Am. J. Hum. Genet. 63:1663–1674, 1998

Functional Characterization of Missense Mutations in *ATP7B:* Wilson Disease Mutation or Normal Variant?

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Summary

Wilson disease is an autosomal recessive disorder of copper transport that causes hepatic and/or neurological disease resulting from copper accumulation in the liver and brain. The protein defective in this disorder is a putative copper-transporting P-type ATPase, ATP7B. More than 100 mutations have been identified in the *ATP7B* gene of patients with Wilson disease. To determine the effect of Wilson disease missense mutations on ATP7B function, we have developed a yeast complementation assay based on the ability of ATP7B to complement the high-affinity iron-uptake deficiency of the yeast mutant *ccc2*. We characterized missense mutations found in the predicted membrane-spanning segments of ATP7B. Ten mutations have been made in the *ATP7B* cDNA by site-directed mutagenesis: five Wilson disease missense mutations, two mutations originally classified as possible disease-causing mutations, two putative *ATP7B* normal variants, and mutation of the cysteine-proline-cysteine (CPC) motif conserved in heavy-metal–transporting P-type ATPases. All seven putative Wilson disease mutants tested were able to at least partially complement *ccc2* mutant yeast, indicating that they retain some ability to transport copper. One mutation was a temperature-sensitive mutation that was able to complement *ccc2* mutant yeast at 30°C but was unable to complement at 37°C. Mutation of the CPC motif resulted in a nonfunctional protein, which demonstrates that this motif is essential for copper transport by ATP7B. Of the two putative *ATP7B* normal variants tested, one resulted in a nonfunctional protein, which suggests that it is a disease-causing mutation.

Received July 31, 1998; accepted for publication October 1, 1998; electronically published November 23, 1998.

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0002-9297/98/6306/0013$02.00

Introduction

Integral membrane P-type ATPases have been shown to be important for heavy-metal homeostasis in eukaryotes and prokaryotes (Brown et al. 1992; Silver 1992; Bull and Cox 1994; Solioz 1998). Prokaryotic heavy-metal–transporting P-type ATPases are thought to have evolved as a defense against toxic metals in the environment. In eukaryotes, copper-transporting P-type ATPases are involved in copper and iron homeostasis. Defective copper transporters are responsible for human Menkes disease and Wilson disease.

Wilson disease is an autosomal recessive disorder of copper transport mapped to 13q14.3 (Farrer et al. 1991). The disease has a worldwide frequency of ~1/35,000, is characterized by chronic liver and/or neurological disease, and is often accompanied by kidney malfunction (Cox and Roberts 1998). Symptoms are caused by deposition of copper in the liver, brain, and kidneys as a result of severely impaired hepatic biliary excretion of copper. It is unclear whether deposition of copper in the brain and kidneys is specific or secondary to hepatic copper overload (Danks 1995). Incorporation of copper into ceruloplasmin is impaired, although Wilson disease patients may have normal levels of serum ceruloplasmin.

The Wilson disease gene (*ATP7B*) has been cloned and is predicted to encode a copper-transporting P-type ATPase with a molecular mass of 159 kD (Bull et al. 1993; Tanzi et al. 1993). On the basis of northern blots, *ATP7B* has been found to be expressed at high levels in the liver and kidneys and at low levels in the brain. Experiments with rats and in hepatic cell cultures have shown that ATP7B is required for biliary copper excretion via the lysosomes and for incorporation of copper into ceruloplasmin within the secretory pathway (Gross et al. 1989; Schilsky et al. 1994; Murata et al. 1995). ATP7B has been localized to the trans-Golgi network (TGN) of hepatocytes and has been shown to undergo copper-dependent trafficking to an undefined vesicular compartment (Hung et al. 1997). One study suggests that ATP7B may also be localized to the mitochondria (Lutsenko and Cooper 1998). ATP7B shares a high degree of similarity with the copper-transporting P-type ATPase defective in Menkes disease (ATP7A; Bull et al. 1993),

which undergoes a copper-dependent trafficking event from the trans-Golgi to the plasma membrane of Chinese hamster ovary cells (Petris et al. 1996).

More than 100 mutations have been found in the *ATP7B* gene of Wilson disease patients (Krawczak et al. 1997). Nonsense, frameshift, and splice-site mutationshave been found throughout the gene. Missense mutations, however, tend to be clustered within the putative ATP-binding domain and the membrane-spanning segments, which supports the importance of these structures for the proposed ATP-dependent copper transport function of ATP7B. No disease-associated missense mutations have been found in the metal-binding motifs of ATP7B (or ATP7A), perhaps because of functional redundancy among the six repeated motifs. A base-pair substitution generally is considered to be disease causing if it is not found on $\geq$50 normal chromosomes from the same population, if no other mutations can be detected, if the amino acid substitution is nonconservative, or if the substituted amino acid is conserved in functionally related proteins (e.g., ATP7A). There may be difficulty in distinguishing disease-causing missense mutations from rare normal variants not yet found on normal chromosomes, especially when the amino acid change appears to be conservative. Therefore, it is desirable to have a functional means to distinguish disease mutations from normal variants.

Wilson disease symptoms are highly variable, even in patients homozygous for the common northern European mutation histidine1069glutamine (H1069Q; D. W.C., unpublished data). Environmental effects such as dietary copper intake may be a factor. Genetic background may also affect disease severity. For example, among patients, allelic differences in metallothionien and superoxide dismutase genes could alter the ability of tissues to cope with copper overload. Most Wilson disease mutations occur at low frequency; therefore, most patients are compound heterozygotes. These factors combine to make genotype/phenotype correlations difficult. The ability to determine the degree to which a missense mutation affects protein function may be helpful for the interpretation of the phenotype of patients and may give insight into the biochemical basis of disease variability.

The yeast (*Saccharomyces cerevisiae*) homologue of *ATP7B* is *CCC2* (Yuan et al. 1995). In yeast, the protein product, Ccc2p, is part of the pathway required for high-affinity iron uptake (fig. 1). We have developed a functional assay for ATP7B, on the basis of the complementation of the yeast mutant *ccc2*. Expression of ATP7B in *ccc2* mutant cells complements the mutation and restores the cells' ability to grow on iron-limited medium. Similiar functional assays recently have been reported and used to study ATP7A (Payne and Gitlin 1998), ATP7B (Hung et al. 1997; Iida et al. 1998), and CUA-1, the *Caenorhabditis elegans* homologue of ATP7A/ATP7B (Sambongi et al. 1997; Yoshimizu et al. 1998). We are using complementation of the high-affinity iron-uptake–deficiency phenotype of the *ccc2* mutant yeast as an indirect assay to test the degree to which Wilson disease mutations found in the membrane-spanning segments of ATP7B affect protein function.

