

A New Mouse Liver-specific Gene, Encoding a Protein Homologous to Human Antimicrobial Peptide Hepcidin, Is Overexpressed during Iron Overload*

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Christelle Pigeon^{‡§}, Gennady Ilyin[‡], Brice Courselaud[‡], Patricia Leroyer[‡], Bruno Turlin^{‡¶},
Pierre Brissot^{‡||}, and Olivier Loréal[‡]

From the [‡]INSERM U522, CHRU Pontchaillou, Rennes, [¶]Laboratoire d'Anatomo-Pathologie B, CHRU Pontchaillou, Rennes, and ^{||}Service des Maladies du Foie, CHRU Pontchaillou, 35033 Rennes, France

Considering that the development of hepatic lesions related to iron overload diseases might be a result of abnormally expressed hepatic genes, we searched for new genes up-regulated under the condition of iron excess. By suppressive subtractive hybridization performed between livers from carbonyl iron-overloaded and control mice, we isolated a 225-base pair cDNA. By Northern blot analysis, the corresponding mRNA was confirmed to be overexpressed in livers of experimentally (carbonyl iron and iron-dextran-treated mice) and spontaneously (β_2 -microglobulin knockout mice) iron-overloaded mice. In addition, β_2 -microglobulin knockout mice fed with a low iron content diet exhibited a decrease of hepatic mRNA expression. The murine full-length cDNA was isolated and was found to encode an 83-amino acid protein presenting a strong homology in its C-terminal region to the human antimicrobial peptide hepcidin. In addition, we cloned the corresponding rat and human orthologue cDNAs. Both mouse and human genes named *HEPC* are constituted of 3 exons and 2 introns and are located on chromosome 7 and 19, respectively, in close proximity to *USF2* gene. In mouse and human, *HEPC* mRNA was predominantly expressed in the liver. During both *in vivo* and *in vitro* studies, *HEPC* mRNA expression was enhanced in mouse hepatocytes under the effect of lipopolysaccharide. Finally, to analyze the intracellular localization of the predicted protein, we used the green fluorescent protein chimera expression vectors. The murine green fluorescent protein-prohepcidin protein was exclusively localized in the nucleus. When the putative nuclear localization signal was deleted, the resulting protein was addressed to the cytoplasm. Taken together, our data strongly suggest that the product of the new liver-specific gene *HEPC* might play a specific role during iron overload and exhibit additional functions distinct from its antimicrobial activity.

The understanding of iron metabolism in mammals especially of the mechanisms involved in cellular iron transport and in the regulation of intracellular iron amount is of a great importance since diseases associated with iron overload are common in humans and can be responsible for the shortening of life expectancy. Genetic hemochromatosis is characterized by digestive hyperabsorption of iron. In 1996, Feder *et al.* (1) reported that the C282Y mutation of *HFE* gene was responsible for this disease. Other iron overload diseases include β -thalassemias, genetic diseases characterized by hemolytic anemia, in which iron overload results both from digestive hyperabsorption of iron and blood transfusions (2, 3). In addition, it has been shown that iron excess may occur in chronic liver diseases such as alcoholic liver diseases (4) or hepatitis B and C infections (5, 6). For these various diseases, clinical data suggest that iron excess favors the development of hepatic lesions likely due to the preponderant role of the liver in iron storage. This is particularly true in untreated genetic hemochromatosis in which many authors demonstrated a relationship between the increase in liver iron concentration and the risk of cirrhosis and hepatocellular carcinoma (7–12).

Recently, additional elements involved in iron metabolism have been characterized as follows: (i) *HFE* potentially modulating iron absorption by its interaction with transferrin receptor (13, 14); (ii) *Nramp2* (also named *DCT1* or *DMT1*) involved in iron uptake at the apex of the enterocyte (15, 16); (iii) transferrin receptor 2 involved in hepatic iron uptake (17); (iv) hephaestin, implicated in iron transfer from enterocyte to the plasma (18), and frataxin which play a role in the mitochondrial iron metabolism (19).

Despite these recent advances, molecular mechanisms leading to hepatic iron overload remain poorly understood. In addition, molecular mechanisms involved in the development of iron-related lesions of the liver are not well known. However, it is clear that modulation of hepatic gene expression, related directly or indirectly to iron excess, takes place during the development of hepatic lesions. Several specific mRNAs are known to be modulated by the iron amount of the liver. Thus, transferrin receptor mRNA, presenting an iron-responsive element (IRE)¹, is repressed during iron overload. Moreover, even if not yet fully demonstrated, genes containing an IRE in their 3'-untranslated region may be good candidates to be

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF297664, AF309489, and AF344185.

§ To whom correspondence should be addressed: INSERM U522, CHRU Pontchaillou, 35033 Rennes Cedex, France. Tel.: 33 299543737; Fax: 33 299540137; E-mail: christelle.pigeon@rennes.inserm.fr.

¹ The abbreviations used are: IRE, iron-responsive element; aa, amino acid; DFO, desferrioxamine; EST, expressed sequence tag; FCS, fetal calf serum; GFP, green fluorescent protein; LIC, liver iron concentration; LPS, lipopolysaccharide; PCR, polymerase chain reaction; RT, reverse transcription; SSH, suppressive subtractive hybridization; FGF-2, fibroblast growth factor 2; bp, base pair; NLS, nuclear localization signal.

repressed by iron excess in the liver. This is especially the case for Nramp2 (15) and glycolate oxidase (20). For genes devoid of IRE, it is known that collagen α 1(I) chain (21–24) and transforming growth factor- β 1 mRNAs (23, 25, 26), encoding two proteins potentially involved in the development of liver fibrosis, are both induced by hepatic iron excess. Other mRNAs, such as γ -glutamyltransferase (27) and heme oxygenase 1 (28), which are involved in oxidative stress, were also found to be up-regulated in the liver by high amounts of iron.

Since only a few genes have been reported to be abnormally expressed in the liver when iron is in excess, it became evident that identification of new hepatic genes up-regulated by iron would be helpful for a better understanding of mechanisms leading to iron overload and/or liver injury. The aim of our work was to identify new genes positively regulated by iron. For this purpose, we used the previously described carbonyl iron overload mouse model (29). We performed a suppressive subtractive hybridization between cDNAs obtained from the liver of 3% carbonyl iron-overloaded and control mice. This methodology allows for the analysis of differential gene expression in two different conditions (30). It has already been shown to be useful for identifying the up-regulation of complement C4 in hepatic stellate cells issued from rats overloaded by carbonyl iron (31). More recently, this technology enabled the characterization and the identification of IREG1, an iron transporter for which expression was up-regulated under the conditions of increased iron absorption in the duodenum (32). By using this method, we were able to isolate and characterize a novel gene strongly induced in the liver of iron overloaded mice.

