Copper-Dependent Iron Assimilation Pathway in the Model Photosynthetic Eukaryote *Chlamydomonas reinhardtii*

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The unicellular green alga *Chlamydomonas reinhardtii* is a valuable model for studying metal metabolism in a photosynthetic background. A search of the *Chlamydomonas* expressed sequence tag database led to the identification of several components that form a copper-dependent iron assimilation pathway related to the high-affinity iron uptake pathway defined originally for *Saccharomyces cerevisiae*. They include a multicopper ferroxidase (encoded by *Fox1*), an iron permease (encoded by *Ftr1*), a copper chaperone (encoded by *Atx1*), and a copper-transporting ATPase. A cDNA, *Fer1*, encoding ferritin for iron storage also was identified. Expression analysis demonstrated that *Fox1* and *Fer1* were coordinately induced by iron deficiency, as were *Atx1* and *Fer1*, although to lesser extents. In addition, *Fox1* abundance was regulated at the posttranscriptional level by copper availability. Each component exhibited sequence relationship with its yeast, mammalian, or plant counterparts to various degrees; *Atx1* of *C. reinhardtii* is also functionally related with respect to copper chaperone and antioxidant activities. *Fox1* is most highly related to the mammalian homologues hephaestin and ceruloplasmin; its occurrence and pattern of expression in *Chlamydomonas* indicate, for the first time, a role for copper in iron assimilation in a photosynthetic species. Nevertheless, growth of *C. reinhardtii* under copper- and iron-limiting conditions showed that, unlike the situation in yeast and mammals, where copper deficiency results in a secondary iron deficiency, copper-deficient *Chlamydomonas* cells do not exhibit symptoms of iron deficiency. We propose the existence of a copper-independent iron assimilation pathway in this organism.

While iron is abundant in the environment, it is present in the insoluble ferric [Fe(III)] state, so that its bioavailability is low (16). Yet iron is an essential micronutrient for all organisms because it functions as a cofactor in enzymes that catalyze redox reactions in fundamental metabolic processes. Iron exhibits stable, redox-interchangeable ionic states with the potential to generate less stable electron-deficient intermediates during multielectron redox reactions involving oxygen chemistry (16). Therefore, organisms are challenged with the acquisition of sufficient iron to meet cellular metabolic requirements while avoiding uncontrolled intracellular chemistry. This is accomplished via the operation of iron homeostatic mechanisms. The essential features of iron metabolism include assimilation and distribution, storage and sequestration, and utilization and allocation. The assimilatory pathway can be further subdivided into reduction of insoluble ferric species to more soluble ferrous species and uptake into the cell, followed by intracellular transport and intraorganellar distribution. The storage and sequestration of iron involve loading of cellular proteins as well as compartmentalization into organelles like vacuoles and plastids, which in turn requires proteins for transport into and out of these compartments and organellar iron binding and metabolizing proteins. Photosynthetic organisms have requirements for iron beyond those of heterotrophs because of the occurrence in many metabolic pathways within the plastid. Therefore, in a eukaryotic phototroph, there must be an additional layer of complexity in iron metabolism.

**Mobilization by redox chemistry.** *Saccharomyces cerevisiae* uses different assimilatory pathways depending on the chemical source of iron and its concentration. Reduction of Fe(III) to Fe(II) (68, 91) is a key step in uptake, either to solubilize the ion or to release it from ferric specific chelates. Under iron-limiting conditions, high-affinity uptake is mediated by the inducible multicopper oxidase (MCO) (Fet3p)/iron permease (Ftr1p) complex at the plasma membrane (6, 91, 100), where Fet3p oxidizes Fe(II) to Fe(III) which is then delivered to Ftr1p for transport of Fe(III) into the cell (91, 100). Highly related pathways operate in other fungi, including *Schizosaccharomyces pombe* (7) and the fungal pathogen *Candida albicans* (21, 35, 56, 92). Under iron-replete conditions, low-affinity systems operate, such as Fet4p (19, 37) or Smf1/Smf2 (15). Iron can also enter the cell complexed with siderophores, mediated by the ARN family of siderophore transporters through an endocytic pathway (40, 41, 62, 63, 86, 118, 119).

In mammals, dietary Fe(III) is reduced to Fe(II) by Dcytb (69) and perhaps other as-yet-unidentified ferrireductases and transported across the apical surface of enterocytes by the
divalent metal ion transporter DMT1 (DCT1, Nramp2) (2, 3, 97, 101). The basolateral transporter ferroportin1/IREG1/MTF1 (1, 20, 70) mediates the transport of iron out of enterocytes into the blood for distribution to other organs. Iron is moved through the circulatory system bound to the plasma protein transferrin. The loading of iron onto transferrin requires oxidation from Fe$^{2+}$ to Fe$^{3+}$, which may be mediated by either or both of the MCOs hephaestin and ceruloplasmin. Hephaeatin may act together with ferroportin1 at the basolateral surface of enterocytes to oxidize Fe$^{2+}$ to Fe$^{3+}$ prior to export into the plasma (53). Alternatively or in addition, plasma ceruloplasmin oxidizes Fe$^{2+}$ to Fe$^{3+}$ subsequent to export into blood plasma for loading onto transferrin. An additional important role of ceruloplasmin is the mobilization of iron from organs like the liver where ceruloplasmin is synthesized (24). Therefore, unlike yeast, where the MCO/iron permease complex mediates iron uptake, the analogous complex(es) in mammals is involved in iron release from cells. Nevertheless, the principle of transport in conjunction with redox chemistry holds.

Iron acquisition by all plants begins with the reduction of Fe$^{3+}$ from insoluble Fe$^{3+}$ complexes in the soil (8, 33, 93, 115). In addition to iron reduction, iron solubility in the rhizosphere is increased through iron-deficiency-induced activation of a specific H$^+$-ATPase (29). In dicotyledons and nongraminaceous monocotyledons, IRT1 (23, 32, 57) and members of the NRAMP family (17, 103) are responsible for iron uptake. Grasses secrete phytosiderophores, which chelate Fe$^{3+}$, and the resulting complex is taken up by iron-deficiency-induced siderophore transporters represented by the prototypical member YS1 (9, 18).

**Ferritin.** Ferritin has long been known as an iron storage protein in vertebrates. Accordingly, its expression is increased in cells supplied with high iron through translational regulation (38). Vertebrate ferritin is composed of multiple subunits of two types of chains, called heavy and light. Together these chains function to oxidize iron and bind up to 4,500 ferric atoms within the core of a multicentric structure. In plants, the 24-subunit ferritin multimer consists of only one type of subunit, whose sequence is most similar to that of the heavy subunit of vertebrate ferritins that contains the ferroxidase active site, although the carboxylates of the light subunit that promote and stabilize the mineralized core are also present (reviewed in reference 9). Ferritin is found in plastids and is a source of iron during plastid development (reviewed in reference 9). Preferrittin, with a plastid targeting sequence, is encoded by a multigene family in plants, as is the case in animals (113). Increased ferritin production in high-iron-supplied cells is accomplished, at least in part, by transcriptional regulation of one or more ferritin genes (10, 61). This pattern of expression is consistent with a role for ferritin as an iron storage molecule under conditions of iron overload.

