GASTROENTEROLOGY 2004;126:1400–1408

Multigenic Control of Hepatic Iron Loading in a Murine Model of Hemochromatosis

MOUNIA BENSAID,* SÉVERINE FRUCHON,* CHRISTINE MAZÈRES,* SEIAMAK BAHRAM,† MARIE–PAULE ROTH,* and HÉLÈNE COPPIN*

*INSERM Unité 563, Centre Hospitalier Universitaire Purpan, Toulouse, France; and †INSERM-CreS, Centre de Recherche d'Immunologie et d'Hématologie, Strasbourg, France

Background & Aims: **Hereditary hemochromatosis is a common disorder of iron homeostasis characterized by increased dietary iron absorption and progressive iron accumulation, mainly in the liver. Most patients are homozygous for the C282Y mutation in the *HFE* gene. However, not all individuals carrying the hemochromatosis-predisposing genotype in the general population become iron loaded. Genetic modifiers have been shown to influence disease penetrance, but their number and chromosomal locations remain unknown, and their identification is hampered by complex interactions with environmental factors. To circumvent these difficulties, we used 2 strains of mice made deficient for the *Hfe* gene that strongly differ in their propensity to develop hepatic iron loading.** ***Methods:*** **To localize the loci controlling hepatic iron loading in this murine model of hemochromatosis, we produced 1028 mice by an F2 intercross between the C57BL/6 and DBA/2 *Hfe*-deficient strains. We selected the 276 mice that contributed the most to the total linkage information for genotyping with 145 microsatellite markers.** ***Results:*** **We mapped 4 modifier loci on chromosomes 7, 8, 11, and 12, with logarithm of odds scores of 14.47, 12.96, 6.04, and 6.72, respectively, in regions containing several genes recently shown to exert important roles in the regulation of iron metabolism.** ***Conclusions:*** **Our data provide a clear demonstration of the polygenic pattern of hepatic iron loading inheritance in *Hfe*-deficient mice. Examination of candidate genes residing at the loci identified in this study and genetic analysis of the syntenic chromosomal regions in humans may provide important insight into the heterogeneous disease presentation observed among *HFE* C282Y homozygotes.**

Hereditary hemochromatosis (HH) is a common autosomal recessive disorder of iron homeostasis characterized by increased dietary iron absorption and progressive iron accumulation, mainly in the liver. If untreated, iron accumulation can result in tissue damage, with clinical manifestations that include cirrhosis, hepatic carcinoma, congestive heart failure, and premature death.[1] In northern Europe, most patients with HH are homozygous for a single mutation (C282Y) in the *HFE* gene,[2] and approximately 1 person in 200 in the general population carries the HH-predisposing genotype.

However, not all C282Y homozygotes become iron loaded. Several families in which C282Y-homozygous members have a transferrin saturation and serum ferritin within the reference range and no obvious signs or symptoms of HH have been discovered through pedigree testing.[3–6] In addition, C282Y homozygosity is not underrepresented in the elderly population[7] or among centenarians,[8] suggesting that life-threatening HH-related complications do not occur in most subjects. There is also a large discrepancy between the prevalence of the HH-predisposing genotype in the population and the number of patients effectively diagnosed and treated with HH,[9,10] further suggesting that the C282Y homozygous genotype is not fully penetrant.

The true prevalence of nonexpressing homozygotes has proven difficult to estimate. It was first assessed in population screening studies in which both genotyping and phenotyping were performed.[11–16] In these studies, the prevalence of C282Y homozygotes detected with a normal serum ferritin not requiring therapy ranged from 25% to 81%. However, there is still scant information about the number of people in the general population with the HH-predisposing genotype who have clinical manifestations caused by the mutation and not merely associated with it. Beutler et al.[17] underlined the need for controlled studies to assess the clinical penetrance and expressivity of HH, and they estimated in a large case-control study in California that the clinical penetrance of the HH-predisposing genotype was less than 1%. Although this value of disease penetrance cannot be considered as conclusively established,[18–20] the data avail-

Abbreviations used in this paper: $\beta 2m^{-/-}$, β_2-microglobulin knockout mice; Hmox1, heme oxygenase 1; HH, hereditary hemochromatosis; LOD, logarithm of odds; QTL, quantitative trait loci.

0016-5085/04/$30.00
doi:10.1053/j.gastro.2004.01.021

able today indicate that it is much lower than initially appreciated.

A question of great interest is understanding why the severity of iron loading and clinical disease is variable in individuals homozygous for the C282Y allele. Although age, sex, and environmental factors have been shown to influence the disease, genetic factors may also enhance or reduce the phenotypic expression in HH.[21] For many single-gene disorders, patients with the same genotypes often differ markedly with respect to disease expression because of the effects of additional independently inherited genetic variations.[22] It is interesting to note that there is a higher incidence of HH-related conditions in relatives, male and female, of clinically affected probands than in relatives of probands identified because of increased transferrin saturation values, even though the 2 groups of relatives do not differ in any relevant feature.[23] This is not unexpected if mutations in modifier genes determine whether clinical expression will occur. The segregation of these mutations in relatives of patients would then explain their higher risk of developing clinical HH. Despite clear indications that genetic modifiers exist,[23–26] surveys of multiple genes of iron metabolism performed in patients homozygous for the C282Y mutation and in nonexpressing controls have not shown polymorphisms or mutations associated with increased iron loading.[27] The identification of modifier loci that control susceptibility to iron overload in at-risk C282Y-homozygous individuals may be hampered by several factors, including multiple genetic loci, complex interactions of environmental factors with the predisposing genetic background, and genetic heterogeneity.

Murine models of iron loading thus provide a useful alternative to human models to identify modifier loci and to better understand the physiological pathways involved in the disease process. β_2-Microglobulin knockout mice ($\beta 2m^{-/-}$), which do not express Hfe, and *Hfe* knockout mice (*Hfe*$^{-/-}$) have, like HH patients, augmented duodenal iron absorption, abnormally high plasma transferrin saturations, increased iron deposition in hepatic parenchymal cells with periportal predominance, and relative resistance of the spleen to iron loading.[28–31] Although $\beta 2m^{-/-}$ and *Hfe*$^{-/-}$ mice differ from HH patients because they lack most of the clinical manifestations of the disease—such as hypogonadism, hypopituitarism, diabetes mellitus, cardiomyopathy, joint disease, or frank cirrhosis—they have proven to be valuable tools for investigating iron homeostasis. In particular, they have been shown to have, like HH patients, impaired iron uptake of transferrin-bound iron by the duodenum[32] and up-regulation of the expression of the duodenal iron transporters.[33,34] Of particular interest, strain background was found to be a major determinant in iron loading, both in $\beta 2m^{-/-}$[35] and in *Hfe*$^{-/-}$[36] mice, suggesting the existence of genes other than *Hfe* that modify the gravity of iron accumulation. We previously reported that, compared with the C57BL/6 strain, the DBA/2 strain was particularly susceptible to iron loading in response to *Hfe* disruption.[34] In this study, we have taken advantage of the marked phenotypic differences between these 2 strains to localize quantitative trait loci (QTLs) that control hepatic iron loading in this murine model. We first generated a cross between the C57BL/6 and DBA/2 *Hfe* knockout mouse strains and produced a segregating progeny of more than 1000 mice by an F2 intercross (F1 × F1). To increase the power of mapping quantitative traits, we then selected the 276 F2 mice that contributed the most to the total linkage information for genotyping with 145 microsatellite markers covering the entire mouse genome.

