Multiple Polymorphic Loci Determine Basal Hepatic and Splenic Iron Status in Mice

Gemma R. Grant,[1] Susan W. Robinson,[1] Richard E. Edwards,[1] Bruce Clothier,[1] Reginald Davies,[1] David J. Judah,[1] Karl W. Broman,[2] and Andrew G. Smith[1]

Polymorphisms of genes linked to iron metabolism may account for individual variability in hemochromatosis and iron status connected with liver and cardiovascular diseases, cancers, toxicity, and infection. Mouse strains exhibit marked differences in levels of non-heme iron, with C57BL/6J and SWR showing low and high levels, respectively. The genetic basis for this variability was examined using quantitative trait loci (QTL) analysis together with expression profiling and chromosomal positions of known iron-related genes. Non-heme iron levels in liver and spleen of C57BL/6J × SWR F_2 mice were poorly correlated, indicating independent regulation. Highly significant ($P < .01$) polymorphic loci were found on chromosomes 2 and 16 for liver and on chromosomes 8 and 9 for spleen. With sex as a covariate, additional significant or suggestive ($P < 0.1$) QTL were detected on chromosomes 7, 8, 11, and 19 for liver and on chromosome 2 for spleen. A gene array showed no clear association between most loci and differential iron-related gene expression. The gene for transferrin and a transferrin-like gene map close to the QTL on chromosome 9. Transferrin saturation was significantly lower in C57BL/6J mice than in SWR mice, but there was no significant difference in the serum level of transferrin, hepatic expression, or functional change in cDNA sequence. β2-Microglobulin, which, unlike other loci, was associated with C57BL/6J alleles, is a candidate for the chromosome 2 QTL for higher iron. In conclusion, the findings show the location of polymorphic genes that determine basal iron status in wild-type mice. Human equivalents may be pertinent in predisposition to hepatic and other disorders. (HEPATOLOGY 2006;44:174-185.)

Fundamental processes such as oxygen transport, respiration, cell cycle and signaling and metabolism of endogenous and exogenous chemicals are dependent on the redox potential of iron.[1] At the same time redox activity occurring in tissues such as liver, kidney, brain, and heart may have pathological consequences in circumstances of abnormal iron deposition, malfunctions of iron homeostasis, or oxidative stress.[2] Iron overload of hereditary hemochromatosis as a result of homozygosity for HFE mutations is associated with cirrhosis, diabetes, cardiomyopathy, arthritis, cancer, and sterility. However, penetrance of these mutations is low and probably depends on modifier genes.[1,3-5] More subtle differences of higher iron burden in populations have also been linked with liver and cardiovascular disease, diabetes, cancer, increased oxidative DNA damage, and neurodegenerative disorders.[6-11] In addition, the iron status of a host may be a major factor in infections,[12] and iron deficiencies associated with anemias are of wide concern.[13]

The absorption of iron and its subsequent use and turnover are highly regulated and likely to be influenced by individual variation.[1,3] Mice null for specific iron metabolism genes have greatly facilitated the understanding of iron homeostasis, but the severity of the hepatic phenotype of these genes may depend considerably on other genes that have yet to be identified.[14-16] In particular, the C57BL/6J mouse background confers resistance to hepatic iron loading.[14-17] However, the influence of mouse genes explored with null gene technology may not correspond to polymorphic susceptibility loci observed in non–genetically manipulated animals. Many important genetic traits in humans and experimentally, such as blood pressure and survival from infection, are recognized

Abbreviations: QTL, quantitative trait loci; EST, expressed sequence tag; RT-PCR, reverse-transcriptase PCR; LOD, log odds ratio.

From the [1]MRC Toxicology Unit, University of Leicester, Leicester, UK, and [2]Department of Biostatistics, Johns Hopkins University, Baltimore, MD.

Received November 8, 2005; accepted April 14, 2006.

Address reprint requests to: Dr. A.G. Smith, MRC Toxicology Unit, Hodgkin Building, University of Leicester, Lancaster Road, Leicester LE1 9HN, UK. E-mail: ags5@le.ac.uk; fax: (44) 116-252-5616.

Published online in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/hep.21233

Potential conflict of interest: Nothing to report.

to be complex and quantitative, with polymorphism of modifier genes having profound effects on the dominance of a primary gene defect.[18] So far most investigations of iron-overload diseases have focused on the locations of modifier genes for hepatic iron loading in *Hfe*-null mice.[17,19] Variation also occurs in the basal iron status of common inbred mouse strains.[20-27] C57BL strains show much lower iron than some others, with SWR mice having much higher levels of hepatic and splenic iron, which may explain their different responses to toxins, infection, and age-related changes.[22-24,28]

In this article, we show the location and contribution of polymorphic genes that determine basal iron status in apparently normal individuals using quantitative trait loci (QTL) analysis of an intercross between C57BL/6J and SWR mice.

Materials and Methods

Generation of F_2 Mice. C57BL/6J/Ola and SWR/Ola were obtained from Harlan UK. They were fed an RM1 diet (Special Diet Services, Witham, UK) for at least 2 weeks before use and maintained at 21°C with a 12-hour light/dark cycle. (C57BL/6 × SWR) × (C57BL/6 × SWR) and (SWR × C57BL/6) × (SWR × C57BL/6) F_2 mice were bred from F_1 mice with either C57BL/6J/Ola or SWR/Ola as paternal grandmothers. A total of 284 male and female F_2 mice were bred and culled at approximately 8 weeks of age. Livers and spleens were frozen at −80°C. Tissues from mice for histological and immunohistochemical analyses were fixed in buffered formalin.[29] Antimouse DCYTB11-A peptide antibody was from Alpha Diagnostic (San Antonio, TX).

Non-Heme Iron. Non-heme iron in liver and spleen was quantitated by the method of Torrance and Bothwell as previously reported[22,23] Levels are expressed as micrograms of Fe per gram of wet tissue. The non-heme iron level in the diet was 143 μg Fe/g. The manufacturer's estimate of total iron content was 159 μg Fe/g. The distribution of non-heme iron in tissues was shown by Perls' stain.

Analysis of Serum Transferrin. Blood was collected by cardiac puncture under terminal anesthesia (Home Office license 40/2571). Serum iron, total iron-binding capacity (TIBC), and transferrin saturation were determined with a kit (Diagnostic Chemicals Ltd., Canada). Analysis of serum transferrin by electrophoresis was performed on 10% SDS polyacrylamide gels followed by immunoblotting using a rabbit polyclonal anti–human transferrin antibody (Stressgen Biotechnologies Corp., Canada) and mouse transferrin as a control (US Biological, MA) with detection by a chemiluminescence system (Amersham Biosciences, Amersham, UK).

Genotyping. Genomic DNA was isolated from mouse liver by phenol-chloroform extraction. Individuals were genotyped using polymerase chain reaction (PCR) amplification of polymorphic microsatellite markers.[30] Positions of markers were based on data of the Whitehead Institute (WI; http://www.broad.mit.edu/cgi-bin/mouse/index/). In many cases these were <2 cM different from those of Mouse Genome Informatics (MGI; http://www.informatics.jax.org/). Where positions differed markedly, this was taken into account when considering candidate genes. Information about SWR allele sizes was obtained as previously described, or primers were selected and tested for polymorphisms[30] and can be obtained from the corresponding author. Pairs of fluorescently labeled primers were purchased from Applied Biosystems (Warrington, UK), and unlabeled primers were synthesized in the University of Leicester. Fluorescent PCR products were analyzed on an ABI 377 sequencer using GeneScan and Genotyper (Applied Biosystems) or were separated on 4% MetaPhor agarose (Cambrex) for unlabeled products. The extreme iron burden cohorts representing the most useful information for linkage analysis were genotyped for markers spread across the genome (2 or 3 per chromosome at 20-30 cM).[30] Where the evidence was strongest for a QTL or a chromosomal region was of particular interest, further markers were included (if available) and the remainder of the mice genotyped.

