Severe iron deficiency anemia in transgenic mice expressing liver hepcidin

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We recently reported the hemochromatosis-like phenotype observed in our *Usf2* knockout mice. In these mice, as in murine models of hemochromatosis and patients with hereditary hemochromatosis, iron accumulates in parenchymal cells (in particular, liver and pancreas), whereas the reticuloendothelial system is spared from this iron loading. We suggested that this phenotypic trait could be attributed to the absence, in the *Usf2* knockout mice, of a secreted liver-specific peptide, hepcidin. We conjectured that the reverse situation, namely overexpression of hepcidin, might result in phenotypic traits of iron deficiency. This question was addressed by generating transgenic mice expressing hepcidin under the control of the liver-specific transthyretin promoter. We found that the majority of the transgenic mice were born with a pale skin and died within a few hours after birth. These transgenic animals had decreased body iron levels and presented severe microcytic hypochromic anemia. So far, three mosaic transgenic animals have survived. They were unequivocally identified by physical features, including reduced body size, pallor, hairless and crumpled skin. These pleiotropic effects were found to be associated with erythrocyte abnormalities, with marked anisocytosis, poikylocytosis and hypochromia, which are features characteristic of iron-deficiency anemia. These results strongly support the proposed role of hepcidin as a putative iron-regulatory hormone. The animal models devoid of hepcidin (the *Usf2* knockout mice) or overexpressing the peptide (the transgenic mice presented in this paper) represent valuable tools for investigating iron homeostasis *in vivo* and for deciphering the molecular mechanisms of hepcidin action.

Iron is an essential element that is required for growth and survival. However, in excess, the metal is toxic to the cell. To deal with this problem, elaborate cellular mechanisms have evolved in virtually all organisms to meet the iron needs of the body. In adult mammals, iron homeostasis depends upon regulated absorption of iron by the enterocyte, a highly specialized cell of the duodenum that coordinates dietary iron uptake and transport into the body. In the fetus, the mechanisms involved in placental maternofetal iron transport are also highly efficient and tightly regulated. Indeed, although fetal serum iron concentrations generally exceed those in the maternal circulation, only rare newborns exhibit evidence of iron toxicity. Although considerable progress has been made these last years in identifying key components of the iron transport molecular pathway (for review, see refs. 1–3), important questions are still unanswered. In particular, the molecular nature of the signals that programs the enterocyte cells of the duodenum and the syncytiotrophoblast cells of the placenta to adjust iron absorption and iron transport still remains to be identified.

In this context, we recently identified a putative key component of iron homeostasis that could act as a central player in the programming pathway from body iron stores to iron absorptive cells (4). It concerns a recently characterized mammalian peptide exhibiting antimicrobial activity, hepcidin (5, 6). The expression of the gene encoding hepcidin is restricted nearly to the liver (5–7). Whereas only one copy of the gene exists in humans, two duplicated hepcidin genes have been reported in mice (4, 7). The human gene encodes a precursor protein of 84 aa, including a putative 24-aa leader peptide. The human circulating form of hepcidin, described by two research groups in blood (6) and in urine (5), consists of the C-terminal 25 aa of the protein. Like many antimicrobial peptides, the mature hepcidin peptide seems to be highly structured with four intrachain disulfide bonds (5, 6). The link between hepcidin and iron metabolism stemmed from our finding that hepcidin gene expression was totally inhibited in mice exhibiting iron overload consequent to target disruption of the *Usf*2 (Upstream Stimulatory Factor 2) gene (4). In these *Usf*2 knockout mice, the development of iron overload was strikingly similar to that observed in human hereditary hemochromatosis and in $Hfe^{-/-}$ mice, the mouse model for hemochromatosis, with preferential loading of iron in parenchymal cells, increased circulating iron and decreased reticuloendothelial (RE) iron (8, 9). Interestingly, whereas our data show that a complete defect in hepcidin expression was linked to progressive tissue iron overload, Pigeon *et al.* (7) demonstrated that hepcidin gene expression was strongly upregulated in the liver of iron-overloaded mice. This latter result highlighted the role of hepcidin as a putative iron regulator that could be induced to decrease dietary iron absorption.