Materials and Methods

Mice

The disrupted *Hfe* allele, initially on a mixed background (129/Ola × C57BL/6),[30] was bred by 10 successive backcrosses onto the DBA/2 and C57BL/6 backgrounds. Mice heterozygous for the disrupted allele were then mated to produce *Hfe*-null (*Hfe*$^{-/-}$) mice. Using the convention of (female × male) to indicate strain parentage, 671 *Hfe*$^{-/-}$ F2 mice (321 males and 350 females) were generated from 6 ([C57BL/6 × DBA/2]F1 × [C57BL/6 × DBA/2]F1) mating pairs, 284 *Hfe*$^{-/-}$ F2 mice (144 males and 140 females) were generated from 8 ([DBA/2 × C57BL/6]F1 × [DBA/2 × C57BL/6]F1) mating pairs, 33 *Hfe*$^{-/-}$ F2 mice (16 males and 17 females) were generated from 1 ([C57BL/6 × DBA/2]F1 × [DBA/2 × C57BL/6]F1) mating pair, and 40 *Hfe*$^{-/-}$ F2 mice (18 males and 22 females) were generated from 1 ([DBA/2 × C57BL/6]F1 × [C57BL/6 × DBA/2]F1) mating pair. The studied population consisted of *Hfe*$^{-/-}$ C57BL/6 mice (45 males and 36 females), *Hfe*$^{-/-}$ DBA/2 mice (35 males and 43 females), *Hfe*$^{-/-}$ F1 mice (26 males and 30 females), and the 1028 *Hfe*$^{-/-}$ F2 mice (499 males and 529 females). The mice were housed in the Institut Fédératif de Recherche 30 animal facility and had free access to water and R03 diet (UAR, Epinay-sur-Orge, France) containing iron 280 mg/kg. All mice were analyzed at 7 weeks of age and were fasted for 14 hours before blood sampling. Liver and spleen were taken from each mouse and were frozen at −70°C. Experimental protocols were approved by the IFR 30 Animal Care and Use Committee.

Nonheme Iron Quantitation of Liver Tissue

Samples of approximately 100 mg were crushed to pulp, dried overnight in a 65°C oven, and weighed. The dried liver was then transferred in 1 mL of acid mixture (3 mol/L

HCl/10% trichloroacetic acid) and heated in a 65°C oven for 20 hours. After cooling to room temperature, 50 μL of the clear acid extract was brought to 500 μL with iron-free water, placed in 2 mL of the working chromagen reagent (1 volume of 0.1% bathophenanthroline sulfonate/1% thioglycolic acid added to 5 volumes of saturated sodium acetate and 5 volumes of iron-free water) and vortex-mixed. Working chromagen reagent was prepared fresh daily. A total of 250 μL of a 200 μmol/L iron standard solution (Sigma Aldrich, Saint Quentin Fallavier, France) was treated the same way and used for calibration. Color was allowed to develop for 15 minutes, and the absorbance was measured at a wavelength of 535 nm on a Pharmacia Biotech Ultrospec 2000 spectrophotometer.

Genetic Typing

DNA was prepared from frozen spleens by using the Nucleospin Tissue kit (Macherey-Nagel, Hoerdt, France). A total of 145 microsatellite markers were selected to genotype the extreme 276 F2 mice and were chosen at 10-to 20-cM intervals based on the genetic map of the mouse.[37] Polymerase chain reaction amplifications (total volume, 10 μL) were performed in PCR Master Mix 1X (Promega, Charbonnières-les-Bains, France), with 0.25 to 1 μmol/L of each primer. An oligonucleotide of each pair was labeled with 1 of the fluorescent dyes 6-FAM or HEX. Primers were obtained from MWG-Biotech AG (Ebersberg, Germany). Thirty cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds were generally used, although some primers required a slightly higher or lower annealing temperature for optimum amplification. After electrophoresis of pools of 4 to 9 amplification products and an internal lane size standard (Genescan 400 HD) labeled with the ROX dye (Applied Biosystems, Courtaboeuf, France) on a 96-capillary ABI PRISM 3700 DNA Analyser (CRGS platform; Genopole, Toulouse, France), fluorescent-labeled fragments were sized by using the fragment analysis software packages Genescan and Genotyper (Applied Biosystems).

Data Analysis

Map orders and intermarker distances were obtained from the genotypes of the F2 progeny by using MAPMAKER/EXP 3.0 (available at http://www.broad.mit.edu/genome_software/other/mapmake.html).[38] All genotypes with a logarithm of odds (LOD) of error >1% were rescored. Interval mapping of the QTLs affecting hepatic iron concentration was performed with the MAPMAKER/QTL program (available at http://www.broad.mit.edu/genome_software/other/qtl.html).[39] The genotypes of the 752 nonextreme F2 mice were handled as missing data to avoid an overestimation of the phenotypic effects due to a biased selection of the progeny.[39] Individual nonheme hepatic iron values were log-transformed before genetic analysis, and multipoint analysis was performed by using recessive, dominant, additive, and free models of inheritance. LOD scores were calculated at 2-cM intervals throughout the genome. A LOD score ≥2.8 was the threshold for suggestive evidence of linkage, and a LOD score ≥4.3 was interpreted as significant evidence of linkage.[40] Epistatic interactions between the 4 loci achieving LOD scores indicative of significant linkage to the level of hepatic iron loading were evaluated with a 6-*df* likelihood ratio test comparing logistic regression models with and without interaction terms.

