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β_2 Knockout mice develop parenchymal iron overload: A putative role for class I genes of the major histocompatibility complex in iron metabolism

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ABSTRACT **Hemochromatosis (HC) is an inherited disorder of iron absorption, mapping within the human major histocompatibility complex (MHC). We have identified a multigene system in the murine MHC that contains excellent candidates for the murine equivalent of the human HC locus and implicate nonclassical class I genes in the control of iron absorption. This gene system is characterized by multiple copies of two head-to-head genes encoded on opposite strands and driven by one common regulatory motif. This regulatory motif has a striking homology to the promoter region of the β-globin gene, a gene obviously involved in iron metabolism and hence termed β-globin analogous promoter (βGAP). Upstream of the βGAP sequence are nonclassical class I genes. At least one of these nonclassical class I genes, Q2, is expressed in the gastrointestinal tract, the primary site of iron absorption. Also expressed in the gastrointestinal tract and downstream of the βGAP motif is a second set of putative genes, termed Hephaestus (HEPH). Based on these observations, we hypothesized that the genes that seem to be controlled by the βGAP regulatory motifs would be responsible for the control of Fe absorption. As a test of this hypothesis, we predicted that mice which have altered expression of class I gene products, the β_2-microglobulin knockout mice, [β_2m(−/−)], would develop Fe overload. This prediction was confirmed, and these results indicate β_2m-associated proteins are involved in the control of intestinal Fe absorption.**

A genetic malady involving increased iron absorption is idiopathic hemochromatosis (HC), a naturally occurring Fe overload disease. The untreated disease is characterized by Fe overload of parenchymal cells and various complications, including hepatopathy, arthropathy, hypogonadotropic hypogonadism, cutaneous hyperpigmentation, diabetes mellitus, and cardiomyopathy (1). Largely unrecognized by the scientific community, this is the most common genetic disease in humans, far exceeding cystic fibrosis, phenylketonuria, and muscular dystrophy combined (2). Within the Caucasian population, 1 in 200 individuals is homozygous and 1 in 8 is heterozygous for the HC trait. The incidence of this genetic disease exceeds the occurance of AIDS in the United States. The responsible genes are linked to the human major histocompatibility complex (MHC) (HLA complex), located on chromosome 6 (3). Linkage to human HLA-A3 has been documented in ≈73% of cases. Other genetic loci have also been implicated in increased iron absorption, especially in African (4) and African-American populations (5). However, these conditions, as of yet, have not been shown to be related to HC.

Here we report an animal model for HC, the β_2-microglobulin (β_2m) knockout mouse (30, 31), and a candidate gene system for the mouse equivalence of HC. In addition, our results implicate class I genes in Fe metabolism and provide a plausible explanation why an animal deficient in β_2m would develop Fe overland. Finally, we have identified by Northern blot analysis and S1 protection assays a multigene system in the MHC termed Hephaestus (HEPH).

MATERIALS AND METHODS

Nucleotide Sequencing. DNA sequences were determined by the dideoxy chain-termination method using ATP[^{35}S] and analyzed using the University of Wisconsin Genetics Computer Group programs. Sequence homologies were identified using the GenBank data base and the methods as outlined by Doolittle (6).

Northern Blotting. Total RNA was isolated using Life Technologies (Gaithersburg, MD) protocol for the TRIzol method. Twenty milligrams of total RNA was analyzed on formaldehyde/agarose gels and transferred to Hybond-N (Amersham) nylon membranes. An 800-bp *Xba* I, *Nco* I fragment, isolated from the λ cosmid LSHT36, was used as a probe. This fragment encompasses the 500-bp β-globin analogous promoter (βGAP) sequence and an additional 300 bp immediately 3′. Subsequent sequencing confirmed that this sequence does not contain any known repeat sequences. The 800-bp fragment was labeled to a specific activity of 1×10^8 cpm with [^{32}P]CTP using the Prime-It RmT kit (Stratagene). The membrane was hybridized with this probe for 18 h at 42°C and washed at high stringency, 0.1× SSC at 65°C.

