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Human Mitochondrial Ferritin Expressed in HeLa Cells Incorporates Iron and Affects Cellular Iron Metabolism*

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Mitochondrial ferritin (MtF) is a newly identified ferritin encoded by an intronless gene on chromosome 5q23.1. The mature recombinant MtF has a ferroxidase center and binds iron *in vitro* similarly to H-ferritin. To explore the structural and functional aspects of MtF, we expressed the following forms in HeLa cells: the MtF precursor (~28 kDa), a mutant MtF precursor with a mutated ferroxidase center, a truncated MtF lacking the ~6-kDa mitochondrial leader sequence, and a chimeric H-ferritin with this leader sequence. The experiments show that all constructs with the leader sequence were processed into ~22-kDa subunits that assembled into multimeric shells electrophoretically distinct from the cytosolic ferritins. Mature MtF was found in the matrix of mitochondria, where it is a homopolymer. The wild type MtF and the mitochondrially targeted H-ferritin both incorporated the ^{55}Fe label *in vivo.* The mutant MtF with an inactivated ferroxidase center did not take up iron, nor did the truncated MtF expressed transiently in cytoplasm. Increased levels of MtF both in transient and in stable transfectants resulted in a greater retention of iron as MtF in mitochondria, a decrease in the levels of cytosolic ferritins, and up-regulation of transferrin receptor. Neither effect occurred with the mutant MtF with the inactivated ferroxidase center. Our results indicate that exogenous iron is as available to mitochondrial ferritin as it is to cytosolic ferritins and that the level of MtF expression may have profound consequences for cellular iron homeostasis.

Ferritins are ubiquitous proteins made of 24 subunits that form a spherical shell that can accommodate up to 4,000 iron atoms (reviewed in Ref. 1). In mammals, nearly all of the ferritin is found in cytoplasm, where its expression is controlled translationally by iron through an iron regulatory element in the mRNA (2, 3). This ferritin is composed of two subunit types, H and L, with ~50% sequence identity and very similar three-dimensional structures made of a bundle of four α-helices. H-ferritin shells have ferroxidase activity that results in the conversion of soluble ferrous ions into inert aggregates of ferric hydroxides (4–6). This ferroxidase activity is associated with di-iron binding sites coordinated by seven residues that are conserved in ferritins from animals, plants, and bacteria (1, 7). These sites catalyze Fe(II) oxidation, a rate-limiting step in iron incorporation, in a reaction that consumes one dioxygen molecule per two Fe(II) ions and produces hydrogen peroxide (1, 6, 8). The L-subunit lacks the ferroxidase center, and L-homopolymers do not incorporate iron *in vivo.* However, the L-subunit provides efficient sites for iron nucleation and mineralization and somehow increases turnover at the H-ferroxidase centers (4–6).

The ferroxidase activity of the H-chain is largely responsible for the biological activity of mammalian ferritins. Inactivation of H-chains in knockout mice is lethal at early stages of embryogenesis (9). Overexpression of H-chains in stable transfectants of the mouse erythroleukemic (MEL) cell line (10–12) and HeLa cells results in an iron-deficient phenotype (13). This is accompanied by reductions in heme and hemoglobin synthesis and also in proliferation rate, a reduction multidrug resistance, and a reduction of oxidative damage from free iron (10–13). These effects are abolished by iron supplementation or by the mutational inactivation of the ferroxidase center (13). Other than facilitating iron deposition, little is yet known of the biological role of L-chains. Large increases in L-ferritin levels occur as a result of mutations in the iron regulatory element. These increases cause cataracts but no apparent abnormalities in body iron metabolism (14, 15). However, a mutation in the C-terminal sequence of the L-chain causes a neurological disorder with increased deposition of ferritin and iron in the basal ganglia of the brain (16).

We have recently identified a new human ferritin, MtF,[1] that is encoded by an intronless gene on chromosome 5q23.1 and a mouse ortholog (17). Human MtF is synthesized as a 242-amino acid precursor with a long N-terminal sequence for mitochondrial import (17). Experiments with transfectant cells showed that this precursor is efficiently targeted to mitochondria and processed into typical ferritin shells. The amino acid sequence of the predicted mature protein overlaps the H sequence with 77% identity and contains all the residues of the

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[1] The abbreviations used are: MtF, mitochondrial ferritin; T-MtF, truncated MtF; Mt, mitochondrial ferritin; HF, H-ferritin; LF, L-ferritin; DMEM, Dulbecco's modified Eagle's medium; FAC, [^{55}Fe]ferric ammonium citrate; ELISA, enzyme-linked immunosorbent assay; DFO, desferrioxamine.

ferroxidase center. The mature protein produced in *Escherichia coli* incorporated iron i*n vitro*, indicating that it has ferroxidase activity (17). As judged from mRNA levels, MtF is expressed at low levels in most cells except testis. MtF is present at a low level in normal erythroblasts, but this level increases dramatically in iron-loaded erythroblasts from patients with sideroblastic anemia (17). This increased expression does not appear to be due to the typical translational control since MtF mRNA lacks the classical stem-loop iron regulatory element.

The function and regulation of this new ferritin have not been established. Mitochondria are exposed to a heavy traffic in iron for the synthesis of heme and Fe/S clusters. Mitochondria are also the major sites of reactive oxygen species production (18–20) and presumably must have efficient mechanisms to segregate Fe(II) from reactive oxygen species (particularly H_2O_2) to prevent the production of highly toxic hydroxyl radicals in Fenton-type reactions. Iron homeostasis in mitochondria also differs from that in the cytoplasm. Iron deprivation affects mitochondrial iron enzymes less than cytosolic iron enzymes (21). By contrast, excess iron is not usually deposited in mitochondria but is deposited in the cytosol as ferritin.

Although iron does not normally accumulate in mitochondria, defects in its transport or utilization in mitochondria can result in mitochondrial iron loading. Visible granular iron deposits are formed inside the mitochondria of erythroblasts with defective heme synthesis as in subjects with sideroblastic anemia (22, 23). Much of this iron is probably present as MtF (17). Iron also accumulates in the mitochondria of patients with Friedreich's ataxia resulting from defects in the synthesis of frataxin (24) or in sideroblastic anemia with ataxia from defects in the Fe/S transporter ABC7 (25). The form of this iron is not known, but the iron overload is associated with a decrease in respiratory chain and aconitase activity, probably from iron-induced oxidative damage (26).