MATERIALS AND METHODS

Animals

5-Week-old BALB/cJ male mice purchased from CERJ (Le Genet St. Ile, France) were used for experimental iron overload (carbonyl iron and iron-dextran). For liver perfusion, BALB/cJ mice and 10-week-old Sprague-Dawley rats were used.

C57 BL/6 β_2 -microglobulin ($-/-$) male mice, 8 weeks old, developing spontaneous iron overload (33, 34) were obtained from CDTA (Orleans, France). Wild type mice C57 BL/6 littermates were used as controls. Another group of C57 BL/6 β_2 -microglobulin ($-/-$) male mice from the Jackson Laboratory (Bar Harbor, ME) was used for performing the iron deprivation experiment.

All these animals were maintained in accordance to French law and regulations in a temperature- and light-controlled environment. They were given free access to tap water and food.

Modulation of Liver Iron Concentration in Vivo

Carbonyl Iron Overload—Iron overload was performed as described previously (29). Briefly, mice were iron-overloaded by 0.5, 1.5, and 3% carbonyl iron supplemented in the diet (AO3, UAR, France) for 8 months. Control mice have a carbonyl iron-free diet. For 3% carbonyl iron-overloaded mice, liver iron concentration was on average 10-fold higher than the control value (115 μ mol of iron/g of dry weight liver versus 10 μ mol, respectively). Liver iron concentration increased 4- and 2-fold, respectively, over the control values for 1.5 and 0.5% carbonyl iron-overloaded mice after 8 months. To study mRNA expression during early stages of iron overload, 3% carbonyl iron was given for only 2 months. Results were compared with mice overloaded by 3% carbonyl iron for 8 months.

Iron-Dextran Overload—A single subcutaneous injection of iron-dextran was performed at the dose of 1 g of iron/kg of body weight (Sigma, France) according to Carthew *et al.* (35). Control mice were injected with a mixture containing dextran and phenol at the same concentration as in iron-dextran solution. Animals, fed with an AO3 diet (UAR, France), were sacrificed 2 months later. In another experiment, a time-dependent iron overload was performed, and animals were sacrificed 1–4 and 8 weeks after the iron-dextran injection.

Spontaneous Iron Overload— β_2 -Microglobulin-deficient and the corresponding control mice, 8 weeks old, were fed with 113 diet (UAR, France) for 12 months and then sacrificed. Ten β_2 -microglobulin-deficient mice, 11 weeks old, were used to study the effect of iron depletion on HEPC mRNA expression. They were divided into two groups. The

first group was given a low iron diet (UAR, France; 13 mg/kg). The control group was given a similar food containing 230 mg of iron/kg. Mice were sacrificed 2 months later.

Liver Iron Concentration

Liver iron concentration (LIC) was evaluated biochemically according to the method of Barry and Sherlock (36) on liver specimens that have been previously fixed in 4% formalin.

Total RNAs and Poly(A)⁺ RNA Preparations

Total RNAs were extracted from liquid nitrogen-frozen tissue specimens using SV total RNA isolation system (Promega, Madison, WI). For suppressive subtractive hybridization, poly(A)⁺ RNAs were isolated separately from livers of 4 mice overloaded with 3% of carbonyl iron for 8 months and from 4 control mice using Invitrogen's FastTract kit (Invitrogen, Groningen, The Netherlands). mRNAs were then pooled per each group of mice.

Suppressive Subtractive Hybridization

Suppressive subtractive hybridization (SSH) was performed between control and iron-overloaded group mice according to the manufacturer's instructions using "PCR-Select cDNA Subtraction kit" (CLONTECH, Palo Alto, CA) (30). The subtractive product, corresponding to overexpressed genes in condition of iron excess, was recovered by PCR. Iron-induced cDNAs were cloned into pCRII vector using a T/A cloning kit (Invitrogen). Each cDNA was sequenced using Thermosequenase kit (Amersham Pharmacia Biotech) with α -³²P-radiolabeled dideoxynucleotides (1500 Ci/mmol). Samples were analyzed on a 5% urea-polyacrylamide gel. Nucleic and amino acid homology searches were performed using the BLAST program (37).

Northern Blot Analysis

Total RNAs were loaded on a denaturing 1.2% agarose gel and transferred onto Hybond N+ filters (Amersham Pharmacia Biotech). Filters were hybridized with the cDNA of interest labeled with [α -³²P]dCTP (3000 Ci/mmol) (Rediprime™, Amersham Pharmacia Biotech). Equal mRNA loading was estimated by methylene blue staining or hybridization with 553-bp mouse cytoskeletal β -actin cDNA probe generated by RT-PCR using following oligonucleotides: 5'-TGTGCTGTCCCTGTATGCCT-3' and 5'-TAGGAGCCAGAGCAGTAATC-3'. Mouse albumin cDNA probe was a 297-bp fragment obtained by SSH.

Cloning of the Full-length cDNA

A 5' stretch cDNA library (CLONTECH) derived from BALB/cJ liver was screened with the [α -³²P]dCTP-radiolabeled 225-bp cDNA obtained by SSH to isolate the full-length murine cDNA. The final isolated 410-bp cDNA was sequenced in both strands. Rat and human orthologue cDNAs were generated, respectively, by RT-PCR from rat liver and human hepatoma HepG2 mRNAs using the following primers: 5'-CACGAGGGCAGGACAGAAGGCAAG-3' and 5'-CAAGGTCATTGCTGGGGTAGGACAG-3' for rat cDNA and 5'-GACTGTCACTCGGTCC-CAGACACCAG-3' and 5'-GGGGCAGGAATAAATAAGGAAGGG-3' for the human cDNA. After an initial denaturation at 94 °C for 5 min, PCRs were set out as follow: 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min for 30 cycles. All cDNAs were then cloned into a pCRII-TOPO vector (Invitrogen) and were sequenced.

Identification of Genomic Structure of the Mouse and Human Genes

The mouse genomic clone CT7-8N15 and the human genomic clone R30879 (GenBank™ accession numbers AC020841 and AD000684, respectively) were used to elucidate the genomic organization of the mouse and human HEPC genes by sequence comparison with the mouse and human cDNAs. Search for transcription binding sites was performed using MatInspector (38).