Mapping and Statistical Analysis. The R/qtl program (http://www.biostat.jhsph.edu/~kbroman/qtl) was used for QTL mapping.[31] As the distributions were skewed from normality, the phenotypes were analyzed as the $\log_{10}$ values. Untransformed data gave similar QTL results. Males and females were considered separately and also combined (in which case sex was included as an additive covariate). Genomewide thresholds of 10% and 1% significance were derived from 10,000 permutations of shuffled genotypes.[32] Significant differences between groups were assessed by the Student t test or by one-way analysis of variance and correlation coefficients calculated using the Statistica 6 package.

Gene Expression Profiling. The mouse cDNA microarray consisted of approximately 9,000 candidate EST clones obtained from Research Genetics (http://www.resgen.com) and the IMAGE collection and analyses performed as previously described.[29,33] mRNA was reverse-transcribed using aminoallyl dUTP. The resulting cDNA from C57BL/6 tissue was labeled with Alexa Fluor 555 Reactive Dye (Invitrogen, Paisley, UK) and cDNA from SWR mouse tissue labeled with Alexa Fluor 647. The two

Table 1. Primers Used for Real-Time RT PCR

Gene	Symbol	Primer
β-Actin	*Actb*	Forward: GATTACTGCTCTGGCTCCTAGCA
		Reverse: GTGGACAGTGAGGCCAGGAT
γ-Actin	*Actg*	Forward: CCCTAGCACCTAGCACGATGA
		Reverse: GCCACCGATCCA ACTGAGTAC
Heme oxygenase 1	*Hmox1*	Forward: CACTTCGTCAGAGGCCTGCTA
		Reverse: GTCTGGGATGAGCTAGTGCTGAT
Hepcidin	*Hamp*	Forward: TTGCGATACCAATGCAGAAGAG
		Reverse: ACTGGGAATTGTTACAGCATTTACAG
β2-Microglobulin	*B2m*	Forward: CATACGCCTGCAGAGTTAAGCA
		Reverse: GATCACATGTCTCGATCCCAGTAG
Transferrin*	*Trf*	Forward: TGAGGACTGCATTGAAAAGATTGT
		Reverse: GTAGGCATGTCCTCCATCCAA
Transferrin†		Forward: ACAACAAGGTATCTTTCCTAAAGGGT
		Reverse: GGTTGTTCCAGGTGATGCTAGTG
Transferrin-like‡	*Tflg*§	Forward: GGTCCCTCGAAAGATAGACATCA
		Reverse: GGGAGTCTTCCAGACCTCTTTTAA

*Primers span exons 9 and 10.
†Primers span exons 11 and 12.
‡*Tflg* primers were designed to a unique region to avoid detection of *Trf*.
§Preliminary nomination.

were then mixed and hybridized against the ESTs on the cDNA arrays. The procedure was repeated by reversing the dye labeling. Data from the arrays were normalized and averaged for each pair[34] and can be found at ArrayExpress (E-MEXP-486; www.ebi.ac.uk/arrayexpress/).

Real-Time RT-PCR. Reverse-transcriptase PCR (RT-PCR) was performed with 400 ng of mRNA, Superscript III Reverse Transcriptase (Invitrogen), and random hexamers (Amersham Biosciences). Primer Express software (Applied Biosystems) was used to design real-time RT-PCR primers (Table 1). Reactions were performed in triplicate using SYBR green PCR Master Mix (Applied Biosystems) on an ABI 7000 sequence detection system under standard conditions.[35] Results were normalized to reference genes β- or γ-actin using the comparative C_T method.

Sequencing of cDNA. Using the sequence for C57BL/6J (accession number AK085754), primers for the amplification of transferrin cDNA were forward 5′-CTGTACTCCCCGCTCCTCGC-3′ positioned 50 bp 5′ to the translation start site, and reverse 5′-GAGCATCTGTCTCCACCACAG-3′ approximately 40 bp 3′ of the stop codon. Amplicons were made by pooling eight PCRs for each mouse with three mice per strain. Sequencing reactions and analyses were performed using the above primers and a Big Dye terminator v3.1 cycle sequencing kit (Applied Biosystems). New primers were designed to extend the sequence to the center of the amplicons (not shown). The data were aligned and edited using BioEdit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). A similar strategy was used for the transferrin-like gene (1300017J02Rik). Primers used to make the PCR amplicon were forward 5′-GAGAGCAAGCAGAACGCAGT-3′ and reverse 5′-ACATGGAGGCAAGCAGTTG-3′.

Table 2. Phenotypes in C57BL/6J and SWR Mice and Their F_2 Crosses

Strain	N	Sex	Liver Iron (μg/g)	Spleen iron (μg/g)
C57BL/6J	5	Male	40 ± 5†	100 ± 9†
	5	Female	57 ± 8	157 ± 75
SWR	5	Male	234 ± 40‡	337 ± 128‡
	5	Female	254 ± 15‡	342 ± 65‡
C57BL/6 × SWR F_1	10	Male	53 ± 14†	102 ± 25†
	13	Female	84 ± 16	192 ± 75
C57BL/6 × SWR F_2*	145	Male	78 ± 34†	317 ± 172†
	139	Female	112 ± 35	427 ± 201

Values are means ± SDs from mice 6-8 weeks of age.
*Consisted of both (C57BL/6 × SWR) × (C57BL/6 × SWR) F_2 and (SWR × C57BL/6) × (SWR × C57BL/6) F_2 progeny.
†Significantly less than females ($P < .05$, two-tailed Student *t* test).
‡SWR significantly greater than the C57BL/6J strain ($P < .05$). The C57BL/6J and SWR mice shown are not the parents of the F_2 generation reported.