Very little is yet known about how iron is delivered to mitochondria and whether it is normally accessible to MtF. It is also not known whether MtF responds to changes in cellular iron or whether its level affects the partitioning of cellular iron. This report explores some of these issues through analyses of different forms of MtF and H-ferritins transfected into HeLa cells. We show that MtF readily incorporates iron inside mitochondria by a process similar to that of H-ferritins. Unlike cytoplasmic ferritins, the levels of MtF are not increased by exogenous iron. However, when increased by transfection, MtF retains a high proportion of available iron, and cells show signs of iron deficiency. We conclude that iron is potentially as accessible to MtF as it is to cytosolic ferritin and that the control of MtF levels may offer a powerful method for regulating cellular iron homeostasis.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—The vector pcDNA3.1 was purchased from Invitrogen. Monoclonal antibodies, rH02 and LF03, prepared against human ferritin H- and L-chains, respectively, have been described previously (27, 28) as has the rabbit antiserum, anti-rΔ9MtF elicited by a truncated form of MtF corresponding to residues 10–182 of the H-chain (17). A more potent antiserum, anti-MtF, was elicited in mice by injecting the full mature form of recombinant MtF. Monoclonal rH02 recognizes H- but not L-ferritins and also cross-reacts with MtF (17), whereas LF03 is specific for L-ferritins. Both antisera recognized MtF, but neither recognized H- or L-ferritins. Anti-transferrin receptor antibody was purchased from Zymed Laboratories Inc.(San Francisco, CA).

Plasmid Construction and Cell Culture—The pcDNA3MtF vector, encoding the entire precursor MtF protein, was described in Ref. 17. The MtF_{222} mutant (E62K, H65G, H-chain numbering) with an inactivated ferroxidase activity was produced by oligonucleotide-directed mutagenesis of pcDNA3MtF. The chimera Mt-HF was constructed by fusing the mitochondrial leader peptide of MtF (residues 1–60) to the full human H-ferritin chain sequence. The plasmid for the truncated MtF (T-MtF) was constructed by subcloning into pcDNA3 the sequence encoding residues −2 to 182 (H-chain numbering). To obtain stable transfectants, the full coding regions of MtF and of the MtF_{222} mutant were subcloned into pUDH10–3 vector (CLONTECH) (29) under the control of the tTA promoter to obtain pUD-MtF and pUD-MtF_{222} plasmids.

HeLa cells were transfected with calcium phosphate as in Ref. 30 and grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 1 mM L-glutamine. Typically in transient experiments, 10^6 cells were transfected with 10 μg of pcDNA3 plasmid containing the ferritin cDNAs or with the pcDNA3 vector for a control. Transfection efficiency was monitored by immunofluorescence staining with anti-Δ9MtF antiserum and ranged between 20 and 30% of cells. A stable HeLa-tet Off cell line was generated and selected as described in Ref. 13. The HeLa-tet Off cells (CLONTECH) were co-transfected with 3.8 μg of pUD-MtF or pUD-MtF_{222} plasmids and with 1 μg of pTK-Hyg plasmid (5:1 molar ratio) (CLONTECH). Clones expressing MtF and MtF_{222} were selected and maintained in DMEM supplemented with 10% fetal bovine serum, 100 μg/ml G418 (Geneticin, Sigma), 150 μg/ml hygromycin D (CLONTECH), 100 units/ml penicillin, 100 μg/ml streptomycin, 1 mM L-glutamine. In the presence of doxycycline (2 ng/ml, Sigma), the protein synthesis was repressed, whereas in its absence, the synthesis was induced.

Ferritin Evaluation and Immunoblotting—The levels of cytosolic ferritins were assayed in extracts of 10^6 cells with ELISA assays using the monoclonal antibody rH02 calibrated on the recombinant homopolymer (28). Purified recombinant MtF was not recognized by L-ferritin ELISA, but it gave a signal in the H-ferritin ELISA that corresponded to 1% of the ferritin content and was also recognized by this antibody in Western blots. Protein concentration was evaluated by the BCA method (Pierce) calibrated on bovine serum albumin. In immunoblot experiments, 30 μg of soluble proteins were separated by PAGE in 7% non-denaturing gels. Nitrocellulose filters from the blotted gel were incubated with rabbit anti-Δ9MtF antiserum (dilution 1:2,000) or rHO2 monoclonal antibody (dilution 1:1,000) followed by peroxidase-labeled antibody (Sigma). The bound peroxidase was revealed by ECL (Amersham Biosciences).

Cellular ^{55}Fe Incorporation—In experiments with transient transfectants, the cells (2×10^5) were transfected with 2 μg of DNA plasmid and grown for 30 h in complete medium. Stable transfectants were induced to express ferritin by omitting doxycycline for 7 days. The cells were then incubated for 18 h, or the indicated time, with 2 μCi/ml [^{55}Fe]ferric ammonium citrate (FAC) (ratio 1:2), 200 μM ascorbic acid, or 1 μM ^{55}Fe-labeled transferrin in DMEM, 0.5% fetal calf serum, 0.5% bovine serum albumin. The cells were washed and lysed in 0.3 ml of lysis buffer. After centrifugation, 10 μl of the soluble fraction were mixed with 0.3 ml of Ultima Gold (Packard) and counted for 1 min in a scintillation counter (Packard). The soluble proteins were analyzed also by PAGE in 7% non-denaturing gels directly or after immunoprecipitation with anti-Δ9MtF or LF03 (13, 17). Gels were dried and exposed to autoradiography. The intensity of ferritin subunit bands was quantified by densitometry in the linear range.

Mitochondrial Enrichment—Transfectant cells were grown for 18 h in the presence of 2 μCi/ml FAC (ratio 1:2), 200 μM ascorbic acid, and mitochondrial fraction enriched as described previously (31). Briefly, the cells were washed twice in phosphate-buffered saline and lysed on the plate using 0.007% digitonin in 0.25 M sucrose, 10 mM Hepes, pH 7.4, 0.15% bovine serum albumin. Unbroken cells and nuclei were first cleared by centrifugation at $1,000 \times g$ for 10 min, and the mitochondria were precipitated by a further centrifugation at $3,000 \times g$ for 10 min at 4 °C. The cytosolic supernatants (post-mitochondrial fractions) and the mitochondrial pellet (mitochondrial fractions) were analyzed directly or heated at 75 °C for 10 min for ferritin enrichment. The heat-stable proteins were separated by PAGE in 7.5% non-denaturing gels and exposed to autoradiography.

