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Comparative analysis of mouse hepcidin 1 and 2 genes: evidence for different patterns of expression and co-inducibility during iron overload[1]

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Abstract **In contrast to the human genome, the mouse genome contains two HEPC genes encoding hepcidin, a key regulator of iron homeostasis. Here we report a comparative analysis of sequence, genomic structure, expression and iron regulation of mouse HEPC genes. The predicted processed 25 amino acid hepcidin 2 peptide share 68% identity with hepcidin 1 with perfect conservation of eight cysteine residues. Both HEPC1 and HEPC2 genes have similar genomic organization and have probably arisen from a recent duplication of chromosome 7 region, including the HEPC ancestral gene and a part of the adjacent USF2 gene. Insertion of a retroviral intracisternal A-particle element was found upstream of the HEPC1 gene. Both genes are highly expressed in the liver and to a much lesser extent in the heart. In contrast to HEPC1, a high amount of HEPC2 transcripts was detected in the pancreas. Expression of both genes was increased in the liver during carbonyl–iron and iron–dextran overload. Overall our data suggest that both HEPC1 and HEPC2 genes are involved in iron metabolism regulation but could exhibit different activities and/or play distinct roles.**

Key words: Hepcidin; Genomic structure; Expression; Iron overload

1. Introduction

In search of hepatic genes induced during iron overload we identified a novel mouse gene named HEPC [1] encoding a small 83-amino-acid (aa) protein that shared significant homology in its C-terminal region with human cysteine-rich circulating peptide hepcidin (also termed LEAP-1) isolated in two parallel studies from human plasma ultrafiltrate [2] and urine [3]. Human hepcidin was reported to exhibit antibacterial and antifungal activities [2,3]. However, in contrast to the mammalian antimicrobial peptides described to date, it displays several original characteristics including a distinctive cysteine structural motif. Specifically, all eight cysteine residues of 25-aa hepcidin form intramolecular bonds, with six of them involved in maintaining interstrand connectivity. One unusual vicinal disulfide bridge was suggested to have a functional importance [4]. Another peculiar characteristic of human hepcidin is its predominant hepatic expression pattern [1–3]. Similarly, mouse HEPC transcripts were found essentially in the liver [1].

Besides its potential antimicrobial activity, recent reports argue in favor of a role of hepcidin in iron metabolism. Indeed, the analysis of upstream stimulatory factor 2 (USF2) knockout mice lacking HEPC transcripts revealed a development of iron overload in the liver, pancreas and heart [5]. Inversely, early liver expression of hepcidin in transgenic mice resulted in phenotypic traits of iron deficiency [6]. These observation support the idea that hepcidin is involved in the regulation of iron homeostasis. By controlling the level of duodenal iron absorption and iron release from macrophages, hepcidin could act as a hormonal regulator of iron storage or recycling [5,7]. Such a role of hepcidin in the regulation of iron metabolism was confirmed in humans. Indeed, a refractory anemia similar to that observed in chronic inflammatory diseases was described in patients with large hepatic adenomas overexpressing hepcidin [8]. Furthermore, mutations in the HEPC gene were reported in two families with severe form of juvenile hemochromatosis [9].

Only one hepcidin gene was found in the human genome. However, a previous analysis of GenBank databases of mouse expressed sequence tags (ESTs) and genomic sequences revealed that in addition to the originally identified HEPC gene, the mouse genome contains another highly related gene named HEPC2 [1]. The presence of two mouse hepcidin genes was confirmed subsequently [5]. One might expect, based on the high degree of identity between HEPC1 and HEPC2, that these two genes have a similar regulation and are functionally redundant; however, it has not yet been demonstrated formally. Considering that the mouse is a useful animal model for the understanding of human iron metabolism dysfunction [10] and that hepcidin plays an important role in iron store regulation, we performed a comparative analysis of sequences, genomic organization, expression and iron regulation of mice HEPC1 and HEPC2 genes.

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[1] The nucleotide sequence reported in this paper has been submitted to the GenBank Data Bank with accession number AY232841.

