTTAGCTATTACTATTACTGCTATTTTATGT
-908  TTTATAGACAGGGTCTCACTCTGTCACCCAGGCTGGAGTGCAGTCACACA
-858  ATCATAGCTCACTGCAACCTCAGCCTCCTGGGCTTAAGCGATCTGCCTCA
-808  GCCTCCCAAGTAGCTGGGACTACAGATGTGTGCCACCACGCCTGGCTAAA

-758  TTTGTTTAAAATTTTTTTTGTAGAGATGGGGTCTCCCTATGTTGCCCAGG

-708  CTAGTCTTGAACTTCTGGGCTCAAGCGACCCTCCTGCCTTGGCCTCCCAA

-658  ATTGCTGGGATTACAGGCATAAGCCACTGTGCTGGGCCATATTACTGCTG
                    HNF4
-608  TCATTTATGGCCAAAAGTTTGCTCAAACATTTTCCAGTTACCAGAGCCAC

-558  ATCTCAAGGGTCTGACACTGGGAAAACACCACGTGCGGATCGGGCACACG

-508  CTGATGCTTGCCCTGCTCAGGGCTATCTAGTGTTCCCTGCCAGAACCTAT

-458  GCACGTGTGGTGAGAGCTTAAAGCAATGGATGCTTCCCCCAACATGCCAG

-408  ACACTCCTGAGGAGCCTGGCGGCTGCTGGCCATGCCCCGTGTGCATGTAG

-358  GCGATGGGGAAGTGAGTGGAGGAGAGCGGAACCTTGATTCTGCTCATCAA
                    C/EBP
-308  ACTGCTTAACCGCTGAAGCAAAAGGGGGAACTTTTTTCCCGATCAGCAGA
                C/EBP
-258  ATGACATCGTGATGGGGAAAGGGCTCCCCAGATGGCTGGTGAGCAGTGTG

-208  TGTCTGTGACCCCGTCTGCCCCACCCCCTGAACACACCTCTGCCGGCTGA

-158  GGGTGACACAACCCTGTTCCCTGTCGCTCTGTTCCCGCTTATCTCTCCCG
                  STAT C/EBP                  HNF4
-108  CCTTTTCGGCGCCACCACCTTCTTGGAAATGAGACAGAGCAAAGGGGAGG
                                      TATA box
 -58  GGGCTCAGACCACCGCCTCCCCTGGCAGGCCCCATAAAAGCGACTGTCAC
        *
  -8  TCGGTCCCAGACACCAGAGCAAGCTCAAGACCCAGCAGTGGGACAGCCAG

 +43  ACAGACGGCACGATGGCACTGAGC
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FIG. 3. **Nucleotide sequence of 5′-flanking region of the human *HEPC* gene.** The putative start of transcription is indicated by an *asterisk* and is designated as +1 and the translation initiation codon ATG is shown in *bold*. *Underlined sequences* indicate potential binding sites for transcription factors.

respectively. Transfection of reporter vector containing 950 bp of the HEPC 5′-flanking region (pHEPC−960/−9) resulted in high luciferase activity in HuH-7 cells whereas the promoter activity was very low in non-hepatic U-2OS cells. Likewise, the truncated 624-bp *HEPC* promoter construct exhibited a very low luciferase activity in U-2OS cells; however, in HuH-7 cells it was even more active than the 950-bp construct (Fig. 4*A*) suggesting that the region between −960 and −633 contains negative cis-acting elements.

Similar to the human *HEPC* promoter, a reporter vector pmHEPC−783/+5 containing 789 bp of the mouse *HEPC1* gene 5′-flanking region exhibited low luciferase activity in U-2OS cells. In contrast, transfection assays with primary mouse hepatocytes showed high reporter activity (Fig. 4*B*). Furthermore we performed a more detailed analysis of the mouse *HEPC1* promoter activity. For this purpose several promoter-luciferase reporter plasmids were constructed and transfected into primary mouse hepatocytes. As shown in Fig. 4*C*, deletion of the 5′-flanking DNA from bp −783 to bp −554 that contains a distal putative C/EBP binding site had no significant effect on the activity of the mouse hepcidin promoter. Deletion to bp −338 resulted in moderate increase of luciferase activity and, finally, further deletion of DNA between −338 and −267 containing second putative C/EBP binding site, strongly (~12-fold) decreased promoter activity.