Metabolic Labeling and Immunoprecipitation—After transient transfection, the cells (5×10^5) were grown for 30 h, or the stable clones were grown for 7 days in the absence of doxycycline. Then, they were incubated for 1 h in DMEM, methionine, and cysteine-free (ICN) 0.5% fetal calf serum, 0.5% bovine serum albumin and were labeled for 18 h with 50 μCi/ml [^{35}S]methionine, [^{35}S]cysteine (ICN) in the same medium (13). The cells were washed with phosphate-buffered saline and then lysed with 500 μl of lysis buffer (20 mM Tris-HCl, pH 8.0, 200 mM LiCl, 1 mM EDTA, 0.5% Nonidet P-40). Total radioactivity associated with the soluble proteins was determined by trichloroacetic acid precipitation. For immunoprecipitation studies, 4×10^6 cpm of cytosolic lysates were precleared by incubation with 30 μl of protein A-Sepharose 50% v/v (Sigma) for 1 h at 4 °C with gentle shaking and centrifuged for

1 min at 14,000 rpm. Then, anti-ferritin L-chain monoclonal antibody (LF03) or mouse anti-MtF antibody was added, incubated for 1 h followed by protein A-Sepharose (30 μl). The samples were then incubated for 1 h at 4 °C, and the precipitates were collected. The soluble fractions were further incubated for 1 h at 4 °C with 30 μg of anti-ferritin H-chain antibody (rH02) and protein A-Sepharose (30 μl) and precipitated (13). The immunobeads were washed, resuspended in SDS buffer, boiled for 10 min, and loaded on 12% SDS-polyacrylamide gel. The gels were treated with autoradiography image enhancer (Amplify, Amersham Biosciences), dried, and exposed.

Immunofluorescence and Immunoelectron Microscopy—Cells expressing MtF and its mutants were fixed and permeabilized (17). The preparations were then overlaid with rH02 (0.5 μg/ml) antibody followed by rhodamine-conjugated anti-rabbit IgG and washed as described previously. Fluorescence was visualized on an Axiophot microscope (Zeiss) with a 554-nm filter for rhodamine. For immunoelectron microscopy, the cells were fixed for 15 min with 4% paraformaldehyde and 0.25% glutaraldehyde mixture, detached by scrubbing, and centrifuged. The pellets were infiltrated in 0.6 M sucrose mixed with 7% polyvinylpyrrolidone and then brought to 1.86 M sucrose and 20% polyvinylpyrrolidone by successive increases of the infiltrating solution. Freezing was in a 3:1 mixture of propane and cyclopentane cooled with liquid nitrogen. Ultrathin cryosections (50–100 nm) were cut using an Ultracut ultramicrotome equipped with a Reichert FC4 cryosectioning apparatus and processed as described previously (32). In brief, the cryosections were collected over nickel grids and covered with 2% gelatin. After treatment with 125 mM phosphate-buffered saline supplemented with 100 mM glycine, the sections were exposed for 2 h at 37 °C to anti-Δ9MtF in phosphate-glycine buffer, then washed with the buffer, and finally labeled with anti-IgG-coated gold particles (6 nm, dilution 1:60 in the same buffer). Cryosections were then examined by electron microscopy.

RESULTS

Characterization of Transfected MtF—The cDNA for the human MtF precursor was subcloned into pcDNA3 vector to transiently transfect HeLa cells. MtF protein expression was first analyzed using anti-Δ9MtF antibodies by Western blot after separating cell extracts on non-denaturing PAGE. No MtF was detected in the untransfected HeLa cells, but high levels were found in the transfectants (Fig 1*A*, *lanes 1* and *2*). The single band in the transfectants had a similar, but slightly slower, mobility than that of the cytoplasmic ferritin shown in Fig. 1*A* (*lane 3*), indicating that MtF has a similar multimeric structure. To explore iron uptake into MtF, cells were incubated with the ^{55}Fe label, supplied as FAC, for 18 h. Cells and organelles were lysed with 0.5% Nonidet P-40, and the proteins in supernatant fractions were separated on non-denaturing gels and then exposed to autoradiography. The untransfected parent cells gave a single radioactive band corresponding to the cytosolic ferritin and none in the position of MtF (Fig 1A, *lane 3*). The MtF transfectants showed uptake into cytosolic ferritin but also into a slower band in the position of MtF (Fig. 1*A*, *lane 6*). To confirm the identity of the bands, cytosolic ferritin heteropolymers were first precipitated from the cell extracts with an excess of anti-L-chain LF03 antibody (13). This treatment eliminated the band corresponding to the cytosolic ferritin but left the MtF band (Fig. 1*A*, *lanes 5* and *8*). In contrast, anti-Δ9MtF antibody essentially eliminated the upper band specific to the transfected cells but had no effect on the lower band (Fig. 1*A*, *lanes 4* and *7*). These results identify MtF and show that it is a homopolymer, as predicted from its compartmentalization, and that it actively incorporates iron *in vivo*.

To confirm the cellular compartmentalization of the ferritins, the plasma membranes of the cells were lysed with digitonin, and the mitochondrial fractions of ^{55}Fe-labeled cells were separated from the cytosolic fraction by differential centrifugation (31). Ferritin was partially purified from both fractions by heat extraction and identified by its electrophoretic mobility and the bound radioactive iron. Autoradiography showed that the MtF band was highly enriched in the mitochondrial fraction