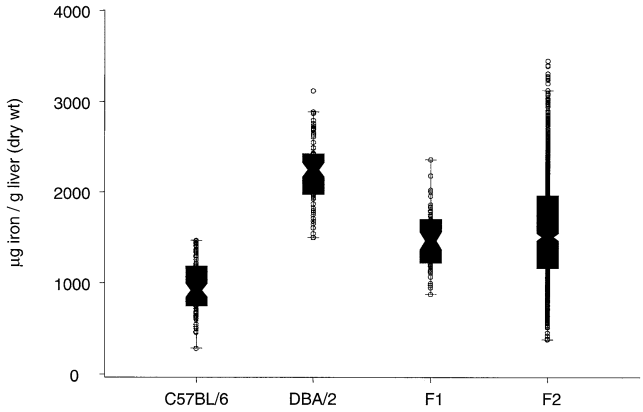

Figure 1. Box-and-whisker plots of nonheme iron concentrations in the liver of the *Hfe*-deficient parental strains—C57BL/6 (n = 81) and DBA/2 (n = 78)—and their F1 (n = 56) and F2 (n = 1028) progenies. Notches indicate the median for each group of mice. The lower and upper limits of the boxes represent the 25th and the 75th percentiles, respectively. Mice with iron concentrations below the 25th percentile or above the 75th percentile are displayed individually. Whisker lines extend from below and above the box limits to the minimum observation above the lower fence (1.5 times the interquartile range below the 25th percentile) and the maximum observation below the upper fence (1.5 times the interquartile range above the 75th percentile), respectively. Outliers are observations above the upper fence.

Results

Hepatic Iron Concentration in the Parental Strains and in the F1 and F2 Progenies

To investigate the genetic nature of susceptibility to iron loading in $Hfe^{-/-}$ mice, the liver iron content of 1028 F2 progeny was analyzed when mice were 7 weeks old. The results were compared with results from 81 C57BL/6 $Hfe^{-/-}$, 78 DBA/2 $Hfe^{-/-}$, and 56 F1 $Hfe^{-/-}$ mice. As previously reported,[34] iron concentrations of livers from C57BL/6 $Hfe^{-/-}$ mice (962 ± 292 μg of iron per gram of dry weight) were significantly lower than those from DBA/2 $Hfe^{-/-}$ mice (2207 ± 362 μg of iron per gram of dry weight; $P < 0.0001$), thus confirming that the severity of iron loading in *Hfe*-deficient mice is influenced by the strain background. Results in Figure 1 show that the 56 F1 mice had intermediate levels of hepatic iron loading (1480 ± 321 μg of iron per gram of dry weight) compared with parental mice. The relative level of susceptibility observed in parental strain mice was thus inherited by their progeny as an additive trait. The 1028 F2 mice had levels of iron loading that were also intermediate between the parental strains (1591 ± 581 μg of iron per gram of dry weight), but with a larger

Table 1. Description of the Modifier Loci Affecting the Severity of Hepatic Iron Loading Detected by Linkage Analysis

Chromosome	QTL position (marker + cM)[a]	Maximum LOD scores under specific model				Phenotypic effect of DBA/2 alleles	Variance explained (%)
		Free	Dominant	Recessive	Additive		
1	D1Mit231 + 5.4	3.05[b]	1.29	2.89	2.74	Decrease	2.2
1	D1Mit206 + 3.8	2.95[b]	0.23	2.48	1.29	Increase	2.1
3	D3Mit32	2.84[b]	2.27	1.91	2.82	Increase	2.3
7 (*Hfe*-modifier 1)	D7Mit246 + 1.1	14.47[c]	9.55	10.11	14.46	Decrease	9.6
8 (*Hfe*-modifier 2)	D8Mit211 + 0.1	12.96[c]	10.52	6.60	12.63	Increase	8.3
11 (*Hfe*-modifier 3)	D11Mit86 + 1.5	6.04[c]	3.51	5.40	5.85	Increase	5.3
12 (*Hfe*-modifier 4)	D12Mit158 + 1.1	6.72[c]	5.91	2.95	6.32	Increase	4.6

[a]Distance in cM from the specified marker.
[b]Suggestive evidence of linkage.
[c]Significative evidence of linkage.

variance than the F1 mice. The proportion of the total phenotypic variance within the F2 progeny due to genetic effects amounts to 62%, indicating that susceptibility to iron overload in the *Hfe*$^{-/-}$ mouse model of HH is heritable and can be explained by polymorphic autosomal loci. To determine whether sex influences iron loading in *Hfe*-deficient mice, we compared hepatic iron concentrations in males and females of the parental strains and of the F1 and F2 progenies. Female mice had slightly higher hepatic iron concentrations than males of the same strains/progenies, but the difference was significant only for the C57BL/6 strain ($P = 0.012$). Nevertheless, in addition to the genetic markers, we added sex as a covariate in the models used in MAPMAKER/QTL to predict hepatic iron loading.

Linkage Analysis of Susceptibility to Hepatic Iron Loading in the F2 Progeny

To increase the power of mapping modifier loci that regulate hepatic iron loading, we selectively genotyped only the extreme progeny, i.e., the subset of 276 F2 animals with the highest (n = 137) or the lowest (n = 139) liver iron concentrations. We identified 7 regions—2 on chromosome 1 and 1 on each of chromosomes 3, 7, 8, 11, and 12—with LOD scores $\geq$2.8 (Table 1). Genomic segments on chromosomes 7, 8, 11, and 12 had LOD scores $>$4.3 (Figure 2), indicating significant linkage[40] of each of these chromosomal segments to the hepatic iron loading severity trait. These QTLs on chromosomes 7, 8, 11, and 12 were designated *Hfe*-modifier 1, 2, 3, and 4, respectively. Of interest, the F2 mice homozygous for the DBA/2 allele at *Hfe*-modifier 1 on chromosome 7 and for 1 of the suggested QTLs on chromosome 1 developed less severe hepatic iron overload than the F2 animals homozygous for the C57BL/6 allele (Table 1). The fact that 1 parental strain shows larger phenotypic values than the other does not guarantee that all QTLs segregating in that cross increase the phenotypic value in that strain.[41] In fact, there are many examples of QTLs decreasing the phenotypic value,[42–44] and it is therefore not surprising to detect QTLs derived from the DBA/2 strain that decrease hepatic iron loading severity. As shown in Table 1, the QTLs on chromosomes 7 and 8 seem to be inherited in an additive fashion, whereas the QTL on chromosome 11 may be inherited in either a recessive or an additive manner and the QTL on chromosome 12 may be inherited in either a dominant or an additive manner. Of the weaker linkages on chromosome 1, 1 is probably inherited in a recessive manner, whereas no distinction between recessive and additive modes of inheritance can be made for the other. Together, these loci explain 34.4% of the phenotypic variation and roughly 55% of the genetic variation in hepatic iron loading among the F2 generation. The relationship among the 4 loci with significant evidence for linkage was assessed with logistic regression analysis by using the nearest markers to the LOD score peak in each of the identified genomic intervals as explanatory variables and by treating hepatic iron loading as a binary response variable (high vs. low). No evidence for interactions among the 4 loci was found ($\chi^2_6 = 9.90$ for testing all 2-loci interactions; $P = 0.13$), strongly suggesting that the 4 loci act additively, without epistasis.