Liver-enriched Transcription Factor C/EBPα but Not HNF4α Can Transactivate Human and Mouse HEPC Gene Promoters in Vitro—Because sequence analysis of 5′-flanking region of human and mouse *HEPC* genes revealed several putative C/EBP and HNF4 binding sites we studied the involvement of C/EBPα and β, members of C/EBP family of transcription factors, and HNF4α on its activity. While co-transfection of

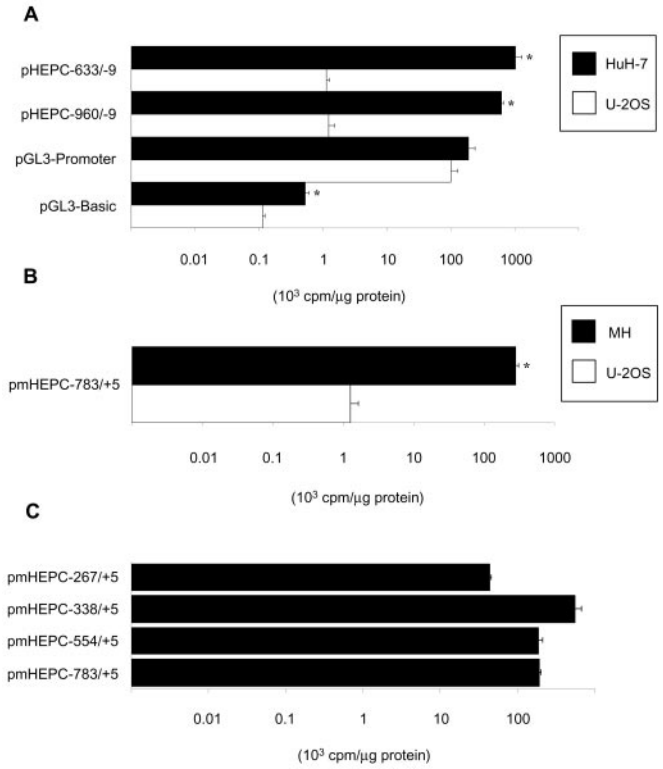

FIG. 4. **Hepatocyte-specific activity of human and mouse *HEPC* promoters.** *A*, luciferase reporter vectors pHEPC−960/−9 and pHEPC−633/−9 containing 5′-flanking regions of the human *HEPC* gene were transfected into hepatoma HuH-7 and non-hepatic U-2OS cells. pGL3-Basic and pGL3-Promoter vectors were used as negative and positive controls, respectively. In all cases, transfection efficiency assessed by co-transfected vector pEGFP-C3-encoding GFP was ~10–15%. *B*, the reporter vector pmHEPC-783/+5 containing 789 bp of the 5′-flanking region of the mouse *HEPC1* gene was transfected into primary mouse hepatocytes (*MH*) and U-2OS cells. *C*, various luciferase reporter plasmids containing the mouse *HEPC1* promoter (−267, −338, −554, and −783/+5 bp from transcription initiation codon) were transfected into primary mouse hepatocytes. Transfection efficiency assessed by expression of GFP in mouse hepatocytes was ~15–20%. Luciferase activity for each condition is presented as cpm/μg of cellular protein. Transfections were performed in triplicates, and results are presented as means ± S.D. Similar results were obtained from three independent experiments for the human *HEPC* promoter activity and from two independent experiments for mouse promoter activity in mouse hepatocytes. *, $p < 0.01$ between promoter activity in HuH-7 (or mouse hepatocytes) and U-2OS.