**Role of copper in iron assimilation.** Since the MCOs Fet3p and ceruloplasmin and hephaestin in *S. cerevisiae* and mammals, respectively, require copper for activity, copper is an essential cofactor for high-affinity iron uptake. Also essential are copper-metabolizing components such as the copper chaperone ATX1 or ATOX1 in *S. cerevisiae* and mammals, respectively, that deliver copper to the copper-transporting ATPase Ccc2p or the Wilson (WND)/Menkes (MNK) proteins, respectively. The ATPases transport copper into the lumen of secretory pathway vesicles for incorporation into apo-Fet3p (66, 117) or apoceruloplasmin and apohephaestin, respectively. Therefore, in *S. cerevisiae* or mammals, copper deficiency leads to iron deficiency. In contrast, plants do not appear to manifest a similar dependence on copper for iron assimilation. In *Arabidopsis* species, both types of iron transporters, IRT1 and NRAMP, take up Fe$^{2+}$, so that a role for a ferroxidase is not evident, nor has one been identified. However, ATX1 and WND/MNK homologues occur in *Arabidopsis* and *Synechocystis* species, and they carry out analogous functions (44, 45, 47, 105, 106).

**Chlamydomonas model.** Significant insights into plant metal metabolism can be gained from studying other model photosynthetic organisms such as the unicellular green alga *Chlamydomonas reinhardtii*. With its simple growth requirements *C. reinhardtii* is a valuable experimental model for the study of metalloprotein biosynthesis (72, 75) and metal-responsive gene regulation in photosynthetic organisms (71). As in other organisms, iron uptake by *Chlamydomonas* involves reductases (22, 42, 67, 114) that are induced in iron deficiency and may be the same enzyme as that induced in copper-deficient (−Cu) cells (42). Iron uptake is also induced, although to a lesser extent than Fe$^{2+}$ reduction (22). The molecular components of iron assimilation in *C. reinhardtii* have not as yet been identified.

In this work, we report the identification, expression, and functional characterization of *Chlamydomonas* homologues of a multicopper ferroxidase, an iron permease, a copper chaperone, and ferritin and propose that together these components form part of an iron assimilation pathway related to the high-affinity iron uptake pathway of *S. cerevisiae*. Yet, while copper-requiring components appear to be required for iron assimilation in *C. reinhardtii*, copper deficiency in *Chlamydomonas* does not lead to a secondary iron deficiency, indicating that backup systems are in place to accommodate simultaneous copper and iron deficiency.

**MATERIALS AND METHODS**

**Strains and culture conditions.** *Escherichia coli* DH5α was used for cloning, maintenance, and propagation of plasmids and was cultured in Luria-Bertani medium (U.S. Biologicals) at 37°C. Ampicillin was added to a 100-μg/ml concentration where appropriate. *E. coli* clones containing plasmid-borne expressed sequence tags (ESTs) were obtained from the Kazusa DNA Research Institute, Chiba, Japan. *S. cerevisiae* strains YPH490 (wild type [wt]) (98) and SL215 (aΔΔ) (66) (kind gifts from V. C. Culotta) and DBY746 (wt) and EG118 (sod1Δ) (kind gifts from J. S. Valentine and E. Gralla) were maintained on standard yeast extract-peptone-dextrose medium at 30°C (14). Tests for iron-dependent growth were carried out as described previously (100) with synthetic dextrose (SD) minimal medium plates containing 1.5 mM ferrozine [3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazene; Sigma] and supplemented with 350 μM ferrous ammonium sulfate where required. Agarose was used instead of agar to minimize contamination with iron. Plates were incubated at 30°C for 5 days. Tests for complementation of lysine and methionine auxotrophy of the sod1Δ mutant were carried out on SD plates lacking either lysine or methionine. Plates were incubated at 30°C for 5 days. *C. reinhardtii* strain CC125 was used for growth experiments and was cultured in Tris-acetate-phosphate (TAP) medium prepared with copper-free, iron-free trace elements (89) and supplemented with either Fe-EDTA prepared as described previously (79) or CuCl2 at the required concentrations where appropriate. Cell density was determined by counting cells, and the chlorophyll content was measured spectrophotometrically at 652 nm as described previously (79).

**Chemicals and reagents.** Enzymes were from New England Biolabs, and vectors were from Stratagene. Chemicals were purchased from Fisher, Sigma,
and Gibco BRL. DNA purification kits were from Qiagen. Oligonucleotide primers were synthesized by Genosys or Gibco BRL.

DNA manipulation and sequence analysis. Standard procedures were used for plasmid DNA isolation from E. coli and for DNA cloning and manipulation (95).

Amplification. Oligonucleotide primers used in amplification reactions were derived from ESTs (Table 1). For preparation of first-strand cDNA, C. reinhardtii total RNA was used as the template in a reaction mixture containing random hexamers or oligo(dT) 

hybridization buffer, and RNasin RNase inhibitor (Promega). For amplification of specific sequences from the C. reinhardtii cDNA template, the reaction mixture contained 1.25 μM concentrations of the appropriate primers, 0.2 μM deoxynucleotides triphosphates, 1.5 mM MgCl2, 10× reverse transcription buffer, and Taq polymerase purified as described previously (25). The same conditions were used with primers 5′-gcccgcggccgcT GTAGTGCATTTTTGAGCTTC-3′ and 5′-gcccgcggccgcTGGCATTTTTTGACGCTC-3′ to amplify S. cerevisiae ATX1 from genomic DNA.

Thermal cycling conditions for Atx1, Fox1, and the copper ATPase were as follows: 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min (4 times); 94°C for 1 min, 55°C for 45 s, and 72°C for 1 min (26 times); and 72°C for 10 min (1 time). For Fox1 and Frl1 the conditions were the same except that annealing temperatures of 45°C and 55°C, respectively, were used. PCR products were purified with Qiaquick columns (Qiagen) and cloned into the BamHI site of pBluescript SK II (+) (Stratagene), PGEM-T Easy (Promega) in the case of the copper ATPase, or the NotI site of pPF61 (78) in the case of C. reinhardtii Atx1 and S. cerevisiae ATX1.

All plasmids are available from the Chlamydomonas culture collection under the name pFox1-594, pFox1-852, pFox1-TrxA, pFtr1-358, pAtx1-410, pFer1-399, and pCcc2-229.

**5′-RACE.** To amplify the 5′ end of the Fox1 mRNA, the 5′ rapid amplification of cDNA ends (RACE) system from Gibco BRL was used. Gene-specific primer 1 (5′-CCACACGGGCCTGCAGTGACGTTGAGCCTAGTTG-3′) designed to correspond to positions 1027 to 1000 of the cDNA sequence shown in Fig. 6, was used to prime first-strand cDNA synthesis from total RNA. Gene-specific primer 2, corresponding to positions 853 to 829 and containing an EcoR1 site at the 5′ end to facilitate cloning (Table 1), was designed for amplification of the desired fragment from the cDNA with the universal amplification primer from the kit. The reactions were carried out according to the manufacturer’s instructions. A diffuse band of approximately 8×10^2 bp was purified and cloned into the SalI (5′ end of PCR product) and EcoRI (3′ end of PCR product) sites of pBluescript KS II (+) (Stratagene), and DNA from individual clones was sequenced with the T7 primer to determine if the clones overlapped with the 5′ end of clone CL4810 corresponding to EST accession no. AV395796. The longest overlapping clone, designated Fox1-RACE9 (8×10^2 bp), was sequenced completely on both strands by Qiagen genomics and assembled with the sequence of the cDNA insert in clone CL4810 to generate the sequence shown in Fig. 6.