Tissue-specific Expression

Total mRNA was isolated from the following 8-week old BALB/cJ mice tissues: liver, stomach, duodenum, intestine, ileum, colon, lungs, kidneys, brain, heart, testis, ovaries, thymus, pancreas, bladder, muscle, uterus, and bone marrow, and subjected to Northern blot analysis using the murine 225-bp cDNA as a probe. The expression pattern of the human mRNA was analyzed by RNA blot assay using multiple tissue expression array (CLONTECH), a membrane to which poly(A)⁺ RNAs from different fetal and adult human tissues and cell lines have been immobilized in separate dots. Hybridization was carried out in the

ExpressHyb hybridization solution (CLONTECH) in the presence of ^{32}P -labeled human-specific cDNA probe according to the manufacturer's instructions.

Cell Isolation

Hepatocytes were isolated by enzymatic dissociation from the liver of normal BALB/cJ mice or Sprague-Dawley rat according to Guguen *et al.* (39). Briefly, after cannulation of the portal vein, the liver was perfused with calcium-free Hepes buffer (0.33 mM, pH 7.6) for 5 min for the mouse and 15 min for the rat. Then the liver was perfused with Hepes buffer (0.33 mM, pH 7.6) containing collagenase 0.025% and calcium chloride (0.075%) for 8 min and delivered at 10 ml/min for the mouse and 30 ml/min for the rat. After enzymatic digestion, hepatocytes were obtained by sedimentation (20 min) and then by centrifugation (700 rpm; 1 min) in a Leibovitz medium (Eurobio, France). The supernatant corresponding to the sedimentation step of rat or mouse hepatocytes was centrifuged (1400 rpm; 4 min) to obtain enriched hepatic non-parenchymal cell fraction as adapted from Doolittle and Richter (40). The presence of contaminating hepatocytes evaluated by light microscopy (hepatocytes are bigger cells than non-parenchymal cells) was lower than 5%. Freshly isolated non-parenchymal cells and hepatocytes were frozen immediately in liquid nitrogen before total RNA extraction.

Cell Cultures

Mouse hepatocytes have been seeded in a mixture of minimum essential medium (75%) and M199 (25%) (Eurobio, France) supplemented with 100 IU/ml penicillin, 30 $\mu\text{g}/\text{ml}$ streptomycin sulfate, 1 mg/ml bovine albumin serum, 5 $\mu\text{g}/\text{ml}$ bovine insulin, and 10% fetal calf serum (FCS). Four hours later, medium was renewed by an identical medium containing hydrocortisone hemisuccinate, 5×10^{-5} or 1×10^{-6} M. The day after, hepatocytes have been maintained in the same medium supplemented or not with 10% FCS. Thereafter, hepatocytes were maintained in culture for 2 or 4 days. The medium was renewed every day. In addition, we used human HepG2 (41) rat FAO (42) hepatocarcinoma-derived cell lines that were maintained in Dulbecco's modified Eagle's medium supplemented with 100 IU/ml penicillin, 30 $\mu\text{g}/\text{ml}$ streptomycin, and 1×10^{-6} M hydrocortisone hemisuccinate sulfate or a mixture of Ham's F-12 and NCTC 135 medium (v/v) supplemented with 10% FCS, 100 IU/ml penicillin, and 30 $\mu\text{g}/\text{ml}$ streptomycin sulfate, respectively. Mouse hepatic cell line (43) derived from immortalized adult mouse hepatocytes was maintained accordingly to Paul *et al.* (43).

In Vitro Iron Exposure and Iron Depletion of Hepatocytes

During the first 24 h, hepatocytes were maintained under conditions identical to those described previously. The next day, the culture medium was changed by a similar medium devoid of FCS and supplemented with 1×10^{-6} M hydrocortisone hemisuccinate. Iron exposure was performed by the addition of citrate-iron (5 and 10 μM of iron) in the medium according to the procedure of Azari and Baugh (44). We chose iron-citrate for performing iron exposure because it is a physiologic form of iron that is found in the serum of hemochromatotic patients (45) and because the internalization of iron-citrate into hepatocytes is a well described process (46, 47). The ratio was 1/10 for iron as compared with citrate. Control cultures were incubated in the absence or in the presence of citrate alone (50 and 100 μM). Iron depletion was performed by addition of desferrioxamine (DFO, Ciba Geigy) at the concentration of 10 μM (48, 49). In addition, a culture was carried out in presence of both DFO 10 μM and citrate-iron 10 μM . All the cultures were maintained for 2 days. A similar experiment was performed except that the culture medium was supplemented with 10% FCS.

LPS Treatments

For *in vivo* experiments, 5-week-old mice BALB/cJ mice were used. LPS (Westphal preparation from *Escherichia coli* 055:B5; Sigma) was given intraperitoneally in 0.2 ml of sterile, pyrogen-free saline (0.9% NaCl) at the dose of 0.1 mg/kg to mice fasted overnight (50). Animals were sacrificed 90 min later, and their livers were frozen in liquid nitrogen. For *in vitro* experiments, the primary cultures of mouse hepatocytes, maintained in absence of FCS, were exposed to LPS (20 $\mu\text{g}/\text{ml}$ diluted in pyrogen-free saline, 0.9% NaCl) for 24 h according to Griffon *et al.* (51).

Construction of GFP-Prohepcidin Protein Chimeras

The cDNA containing the entire open reading frame of prohepcidin was generated by PCR using the following primers 5'-CGGGATC-CGATGATGGCACTCAGCACT-3' and 5'-CCCAAGCTTCTATGTTTT-GCAACAGAT-3' and full-length mouse prohepcidin cDNA as template.

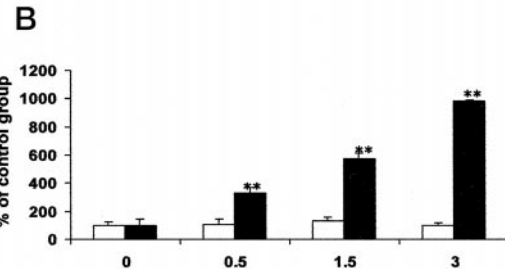
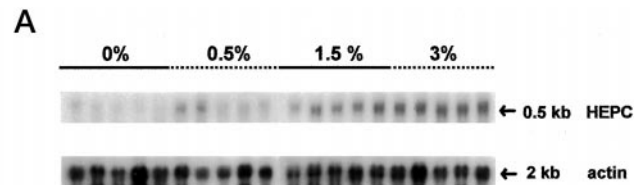


FIG. 1. Identification of mRNA overexpressed in the liver of iron-overloaded mice. A, total RNA was extracted from livers of mice overloaded by 0.5, 1.5, and 3% carbonyl iron for 8 months and control mice (five animals for each group) and analyzed by Northern blot using 225-bp cDNA as a probe. Hybridization with actin cDNA probe was performed to assess the equivalence of RNA loading. B, densitometry analysis. The average for each group of iron-overloaded animals is shown in histograms: values for actin and HEPC mRNAs are shown as open and solid boxes, respectively. Densitometry values obtained for control mice were arbitrary set as 100% for each mRNA. Statistical significance was reported as **, $p < 0.01$ between iron-overloaded and control mice. kb, kilobase pairs.