Mice. β_2m(−/−) as well as heterozygous β_2m(−/+) and C57BL/6 mice were maintained on a standard laboratory mouse chow diet containing 350 mg of ferric carbonate per kg. Mice were sacrificed from the ages of 3 months to 2 years. Each data point in the age-dependent study of iron stores represents 3 or 4 β_2m(−/−) or C57BL/6 mice. For the diet-dependent studies, 13 β_2m(−/−) and 5 C57BL/6 females, approximate age of 1 year, were placed on a "breeder" diet (normal mouse chow supplemented with 10 mg of ferrous sulfate per kg).

All mice were housed under similar conditions with the exception that the β_2m(−/−) and β_2m(−/+) mice were maintained in cages with microisolators. Mouse hepatitis virus (MHV) screens are performed monthly. Our colony has been free of MHV for 4 years.

Histology. All tissues were fixed in 10% buffered formalin. Tissues were subjected to routine histologic processing, and paraffin sections were stained as indicated (Prussian blue iron Stain, ×250).

Quantition of Iron Levels. Four to 5 g of liver was snap frozen. The liver samples were thawed and ashed, and the

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Abbreviations: HC, hemochromatosis; MHC, major histocompatibility complex; βGAP, β-globin analogous promoter; GI, gastrointestinal; HEPH, Hephaestus gene; β_2m, β_2-microglobulin; βDRE, β-globin direct repeat elements.

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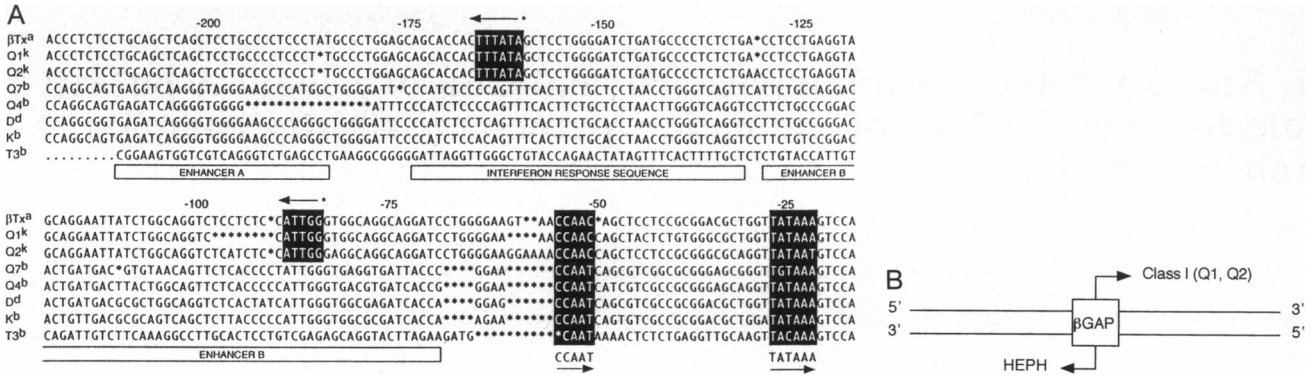

FIG. 1. βGAP sequences replace the 5′ regulatory sequences of class I genes. (*A*) The CAAT box of the class I gene is ≈25 bp from the CAAT box of the HEPH gene. The 5′ regulatory sequences of Q1, Q2, and a TL gene have been replaced by the βGAP sequences. Boxes denote CAAT and TATA boxes. Arrows signify orientation of promoter and direction of transcription. An asterisk (*) indicates the orientation of the HEPH gene on the opposite strand. Enhancer A and B elements, and the interferon response sequence, are indicated. (*B*) The βGAP promoter is situated between two head-to-head genes coded on opposite DNA strands.