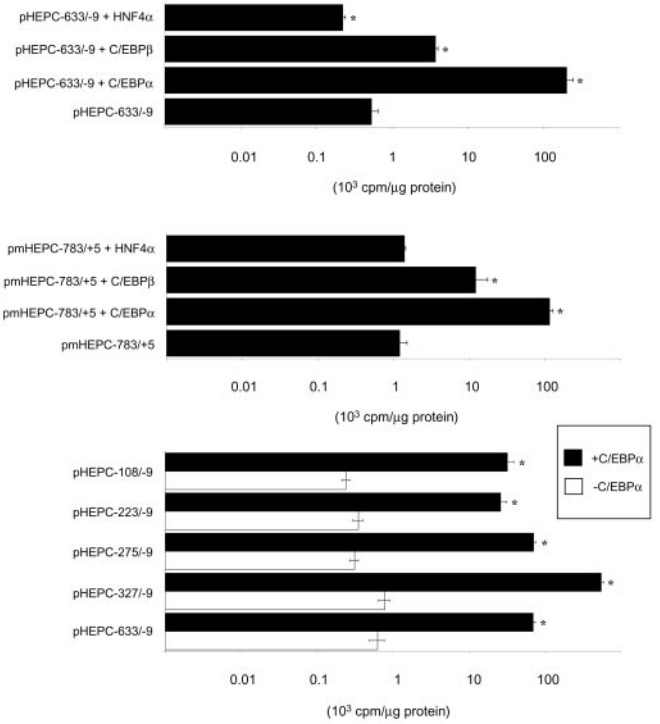

FIG. 5. **Analysis of transactivation of the human and mouse *HEPC* promoters by C/EBPα, C/EBPβ, and HNF4α.** Expression vectors encoding C/EBPα, C/EBPβ, and HNF4α or empty vectors were co-transfected with human *HEPC* promoter-luciferase vector pHEPC−633/−9 (*A*) or mouse *HEPC1* promoter-luciferase plasmid pmHEPC-783/+5 (*B*) into U-2OS cells. *C*, a series of reporter plasmids fusing variable amounts of human *HEPC* promoter (−108, −223, −275, −327, and −633/−9 bp from transcription initiation codon) to luciferase were co-transfected with or without C/EBPα into U-2OS cells. In all cases, transfection efficiency assessed by expression of GFP was ~10–15%. Luciferase activity is presented as cpm/μg of cellular protein. Transfections were performed in triplicates, and results are presented as means ± S.D. * indicates significant modulation of promoter activity by co-transfected expression vectors ($p < 0.01$).

C/EBPα with the human hepcidin promoter reporter vector pHEPC−633/−9 into U-2OS cells very strongly increased luciferase activity, the C/EBPβ-mediated induction of promoter activity was relatively weak. In contrast, co-expression of HNF4α had ~2-fold inhibitory effect on luciferase activity (Fig. 5*A*). In a similar manner, co-transfection of C/EBPα or C/EBPβ with pmHEPC−783/+5 reporter vector that contains 5′-flanking region of the mouse *HEPC1* gene, resulted respectively in relatively strong and weak increase of luciferase activity. HNF4α co-expression had no significant effect on the mouse *HEPC* promoter activity (Fig. 5*B*).

Finally, to analyze precisely the involvement of C/EBPα in the transcriptional control of *HEPC* promoter, a series of plasmids fusing 5′-flanking DNA of the human *HEPC* gene were constructed and tested for promoter activity in the absence or presence of C/EBPα in U-2OS cells. Deletion of the 5′-flanking DNA between −633 and −327 resulted in an increase on the magnitude of C/EBPα induction. Deletion of the DNA region (between −327 and −275) that contains the distal putative C/EBPα binding site led to the decrease of C/EBPα inductibility to levels observed with the pHEPC (−633/−9) reporter vector. Cotransfection of C/EBPα with pHEPC (−223/−9) reporter vector lacking the second putative C/EBPα binding site had an important inhibitory effect on C/EBPα-mediated modulation of promoter activity. Finally, further deletion to −108 had no significant effect on the magnitude of the C/EBP induction (Fig. 5*C*).