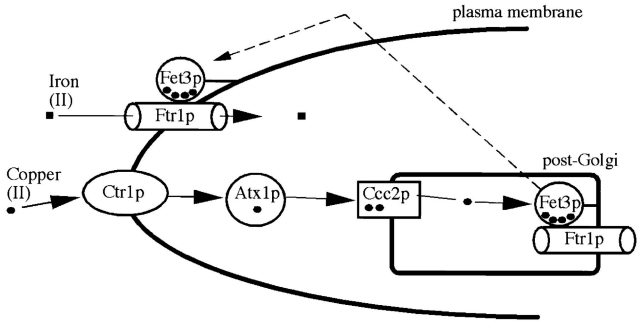

Figure 1 Model of yeast copper and iron homeostasis. The plasma membrane protein Ctr1p transports copper with high affinity into the cytoplasm (Dancis et al. 1994). Copper is carried in the cytoplasm by the copper chaperone Atx1p (Lin et al. 1997), which delivers copper to Ccc2p. Copper is supplied by Ccc2p, within a post-Golgi compartment (Yuan et al. 1997), to the plasma membrane oxidase Fet3p, which functions with the high-affinity iron transporter Ftr1p to import iron (Stearman et al. 1996). When yeast cells lack Ccc2p, copper is not incorporated into Fet3p, and subsequently the cells lack high-affinity iron uptake (Yuan et al. 1995). In the absence of high-affinity iron uptake, *ccc2* mutant yeast cells are unable to grow on iron-limited medium. This diagram has been adapted from recent publications (Stearman et al. 1996; Lin et al. 1997).

Material and Methods

Yeast Strains

The wild-type strain used in all experiments was the protease-deficient *S. cerevisiae* strain BJ2168 (*MAT***a***-pep4–3 prcl–407 prb1–1122 ura3–52 trp1 leu2;* Zubenko et al. 1980). This strain was chosen to minimize potential proteolysis during procedures such as the oxidase assay in which samples are not heated prior to electrophoresis. Yeast mutants *fet3* and *ccc2,* which lack functional *FET3* and *CCC2* genes, were made by the transformation of BJ2168 with the linearized plasmids pΔfet3 and E5-URA3.4 (plasmid described by Yuan et al. 1995), respectively. Integrations were selected on synthetic dextrose (SD) medium lacking uracil. Transformants were plated on nonfermentable glycerol carbon-source medium either unsupplemented or supplemented with 500 μM copper sulfate or 1 mM ferrous ammonium sulfate, to confirm the metal-dependent respiration-deficiency phenotype of the mutant strains as described by Yuan et al. (1995). All transformations were performed

by use of a modified lithium acetate method (Elble 1992).

Construction of ATP7B *cDNA and Mutagenesis*

Total human liver RNA was used for reverse transcription (RT) using random hexamer primers and molony murine leukemia–virus reverse transcriptase, in accordance with the protocol of the manufacturer (Pharmacia). cDNA obtained by this method was used as a template for PCR amplification of five overlapping fragments that contained the coding region of *ATP7B,* by use of *pfu* polymerase in the buffer supplied by the manufacturer (Stratagene). Primers were chosen in such a way that unique restriction sites were present at the ends of each fragment, which facilitated the construction of the full-length cDNA. A 5′ *Bam*HI site was added to the PCR primer sequence immediately preceding the initiating ATG codon. PCR fragments were A-tailed by incubation with *Taq* polymerase. A-tailed PCR fragments were cloned by use of T/A-cloning vectors (Promega, Invitrogen). The two most 5′ cDNA fragments encoding the copper-binding domain proved to be unstable when transformed into bacteria, which resulted in plasmid mutation and rearrangements. This was corrected by the addition of 100 μM $CuSO_4$ to the culture medium. Each fragment was sequenced (Sequenase, Amersham) to ensure that no sequence errors were present, and the full-length cDNA was constructed by ligating the fragments together. The final cDNA construct was cloned into pUC19 by use of the 5′ *Bam*HI site and a 3′ *Sal*I site from the polylinker of the Promega T/A vector.

To create mutations in the *ATP7B* cDNA, oligonucleotides carrying the desired codon changes were synthesized. Mutant cDNAs were made by use of the QuickChange method (Stratagene). The fragments were sequenced to ensure that there were no secondary mutations and then were ligated back into an unmanipulated cDNA, to create the mutant constructs.

Expression Vectors

For high-level expression of ATP7B in yeast, cDNAs were cloned into the multicopy 2μ replication-origin vector pG3 (Schena et al. 1991). This vector utilizes a constitutive glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter, a phosphoglycerate kinase (PGK) terminator and polyadenylation sequence, and a tryptophan-selectable marker. A single-copy integrating vector named "pG4" was derived from pG3. The pG3 2μ replication origin, carried on a single *Eco*RI fragment, was removed by use of restriction-enzyme digestion. The vector lacking its yeast origin of replication was agarose-gel purified (Qiagen) and ligated to form pG4. Expression vectors were transformed into yeast, and transformants were selected on SD medium lacking tryptophan. Genomic DNA isolated from yeast strains that carried pG4 constructs were analyzed by use of Southern blot analysis, to confirm that the constructs were correctly integrated as single copy.

Complementation Assay

The assay medium consisted of SD medium made with a yeast nitrogen base lacking iron and copper (Bio-101), supplemented with all amino acids, except tryptophan, and 50 mM MES buffer, pH 6.1. The iron-limited medium was SD medium with 1 mM ferrozine (an iron-specific chelator), 50 μM ferrous ammonium sulfate, and 1 μM copper sulfate. The iron-limited medium was supplemented to 500 μM copper sulfate or 350 μM ferrous ammonium sulfate, to make the copper- or iron-sufficient control media, respectively. For the plating assay, the medium contained 2% Bacto agar (Difco). Cells were prepared for growth-curve analysis and plating assays in the following manner: stationary yeast cultures grown in SD liquid culture were washed with ice-cold distilled water, resuspended in iron-limited medium, and then grown to saturation overnight. For the plating assay, these cultures were diluted to an optical density of OD_{600} = 0.1 in 1 ml sterile deionized water; 5 μl of this cell suspension was streaked onto plates containing the assay media. Cells were grown at 30°C or at 37°C and were photographed after 48 h. To measure the growth curves of yeast strains, saturated yeast cultures grown in the iron-limited medium were pelleted and resuspended in fresh iron-limited medium. This was used to inoculate cultures at a density of OD_{600} = 0.1. Cultures were grown at 30°C for 24 h while growth was monitored spectroscopically after 0, 3, 6, 12, and 24 h.