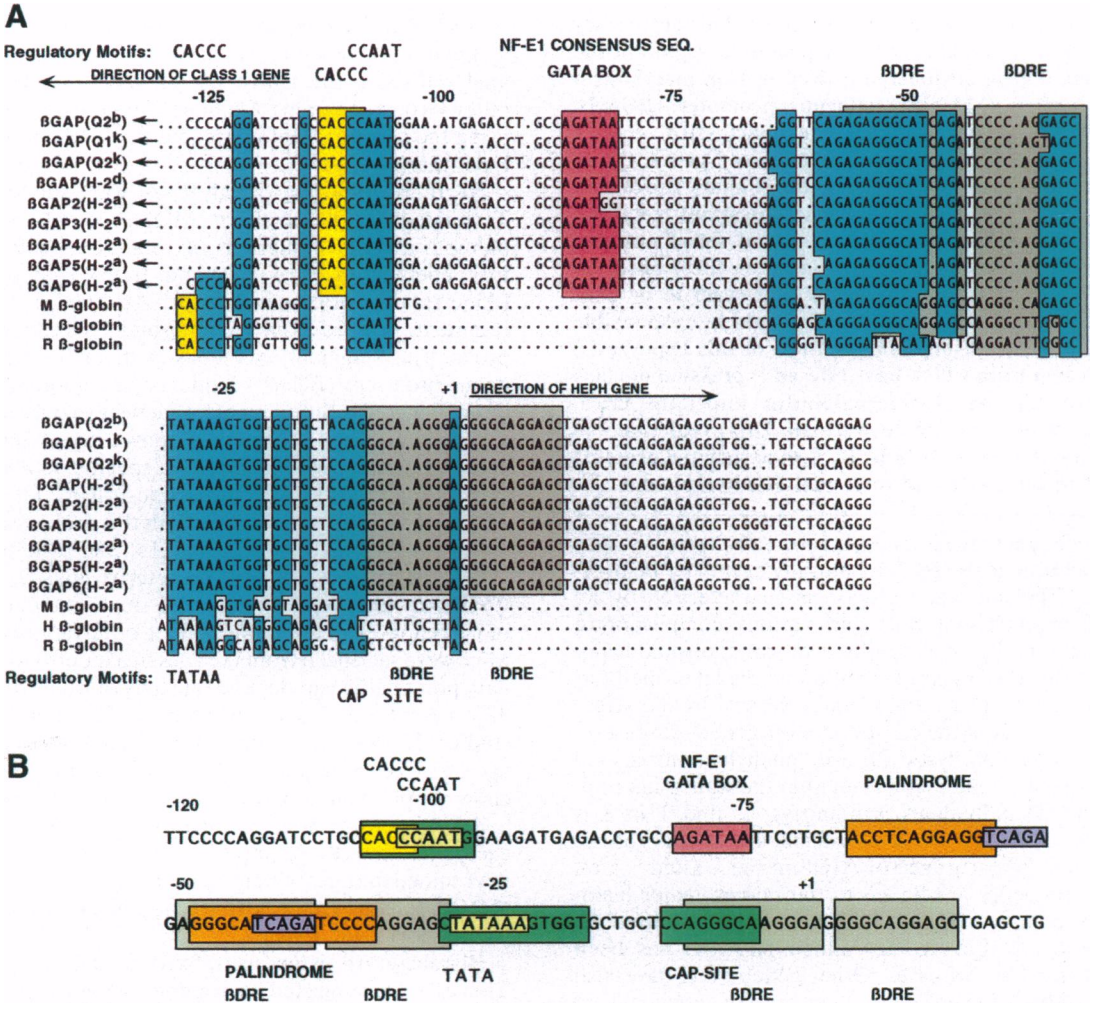

FIG. 2. (*A*) Sequence comparison of 9 βGAP sequences with the β-globin promoters of different species. βGAP(Q1^k) and βGAP(Q2^k) are from Watts *et al.* (16). All other βGAP sequences were obtained by sequencing of indicated cosmid clones. Mβglobin, mouse β-globin; Hβglobin, human β-globin; and Rβglobin, rabbit β-globin. ←, Direction of transcription of the upstream class I gene. Regulatory motifs are designated as CACCC, CCAAT, and TATA boxes, NF-E1 (erythroid-specific binding factor), βDRE (β-globin direct repetitive element), and the cap site consensus sequence. Blue boxes denote regions of homology of the β-globin regulatory regions to the various βGAP promoters, the pink box highlights the NF-E1 binding site found in the βGAP promoters, and the β-globin and putative βGAP CACCC boxes are highlighted in bright yellow. Gray shaded boxes contain βDRE. Green areas denote consensus sequences encompassing the CCAAT and TATA box and putative cap site. There are no further homologies seen between the β-globin and βGAP sequences after the β-globin transcription start site. (*B*) Regulatory elements in the βGAP sequence. In addition to the regulatory elements described in *A*, the βGAP sequence also contains two sets of palindromes which may have regulatory function. Large palindromes are in orange, and two small palindromes are in light blue. Other regulatory motifs are as in *A*. β-Globin regulatory sequences are from Stuve and Myers (17). Sequence homologies to β-globin were identified using the GenBank data base.

sample was analyzed in an atomic absorption spectrophotometer using an iron lamp.