Our previous results left open the role of the transcription factor USF2 in the iron-overload phenotype and did not directly prove that the absence of hepcidin was responsible for it. In the present work, we address these two questions. Because the hepcidin genes are located directly downstream of the *Usf*2 gene in the genome, we hypothesized that extinction of hepcidin gene activities in our $Usf2^{-/-}$ mice could result from a specific *cis* effect of the targeting construct. The role of USF2 in the process of iron loading was evaluated by studying the iron status in another mouse model where the *Usf*2 gene was inactivated by using a different targeting approach (10). We show that in this alternative model the iron metabolism is normal, thus demonstrating that the lack of USF2 is probably not involved in the iron-overload phenotype in our previously reported $Usf2^{-/-}$ mice. Secondly, to address the question of whether hepcidin could directly fulfill the role of a sensor for iron homeostasis, we sought to generate transgenic mice overexpressing hepcidin in the liver. In good agreement with our proposed role for hepcidin in iron metabolism, we report that transgenic animals overex-

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Abbreviations: TTR, transthyretin; E*n*, embryonic day *n*; P*n*, postnatal day *n*; TFR, transferrin-receptor.

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pressing hepcidin died perinatally with severe iron-deficiency anemia.

Taken together, our previous data and the results presented in this paper strongly suggest that hepcidin is a secreted factor acting as a signaling molecule required to regulate iron homeostasis. Any pathophysiological situations leading to variation of circulating hepcidin are predicted to produce either iron deficiency or iron overload.

Materials and Methods

Animals. All animals used in these experiments were cared for in accordance with criteria outlined in the European Convention for the Protection of Vertebrate Animals. Animals were maintained in a temperature- and light-controlled environment and were given free access to tap water and food (standard laboratory mouse chow, AO3, Usine d'Alimentation Rationnelle, Epinay-S/Orge, France).

Where indicated, iron-dextran was injected s.c. at the dose of 0.5–1 mg of iron per g of body weight (Sigma).

Generation of Transgenic Mice. The Paris and Houston knockout *Usf*2 mice have been described (10, 11). Transgenic mice had a mixed genetic background that included contributions from C57BL/6, 129/Sv, and B6D2 strains as described (12). Full-length cDNA of the murine *HEPC1* cDNA was amplified by using primers 5′-GGGGGATATCAGGCCTCTGCACAGCA-GAACAGAAGG-3′ and 5′-GGGGGATATCAGGCCTC-TATGTTTTGCAACAGATACC-3′. The *HEPC1* PCR fragment was introduced between the transthyretin (TTR) sequences (consisting of the 3 kb of the mouse TTR regulatory regions 5′ to the cap site, the first exon, first intron, and most of the second exon) and the SV40 small-T poly(A) signal sequence (13). The construct was checked by DNA sequencing. The 4.7-kbp TTR-*HEPC1* transgene was separated from the plasmid sequence by digestion with *Hin*dIII and used for pronuclear microinjection.

Genotyping of Transgenic Mice by PCR and Southern Blotting. Southern blotting was done as described (4). *Bam*HI-digested DNA was electrophoresed, transferred to a nylon membrane (Hybond-N+, Amersham Pharmacia), and hybridized to a probe corresponding to the 1.7-kbp *Bgl*II-*Hin*dIII fragment of the TTR plasmid (13). The probe was labeled with [^{32}P]dCTP by random priming with a commercially available kit (Megaprime DNA labeling systems, Amersham Pharmacia). The 5.3-kbp-labeled fragment corresponds to endogenous TTR gene, and the 4.7-kbp-labeled fragment corresponds to the transgene.

For PCR, genomic DNA (0.5–1 μg) was used in 50-μl reactions that included three primers. The TTR-*HEPC1* transgene was amplified by using the forward primer 5′-GAGTCAG-GAAGTATGTGAGGG-3′ (annealing in intron 1 of TTR) and the reverse primer 5′-AACAGATACCACACTGGGAA-3′ (annealing in *HEPC1* cDNA). The endogenous TTR gene was amplified by using the same forward primer 5′-GAGTCAG-GAAGTATGTGAGGG-3′ and the reverse primer 5′-CTTTCCGGTGATAGACTCTGG-3′, both annealing in intron 1 of TTR. The PCR was performed as follows: 34 cycles (each cycle consisting of 40 s at 94°C, 40 s at 53°C, and 40 s at 72°C), with an initial denaturation step at 94°C for 4 min and a final elongation step at 72°C for 5 min, in 20 mM Tris·HCl (pH 8.4)/50 mM KCl/0.05% W-1/2 mM $MgCl_2$/0.2 mM each dNTP/0.2 μM each primer, and 4 units of *Taq* polymerase (GIBCO/BRL). The 705-bp-specific product corresponds to the TTR-*HEPC1* transgene and the 364-bp product corresponds to the endogenous TTR gene. The reaction was analyzed on 1.5–2% agarose gel containing ethidium bromide. This PCR method for mouse genotyping was found to give the same results as the Southern blot method.