Abbreviations: EST, expressed sequence tags; IAP, intracisternal A-particle; USF2, upstream stimulatory factor 2

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2. Materials and methods

2.1. Animals

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

2.2. Iron overload

Carbonyl–iron and iron–dextran overloads were performed as previously described [1,11]. Briefly, a group of five mice was iron overloaded by 3% carbonyl–iron supplemented in the diet (AO3, UAR, France) during 8 months. Control mice had a carbonyl–iron-free diet. For iron–dextran overload, a single subcutaneous injection of iron–dextran (Sigma, France) was performed at a dose of 1 g/kg body weight. Control mice received a single injection of a mixture containing dextran at the same concentration as in the iron–dextran solution. Animals were killed 2 months after iron injection.

2.3. Determination of genomic organization of mouse HEPC1 and HEPC2 genes

Mouse genomic clones CT7-8N15, RP23-22G9 and RP23-28D21 (GenBank accession numbers AC020841, AC087143 and AC021477, respectively) were used to elucidate the genomic organization of HEPC1, HEPC2 and USF2 genes by sequence comparison with the corresponding cDNAs. The presence of splice donor and acceptor sites flanking the alignment matches helped to predict the exact positions of exons.

2.4. RNA isolation and real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from livers of iron-overloaded and control mice using SV total RNA isolation system (Promega, Charbonnières, France). Mouse BALB/c liver and pancreas poly(A)$^+$ RNA were purchased from Clontech Laboratories (Palo Alto, CA, USA). cDNA first-strand synthesis was performed with Advantage RT-for-PCR kit (Clontech, Palo Alto, CA, USA). A set of first-strand cDNAs generated from different 8–12-week-old male BALB/c mice organs and from whole embryos (Mouse Multiple Tissue cDNA Panel I) was obtained from Clontech.

Primers and probes for real-time PCR analysis were designed with the assistance of Primer Express Software (PE Biosystems). The following primers were selected to amplify HEPC1 and HEPC2: forward for both HEPC1 and HEPC2, 5′-CCTATCTCCATCAACAGATG-3′; reverse for HEPC1 5′-AACAGATACCACACTGGGAA-3′ and reverse for HEPC2, 5′-AACAGATACCACAGGAGGGT-3′. To avoid co-amplification of genomic DNA the designed forward HEPC1 and HEPC2 primers overlapped exons I and II and reversed primers positioned on exon III. The specificity of these primers was demonstrated previously [5]. To amplify mouse β-actin we used the following forward and reverse primers: 5′-GACGGCCAAGTCATCACTATTG-3′ and 5′-CCACAGGATTCCATACCCAAGA-3′, as described by Dupic et al. [12]. To detect 171-bp mouse HEPC1 and HEPC2, and 68-bp β-actin amplification products we used the following probes: 5′-FAM-CCCTGCTTTCTTCCCCGTGCAAAG-TAMRA-3′ and 5′-FAM-TACGAGGGCTATGCTCTCCCTCACGC-TAMRA-3′, respectively. The relative expression of HEPC1 and HEPC2 in the different samples was determined by standardization to its expression in normal adult mouse liver. For this purpose, we used a two-step calculation method. First, for each sample, the Ct_{actin} value was subtracted from the corresponding Ct_{HEPC} to obtain normalized ΔCt values. Then, these ΔCt values were used to calculate relative levels of HEPC1 or HEPC2 expression as follows: HEPC expression $ratio_{(sample/reference)} = 2^{\Delta\Delta Ct}$, where $\Delta\Delta Ct = (\Delta Ct_{reference} - \Delta Ct_{sample})$. The HEPC1 and HEPC2 expression levels in adult normal liver were used as reference and were set as 100% (Fig. 3) or as one-fold expression (Fig. 4). All quantitative real-time PCR assays were performed in triplicate in a 96-well microplate using the qPCR™ Core reagent kit (Eurogentec, Seraing, Belgium) and the ABI PRISM 7700 Sequence Detection System (PE Biosystems). For each microplate the reactions contained target cDNAs, two standard curves prepared from normal adult mouse liver cDNA and negative no-template control. PCR products were assessed by electrophoresis on 2% agarose gel.