RESULTS

Structure of the βGAP Regulatory Motif. In mapping the MHC region of the A/J strain of mouse, a unique 500-bp *Bam*HI fragment (BB500), encoding the βGAP sequence, was identified in 5 of 17 independent cosmid clones, containing nonclassical class I genes. Sequence analysis revealed 93% homology among these five fragments (7). Additional homologous sequences have been identified in BALB/c, C3H, and C57BL/6. Restriction fragment length polymorphism analysis of these and other murine strains revealed that there are four to six copies of the βGAP sequence, depending on the mouse strain (7). Southern blot analysis also indicates multiple copies of the βGAP sequence in the rat (data not shown).

Further analysis mapped all of the βGAP sequences to chromosome 17 in the mouse (7). In C57BL/6, two of the βGAP fragments are located in the TL region, and two others are in a region between Q1 and Q4 (7). Sequence analysis of all of the Q region genes in C57BL/6 shows that the βGAP sequence is encoded immediately 5′ to only the Q1 and Q2 genes. The βGAP motif replaces the regulatory sequences found 5′ of at least six nonclassical class I genes, including Q1, Q2, and two TL genes (Fig. 1*A*).

At approximately 25 and 110 bp upstream from the CAAT box of these nonclassical class I genes and on the opposite strand, a second set of CAAT and TATA motifs was identified (7). This structure suggested a head-to-head promoter arrangement with a single regulatory region controlling two genes (Fig. 1*B*). Similar genomic structures have been seen in many other gene systems (8–13). In addition, a plasmid containing a TL and HEPH gene and the βGAP motif has been shown to express both genes after being transfected into various cell lines (7). This prompted the search for additional regulatory motifs in the βGAP sequence.

βGAP Promoter Homologies. Q1 and Q2 had previously been reported to be missing their 5′ regulatory sequence and the control of their expression has remained a puzzle (14–16). Inspection of this sequence immediately upstream from Q1 and Q2 and several other nonclassical class I genes revealed motifs on the opposite strand with striking homology to the 5′ regulatory region of the β-globin gene (7) (Fig. 2*A*). The region of homology encompassed ≈120 bp and contained numerous regulatory elements known to be essential for β-globin expression (Fig. 2*B*). These include a putative

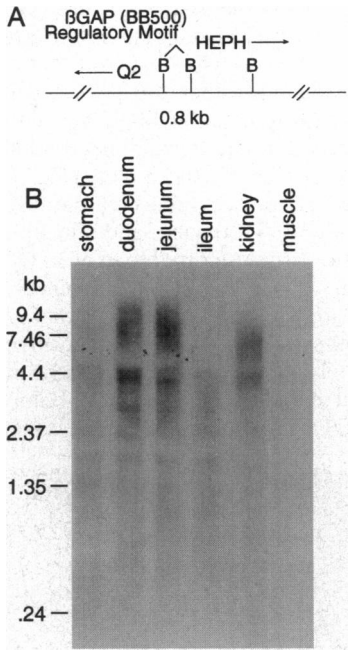

FIG. 4. Northern blot demonstrating expression of βGAP sequences in the GI tract. (*A*) A probe was generated by *Bam*HI digestion and gel purification of a cosmid clone containing the Q2 class I gene and βGAP motif. A 0.8-kb fragment containing the βGAP regulatory element was used as a probe. Sequence analysis has demonstrated that this probe does not contain class I sequences or repeats. (*B*) Total RNA from the indicated tissues was probed with the 0.8-kb fragment shown above.

CACCC box, a GATA box, and what have been termed β-globin direct repeat elements (βDRE) (17). The positions of two of the βDREs are conserved in the βGAP sequence (Fig. 3).

