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 perportal 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 microbial substances named antimicrobial peptides that behave as natural antibiotics of the organism (1, 2). These molecular effectors, universally found in proaryotes, 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 (USP2) knockout mice revealed a development of iron overload

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† 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.

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41163

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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 USP2 gene, were transcriptionally inactive, and livers of USP2 1/2–/– 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 USP2 1/2–/– mice (15, 16). Inversely, early liver expression of 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-lès-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. Ille, 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% carboxyl iron supplemented in the diet (AOS, UAR, France) over 8 months. Control mice had a carboxyl 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% 50% Ham’s F-12 and 50% NCTC135 and in DMEM, respectively, supplemented with 10% FCS, 1 mg of bovine serum albumin, 5 μg of bovine insulin, and 2 mM L-glutamine.

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, m M 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 32P-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 EcoRI 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-CTGGCGGCTGTCATCAGTCA-TAMRA-3′ and 5′-FAM-ATGGAGACATGGAAAAACTCGGC-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.

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 (60 μg) were resolved in MOPS-PAGE, transferred to a 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 secondary 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 genomic DNA isolated from peripheral blood mononuclear cells following forward primers: 5′-GGCTCGAGATCTCAGCTGACGTATGTC TTAGC-3′ and 5′-GGCTCGAGAATTCGGCGTAGACCT-3′, respectively, and the reverse primer: 5′-GGAGAAGGTTGTCGGAC TGCATTGTTATGGGGCCCCTCG-3′ (the incorporated XhoI and HindIII sites are underlined). PCR fragments were digested with XhoI and HindIII and inserted into the multiple cloning site of the pGL3 Basic 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 −106/−9) were generated by PCR using pHEPC−633/−9 as template and specific primers containing XhoI and HindIII restriction sites inserted between the PCR product and XhoI site on 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′-GGCTCGAGAATTCATGTCGTCAAGCAG-3′ and the reverse...
verse primer, 5′-GCGAACGTCTGTCGGTGCTCCTAGGACG-3′ (the incorporated XhoI and HindIII sites are underlined). The PCR fragment was digested with XhoI and HindIII and was inserted into the pGL3-Basic vector. The obtained construct was designated pmHEPC—738/+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/EBPa, 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 transduced 24 h after seeding using GB12 reagent as previously described (25). The following expression plasmids were used for co-transfection: pCMV-C/EBPa and pCMV-NF-IL6 encoding C/EBPa and C/EBPβ, respectively (kindly provided by G. Darlington, Baylor College of Medicine, Houston) and a HNF4α expression vector pSG5/HNF4. 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 MgCl2, 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′-CTAGGCTTGGCAACTTATATCCG-3′ and 3′-GATCCCCAGGTGGAATAGA-5′. The mutant form of the C/EBP consensus sequence element: 5′-CTAGGCTTGGCCTCCCTATATCCG-3′ and 3′-GATCCCCAGGTGGAATAGAAGATAGAAG-5′. The oligonucleotide probe encompassing first putative C/EBP binding site in 5′-flanking region of the human HEPC gene, HEPC—(300—280), 5′-CTTAAACCGCTGAGGAAAGGGGGA-3′ and 3′-TGCGGACTCTGGTCTGAGG-5′. Second putative C/EBP binding site, HEPC—(250—230), 5′-CATCGTGAGGCGAACGCCGCC-3′ and 3′-GATCCCCAGGTGGAATAGA-5′. The mutant form of the C/EBP binding site, HEPC—(92—72), 5′-GATCGTGTTGGCCTTATATCCG-3′ and 3′-GATCCCCAGGTGGAATAGAAGATAGAAG-5′. The mutant form of the C/EBP binding site, HEPC—(92—72), 5′-GATCGTGTTGGCCTTATATCCG-3′ and 3′-GATCCCCAGGTGGAATAGAAGATAGAAG-5′.

**RESULTS**

**HEPC Gene Expression in Developing Rat Liver**—**Regulated 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. 1A). 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. 1B). Furthermore, HEPC mRNAs could not be detected in rat hepatoma cell lines HCTc and FAZA after a 2-week exposure.

The survey of four human hepatoma cell lines (HepG2,
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. 1C).

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 TTCTGGAAAATGGA overlaps with the STAT consensus binding site TCTCTGTCTCCCGAC.

Sequence analysis of a 789-bp nucleotide fragment of the 5'-flanking 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 STAT consensus binding site TATATCTCCCGDG.

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, 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. 4A) 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. 4B). 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. 4C, 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
C/EBPα Regulates Transcription of Hepcidin

**Fig. 4.** Hepatocyte-specific activity of human and mouse HEPC promoters. A, luciferase reporter vectors pHEPC–633/–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.

C/EBPs with the human hepcidin promoter reporter vector pHEPC–633/–9 into U-2OS cells very strongly increased luciferase activity, while in contrast, co-expression of HNF4α had ~2-fold inhibitory effect on luciferase activity (Fig. 5A). 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. 5B).

**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/EBPs 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).

Cotransfection of C/EBPs 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. 5C).

C/EBPα Binds to the HEPC Promoter—Furthermore, in order to demonstrate that the positive effect of C/EBPs on HEPC promoter activity was direct, we performed EMSA using adult rat liver nuclear extracts, together with three 32P-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-
C/EBPα Regulates Transcription of Hepcidin

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 32P-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, HEPC (−300/−280), HEPC (−250/−230), and HEPC (−92/−72) double-stranded oligonucleotides. For the supershift assay, rat liver nuclear extracts were incubated with anti-C/EBPα and anti-C/EBPβ antibodies as indicated above the lanes.

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/EBPαfl/fl mice (C/EBPαfl/fl × albumin-Cre−/−) (lanes 1 and 2) and from C/EBPαLivKO mice (C/EBPαfl/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 (HNF4αfl/fl × albumin-Cre−/−) mice and from liver-specific HNF4α-null mice HNF4αKoLivKO (HNF4αfl/fl × albumin-Cre−/−). 10 μg of RNA were separated by electrophoresis, transferred to a nylon membrane, and hybridized with the indicated 32P-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 perportal 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 perportal 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).
C/EBPα Regulates Transcription of Hepcidin

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 USP2−/− 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 metabolic, 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/EBP 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 USP22+/− mice exhibited strong iron accumulation in the liver and pancreas. It is worthy noting that although homozygote USP22−/− mice with undetectable expression of HEPC gene transcripts increased in mice with liver-specific C/EBPα−/− animals exhibited a strong decrease of HEPC gene 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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