3. Results

3.1. Identification and nucleotide sequence of mouse HEPC2 cDNA

Careful analysis of mouse EST GenBank database revealed the existence of two sets of highly homologous mice ESTs: those identical to originally isolated HEPC1 cDNA [1] and a set of ESTs showing approximately 85–92% identity and corresponding to HEPC2. One of these EST clones, IMAGE clone 1889041 (EST AI255961) was completely sequenced. This cDNA fragment was 406 bp in length and was found to exhibit 92% identity with HEPC1 cDNA at the nucleotide level (Fig. 1A). Similarly to HEPC1, HEPC2 cDNA contains an open reading frame for the predicted polypeptide of 83 aa. This hepcidin 2 proform exhibits 89% identity with prohepcidin 1 at the amino acid level (Fig. 1B). The 58-aa N-terminal part of hepcidin precursor 2 including the peptide signal sequence showed 98% identity with that of prohepcidin 1 with perfect conservation of putative cleavage sites for furin-like propeptide convertases. However, the 25-aa C-terminal peptide corresponding to the putative mature chain of mouse hepcidin 2 shares with hepcidin 1 only 68% identity (8 aa from 25 are different). It is noteworthy that all 8 cysteine residues in the 25-aa mature hepcidin 1 and hepcidin 2 peptides are conserved.

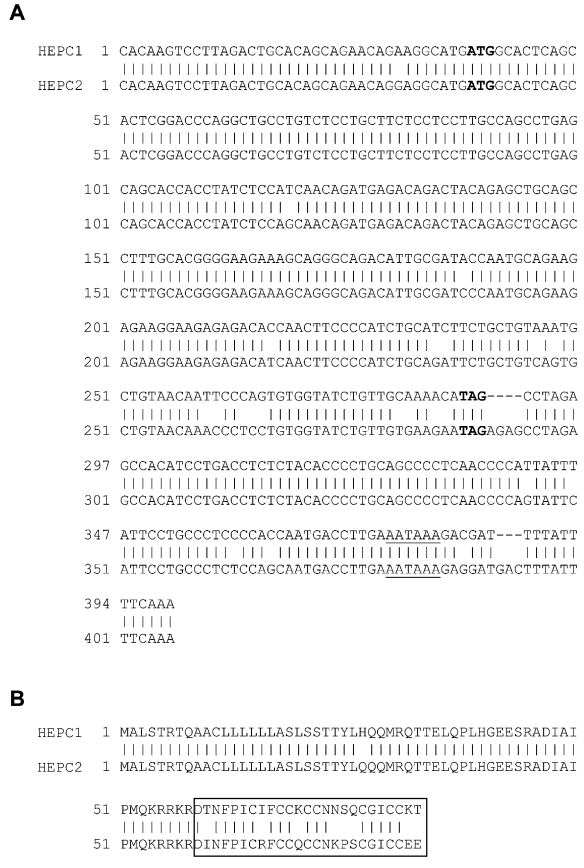

Fig. 1. The nucleotide and predicted amino acid sequences of mouse HEPC2. A: Alignment of full-length HEPC1 and HEPC 2 cDNAs. Start and stop codons are shown in bold and polyadenylation signals in the 3′-untranslated region are indicated by thin underlining. B: Alignment of hepcidin 1 and hepcidin 2 proteins. Sequences of putative 25-aa mature peptides are boxed.

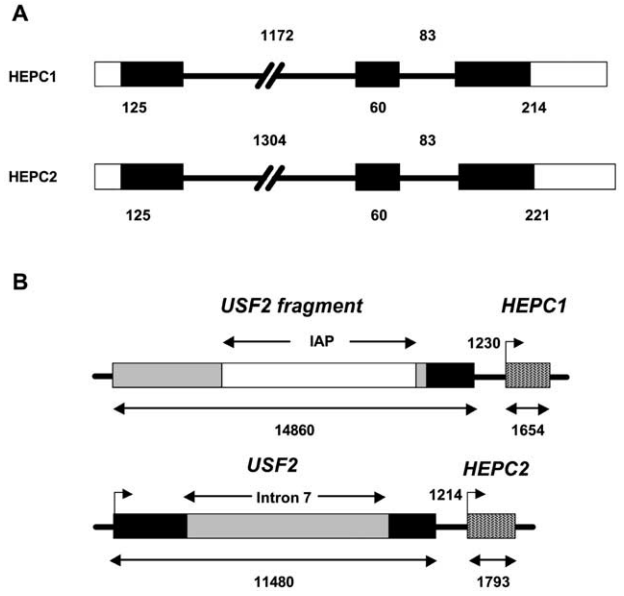

Fig. 2. Genomic organization and location of mouse HEPC1 and HEPC2 genes. A: Schematic representation of exon–intron organization of HEPC 1 and HEPC 2 genes. Exons are indicated as boxes. Closed and open boxes correspond to coding and untranslated regions, respectively. The lengths of exon and intron are shown in bp. B: Genomic location of HEPC1, HEPC2 and USF2 genes on mouse chromosome 7. The lengths of genes and intergenic regions are shown in bp. Arrows indicate the site of initiation and direction of transcription.