C/EBPα Binds to the HEPC Promoter—Furthermore, in order to demonstrate that the positive effect of C/EBPα on *HEPC* promoter activity was direct, we performed EMSA using adult rat liver nuclear extracts, together with three ^{32}P-labeled double-stranded oligonucleotides, HEPC(−300/−280) HEPC-(−250/−230), and HEPC(−92/−72) whose sequences encompass putative C/EBP binding sites in the 5′-flanking region of the human *HEPC* gene. As expected, C/EBP binding activity was detected by EMSA in rat liver nuclear extracts using reference C/EBP oligonucleotide probes. The DNA binding was specific since it was efficiently suppressed by addition of 100-fold molar excess of the unlabeled wild-type but not mutant C/EBP probe. Preincubation of nuclear extracts with specific antibodies to C/EBPα resulted in partial supershifting of binding complexes (Fig. 6). HEPC(−300/−280) probe that contains the distal potential C/EBP binding site showed low levels of binding activity with rat liver nuclear extracts. In contrast, using labeled HEPC(−250/−230) probe representing the second C/EBP binding site we found that liver nuclear extracts contained strong binding activity, and the migration profile was similar to that obtained with reference wild-type C/EBP oligonucleotide probe. The DNA binding was efficiently com-

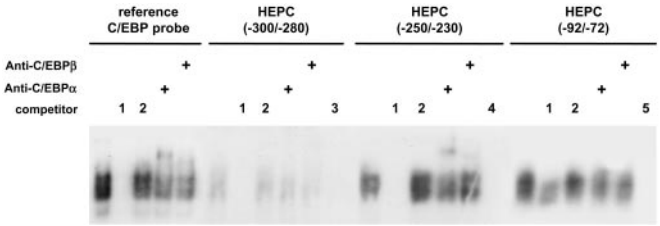

FIG. 6. **Characterization of C/EBP-binding sites in the human *HEPC* promoter.** Electrophoretic mobility shift assay was performed with rat liver nuclear extracts and ^{32}P-labeled reference wild-type C/EBP, HEPC (−300/−280), HEPC (−250/−230), and HEPC (−92/−72) double-stranded oligonucleotide probes. Competition experiments were carried out by addition of 100-fold molar excess of the unlabeled reference wild type C/EBP (1), mutant C/EBP (2), HEPC (−300/−280) (3), HEPC (−250/−230) (4), or HEPC (−92/−72) (5) double-stranded oligonucleotides. For the supershift assay, rat liver nuclear extracts were preincubated with anti-C/EBPα and anti-C/EBPβ antibodies as indicated above the lanes.

peted with 100-fold molar excess of unlabeled reference wild-type C/EBP and homologous HEPC (−250/−230) oligonucleotide probes but not with mutant C/EBP probe. Furthermore, a supershift reaction was evidenced when anti-C/EBPα but not anti-C/EBPβ antibodies were added to the binding mixture. Finally, the HEPC (−92/−72) probe that contains the overlapping C/EBP and STAT sites bound rat liver nuclear proteins. All binding was efficiently suppressed by addition of the unlabeled homologous oligonucleotide probe. However, competition performed with reference wild-type C/EBP probe resulted in partial blocking of binding activities. In addition, no supershifting was detected by addition of either C/EBPα or C/EBPβ antibodies (Fig. 6). Taken together, the results of transfection experiments and gel shift assays suggest that the HEPC (−250/−230) C/EBP binding site is more important for activation of the human *HEPC* promoter by C/EBPα than two other sites.

C/EBPα and HNF4α Control Expression of HEPC Transcripts in Mice Livers in an Opposite Manner—To validate the data obtained from *in vitro* experiments and to determine whether liver-enriched transcription factors C/EBPα and HNF4α can control transcription of the *HEPC* gene in the same manner *in vivo*, we studied the role of these proteins in the regulation of hepcidin expression in an intact mouse model. Because the standard HNF4α and C/EBPα gene knockout led to early embryonic lethality or to death shortly after the birth, respectively (29, 30) we investigated expression of hepcidin in mice with conditional liver-specific disruption of *C/EBPα* or *HNF4α* genes. Specifically, amounts of HEPC transcripts were determined by Northern blotting in mice in which *C/EBPα* gene was specifically disrupted in the liver by Cre-mediated recombination (C/EBPαLivKO) and in control mice containing a functional *C/EBPα* gene (C/EBPαFlox). As shown in Fig. 7*A*, control mice expressed C/EBPα transcripts (*lanes 1* and *2*) and contained also high amounts of liver HEPC mRNA. In contrast, the hepatic *C/EBPα* gene-deficient mice (*lanes 3* and *4*) showed decreased amounts of HEPC transcripts in the liver. Specifically, the mouse with undetectable expression of *C/EBPα* gene (*lane 3*) contained the lowest amount of HEPC transcripts and those expressing some C/EBPα mRNA (*lane 4*) exhibited elevated expression of hepcidin.