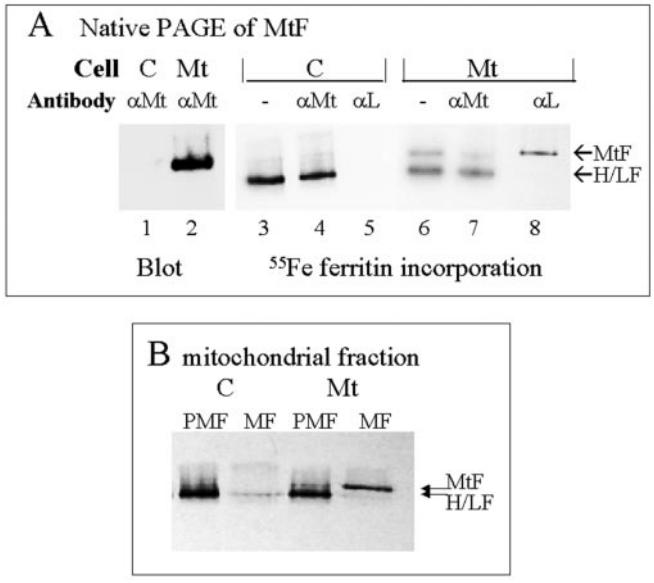

FIG. 1. **Expression and iron incorporation of MtF in transiently transfected HeLa cells.** HeLa cells were transiently transfected with pcDNA3Mt plasmid for MtF expression (*Mt*) and harvested 30 h after transfection. Proteins from lysates of control (*C*) and transfected cells were separated by PAGE in 7% polyacrylamide non-denaturing gels. *A*, *lanes 1* and *2*, blotting with anti-Δ9MtF polyclonal antibody specific for MtF (dilution 1:2,000), ECL development; *lanes 3–8*, cells grown for 18 h in medium containing the ^{55}Fe label, as ferric ammonium citrate, in the presence of 200 μM ascorbate. 10 μg of the soluble proteins were loaded on PAGE before (−) and after immunosequestration with saturating amounts of anti-Δ9MtF (*αMt*) or of anti-L-ferritin antibody (*αL*). Bound radioactivity was revealed by autoradiography. *B*, cells labeled with ^{55}Fe as described in *panel A* were lysed with digitonin, and the mitochondrial fraction (*MF*) was separated from the post-mitochondrial fractions (*PMF*) by sequential centrifugation. The two fractions were heated at 75 °C, and 10 μg of the heat-stable proteins were resolved on non-denaturing PAGE and exposed to autoradiography. The *arrows* indicate the mobility of MtF and cytosolic ferritin (*H/LF*).

(Fig. 1*B*, *MF*) of the transfected cells, whereas cytosolic ferritins were almost exclusively associated with the post-mitochondrial fractions (Fig. 1*B*, *PMF*) of the control and transfected cells (Fig. 1*B*). We conclude that MtF is restricted to mitochondria, where it assembles into ferritin-like structures that incorporate iron.

Analyses of MtF Mutants—To explore structural and functional elements for iron uptake into MtF, different constructs were expressed in HeLa cells. MtF_{222} has Glu-62 → Lys and His-65 → Gly (H-chain numbering), which inactivate the ferroxidase activity of human H-ferritin (13). T-MtF represents the predicted mature protein lacking the mitochondrial targeting sequence and starting at position −2 (H-chain numbering). Finally, Mt-HF has the N-terminal MtF sequence (residues 1–60) fused to the H-chain and predicted to be cleaved at residue 58. Transfectant ferritins were identified with monoclonal rH02 that reacts with human H-ferritin and also with MtF (17). Western analyses of cell lysates showed that all four transfected ferritins were detected with this antibody and are therefore expressed. MtF, MtF_{222}, and Mt-HF had a similar mobility, whereas T-MtF was faster and co-migrated with the cytosolic ferritin (Fig. 2*A*). In addition, this blotting and that of Fig. 2*C* (*bottom panel*) indicate that the transfectant ferritins accumulate in the cells at levels much higher than those of the endogenous cytosolic H-ferritins. In the absence of an ELISA assay, it could not be quantitated.

Iron Uptake—To compare iron uptake into the transfected ferritins, the soluble fractions of the lysates of transfectant cells labeled with FAC were separated on non-denaturing

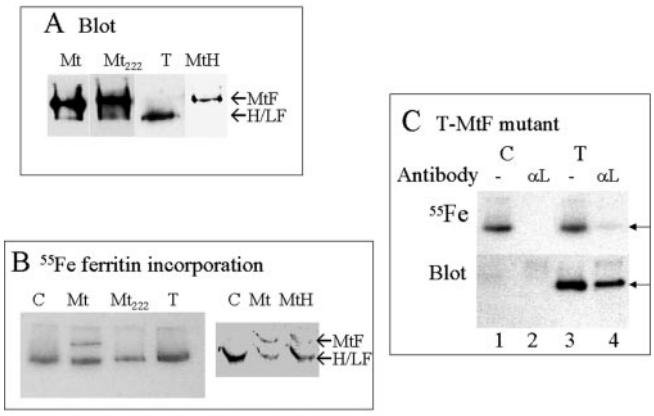

FIG. 2. **Expression and iron incorporation of MtF mutants.** HeLa cells (2×10^5) were transiently transfected with 2 μg of the plasmids encoding for MtF (*Mt*), its mutant with substitutions E62K and H65G (H-chain numbering) to inactivate the ferroxidase center (Mt_{222}), its mutant deleted of the mitochondrial leader sequence (*T*), and the chimeric construct for the mitochondrial leader sequence fused to H-subunit (*MtH*). Soluble proteins from cell homogenates were analyzed on non-denaturing gels as described in the legend for Fig. 1. *A*, cells were harvested 48 h after transfection. 10 μg of the soluble proteins were resolved by PAGE, and the proteins were revealed by blotting with rH02 antibody, which recognizes HF and MtF using ECL development. *B*, after transfection, the cells were metabolically labeled for 18 h with ^{55}Fe as described in the legend for Fig. 1 (*panel A*) and homogenized, and 10 μg of protein soluble extracts were resolved by PAGE and exposed to autoradiography. *C*, control; *H/LF*, cytosolic ferritin. *C*, the homogenates of T-MtF transfectant cells metabolically labeled with ^{55}Fe were analyzed on non-denaturing PAGE before (−) or after incubation with an excess of anti-L-ferritin antibody (*αL*) to sequester cytosolic ferritins. Ferritin-bound iron was revealed by autoradiography (*upper*) and ferritin protein by blotting with the rH02 antibody (*lower*).

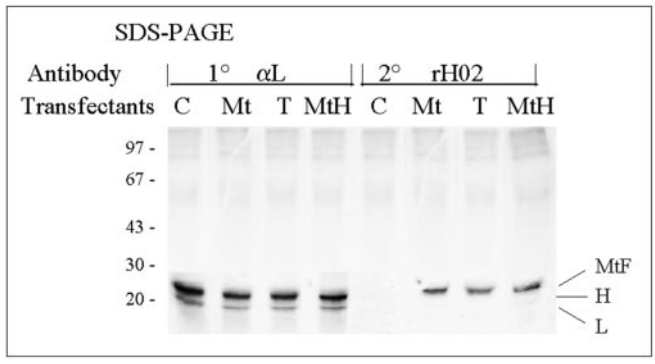

FIG. 3. **Immunoprecipitation of the transient transfectant cells.** After transient transfection, the cells (5×10^5) were grown for 30 h and then metabolically labeled for 18 h by incubation in medium containing 50 μCi/ml [^{35}S]methionine and [^{35}S]cysteine. Aliquots of the soluble fractions containing 4×10^6 cpm were sequentially precipitated first with a saturating amount of anti-L-ferritin antibody (*1° αL*) to collect cytosolic ferritins and then with saturating amounts of rH02 antibody (*2° rH02*) to precipitate the remaining ferritins. The precipitates were separated on 12% SDS-polyacrylamide gels and exposed to autoradiography. The *arrows* indicate the mobility of MtF and of the cytosolic H- and L-ferritin subunits. *T*, MtF mutant deleted of the mitochondrial leader sequence; *MtH*, chimeric construct for the mitochondrial leader sequence fused to H-subunit.