3.2. Organization of HEPC1 and HEPC2 genes in the mouse

Previously, using the sequence information derived from mouse genomic clone CT7-8N15 (GenBank accession number AC020841) we determined that HEPC1 gene is composed of three exons and two introns and is located on the chromosome 7 [1]. We have reexamined the genomic organization of mouse HEPC1 and HEPC2 genes using sequence information derived from several contigs of genomic clones CT7-8N15, RP23-22G9 and RP23-28D21 (GenBank accession numbers AC020841, AC087143 and AC021477, respectively) and previously reported partial nucleotide sequence of mouse USF2 gene [13]. Although sequence analysis of overlapping regions of contigs from two distinct genomic clones CT7-8N15 and RP23-22G9 revealed several discrepancies, we were able to determine the following: (1) The HEPC1 and HEPC2 genes co-localize on the same genomic clone. (2) The HEPC2 gene comprises three exons and two introns and shares with the HEPC1 gene a very high level of similarity (approximately 94% identity). The intron 2 of the HEPC2 gene but not of HEPC1 contains insertion of several copies of simple tandem repeat GAGAG (Fig. 2A). (3) 18 300-bp contig from genomic clone RP23-22G9 includes full-length USF2 and HEPC2 genes lying in a tail-to-head orientation. (4) The HEPC1 gene lies directly downstream of the fragment of the USF2 gene encompassing exons 8–10 and a mouse retroviral intracisternal A-particle (IAP) element inserted in the intron 7 of the truncated USF2 gene (Fig. 2B). This IAP sequence possesses both 5′ and 3′ long terminal repeats.

3.3. Expression of HEPC1 and HEPC2 genes in mouse tissues

Because of the very high level of homology between HEPC1 and HEPC2 mRNA and similar lengths of transcripts, it was not possible to study the specific expression of corresponding mRNAs by Northern blot analysis. Previous Northern blot data most likely represented cumulative amounts of both HEPC1 and HEPC2 transcripts. To assess the levels of HEPC1 and HEPC2 mRNA expression, we developed a quantitative real-time RT-PCR analysis using specific primers for HEPC1 and HEPC2 transcripts. To test the specificity of the amplification reaction we used HEPC1 and HEPC2 cDNA. Neither HEPC1- nor HEPC2-specific primers were able to amplify DNA fragments using, respectively, 0.5 ng of HEPC2 or HEPC1 cDNA as templates after 25 cycles (data not shown), demonstrating a high specificity of the PCR reaction.

The analysis of HEPC1 and HEPC2 gene expression in eight mouse tissues including liver, spleen, skeletal muscles, kidney and heart revealed that transcripts of both genes were predominantly expressed in the liver and were weakly detectable in the heart, skeletal muscles, lung and brain (Fig. 3A). Additionally, we studied expression of HEPC1