**Sequence analysis.** The cloned inserts were used by primer pairs derived from the multiple cloning site of pBluescript (M13 reverse, 5′-GGGATCC CACACGCTGTGTAAGA-3′, and M13 forward, 5′-GGGATCC TCGAGGCTTTGTTAAGA-3′) and gene-specific primers that were designed from known sequences. Reactions for automated sequencing were carried out with the BigDye Terminator reaction mix (Perkin-Elmer Applied Biosystems), and unincorporated dye terminators were removed by gel filtration cartridges (Edge BioSystems). Reaction mixes were analyzed on an automated sequencer (AB Prism 377 DNA sequencer). Raw sequence data were analyzed with Sequence Analysis 2.0.1 for Macintosh, and contigs were assembled with AutoAssembler 1.4.0 for Macintosh (Perkin-Elmer Applied Biosystems). The insertions in clone CM01797 (Kazusa DNA Research Institute) corresponding to the Atx1 cDNA, clone CL42d10 (Kazusa DNA Research Institute) corresponding to the Frl1 cDNA, and clones Fox1-RACE9-852 and CL4810 (Kazusa DNA Research Institute) corresponding to positions 1 to 852 and 787 to 4908 of the Fox1 cDNA, respectively, were sequenced by Qiagen Genomics. Database searches were carried out with the BLAST software on the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST/).

Protein alignments were carried out with ClustalW (http://www.ebi.ac.uk/clustalw/). Protein sequences were analyzed with BioEdit software (34), Prosite (13, 48), SignalP V1.1 (80), TMpred (49), and TopPred2 (110).

**RNA blot analysis.** C. reinhardtii total RNA was isolated and analyzed by hybridization as described previously (43). Five micrograms of RNA was loaded per lane. Gene-specific probe fragments were isolated after BamHI or NotI digestion of the cloned amplification products described above (Table 1), purified with Qiagextract columns (Qiagen), and labeled with [α-32P]-dCTP by random priming. The probes for Fox1, Atx1, and Frl1 recognized a single band in Southern hybridization analysis of digested genomic DNA. The probe used to detect Frl1

**TABLE 1.** Oligonucleotides used for PCR amplification of gene-specific sequences.

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<th>Gene</th>
<th>Accession no., source, or reference</th>
<th>Insert*</th>
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<th>primer 2,</th>
<th>Upstream primer (3′→5′) primer 1</th>
<th>primer 2,</th>
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<tr>
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<td><strong>(Trox-Fox1 fusion)</strong></td>
<td>AV395796, Gibco BRL, pCcc2-229</td>
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*Accession nos. correspond to sequences shown in Fig. 2, 3, 4, 6, and 9. For the Cu ATPase, the gene-specific sequence is uppercased.

Downloaded from http://ec.asm.org/ on October 24, 2017 by guest
transcripts was generated by Nor1 digestion of clone CM080802 (Kazusa DNA Research Institute; GenBank accession no. AV92930). The probe used to monitor RbcS2 encoding the small subunit of ribulose-bisphosphate carboxylase- oxygenase or the Cblp gene (for normalization between samples) was described previously (90, 96). Specific activities of probes ranged from 3 x 106 to 6 x 109 cpm/μg of DNA. Hybridization signals were detected by exposure of membranes to Biomax MS film (Eastman-Kodak Co.) at ~80°C with two intensifying screens and developed typically after overnight exposure. Hybridization signals from the same blots were quantitated with a Molecular Dynamics PhosphorImager and Image QuaNT (version 4.2a) software (Sunnyvale, Calif.).

Yeast complementation experiments. PCR products encoding C. reinhardtii Atxl and S. cerevisiae Atx1p were cloned into the Nor1 site of the yeast expression vector pFL61 (78) under the control of the PGK1 promoter. Plasmid DNA was transformed into the appropriate strain of S. cerevisiae (50). Transformants were selected on SD plates lacking uracil. Several independent transformants were analyzed for each experiment.

Preparation of Fox1 antisera. A thiorodoxin (Trx)-Fox1 fusion protein was generated for the preparation of Fox1 antisera. A PCR with Fox1-specific primers (Table 1) resulted in the amplification of a 761-bp product (nucleotides 1476 to 2237; amino acids His394 to Val463) that was digested with BamHI, cloned in frame into the 3′ terminus of the TrxA-encoding sequence of the expression vector pTrxFus, and introduced into E. coli for tryptophan-inducible expression (Invitrogen). The majority (approximately 90%) of the expressed TrxA-Fox1 fusion protein was present in inclusion bodies. E. coli cells expressing the fusion protein were cultured and harvested as described in the manufacturer’s protocols. Briefly, flasks containing 100 ml of prewarmed induction medium were inoculated with 2 ml of an overnight culture of the TrxA-expressing strain, grown to an A600 of 0.7 (30°C, 200 rpm), and then induced with (tryptophan) for 4 h at 37°C. Cultures were chilled in ice-water; collected by centrifugation (4,300 x g, 15 min); washed once with a solution of cold 10 mM Tris-Cl (pH 8.0), 1 mM EDTA (pH 8.0), and 100 mM NaCl (TEN); resuspended in 10 ml of cold 50 mM Tris HCl (pH 7.5) containing 5 mM EDTA; subjected to three quick-freeze (dry ice ethanol)-quick-thaw (37°C) cycles; and disrupted by sonication (Fisher Scientific model 550 Sonic Dismembrator; micropor probe; amplitude setting 4, 12 cycles of 30 s of sonication followed by 60 s of cooling). Samples were kept cold by immersion in an ice-water bath. The inclusion bodies were collected by centrifugation (5 min at 14,000 x g, 4°C), washed three times with cold TEN, solubilized with sample buffer (50 mM Tris-HCl [pH 6.8], 5% [vol/vol] 2-mercaptoethanol, 2% [wt/vol] sodium dodecyl sulfate [SDS], 0.1% [wt/vol] bromophenol blue, 10% [wt/vol] glycerol), and subjected to preparative SDS-polyacrylamide gel electrophoresis (12% acrylamide). The region of the gel containing the fusion protein (visualized by zinc-imidazole staining) was excised and used directly for antisera production in rabbits (service provided by Covance Research Products, Denver, Pa.).