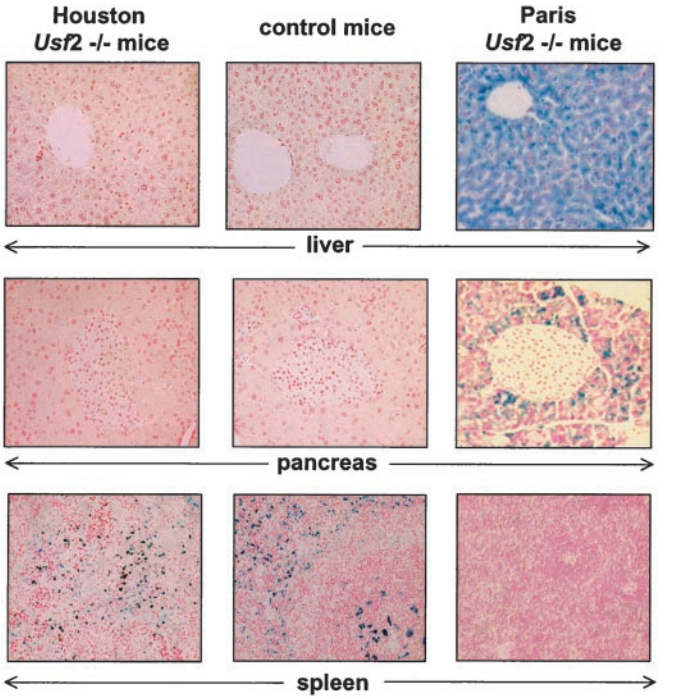

Fig. 1. Iron content in liver, pancreas, and spleen of Houston *Usf2*$^{-/-}$ mice. Liver, pancreas, and spleen were fixed in formaldehyde and stained with the Perls' stain for iron. Non-heme iron stains blue. Liver, pancreas, and spleen sections from a representative 10-month-old Houston *Usf2*$^{-/-}$ mouse, 8-month-old control mice, and an 8-month-old Paris *Usf2*$^{-/-}$ mouse (×20).

Northern Blotting. Twenty micrograms of RNA from each source was denatured in formaldehyde-containing buffer and electrophoresed in 1% agarose/2.2 M formaldehyde gels. Northern blot was performed as described (4). After electrophoresis, RNA was transferred to a nylon membrane (GeneScreen Plus, Dupont/NEN) in 20 × SSC buffer. The probe used to detect hepcidin mRNAs was prepared from the plasmid isolated by suppressive subtractive hybridization pT-Adv/*HEPC1* (4). To detect TTR mRNAs, we used a 600-bp cDNA fragment (a gift from Virginie Joulin, Institut Cochin, Paris, France). Because of the presence of the SV40 small-T poly(A) sequence, the size of the transgenic hepcidin transcript is greater than that of the endogenous one. Each blot was stripped and reprobed with ribosomal 18S cDNA to check for the integrity and the amount of loaded RNAs.

Iron Measurements and Histology. Quantification of iron level was performed as described by Torrance and Bothwell (14) on fragments or total organs by using an IL test (Instrumentation Laboratory, Lexington, MA). For histology, tissues were fixed in 4% (vol/vol) formaldehyde, embedded in paraffin, mounted onto slides, and stained with Prussian blue and nuclear red counterstains by using standard procedures.

Results

The Iron-Overload Phenotype Is Not Related to USF2 Deficiency. To assess the role, if any, of the transcriptional factor USF2 in the establishment of the iron-overload phenotype observed in our *Usf2*-null mice (ref. 4, referred to as the Paris KO), we sought to determine whether the same iron-related phenotype could be observed in another KO mouse model. To this end, we used the *Usf2*-null mice (ref. 10, referred to as the Houston KO) and analyzed the iron status in this model by Perls' Prussian blue staining in the liver, pancreas, and spleen of these mice.

As clearly demonstrated in Fig. 1, whereas the Paris *Usf2*$^{-/-}$

KO mice exhibit marked iron overloading in the liver and pancreas, iron did not accumulate in these tissues from Houston *Usf2*$^{-/-}$ KO mice. In the spleen, the normal positive reaction with the Perls' Prussian blue staining observed between the red pulp cells was also clearly present in the Houston KO mice. In contrast, as previously demonstrated, the spleen reticuloendothelial system from Paris *Usf2*$^{-/-}$ KO mice is spared from this iron loading (4).

In agreement with this normal iron metabolism phenotype in the Houston *Usf*2-null mice, we found normal amounts of both *HEPC1* and *HEPC2* transcripts in the liver of these mice by Northern blot and reverse transcription (RT)-PCR (data not shown).