Oxidase Assay

Fet3p oxidase activity was measured by use of a modified version of the assay described by Yuan et al. (1995). Cells were grown to mid–log phase (at 30°C) in SD media lacking tryptophan, iron, and copper and supplemented with 50 mM MES buffer, pH 6.1, and 0.5 μM copper sulfate. The buffer for homogenization consisted of 25 mM HEPES-NaOH, pH 7.4, 150 mM NaCl; and a protease-inhibitor cocktail of 30 μM leupeptin, 10 μM pepstatin A, and 5 μM aprotinin. The buffer was supplemented during homogenization, with either 1 mM bathocuproinedisulfonate (BCS, a copper chelator) and 1 mM ascorbate, to prevent copper-loading of Fet3p during homogenization, or with 50 μM $CuSO_4$, to reconstitute apo-Fet3p (Fet3p lacking bound copper) in vitro during homogenization, as a positive control. Cells from 25 ml of culture were washed twice with ice-cold distilled water and once with 800 μl supplemented buffer and then were resuspended in 200 μl of the same. Cells

were broken by vortexing with glass beads for 5 min. Yeast membranes were recovered by the addition of 800 μl buffer supplemented as above, and the supernatants were cleared of unbroken cells and heavy organelles by centrifugation at 10,000 *g* for 30 s. This step was repeated once. Membranes were collected by centrifugation at 20,000 *g* for 30 min, and then were washed with buffer containing 1 mM BCS. The final membrane pellet was solubilized in 100 μl buffer that contained 1mM BCS and 1% Triton X-100. Insoluble material was removed by centrifugation at 20,000 *g*. Protein was quantified by use of the Enhanced Bradford assay (Pierce Chemical). Solubilized membrane proteins (30 μg) were dissolved in Laemmli loading buffer (Laemmli 1970) lacking DTT and were run on 7.5% SDS-PAGE gels, without prior heating. Gels were equilibrated for 1 h in 20 gel volumes of oxidase buffer containing 100 mM sodium acetate, pH 5.7, 10% glycerol, 1 mM sodium azide, and 0.05% Triton X-100. Equilibrated gels were soaked in buffer containing 100 mM sodium acetate, pH 5.7, 0.5 mg/ml p-phenylenediamine dihydrochloride substrate, and 1 mM sodium azide, for 1 h in the dark. Gels were incubated overnight in the dark between cellophane sheets in a humidified box to develop bands of oxidase activity. All glassware was washed with hydrochloric acid and well rinsed with deionized water, to eliminate copper contamination.

Polyclonal Antibody against ATP7B

A histidine-tagged fusion protein corresponding to the C-terminal fragment of ATP7B (amino acids 1375–1465) was expressed in *Escherichia coli*. The protein was very insoluble and was purified by nickel-affinity chromatography under denaturing conditions in phosphate-buffered saline (PBS) that contained 8 M urea. Protein bound to the affinity columns was washed with buffer solution containing increasing concentrations of imidazole, and the fusion protein was eluted with 100 mM imidazole. Purified protein was judged to be >95% pure by SDS-PAGE analysis. Purified protein was dialyzed against several changes of PBS, to remove the urea. The insoluble precipitate was lyophilized. Lyophilized protein was resuspended, conjugated to keyhole limpet hemocyanin (KLH), and injected into rabbits, by Dr. Gregory Lee at the University of British Columbia. The antiserum obtained was affinity purified by running 250 μg fusion protein on 15% SDS-PAGE gels. Protein was electrophoretically transferred to polyvinylidene difluoride (PVDF) membrane, and the antigen band was cut out. Several antigen strips were blocked with 3% BSA in PBS containing 0.1% Tween-20 (PBST), were incubated at 4°C with 500 μl rabbit antiserum diluted in PBST, and then were washed. Bound antibody was eluted with 50 mM glycine, pH 2.7. The eluate was neutralized with Tris buffer and was concentrated by use of a centricon-30 (Amicon) ultrafiltration device. The final antibody, designated "anti-ATP7B.C10," was made to 1× PBS and 0.1% BSA, for storage at −20°C.

Protein Preparations and Western Blotting

Homogenization buffer consisted of 25 mM HEPES-NaOH, pH 7.4; 150 mM NaC1; 1 mM DTT; and a protease-inhibitor cocktail that contained 30 μM leupeptin, 10 μM pepstatin A, 5 μM aprotinin, and 1 mM EDTA. Cells from 10 ml stationary culture were washed twice with ice-cold distilled water, and once with homogenization buffer, and were resuspended in 200 μl buffer. Cells were broken by vortexing in the presence of glass beads for 5 min. The homogenate was centrifuged for 30 s at 10,000 *g* to remove unbroken cells and heavy organelles. Protein content was estimated by use of the Enhanced Bradford assay (Pierce Chemical). Protein was mixed with Laemmli loading buffer (Laemmli 1970) that contained DTT and was heated for 5 min at 75°C. SDS-PAGE was performed by use of 7.5% polyacrylamide gels. Protein was transferred electrophoretically to PVDF membrane for 450 V-h in Towbin buffer (Towbin et al. 1979) that contained 15% methanol and 0.01% SDS. Western blotting was performed by use of anti-ATP7B.C10 as the primary antibody, at a 1/3,000 dilution. The secondary antibody was horseradish peroxidase–conjugated goat antirabbit antibody, at a dilution of 1/10,000 (Pierce Chemical). Bound antibody was detected by enhanced chemiluminescense using Supersignal substrate (Pierce Chemical).

Results

Construction of ATP7B *cDNA*

ATP7B cDNA was constructed by the ligation of five overlapping fragments generated by RT-PCR. The two fragments that encode the copper-binding domain proved to be unstable and prone to frequent deletion and mutation when grown in *E. coli*. We hypothesized that expression of these fragments during growth could produce a polypeptide capable of binding copper, which would result in copper depletion within bacterial cells in such a way that only cells harboring deleted or mutated cDNAs were capable of growth. These clones were stabilized by supplementing the growth medium with copper. The full-length cDNA was ligated into pUC19 vector, with the coding region in reverse orientation to the vector's *lacZ* promoter. *ATP7B* cDNA cloned in this manner was stable in *E. coli* without added copper.

Antibody against ATP7B

A rabbit polyclonal antiserum was prepared against a histidine-tagged fusion protein containing the 10-kD

C-terminal fragment (residues 1375–1465) of ATP7B. The fusion protein, poorly antigenic in rabbits, was conjugated to KLH as a carrier protein and was used in this form to produce a polyclonal antiserum. The antiserum obtained recognized the fusion protein on western blots, whereas the preimmune serum did not (data not shown). The antiserum, designated "anti-ATP7B.C10," was affinity purified to reduce nonspecific signals on western blots.