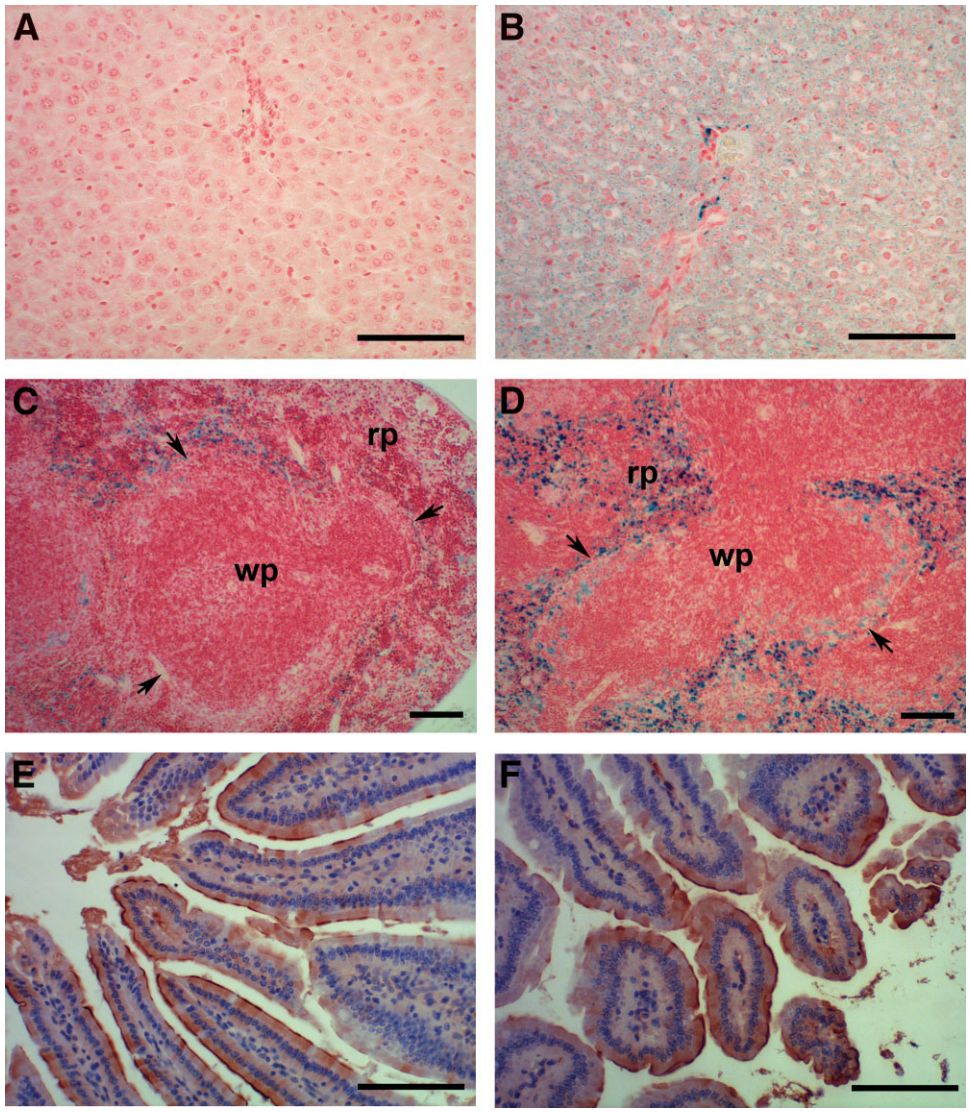

Fig. 1. Iron staining and immunohistochemistry of tissue from 12-week-old female C57BL/6J and SWR mice. (A) and (B) Hepatic non-heme iron by Perls' stain in C57BL6/J and SWR tissue, respectively. All hepatocytes of SWR tissue showed blue staining, whereas little was seen in C57BL/6J tissue. There was no consistent lobular distribution of iron in SWR tissue. In some sections, iron staining of Kupffer cells was also observed. Original magnification: 200×. (C) and (D) Splenic iron by Perls' stain in C57BL/6J and SWR tissue, respectively (**wp,** white pulp; **rp,** red pulp; arrows show marginal zone). Original magnification ×100. (E) and (F) Immunohistochemistry of duodenum for ferric reductase (DCYTB) in C57BL6/J and SWR tissue, respectively. Sections with immunoglobulin G as control showed no brown apical staining. Original magnification ×200. Bar size is 100 μ.

Results

Distribution of Iron in Parental Strains. Non-heme iron in liver and spleen of C57BL mice is significantly lower than that in SWR mice (Table 2).[22-24,36] Figure 1 illustrates the distribution of non-heme iron by Perls' stain in the liver and spleen of fully adult mice. Whereas there was little staining of hepatocytes and only positive-staining of occasional Kupffer cells in C57BL/6J mouse liver, there was pronounced staining in SWR mouse liver, mainly of hepatocytes but also of some Kupffer cells (Fig. 1A-B). Iron in the spleen was particularly pronounced in the SWR mice, mainly in the macrophages of the red pulp (Fig. 1C-D). Perls' staining in the SWR mice was also observed in kidney proximal convoluted tubules at the apex of the columnar epithelial cells and their brush border lining the tubule. It was far less intense in kidneys from C57BL/6J mice. Although expression was variable, no consistent strain difference for duodenal ferric reductase (DCYTB; *Cybrd1* gene) was observed by immunohistochemistry (Fig. 1E-F).

Non-Heme Iron Levels in Genetic Crosses. To investigate the genetic basis of the strain variation in levels of liver and spleen iron, an F_2 intercross between the parent C57BL/6J and SWR strains was examined at 6-8 weeks (Table 2), when divergence in iron status was still developing.[23] In addition to a marked difference between the parental strains, there was a propensity for female C57BL/6J F_1 and F_2 mice to have greater iron contents than males. This was not detected at this age in the SWR parental animals. In other studies a greater distinction between the sexes was observed, perhaps reflecting contributions from age and diet. Figure 2A-B shows the distribution of iron levels for liver and spleen. The low correlation ($r < 0.3$) between liver and spleen iron (Fig. 2C) implies that genetic and environmental factors deter-

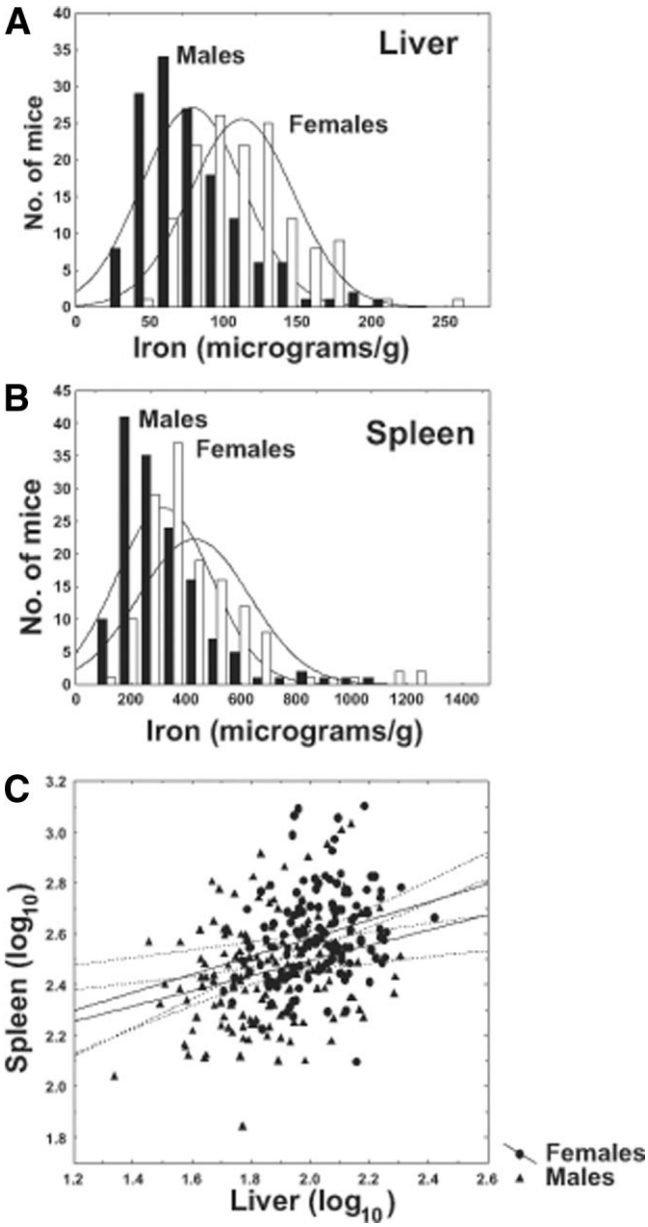

Fig. 2. Distribution plots of tissue non-heme iron levels in 284 6- to 8-week-old male and female C57BL/6J × SWR F_2 mice: (A) liver iron; (B) spleen iron (shading, males; no shading, females). (C) Correlation analysis (showing 95% confidence limits) of $\log_{10}$ iron levels in liver and spleen between males and females. Results of analyses by Pearson correlations and Bonferroni probabilities of difference were: females, $r = 0.270$, $P = .027$; males, $r = 0.259$, $P = .035$.

mining levels in the two tissues were largely, but not totally, independent.

Quantitative Trait Locus Analysis. F_2 mice representing the extreme 17th percentiles for male and female liver and spleen phenotypes (24 per sex) were genotyped using microsatellite markers polymorphic between the parent strains and distributed across the genome. These represented a total of 55% of all F_2 progeny. Further markers and mice were added to strengthen potential quantitative trait locus (QTL) positions. Combined data from the sexes were analyzed in a genomewide scan using R/qtl (Fig. 3). Permutation analyses of the two phenotypes were set to define genomewide LOD thresholds of 10% and 1% (termed here suggestive and highly significant, respectively).[32] QTL locations, microsatellite markers nearest the calculated peak of the QTL, and LOD scores and confidence intervals of the QTL are shown in Table 3.