Discussion

In this study, we used interval mapping to localize modifier genes that modulate hepatic iron loading in *Hfe*-deficient mice. We showed that susceptibility to hepatic iron loading in these mice has a significant genetic component and is inherited as a complex trait to which several genetic determinants, together with environmental factors, contribute. We identified 4 genomic intervals on chromosomes 7, 8, 11, and 12 that are

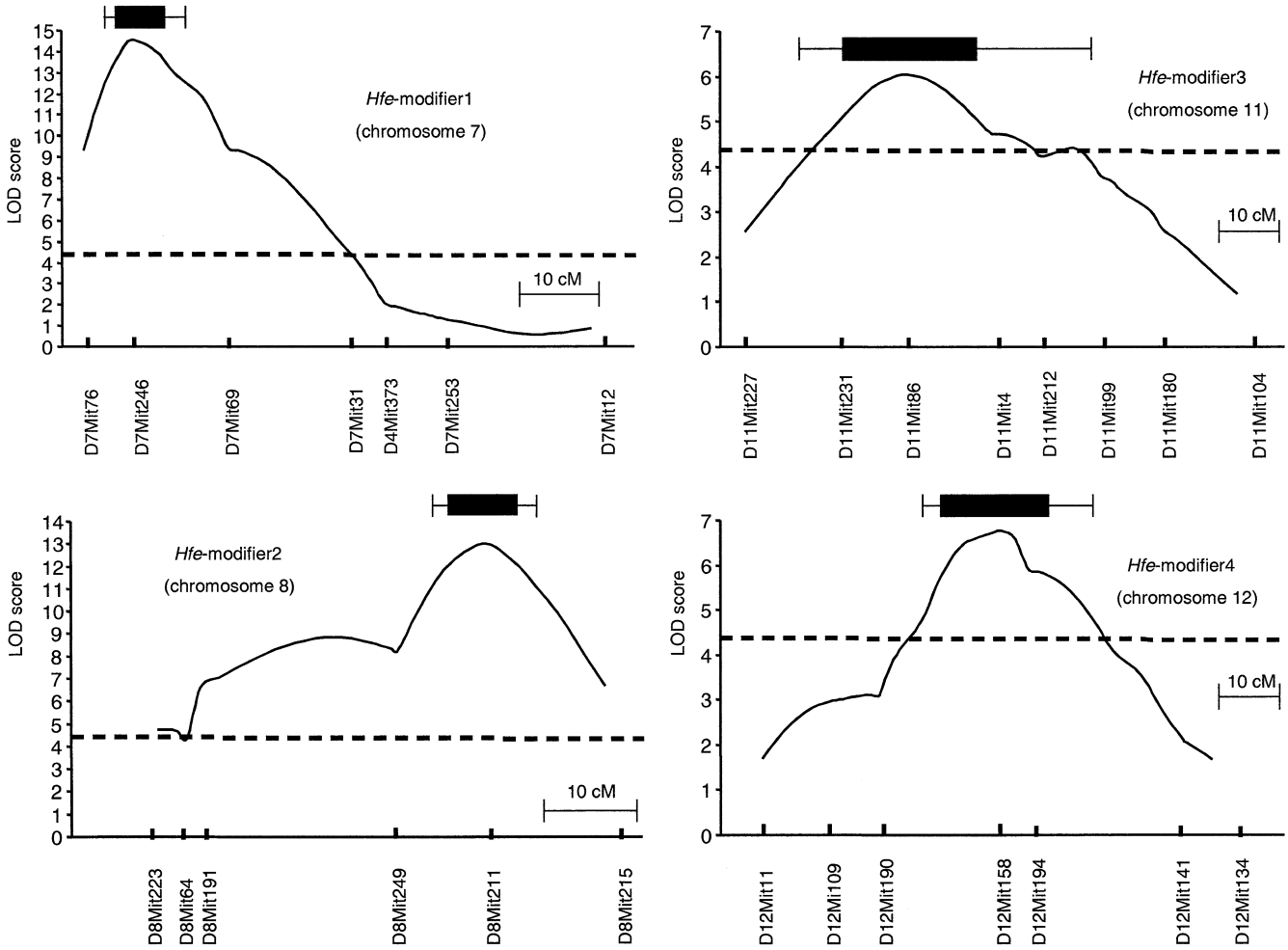

Figure 2. LOD scores for the quantitative traits that modulate hepatic iron loading in *Hfe*-deficient mice. The chromosomes harboring the 4 QTLs with LOD scores exceeding the threshold value of 4.3 (dotted line) required for significant linkage are displayed. The black bars indicate the 1-LOD support intervals for the position of the QTLs: outside these regions, the odds ratio decreased by a factor of 10. The thin lines extending from the black bars indicate the 2-LOD support intervals: outside these regions, the odds ratio decreased by a factor of 100. Data were analyzed with the MAPMAKER/QTL computer package.[39]

strongly linked to severe iron loading, and we showed that each acts in an independent, additive fashion. In addition, 3 genome segments—2 on chromosome 1 and 1 on chromosome 3—met the suggestive level of significance for linkage. However, although these different regions of the mouse genome show cosegregation with hepatic iron concentration, none of these loci is necessary or sufficient for the development of severe iron loading. Rather, the frequency of severe iron loading increases as a function of the number of susceptibility alleles at *Hfe*-modifier loci present in individual progeny. This is illustrated in Figure 3, in which the liver iron concentration of the 276 F2 mice genotyped for this study is presented as a function of the genotypes of these mice at the markers nearest to the LOD score peaks on chromosomes 7 and 8. This mode of inheritance, consistent with the predictions of a threshold model, is typical of a polygenic trait.

Genes involved in iron homeostasis and located within the identified intervals are good candidates for modulation of hepatic iron loading in *Hfe*-deficient mice. The characterization of polymorphisms within the coding and regulatory regions of these genes between parental C57BL/6 and DBA/2 strains and the analysis of their segregation together with hepatic iron loading in the F2 progeny will be an essential step toward the demonstration of their implication as modifier genes in this murine model. It is noteworthy that the *Hfe*-modifier 1 support interval on chromosome 7 contains several genes that have recently been shown to exert important roles in the regulation of iron metabolism. The best candidates are the 2 highly related mouse hepcidin genes, *Hamp1* and *Hamp2*. Hepcidin is a peptide hormone synthesized predominantly in the liver and whose expression is induced by dietary iron loading.[45–47] *Hepcidin*-deficient mice accumulate iron in parenchymal cells because of greater