The nuclear localization signal (NLS) deletion mutants was constructed by two-step PCR method as described by Le Seyec *et al.* (52). Briefly, in a first step, two parallel reactions were performed using the following two sets of primers: P1, 5'-CGGGATC-CGATGATGGCACTCAGCACT-3', and P2, 5'-GATGGGAAGTTGGTGTCTCTGCAATTGGTATCGC-AATGT-3', for the first reaction and P3, 5'-ACATTGCGATACCAATG-CAGAGAGACACCAACTTCCCCATC-3', and P4, 5'-CCCAAGCTTCT-ATGTTTTGCAACAGAT-3', for the second reaction. In a second step, the two PCR products were purified, mixed, and used as template for PCR amplification using P1 and P4 primers. These two PCR products corresponding to wild type and NLS-deleted mutant forms of prohepcidin were digested with *Bam*HI and *Hind*III and subcloned into with *Bgl*II-*Hind*III digested pEGFP-C3 plasmid (CLONTECH, Palo Alto, CA).

Cell Transfections

The human osteosarcoma cell line U-2OS (53) was obtained from ATCC (Manassas, VA) and grown in Dulbecco's modified Eagle's medium containing 10% FCS. Then these cells were transiently transfected using Lipofectin (Life Technologies, Inc.) according to the manufacturer's instructions. Analysis of protein localization was performed by fluorescence microscopy.

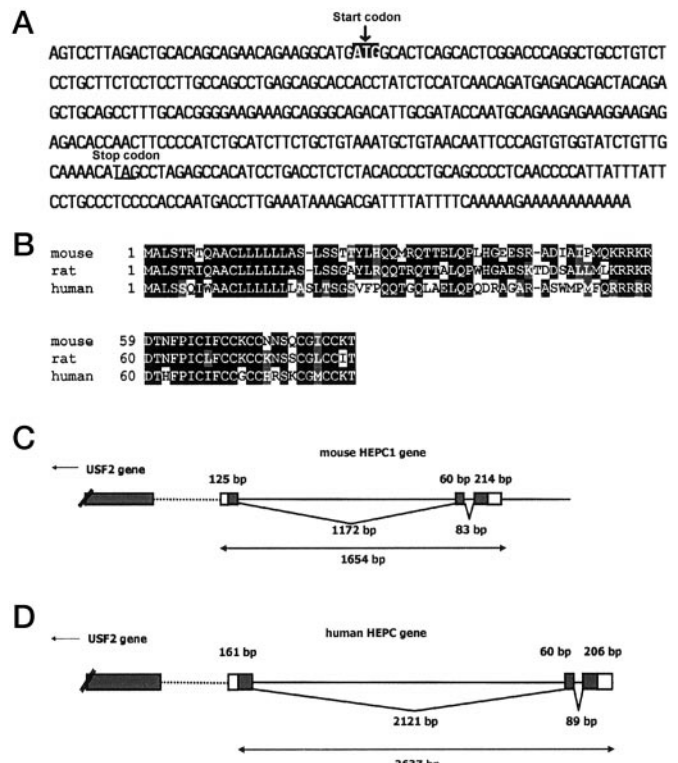
Statistical Analysis

Results were expressed as mean \pm S.D. Mann-Whitney test was used for estimation of statistical significance when appropriate. A p value less than 0.05 was considered statistically significant.

RESULTS

Isolation of an Iron-induced Gene—SSH was performed between livers of mice overloaded by 3% of carbonyl iron during 8 months and control mice. Among clones obtained, we isolated a 225-bp cDNA presumably overexpressed under the condition of iron overload. By using this cDNA as a probe, we studied the expression of the corresponding mRNA in the liver of mice overloaded with carbonyl iron during 8 months and in the liver of control mice. As presented in Fig. 1, the size of mRNA hybridizing with the cDNA probe was about 500 bp. The amount of this mRNA was increased in a dose-dependent manner by the carbonyl iron treatment. Its expression was 9.8-fold higher ($p < 0.01$) in the liver of 3% carbonyl iron-overloaded mice compared with the control mice. This overexpression was

FIG. 2. *A*, sequence of the full-length (410 bp) murine HEPC cDNA. *B*, alignment of putative mouse prohepcidin1 with its rat and human counterparts using ClustalW 1.7 program. Black color corresponds to identical residues and gray color to similar residues. *C* and *D*, schematic representation of the genomic organization of (*C*) mouse *Hepc1* gene and (*D*) human *HEPC* gene. Boxes represent exons. Gray and white colors correspond to coding and to untranslated regions, respectively.



confirmed in a second independent experiment where mice have been overloaded with 3% carbonyl iron for 8 months (8.6 times; $p < 0.05$) (data not shown).

Cloning of Full-length cDNA and Analysis of HEPC Gene Organization—To obtain a full-length clone, we screened a mouse liver cDNA library with the 225-bp probe obtained by SSH. The isolated cDNA fragment was 410 bp in length that is similar to previously estimated mRNA size. Thus, this clone likely represents the full-length cDNA (Fig. 2A). By using the sequence of this mouse cDNA, we searched for homologous sequences in EST GenBank™ data base and found at least two apparently full-length mouse EST clones W12913 (419 bp) and AI255961 (429 bp) showing 100 and 93%, respectively, identity in the overlapping region with our cDNA. Further data base screening led us to identify highly related 412-bp rat and 430-bp human ESTs (GenBank™ accession numbers AW534367 and AI937227, respectively). The last two cDNAs were isolated by RT-PCR and verified by sequencing.

Sequence analysis of the cloned mouse cDNA corresponding to the EST W12913 revealed the presence of consensus Kozak start site (54) at position 35 and open reading frame for a predictive protein of 83 amino acids (aa) (Fig. 2A). A protein data base search allowed us to identify a significant homology (76% of identity) between the C-terminal region of predictive mouse protein and the 25-aa mature chain of human hepcidin (GenBank™ accession number P81172), a secreted peptide purified from human urine. Thus, it is likely that we cloned mouse orthologue of human hepcidin. Because the isolated cDNA encodes a proform of this peptide, it was named prohepcidin1. Consequently, the second mouse EST clone AI255961 encoding an 83-aa protein that exhibits 90% identity with prohepcidin1 was referred to as prohepcidin2. The corresponding mouse genes were named *Hepc1* and *Hepc2*. Analysis of the deduced amino acid sequences of human and rat cDNA clones revealed that both encode 84-aa proteins and showed 54 and 77%, respectively, identity with mouse prohepcidin1. Alignment of the predicted mouse, rat, and human amino acid sequences is presented in Fig. 2B. We noted the presence of

repeated 6 leucines and the conserved locations of 8 cysteines. Moreover, these proteins are rich in positively charged residues (10 from 83) represented by arginine and lysine.