Highly significant liver QTL for the combined sexes were found on chromosome 2 and, particularly, on chromosome 16, and suggestive QTL were found on chromosomes 7 and 15 (Fig. 3A, Table 3). When the sexes were considered separately, highly significant QTL were detected on chromosomes 7 and 16 (males) and suggestive QTL on chromosomes 8, 11, and 19 (females) (Table 3). For the combined spleen data two highly significant QTL were detected on chromosome 8 and, particularly, on chromosome 9 (Fig. 3B). The spleen chromosome 8 QTL was at a different position than that for the liver (Table 3). A QTL on chromosome 2 only reached the 90% confidence threshold for males but appeared to be identical to that observed for the liver (Table 3). Sex explained 15.4% of total variance for liver and 11% for spleen. There was no evidence of linkage on the X chromosome for either

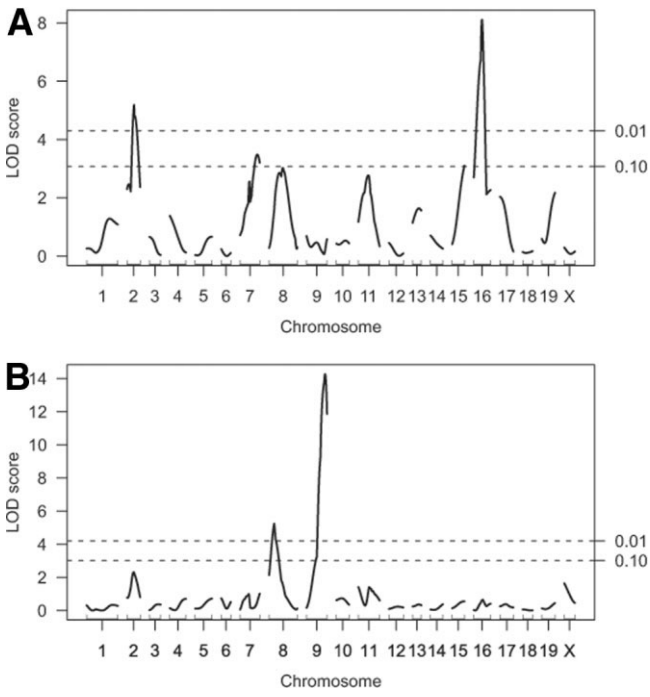

Fig. 3. Genome scans using R/qtl for the iron status of mice of both sexes combined: (A) liver; (B) spleen. Horizontal lines represent genomewide significance thresholds of 10% (suggestive) and 1% (highly significant), as determined by permutation tests. The broad bands under each chromosome represent the relative width between the first and last markers.

Table 3. Significant QTL for Iron Status Observed in C57BL/6J × SWR F_2 Mice by Sex and Overall*

Organ	Sex	Chr	Location (cM)	Peak Marker	LOD	*P*	Confidence Interval (cM)
Liver	Combined	2	57	D2Mit17	5.2	.002	51-68
		7	47	D7Mit71	3.5	.048	23-54
		15	49	D15Mit159	3.1	.096	31-49
		16	29	D16Mit30	8.1	< .001	25-34
	Male	2	56	D2Mit17	3.4	.040	38-73
		7	47	D7Mit71	4.4	.004	34-54
		16	29	D16Mit30	5.6	< .001	17-35
	Female	8	38	D8Mit195	3.1	.084	27-46
		11	50	D11Mit36	3.4	.047	27-66
		19	38	D19Mit37	3.3	.062	16-38
Spleen	Combined	8	14	D8Mit4	5.2	.001	6-23
		9	56	D9Mit182	14.3	< .001	49-61
	Male	2	55	D2Mit17	3.3	.061	45-69
		9	56	D9Mit182	10.8	< .001	49-61
	Female	9	57	D9Mit182	4.4	.012	41-61

*Genomewide significance was based on permutation analysis (see Materials and Methods section) giving 10% (suggestive) and 1% (highly significant) LOD score thresholds for liver of 3.07 and 4.30, respectively; the thresholds for spleen were 3.01, and 4.20 respectively. The locations of QTL are given at calculated maximum LODs with markers closest to these. Confidence intervals for the QTL were calculated as the chromosomal region with LOD score within 1.5 units of its maximum for the chromosome using WI, but ranges and peak marker positions may differ to some extent depending on use of WI or MGI databases, most notably D2Mit17 and D7Mit71.

tissue. Although most of the time the same QTL was found for males and females, relative strength varied considerably by sex (Figs. 4 and 5). Only on chromosome 2 for spleen (D2Mit304) was the sex difference statistically significant ($P = .009$).

Plots of iron levels versus allele genotypes at markers nearest to peak LOD scores showed approximately additive effects (Figs. 4 and 5). Two-dimensional, two-QTL scans showed no evidence of epistasis, in which the effect of the alleles at one locus depend on the alleles at other loci. For most of the QTL the SWR allele was associated with higher iron levels. In contrast, the higher iron phenotype QTL on chromosome 2, for both tissues, was associated with the C57BL/6J allele (Figs. 4E and 5D).

Iron-Related Gene Profiling. We used our cDNA arrays[29] to compare the expression of many known genes associated with iron metabolism in the liver, spleen, and duodenum of males of both mouse strains (Table 4). These cDNA arrays broadly contain those genes linked to iron and heme metabolism and were first reported by Muckenthaler.[37] No significant differences in the expression of most genes according to major QTL were detected. These included hepatic L ferritin (*Ftl1*) and IRP1 (*Aco1*), genes that previously showed strain variation,[23] which may have been a result of posttranscriptional responses. A notable exception for all three tissues was the β2-microglobulin (*B2m*) gene (chromosome 2), which apparently was expressed to a greater degree in the C57BL/6J mice than in the SWR mice. A small but significant elevation of the heme oxygenase 1 (*Hmox1*) gene in liver and spleen was observed for the SWR mice, which was confirmed by RT-PCR (Table 5).

Candidate Genes for QTL. Particular consideration was given to candidate genes for highly significant QTL. There was a trend toward higher hepatic expression of hepcidin (*Hamp*) genes in SWR mice compared with that in C57BL/6J mice, although not significantly so, as judged by the array (Table 4) and RT-PCR (Table 5). A similar finding (data not shown) was made for *Hamp2*, whose role in iron metabolism is questionable.[38-40] However, the location of the *Hamp* genes (11 cM) is markedly different from that of the chromosome 7 QTL (Table 3). Although transferrin receptor (21 cM) is a candidate gene for the chromosome 16 QTL, it lies on the edge of the confidence interval, some distance from the peak marker D16Mit30 (Whitehead, 30.6 cM; MGI, 36.5 cM). A hypothetical gene much closer, E330017A01Rik, has a ferritin domain, but we failed to amplify cDNA of this gene from tissue samples.