Expression of the βGAP Genes. The striking homology of the βGAP sequence to genes known to be involved in Fe metabolism (i.e., β-globin) suggests that the genes driven by the βGAP regulatory motif should be involved in Fe metabolism. The fact that these motifs are located in the nonclassical class I region of the murine MHC and that the genes thought to be responsible for HC map to a similar region in humans suggested that the βGAP-associated genes in the mouse would be involved in Fe absorption (7). If the βGAP sequences were driving genes involved in Fe absorption, then their products

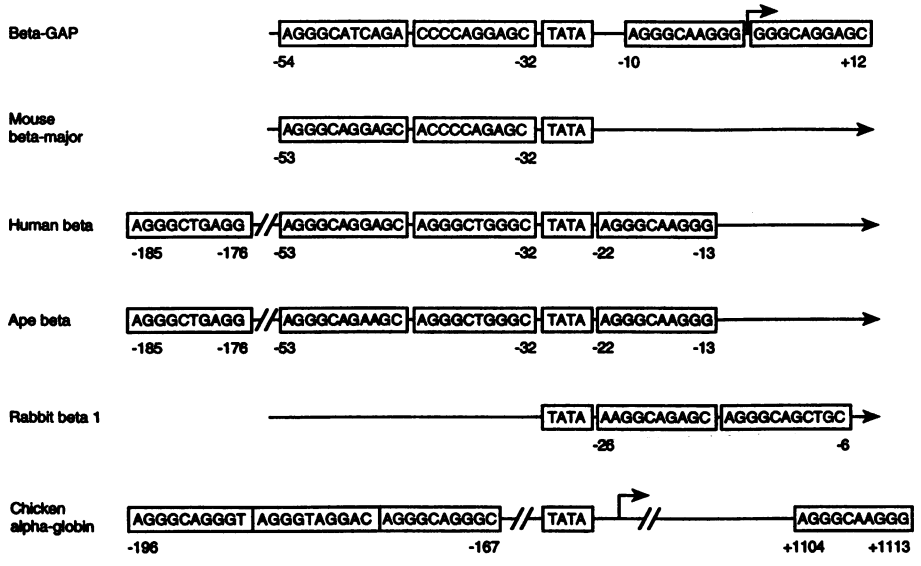

FIG. 3. Conservation of βDRE in the βGAP sequences. The βDRE are very old motifs. The evolutionary conservation of the βDRE motif in βGAP and globin genes is shown in boxes. Numbering refers to nucleotide position relative to the transcription start site +1 (for βGAP this is a putative start site). TATA designates the location of the TATA box consensus sequence. //, Regions of nonlinearity; →, direction of transcription. Sequences of the β-globin promoter of various species were obtained from various references (reviewed in ref. 17).

should be expressed in the gastrointestinal (GI) tract. In fact, it had been previously reported that Q2 is expressed in the GI tract (14).

To test the possibility that the HEPH gene, like Q2, is expressed in the GI tract, an 800-bp fragment containing the βGAP sequence was isolated from the cosmid clone LSHT36 and used as a probe on Northern blots (Fig. 4*A*). The probe recognizes a polydispersed family of putative HEPH gene messages in several GI tissues, and the kidney (Fig. 4*B*), corresponding to tissues reported to express Q2. These results indicate that the βGAP regulatory sequences are functional and that there are two genes linked to it that are expressed in the GI tract. Other polydispersed bands occurring between 2.5 and 8 kb are visible after longer exposure in the stomach, duodenum, and jejunum. These results also imply that the Q2, and probably Q1, genes are involved in Fe metabolism.

β_2 Knockout Mice Develop Parenchymal Fe Overload. If nonclassical class I genes are involved in the control of Fe absorption, then disruption of the class I gene products should affect Fe metabolism. The β_2m knockout mouse is an animal model in which class I gene function is disrupted. Based on several facts, we predicted the β_2m(−/−) mice would develop Fe overload: (*i*) our observation that some of the β_2m(−/−) mice (7%) spontaneously developed hepatomas as is the case in HC patients; (*ii*) the reported expression of several nonclassical class I antigens on intestinal epithelium facing into the lumen; and (*iii*) the molecular and genetic data described above. These animals were therefore examined for Fe deposition.