PAGE and exposed to autoradiography. Ferritins were initially identified from their electrophoretic mobilities. As before, untransfected cells showed incorporation of iron only into the cytosolic ferritin. The same occurred in the cells transfected with T-MtF and MtF_{222}. However, cells transfected with MtF and Mt-HF showed uptake also into a slower band corresponding to MtF or to Mt-HF (Fig. 2*B*). The results show that the mitochondrially targeted MtF and Mt-HF both took up iron, whereas MtF_{222} did not, consistent with the mutational inactivation of the ferroxidase center (13). No statement could be made at this point regarding T-MtF since it co-migrates with cytoplasmic ferritins. Cytosolic ferritins are hybrids of H- and L-chains, and these molecules can be selectively removed with antibodies specific for L-chains. This treatment removed essentially all of the ^{55}Fe-labeled ferritin in both the control and the T-MtF transfectants (Fig. 2*C*, *upper gel*, *lanes 2* and *4*). However, it left in solution most of the ferritin protein in the T-MtF transfectant cells, as shown by blotting with antibody rH02 (Fig. 2*C*, *lower gel*, *lanes 3* and *4*). This finding shows that the transfected T-MtF in the cytosol did not form heteropolymers with endogenous L-chains and is probably a homopolymer. In addition, these results indicate that T-MtF shells do not incorporate significant amounts of iron in this transient expression system. Possible reasons for this apparent anomaly are discussed later.

To compare relative synthesis rates of cytosolic and mitochondrial ferritins, the transfected cells were metabolically labeled with [^{35}S]methionine. The labeled lysates were treated first with LF03 to precipitate L-containing ferritins and then with the rH02 antibody to precipitate any remaining ferritins of the H or MtF type. The pellets were analyzed by autoradiography after SDS-PAGE (Fig. 3). The first immunoprecipitates contained only H- and L-chains at ratios that were remarkably similar in the control and the three transfectants, a further evidence that the transfected MtF chains did not associate with L-subunits. The subsequent addition of rH02 antibody gave no further precipitate from the control cells, confirming that the endogenous cytosolic ferritins were removed with the anti-L antibodies and supporting previous indications that HeLa cells contain few if any H-chain homopolymers (13). This result also confirms the essential lack of MtF in normal HeLa cells. In contrast, the addition of rH02 antibody to the anti-L-supernatant fractions of the three transfectants gave additional precipitates, each giving a single band of similar size. Thus the cell-processed MtF and Mt-HF peptides are slightly larger than the H-chain but similar in size to the T-MtF subunit whose leader had been experimentally truncated at residue 58, corresponding to H-2. This result indicates that this is the natural cleavage site of MtF. Since MtF and T-MtF subunits have the same size, the slower electrophoretic mobility of MtF shells as compared with T-MtF shells (Fig. 2*A*) is more likely due to differences in surface charge, perhaps from *N*-acetylation of the N terminus of cytosolic ferritins. A similar phenomenon has been described for the recombinant human H-ferritin expressed in *E. coli*, which has a free N terminus and slower electrophoretic mobility than the natural H-ferritins (33).

Cell Localization—Transfected cells were examined by *in situ* immunostaining using rH02 antibody. In addition to a faint background from cytosolic ferritins, MtF, MtF_{222}, and Mt-HF transfectants all showed strong staining of filamentous and perinuclear intracellular bodies in 10–20% of the cells, whereas the T-MtF transfectants showed a strong diffuse and cytosolic staining (Fig. 4*A*) similar to that obtained with H-ferritin wild type transfectants (not shown). For a more precise localization, the MtF transfectants were frozen, sliced, and subjected to immunostaining with the anti-Δ9MtF-specific antibody followed by immunogold secondary antibody. Electron microscopic imaging showed that most of the signal accumulated inside the mitochondria with sparse background signals elsewhere, possibly from damage during slide preparation (Fig. 4*B*). The gold granules in the mitochondria appeared to be in the soluble matrix and were not associated with the crystae or membranes.

MtF Expression and Cell Iron Metabolism—To analyze whether MtF expression affected cellular iron metabolism, we

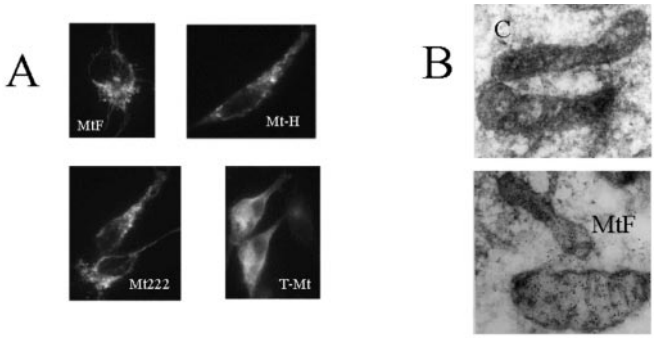

FIG. 4. **Immunostain of the transient transfectant cells.** *A*, the HeLa cells transfected with the plasmids encoding the four different ferritins were grown for 30 h, fixed, and permeabilized. The preparations were overlaid with rH02 antibody followed by secondary fluorescein isothiocyanate antibody, and the fluorescence image was captured. The filamentous bodies stained in MtF, MtF_{222} (*Mt222*), and Mt-HF (*Mt-H*) cells are analogous to those described in Ref. 17 and identified as mitochondria, whereas the diffuse cellular stain of T-MtF (*T-Mt*) cells is consistent with a cytosolic distribution. *B*, immunogold staining of the untransfected (*C*) and MtF-transfected HeLa cells (*MtF*) with anti-Δ9MtF antiserum for MtF recognition followed by secondary anti-IgG-coated gold particles. Cryosections were then examined by electron microscopy.