The GenBank™ search revealed the presence of both mouse *Hepc1* and *Hepc2* genes on mouse genomic clone CT7-8N15 (GenBank™ accession number AC020841). Comparison of mouse prohepcidin1 cDNA sequence to that of CT7-8N15 clone enabled us to determine the exon/intron organization of the mouse gene. We deduced that this gene was small (1654 bp) and composed of 3 exons and 2 introns (Fig. 2C). Just on the 5' upstream flanking region of this gene, we found the gene *Usp2* (upstream stimulatory factor 2) that was located in tail-to-head orientation. The 1240-bp region separating the end of *Usp2* gene from the start of exon 1 of *Hepc1* gene was analyzed for predictive transcription factor binding sites using MatInspector. This investigation pointed out the presence of putative HNF3 β , C/EBP β , and NF- κ B regulatory element binding sites and a TATA box located about 70 bp upstream of the first start codon. Interestingly, nucleotide sequence and organization of *Hepc2* gene showed a very high degree of similarity to that of *Hepc1* suggesting that mouse *Hepc1* and *Hepc2* genes arose from relatively recent duplication of ancestral gene. As *Usp2* is located on chromosome 7 in mouse (55), we deduced that *HEPC* genes were also on chromosome 7. In addition, we determined the genomic organization of the human gene taking advantage of the human genomic clone R30879 from chromosome 19 that matched 100% with the human cDNA (Fig. 2D). Similar to the mouse counterpart, human *HEPC* gene includes 3 exons, lies in close proximity to the *USF2* gene, and contains potential binding sites for HNF3 β , C/EBP β , and NF- κ B in the upstream regulatory region.

Tissue-specific Expression—After having identified an mRNA encoding a protein belonging to the antimicrobial peptide family, regulated by iron in the liver, we looked for its tissue-specific expression in mice. By Northern blot, using the cDNA of 225 bp obtained by SSH as a probe, we observed that HEPC mRNA was predominantly expressed in the liver and very weakly in stomach, intestine, colon, lungs, heart, and

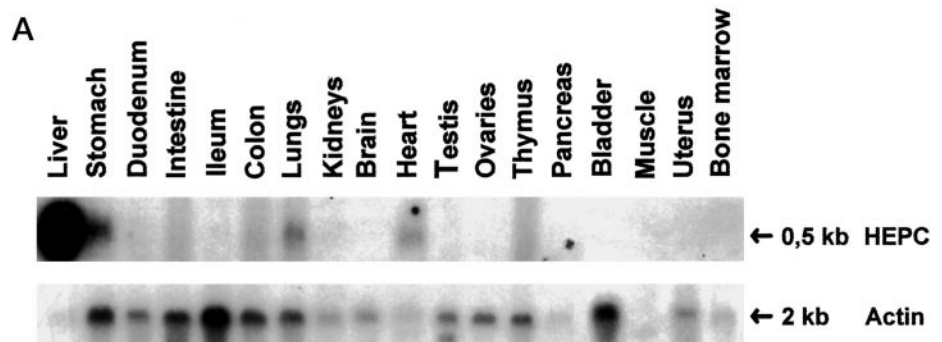
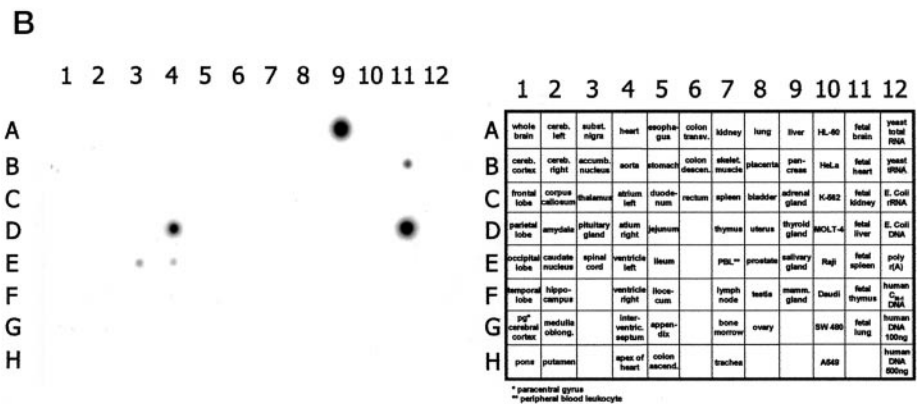


FIG. 3. Liver-specific expression of human and mouse HEPC mRNA. A, Northern blot analysis of HEPC mRNA expression in different murine tissues using murine probe. B, dot-blot analysis of HEPC mRNA expression in human tissues and cell lines using human cDNA as a probe (left panel). The diagram shows the types and positions of poly(A)⁺ RNAs and controls dotted on nylon membrane (right panel). kb, kilobase pairs.



thymus (Fig. 3A). By using multiple tissue expression array membrane and human cDNA as a probe, we studied mRNA expression in human adult and fetal organs and found that it was very strongly expressed in the adult and fetal liver and to a lesser extent in adult left atrium of heart, fetal heart, and adult spinal cord (Fig. 3B). Non-hepatic cell lines which were investigated did not express HEPC mRNA (Fig. 3B). Since HEPC mRNA was specifically expressed in the liver of both humans and mice, we sought the hepatic cellular types expressing HEPC mRNA. By using the mouse fragment of cDNA as a probe, we found that HEPC mRNA was expressed in freshly isolated hepatocytes both in mouse and in rat (Fig. 4, A and B). In enriched non-parenchymal cells isolated from mouse and rat livers, this mRNA expression was much weaker (Fig. 4, A and B) as compared with hepatocytic expression. As expected, albumin mRNA was strongly expressed in hepatocytes and was undetectable in nonparenchymal cells. The amount of HEPC mRNA rapidly and dramatically decreased in mouse hepatocytes maintained under serum-free conditions, and addition of FCS partially stabilized expression of HEPC transcripts (Fig. 4C). Furthermore, HEPC transcripts were undetectable in human, rat, and mouse hepatic cell lines that we studied by Northern blot (data not shown). However, we evidenced a PCR product generated from reverse-transcribed HepG2 mRNA using human HEPC-specific primers. These data suggest that expression of HEPC mRNA is likely related to hepatocyte differentiation status.