Immunoblot analysis. Immunoblotting was performed as described previously (79). Chlamydomonas cells (107 cells ml−1 in 200 ml) were collected by centrifugation (3,800 x g, 5 min), washed once with 0.01 M sodium phosphate (pH 7.0), and resuspended in the same buffer such that samples were matched for number of cells per unit volume. For Fox1 immunoblotting, aliquots of 50 μl were subjected to three cycles of freezing to −80°C and thawing to 4°C and centrifuged (5 min at 14,000 x g, 4°C). The supernatant was discarded, and the pellets were washed once with 100 μl of ice-cold phosphate-buffered saline before resuspension in 50 μl of 0.01 M sodium phosphate. For analysis of Fox1 expression in cells grown under various iron concentrations, washed and resuspended cells were analyzed for CF1 by immunoblotting and densitometric quantitation of the signals for the α and β subunits. Sample loadings for the Fox1 immunoblot were then adjusted such that samples were matched for CF1 content. The abundance of CF1 in C. reinhardtii is not affected by iron nutritional status.

Nucleotide sequence accession number. The newly published sequences and their GenBank accession numbers are as follows: Fox1, AF450137; Ftr1, AF874811; Atxl, AV129036 and AF280056; Fer1, AF503338; and Cox17, AF280543.

RESULTS

To identify components of iron assimilation pathways in C. reinhardtii, the EST database was searched by BLAST for candidate homologues of the proteins that are known to be involved in iron or copper metabolism in fungi, plants, or animals. Sequences corresponding to ferritin and copper chaperones (Atxl and Cox17) were identified with excellent probability scores (AV395232, 1e−31 for soybean ferritin input; AV388156, 5e−15 for Arabidopsis CCH input; and AV392030, 4e−15 for human Cox17 input). A candidate Ftr1-like sequence with a weak probability score (0.004 for S. cerevisiae Ftr1p input) but containing a conserved RExxE motif was identified (accession no. AV395492), as were several sequences corresponding to the copper binding sites of MCOs (input sequence, laccase or ascorbate oxidase) or the ATP binding sites of P-type ATPases (input sequence, Ccc2p, MNK, or WND). The genes corresponding to these sequences were eventually named as follows: Ftr1 for ferritin; Atxl, Cox17, Ftr1, and Ccc2 on the basis of a sequence relationship to the equivalent yeast proteins; and Fox1 for ferrodoxins. The relevance of candidate sequences to iron metabolism was tested by RNA blot analysis (Fig. 1). RNA was prepared from cells grown in TAP medium containing either 0.1 μM supplemental iron, under which condition they were evidently iron deficient as assessed by chlorophyll accumulation (i.e., Fe-deficiency chlorosis), or 1 μM supplemental iron, under which condition chlorosis was not evident. Cox17 mRNA was not affected by medium iron concentrations, but Fer1, Atxl, Ftr1, and Fox1 RNAs were each induced severalfold (Fig. 1) relative to total RNA and were, therefore, of interest for further analysis.

Ferritin (Ftr1). The cDNA clone (LC007f05) corresponding to EST AV619384 was obtained from the Kazusa DNA Research Institute. The 1.4-kb cDNA insert was sequenced completely (AF503338) (Fig. 2A). A 750-bp open reading frame (ORF) encoding a predicted polypeptide of 249 amino acids was identified and was flanked by 52 bp of 5′ untranslated region (UTR) and 593 bp of 3′ UTR with a putative polyadenylation signal (TGTAAG) located 15 nucleotides upstream of the poly(A) tail. The corresponding gene was designated Fer1.

FIG. 1. Increased accumulation of RNAs encoding iron metabolism components in iron-deficient Chlamydomonas cells. C. reinhardtii cells from a late-log culture in copper-free TAP medium were harvested and resuspended in 90 ml of −Cu TAP with 0.1 μM iron chelate. One milliliter was used to inoculate 100-ml cultures of +Cu TAP with either 0.1 μM iron chelate (cells severely chlorotic) or 1 μM iron chelate (cholorophyll content relatively unaffected compared to that of iron-replete cells). Cultures were grown to late log phase and transferred, and this process was repeated twice more to adapt cells to 1 or 0.1 μM iron chelate. Total RNA was prepared from late-log-phase cultures after three rounds and analyzed by hybridization with gene-specific probes as indicated. For quantitation, the signals were normalized to total RNA loaded. Specifically, the relative intensities (10−6) after object average background correction were as follows: Fox1, 25.2 and 8.7; Ftr1, 5.2 and 1.2; Fer1, 3.7 and 0.9; Atxl, 1.2 and 0.5; Cox17, 0.05 and 0.05. For RbcS2, the signal was actually decreased in 0.1 μM iron samples. The relative intensities (10−4) were 20.6 and 44.8. Transcript sizes were as follows: Fox1, 5.2 kb; Ftr1, 2.8 kb; Fer1, 1.3 kb; and Atxl, 1.0 kb. They were estimated from a standard curve of the relative mobility of each marker (Gibco BRL; 0.24- to 9.5-kb RNA marker) versus log10 of its size in bases.
### A) ATG Translation Table

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The putative polypeptide encoded by the ORF identified within the assembled sequence showed a high level of similarity to mammalian and plant ferritins (Fig. 2B), which increased slightly if only the mature subunits were considered (28 and 47% amino acid identity, respectively). All of the plant ferritins showed greater similarity with the human heavy chain subunits than with the light chain subunit. Not surprisingly, the C. reinhardtii mature sequence showed less similarity with either of the Arabidopsis thaliana and Glycine max sequences (47%) than the similarity that exists between these latter two sequences (72%). In addition, there was less similarity between the C. reinhardtii sequence and the human ferritin subunits than between the other plant sequences and the human subunits. The N terminus had features that are consistent with the presence of a chloroplast transit peptide (12), in agreement with the ChloroP software, which predicted its targeting to the plastid. The C. reinhardtii sequence also appeared to possess an extension peptide, a plant-specific sequence thought to be involved in stabilizing the protein in vitro (59, 109). This peptide, from approximately amino acid position 30 to 69, is located between the predicted transit peptide cleavage site and the region that shows a high level of conservation with both plant and human sequences, and it is less conserved in the C. reinhardtii sequence than in the other plant sequences.

Fer1 contained the highly conserved iron-binding motif RExxE found in other ferritins and, like other plant ferritins, contained the conserved amino acids required for ferroxidase activity (Fig. 2). Note that His77 (C. reinhardtii numbering) is conserved between the C. reinhardtii and human sequences but is replaced by Ala in the plant sequences, where it was shown previously to have a role in the ferroxidase activity of the plant proteins (109). We conclude that Chlamydomonas contains at least one plastid-localized ferritin.

The abundance of Fer1 transcripts increases in iron deficiency, up to 10-fold, rather than under conditions of iron excess (up to 200 μM as Fe-EDTA tested [data not shown]), suggesting that the plastid Fer1 gene product may be important for iron buffering (see Discussion). Nevertheless, the protein abundance was not changed noticeably in −Fe relative to +Fe cells. It is well known that ferritin abundance is controlled by translational mechanisms in animals (104), and such mechanisms may operate also in C. reinhardtii. A search of the Chlamydomonas dbEST (with Arabidopsis Fer1 as input) database reveals more than a dozen ESTs (July 2002), all of which appear to arise from a single gene. Comparisons between the sequenced clone LC007f05 and the ESTs also suggest that all the Fer1 sequences represent a single gene or highly related gene sequences.