Taken together, these data, which demonstrate that the Houston *Usf2*-null mice have a normal iron-related phenotype with normal amounts of liver hepcidin, are strongly in favor of the hypothesis that the lack of USF2 protein is not responsible for the increased iron accumulation observed in the Paris *Usf*2 KO, and that the absence of hepcidin gene expression could indeed explain the expression of the hemochromatosis-like phenotype in these mice. This supposition led us to conjecture that the reverse situation, namely an increase in hepcidin gene expression, could result in pathologies associated with iron deprivation. To investigate this hypothesis, we created transgenic mice overexpressing hepcidin.

Generation of TTR-*HEPC1* Transgenic Mice. Because of the highly liver-specific pattern of hepcidin gene expression (5–7), we used a liver-specific promoter to direct selective expression of hepcidin into hepatocytes. Although there is only one copy of the hepcidin gene in the human genome, two highly related copies, *HEPC1* and *HEPC2*, have been reported in the mouse. So far, however, there is no data assessing differential functional roles for *HEPC1* and *HEPC2* in mice. In this study, the HEPC1 peptide was chosen rather than HEPC2, because it is the peptide most closely related to that found in humans. Indeed, as shown in Fig. 2*A*, there is a 76% sequence identity of the human 25-residue peptide with HEPC1 and only a 58% identity with HEPC2.

The transgenic construct was made by introducing a murine PCR-made *HEPC1* cDNA fragment between the −3 kb mouse TTR promoter region and the SV40 small-T poly(A) signal (13). The construction is schematized in Fig. 2*B*. After standard microinjection of the linearized construct, a total of 14 independent transgenic F0 was obtained. Fig. 2*C* shows the Southern blot of the *Bam*HI-digested genomic DNA from these different F0 mice. The probe revealed a band at 4.7 kbp corresponding to the transgene (Tg) and one at 5.3 kbp corresponding to the transthyretin endogenous gene (End). This latter band was used as the internal reference corresponding to two allele copies. The transgenic F0 mice could be separated into two groups according to their phenotype. The first one corresponded to transgenic mice that were born with a pale skin and died within a few hours after birth (Fig. 2*C*, newborn mice transgenic for hepcidin—THnb—including THnb 2, 3, 4, 10, 25, and 29). In this case, a transgene band at the expected size and representing more than two copies was always detected. The second group was characterized by the same genomic pattern—i.e., at least two copies of the transgene—and corresponded to three transgenic mouse founders (Fig. 2*C*, group 2, TH5, TH35, and TH61). These animals all had an unequivocal phenotype with small size, apathy, poor coat, and crumpled skin. This phenotype appeared rapidly after birth, except for TH61, whose phenotype was only clearly established after 2 weeks. The health of TH5 and TH35 founders deteriorated significantly after 2–3 weeks of life. Because hepcidin was hypothesized to impair iron absorption, we sought to rescue these animals by directly administrating iron by means of s.c. injection of iron-dextran. This iron therapy caused