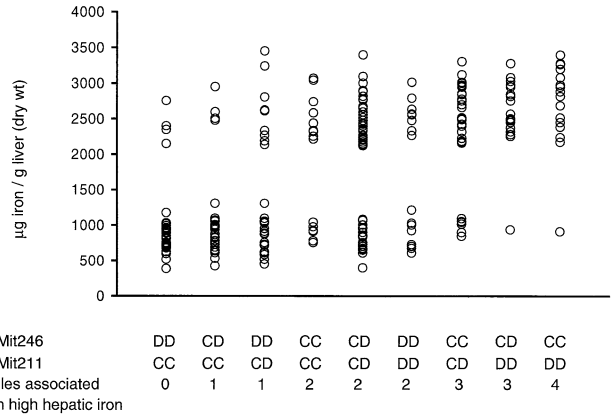

Figure 3. Relative contribution of *Hfe*-modifier 1 and *Hfe*-modifier 2 to the nonheme iron concentration in the liver of the 276 F2 progeny genotyped for this study. To visualize the contributions of the 2 loci in chromosomes 7 and 8 to hepatic iron loading, mice of the F2 progeny were separated according to their genotypes at markers D7Mit246 and D8Mit211. C, alleles of C57BL/6 origin; D, alleles of DBA/2 origin. Each dot represents 1 mouse. None of these loci is necessary or sufficient for the development of severe iron loading. However, the frequency of severe iron loading increases as a function of the number of susceptibility alleles in individual mice.

intestinal absorption and impaired retention of iron by reticuloendothelial macrophage cells.[48] Recently, humans with severe HH have been shown to have null mutations in the gene encoding hepcidin.[49] In contrast, transgenic mice overexpressing hepcidin have markedly lower iron stores, resulting in severe anemia.[50] Thus, hepcidin seems to be a negative regulator of iron release from both reticuloendothelial macrophages and from enterocytes that mediate intestinal absorption of dietary iron. Of further interest, a lower hepcidin expression has recently been observed in *Hfe*$^{-/-}$ mice[51,52] and in individuals with HFE-associated HH.[53] Genetic variability in the expression of hepcidin could thus contribute to the wide range in phenotypic expression observed in *Hfe*-deficient mice of different genetic backgrounds and in individuals who are homozygous for the C282Y allele in the *HFE* gene. The recent report of 2 families in which there was concordance between the severity of iron overload and heterozygosity for mutations in the hepcidin gene when present with the *HFE* C282Y mutation[54] prompted us to sequence the 2 hepcidin genes in the C57BL/6 and DBA/2 strains. By comparison with the C57BL/6 strain, 3 amino acid variants were found in the *Hamp1* gene of the DBA/2 strain (H27Q, E43G, and N73K), and 1 variant was found in the *Hamp2* gene (S76F). Amino acids 73 and 76 are located between the fifth and the sixth cysteines in the active peptide comprising the 25–amino acid C-terminal portion of the molecule. The N73K variant changes the charge of residue 73 from neutral to basic, and the S76F variant substitutes an aromatic hydrophobic residue for a small polar amino acid. Whether these changes are functionally significant remains to be established. C/EBPα has recently been implicated in hepcidin gene regulation in response to iron status.[55] Iron overload indeed leads to a significant increase of C/EBPα protein and hepcidin transcripts in mouse liver. It is interesting to note that the gene encoding C/EBPα maps to the *Hfe*-modifier 1 region on chromosome 7 and is therefore another candidate modifier gene in this region.

The region encompassing *Hfe*-modifier 2 on chromosome 8 also contains potential candidate genes. Among them is heme oxygenase 1 (Hmox1) which is an important enzyme in the salvage of iron from heme.[56] *Hmox1*-deficient mouse fibroblasts accumulate iron by increasing iron uptake and decreasing iron release,[57] and it was recently shown that a relative deficiency in Hmox1 could have a role in hepatic iron accumulation in *Hfe*-deficient mice.[52] Calreticulin is also encoded within this interval. Expression of calreticulin was recently shown to be diminished in *Hfe*-deficient mice.[52] It is interesting to note that calreticulin interacts with C/EBPα messenger RNA and represses the translation of C/EBP proteins.[58] Because C/EBPα stimulates hepcidin transcription,[55] genetic variability in the expression of C/EBPα could also contribute to the modulation of phenotypic expression observed in *Hfe*-deficient mice. Other candidate genes within the *Hfe*-modifier 2 support interval encode metallothioneins 1 and 2, which are cysteine-rich metal-binding proteins that exert cytoprotective effects against metal toxicity.[59] The last candidate gene encodes haptoglobin, whose best-known biological function is to capture free hemoglobin in plasma to allow hepatic recycling of heme iron. A common duplication polymorphism in the haptoglobin gene was shown to affect iron metabolism in humans, and carriers of the haptoglobin 2-2 genotype have higher serum iron, transferrin saturation, and ferritin than other subjects.[60] It is interesting to note that individuals carrying this genotype are overrepresented among C282Y-homozygous patients and have more pronounced iron overload.[61] Genetic variability in the haptoglobin gene could therefore also contribute to the wide range in phenotypic expression observed in *Hfe*-deficient mice of different genetic backgrounds.

The region around D1Mit231 on chromosome 1, which met the threshold for suggestive evidence of linkage, contains the *Slc39a1* gene, which encodes the mouse homologue of human ferroportin-1, in which several mutations/deletions have been shown to be responsible for an autosomal dominant inheritance of increased body iron stores characterized by increased serum ferritin con-

centration and normal transferrin saturation.[62–65] The *Slc39a1* gene can thus be considered a candidate gene on mouse chromosome 1.

No obvious candidate genes were identified in the telomeric region of chromosome 1 or on chromosomes 3, 11, or 12. It is possible, however, that genes not yet known to play a role in iron homeostasis modulate disease expression. It is noteworthy that the region encompassing *Hfe*-modifier 3 on chromosome 11 contains the gene that encodes a novel antimicrobial peptide expressed in the liver, LEAP-2, which has some similarities with LEAP-1/hepcidin.[66] Its physiological role must be unraveled in the near future, together with its possible implication in the regulation of iron homeostasis. Furthermore, the telomeric region of chromosome 1, which met the threshold for suggestive evidence of linkage, is homologous to human chromosome 1q21, to which the gene that causes juvenile HH maps.[67–69] It is possible that mutations of the same gene may help to explain why only a few *Hfe*-deficient mice develop severe iron-storage disease. Alternative strategies based on microarray analysis of target organ gene expression and genotyping of single nucleotide polymorphisms must be implemented to identify the *Hfe* modifiers that account for the variable disease expression in these regions. Their discovery is likely to shed new light on the mechanisms that control total body iron content.