Expression of ATP7B *in Yeast*

To express *ATP7B* in yeast, the cDNA was cloned into two expression vectors: a multicopy vector (pG3) and a single-copy integrating vector (pG4). These vectors were used so that the level of *ATP7B* expression could be controlled by plasmid copy number. *ATP7B* expression in yeast protein extracts was analyzed by western blotting using anti-ATP7B.C10 (fig. 2). ATP7B protein produced in yeast migrated as a doublet of ~163 and ~177 kD. The 163-kD band was the predominant product, since the larger band was not detected when produced at lower levels from the single-copy vector. The molecular weight of the 163-kD band agrees well with the predicted molecular weight of 159 kD for ATP7B and with the molecular weights determined previously by other groups (Hung et al. 1997; Iida et al. 1998; Lutsenko and Cooper 1998). The larger protein band probably represents a glycosylated form of ATP7B produced in yeast. ATP7B was found entirely in yeast membrane pellets, which is consistent with its predicted integral membrane structure (data not shown).

Functional Replacement of Ccc2p by ATP7B In Vivo

To test the function of normal and mutant ATP7B proteins, we exploited the high-affinity iron-uptake deficiency of the yeast mutant *ccc2*. In the absence of high-affinity iron uptake, *ccc2* mutant yeast harboring empty expression vectors were unable to grow on iron-limited medium (fig. 3*A*). Both single-copy and multicopy expression of ATP7B were able to complement the *ccc2* mutation, which allowed the cells to grow. Growth rescue of *ccc2* mutant yeast can be accomplished by the addition of high concentrations of copper or iron to the iron-limited growth medium (Yuan et al. 1995; Stearman et al. 1996). Under these conditions, iron enters the cells by low-affinity pathways, or copper binds to apo-Fet3p at the cell surface, which reconstitutes its activity and restores high-affinity iron uptake. All yeast strains tested were able to grow on culture plates that contained copper- or iron-sufficient media, demonstrating that the *ccc2* mutant strains that contain empty vectors were viable. Expression of ATP7B had no phenotypic effect on *fet3* or *ctr1* mutant yeast strains (data not shown).

Fet3p oxidase activity, which is dependent on the copper delivered to it by Ccc2p, serves as a marker enzyme for the putative copper-transporting function of Ccc2p (Yuan et al. 1995). Gel-based Fet3p oxidase assays were used to provide biochemical evidence that ATP7B can functionally replace Ccc2p in vivo. Fet3p activity was absent in BCS/ascorbate protein extracts from the *ccc2* mutant yeast–harboring vector only (fig. 3*B*). BCS and ascorbate were included in the homogenization buffer, to prevent adventitous copper incorporation into Fet3p, so that only holo-Fet3p (Fet3p with copper bound), copper loaded in vivo during biosynthesis, was detected. Expression of ATP7B in *ccc2* mutant yeast restored holo-Fet3p activity in vivo. As a positive control, Fet3p assays were performed on protein extracts from yeast homogenized in the presence of copper. Copper is able to reconstitute apo-Fet3p in vitro, restoring its oxidase activity such that total cellular Fet3p activity is detected (Yuan et al. 1995). Although Fet3p activity was absent in the *ccc2* strain, apo-Fet3p was still produced and was capable of functioning when reconstituted with copper (fig. 3*B*). Reconstituted total-Fet3p activity was considerably higher in the *ccc2* strain than in wild-type or ATP7B-complemented strains. In *ccc2* mutant cells, copper is not incorporated into Fet3p, and, therefore, high-affinity iron uptake is abolished (Yuan et al.1995). In the absence of high-affinity iron uptake, *FET3* expression is induced to high levels (Askwith et al. 1994), resulting in the accumulation of apo-Fet3p, which was detected by copper reconstitution in vitro. These data show that ATP7B was able to functionally replace Ccc2p. ATP7B provided the putative copper transport activity required to deliver copper to Fet3p and thereby restored the ability of *ccc2* mutant yeast to grow on iron-limited medium.

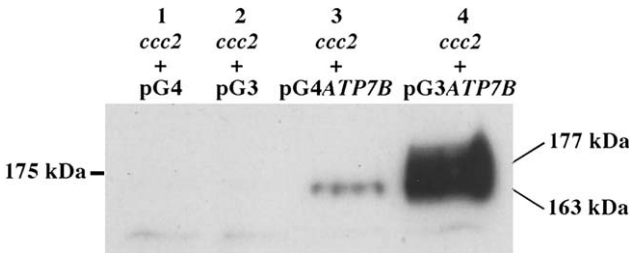

Figure 2 Expression of ATP7B in yeast. ATP7B was expressed in *ccc2* mutant yeast and analyzed by western blotting. Analysis of 40 μg total yeast protein was performed by enhanced chemiluminescense using an affinity-purified rabbit polyclonal antibody against the C-terminal 10-kD fragment of ATP7B (anti-ATP7B.C10). Lanes 1 and 2, Yeast protein from strains that harbored an empty expression vector only. Lanes 3 and 4, Yeast protein from strains that harbored the single-copy or multicopy ATP7B expression constructs, respectively. ATP7B migrated as a doublet of ~163 and ~177 kD. The multicopy vector (pG3) produced ~30-fold more ATP7B protein than did the single-copy vector (pG4), as estimated by densitometry (data not shown).