Tissues from 20 β_2m(−/−) mice between 3 and 24 months of age, maintained on a routine diet, were examined and found to have increased levels of Fe in the parenchyma of their livers as

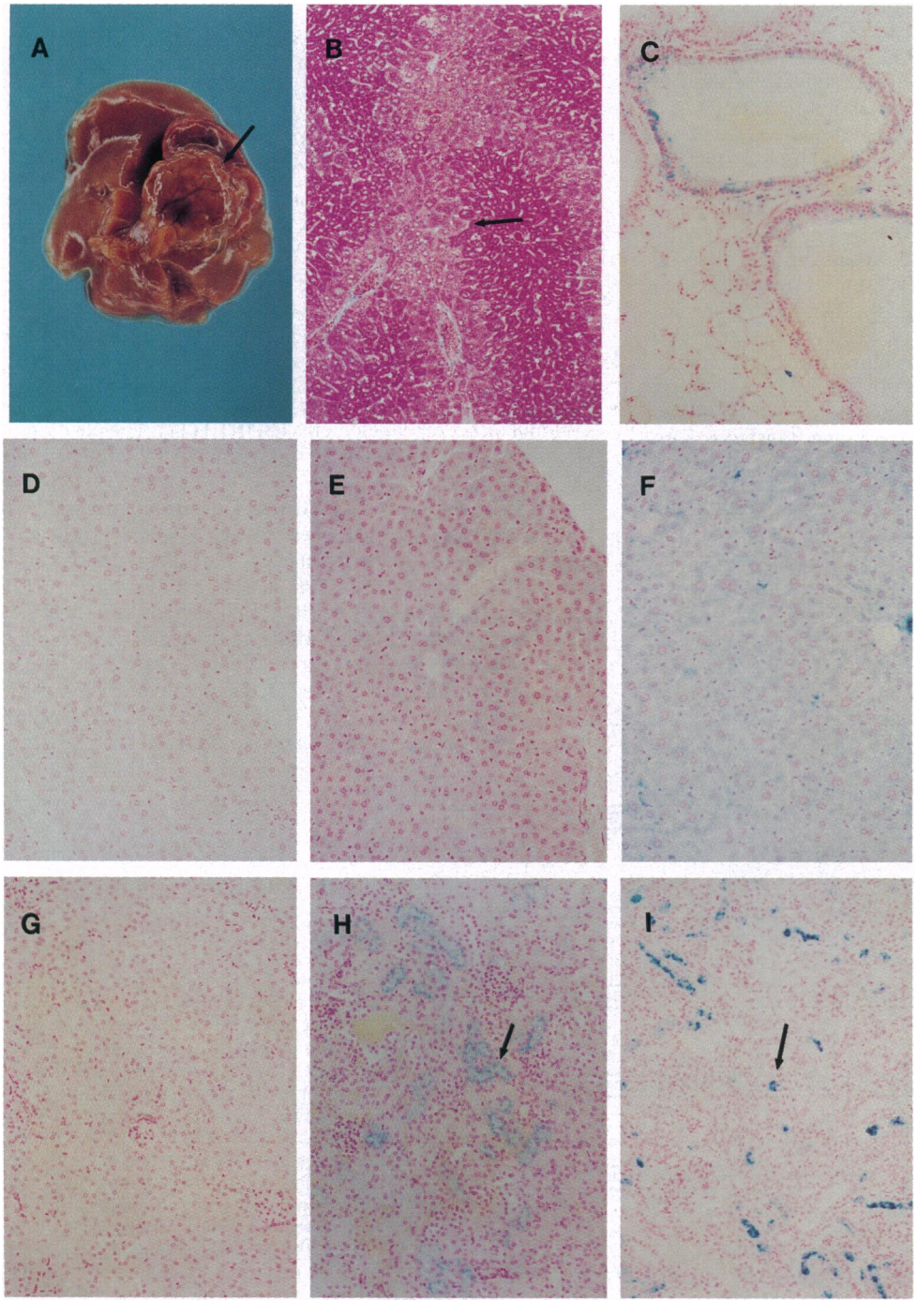

FIG. 5. Pathology and histochemistry of Fe overload in β_2m(−/−) mice. (*A*) β_2m(−/−) mice develop spontaneous hepatomas (arrow). (*B*) Liver from 1-year-old β_2m(−/−) mouse. The liver was pale and had a granular appearance. Histologically, there were multiple bands of necrosis running through the liver (arrow). There was also increased sinusoidal fibrosis. (Mallory trichrome stain; ×50.) (*D* and *G*) Iron stain of liver (*D*) from a β_2m(+/−) littermate and kidney (*G*) from 12-month-old C57BL/6 mouse maintained on the breeder diet. Despite the higher levels of iron in the diet, no parenchymal iron could be detected in any tissue examined. (Prussian blue-reactive iron stain; ×125.) (*E* and *H*) Iron stain of the liver (*E*) and kidney (*H*) from 12-month-old β_2m(−/−) mice maintained on a standard diet. Hemosiderin deposition could be detected in the cytoplasm of the hepatocytes as well as the proximal convoluted tubules of the kidneys (arrow). The livers of all mice examined demonstrated varying degrees of iron deposition. (Prussian blue-reactive iron stain; ×125.) (*C*, *F*, and *I*) Iron stain of the lung (*C*), liver (*F*), and kidney (*I*) from β_2m(−/−) mice maintained on the breeder diet. Increased ferrous iron in the diet resulted in significant increases in the amount of iron seen in the tissues. Essentially all of the hepatocytes demonstrated hemosiderin in the cytoplasm. The proximal convoluted tubules of the kidneys were again affected (arrow) as was the bronchial epithelium of the lung. The Prussian blue stain used detects only ferric iron (Fe^{3+}), which stains blue (18). It does not detect ferrous iron (Fe^{2+}) contained in hemoglobin, myoglobin, or cytochromes.

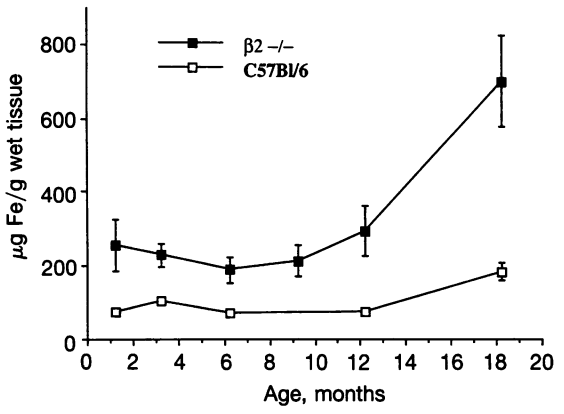