In conclusion, this study is the first reported genome-wide linkage scan for *Hfe*-modifier genes in the mouse. Our data provide a clear demonstration of the polygenic pattern of hepatic iron loading inheritance in *Hfe*-deficient mice. Finding the genetic modifiers that are required in addition to *Hfe* invalidation to produce significant iron-storage disease will be an important step forward and is likely to provide substantial insight into the pathogenic mechanisms that lead to iron overload. In humans, the *HFE* C282Y mutation is not necessarily accompanied by an increase in the concentration of serum ferritin,[11–16] and family studies indicate that genetic modifiers explain, at least in part, this reduced penetrance. Although the $Hfe^{-/-}$ mouse model does not entirely reflect HH, especially because $Hfe^{-/-}$ mice do not develop hypogonadism, hypopituitarism, diabetes mellitus, cardiomyopathy, joint disease, or frank cirrhosis, they have excessive iron accumulation in the liver. The severity varies according to the strain. Because serum ferritin levels in humans closely mirror hepatic iron stores, at least some of the genetic modifiers responsible for variable disease expression in humans may be either direct homologues of loci identified in the mouse or genes encoding other proteins involved in the same biological pathways.[22] Their identification should be facilitated by this preliminary study and will provide us with further insights into the mechanisms by which organisms modulate iron homeostasis to accommodate the adverse effects of the *HFE* C282Y mutation.

References

1. Bothwell TH, MacPhail AP. Hereditary hemochromatosis: etiologic, pathologic, and clinical aspects. Semin Hematol 1998;35:55–71.
2. Feder JN, Gnirke A, Thomas W, Tsuchihashi Z, Ruddy DA, Basava A, Dormishian F, Domingo R Jr, Ellis MC, Fullan A, Hinton LM, Jones NL, Kimmel BE, Kronmal GS, Lauer P, Lee VK, Loeb DB, Mapa FA, McClelland E, Meyer NC, Mintier GA, Moeller N, Moore T, Morikang E, Prass CE, Quintana L, Starnes SM, Schatzman RC, Brunke KJ, Drayna DT, Risch NJ, Bacon BR, Wolff RK. A novel MHC class I-like gene is mutated in patients with hereditary haemochromatosis. Nat Genet 1996;13:399–408.
3. Rhodes DA, Raha-Chowdhury R, Cox TM, Trowsdale J. Homozygosity for the predominant Cys282Tyr mutation and absence of disease expression in hereditary haemochromatosis. J Med Genet 1997;34:761–764.
4. Adams PC, Chakrabarti S. Genotypic/phenotypic correlations in genetic hemochromatosis: evolution of diagnostic criteria. Gastroenterology 1998;114:319–323.
5. Mura C, Le Gac G, Scotet V, Raguenes O, Mercier AY, Ferec C. Variation of iron loading expression in C282Y homozygous haemochromatosis probands and sib pairs. J Med Genet 2001;38:632–636.
6. Sachot S, Moirand R, Jouanolle AM, Mosser J, Fergelot P, Deugnier Y, Brissot P, le Gall JY, David V. Low penetrant hemochromatosis phenotype in eight families: no evidence of modifiers in the MHC region. Blood Cells Mol Dis 2001;27:518–529.
7. Willis G, Wimperis JZ, Smith KC, Fellows IW, Jennings BA. Haemochromatosis gene C282Y homozygotes in an elderly male population. Lancet 1999;354:221–222.
8. Coppin H, Bensaid M, Fruchon S, Borot N, Blanche H, Roth MP. Longevity and carrying the C282Y mutation for haemochromatosis on the HFE gene: case control study of 492 French centenarians. BMJ 2003;327:132–133.
9. Merryweather-Clarke AT, Worwood M, Parkinson L, Mattock C, Pointon JJ, Shearman JD, Robson KJ. The effect of HFE mutations on serum ferritin and transferrin saturation in the Jersey population. Br J Haematol 1998;101:369–373.
10. McCune CA, Al-Jader LN, May A, Hayes SL, Jackson HA, Worwood M. Hereditary haemochromatosis: only 1% of adult HFEC282Y homozygotes in South Wales have a clinical diagnosis of iron overload. Hum Genet 2002;111:538–543.
11. Burt MJ, George PM, Upton JD, Collett JA, Frampton CM, Chapman TM, Walmsley TA, Chapman BA. The significance of haemochromatosis gene mutations in the general population: implications for screening. Gut 1998;43:830–836.
12. McDonnell SM, Hover A, Gloe D, Ou CY, Cogswell ME, Grummer-Strawn L. Population-based screening for hemochromatosis using phenotypic and DNA testing among employees of health maintenance organizations in Springfield, Missouri. Am J Med 1999;107:30–37.
13. Olynyk JK, Cullen DJ, Aquilia S, Rossi E, Summerville L, Powell LW. A population-based study of the clinical expression of the hemochromatosis gene. N Engl J Med 1999;341:718–724.
14. Adams PC, Kertesz AE, McLaren CE, Barr R, Bamford A, Chakrabarti S. Population screening for hemochromatosis: a comparison of unbound iron-binding capacity, transferrin saturation, and C282Y genotyping in 5,211 voluntary blood donors. Hepatology 2000;31:1160–1164.