Iron Regulation of HEPC mRNA Expression—To confirm that HEPC mRNA expression was linked to the amount of iron in the liver, two other types of *in vivo* iron overload were studied as follows: experimental iron overload using iron-dextran, and spontaneous iron overload observed in β_2 -microglobulin ($-/-$) mice. In addition, to ascertain that a decrease of liver iron concentration leads to a drop of HEPC mRNA expression, β_2 -microglobulin-deficient mice were fed with an iron-poor diet. In the liver of mice injected by iron-dextran and sacrificed 2

months later, the amount of HEPC mRNA was 4 times higher compared with the control mice ($p < 0.01$) (Fig. 5A). In this experiment, LIC was $170.6 \pm 29 \mu\text{mol}$ of iron/g of dry liver for iron-overloaded mice and $7 \pm 0.8 \mu\text{mol}$ of iron/g of dry liver of control mice. After 4 months, mRNA expression was induced 6 times ($p < 0.05$) in iron-overloaded mice compared with controls (data not shown). In 12-month-old β_2 -microglobulin ($-/-$) mice (developing spontaneous iron overload), hepatic expression of HEPC mRNA was also found to be overexpressed (2 times, $p < 0.05$) compared with the control wild type mice (Fig. 5B). In these mice, the LIC reached $23 \mu\text{mol}$ of iron/g of dry liver for β_2 -microglobulin knockout mice *versus* $2.5 \mu\text{mol}$ for control mice.

In the liver of β_2 -microglobulin ($-/-$) mice fed a low iron diet, the liver iron concentration was 4 times lower in comparison with β_2 -microglobulin ($-/-$) mice fed a normal diet. By Northern blot analysis, this decrease of LIC was accompanied by a 5.5 times ($p < 0.03$) reduction of HEPC mRNA in the liver of iron-deprived mice compared with spontaneous iron-overloaded mice (Fig. 5C).

Then, to determine whether overexpression was observed in earlier steps of iron overload, we looked for hepatic mRNA expression in mice overloaded with 3% carbonyl iron for 2 months and compared it with that observed after 8 months of treatment. Under these conditions, HEPC mRNA expression was induced as soon as 2 months of carbonyl iron treatment (10 times, $p < 0.03$). Its amount was comparable with that observed at 8 months of 3% carbonyl iron administration. Likewise, in the iron-dextran model, overexpression was observed in an independent experiment, as soon as 1 week after iron injection (2.5 times) and was similar to that observed after 2 months (2-fold increase) (data not shown).

Next, to evaluate the direct effect of iron on HEPC mRNA expression, we studied mRNA expression *in vitro* in mouse hepatocytes either exposed to iron or depleted of iron. Whatever the presence or absence of FCS, the addition of iron citrate

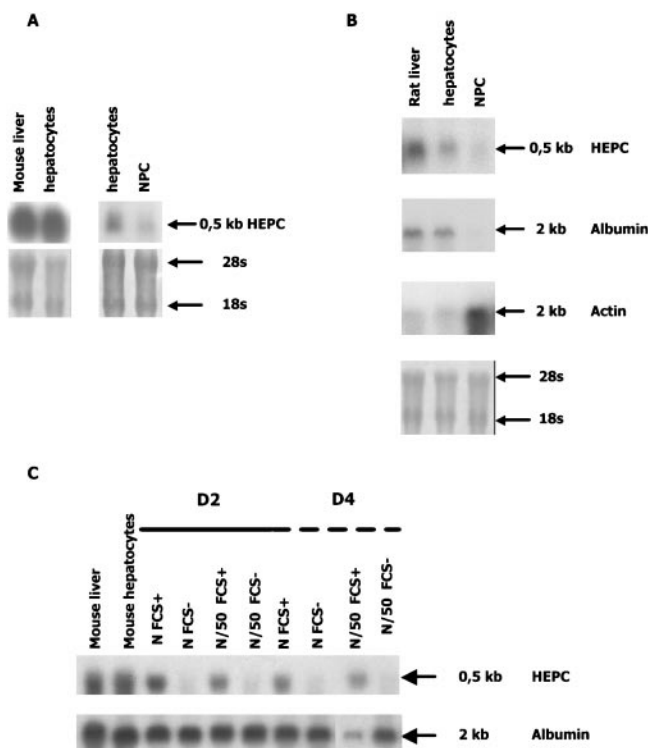


FIG. 4. Expression of HEPC in hepatocytes. Northern blot analysis of HEPC mRNA expression in liver and freshly isolated hepatocytes and non-parenchymal cells (NPC) in mouse (A) and in rat (B). Corresponding expression of albumin and actin mRNAs as well as methylene blue staining of total RNAs are presented. C, Northern blot analysis of HEPC mRNA expression by mouse hepatocytes maintained under different culture conditions. Mouse hepatocytes were maintained in the presence (FCS+) or absence (FCS-) of fetal calf serum and at two concentrations of hydrocortisone hemisuccinate as follows: 5×10^{-5} M (N) and 1×10^{-6} M (N/50) for 2s (D2) and 4 days (D4). Albumin mRNA expression is shown in parallel. kb, kilobase pairs.

or iron chelator (DFO) to the culture medium did not induce significant changes in HEPC mRNA expression (data not shown).

Induction of HEPC mRNA Expression by LPS—Based on the similarity of prohepcidin to antimicrobial peptides and considering that some of them are induced by LPS, we tested the effect of LPS on HEPC mRNA expression *in vivo* in mouse liver and *in vitro* using hepatocytes cultures. As shown in Fig. 6, the steady state level of mRNA was 4.3 times higher ($p < 0.05$) in the liver of mice injected with LPS. This result was confirmed by *in vitro* experiments that showed a 7-fold increase of HEPC mRNA in mouse hepatocytes treated with LPS compared with control culture (Fig. 6).

Localization of the GFP-Prohepcidin Chimera—To determine the cellular localization of prohepcidin protein, it was expressed as a fusion with GFP. In contrast to GFP that exhibited a homogeneous intracellular pattern of fluorescence (Fig. 7A), the fluorescence of cells transfected with expression vector encoding the GFP-prohepcidin chimera was specifically localized in nuclei (Fig. 7B). Predictive studies using PSORT II program (56) enabled us to identify a putative nuclear localization signal (KRRK) in prohepcidin protein. To verify the functional role of this sequence, these four amino acids were deleted from prohepcidin protein, and the resulting mutant was fused to GFP. In this configuration, in most of the transfected cells, fluorescence was predominantly found in the cytosol, and the nuclei appeared devoid of fluorescence (Fig. 7C).