To investigate whether the expression of hepcidin was affected *in vivo* by disruption of the hepatic *HNF4α* gene, RNA from 45-day-old male HNF4αLivKo mice (21) lacking HNF4α in the liver and control animals was analyzed for the presence of HEPC transcripts. While mRNA levels for apolipoprotein C-III (ApoC-III) were drastically decreased in liver-specific HNF4α-null mice in accordance with previous data (21), liver expression of hepcidin was 4.1-fold higher ($p < 0.01$) in these animals (Fig. 7*B*).

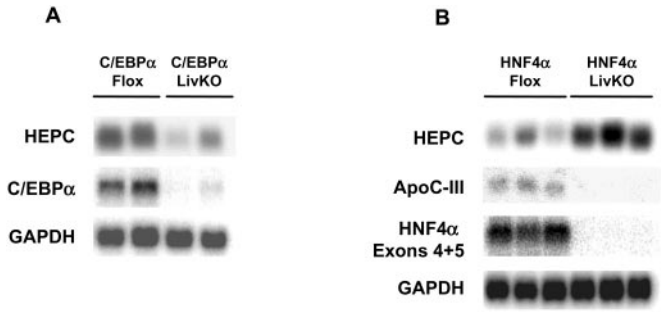

FIG. 7. **Expression of HEPC transcripts in livers of C/EBPα- or HNF4α-deficient mice.** *A*, total RNA was extracted from livers of 6-week-old control C/EBPαFlox mice (C/EBPα$^{fl/fl}$ × albumin-Cre$^{-/-}$) (*lanes 1* and *2*) and from C/EBPαLivKO mice (C/EBPα$^{fl/fl}$ × albumin-Cre$^{+/-}$) in which the *C/EBPα* gene was specifically disrupted in the liver (*lanes 3* and *4*). 10 μg of RNA were separated by electrophoresis and subjected to Northern blot analysis using mouse hepcidin and C/EBPα cDNA probes. *B*, total RNA was extracted from livers of 45-day-old control HNF4αFlox (HNF4α$^{fl/fl}$ × albumin-Cre$^{-/-}$) mice and from liver-specific HNF4α-null mice HNF4αLivKO (HNF4α$^{fl/fl}$ × albumin-Cre$^{+/-}$). 10 μg of RNA were separated by electrophoresis, transferred to a nylon membrane, and hybridized with the indicated ^{32}P-labeled cDNA probes.

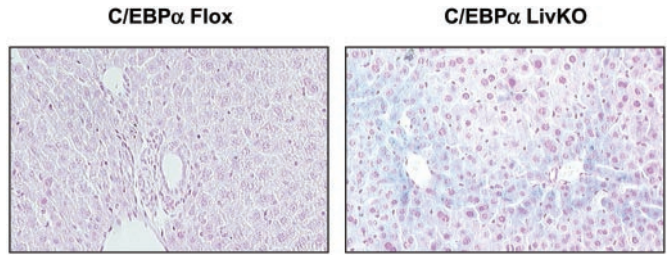

FIG. 8. **Iron accumulation in liver of mice lacking hepatic C/EBPα expression.** Liver sections were prepared from control (C/EBPαFlox) or from liver-specific C/EBPα-null mice (C/EBPαLivKO) and stained for iron. Positive *iron blue staining* is localized in periportal hepatocytes.

Liver-specific Deletion of the C/EBPα Gene Results in Hepatic Iron Overload in Mice—Nicolas *et al.* (15) demonstrated previously that *USF2*$^{-/-}$ mice had undetectable expression of hepcidin mRNA in the liver and exhibited massive iron overload in the liver. We investigated whether the decreased expression of hepcidin in hepatic C/EBPα-deficient mice could also result in alteration of iron metabolism. Iron staining was performed on livers of 2-month-old control (C/EBPαFlox) and liver-specific C/EBPα-null mice (C/EBPαLivKO). While no iron accumulation was detected in the group of control mice, 4 of 5 C/EBPα-null mice exhibited the presence of iron deposits, visible as blue staining, mainly in periportal hepatocytes (Fig. 8).