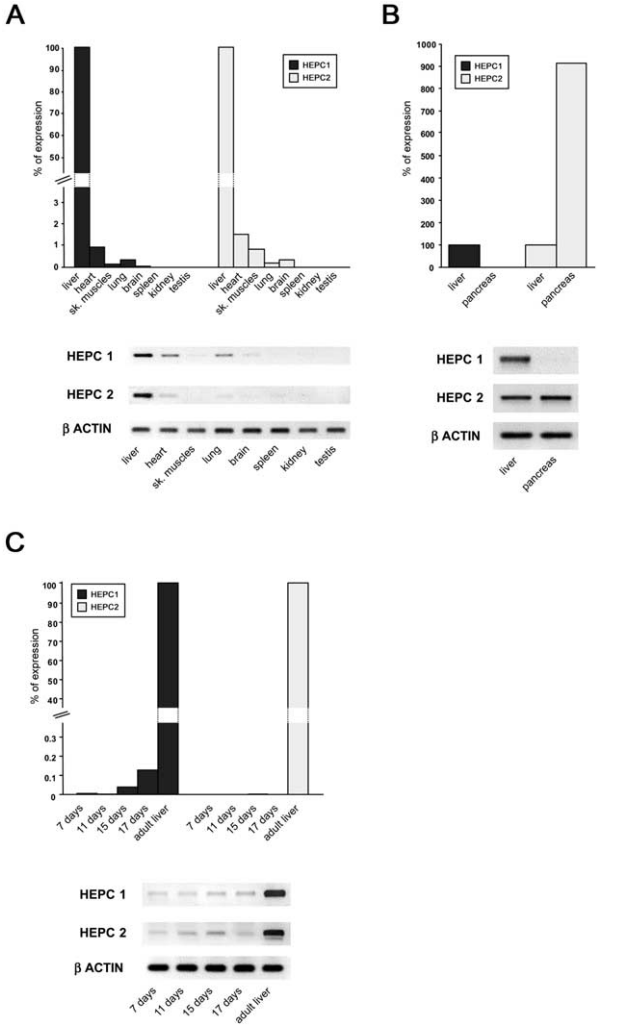

Fig. 3. Expression of hepcidin genes in mouse adult and fetal tissues. Amount of specific HEPC1 and HEPC2 transcripts were determined by real-time RT-PCR analysis as described in Section 2. Relative levels of HEPC1 and HEPC2 gene expression in eight adult mouse tissues (A), in adult liver and pancreas (B), and in whole mouse embryos and in adult liver (C). Following RT-PCR, the HEPC1, HEPC2 and β-actin amplification products were separated on 2% agarose gel (lower panels).

and HEPC2 genes in the liver and pancreas, two organs that share a common embryonic origin, arising from the endomesodermal rudiment of foregut. We found that the HEPC1 gene was very weakly expressed in the pancreas. In contrast, the amounts of hepcidin 2 transcripts in this tissue proved to be at least nine times higher than that found in the liver (Fig. 3B). The specificity of the amplification reaction was confirmed by direct sequencing of PCR products obtained with HEPC1 or HEPC2 primers.

The study of hepcidin gene expression during mouse fetal development revealed that whole embryos contained very low amounts of HEPC1 and HEPC2 transcripts and, in a general manner, expression of both genes increased during development (Fig. 3C).

3.4. Iron regulation of HEPC1 and HEPC2 genes

Previously we demonstrated by Northern blot analysis using mouse HEPC1 probe that the amounts of hepcidin transcripts increased in the livers of mice overloaded by carbonyl–iron or iron–dextran [1,14]. By quantitative real-time RT-PCR we detected, respectively, a 4.7- and 5.8-fold increase of HEPC1 and HEPC2 transcripts in the livers of carbonyl–iron-overloaded mice. Similarly, liver expression of HEPC1 and HEPC2 genes was,respectively, 2.8 and 3.0 times higher in iron–dextran-treated mice in comparison to the control group (Fig. 4A,B).

4. Discussion

In this report we describe the mouse HEPC2 gene and provide a comparative analysis of its genomic organization, expression and iron regulation with that of the HEPC1 gene. Previous transgenic mice experiments evidenced a central role of hepcidin in iron metabolism. Specifically, the absence of both hepcidin transcripts was associated with iron-overload phenotype [5]. In contrast, overexpression of HEPC1 cDNA resulted in development of severe iron deficiency anemia [6]. Although it was not formally shown that hepcidin 2 has a similar functional activity, it has been suggested that HEPC1 and HEPC2 gene functions are redundant in the mouse [5].

As a first step to understand the regulation and the role of hepcidin 2, we isolated HEPC2 cDNA and demonstrated that it shares a very high similarity with HEPC1 both at the nucleotide and at the amino acid level in the corresponding translated regions (92% and 89% identity, respectively). Interestingly, although the positions of all eight cysteine residues are conserved, the sequence of the 25-aa C-terminal part of hepcidin 2 corresponding to the putative processed circulated peptide is considerably divergent from that of hepcidin 1 with only 68% identity, suggesting that these peptides could exhibit different levels of iron-regulating activities.