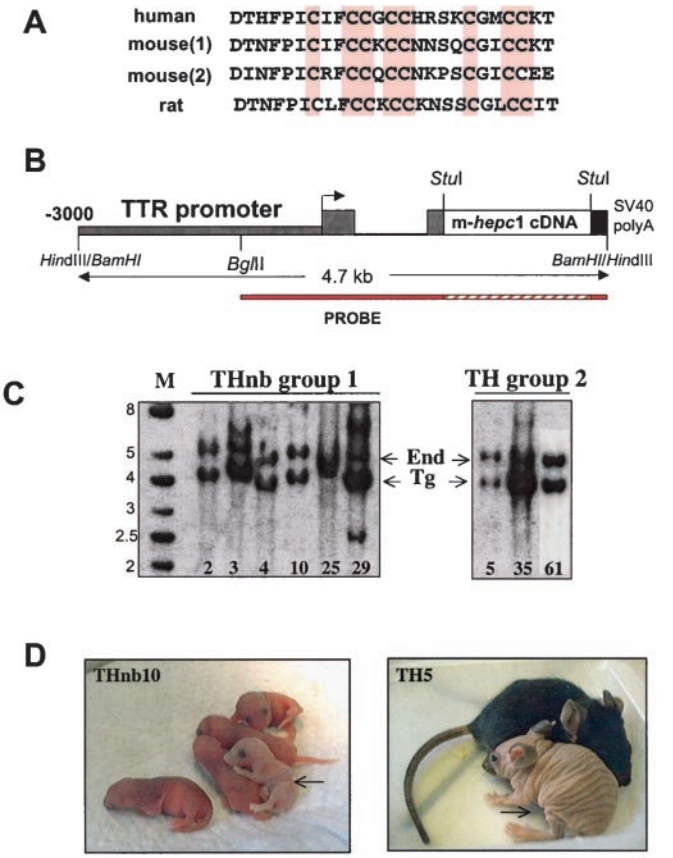

Fig. 2. Generation of TTR-*HEPC1* transgenic mice. (*A*) Comparison of the 25-aa peptide sequences from human, mouse, and rat. Conserved cysteine residues are shaded. (*B*) Schematic representation of the TTR-*HEPC1* construct. The murine *HEPC1* cDNA was introduced between the transthyretin sequences (consisting of the 3 kb of the mouse TTR regulatory regions 5′ to the cap site, the first exon, first intron, and most of the second exon) and the SV40 small-T poly(A) signal sequence (13). (*C*) Southern blot analysis of tail DNA from transgenic founders. The founders were distributed among two groups according to their phenotype. Genomic DNA was digested by *Bam*HI and hybridized with the TTR probe represented in *B*. Two bands of the expected size, 5.3 kbp and 4.7 kbp, representing the endogenous TTR gene (End) and the transgene (Tg), respectively, were detected. (*D*) Phenotypic features of transgenic mice harboring hepcidin. The arrows indicate the pale newborn THnb10 (arrow) at birth among the nontransgenic littermates and the hairless and crumpled TH5 founder at 4 weeks, as compared with a nontransgenic littermate.

improvements in both founders; several weeks after injection, they were indistinguishable from their normal coat littermates (not shown). However, it cannot definitively be concluded that this renewal was caused by iron-dextran therapy because no controls, i.e., noninjected transgenic founder mice, were available. The transgenic male founder TH5 and the two females TH35 and TH61 were mated to give F1 offspring. In all cases, phenotypes of the progeny were much more severe than those of the parent founders. Indeed, all transgenic mice from TH5 died perinatally with marked pallor (to date, from nine independent litters, 13 F1 transgenic newborns died at birth). Female TH35 gave birth only once, with only one transgenic pup in the litter. This pup, which was small and had a pale and crumpled skin, died at 13 days. The TH61 female gave birth to two transgenic F1 pups in one litter. One transgenic pup died at birth with pallor, whereas the other died at 3 weeks.

The phenotype of the transgenic mice from the two groups is shown in Fig. 2*D*, where THnb10 at birth and TH5 at 3 weeks are presented next to unaffected littermates.

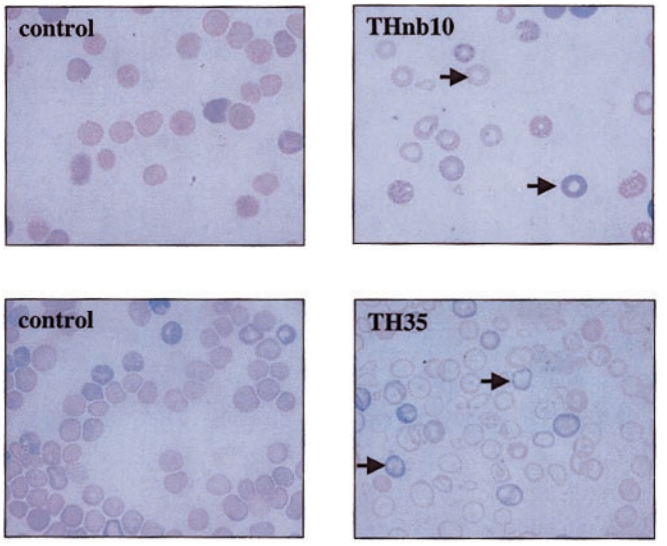

Fig. 3. Hematological phenotype of the transgenic mice. Wright-Giemsa-stained blood smears were performed on control (*Left*) and transgenic mice (*Right*). Compared with normal erythrocytes, transgenic erythrocytes from THnb10 at birth and TH35 at 4 weeks show marked anisocytosis, poikylocytosis, and hypochromia. Arrows indicate the typical ringed red cells characteristic of iron deficient anemia.

Severe Microcytic Anemia in Transgenic Mice Overexpressing Hepcidin. If hepcidin compromises iron transport, then erythropoiesis should be altered because most iron is directed to hemoglobin synthesis. Therefore, to directly assess the consequences of exogenous hepcidin on erythropoiesis, we performed Wright-Giemsa-stained blood smears on newborn transgenic F0 mice and on TH5. Fig. 3 shows that, in comparison to control erythrocytes, erythrocytes from both THnb10 and TH5 exhibited evidence of poikylocytosis, hypochromia, and microcytosis. Most red cell centers were flattened with the hemoglobin being extruded to the periphery, leading to the typical ring aspect of hypochromic red cells. The same erythrocyte abnormalities were found in F1 progenies from TH5, TH35, and TH61. Because of the early lethality and, as a result, the very small amount of plasma available, it was difficult to characterize further the anemia by blood parameters (especially ferritin, which is a good indicator and whose level drops substantially during iron deficiency). On the other hand, total iron deficiency was evaluated in F1 progeny from TH5 by directly measuring iron content in the whole embryo at embryonic day 15.5 (E15.5). The results indicated that there was 4-fold less total iron in a transgenic fetus as compared with a control fetus (5.3 ± 0.4 μg of iron per gram wet tissue in transgenic F1 progeny from TH5 [$n = 5$] vs. 19.7 ± 2.6 μg in control fetuses [$n = 19$] $P < 0.0001$). The classical hematological features of iron deficiency anemia associated with the severe iron deficit in transgenic mice strongly support the idea of hepcidin acting as a negative regulator of iron transport.