Iron Overload Induces C/EBPα Expression—Iron overload observed in liver-specific C/EBPα-null mice strongly indicates a possible involvement of C/EBPα in the regulation of iron metabolism. The positive effect of C/EBPα on the *HEPC* gene transcription *in vitro* and *in vivo*, and previously described up-regulation of hepcidin expression in the liver by iron excess suggested that this effect is mediated by C/EBPα and prompted us to investigate expression of this transcription factor during iron overload. In accordance with our previous observations, amounts of HEPC mRNA in liver tissue strongly increased in all animals overloaded by 3% carbonyl iron for 8 months (Fig. 8). Interestingly, these mice also showed a 2.1-fold increase ($p < 0.01$) of the C/EBPα protein in the liver compared with the control ones (Fig. 9).

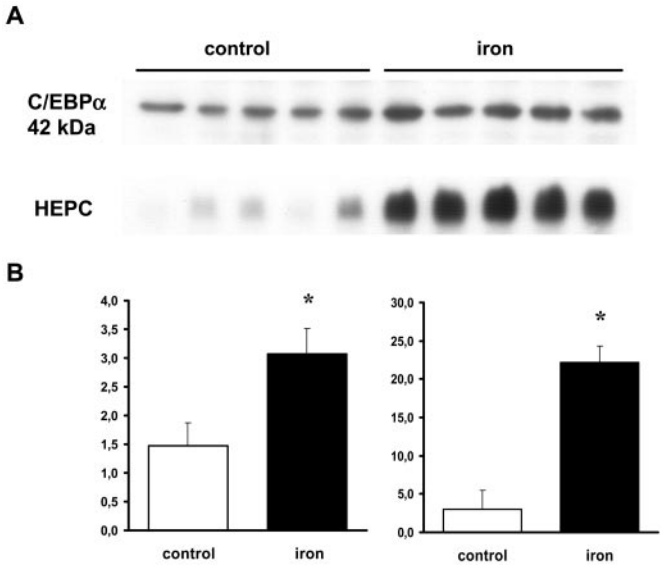

FIG. 9. **Iron overload leads to an increase of C/EBPα protein expression in mouse liver.** *A*, liver samples from mice iron-overloaded by 3% carbonyl iron for 8 months and control animals were used for extraction of total RNA and for preparation of tissue lysates. Expression of C/EBPα and hepcidin was studied by Western blot and Northern blot analysis, respectively. *B*, densitometry analysis. The average for the group of control and iron-overloaded mice is shown in histograms; *left panel* represents data from Western blot analysis of C/EBPα and *right panel* from Northern blot analysis of hepcidin. The lowest densitometry value from the group of control mice was arbitrarily set as 1. *, $p < 0.01$.

DISCUSSION

Hepcidin, a small circulating cysteine-rich peptide, exhibits a broad range of antimicrobial and antifungal activities (8, 9). However, several lines of evidence including pattern of expression and regulation by iron overload strongly suggest that hepcidin differs from described antimicrobial peptides (7, 8). This hypothesis is reinforced by recent data describing the association of iron-overload phenotype with lack of hepcidin expression in *USF2*$^{-/-}$ mice (15, 16). After submission of this manuscript, Nicolas *et al.* (17) reported development of phenotypical traits of severe iron deficiency anemia in transgenic mice expressing hepcidin under control of the liver-specific transthyretin promoter. This observation reinforced the idea that this peptide plays an essential role in regulating liver metabolism.

Here we confirm and extend previous data on hepatocyte-specific expression of the hepcidin gene and the relation of its expression on hepatocyte differentiation phenotype in humans, mice, and rats. In addition to its predominant hepatic expression, the *HEPC* gene is likely one of the most highly expressed genes in adult liver. Indeed, as assessed by Northern blot analysis of mice, rat, and human adult liver mRNA, hybridization signal was detected after a very short time of exposure to film (as early as 2–4 h). In addition the real-time RT-PCR analysis revealed *HEPC* gene expression in human adult liver in a range compatible to that of β-actin gene supporting the previously reported analysis of hepcidin expression in human tissues (9). Considering a strong liver-specific pattern of hepcidin expression, activation of its expression by iron and implication in the control of iron metabolism, we analyzed the molecular mechanisms involved in hepcidin gene transcription regulation.