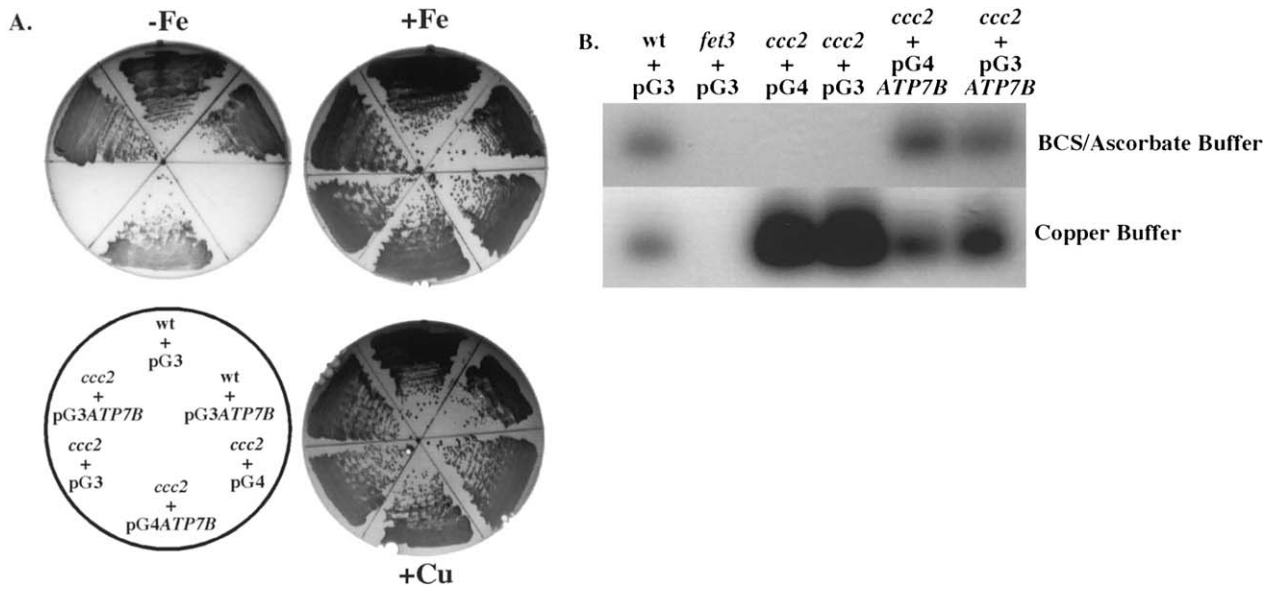

Figure 3 Complementation of *ccc2* mutant yeast by ATP7B. *A*, Plating assay. *B*, Fet3p oxidase assay. Oxidase activity in the BCS/ascorbate buffer represents holo-Fet3p copper loaded in vivo during biosynthesis. Oxidase activity in the copper buffer represents total-Fet3p activity reconstituted in vitro during homogenization.

Complementation of ccc2 *Mutant Yeast with* ATP7B *Mutants*

Site-directed mutagenesis was used to create mutations in the *ATP7B* cDNA that correspond to those found in the putative transmembrane segments of ATP7B from Wilson disease patients. Five proposed disease-causing mutations were made: aspartate765asparagine (D765N), arginine778leucine (R778L), arginine778glutamine (R778Q), glycine943serine (G943S), and proline992leucine (P992L). Two mutations, methionine769valine (M769V) and leucine776valine (L776V), originally designated as possible mutations because of the conservative nature of the mutations, were made, as well as two proposed normal variants, threonine977methionine (T977M) and valine995alanine (V995A) (Thomas et al. 1995). The cysteines of the cysteine-proline-cysteine (CPC) motif in transmembrane segment six were mutated to serine (mutant construct designated "CPC/S"). These mutant ATP7B proteins were expressed in *ccc2* mutant yeast from the single-copy expression vector. The genomic DNA of all recombinant strains was checked by Southern blotting, to ensure single-copy integration of the expression constructs. Complementation of *ccc2* mutant yeast with these constructs is shown in figures 4*A* and 5*A*. Mutant proteins D756N, M769V, L776V, G943S, and V995A appeared to fully complement *ccc2* mutants, as did normal ATP7B. Mutations R778L and P992L weakly rescued *ccc2* mutant yeast, and R778Q partially rescued the mutant yeast. As expected, mutation of the CPC motif abrogated function of ATP7B. The proposed normal variant T977M was the only Wilson disease mutation tested that resulted in a protein completely unable to complement *ccc2* mutant yeast.

These results were supported by the Fet3p oxidase-assay data (figs. 4*B* and 5*B*). When the oxidase assay results were interpreted, the ratio of holo-Fet3p activity to total-Fet3p activity was informative. Wild-type yeast and ATP7B-complemented *ccc2* mutant yeast had similar amounts of holo-Fet3p and total-Fet3p activity, which indicates that there was little apo-Fet3p present. However, yeast that expressed R778L, R778Q, and P992L mutant ATP7B proteins exhibited reduced holo-Fet3p activity but greatly increased total-Fet3p activity, which indicates the presence of large amounts of apo-Fet3p. This implies that ATP7B mutants with reduced function could not deliver enough copper to Fet3p during its biosynthesis in vivo, which led to an accumulation of the apo-enzyme form. Therefore, the greater the amount of total-Fet3p activity, in comparison with holo-Fet3p activity, the greater the defect in mutant ATP7B function. For example, R778L had a higher ratio of total-Fet3p to holo-Fet3p activity than did R778Q, which indicates that the R778L mutation affected ATP7B function more severely. Oxidase-assay results were reproduced in several experiments.

Growth curves of yeast grown in iron-limited medium were used to quantify the relative ability of Wilson disease mutant proteins, expressed from single-copy constructs, to complement *ccc2* mutant yeast (table 1 and