DISCUSSION

To identify novel hepatic iron up-regulated genes, we took advantage of the carbonyl iron overload model that we previ-

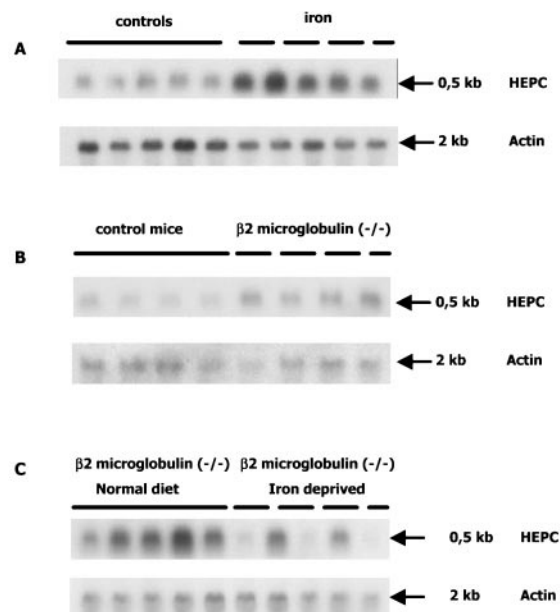


FIG. 5. Iron-dependent expression of HEPC mRNA in the liver of mice. A, HEPC mRNA expression in an iron-dextran model of iron overload. HEPC mRNA was 4 times higher ($p < 0.01$) in the liver of mice injected 2 months previously with iron-dextran than in control mice. B, Northern blot analysis of HEPC mRNA in the livers of β_2 -microglobulin ($-/-$) mice, a spontaneous iron overload model. HEPC mRNA was increased 2-fold ($p < 0.03$) in the liver of β_2 -homozygous microglobulin ($-/-$) mice as compared with the control wild type strain. C, HEPC mRNA in the liver of β_2 -microglobulin ($-/-$) mice under the effect of iron deprivation. Northern blot analysis revealed that a 4-fold decrease of liver iron concentration is accompanied by a 5.5-fold decrease ($p < 0.03$) of HEPC mRNA in the liver of β_2 -microglobulin ($-/-$) mice fed a low iron diet. Expression was compared with β_2 -microglobulin ($-/-$) mice fed a normal iron diet. Each loading represents samples of total RNAs extracted from the liver of each mouse. Hybridization was performed with the mouse cDNA probe. For each experiment, actin mRNA expression is presented to show equal RNA loading. kb, kilobase pairs.

ously developed in mice (29). We performed an SSH between cDNAs obtained from livers of control and iron-overloaded mice, and we isolated a 225-bp cDNA corresponding to a gene that was overexpressed in the liver of carbonyl iron-overloaded mice.

To confirm that the observed mRNA overexpression was directly related to iron overload itself and not to other parameters related to the carbonyl iron model, we investigated hepatic mRNA expression in other *in vivo* models of iron overload.

The fact that the HEPC mRNA was induced in all these different models led us to conclude that liver iron excess itself is responsible for the up-regulation of mRNA expression. In addition, the amount of mRNA was directly linked to liver iron concentration as evidenced by the dose dependence of mRNA induction in carbonyl iron-overloaded mice and the decrease of HEPC mRNA expression in β_2 -microglobulin ($-/-$) mice fed with a low iron content diet. Thus, iron overload triggers HEPC mRNA overexpression, and iron depletion leads to a decrease of its expression. However, it seems that mRNA expression was not dependent on the duration of iron overload. Indeed, HEPC mRNA was strongly up-regulated as early as 2 months and 1 week in carbonyl iron and iron-dextran models, respectively, and similar levels were maintained thereafter.

In contrast to *in vivo* situation where HEPC mRNA was strongly overexpressed during iron overload, the amount of transcript was not modified by *in vitro* exposure of primary mouse hepatocytes (hepatic cells that predominantly expressed HEPC mRNA) to iron citrate. Cultured hepatocytes are widely used as a tool to perform metabolic and pharmacologic studies

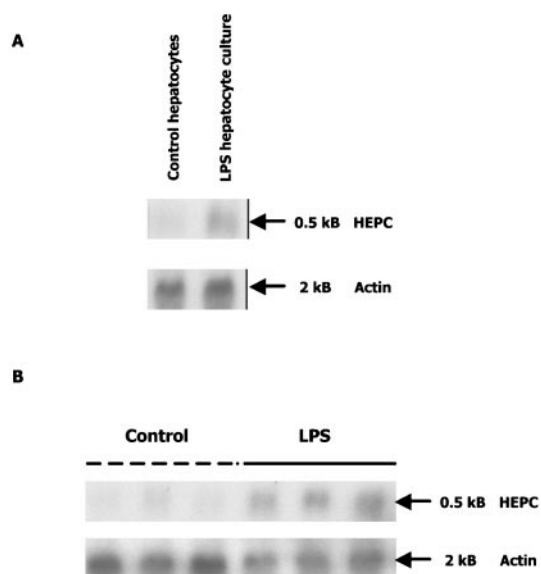


FIG. 6. The effect of LPS on hepatic HEPC mRNA expression. A, total mRNAs were extracted from control and LPS-treated primary mouse hepatocytes and subjected to Northern blot analysis. B, total mRNAs were extracted from the livers of control and LPS-injected mice and analyzed by hybridization for the presence of HEPC transcripts. *kb*, kilobase pairs.

as an alternative to animal models (57). However, it is likely that the absence of HEPC mRNA induction by iron was related to hepatocyte phenotypic changes that occur with time in culture. Indeed, when maintained for a few days in primary cultures without differentiation promoting factors, hepatocytes lost the expression of several liver-specific genes (58). In particular, hepatocytes in conventional culture failed to maintain expression of genes encoding members of the CYP2B subfamily of the cytochromes P450 and lost their inducibility by phenobarbital (59). The fact that HEPC mRNA expression (i) is higher in human adult liver compared with fetal liver, (ii) decreases spontaneously in conventional mouse hepatocyte culture, and (iii) is not detected in hepatic cell lines could support the hypothesis that expression of HEPC mRNA is dependent of hepatocyte differentiation status.