A study of *HEPC* gene expression during rat liver development, which is an *in vivo* model of hepatocyte differentiation, revealed very low amounts of HEPC transcripts in fetal liver, and its relatively late accumulation from the 28th day of postnatal development. Interestingly, we found a strong and transient induction of HEPC mRNA shortly after birth. After submission of this manuscript, Nicolas *et al.* (17) published a report describing expression of hepcidin mRNA during liver development in wild type C57BL/6 mice. In these mice it appeared that hepcidin genes were strongly and transiently induced at birth and at first and second days of postnatal development. Thus, at least in rodents, hepcidin follows the similar pattern of developmental regulation. Previously it has been reported that a number of genes, mainly involved in the regulation of hepatic metabolism, are activated near the time of birth (31). The shift from fetal to neonatal life is manifested by dramatic metabolic changes in the liver, including mobilization of stored glycogen and development of hepatic gluconeogenesis in order to maintain glucose homeostasis during the postnatal period. Thus, an increase of hepcidin shortly after birth is probably a part of metabolic patterning of the liver that occurs during the perinatal period and might have an adaptive role to prevent microbial infection and/or to modulate iron metabolism after birth.

Analysis of sequences of 5′-flanking regions of human and mouse *HEPC* genes and measurement of promoter activities provided important elements that help to understand the phenomenon of predominant expression of this gene in the liver and mechanisms of its regulation during various physiopathological situations. Reporter vectors containing nucleotide fragments of 5′-flanking genomic region of human and mouse *HEPC* genes exhibited strong basal promoter activity in HuH-7 hepatoma cells and primary mouse hepatocytes, respectively. However, activity of both human and mouse *HEPC* promoters was very low in non-hepatic U-2OS cells suggesting that expression of hepcidin is regulated in the liver primarily at transcriptional level. Sequence analysis of the 5′-flanking region of the human and mouse *HEPC* gene identified several binding sites for liver-enriched transcription factors C/EBP and HNF4 known to be involved in the regulation of liver-specific genes (18, 32, 33). Interestingly, four putative C/EBP binding sites were also described in 5′-flanking region of bass hepcidin gene (11). The C/EBP family of transcription factors includes at least six members, although two of them, C/EBPα and C/EBPβ, are enriched in the liver. The important functional role of C/EBPα in *HEPC* gene transcription was confirmed in cotransfection experiments. The promoter activity of both the human and mouse *HEPC* gene was dramatically increased by C/EBPα in U-2OS cells. However, C/EBPβ isoform had a relatively weak capacity to transactivate HEPC promoters. Characterization of three putative C/EBP binding sites in the human *HEPC* promoter revealed high DNA binding activity of rat liver nuclear extracts to one site (HEPC−250/−280) and the predominant presence of C/EBPα in the bound complex. Together with the results of cotransfection of C/EBPα expression vector with various human HEPC promoter-luciferase reporter plasmids demonstrated that the HEPC−250/−280 site represents a functional C/EBP site and is likely the most important for induction of the human *HEPC* promoter by C/EBPα.

It is interesting to note that despite the similar binding specificities of C/EBPα and C/EBPβ to CAAT sites, they regulate different genes in liver. C/EBPα also specifically controls the retinaldehyde dehydrogenase gene 4 (34). In contrast, the *Cyp2d5* gene is controlled by C/EBPβ and not by C/EBPα (35). While the mechanisms governing this specificity between C/EBPα and C/EBPβ is presently unclear, it is interesting to note that replacement of the *C/EBPα* gene with the C/EBPβ does not change the phenotype of liver (36).