Iron permease (Ftr1). The cDNA clone (CL42d10) corresponding to EST AV395492 was obtained from the Kazusa DNA Research Institute. The 2.9-kb cDNA insert, probably corresponding to the full-length mRNA based on comparison with the size of the mRNA transcript (Fig. 1), was sequenced completely (GenBank accession no. AF478411), and found to contain an ORF encoding 541 amino acids flanked by 264 bp of the 5′ UTR and 1,059 bp of the 3′ UTR with a canonical polyadenylation signal, TGTTAA, at the expected position relative to the poly(A) tail (Fig. 3). A BLAST search of the nonredundant sequence database with the complete Ftr1 reading frame revealed its relationship to S. cerevisiae, S. pombe, and Candida albicans Ftr1p and Ftr1p homologues, each of which has a demonstrated role in iron metabolism (7, 92, 100), with scores ranging from 1e−12 to 3e−08. All other sequences show weak similarities and carrying RExxE motifs, including Synechocystis sp. strain PCC 6803 (BAAA16870, ORF slr0964, 0.001), were of prokaryotic origin. A search of the dbEST database revealed several sequences in Physcomitrella patens (moss) that contained two RExxE motifs (expected value, 10−9 to 10−8). These could represent a plant Ftr1 homologue. A multiple alignment of C. reinhardtii Ftr1 with S. cerevisiae Ftr1p and Fth1p, S. pombe Fip1p, Candida albicans CaFtr1 and CaFtr2, and Synechocystis sp. strain PCC 6803 ORF slr0964 is shown in Fig. 4. Although the C. reinhardtii and Synechocystis sequences display less than 20% amino acid identity overall, the latter is included in the alignment because of the relevance of cyanobacterial metabolism to chloroplast biology. Like the fungal sequences, C. reinhardtii Ftr1 is predicted to have a cleavable N-terminal leader sequence (SignalP V1.1 [80]). Six transmembrane domains at positions that correspond to those shown previously for S. cerevisiae Ftr1p are also predicted (58). Two RExxE motifs were present within all sequences: the first one, within the putative N-terminal leader sequence, is less conserved; the other, within the hypothesized third transmembrane domain, is more conserved. In all of the fungal sequences, this motif is REGLE, whereas in the C. reinhardtii and Synechocystis sequences, the L was replaced by I and F, respectively. ExxE has been identified as a potential iron-binding motif in Ftr1p and CaFTR1 and CaFTR2 (92, 100); two such motifs are found in the C-terminal region. Although the predicted C. reinhardtii transmembrane regions corresponded closely with those predicted for the S. cerevisiae Ftr1p sequence, there was a region of 151 amino acids between
transmembrane domains 2 and 3 that was unique to C. reinhardtii. This region was hydrophobic and rich in proline residues and perhaps may represent a highly folded structure with a hydrophobic interior.

RNA blot analysis had indicated that Ftr1 mRNA accumulated to higher levels in cells displaying iron-deficiency chlorosis than in cells not displaying iron-deficiency chlorosis (Fig. 1). To investigate the pattern of iron-dependent expression of Ftr1, total RNA from C. reinhardtii grown in medium supplemented with various amounts of copper and iron (from 0 to 6 \( \mu \)M Cu and from 0.1 to 18 \( \mu \)M Fe) was analyzed by hybridization. Ftr1 mRNA accumulation increased steadily as the medium iron concentration decreased from 18 \( \mu \)M (concentration in standard TAP medium [36]) to 0.1 \( \mu \)M (concentration where cell growth was inhibited and chlorosis was evident [see below and Fig. 12]) (Fig. 5). The magnitude of Ftr1 induction relative to total RNA between the maximum and minimum medium iron concentrations tested was at least 102-fold. The effect of copper deficiency, at most a twofold increase in copper-deficient cells (no added copper) over that in copper-supplemented cells (6 \( \mu \)M supplemental copper), was not consistent, nor did it display a reproducible pattern with respect to copper concentration (Fig. 5). The extent of copper deficiency of the culture was verified routinely by analyzing the expression of the Cyc6 gene (89). When medium iron concentration was increased about 10-fold to 200 \( \mu \)M, Ftr1 mRNA abundance was decreased further (data not shown).

Based on the overall sequence relationship between C. reinhardtii Ftr1 and Ftr1 homologues, the presence of two conserved RExxE motifs, a predicted membrane topology similar to that of S. cerevisiae Ftr1p, and its iron-regulated expression, we conclude that Ftr1 encodes an iron permease with a function related to S. cerevisiae FTR1. Hence, the name Ftr1, for ferric transporter, was adopted. The C. reinhardtii dbEST database contains only five clones (six ESTs).

FIG. 3. Analysis of the Ftr1 cDNA. The nucleotide sequence of the 2.9-kb cDNA from clone CL42d10 corresponding to EST AV395492 (Kazusa DNA Research Institute) is shown with the deduced amino acid sequence of the longest ORF given below the nucleotide sequence. The numbers on the left refer to the nucleotide sequence, which is numbered from the first nucleotide of the GenBank entry. The numbers on the right indicate the positions of the amino acids in the reading frame. The polyadenylation signal is double underlined. The putative N-terminal signal peptide is underlined. Boxes denote sequences corresponding to putative transmembrane regions. The iron-binding RExxE motifs are given in boldface and shaded gray. The ExxE motifs are double underlined. Half-arrows denote the sequences of primers used for PCR.

transmembrane domains 2 and 3 that was unique to C. reinhardtii. This region was hydrophobic and rich in proline residues and perhaps may represent a highly folded structure with a hydrophobic interior.
FIG. 4. Alignment of *C. reinhardtii* Ftr1 with Ftr1 homologues from other organisms. The alignment was generated by using the ClustalW algorithm and BioEdit software (34). Residues that are similar or identical in a majority (four) of sequences are shaded gray and black, respectively. A line above the alignment indicates the conserved RExxE motifs. GenBank accession numbers: *C. reinhardtii* Ftr1, AF478411; *Synechocystis*, BAA16870; *S. cerevisiae* Ftr1p, NP_011072; *S. cerevisiae* Fth1p, AF177330; *S. pombe* Fip1, CAA91954; *C. albicans* CaFTR1, AF195775; and *C. albicans* CaFTR2, AF195776.
representing this Ftr1 gene, and these appear to be the only Ftr1-related sequences in the dbEST database (July 2002).

**Ferrooxidase (Fox1).** In the fungi, Ftr1p and its homologues function in obligate partnership with an MCO with ferroxidase activity, Fet3p and its homologues (7, 21, 100). Together the function in obligate partnership with an MCO with ferroxidase (1e

A putative N-terminal signal peptide was identified (80), which if cleaved around position 59 would yield a mature protein with a mass of 117 \times 10^3\text{Da}.