No candidate for the spleen chromosome 8 QTL was identified. The most significant QTL detected was that on chromosome 9 for the spleen with a maximum LOD score within 1 cM of the transferrin gene (*Trf*) at 56 cM (Table 3). Consistent differences in transferrin expression in liver or spleen were not detected by gene array or by real time RT-PCR (Tables 4 and 5). Transferrin levels in serum estimated as TIBC (Table 6) and by immunoblotting (Fig. 6) were not significantly different according to mouse strain. In contrast, serum iron level and saturation of transferrin were significantly lower in C57BL/6J mice compared with SWR mice, similar to the findings for AKR.[20,26] The cDNA for transferrin showed polymorphisms between C57BL/6J and SWR mice in codons for amino acids 203 (nt 609, A→G)

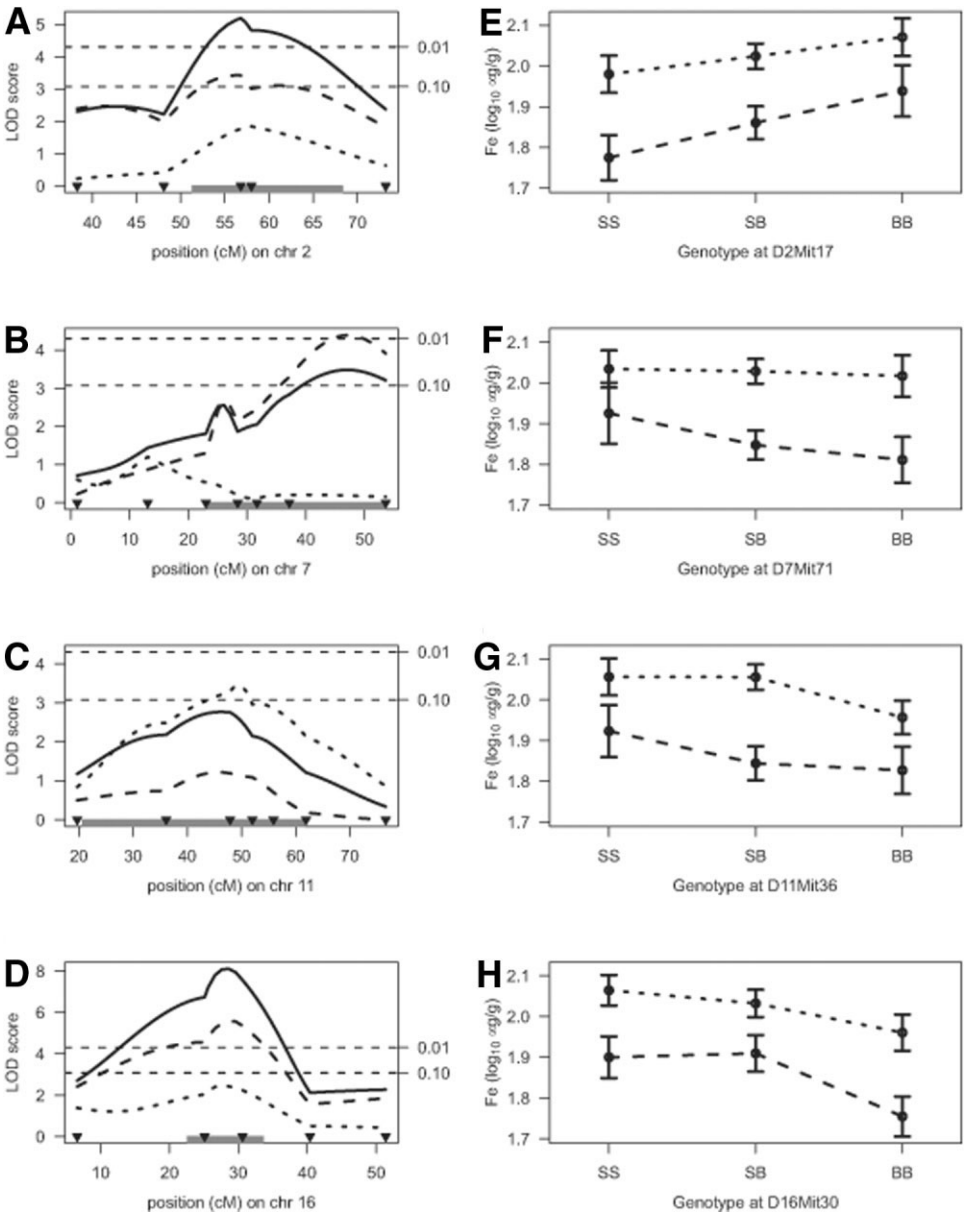

Fig. 4. Chromosome scans and allele effects for major liver QTL: (A-D) chromosome scans; (E-H) effect of genotype on iron levels (μg/g liver) at markers closest to peak LOD scores. Continuous line shows combined data. The dotted line represents females, and the dashed line shows males. Horizontal lines correspond to genome-wide LOD thresholds of 10% and 1%. Shaded bands show the confidence intervals for the combined data. Triangles show marker positions. SS, SB, and BB represent homozygous and heterozygous alleles of SWR and C57BL/6J for markers closest to the peak LOD scores. QTL on chromosomes 2 and 16 explained 6.7% and 7.5%, respectively, of total variance.

and 508 (nt 1524, C→T), respectively, but these are synonymous. A gene highly homologous to transferrin (1300017J02Rik) lies approximately 20 kb downstream from *Trf*. [41] This transferrin-like gene, which we termed *Tflg,* shares 67.9% of its identity with *Trf* and 55.3% of its amino acid identity with the protein *Trf* encodes, as determined by EMBL-EBI sequence alignment. RT-PCR showed greater expression of *Tflg* in SWR mice than in C57BL/6J mice, but this difference was statistically significant only for the spleen (Table 5). A cDNA polymorphism between the strains (nt 107 C→T) corresponded to a change in amino acid 36 from threonine (C57BL/6J) to isoleucine (SWR).

The β2-microglobulin (*B2m*) is a candidate for the chromosome 2 QTL. *B2m* showed significantly greater expression in the spleen of C57BL/6J male mice than in that of SWR mice according to gene array and RT-PCR (Tables 4 and 5). The *B2m* gene (69 cM) does not appear to be close to the two peak markers, D2Mit17 and D2Mit304, 56.8 and 59 cM, respectively (Table 3, Figs. 3 and 4), in the WI database but is much closer to MGI (69 and 73 cM, respectively).

Discussion

In humans, individual differences in apparently normal iron status may confer important predispositions for some diseases and responses to toxins.[6,12] In the present study we used an intercross of SWR and C57BL/6J mice to show basal levels of hepatic and splenic non-heme iron