Full-length mouse cDNA as well as human and rat orthologues were cloned, and genomic organization of mouse and human genes was established. In addition, we determined that mouse genome contains two highly related *Hepc* genes. Both *Hepc* genes, which exhibit similar genomic organization, are located in tandem on chromosome 7 and likely arose from duplication of an ancestral gene. In humans, *HEPC* gene was found on chromosome 19 in a locus that is syntenic to the region containing *Hepc* genes on chromosome 7 in mouse (60).

The translation of both murine cDNAs leads to an 83-aa protein that shows strong homology in its C-terminal region to mature forms of a human antimicrobial peptide, the hepcidin, suggesting that we have identified the murine proforms of hepcidin. As shown in the accompanying paper by Ganz and co-workers (86), peptides of different length (20, 22, and 25 aa) corresponding to mature forms of hepcidin were purified from human urine. It should be noticed that Krause *et al.* (61) also reported in a recent paper, published after submission of our manuscript, a 25-aa peptide that is similar to the 25-aa hepcidin form described by Park *et al.* (86). The human peptides of 20 and 25 aa, resulting from the processing of the 84-aa proform, exhibited antimicrobial activities against bacteria and fungi. The high number of cationic residues in the mouse protein and the conserved position of cysteines between human and mouse proteins argue in favor of an antimicrobial role of

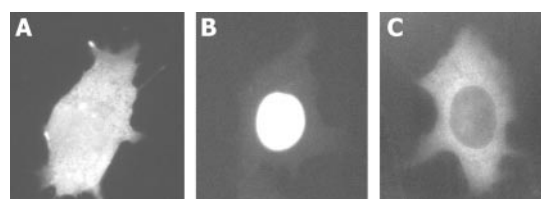


FIG. 7. Intracellular localization of GFP-prohepcidin protein. U2OS cells were transfected with expression vectors encoding GFP (A), GFP fused to 83-aa murine prohepcidin protein (B), and GFP fused to the prohepcidin protein lacking the putative nuclear localization signal (C).

mature forms of hepcidin in mice.

Interestingly, both in mice and in humans, HEPC transcripts were found exclusively in the liver. The presence of potential binding sites for liver-enriched transcription factors C/EBP β (62, 63) and HNF3 β (64), known to regulate expression of several hepatic genes, indicates that these transcription regulators could confer liver specificity to hepcidin. To our knowledge, it is the first demonstration of a liver-specific mammalian antimicrobial peptide that is reminiscent of insect antimicrobial peptides in the fat body, the functional equivalent of mammalian liver (65, 66). However, we did not find any structural homologues of mammalian prohepcidin in the *Drosophila* genome.

Considering that mature forms of human hepcidin were purified from urine, it was unexpected to find that mouse GFP-prohepcidin chimera was localized in the nuclei of mammalian cells. We demonstrated that nuclear localization of this protein was related to the presence of an NLS because its deletion led to a cytoplasmic expression of prohepcidin mutant form. This confirmed the functional role of this sequence. However, the mechanism involved in the transport of the mature form of prohepcidin to the extracellular zone is not fully understood. The presence of hepcidin in biological fluids is unlikely resulting from necrosis of hepatocytes when considering that normal liver is characterized by a very low level of renewal and that this peptide was isolated from the urine of healthy persons (see accompanying article (86)). At least two mechanisms could be taken into consideration. The prohepcidin could be transiently addressed to the nucleus before relocation to the cytoplasm and secretion. This process has been described for at least one secreted protein, the fibroblast growth factor 2 (FGF-2). It has been shown that the GFP low molecular weight FGF-2 form chimera was rapidly translocated to the nucleus after exposure of cells to the expression vector and then retro-transported to the cytoplasm, after which this molecule was secreted (67). Alternatively, the GFP-prohepcidin might be processed in the cytoplasm releasing the secreted mature C-terminal hepcidin, and the N-terminal part of prohepcidin containing NLS could be addressed to the nucleus. Indeed, the prohepcidin potential NLS sequence also matches the signal sequence cleavage site (RX(R/K)R↓X) for furin and related mammalian subtilisin/Kex2p-like propeptide convertases that could process prohepcidin to the secreted forms (68). It is tempting to speculate that under normal physiological conditions most of prohepcidin would be cleaved in the liver by furin-like convertases to produce mature chains of hepcidin. Indeed, at least three enzymes of this family, namely furin, PACE4, and PC8, are expressed in liver (69). However, considering our data, we cannot exclude that, due to the presence of functional NLS, at least some amount of prohepcidin could be addressed to the nucleus. In addition, in pathological situations in which expression or activity of processing enzymes would be reduced or inhibited, the localization and the fate of prohepcidin could be modified.

Patients developing iron overload have been reported to be

more sensitive to bacterial infections, suggesting a relationship between iron and infection (70–72). In addition, it has been demonstrated that lactoferrin, a protein that binds iron in milk, has antimicrobial activity after cleavage (73). However, why an mRNA encoding the antimicrobial peptide hepcidin is induced during iron overload remains an open question. It is well known that expression of several antimicrobial peptides in mammals can be enhanced by exposure to bacterial cells or components such as LPS (74–76). In our work, we also found a strong positive effect of LPS both *in vivo* and *in vitro* on HEPC mRNA expression. Moreover, we found that, *in vitro*, HEPC mRNA was maintained only in the presence of FCS suggesting that this effect could be linked to the presence of traces of endotoxin in the serum (77). All these data suggest that iron induction of HEPC mRNA probably resulted from inflammation related to chronic iron overload. In our previous report (29), histological studies did not detect important inflammation in the liver of iron-overloaded mice. However, a subtle inflammatory process has been noticed in genetic hemochromatosis patients with heavy iron overload (78). In addition, heavy iron deposits that are observed during iron overload in the Kupffer cells of long term carbonyl iron-overloaded mice (29) and iron-dextran mice very soon after iron injection, but also during heavy iron overload in human diseases, could modify cytokine expression in the liver. Alternatively, it cannot be excluded that the LPS effect on HEPC mRNA expression could be at least partially iron-mediated, since some data have reported that endotoxin and endotoxin-induced cytokines tumor necrosis factor- α , interleukin-1 β , and interleukin-6 (79–81) enhanced liver cell uptake of transferrin-bound iron.

Finally, our data do not exclude the possibility that murine prohepcidin could play a distinct role in addition to its antimicrobial activity. Indeed, antimicrobial peptides are also known to prevent the oxidative stress (82), to exert antitumor activity (83, 84), and to be involved in regulation of angiogenesis (85).

In conclusion, we isolated a novel liver-specific murine mRNA that is overexpressed during iron overload in mice and that encodes an 83-aa protein homologous to the human antimicrobial peptide hepcidin. Further studies are required to determine the physiological role of prohepcidin and its mature forms during iron overload.

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