FIG. 5. Increased abundance of Fox1, Ftr1, and Atx1 transcripts as medium iron is reduced. A 100-ml culture of *C. reinhardtii* was adapted to low iron (0.1 \mu M in copper-supplemented TAP medium) at late log phase and was used to inoculate cultures with the indicated iron concentrations and copper to either 6 \mu M (normal copper-supplemented TAP medium), 0.4 \mu M (saturating for *Cyc6* repression and plastocyanin biosynthesis (76)), or 0 \mu M. RNA was harvested the following day when the cultures were at mid-log phase (2 \times 10^6 to 3 \times 10^6\text{cells}\text{ml}^{-1}) and analyzed by RNA hybridization. Parallel samples were probed for *RbcS2* expression for quantification of the data (tabulated). The relative intensities were obtained after object average background correction. The values in the table represent the signal for each sample relative to the maximum intensity for each probe, which was arbitrarily set at 100.
FIG. 6. Sequence analysis of Fox. The nucleotide sequence shown is derived from the 5'-RACE product (positions 1 to 852) and the insert in clone CL48f10 corresponding to EST AV395796 (Kazusa DNA Research Institute) (positions 787 to 4908). The numbers on the left refer to the nucleotide sequence, which is numbered from the first nucleotide of the GenBank entry. The deduced amino acid sequence of the longest ORF is given below the nucleotide sequence. The numbers on the right indicate the positions of the amino acids in the reading frame. The first methionine is numbered 1. The first 40 amino acids are shown in lowercase since the relationship of Fox with other MCOs suggests that the second methionine residue is likely to represent the initiator methionine. The putative N-terminal signal peptide is underlined, and the C-terminal transmembrane region is boxed. The polyadenylation signal is double underlined. Gray shading denotes sequences corresponding to MCO signature 1, while MCO signature 2 is given in boldface. The His-Xaa-His motifs are given in boldface and underlined. Half-arrows denote the sequences of primers used for 5'-RACE and PCR.
form the type II and type III copper binding sites (Fig. 7A). However, the position of these putative copper ligands within the *C. reinhardtii* Fox1 sequence was different from their relative locations within the other sequences.

The amino acid sequence of human ceruloplasmin can be divided into three contiguous similar units of approximately 350 residues (77). If the predicted Fox1 mature sequence was similarly divided into units of approximately 360 residues and the units were aligned with each other, a high degree of sequence identity was revealed (26 to 29%) (Fig. 7B). Some regions were very highly conserved among the three domain-like units. Each putative domain contained the four ligands (His, Cys, His, and Met) that characterize a type I Cu binding site. The numbers on the left indicate the amino acid position within the sequence.

FIG. 7. Domain-like structure of the Fox1 product with candidate copper binding ligands. The potential ligands for type I, type II, and type III copper binding sites are designated 1, 2, and 3, respectively. (A) Alignment of amino acid sequences of the putative copper binding sites in Fox1 with those of other MCOs. GenBank accession numbers: *C. reinhardtii* Fox1, AF450137; human hephaestin, AF148860; human ceruloplasmin, XM011006; ascorbate oxidase, A51027; plant laccase, U12757; fungal laccase, 17943174; *S. cerevisiae* Fet3p, P38993; *S. cerevisiae* Fet5p, P43561; *S. pombe* Fio1p, CAA91955; *P. putida* CumA, AF326406; and *E. coli* CueO (yacK), P36649. (B) Alignment of Fox1 domains. The putative ligands for type I, II, and III copper binding sites within each domain are shown above the alignment. Each domain contained the four ligands (His, Cys, His, and Met) that characterize a type I Cu binding site.

**Regulation of ferroxidase abundance by iron.** To address the hypothesis that Fox1 encodes an MCO involved in iron metabolism, we examined Fox1 mRNA as a function of iron nutrition (Fig. 5). As expected for a component of iron assimilation, Fox1 mRNA accumulation is increased as iron in the medium is decreased. The pattern of expression appears to be coordinate with that for *Fir1*, with most of the mRNA increase evident at a 1 μM medium iron concentration when the phys-
iological symptoms of iron deficiency are not yet strongly evi- 
dent. The extent of regulation depends strongly on the cell 
density. At $2 \times 10^6$ to $3 \times 10^6$ cells/ml (Fig. 5), there is a 10- 
to 20-fold increase (relative to total RNA loaded) in the abun-
dance of Fox1 mRNA at low iron versus 18 $\mu$M iron in the 
medium. At a higher cell density, the relative difference can be 
as high as 1 $\times 10^2$- to 4 $\times 10^2$-fold (data not shown). As for 
Ftr1 regulation, copper did not have a significant effect on Fox1 
mRNA abundance, a two- to threefold change at most and 
without a reproducible pattern. The coordinate iron-depen-
dent expression of Ftr1 and Fox1 is consistent with the hypo-
thesis that a permease-oxidase complex is involved in iron assim-
ilation in Chlamydomonas.

Antibodies raised against one of the three domains of Fox1 
(see Materials and Methods) were used to monitor the loca-
tion and abundance of Fox1 in Chlamydomonas. Immunoblot 
analysis identified a prominent signal corresponding to migra-
tion at 138 kDa, which compares well with the migration of 
ceruloplasmin at 132 kDa (77) (Fig. 8).

The discrepancy between the apparent and the predicted 
sizes of Fox1 may be due to glycosylation, as is the case with 
other MCOs (26, 77, 81). Based on its presence in the pellet 
rather than the soluble fraction, we conclude that Fox1 is 
probably membrane bound. The signals with higher mobility 
correspond possibly to degradation products because their 
presence is completely correlated with the intensity of the 
138-kDa signal. To test whether the protein is induced in iron-deficient cells, cells cultured in media containing 200 $\mu$M 
added Fe-EDTA were transferred to fresh media supple-
mented with 0.1, 0.25, 1, 18, or 200 $\mu$M Fe-EDTA; sampled 
after 5 days of growth; and examined by immunoblotting for 
Fox1 accumulation. Fox1 accumulation as a function of me-
dium iron nutrition clearly mirrors the pattern noted for its 
mRNA (Fig. 8A). Fox1 abundance was substantially increased 
as the medium iron concentration was reduced from 18 $\mu$M 
(normal TAP medium) and was already increased maximally in 
medium containing 1 $\mu$M iron. When the normal iron supple-
ment was increased about 10-fold to 200 $\mu$M, the amount of 
Fox1 was reduced. To monitor the kinetics of the response to 
iron deficiency, cells grown in medium containing 200 $\mu$M iron 
were transferred to fresh-iron-supplemented (200 $\mu$M) or iron-
depleted (0 $\mu$M) medium and sampled each day for 5 days. 
The response to iron depletion was rapid. Fox1 abundance was 
maximal within 24 h (Fig. 8B) even though cell division had not 
ocurred during this time (data not shown). The change in 
RNA, although transient, was even more rapid and was 
noticeable at 5 h (Fig. 8C). The rapidity of the response suggests 
either that the cells can measure external iron or that an 
internal signaling pool is depleted rapidly when the external 
iron supply is reduced.