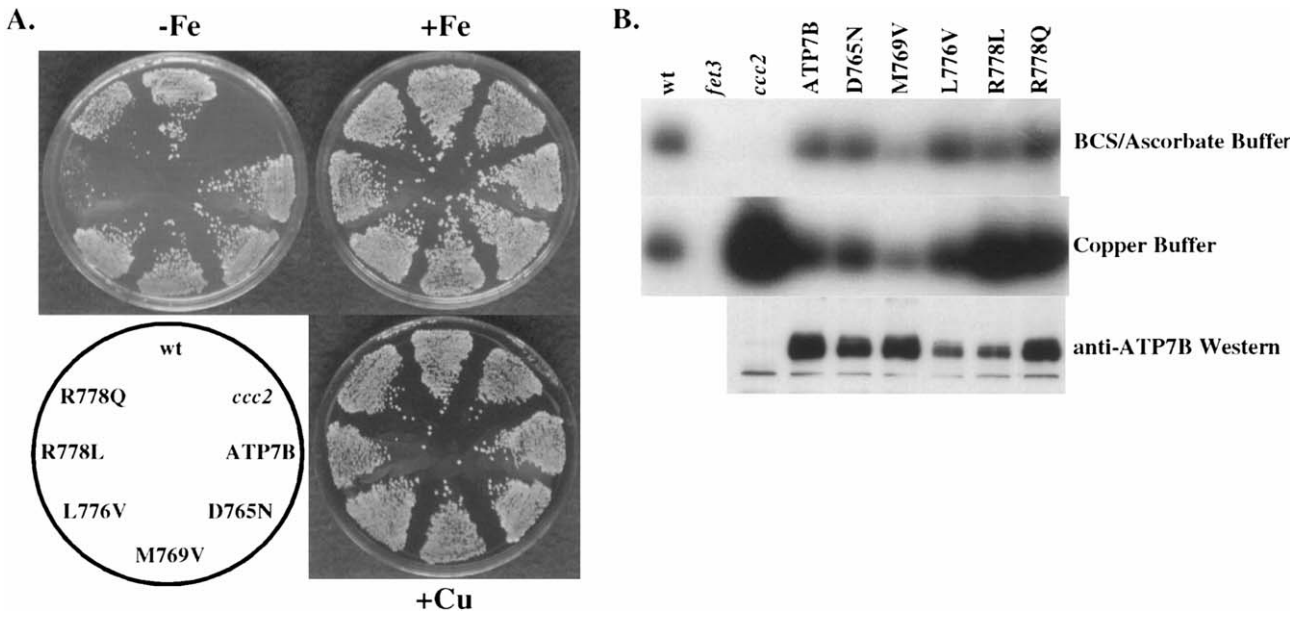

Figure 4 Complementation of *ccc2* mutant yeast by ATP7B mutant proteins. *A*, Plating assay. Disease-mutation constructs were expressed in the *ccc2* mutant background and are designated in the legend by mutation. Wild-type (wt), *Fet3*, and *ccc2* mutant strains harbor empty expression vectors. *B*, Fet3p oxidase assay. Oxidase activity in the BCS/ascorbate buffer represents holo-Fet3p copper loaded in vivo during biosynthesis. Oxidase activity in the copper buffer represents total-Fet3p activity reconstituted in vitro during homogenization.

fig. 6). The growth-rate data agreed well with the Fet3p oxidase-assay results, which indicates that growth was dependent on the ability of mutant ATP7B proteins to incorporate copper into Fet3p in vivo.

Since all of the proposed Wilson disease mutations were able to at least partially complement *ccc2* mutant yeast in single copy, cells were subjected to the further stress of heat shock at 37°C (table 1). Mutations R778L, R778Q, and P992L, which were already affected at 30°C, showed a more severe phenotype when grown on iron-limited medium at 37°C. R778L and P992L were unable to complement *ccc2* mutant yeast, whereas R778Q did so very weakly. The only mutant profoundly affected by heat stress was M769V, which was unable to complement *ccc2* mutant yeast at 37°C. All other mutants tested were unaffected by heat stress. When overexpressed from the multicopy vector, all mutations tested, with the exception of T977M and CPC/S, were able to fully complement *ccc2* mutant yeast at both 30°C and 37°C (data not shown).

Discussion

We have demonstrated that the human protein ATP7B is able to replace functionally the yeast protein Ccc2p. ATP7B provided the putative copper-transporting activity required for delivery of copper to Fet3p, thereby restoring its activity and correcting the high-affinity iron-uptake–deficiency phenotype of the yeast *ccc2* mutant strain. We were able to quantify differences in the ability of these proteins to complement *ccc2* mutant yeast by using growth rates of yeast that expressed ATP7B mutant proteins, which agreed well with the degree to which they were able to incorporate copper into Fet3p.

The Wilson disease mutations that we analyzed are found within the transmembrane segments of ATP7B. These regions of ATP7B harbor a large proportion of Wilson disease–causing missense mutations, which underscores their importance for the structure and function of this protein. On the basis of extensive biochemical work done on the related sarcoplasmic reticulum calcium-transporting P-type ATPase (MacLennan et al. 1992), the transmembrane segments of ATP7B are likely involved in the binding of copper, perhaps via the conserved CPC motif, prior to its translocation across the membrane bilayer. In addition to providing the topological framework for proper protein folding, the transmembrane segments of P-type ATPase undergo conformational changes during cation transport that result in the delivery of cations across membranes. Mutations in the transmembrane segments that affect any of these potential functions would be deleterious to ATP7B function.

We have analyzed five mutations classified as Wilson disease mutations, D765N, R778L, R778Q, G943S, and P992L. D765N, a rare mutation found in patients of

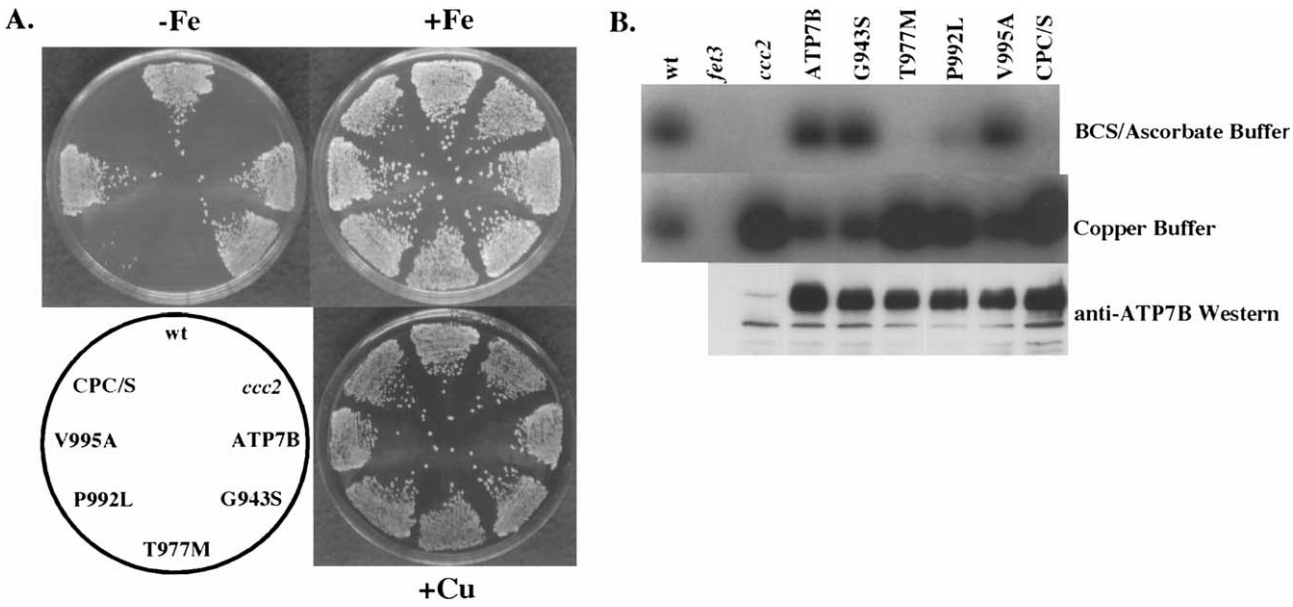

Figure 5 Complementation of *ccc2* mutant yeast by ATP7B mutant proteins. *A*, Plating assay. Disease-mutation constructs were expressed in the *ccc2* mutant background and are designated in the legend by mutation. Wild-type (wt), *Fet3*, and *ccc2* mutant strains harbor empty expression vectors. *B*, Fet3p oxidase assay. Oxidase activity in the BCS/ascorbate buffer represents holo-Fet3p copper loaded in vivo during biosynthesis. Oxidase activity in the copper buffer represents total-Fet3p activity reconstituted in vitro during homogenization.