FIG. 6. Iron deposition in the liver of β_2m(−/−) mice is age dependent. Liver tissue from β_2m(−/−) mice and C57BL/6 control mice, maintained on a standard diet, were analyzed by atomic absorption spectrophotometry to determine quantitative iron levels. Iron levels in the β_2m(−/−) mice were always elevated as compared to control mice, and the levels of iron in the liver of the β_2m(−/−) mice increased with age. Atomic absorption spectrophotometry cannot distinguish between Fe^{2+} and Fe^{3+}.

compared to age-matched C57BL/6 controls and β_2m(+/−) heterozygous littermates (Fig. 5). Four of the animals also had Fe deposition in the kidneys, primarily in the proximal convoluted tubules (Fig. 5). Additional Fe deposition was seen in the spleen in the majority of the animals and in the lungs of two of the animals.

Age-dependent Fe deposition was observed in the β_2m(−/−) mice (Fig. 6), with the highest Fe levels seen in the oldest animals. Reticulocyte cell counts on 14-month-old β_2m(−/−) animals showed no evidence of increased numbers of reticulocytes as compared to aged-matched controls [β_2m(−/−), 3.5% ± 0.2%; C57BL/6, 4.8% ± 0.1%; results represent 1000 cell counts from four mice each], suggesting that the increased levels of Fe were not due to a hemolytic anemia resulting in hemosiderosis.

Enhanced intestinal absorption of Fe is the underlying defect in primary human HC, and diets rich in Fe exacerbate the disease (19). To see if iron accumulation in this animal model was diet dependent, 22 β_2m(−/−) mice between 8 and 15 months of age were maintained for 6–8 months on a "breeder" diet. These animals were subsequently sacrificed and their tissues were examined for iron accumulation. All of the β_2m(−/−) mice had markedly increased deposition of Fe in the liver (Fig. 6), with some mice accumulating Fe levels of 1 mg/g of wet tissue vs. 0.2 mg/g for controls. Two of the animals also had increased Fe deposition in the kidneys, and one had Fe deposition in the lung. Control C57BL/6 and β_2m(+/−) littermates maintained on the breeder diet showed no significant parenchymal Fe or evidence of necrosis. These observations indicate that Fe overload in the β_2m(−/−) mice is related to diet. Similar results have been reported (20, 21).