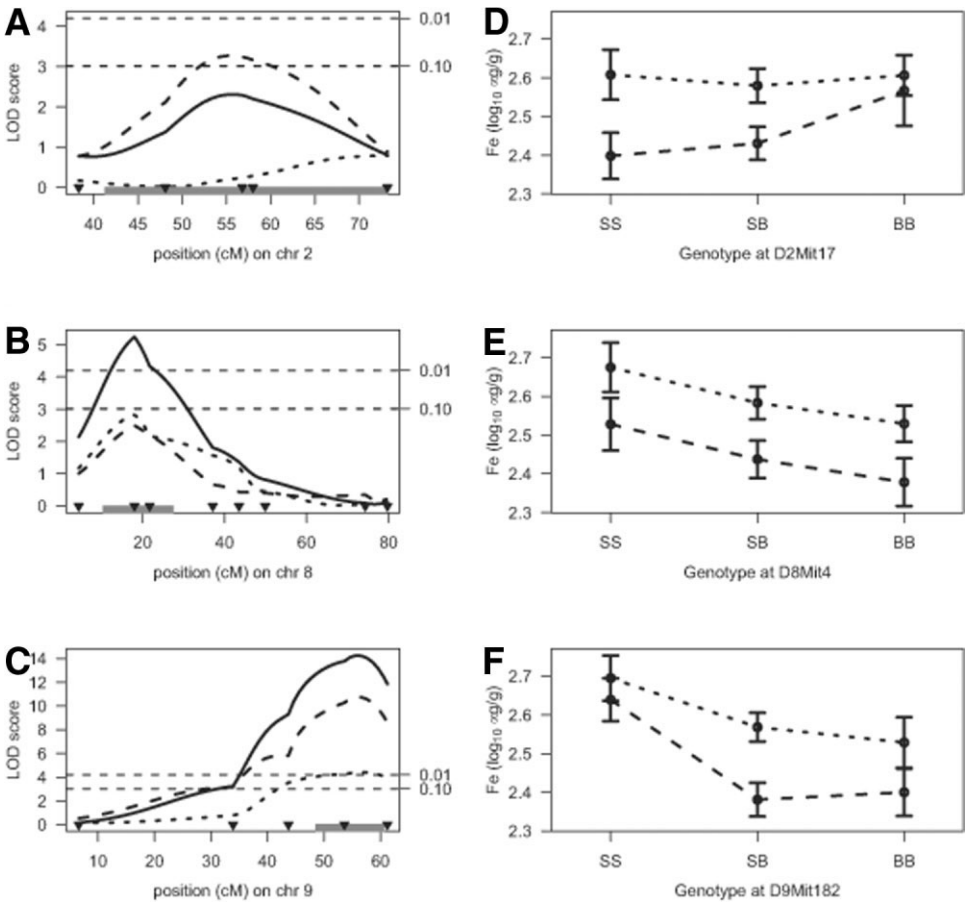

Fig. 5. Chromosome scans and allele effects for spleen QTL: (A-C) chromosome scans; (D-F) effect of genotype on iron levels (μg/g liver) at markers closest to peak LOD scores. Continuous line shows combined data. The dotted line represents females, and the dashed line shows males. Horizontal lines correspond to genomewide LOD thresholds of 10% and 1%. Shaded bands show the confidence intervals for the combined data. Triangles show marker positions. SS, SB, and BB represent homozygous and heterozygous alleles of SWR and C57BL/6J for markers closest to the peak LOD scores. QTL on chromosomes 2, 8, and 9 explained 3.4%, 5.3%, and 14.5%, respectively, of the total variance.

in non–genetically manipulated mice are determined by complex genetic traits that are tissue- and sex-dependent. SWR mice are resistant to the hepatic protoporphyria caused by some iron-chelating drugs,[22] but differences in iron metabolism between SWR and C57BL/6J mice may be a factor in their susceptibility to uroporphyria, a model of the human iron-dependent hepatic disorder sporadic porphyria cutanea tarda, and in aging and oxidative stress.[23,24,36,42] We identified highly significant QTL on chromosomes 2 and 16 for the liver and chromosomes 8 and 9 for the spleen. In addition, other QTL were observed, in particular, on chromosomes 7 and 11 for the liver and chromosome 2 for the spleen, that were either significant only for both sexes or were significant for one sex. The QTL we have observed potentially represent some of the same genes as those of the QTL on chromosomes 1, 3, 7, 8, and 11, reported by Bensaid et al. to be modifiers of the *Hfe* null gene.[17] On the other hand, because loci may be on the same chromosome does not mean they necessarily are associated with identical genes. However, they provide important information about the mechanisms for individual variation.

Although we found no evidence for an X chromosome–linked gene, there was a tendency for greater levels of iron in females, which was especially noted in the C57BL/6J parent strain and the F_1 and F_2 offspring. This is probably related to sexual dimorphism in the penetrance of some QTL, for example, liver chromosome 7. Sex-modified expression of an autosomal locus can occur by imprinting during development and in response to hormonal differences.

Thus far, QTL are not obviously explicable by differences in total duodenal, hepatic, or splenic tissue expression of many known iron-related genes. The QTL may represent polymorphisms and differences in expression of unknown genes as well as those currently characterized. They should not represent posttranscriptional regulation unless there is a genetic component. Loci for one organ also may not necessarily reflect differences detectable by expression in that tissue. In previous work no marked difference between SWR and C57BL/6J mice was found in iron absorption from the intestine, and the major hepatic difference seemed to be in clearance of iron from the circulation.[23] The demonstration that iron accumulation in the spleen occurs in the red pulp in particular is consistent with polymorphisms associated with degradation of red cells and recirculation of iron from this pool by macrophages.

Table 4. Comparison Between SWR and C57BL/6J Mice of Expression of Some Known Iron-Related Genes

Gene	Symbol	Chromosome	Position (cM or Band)	Liver	Spleen	Duodenum
ATP binding cassette, B7	*Abcb7*	X	39	1.17	0.99	1.01
Aconitase 1 (IRP1 IREBP)	*Aco1*	4	21	1.19	0.98	1.00
Aconitase 2, mitochondrial	*Aco2*	15	13	0.92	1.01	0.96
Aminolevulinate synthase 1 (H)	*Alas1*	9	F1	0.85	0.99	1.02
Aminolevulinate synthase 2 (E)	*Alas2*	X	63	1.06	1.32*	1.02
β2 -Microglobulin	*B2m*	2	69	0.69*	0.60*	0.61
Ceruloplasmin	*Cp*	3	55	0.80	0.94	1.02
Cytochrome b reductase 1 (DCYTB)	*Cybrd1*	2	C3	1.11	0.95	1.08
Cytochrome b-561	*Cyb561*	11	65	1.15	1.01	1.00
Erythropoietin receptor	*Epor*	9	5	0.99	1.01	1.0
Ferredoxin 1	*Fdx1*	9	B	0.99	0.99	1.01
Ferrochelatase	*Fech*	18	39	1.00	1.18	0.99
Ferritin heavy chain	*Fth1*	19	2	0.88	1.16	1.03
Ferritin light chain	*Ftl1*	7	25	0.88	1.12	0.99
Frataxin	*Fxn*	19	C1	1.10	1.02	1.02
Hepcidin	*Hamp*	7	11	1.64	1.00	0.99
Hephaestin	*Heph*	X	36	1.12	0.96	0.95
Hemochromatosis	*Hfe*	13	15	0.96	0.92	0.98
Hemochromatosis (juvenile)	*Hfe2*	3	F2	0.99	0.94	1.04
Heme oxygenase 1	*Hmox1*	8	35	1.11*	1.43*	0.99
Heme oxygenase 2	*Hmox2*	16	3	1.03	0.94	1.00
Haptoglobin	*Hp*	8	55	0.68	1.02	0.98
Hemopexin	*Hpxn*	7	F1	0.57	0.92	1.08
IRP2	*Ireb2*	9	29	1.17	1.00	0.99
Lipocalin 2	*Lcn2*	2	27	1.10*	1.16*	1.02
Lactotransferrin	*Ltf*	9	70	0.84	1.20*	0.94
Metallothionein 1	*Mt1*	8	45	1.46	1.16	1.19
NRAMP	*Slc11a1*	1	39	1.07	0.95*	1.01
NRAMP2 (DMT2)	*Slc11a2*	15	60	1.16*	0.96	1.15
IREG1 (MTP, ferroportin)	*Slc40a1*	1	B	1.21	1.18	1.10
Transferrin	*Trf*	9	56	0.93†	1.02†	1.0†
Transferrin receptor	*Tfrc*	16	21	1.12	1.11	1.02
Transferrin receptor 2	*Trfr2*	5	G2	1.04	0.96	1.07

The result for the EST of each gene used is expressed as a mean of the ratio of the response from SWR mice relative to that of paired C57BL/6J mice with initial and reverse labeling.

*Significant at $P < .05$ with four or five separate mouse comparisons per group.

†Some EST clones for transferrin that are not shown had a greater response with C57BL/6J.

A polymorphism of the hepcidin (*Hamp*) gene looks like a promising candidate to be a modifier of the *Hfe*-knockout mouse phenotype.[17] As expected, we found suggestions of greater expression in the SWR mice, perhaps to readdress the balance of higher iron in tissues as a result of processes other than iron absorption. However, in the present study the chromosome interval for the chromosome 7 QTL (Table 3, Fig. 4B) did not encom-

Table 5. Expression of Selected Genes as Estimated by Real-Time RT-PCR

Gene	Liver		Spleen	
	SWR:C57BL/6J	*P*	SWR:C57BL/6J	*P*
β2-microglobulin	0.97 ± 0.14	.741	0.67 ± 0.08	< .001
Hepcidin	2.65 ± 0.16	.053	1.59 ± 0.35	.008
Heme oxygenase 1	1.78 ± 0.36	.001	1.79 ± 0.06	< .001
Transferrin†	1.3 ± 0.24	.067	1.04 ± 0.13	.636
Transferrin‡	1.14 ± 0.26	.279	1.1 ± 0.1	.133
Transferrin-like	1.51 ± 4.9	.059	2.31 ± 1.0	.047

Expression of target genes was calculated relative to γ-actin except for the β-2 microglobulin and heme oxygenase 1 genes, which were calculated relative to β-actin. We used both β- and γ-actin for normalization because the reference gene must have approximately the same amplification efficiency as the target gene for the comparative C_T (threshold cycle) calculation to be valid.[35] Results are means ± SDs of 5 male mice per group, and ratios are of SWR mice compared with C57BL/6J mice.