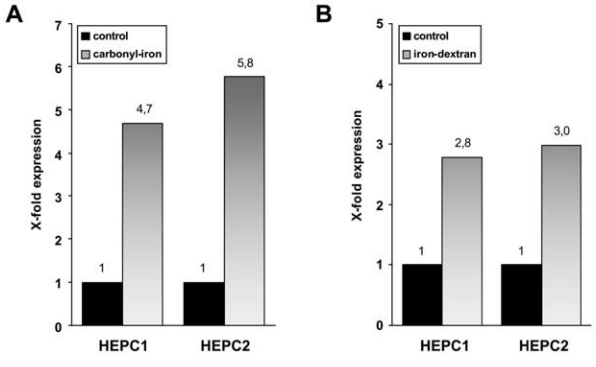

Fig. 4. Iron-mediated induction of HEPC1 and HEPC2 genes. The amounts of specific HEPC1 and HEPC2 transcripts were determined by real-time RT-PCR analysis as described in Section 2. Relative changes of HEPC1 and HEPC2 gene expression in the livers of (A) carbonyl–iron- and (B) iron–dextran-overloaded mice. The relative level of HEPC1 and HEPC2 transcript expression is indicated above the bars.

In this study, we confirm and extend previous observations that mouse genome contains two closely related HEPC genes and that both of them are transcriptionally active. Careful analysis of available mouse chromosome 7 genomic clones suggests the following scenario of HEPC1 and HEPC2 gene evolution: (1) duplication of a chromosome 7 region including the HEPC ancestral gene and a part of the USF2 gene encompassing exons 8–10 and a fragment of a large intron 7 and (2) insertion of the IAP element in the intron 7 of the truncated USF2 gene in the duplicated genomic region. The similar exon–intron organization, high conservation between HEPC1 and HEPC2 genes and the presence of only one HEPC gene in the human and rat genomes indicate that this duplication has likely occurred very recently.

The IAP sequence, located upstream of the HEPC1 gene, is a member of retrovirus-like mobile elements presented at approximately 1000 copies in the mouse genome [15]. IAP transposition frequently resulted in either inactivation or activation of adjacent genes [16]. In addition, retrotransposition of several IAP-like elements is frequently strain-specific, provoking modulation of gene expression activities only in certain strains of mouse [17]. Taking into consideration these observations, we cannot exclude that IAP transposition could also have an influence on hepcidin expression and iron homeostasis in a strain-specific manner. Indeed, different mouse strains exhibit variability in several parameters of iron metabolism including basal serum iron levels and hepatic iron stores [18]. In addition, heritable factors influence considerably iron homeostasis in response to HFE (the gene mutated in the common form of hereditary hemochromatosis) or β2-microblobulin gene disruption [12,19,20]. Therefore, it is tempting to speculate that hepcidin could contribute, at least partly, to strain-specific variability of basal iron status and sensitivity to iron overload.

An interesting finding of this study is the demonstration of a high amount of HEPC2 transcripts in the pancreas. Presently the molecular mechanisms that control pancreatic expression of hepcidin 2 are unknown. Alignment of 800-bp 5′-flanking regions of HEPC 1 and HEPC2 genes revealed a very high level of similarity (92% identity). The HEPC2 gene 5′-flanking regions contains all putative binding sites for transcription factors including C/EBP and HNF4 that have been previously identified in the HEPC1 promoter [14].

Collectively, HEPC1 and HEPC2 genes are expressed essentially in the liver, pancreas and at much lower levels in the heart. Interestingly, the liver and the pancreas were the principal iron-overloaded organs in USF2 (−/−) mice lacking hepcidin transcript expression. Substantial iron accumulation was also observed in the heart [5]. Accumulation of iron in the liver, pancreas and heart was also described in several other rodent models of iron overload including those experimentally provoked [21,22] and in genetic iron overload [23,24]. This striking parallelism between expression of hepcidin transcripts

and localization of iron deposits during iron overload suggests that in addition to the proposed humoral role of hepcidin in regulation of intestinal iron absorption and macrophage iron release, it could be involved in the local control of cellular iron store in the liver and pancreas. Finally, we demonstrated that both HEPC1 and HEPC2 genes were strongly up-regulated in the liver by iron excess.

In conclusion, our data suggests that both HEPC1 and HEPC2 genes are involved in the regulation of iron metabolism. However, we do not exclude that hepcidin 1 and hepcidin 2 could play distinct roles and/or exhibit different physiological activities. Further studies including selective ablation of HEPC1 or HEPC2 genes will be necessary to resolve this issue.

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