Since C/EBPα is highly abundant in adult liver (37), it is likely that this transcription factor is mainly involved in main-

tenance of the hepatic *HEPC* gene expression in mature mouse liver. Moreover, among different regulatory mechanisms, liver-enriched transcription factors that belong to C/EPB family are likely to largely account for developmental changes in liver metabolism after birth. Indeed, C/EBPα appears in the liver during the last trimester of fetal development, reaches the highest levels near the time of birth, decreases during the suckling period, and reaccumulates in adult liver (18). Thus, this transcription regulator is likely to be involved in *HEPC* gene activation in liver both shortly after birth and at the adult stage. In addition, the previous observation of low concentration of C/EBPα in cultured hepatoma cells in comparison with adult hepatocytes (38) is consistent with our data on low or undetectable amounts of HEPC transcripts in rat and human liver-derived cell lines.

Finally, the involvement of C/EBPα in the hepatic regulation of the *HEPC* gene was confirmed *in vivo* in mice lacking C/EBPα expression in the liver. While, as expected, 6-week-old control mice contained high levels of HEPC transcripts, hepatic C/EBPα-null animals exhibited a strong decrease of *HEPC* gene expression.

In addition to well documented antimicrobial activity, it has been hypothesized that hepcidin acts as a key regulator of iron homeostasis (15, 16). Mice with totally silent *HEPC* genes in *USF2*$^{-/-}$ mice exhibited strong iron accumulation in the liver and pancreas. It is worthy noting that although homozygote *USF2*$^{-/-}$ mice with undetectable expression of HEPC gene transcripts had profound abnormalities of iron metabolism, this phenotype was not observed in heterozygote *USF2*$^{+/-}$ mice containing a reduced amount of hepcidin mRNA in the liver (15). We investigated whether a decrease of hepcidin expression in livers of hepatic C/EBPα-null mice could result in modification of liver iron content. Histological examination of liver section from 2-month-old C/EBPαLivKO mice revealed weak but obvious iron staining of periportal hepatocytes. Although we cannot fully exclude that C/EBPα is involved in the regulation of other genes encoding proteins related to iron metabolism, correlation of reduced levels of hepcidin with iron overload in liver-specific C/EBPα-null mice supports the proposed role of hepcidin as a regulator of iron homeostasis.

In contrast to C/EBPα, HNF4α either had no significant effect on the *HEPC* promoter activity (mouse promoter) or moderately decreased its activity (human promoter). *In vivo*, expression of HEPC transcripts increased in mice with liver-specific disruption of the *HNF4α* gene, suggesting that this transcription factor is directly or indirectly involved in the negative regulation of *HEPC* gene transcription.

We have shown previously that expression of hepcidin was strongly up-regulated by iron excess using different models including experimental carbonyl iron and iron-dextran overload (7). However, mechanisms of this regulation remained unknown. Here we found that carbonyl iron overload led to a 2-fold increase of C/EBPα protein expression in mice livers. Thus, taking into consideration the role of C/EBPα in the regulation of *HEPC* gene promoter activity and on expression of HEPC transcripts *in vivo*, it is likely that, at least partly, this transcription factor is involved in an iron-mediated increase of hepcidin expression. Considering that C/EBPα is also involved in the regulation of a large set of liver-specific genes including key glyconeogenic enzymes and is essential for energy homeostasis (18, 30), this nuclear factor could modulate expression of a large number of target genes under condition of iron excess. In this setting, it will be interesting to analyze if iron-induced glucose metabolism abnormalities observed during genetic hemochromatosis (39) are resulted, at least partly, from an increase of C/EBPα expression.

In conclusion, we demonstrated that liver expression of hepcidin is developmentally regulated and is related to hepatocyte functional status. C/EBPα transcription factor is likely to play a key role in regulation of *HEPC* gene transcription. The correlation between expression of C/EBPα and hepcidin and iron accumulation in the liver of mice with hepatic C/EBPα disruption and, on the other hand, the increase of both C/EBPα and hepcidin during iron overload could represent a novel mechanism for cross-talk between the C/EBP signaling pathway and iron metabolism.

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C/EBPα Regulates Hepatic Transcription of Hepcidin, an Antimicrobial Peptide and Regulator of Iron Metabolism: CROSS-TALK BETWEEN C/EBP PATHWAY AND IRON METABOLISM

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