†Primers span exons 9 and 10.

‡Primers span exons 11 and 12. The expression of transferrin and especially hepcidin was far lower in spleen than in liver.

Table 6. Serum Transferrin Saturation in Parent Strains

Strain	Sex	Serum Iron (μg/dL)	TIBC (μg/dL)	TRF Saturation (%)
C57BL/6J	Male	133 ± 37*	343 ± 17	38 ± 9*
	Female	178 ± 19	347 ± 17	51 ± 7
SWR	Male	272 ± 17†	376 ± 50	73 ± 11†
	Female	347 ± 61†	394 ± 63	89 ± 16†
AKR‡	Male	291 ± 4†	391 ± 4	74 ± 4†
	Female	306 ± 20†	444 ± 107	71 ± 15†

Values represent means ± SD of 4 or 5 mice (8 weeks old) per group.
*Significantly different from females ($P < .05$, two-tailed Student *t* test).
†Significantly different from C57BL/6J strain.
‡Spleen and liver non-heme iron contents of AKR mice (data not shown) were not significantly different from the SWR strain shown in Table 2.

pass *Hamp* or the *Hamp* regulator *Cebpa* (12 cM).[43] *Hmox1* seems to be a potential candidate for the suggestive liver chromosome 8 QTL, as judged by position and expression (Tables 3 and 5). The transferrin receptor (*Tfrc*) gene (21 cM) may be a candidate for the major hepatic QTL on chromosome 16. In QTL analyses of the hepatic porphyria induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in combination with iron overload, we found a highly significant locus on chromosome 11.[30] QTL on chromosome 11 for hepatic iron level have been reported in searches for modifier genes in *Hfe*-null mice[17] and in iron levels in chromosome substitution strains.[26] Only a weak hepatic locus was detected in the present study (Fig. 4C), and it remains to be compared more closely with previous findings.

That the QTL observed for spleen were not identical to those for liver was in agreement with the observation that iron level in these tissues, although strain dependent, is predominantly associated with independent factors (see Fig. 3), as was previously observed for transferrin saturation.[20] Most of the difference between strains in the spleen was associated with the highly significant QTL on chromosome 9, which is close to the transferrin gene (56 cM). Differences between mouse strains in transferrins detected by electrophoresis have been reported,[44] and C57BL/6J mice have lower saturation of serum transferrin and greater resistance to iron loading than do other strains (Table 5).[20] We did not find polymorphisms in the transferrin cDNA that would predict amino acid changes that might lead to a difference between C57BL/6J and SWR mice in iron-binding properties. Strain-dependent saturation of transferrin may simply reflect iron status, but polymorphisms of transferrin were linked to iron status in a black population.[45] We did find a polymorphism in a transferrin-like gene positioned nearby that may act as a plasma inhibitor of carbonic anhydrase II.[41]

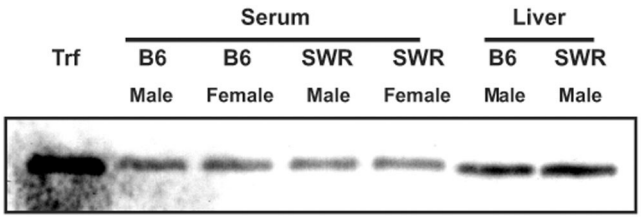

Fig. 6. Levels of transferrin in serum and liver of C57BL/6J and SWR mice by immunoblotting. Lanes contained equal amounts of serum or liver protein. Repeated blots with different samples showed no reproducible differences between strains.

For the QTL on chromosome 2, higher iron level was linked with the C57BL/6J genotype. One candidate is *Cybrd1,* which encodes a Fe^{3+} reductase proposed to be associated with duodenal iron absorption.[46] We did not find consistent strain differences in duodenal expression by immunohistochemistry or gene array or in other studies (not reported here) by immunoblotting and RT-PCR. A more promising candidate for this locus is the *B2m* gene.[14,47,48] Although knockout of the gene leads to iron overload[47,48] we observed indications of greater expression in C57BL/6J mice than in SWR mice. β2-Microglobulin was first detected as a surface antigen in the liver and spleen of C57BL mouse strains but was not observed in SWR mice.[49] Interaction between β2-microglobulin and the HFE protein reportedly modulates iron uptake and recycling of the transferrin receptor and iron uptake by cells.[50] It is interesting to speculate whether the differences between strains in iron status have a basis in immunity[28] in light of many strains originally being selected for immunological investigations.

In summary, we have shown that variability in basal iron status in apparently normal mice is the consequence of complex genetic factors that can be tissue specific. Identification and penetrance of the polymorphic genes, especially those on chromosomes 2, 7, 8, 9, and 16, in conjunction with cellular studies and comparison with syntenic regions in humans should lead to further appreciation of complex iron homeostasis in normal and pathological conditions.

Acknowledgment: We thank the members of the Biomedical Services staff for their expertise and Dr. Tatyana Chernova, Dr. David Dinsdale, Professor Yuri Dubrova, Jenny Edwards, Dr. Timothy Gant, Sam Hoffman, Dr. Katie Ridd, Linda Wilkinson, and Dr. Shu-Dong Zhang for their advice and assistance.