Developmental Expression of Hepcidin Genes in Wild-Type Mice. To understand better the reasons for the early postnatal lethality of most of the transgenic animals, we sought to determine the level of murine hepcidin gene expression during liver development in wild-type C57BL/6 mice. As shown in the Northern blot of Fig. 4, from E15.5 to postnatal day 56 (P56), there was no detectable expression of hepcidin genes apart from a strong transient induction at birth (B) and at P1 and P2. Hepcidin expression only reached a high level in adult liver (P56). This pattern of expression was different to that of transthyretin. Indeed, as shown in the lower part of Fig. 4, transthyretin expression was

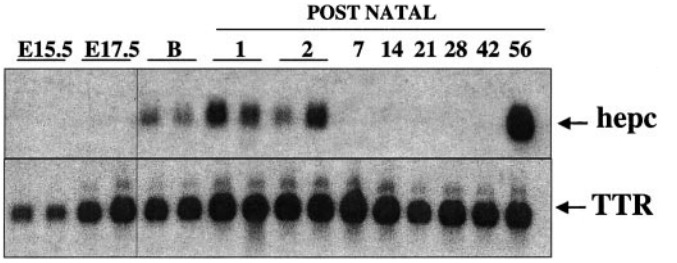

Fig. 4. Hepcidin and transthyretin mRNA content during liver development of wild-type C57BL/6 mice as determined by Northern blot analysis. Twenty micrograms of wild-type total liver RNA, from E15.5 and E17.5, birth (B), and during postnatal development from P1 to P56, was electrophoresed, blotted, and hybridized with hepcidin and TTR ^{32}P-labeled probes.

detectable in the developing liver starting at E15.5 and remained high and constant throughout development.

This leads us to hypothesize that the early death of transgenic mice may be caused by the sustained transgenic hepcidin expression during liver development, at a time when normally the endogenous hepcidin gene is not expressed at detectable levels.

The presence of hepcidin in the fetus at the end of gestation might alter maternofetal iron transport, leading to severely iron-deficient newborn mice. The presence of the transgenic hepcidin transcripts could be confirmed only in the progeny of the male TH5 (TH35 and TH61 were founder females we wanted to keep alive). At E15.5, transgenic F1 animals from TH5 were already visibly anemic, and the expression of the transgene was demonstrated by RT-PCR (data not shown).

The Severity of the TTR HEPC1 Mouse Phenotype Is Correlated with the Expression of the Hepcidin Transgene. The transgenic mice found dead postnatally as well as the progeny from TH5, TH35, and TH61 were analyzed by Northern blot for expression of hepcidin in their livers. Fig. 5 shows a Northern blot revealing both the transgenic (Tg, upper band) and the endogenous (End, lower band) hepcidin transcripts. It is clearly evident that all of the newborn F0 mice, which were readily distinguishable at birth by their pallor, expressed high levels of hepcidin (Fig. 5*A*, THnb10,

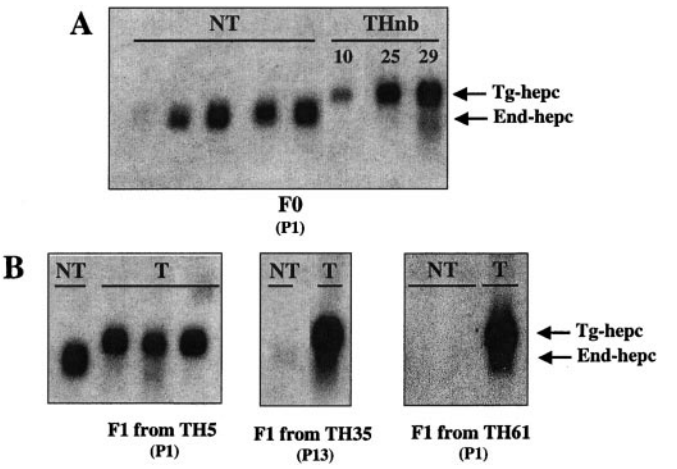

Fig. 5. Hepcidin mRNA level in transgenic mice as determined by Northern blot analysis. Twenty micrograms of total liver RNA was electrophoresed, blotted, and hybridized with the ^{32}P-labeled hepcidin probe made by PCR as described in *Materials and Methods*. This probe reveals both the transgenic hepcidin (Tg-hepc, upper band) and the endogenous hepcidin (End-hepc, lower band). (*A*) Hepcidin mRNA level in F0 transgenic animals (THnb) at P1, as compared with nontransgenic (NT) littermates. (*B*) Hepcidin mRNA level in F1 progeny from TH5 at P1, from TH35 at P13, and from TH61 at P1.