**Copper-dependent accumulation of the ferroxidase.** Since 
copper is an essential cofactor for the ferroxidase, we won-
dered whether copper nutritional status might affect its 
accumulation, by analogy with the effect of copper status on plas-
tocyanin accumulation in Chlamydomonas cells (73). The 
copper dependency of ferroxidase accumulation was tested in 
both iron-deficient and iron-supplemented media to assess 
whether the outcome was influenced by biosynthetic demand 
for copper. Regardless of iron nutritional status and the extent 
of Fox1 mRNA increase, ferroxidase accumulation was 
strongly dependent on copper availability (Fig. 8D). The 
amount in $-$Cu iron-deficient cells was only 10% of that in 
$+$Cu iron-deficient cells. The effect was noted even for iron-
supplemented cells that have a smaller demand for biosyn-
thetic copper because of the lower level of Fox1 expression 
(Fig. 8E). We conclude that copper does not affect the iron-
responsive regulation of Fox1 but is required instead for accum-
ulation of the protein under both iron-deficient and ->suffi-
cient conditions. This effect is executed at the level of protein 
accumulation as evident from the finding that copper defi-
ciency does not reduce the abundance of Fox1 transcripts. As 
noted above (Fig. 5), any effect of copper is minimal and, in 
any case, occurs in the opposite direction of the change in 
protein abundance.

**Other components of the iron assimilation pathway.** The 
involvement of an MCO in iron assimilation prompted us to 
search for copper-metabolizing components analogous to 
Atx1p and Ccc2p of S. cerevisiae (66, 116). S. cerevisiae Atx1p 
is the metallochaperone required for delivery of copper to 
apo-Fet3p via Ccc2p-dependent transport of copper in a post-
Golgi vesicle (66, 116). This copper-requiring step is essential 
for the complete maturation of the Fet3p/Ftr1p complex and 
its passage to the plasma membrane (100). Therefore, loss of 
Atx1p function in S. cerevisiae results in loss of high-affinity 
iron uptake and failure to grow on iron-deficient medium.

**Copper chaperone (Atx1).** Oligonucleotide primers were de-
sign and used in reverse transcription-PCR to amplify a 
411-bp fragment encoding C. reinhardtii Atx1. The amplified 
product was cloned into the Nor1 site of the multicopy yeast 
expression vector pFL61, three independent clones were se-
quenced to validate the accuracy of the amplification, and the 
confirmed sequence for Atx1 was deposited under accession 
no. AF280056. Subsequently, clone CM017g07 (Kazusa DNA 
Research Institute), containing also the 5’ and 3’ UTRs, was 
also obtained and sequenced, and the sequence was deposited 
under accession no. AY120936 (Fig. 9A). A poly(A) tail and 
polyadenylation signal were not identified within the se-
quenced insert. The dbEST database contains about a dozen 
accessions representing this Atx1 gene. The predicted C. rein-
hardtii Atx1 amino acid sequence was aligned with that of S. 
cerevisiae Atx1p and homologues from A. thaliana (CCH), rice 
(Oryza sativa ATX1), soybean (G. max CCH), yeast (S. cerevi-
siae ATX1), human (Homo sapiens HAH1), mouse (Mus mus-
culus Atoxl), rat (Rattus norvegicus Atoxi), and Caenorhabditis 
elegans (CUC-1) (Fig. 9B). An extended C terminus of approxi-
mately 50 to 60 amino acids was present in homologues from 
the three photosynthetic species employed for comparison in 
the alignment (Arabidopsis, rice, and soybean) but not in the C. 
reinhardtii Atx1, which was comparable in length (70 amino 
acids) with the yeast, mammalian, and C. elegans homologues 
(68 to 73 amino acids). C. reinhardtii Atx1 shared the greatest 
sequence identity with S. cerevisiae ATX1 (36%) and less but 
still significant identity with the other sequences (29 to 32%). 
Overall similarity between Atx1 and the other sequences 
ranged from 38 to 40%. All Atx1p homologues contained the 
highly conserved motif MxCxC (where x is any amino acid), 
which has been shown elsewhere to bind copper (88). This 
motif, with a glycine residue immediately preceding the me-
thionine, is found also in the N-terminal domain of the copper-
transporting ATPases, often in multiple copies. Among the
FIG. 8. Fox1 abundance in iron-deficient Chlamydomonas. Protein extracts from C. reinhardtii cells grown in TAP medium containing various concentrations of copper and iron, and collected at a density of $10^7$ cells ml$^{-1}$, were prepared as described in Materials and Methods. Extracts were analyzed after separation by denaturing gel electrophoresis (7.5% acrylamide), transfer to nitrocellulose (1 h, 100 V, in 25 mM Tris–192 mM glycine–0.04% [wt/vol] SDS–20% [vol/vol] methanol), and incubation with anti-Fox1 antiserum (1:300 dilution). Bound antibody was visualized colorimetrically after incubation with alkaline phosphatase-conjugated secondary antibody. (A) The Fox1 gene product accumulates in Fe cells. Cells grown in medium containing 200 mM added Fe-EDTA were transferred to fresh medium supplemented with 0.1, 0.25, 1, 18, or 200 mM Fe-EDTA and sampled after 5 days of growth. Protein extracts from $2.5 \times 10^6$ cells were analyzed as described above. Samples were normalized for loading on the basis of equal cell numbers and verified for accumulation of CF1, which is iron independent (see Materials and Methods). Percentages on the left are the fractional amounts of the “0.1 mM Fe” sample that were loaded. (B) Time course of Fox1 induction in Fe cells. Cells grown with 200 mM added Fe-EDTA were transferred to fresh medium lacking iron (—Fe) or containing 200 mM added Fe-EDTA (+Fe). Cultures were sampled each day for 5 days (d), and ferroxidase abundance was analyzed as described above. Percentages shown on the right are.
Atx1 homologues, *A. thaliana* CCH, rice ATX1, soybean CCH, and *C. elegans* CUC-1 all possess the glycine at this position, whereas the motif in *C. reinhardtii* Atx1 is more similar to the mammalian homologues, which have an aspartic acid residue in this position in place of glycine. The conserved C-terminal lysine-rich region also was present in the *C. reinhardtii* sequence, although the arrangement of the lysines was most similar to the homologues from the photosynthetic species (KTGKK) rather than to those from the nonphotosynthetic ones (KKTGK). Southern analysis reveals a single hybridizing fragment in the genome of *Chlamydomonas*.

**Copper delivery.** A role for *C. reinhardtii* Atx1 in copper delivery to a copper-containing ferroxidase is supported by RNA blot analysis, which indicates an approximately fivefold increase in *Atx1* mRNA accumulation relative to total RNA in cells exhibiting symptoms of iron deficiency (0.1 μM supplemental iron) compared to iron-replete cells (18 μM). Medium copper content, on the other hand, did not affect *Atx1* mRNA abundance (Fig. 5).