References

1. Hentze MW, Muckenthaler MU, Andrews NC. Balancing acts: molecular control of mammalian iron metabolism. Cell 2004;117:285-297.
2. Arosio P, Levi S. Ferritin, iron homeostasis, and oxidative damage. Free Radic Biol Med 2002;33:457-463.
3. Whitfield JB, Cullen LM, Jazwinska EC, Powell LW, Heath AC, Zhu G, et al. Effects of HFE C282Y and H63D polymorphisms and polygenic background on iron stores in a large community sample of twins. Am J Hum Genet 2000;66:1246-1258.
4. 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.
5. 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.
6. Bonkovsky HL, Banner BF, Lambrecht RW, Rubin RB. Iron in liver diseases other than hemochromatosis. Semin Liver Dis 1996;16:65-82.
7. Wolff B, Volzke H, Ludemann J, Robinson D, Vogelgesang D, Staudt A, et al. Association between high serum ferritin levels and carotid atherosclerosis in the study of health in Pomerania (SHIP). Stroke 2004;35:453-457.
8. Salonen JT, Tuomainen TP, Nyyssonen K, Lakka HM, Punnonen K. Relation between iron stores and non-insulin dependent diabetes in men: case-control study. Br Med J 1998;317:727.
9. Stevens RG, Graubard BI, Micozzi MS, Neriishi K, Blumberg BS. Moderate elevation of body iron level and increased risk of cancer occurrence and death. Int J Cancer 1994;56:364-369.
10. Nakano M, Kawanishi Y, Kamohara S, Uchida Y, Shiota M, Inatomi Y, et al. Oxidative DNA damage (8-hydroxydeoxyguanosine) and body iron status: a study on 2507 healthy people. Free Radic Biol Med 2003;35:826-832.
11. Rouault TA. Iron on the brain. Nat Genet 2001;28:299-300.
12. Schaible UE, Kaufmann SH. Iron and microbial infection. Nat Rev Microbiol 2004;2:946-953.
13. Spivak JL. Iron and the anemia of chronic disease. Oncology (Williston Park) 2002;16:25-33.
14. Levy JE, Montross LK, Andrews NC. Genes that modify the hemochromatosis phenotype in mice. J Clin Invest 2000;105:1209-1216.
15. Sproule TJ, Jazwinska EC, Britton RS, Bacon BR, Fleming RE, Sly WS, et al. 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.
16. Fleming RE, Holden CC, Tomatsu S, Waheed A, Brunt EM, Britton RS, et al. 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.
17. Bensaid M, Fruchon S, Mazeres C, Bahram S, Roth MP, Coppin H. Multigenic control of hepatic iron loading in a murine model of hemochromatosis. Gastroenterology 2004;126:1400-1408.
18. Hirschhorn JN, Lohmueller K, Byrne E, Hirschhorn K. A comprehensive review of genetic association studies. Genet Med 2002;4:45-61.
19. Dupic F, Fruchon S, Bensaid M, Borot N, Radosavljevic M, Loreal O, et al. Inactivation of the hemochromatosis gene differentially regulates duodenal expression of iron-related mRNAs between mouse strains. Gastroenterology 2002;122:745-751.
20. Leboeuf RC, Tolson D, Heinecke JW. Dissociation between tissue iron concentrations and transferrin saturation among inbred mouse strains. J Lab Clin Med 1995;126:128-136.
21. Morse AC, Beard JL, Jones BC. A genetic developmental model of iron deficiency: biological aspects. Proc Soc Exp Biol Med 1999;220:147-152.
22. Smith AG, Clothier B, Francis JE, Gibbs AH, De Matteis F, Hider RC. Protoporphyria induced by the orally active iron chelator 1,2-diethyl-3-hydroxypyridin-4-one in C57BL/10ScSn mice. Blood 1997;89:1045-1051.
23. Clothier B, Robinson S, Akhtar RA, Francis JE, Peters TJ, Raja K, et al. Genetic variation of basal iron status, ferritin and iron regulatory protein in mice: potential for modulation of oxidative stress. Biochem Pharmacol 2000;59:115-122.
24. Gerhard GS, Kaufmann EJ, Wang X, Erikson KM, Abraham J, Grundy M, et al. Genetic differences in hepatic lipid peroxidation potential and iron levels in mice. Mech Ageing Dev 2002;123:167-176.
25. Dupic F, Fruchon S, Bensaid M, Loreal O, Brissot P, Borot N, et al. Duodenal mRNA expression of iron related genes in response to iron loading and iron deficiency in four strains of mice. Gut 2002;51:648-653.
26. Ajioka RS, LeBoef RC, Kushner JP. Mapping genes that influence iron metabolism using mouse chromosome substitution strains. Blood 2003;102:756a.
27. Jones BC, Reed CL, Hitzemann R, Wiesinger JA, McCarthy KA, Buwen JP, et al. Quantitative genetic analysis of ventral midbrain and liver iron in BXD recombinant inbred mice. Nutr Neurosci 2003;6:369-377.
28. Turner OC, Keefe RG, Sugawara I, Yamada H, Orme IM. SWR mice are highly susceptible to pulmonary infection with *Mycobacterium tuberculosis*. Infect Immun 2003;71:5266-5272.
29. Davies R, Schuurman A, Barker CR, Clothier B, Chernova T, Higginson FM, et al. Hepatic gene expression in protoporphyic Fech mice is associated with cholestatic injury but not a marked depletion of the heme regulatory pool. Am J Pathol 2005;166:1041-1053.
30. Robinson SW, Clothier B, Akhtar RA, Yang AL, Latour I, Van Ijperen C, et al. Non-ahr gene susceptibility loci for porphyria and liver injury induced by the interaction of 'dioxin' with iron overload in mice. Mol Pharmacol 2002;61:674-681.
31. Broman KW, Wu H, Sen S, Churchill GA. R/qtl: QTL mapping in experimental crosses. Bioinformatics 2003;19:889-890.
32. Churchill GA, Doerge RW. Empirical threshold values for quantitative trait mapping. Genetics 1994;138:963-971.
33. Smith AG, Davies R, Dalton TP, Miller ML, Judah D, Riley J, et al. Intrinsic hepatic phenotype associated with the *Cyp1a2* gene as shown by cDNA expression microarray analysis of the knockout mouse. EHP Toxicogenomics 2003;111:45-51.
34. Zhang SD, Gant TW. A statistical framework for the design of microarray experiments and effective detection of differential gene expression. Bioinformatics 2004;20:2821-2828.
35. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. Methods 2001;25:402-408.
36. Gorman N, Walton HS, Bement WJ, Honsinger CP, Szakacs JG, Sinclair JF, et al. Role of small differences in CYP1A2 in the development of uroporphyria produced by iron and 5-aminolevulinate in C57BL/6 and SWR strains of mice. Biochem Pharmacol 1999;58:375-382.
37. Muckenthaler M, Roy CN, Custodio AO, Minana B, deGraaf J, Montross LK, et al. Regulatory defects in liver and intestine implicate abnormal hepcidin and Cybrd1 expression in mouse hemochromatosis. Nat Genet 2003;34:102-107.
38. Krijt J, Cmejla R, Sykora V, Vokurka M, Vyoral D, Necas E. Different expression pattern of hepcidin genes in the liver and pancreas of C57BL/6N and DBA/2N mice. J Hepatol 2004;40:891-896.
39. Courselaud B, Troadec MB, Fruchon S, Ilyin G, Borot N, Leroyer P, et al. Strain and gender modulate hepatic hepcidin 1 and 2 mRNA expression in mice. Blood Cells Mol Dis 2004;32:283-289.
40. Lou DQ, Nicolas G, Lesbordes JC, Viatte L, Grimber G, Szajnert MF, Kahn A, et al. Functional differences between hepcidin 1 and 2 in transgenic mice. Blood 2004;103:2816-2821.

41. Lambert LA, Perri H, Meehan TJ. Evolution of duplications in the transferrin family of proteins. Comp Biochem Physiol B Biochem Mol Biol 2005;140:11-25.
42. Elder GH. Porphyria cutanea tarda and related disorders. In: Kadish KM, Smith KM, Guilard R, eds. The Porphyrin Handbook; vol 14, Medical Aspects of Porphyrins. Amsterdam, The Netherlands: Elsevier, 2003:67-92.
43. Courselaud B, Pigeon C, Inoue Y, Inoue J, Gonzalez FJ, Leroyer P, Gilot D, et al. 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.
44. Cohen BL. A revised nomenclature for the mouse transferrin locus. Genet Res 1961;2:306-308.
45. Kasvosve I, Delanghe JR, Gomo ZA, Gangaidzo IT, Khumalo H, Wuyts B, et al. Transferrin polymorphism influences iron status in blacks. Clin Chem 2000;46:1535-1539.
46. McKie AT, Barrow D, Latunde-Dada GO, Rolfs A, Sager G, Mudaly E, Mudaly M, et al. An iron-regulated ferric reductase associated with the absorption of dietary iron. Science 2001;291:1755-1759.
47. 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.
48. Rothenberg BE, Voland JR. Beta2 knockout mice develop parenchymal iron overload: a putative role for class I genes of the major histocompatibility complex in iron metabolism. Proc Natl Acad Sci U S A 1996;93:1529-1534.
49. Tada N, Kimura S, Hatzfeld A, Hammerling U. Ly-m11: the H-3 region of mouse chromosome 2 controls a new surface alloantigen. Immunogenetics 1980;11:441-449.
50. Waheed A, Grubb JH, Zhou XY, Tomatsu S, Fleming RE, Costaldi ME, Britton RS, et al. Regulation of transferrin-mediated iron uptake by HFE, the protein defective in hereditary hemochromatosis. Proc Natl Acad Sci U S A 2002;99:3117-3122.