25 and 29). Very interestingly, we noted in these transgenic mice that the normal hepcidin mRNA induction observed at birth (see the nontransgenic littermates) was totally inhibited.

Transgenic TH5, TH35, and TH61 progenies also expressed the hepcidin transgene at a high level, whereas the endogenous hepcidin gene again was found to be silent in these animals (Fig. 5*B*). In the nontransgenic pups from the same litter, the endogenous hepcidin gene was, as expected, expressed 1 day after birth in TH5 male offspring, not expressed at day 13 in TH35 female offspring and, more interestingly, not expressed at day 1 in TH61 female offspring. The difference between TH5 and TH61 offspring is that the mother was wild-type in the first case and transgenic with microcytic anemia in the second.

Discussion

The present study provides substantial evidence for a central role for hepcidin in iron homeostasis. We demonstrate that the iron-overload phenotype previously described in *Usf2/hepcidin*-deficient mice is not related to USF2 deficiency and is thus most likely associated with the lack of hepcidin. Indeed, in another $Usf2^{-/-}$ model (the Houston KO), iron metabolism and hepcidin gene expression are normal. In addition, we show that, when constitutively expressed, hepcidin impairs maternofetal iron transport leading to severe iron deficiency anemia in neonates. This latter observation relies on transgenic mice expressing hepcidin under the control of the TTR promoter. This promoter is known to direct liver-specific expression, but most importantly, this expression starts during liver development. We report here that the endogenous TTR gene is active at E15.5; data in the literature have reported TTR promoter activity as early as E9.5 (15).

The Severity of the Anemia in Hepcidin Transgenic Mice Is Associated with the Fetal Expression of the Transgene. The majority of the transgenic animals we have generated die after birth, presumably from severe anemia. This anemia was revealed by abnormalities of erythrocyte morphology (Fig. 3), and iron deficiency was directly assessed by measuring iron level in transgenic fetuses. Attempts to determine whether some changes occurred in tissue distribution between control and transgenic fetuses were hampered because of the small amount of material available and the very low concentration of iron in fetal fetuses. Today, only three phenotypically affected founders are still alive: the male TH5, and the two females, TH35 and TH61. In fact, Southern blot analysis comparing genomic DNA from parents and progeny showed that TH5, TH35, and TH61 are mosaics (data not shown). This result might explain why these parents survive, whereas their progeny die around birth, and also the low transmission rate of the transgene.

When available, F0 mice, as well as the F1 progeny from TH5, TH35, and TH61, were analyzed for transgenic hepcidin expression (Fig. 5). These transgenic animals, which were all visibly anemic, expressed high levels of hepcidin. For TH5 progeny, hepcidin expression was demonstrated to occur at least at E15.5, a time when pallor was already evident as well as alteration of erythrocyte morphology (data not shown). This result suggests that hepcidin can be correctly processed and secreted in fetal liver. In fact, although we did not detect hepcidin transcription between E15.5 and birth in wild-type mice liver, an earlier fetal expression is conceivable because it was reported by two groups (5, 7) in human liver fetuses between 18 and 24 weeks of gestation. The absence of hepcidin gene expression late in pregnancy is in good agreement with the known high rate of iron transfer from the mother to the embryo during this period. In the postnatal period, hepcidin gene expression is only transiently detected around birth and then in young adults, i.e., after the period of rapid growth in which active iron absorption is crucial. The transient expression at birth probably reflects birth-associated stress. Indeed, the hepcidin gene was reported to be responsive to different stimuli; in particular, it was associated with inflammation (7).

Silencing of endogenous hepcidin gene expression in transgenic neonates is most likely explained by the inhibitory effect of iron deficiency and, perhaps, anemia. The anemia of the TH61 mother also could account for the absence of neonatal hepcidin gene activation even in the nontransgenic offspring. In other words, a normal iron homeostasis could be permissive for the hepcidin gene responding to other stimuli. Conversely, the positive effect of iron on hepcidin gene expression has been well documented by Pigeon *et al.* (7).