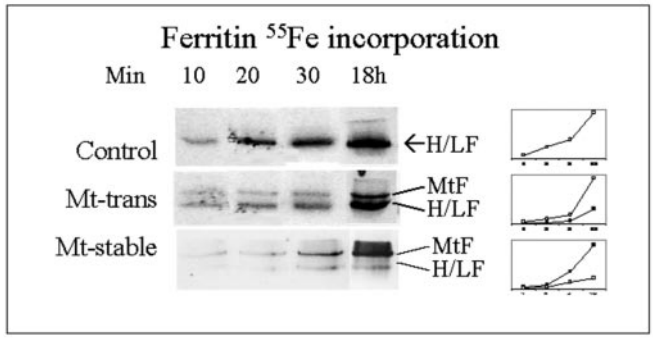

FIG. 5. **Cellular iron incorporation in transient and stable MtF transfectant HeLa cells.** Cells of transfected MtF-tTA clone (*Mt-stable*) were grown for 7 days in the absence of doxycycline to induce MtF expression and then incubated with FAC for the indicated time in parallel with untransfected HeLa cells (*Control*), and transiently MtF transfected cells (*Mt-trans*). The cell extracts (3 μg of total proteins) were separated on non-denaturing PAGE followed by autoradiography to monitor ferritin-bound ^{55}Fe label. Densitometric quantitations of ferritin bands in arbitrary units are shown on the *right side* of each panel (*empty squares*, cytosolic ferritin (H/L); *solid squares*, MtF). The *arrows* indicate the mobility of MtF and cytosolic ferritin (*H/LF*).

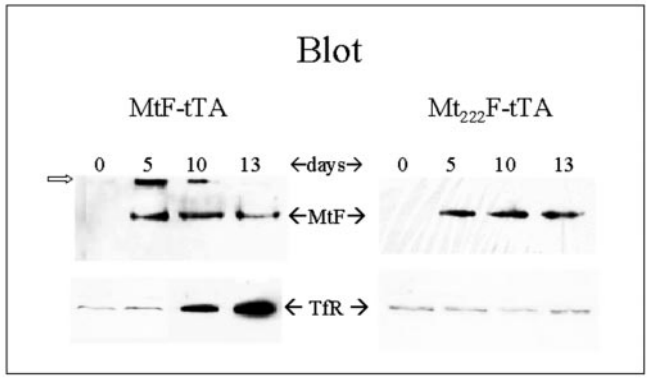

FIG. 6. **Time course of ferritin and transferrin receptor expression in MtF-tTA and MtF_{222}-tTA clones.** Cells of the MtF-tTA and MtF_{222}-tTA clones grown in 2 ng/ml doxycycline were transferred to a medium without doxycycline and harvested at indicated days. After homogenization, the soluble protein extracts (30 μg) were separated and analyzed by blotting using secondary horseradish peroxidase-labeled antibodies and ECL development. In the *upper panel*, the samples were resolved by non-denaturing PAGE and blotted with mouse anti-MtF antibody, and in the *lower panel*, the samples were loaded on 12% SDS-polyacrylamide gels and blotted with anti-human transferrin receptor antibody. The *arrows* indicate the position of MtF and transferrin receptor (*TfR*), and the *empty arrow* indicates the origin of the gel.

first studied iron incorporation from FAC and ^{55}Fe-labeled transferrin in the transiently transfectant cells. The MtF transfectants incorporated similar amounts of iron from FAC or from ^{55}Fe-labeled transferrin (not shown) over an 18-h incubation as the control cells (empty vector transfected). Uptake of iron from FAC into both MtF and cytosolic ferritin was detectable after only a 10-min incubation as shown by non-denaturing PAGE analyses (Fig. 5). In all experiments with transient expression, MtF accounted for ~20% of the total ferritin iron. Since only 20–30% of cells express MtF, the retention of iron as MtF in transfected cells was presumably much higher than 20%.

For a more reliable assessment of the effect of MtF expression on cellular iron metabolism, we produced stable transfectants expressing MtF and MtF_{222} under the regulation of the tetracycline promoter. Clones MtF-tTA and Mt_{222}F-tTA expressed the highest level of transfectant protein in the absence of doxycycline repressor as judged by Western blot with anti-MtF antibody and were selected for further study. A representative time course of accumulation of MtF and MtF_{222} is shown in Fig. 6. No MtF was detected in the transfectants in the presence of doxycycline. After withdrawal of the repressor, MtF was expressed with the highest accumulation occurring between days 5 and 10 (Fig. 6, *upper panel*). The increased expression of MtF was followed by a marked increase in the expression of transferrin receptor (Fig. 6, *lower panel*). By contrast, the expression of MtF_{222} had no detectable effect on transferrin receptor expression (Fig 6, *lower panel*). Since the expression of transferrin receptor is inversely related to the level of free iron in the cytosol, the results in Fig. 6 suggested that the expression of MtF in mitochondria reduces the level of free iron in the cytosol. To test this hypothesis, we examined the effect of MtF expression on cytosolic ferritins whose synthesis is also largely controlled by the levels of free iron. This was done by metabolic labeling of proteins with [^{35}S]methionine followed by autoradiography of the labeled endogenous and transfected ferritin chains. As expected, only the cytosolic H- and L-ferritins were labeled in the presence of the doxycycline repressor in both cell types. Doxycycline withdrawal induced MtF and MtF_{222} expression in the two clones, as indicated by labeling in the Mt chain. The extent of labeling was about 20% of that in the H-chain in the repressed cells as judged by densitometry. However, the synthesis of H- and L-chains was greatly reduced (a range of 50–80% in different experiments) in the MtF clone. On the other hand, the synthesis of H- and L-chains was unaffected in the clone expressing MtF_{222} that does not incorporate iron (Fig. 7*A*). This reduced synthesis of ferritin following the induction of MtF was reflected in the amount of H- and L-ferritin protein. As shown in the ELISA assays in Fig. 7*B*, the level of the cytosolic H-ferritin in cells expressing MtF was about half of that of the control cells or of the MtF_{222} cells, even after 18 h of incubation with 3 μM FAC (Fig. 7*B*).