15. Jackson HA, Carter K, Darke C, Guttridge MG, Ravine D, Hutton RD, Napier JA, Worwood M. HFE mutations, iron deficiency and overload in 10,500 blood donors. Br J Haematol 2001;114:474–484.
16. Deugnier Y, Jouanolle AM, Chaperon J, Moirand R, Pithois C, Meyer JF, Pouchard M, Lafraise B, Brigand A, Caserio-Schoenemann C, Mosser J, Adams P, Le Gall JY, David V. Gender-specific phenotypic expression and screening strategies in C282Y-linked haemochromatosis: a study of 9396 French people. Br J Haematol 2002;118:1170–1178.
17. Beutler E, Felitti VJ, Koziol JA, Ho NJ, Gelbart T. Penetrance of 845G → A (C282Y) HFE hereditary haemochromatosis mutation in the USA. Lancet 2002;359:211–218.
18. Poullis A, Moodie SJ, Maxwell JD. Clinical haemochromatosis in HFE mutation carriers. Lancet 2002;360:411–412.
19. Cox T, Rochette J, Camaschella C, Walker A, Robson K. Clinical haemochromatosis in HFE mutation carriers. Lancet 2002;360:412.
20. Allen KJ, Warner B, Delatycki MB. Clinical haemochromatosis in HFE mutation carriers. Lancet 2002;360:412–413.
21. Bomford A. Genetics of haemochromatosis. Lancet 2002;360:1673–1681.
22. Nadeau JH. Modifier genes in mice and humans. Nat Rev Genet 2001;2:165–174.
23. Bulaj ZJ, Ajioka RS, Phillips JD, LaSalle BA, Jorde LB, Griffen LM, Edwards CQ, Kushner JP. Disease-related conditions in relatives of patients with hemochromatosis. N Engl J Med 2000;343:1529–1535.
24. Moirand R, Jouanolle AM, Brissot P, Le Gall JY, David V, Deugnier Y. Phenotypic expression of HFE mutations: a French study of 1110 unrelated iron-overloaded patients and relatives. Gastroenterology 1999;116:372–377.
25. Ryan E, Byrnes V, Coughlan B, Flanagan AM, Barrett S, O'Keane JC, Crowe J. Underdiagnosis of hereditary haemochromatosis: lack of presentation or penetration? Gut 2002;51:108–112.
26. Whiting PW, Fletcher LM, Dixon JK, Gochee P, Powell LW, Crawford DH. Concordance of iron indices in homozygote and heterozygote sibling pairs in hemochromatosis families: implications for family screening. J Hepatol 2002;37:309–314.
27. Lee P, Gelbart T, West C, Halloran C, Beutler E. Seeking candidate mutations that affect iron homeostasis. Blood Cells Mol Dis 2002;29:471–487.
28. de Sousa M, Reimao R, Lacerda R, Hugo P, Kaufmann SH, Porto G. Iron overload in beta 2-microglobulin-deficient mice. Immunol Lett 1994;39:105–111.
29. Zhou XY, Tomatsu S, Fleming RE, Parkkila S, Waheed A, Jiang J, Fei Y, Brunt EM, Ruddy DA, Prass CE, Schatzman RC, O'Neill R, Britton RS, Bacon BR, Sly WS. HFE gene knockout produces mouse model of hereditary hemochromatosis. Proc Natl Acad Sci U S A 1998;95:2492–2497.
30. Bahram S, Gilfillan S, Kuhn LC, Moret R, Schulze JB, Lebeau A, Schumann K. Experimental hemochromatosis due to MHC class I HFE deficiency: immune status and iron metabolism. Proc Natl Acad Sci U S A 1999;96:13312–13317.
31. Levy JE, Montross LK, Cohen DE, Fleming MD, Andrews NC. The C282Y mutation causing hereditary hemochromatosis does not produce a null allele. Blood 1999;94:9–11.
32. Trinder D, Olynyk JK, Sly WS, Morgan EH. Iron uptake from plasma transferrin by the duodenum is impaired in the Hfe knockout mouse. Proc Natl Acad Sci U S A 2002;99:5622–5626.
33. Fleming RE, Migas MC, Zhou X, Jiang J, Britton RS, Brunt EM, Tomatsu S, Waheed A, Bacon BR, Sly WS. Mechanism of increased iron absorption in murine model of hereditary hemochromatosis: increased duodenal expression of the iron transporter DMT1. Proc Natl Acad Sci U S A 1999;96:3143–3148.
34. Dupic F, Fruchon S, Bensaid M, Borot N, Radosavljevic O, Loreal O, Brissot P, Gilfillan S, Bahram S, Coppin H, Roth MP. Inactivation of the hemochromatosis gene differentially regulates duodenal expression of iron-related mRNAs between mouse strains. Gastroenterology 2002;122:745–751.
35. Sproule TJ, Jazwinska EC, Britton RS, Bacon BR, Fleming RE, Sly WS, Roopenian DC. Naturally variant autosomal and sex-linked loci determine the severity of iron overload in beta 2-microglobulin-deficient mice. Proc Natl Acad Sci U S A 2001;98:5170–5174.
36. Fleming RE, Holden CC, Tomatsu S, Waheed A, Brunt EM, Britton RS, Bacon BR, Roopenian DC, Sly WS. Mouse strain differences determine severity of iron accumulation in Hfe knockout model of hereditary hemochromatosis. Proc Natl Acad Sci U S A 2001;98:2707–2711.
37. Dietrich WF, Miller J, Steen R, Merchant MA, Damron-Boles D, Husain Z, Dredge R, Daly MJ, Ingalls KA, O'Connor TJ, Evans CA, DeAngelis MM, Levinson DM, Kruglyak L, Goodman N, Copeland NG, Jenkins NA, Hawkins TL, Stein L, Page DC, Lander ES. A comprehensive genetic map of the mouse genome. Nature 1996;380:149–152.
38. Lander ES, Green P, Abrahamson J, Barlow A, Daly MJ, Lincoln SE, Newburg L. MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1987;1:174–181.
39. Lander ES, Botstein D. Mapping mendelian factors underlying quantitative traits using RFLP linkage maps. Genetics 1989;121:185–199.
40. Lander E, Kruglyak L. Genetic dissection of complex traits: guidelines for interpreting and reporting linkage results. Nat Genet 1995;11:241–247.
41. Kruglyak L, Lander ES. A nonparametric approach for mapping quantitative trait loci. Genetics 1995;139:1421–1428.
42. Remmers EF, Longman RE, Du Y, O'Hare A, Cannon GW, Griffiths MM, Wilder RL. A genome scan localizes five non-MHC loci controlling collagen-induced arthritis in rats. Nat Genet 1996;14:82–85.
43. Roth MP, Viratelle C, Dolbois L, Delverdier M, Borot N, Pelletier L, Druet P, Clanet M, Coppin H. A genome-wide search identifies two susceptibility loci for experimental autoimmune encephalomyelitis on rat chromosomes 4 and 10. J Immunol 1999;162:1917–1922.
44. Wright FA, O'Connor DT, Roberts E, Kutey G, Berry CC, Yoneda LU, Timberlake D, Schlager G. Genome scan for blood pressure loci in mice. Hypertension 1999;34:625–630.
45. Krause A, Neitz S, Magert HJ, Schulz A, Forssmann WG, Schulz-Knappe P, Adermann K. LEAP-1, a novel highly disulfide-bonded human peptide, exhibits antimicrobial activity. FEBS Lett 2000;480:147–150.
46. Pigeon C, Ilyin G, Courselaud B, Leroyer P, Turlin B, Brissot P, Loreal O. A new mouse liver-specific gene, encoding a protein homologous to human antimicrobial peptide hepcidin, is overexpressed during iron overload. J Biol Chem 2001;276:7811–7819.
47. Park CH, Valore EV, Waring AJ, Ganz T. Hepcidin, a urinary antimicrobial peptide synthesized in the liver. J Biol Chem 2001;276:7806–7810.
48. Nicolas G, Bennoun M, Devaux I, Beaumont C, Grandchamp B, Kahn A, Vaulont S. Lack of hepcidin gene expression and severe tissue iron overload in upstream stimulatory factor 2 (USF2) knockout mice. Proc Natl Acad Sci U S A 2001;98:8780–8785.
49. Roetto A, Papanikolaou G, Politou M, Alberti F, Girelli D, Christakis J, Loukopoulos D, Camaschella C. Mutant antimicrobial peptide hepcidin is associated with severe juvenile hemochromatosis. Nat Genet 2003;33:21–22.
50. Nicolas G, Bennoun M, Porteu A, Mativet S, Beaumont C, Grandchamp B, Sirito M, Sawadogo M, Kahn A, Vaulont S. Severe iron deficiency anemia in transgenic mice expressing liver hepcidin. Proc Natl Acad Sci U S A 2002;99:4596–4601.