Role of Hepcidin in the Iron Transport Pathway. Whereas much progress has been made these last years in the understanding of iron absorption by mature enterocytes of the villus (for review, see ref. 1), the mechanisms of the maternofetal transport of iron through the syncytiotrophoblast cells of the placenta is less well documented. It is known that the growing fetus requires a large supply of iron that is taken up from the maternal blood via transferrin-receptor (TFR)-mediated endocytosis. TFR knockout mice show embryonic death with severe anemia in homozygotes and increased microcytic erythrocytes and decreased tissue iron in heterozygotes (16). Interestingly, Parkkila *et al.* (17) have reported that these TFRs are physically associated with the HFE/β2M complex in the human placental syncytiotrophoblast cells. Therefore, it is tempting to hypothesize that the HFE/β2M/TFR complex, whose pathophysiological role is now well established in the process of iron absorption in the small intestine, could be involved in regulating iron transport in the placenta. The existence of severe neonatal iron deficiency anemia in the microcytic anemia (*mk*) mice defective in the divalent metal iron transporter (DMT1; ref. 18, and see below) indicates that this transporter is also most likely involved in maternofetal iron transport. Efflux of iron in the fetal circulation, whose mechanisms are not yet fully clarified, is thought to operate through the iron exporter IREG1/ferroportin1. Ferroportin1 was identified in the zebrafish mutant *weissherbst* by positional cloning (19), and in a mouse model of increased iron absorption by a subtractive cloning strategy (20). Zebrafish with ferroportin mutations die early in life with severe hypochromic anemia. Investigations of ferroportin1 gene expression in different tissues showed high levels of expression in placenta, with ferroportin1 transcripts being located throughout the labyrinth zone of the mouse inner placenta (19, 20).

How transgenic hepcidin interferes with these placental proteins (HFE, TFR, DMT1, and ferroportin1), leading to down regulation of iron delivery from the mother to the developing fetus, deserves further investigation. We are currently looking at the level of these different proteins in the placenta of F1 transgenic mice. Molecular insights into the mechanism of hepcidin action at the level of intestinal absorption also would be of great interest. However, our model is not suitable for this purpose because of the early lethality of the transgenic mice at a stage when the gastrointestinal tract has not achieved competency for iron absorption. To address this question and to characterize more precisely the steps in hepcidin regulation of iron homeostasis, it is necessary to create inducible transgenic models.

Animal and Human Models of Anemia. The phenotype observed in our transgenic mice is reminiscent of that of the murine model exhibiting severe iron deficiency, namely microcytic anemia (*mk*) mice. These *mk* mice carry mutations in the iron transporter DMT1 (18), thus impairing intestinal iron absorption and iron metabolism in peripheral tissues. In addition, these mice have severe neonatal microcytic anemia, and only a few survive to weaning. Iron injections intended to circumvent the intestinal block in *mk* mice only partially reverse the anemia, suggesting a

further block to iron entry into red blood cell precursors. In our model, iron treatment experiments deserve further investigation to establish clearly whether hepcidin-induced anemia can be fully rescued by this iron therapy.

In any case, the phenotypes of both hepcidin-hyperexpressing (this paper) and hepcidin-deficient mice (4) demonstrate that this peptide plays an essential role in regulating iron transport through both placental and intestinal barriers and is most likely involved at other sites as well, e.g., reticuloendothelial cells.

The anemia of human chronic diseases is particularly interesting because it occurs under conditions such as infection or inflammation, which involve decreased circulating iron, increased reticuloendothelial iron and decreased intestinal iron absorption, all features of iron disorders that could be explained by up-regulation of hepcidin. Fleming and Sly (21) already predicted such an inflammation-induced increase in hepcidin, and this increase is now under investigation with mouse models of inflammation. The putative role of hepcidin in inflammation is reinforced by the interesting finding by Pigeon *et al.* (7) that lipopolysaccharide, a classical inducer of acute-phase proteins involved in response to inflammation and infection, is able to induce hepcidin gene expression both *in vitro* and *in vivo*.

Conclusion

The demonstration that the absence of USF2 protein is not responsible for the iron abnormalities, coupled with the phenotypes of *Usf2* knockout mice and hepcidin overexpressing transgenic mice, clearly permit a key regulatory role in iron homeostasis to be attributed to hepcidin. Further studies are now needed to elucidate the molecular mechanisms of hepcidin action. One of the key questions is whether hepcidin acts by itself or, as a circulating factor, is able to interact with specific receptors. Whatever the mechanisms of hepcidin, we believe that our models of transgenic mice devoid of hepcidin (the *Usf2* knockout mice) or overexpressing hepcidin (the transgenic mice presented in this paper) offer a valuable tool for investigating iron homeostasis *in vivo* and for screening potential drugs for modulating iron absorption.

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