The decreases in synthesis and the levels of cytosolic ferritins resulting from MtF expression seemed likely to be due to a redistribution of free iron from cytosol to mitochondria. This conclusion was confirmed by examining the distribution of exogenous iron between MtF and in these cells. As shown in Fig. 5, exogenous iron appears in cytosolic and mitochondrial ferritin after only 10 min of incubation. However, more iron was found in MtF in the stable line at all periods. After only 30

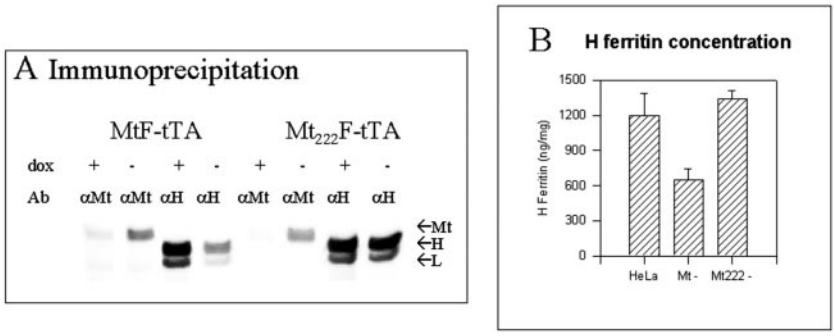

FIG. 7. **Evaluation of the endogenous cytosolic ferritins.** *A*, the uninduced (*dox*+) and induced (*dox*−) HeLa cell clones (MtF-tTA and MtF_{222}-tTA) were metabolically labeled for 18 h with 50 μCi/ml [^{35}S]methionine and [^{35}S]cysteine in methionine and cysteine-free medium. Samples of 10 μg of soluble extract were first immunoprecipitated with saturating amount of anti-MtF antibody (*αMt*), and then the soluble fraction was precipitated again with saturating amount of anti-H-ferritin, rH02 antibody (*αH*). The precipitates were analyzed on 12% SDS-polyacrylamide gels under denaturing conditions and exposed to autoradiography. The *arrows* indicate the mobility of MtF (*Mt*) and of cytosolic H-ferritin (*H*) and L-ferritin (*L*) subunits. *dox*, doxycycline. *Ab*, antibody. *B*, the induced, doxycycline-free HeLa cell clones (MtF-tTA and MtF_{222}-tTA) and control cells (HeLa) were incubated with 3 μM FAC for 18 h. HF concentration was determined with ELISA. Mean and S.D. were from three independent experiments.

min, MtF accounted for about 60% of the total ferritin iron pool and about 75% by 18 h. As expected, no iron was found in MtF_{222} transfectants (not shown). Thus exogenous iron can enter the mitochondrial matrix and be sequestered as MtF as rapidly as it accesses cytosolic ferritins. In addition, the expression of MtF causes a redistribution of cellular iron so that more iron is retained in mitochondria.

We next evaluated the rates of release of this newly bound radioactive iron in the ferritins in cytosol and mitochondria by removing the exogenous radioactive iron and by chelating free cytosolic iron with desferrioxamine (DFO). After 24 h of treatment with DFO, cells were lysed, the ferritins were resolved by non-denaturing PAGE, and their retention of labeled iron was assessed by autoradiography. These experiments showed that essentially all of the newly incorporated iron was lost from cytosolic ferritin in control cells. This occurred even without DFO treatment. By contrast, very little iron was lost from MtF in this 24-h period, and chelation of cytosolic iron by DFO treatment did not greatly affect the retention of iron in MtF (Fig. 8*A*).

The relative stability of the ferritin proteins in this 24-h period following iron removal or DFO treatment was assessed by labeling cells with [^{35}S]methionine for 18 h followed by a 24-h chase. Mitochondrial and cytosolic ferritins were separated by sequential immunoprecipitations, first with anti-MtF and then with anti-H antibodies. Residual radioactivity in the ferritin subunits was assessed by autoradiography after their resolution by PAGE in SDS gels. After a 24-h chase, the cytosolic ferritin subunits decreased below 30% of the initial signal, as before (13), whereas the MtF subunit persisted at about 50% (Fig. 8*B*). This shows that MtF protein and iron turn over at a slower rate than cytosolic ferritin.

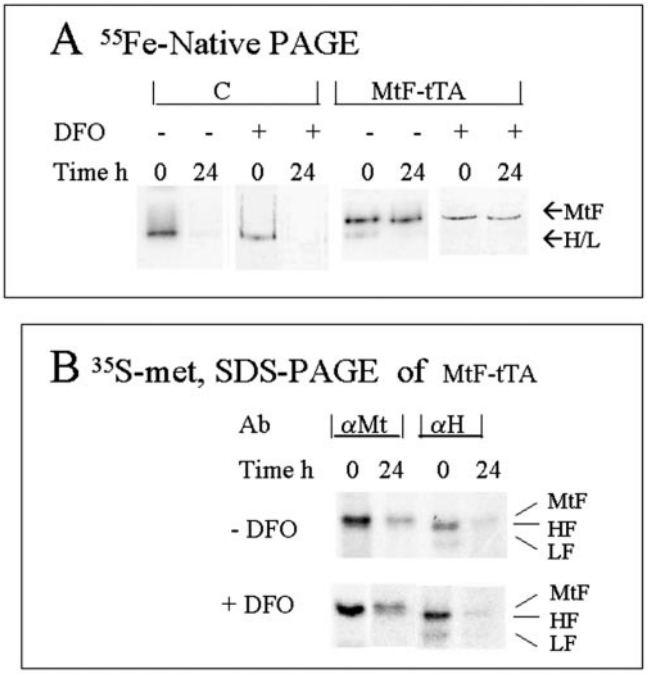

FIG. 8. **Ferritin iron release and protein degradation.** Cells of clone MtF-tTA were induced by for 7-day growth in doxycycline-free medium and analyzed in parallel with control untransfected HeLa cells (*C*). *A*, the cells were labeled for 18h with ^{55}Fe (2 μCi/ml FAC, 200 μM ascorbic acid in serum-free DMEM). Cells were then washed and incubated for a further 24 h in complete medium in the presence or absence of 1 mM DFO. Soluble proteins (5 μg) from cells collected at times 0 and 24 h (of soluble protein) were resolved by non-denaturing PAGE and exposed to autoradiography for detection of ferritin-bound iron. The *arrows* indicate the mobility of MtF and of cytosolic endogenous ferritin (*H*/*L*). *B*, the MtF-tTA cells were metabolically labeled for 18 h with 50 μCi/ml [^{35}S]methionine and [^{35}S]cysteine in methionine and cysteine-free medium, washed, and collected or grown for a further 24 h in complete medium in the presence or absence of 1 mM DFO. Homogenates of cells collected at times 0 and 24 h (10 μg of soluble protein) were first immunoprecipitated with a saturating amount of anti-MtF antibody (*αMt*), and then the soluble fraction was precipitated again with saturating amounts of anti-H-ferritin antibody (*αH*). The precipitates were analyzed on 12% SDS-polyacrylamide gels and exposed to autoradiography. The *arrows* indicate the mobility of Mt, H-, and L-ferritin subunits. *Ab*, antibody.