51. Ahmad KA, Ahmann JR, Migas MC, Waheed A, Britton RS, Bacon BR, Sly WS, Fleming RE. Decreased liver hepcidin expression in the hfe knockout mouse. Blood Cells Mol Dis 2002;29:361–366.
52. Muckenthaler M, Roy CN, Custodio AO, Minana B, DeGraaf J, Montross LK, Andrews NC, Hentze MW. Regulatory defects in liver and intestine implicate abnormal hepcidin and Cybrd1 expression in mouse hemochromatosis. Nat Genet 2003;34:102–107.
53. Bridle KR, Frazer DM, Wilkins SJ, Dixon JL, Purdie DM, Crawford DH, Subramaniam VN, Powell LW, Anderson GJ, Ram GA. Disrupted hepcidin regulation in HFE-associated haemochromatosis and the liver as a regulator of body iron homoeostasis. Lancet 2003;361:669–673.
54. Merryweather-Clarke AT, Cadet E, Bomford A, Capron D, Viprakasit V, Miller A, McHugh PJ, Chapman RW, Pointon JJ, Wimhurst VL, Livesey KJ, Tanphaichitr V, Rochette J, Robson KJ. Digenic inheritance of mutations in HAMP and HFE results in different types of haemochromatosis. Hum Mol Genet 2003;12:2241–2247.
55. Courselaud B, Pigeon C, Inoue Y, Inoue J, Gonzalez FJ, Leroyer P, Gilot D, Boudjema K, Guguen-Guillouzo C, Brissot P, Loreal O, Ilyin G. C/EBPalpha regulates hepatic transcription of hepcidin, an antimicrobial peptide and regulator of iron metabolism. Cross-talk between C/EBP pathway and iron metabolism. J Biol Chem 2002;277:41163–41170.
56. Yoshida T, Migita CT. Mechanism of heme degradation by heme oxygenase. J Inorg Biochem 2000;82:33–41.
57. Ferris CD, Jaffrey SR, Sawa A, Takahashi M, Brady SD, Barrow RK, Tysoe SA, Wolosker H, Baranano DE, Dore S, Poss KD, Snyder SH. Haem oxygenase-1 prevents cell death by regulating cellular iron. Nat Cell Biol 1999;1:152–157.
58. Timchenko LT, Iakova P, Welm AL, Cai ZJ, Timchenko NA. Calreticulin interacts with C/EBPalpha and C/EBPbeta mRNAs and represses translation of C/EBP proteins. Mol Cell Biol 2002;22:7242–7257.
59. Takeda K, Fujita H, Shibahara S. Differential control of the metal-mediated activation of the human heme oxygenase-1 and metallothionein IIA genes. Biochem Biophys Res Commun 1995;207:160–167.
60. Delanghe JR, Langlois MR. Haptoglobin polymorphism and body iron stores. Clin Chem Lab Med 2002;40:212–216.
61. Van Vlierberghe H, Langlois M, Delanghe J, Horsmans Y, Michielsen P, Henrion J, Cartuyvels R, Billiet J, De Vos M, Leroux-Roels G. Haptoglobin phenotype 2-2 overrepresentation in Cys282Tyr hemochromatotic patients. J Hepatol 2001;35:707–711.
62. Njajou OT, Vaessen N, Joosse M, Berghuis B, van Dongen JW, Breuning MH, Snijders PJ, Rutten WP, Sandkuijl LA, Oostra BA, van Duijn CM, Heutink P. A mutation in SLC11A3 is associated with autosomal dominant hemochromatosis. Nat Genet 2001;28:213–214.
63. Montosi G, Donovan A, Totaro A, Garuti C, Pignatti E, Cassanelli S, Trenor CC, Gasparini P, Andrews NC, Pietrangelo A. Autosomal-dominant hemochromatosis is associated with a mutation in the ferroportin (SLC11A3) gene. J Clin Invest 2001;108:619–623.
64. Roetto A, Merryweather-Clarke AT, Daraio F, Livesey K, Pointon JJ, Barbabietola G, Piga A, Mackie PH, Robson KJ, Camaschella C. A valine deletion of ferroportin 1: a common mutation in hemochromastosis type 4. Blood 2002;100:733–734.
65. Devalia V, Carter K, Walker AP, Perkins SJ, Worwood M, May A, Dooley JS. Autosomal dominant reticuloendothelial iron overload associated with a 3-base pair deletion in the ferroportin 1 gene (SLC11A3). Blood 2002;100:695–697.
66. Krause A, Sillard R, Kleemeier B, Kluver E, Maronde E, Conejo-Garcia JR, Forssmann WG, Schulz-Knappe P, Nehls MC, Wattler F, Wattler S, Adermann K. Isolation and biochemical characterization of LEAP-2, a novel blood peptide expressed in the liver. Protein Sci 2003;12:143–152.
67. Roetto A, Totaro A, Cazzola M, Cicilano M, Bosio S, D'Ascola G, Carella M, Zelante L, Kelly AL, Cox TM, Gasparini P, Camaschella C. Juvenile hemochromatosis locus maps to chromosome 1q. Am J Hum Genet 1999;64:1388–1393.
68. Roetto A, Alberti F, Daraio F, Cali A, Cazzola M, Totaro A, Gasparini P, Camaschella C. Exclusion of ZIRTL as candidate gene of juvenile hemochromatosis and refinement of the critical interval on 1q21. Blood Cells Mol Dis 2000;26:205–210.
69. Papanikolaou G, Politou M, Roetto A, Bosio S, Sakelaropoulos N, Camaschella C, Loukopoulos D. Linkage to chromosome 1q in Greek families with juvenile hemochromatosis. Blood Cells Mol Dis 2001;27:744–749.

Received July 30, 2003. Accepted January 15, 2004.

Address requests for reprints to: Marie-Paule Roth, M.D., INSERM U 563, CHU Purpan, F-31059 Toulouse Cedex 3, France. e-mail: roth@cict.fr; fax: (33) 5-61-49-90-36.

Supported by European Union (QLK6-CT-1999-02237 and QLG1-CT-1999-00665), Association pour la Recherche sur le Cancer (to M.B.), and Fondation pour la Recherche Médicale (to S.F.).

M.-P.R. and H.C. share senior authorship.

The authors thank Maryline Calise (Service de Zootechnie, IFR 30) for assistance in the mouse breedings, Michèle Stebenet (Service de Biochimie, Hôpital Purpan) for iron analyses, Claudie Offer and Hélène Brun for help with the sequencing of the hepcidin genes, Cécile Tonon for skilled advice on the Genopole CRGS platform, and Marie-Thérèse Ribouchon and Nicolas Borot for technical assistance.