In addition, four β_2m(−/−) mice in our colony were found to have spontaneously developed liver tumors, which on histologic examination were found to be hepatomas (Fig. 5*A*). Fourteen of 26 mice also developed granular changes in the liver, and histologic examination revealed bands of necrotic hepatocytes extending through the tissue (Fig. 5*B*). The majority of this hepatic necrosis was seen in the livers of mice on the breeder diet (10/22). The only other significant abnormality reported is the development of hyperglycemia in older animals (22). The development of liver tumors, hyperglycemia, and parenchymal Fe deposition indicates that the β_2m(−/−) mice represent an attractive animal model for the study of Fe overload diseases.

DISCUSSION

The genomic structure of the βGAP regulatory motif suggests that it controls the expression of an upstream class I gene and a downstream HEPH gene in the GI tract. The data from the β_2m(−/−) mice, combined with the fact that heterozygous β_2m(+/−) littermates showed no evidence of disease, definitely implicate β_2m-associated proteins in the control of Fe absorption.

Based on the genomic structure of the βGAP regulatory sequences and associated genes, there is a testable receptor–ligand model which explains Fe overload in these animals. The model proposes that Fe is absorbed by a multigene family of β_2m-associated receptors. By necessity, the membrane-bound receptor must be expressed in the GI tract on epithelial tissues and would face into the lumen of the gut. The corresponding ligand would chelate Fe and bind to the receptor. While it is possible that the downstream HEPH genes are these receptors, we believe it is more likely that the nonclassical class I genes fulfill this role. The product of the HEPH gene would then be the Fe chelating ligand. This model supports a receptor–ligand function for some class I molecules that is not dependent on peptide presentation (23). The receptor–ligand model is supported by the observation that some nonclassical class I molecules are located on the intestinal epithelium, facing into the lumen (24, 25). Furthermore there is an analogous model of a β_2m-associated molecule expressed in the gut, which functions as a receptor, the neonatal Fc receptor (FcRn) for maternal immunoglobulin (26, 27). FcRn is homologous to class I proteins.

It should be noted that the β_2m(−/−) mice are β_2m negative, and not class I negative. It has been demonstrated by others that small amounts of classical class I MHC products (D^b) can be detected on the surface of tissues from β_2m(−/−) mice (28). Moreover, in model systems, it has been shown that free heavy chains for several classical class I antigens can reach the surface and can be stabilized by either antibody, free β_2m or ligand.

The above model may offer some explanations for several of the observations that have been made about human HC. First, because class I genes would be directly involved in Fe absorption, the model explains why this disease maps to the MHC. Second, it speaks to why HC is so prevalent. If Fe is absorbed by a multigene family, then a mutation in any one of several genes could lead to HC. This model may also explain why a mouse can accumulate Fe levels in 1 year that take 40 years to accumulate in humans. If there are multiple redundant β_2m-associated Fe receptors, then mutation of one of the receptors in humans could result in gradual Fe accumulation. By removing β_2m we would affect all of the receptors allowing for a much faster accumulation of Fe.

This model has an interesting consequence. The surface of the gut would act as an Fe store. Chelated Fe would be bound to its receptor. When Fe was immediately required, the complex would be internalized. If the Fe was not needed, it would be disposed of when the cells lining the intestinal villi are sloughed into the lumen. In this manner the body could maintain a readily accessible store of Fe, without having to internalize it. Iron is an extremely toxic compound. By immobilizing it in the lumen of the intestine, the internal environment would be protected from the effects of Fe-catalyzed formation of damaging free radicals (29).

Our results implicate at the very least, β_2m, and possibly some class I gene products in the control of Fe absorption. In addition, the β_2m(−/−) mice appear to be a suitable animal model for the study of the pathogenesis, pathology, and ultimately treatment of Fe overload. They will also be valuable in studying the role Fe plays in the pathogenesis of other diseases. Elevated iron levels and hepatic damage affect

immune function. All studies examining the immune system in the β_2m(−/−) mice should be interpreted with caution.

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