Italian descent (Figus et al. 1995), fully complemented the *ccc2* mutant yeast at both 30°C and at 37°C, which indicates normal copper-transport function in this assay. This mutation may be a rare normal variant not yet detected on normal chromosomes. Alternatively, D756N could result in a mutant protein that has normal copper transport activity but is incorrectly localized in hepatocytes.

The Wilson disease mutation R778L is a common mutation in patients of Asian descent (Thomas et al. 1995; Chuang et al. 1996; Nanji et al. 1997), representing ⩽27% of alleles found in this population. In homozygous form, this mutation is associated with the early onset of Wilson disease with hepatic presentation (D.W.C., unpublished data). This correlates well with our functional data. R778L in single copy was able to complement *ccc2* mutant yeast at only 36.1% of that of *ccc2* mutant yeast expressing normal ATP7B, and it could not complement *ccc2* mutant yeast at 37°C. This mutation had severe effects on the function of ATP7B, which confirmed its prediction as a disease-causing mutation. It is interesting to note that the mutation R778Q, found in Taiwanese patients (Chuang et al. 1996), had a less severe effect on ATP7B function than R778L, which is consistent with the fact that glutamine is more closely related to arginine than is leucine. R778Q was able to complement *ccc2* mutant yeast in single copy at 51% of ATP7B-expressing yeast, and at 37°C it complemented *ccc2* mutant yeast very weakly. Homozygous R778Q mutation might be predicted to result in a milder form of Wilson disease than is seen for R778L. Other reported variants of this residue are R778G and R778W (Chuang et al. 1996), for which there are no functional data.

Mutation G943S was found in the Bangladeshi population and resulted in neurologic disease with onset in late childhood, in a homozygous patient (Thomas et al. 1995). Complementation was slightly impaired (86%) when expressed in single copy, compared with normal ATP7B. P992L was found in a Japanese family (Nanji et al. 1997). This change is found in a residue that is highly conserved in copper-transporting P-type ATPases. P992L in single copy had a severely impaired ability to complement *ccc2* mutant yeast, compared with normal (29.1%) at 30°C, and was completely impaired at 37°C, which supports its identity as a Wilson disease–causing mutation.

Two mutations tested, M769V and L776V, originally were not classified with certainty as disease-causing mutations or as normal variants (Thomas et al. 1995). These amino acid changes had been found to be the only mutation on a few Wilson disease chromosomes and are relatively conservative changes. However, these mutations have not been found on normal chromosomes, and the mutated residues are conserved in the Menkes disease protein. When analyzed by complementation, M769V expressed in single copy complemented *ccc2* mutant yeast to a level equivalent to normal ATP7B at 30°C.

Table 1

Growth Rates of *ccc2* Mutant Yeast Strains Expressing Mutant ATP7B Proteins

Strain	Original designation	Growth rate (ΔOD_{600}/h)	Heat sensitive	Functional designation
Wild-type	…	.1146 ± .0025	No	…
fet3	…	.0210 ± .0014[a]	No	…
ccc2	…	.0226 ± .0020[a]	No	…
ATP7B	…	.1140 ± .0012	No	…
CPC/S	…	.0155 ± .0008[a]	No	…
D765N	Mutation	.1110 ± .0020	No	Normal variant or mislocalized
R778L	Mutation	.0411 ± .0016[a,b]	Yes	Mutation
R778Q	Mutation	.0581 ± .0017[a]	Yes	Mutation
G943S	Mutation	.1001 ± .0037[a]	No	Mutation
P992L	Mutation	.0330 ± .0039[a,b]	Yes	Mutation
M769V	Possible mutation	.1094 ± .0011	Yes	Mutation
L776V	Possible mutation	.1131 ± .0013	No	Normal variant or mislocalized
T977M	Possible normal variant	.0220 ± .0024[a]	No	Mutation
V995A	Possible normal variant	.1109 ± .0037	No	Normal variant

NOTE.—Growth rates in iron-limited medium of yeast expressing normal and mutant ATP7B variants at 30°C. Growth curves were generated over a 24-h period. Rates were calculated from the linear exponential growth phase between 3 and 12 h after inoculation. Average rates were calculated from four independent experiments.

[a] Statistically significant difference from ATP7B (*t*-test, $P > .001$).

[b] Statistically significant difference from R778Q (*t*-test, $P > .001$).

However, M769V was a temperature-sensitive mutation unable to complement at 37°C. Since 37°C is the temperature at which ATP7B functions in the human cell, M769V should be considered a disease-causing mutation in patients. L776V was able to fully complement the *ccc2* mutant yeast strain expressed from a single copy and was not affected by heat stress. This mutation may be a rare normal variant not yet identified on normal chromosomes or a mutation that affects the intracellular localization of ATP7B, thereby impairing cellular copper efflux.

We have tested two mutations originally predicted to be normal variants. T977M and V995A are relatively conservative amino acid changes that were found on Wilson disease chromosomes for which a first mutation was already identified, which led to their classification as normal variants (Thomas et al. 1995). However, Waldenstrom et al. (1996) reported that T977M was the only mutation found on seven Wilson disease chromosomes from patients of northern European descent, but no normal chromosomes were tested. By use of our assay, T977M was found to be completely unable to complement *ccc2* mutant yeast, even when overexpressed. These results demonstrate that this mutation resulted in a nonfunctional protein and should be considered a Wilson disease–causing mutation in patients. V995A had normal function in our assay, which is consistent with its classification as a normal variant.

The CPC motif is evolutionarily conserved in heavy-metal transporters (Bull and Cox 1994; Solioz and Vulpe 1996; Solioz 1998) and is predicted to be involved in copper binding within the transmembrane domain of these proteins during transport. The first cysteine and proline of the motif are invariant in all heavy metal–transporting P-type ATPases, which led to the proposed designation "CPx-type ATPase" for this class of proteins (Solioz and Vulpe 1996). The last cysteine residue of the motif is replaced in some CPx-type ATPases by serine or histidine. The functional significance of these changes is unknown, but they presumably do not adversely affect the metal-transporting capacity of these proteins. Mutation of both cysteine residues to serine, within the CPC motif, in the sixth transmembrane segment of ATP7B, resulted in a protein unable to complement *ccc2* mutant yeast when expressed in single copy or when overexpressed from a multicopy vector. Our results complement those published by Hung et al. (1997), who studied ATP7B, and Yoshimizu et al. (1998), who studied CUA-1. In these studies, the CPC motif was replaced with the amino acids CAC or CPA, respectively, and in both cases resulted in a mutant protein unable to complement *ccc2* mutant yeast. These results, together with ours, are consistent with an essential role for this motif in the function of ATP7B, as well as for other CPx-type ATPases. Mutations in this motif have not been found in patients with Wilson disease.