DISCUSSION

In a previous study, we identified a new ferritin, MtF, that is targeted to mitochondria (17). The possible roles of this ferritin are not clear. It is expressed at very low levels in most normal cells expect testis but at very high levels in erythroblasts with disrupted heme synthesis. Since it was impractical to use either type of cell, we transfected HeLa cells with cDNAs for MtF and some engineered variants of MtF and HF-ferritins to explore some aspects of the metabolism of this new protein.

Our results showed that a construct of the H-ferritin with an attached MtF leader sequence is processed like MtF into ~22-kDa peptides, consistent with cleavage of the leader at the predicted site, two residues before the start of the H-subunit. Immunohistochemical staining confirmed that this construct was properly targeted to the mitochondria. This result, along with previous experiments with green fluorescent protein fusions (17), demonstrates that this leader sequence is sufficient for mitochondrial targeting. The demonstration that H-sub-

units with this leader are also targeted to mitochondria and assembled into functional ferritin shells indicates that MtF has no specific structural properties for uptake and processing in the organelle. In addition, the assembly of the processed subunits into shells does not seem to require other mitochondrial components since a construct of MtF lacking the leader and expressed in cytosol also assembled into multimeric shells. We did not detect the MtF precursor by immunoprecipitation in any cell extract (Fig. 3), and *in vitro* translation data showed that the precursor does not assemble in ferritin shells (not shown). These observations suggest that MtF accumulates only inside the mitochondra.

Western analyses and cellular labeling experiments with the ^{55}Fe label confirmed that MtF is not present at detectable levels in normal HeLa cells. However, transfected MtF took up iron and in similar amounts as H-ferritin targeted to mitochondria. This activity of MtF depended on residues identified previously as critical for H-ferroxidase activity. These observations suggest that MtF and H-ferritin can use and process similar iron substrates.

The finding that T-MtF shells transiently expressed in the cytosol do not incorporate iron is puzzling in view of the efficiency of both HF and MtF in incorporating iron when expressed transiently in mitochondria. However, H-ferritin also fails to incorporate iron and does not form hybrids with L-ferritin when transiently expressed in HeLa cells but does both in stable transfectants (13, 30). The reasons for these anomalies are not known. Perhaps cytosol has limiting amounts of required cofactor(s) or only heteropolymers function in cytosol. More important for the present work is the observation that the ferritins take up and retain iron more efficiently when inside the mitochondrion than in the cytosol. In stable transfectants, MtF homopolymers accounted for more than 70% of the total ferritin iron (Figs. 5 and 8*A*), whereas previous studies showed that transfected H-ferritin homopolymers incorporated only a minor amount of the total ferritin iron (13). This suggests that the double membrane of mitochondria does not reduce the diffusion of iron in the organelle. Perhaps the mitochondrion offers more favorable conditions for ferritin iron uptake such as a more suitable redox status.

The activity of MtF has a profound effect on cellular iron homeostasis. In the transient experiments, in which only 20–30% of the cells were transfected, MtF incorporated about 20% of the total ferritin iron. In the stable transfected HeLa cell clone, where all cells express MtF, this ferritin sequestered up to 70% of total ferritin-bound ^{55}Fe label, leaving only a small proportion to the cytosolic endogenous ferritin. In addition, iron supplied either as transferrin or as a soluble chelate (FAC) was taken up as rapidly by MtF in mitochondria as by ferritins in the cytosol. This occurred within only 10 min of incubation both in the transient and in the stable transfectants (Fig. 5). The retention of iron as MtF results in reductions in the labile iron pool, as evidenced by the decrease of cytosolic ferritin levels and the up-regulation of the expression of transferrin receptor. The effects should be concentration-dependent, but without a specific ELISA assay, we could not quantify the levels of MtF expression. However, from immunoblotting signals, we estimated that the levels of MtF in the stable clones were comparable with those obtained previously in the stable H-ferritin HeLa clones, 10–20-fold greater than the background of cytosolic ferritin (13). These high levels may reflect the observed greater stability of MtF shells (Fig. 8*B*).

MtF appears to retain iron better than H-ferritin, even when cells are treated with DFO. High levels of MtF may therefore effectively trap free iron and lead to an iron-deficient phenotype in the cytosol. On the other hand, increased deposition of iron as MtF does not seem to be particularly detrimental to mitochondrial function since the transfected cells could be grown for more than 4 weeks without evident signs of toxicity. In these circumstances, MtF may play a role similar to that of the cytosolic ferritins, *i.e.* buffering and regulating iron availability and protecting the cell from harmful reactive oxygen species (13). This function might be facilitated by the localization of MtF in the matrix, where free iron is converted into heme (34) and other iron molecules.

The emerging picture is that MtF may have specialized functions in only some cells. Although exogenous iron is readily available to mitochondria, excess iron in most cells is not usually retained in MtF but is sequestered in cytosolic ferritin. This arrangement might ensure that MtF does not unnecessarily trap iron needed for the formation of heme and Fe/S clusters. Thus MtF may not normally be required to detoxify excess iron, and mitochondria may have other mechanisms for avoiding iron toxicity. It also seems unlikely that MtF is an obligatory conduit for iron for heme and other iron molecules since MtF levels are low in normal erythroblasts when massive amounts of iron are delivered to mitochondria for heme synthesis.

A different picture emerges if the normal processing of iron in mitochondria is altered. Normal erythroblasts contain low levels of MtF and MtF-bound iron (17). However, when heme synthesis is disrupted, as in sideroblastic anemia, large amounts of iron continue to be imported into mitochondria. This iron is incorporated into MtF as a result of a very large increase in the level of this protein (17). The mechanism of this apparent induction is not known. It does not appear to arise from iron-induced up-regulation of translation since MtF mRNA lacks an iron regulatory element (17), and MtF levels in HeLa cells are not increased by exogenous iron (not shown). Erythroid-specific regulators of transcription also seem an unlikely explanation since the levels of MtF in normal erythroblasts are low. However, down-regulation by the end product heme is possible. In this case, low levels of heme would lead to increases in MtF.

The avidity of MtF for iron may be relevant to other conditions resulting in increases in mitochondrial iron, such as the heart or brain mitochondria of subjects with Friedreich's ataxia (24). The excess iron, particularly the filterable mitochondrial iron, is thought to disturb mitochondrial function (35), but it is not known whether the levels of MtF are increased in these disorders. If not, up-regulation of MtF might prove a useful therapeutic approach to avoid iron toxicity.

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Human Mitochondrial Ferritin Expressed in HeLa Cells Incorporates Iron and Affects Cellular Iron Metabolism

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