Yeast complementation has been used by other groups to study the effect of Wilson disease mutations on ATP7B. Iida et al. (1997) reported that mutations D1027A and T1029A, within the DKTG motif conserved in P-type ATPases, and the Wilson disease mutation N1270S are unable to complement *ccc2* mutant

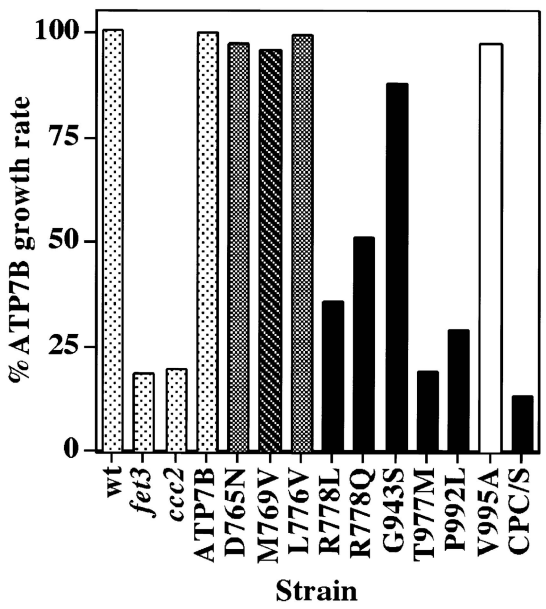

Figure 6 Growth rates of yeast strains in iron-limited medium, expressed as percent of growth rate of yeast that express normal ATP7B. Disease-mutation constructs were expressed in the *ccc2* mutant background and are designated in the legend by mutation. Black bars designate *ATP7B* variants functionally designated as Wilson disease–causing mutations. White bars indicate normal variants. Gray-checkered bars indicate possible normal variants or Wilson disease mutant proteins that are incorrectly localized in hepatocytes. The black-hatched bar indicates an ATP7B variant functionally designated as a mutation because of profound temperature sensitivity.

yeast. H1069Q, the most common Wilson disease mutation in northern European populations (Thomas et al. 1995), had a reduced ability to complement in their assay. This result is in contrast to reports that the H1069Q mutation and the equivalent mutation in ATP7A result in proteins unable to complement *ccc2* mutant yeast (Hung et al. 1997; Payne and Gitlin 1998). We have found that the yeast complementation assay is sensitive to the level of protein expressed, which may explain this discrepancy. H1069Q likely results in a severely impaired but not inactive ATP7B protein.

Most of the putative Wilson disease mutant proteins tested in our complementation assay exhibited partial or normal ability to complement *ccc2* mutant yeast, compared with normal ATP7B proteins. All mutants, with the exception of T977M and CPC/S, were able to fully complement *ccc2* mutant yeast when overexpressed from the multicopy vector, even under conditions of heat stress. These data indicate that these Wilson disease mutant ATP7B proteins retain at least the partial ability to transport copper. However, cellular copper homeostasis, mediated by ATP7B, is likely dependent on both copper-transport activity and localization to the correct intracellular compartment. ATP7B plays a dual functional role in the hepatocyte. One role is biosynthetic, involving the delivery of copper to apo-ceruloplasmin within the Golgi apparatus (Murata et al. 1995). The other role of ATP7B is to transport excess copper out of the cell (Gross et al. 1989; Schilsky et al. 1994). ATP7B, normally localized in the TGN of hepatocytes (Hung et al. 1997), trafficks from the TGN to an undefined vesicular compartment when cells are exposed to elevated copper levels, and it recycles back when copper is removed. The observed copper-dependent trafficking may represent a posttranslational inducible switch from a primarily biosynthetic role in the TGN to a primarily excretory role that involves membrane vesicles, under conditions of copper overload. Wilson disease missense mutations, such as those analyzed in this study, may affect copper-dependent trafficking and may result in a potentially active protein unable to exit the TGN in response to copper, thereby severely reducing biliary copper excretion. However, partially or fully active mutant ATP7B proteins trapped in the secretory pathway could transport sufficient copper to constitute apo-ceruloplasmin during its biosynthesis. This hypothesis may in part explain the phenotype of patients with hepatic copper accumulation but with normal serum ceruloplasmin levels. Our hypothesis is supported by an article that was published after submission of this article. Payne et al. (1998) reported that the H1069Q mutation results in a mutant ATP7B protein, which, unlike normal ATP7B, was unable to rescue the copper-sensitive phenotype of ATP7A-deficient *mottled* mouse fibroblasts, because of temperature-sensitive retention and degradation of ATP7B protein in the endoplasmic reticulum. The cystic fibrosis transmembrane-conductance regulator (CFTR) has been shown to have a similar defect. The common ΔF508 mutation is a temperature-sensitive mutation, producing a mutant CFTR protein that retains some chloride-channel activity, but it is not properly processed and localized to the plasma membrane, which results in defective cellular chloride conductance (Denning et al. 1992). The intracellular localization of Wilson disease mutant ATP7B proteins expressed in mammalian cells must be analyzed to obtain a clear picture of ATP7B function in both normal and disease states.

The application of yeast complementation to the study of ATP7B provides an elegant and sensitive method to assay the putative copper-transporting function of mutant ATP7B proteins, which allows detailed structure/function analysis. This assay avoids the use of radioactive copper or silver isotopes to trace copper-transport function in cells or membrane vesicles. As observed from our growth-rate data, ATP7B is able to replace Ccc2p with 100% efficiency when expressed from a single-copy integrating vector. This demonstrates the validity of yeast complementation as an assay for ATP7B function.

Acknowledgments

We wish to thank Morrie Manolson for providing training in basic yeast techniques, yeast strains, and expression vectors; Daniel Yuan for providing the plasmids E5-URA3.4 and pΔfet3 and protocols for the Fet3p oxidase assay as developed in the laboratory of R. D. Klausner; Gloria Hsi for technical assistance in making mutant cDNA constructs; and Gregory Lee for preparing rabbit polyclonal antiserum. This work was supported by grants from the National Science and Engineering Council, Canada, and the Canadian Genetic Diseases Network. J.R.F. holds a graduate scholarship from the Medical Research Council, Canada.

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