*C. reinhardtii* Atx1 function in copper delivery was also tested by functional complementation of an *S. cerevisiae atx1Δ* strain. Atx1p is essential for high-affinity iron uptake in *S. cerevisiae*. Accordingly, a strain with the *ATX1* gene deleted (*atx1Δ, SL215*) is unable to grow on iron-depleted medium (66). *CCH* (Arabidopsis), *HAH1* (human), and *CUC-1* (*C. elegans*) have each been shown to restore the growth of an *S. cerevisiae* atx1Δ strain on iron-depleted medium, indicating that the heterologous proteins can function in *S. cerevisiae* (45, 55, 66, 112). In this study, *S. cerevisiae* strains YPH250 (wt) and SL215 (atx1Δ) were transformed with either *C. reinhardtii* Atx1 (CtAtx1) or *S. cerevisiae* Atx1 (ScATX1) under the control of the *PGK1* promoter in pFL61 (78). Transformants were plated on SD complete medium containing the iron chelator ferrozine (1.5 mM) in the absence (−Fe) or presence (+Fe) of 350 μM ferrous ammonium sulfate. Only the wt strain and transformants expressing Atx1 of *C. reinhardtii* or *S. cerevisiae* were able to grow on the iron-deficient medium (Fig. 10A). Complementation of the mutant phenotype was plasmid dependent, and similar results were obtained when the experiment was repeated with independent transformants expressing CtAtx1 or ScATX1 (data not shown). The vector by itself did not alter the iron-dependent growth of the atx1Δ strain. *C. reinhardtii* Atx1 was comparable to *S. cerevisiae* ATX1 in the context of this experiment. Differences between the wt strain and the transformants reflect perhaps the difference between ATX1 function at a chromosomal location and that on a plasmid. We conclude that *C. reinhardtii* Atx1 can function to deliver copper to a copper transporter in the secretory pathway.

**Antioxidant function.** *S. cerevisiae* ATX1 was isolated originally for its ability to protect the *sod1Δ sod2Δ* mutant strain against oxygen toxicity, hence the name *ATX1* for antioxidant (65). The *sod1Δ* mutant strain exhibits auxotrophy for lysine and methionine, since the biosynthetic pathways for these amino acids contain steps that are sensitive to reactive oxygen. Overexpression of *ATX1* in a *sod1Δ* mutant can rescue the lysine and methionine auxotrophy of this strain (65) via the antioxidant activity of Atx1p, which is attributed to its copper binding function. To test whether *C. reinhardtii* Atx1 has an analogous function, wt, *sod1Δ* mutant, and *sod1Δ* strains transformed with *C. reinhardtii* Atx1 (CtAtx1), *S. cerevisiae* ATX1 (ScATX1), or pFL61 alone were compared for growth on medium lacking lysine or methionine. *C. reinhardtii* Atx1 was indeed able to restore aerobic growth of the *sod1Δ* strain on medium lacking lysine (−Lys) or methionine (−Met) (Fig. 10B), confirming yet another aspect of its function and the validity of the designation Atx1.

**Copper ATPase.** The functionality of *C. reinhardtii* Atx1 argues in favour of a Ccc2- or MNK/WND-like copper-transporting P-type ATPase in the ferroxidase assembly pathway. A BLAST search of the *C. reinhardtii* EST database (31) with the sequences of *A. thaliana* copper ATPases as the input revealed two *C. reinhardtii* ESTs represented by accessions BE761354 and BG844651 with good probability scores, 2e−13 and 2e−18, respectively. When each is used as the input to search the nonredundant protein database, sequences encoding copper-transporting ATPases are retrieved with the best probability relative to other ATPases. The 3′ ends of the two clones from which the ESTs were derived are identical, indicating that the two ESTs represent a single gene, which increases the likelihood of our assignment of this sequence as a copper-transporting ATPase. The amino acid sequences deduced from the two *C. reinhardtii* ESTs were aligned with those of copper-transporting ATPases from *Synochococcus* strain PCC 7942 (PacS and CtaA), *A. thaliana* (RAN1), *S. cerevisiae* (Cc2p), *Entero- coccus hirae* (CopA), and the human Menkes (MNK) and Wilson (WND) proteins. The protein sequence derived from accession BG844651 showed similarity to a highly conserved region that spans transmembrane domain 7 of the MNK and WND proteins and is close to the C terminus of the copper ATPases. The protein sequence derived from accession BE761354 showed similarity to a less well conserved region between the phosphorylation site and the ATP binding site of MNK and WND that is located within the cytoplasmic loop that lies between transmembrane domains 6 and 7 (Fig. 11). A fragment corresponding to the EST represented by accession BE761354 was amplified and cloned (see Materials and Meth-
ods) and used as a probe in RNA blot analysis with the objective of testing its expression in response to iron deficiency. Unfortunately, a signal was not detected, and this question could not be addressed.

**Is copper-deficient C. reinhardtii also iron deficient?** In yeast and mammals, the requirement of multicopper-containing ferroxidases for iron metabolism results in an obligate link between copper nutritional status and iron metabolism, such that copper deficiency leads to impairment of ferroxidase function and hence secondary iron deficiency. Previously, we have demonstrated that this is not the case for C. reinhardtii based on the distinct, nonoverlapping pattern of responses to copper deficiency. In yeast and mammals, the requirement of multicopper-containing ferroxidases for iron metabolism results in an obligate link between copper nutritional status and iron metabolism, such that copper deficiency leads to impairment of ferroxidase function and hence secondary iron deficiency. Previously, we have demonstrated that this is not the case for C. reinhardtii based on the distinct, nonoverlapping pattern of responses to copper deficiency.
ciencies versus those to iron deficiencies (42). In this work, we provide substantial evidence for the involvement of an MCO in iron metabolism (Fig. 5 and 8), including a pathway for assembly of its copper binding site (Fig. 9 to 11). We also show that the induced accumulation of the ferroxidase in iron deficiency is greatly inhibited in copper deficiency. The lack of effect of copper nutritional status on iron metabolism as suggested previously (42) appeared contrary. We considered, therefore, whether the operation of low-affinity iron assimilation pathways might mask the effect of copper deficiency on iron uptake. If so, one might expect a greater impact of copper deficiency when iron supply is limiting to growth. The effect of copper-deficient growth conditions on the iron status of C. reinhardtii cells was therefore reassessed on wt C. reinhardtii as a function of iron concentration from 0.1 to 200 μM (Fig. 12A). Growth was monitored over a period of 6 days by cell counts, and iron sufficiency was monitored over the same time by chlorophyll content (Fig. 12B). Iron-deficient cells became chlorotic due to degradation of chlorophyll proteins (J. Moseley, S. Merchant, and M. Hippler, unpublished results), and as deficiency became more severe, cell growth was inhibited so that the final cell density of iron-deficient cells was lower than that for iron-replete cells. The medium iron concentration at which these changes were evident was independent of medium copper concentration (Fig. 12), indicating that copper deficiency and loss of ferroxidase function do not affect cellular iron status in C. reinhardtii. By analogy with the occurrence of a copper-independent pathway for photosynthetic electron transfer, we suggest that (i) there must be a backup copper-independent pathway for iron assimilation and (ii) the expression of this pathway must be increased in copper deficiency to compensate for the loss of Fox1 function.

**DISCUSSION**

The molecular components of iron metabolism in green algae are largely unidentified. Physiological experiments implied the involvement of reductases in iron assimilation because this activity is induced in iron-deficient cells (22, 42, 67, 114). The question of whether these reductases are iron specific or whether they function also in copper assimilation is unexplored yet because the identity of the enzymes is unknown. The nature of iron transporters is also largely unknown, although molecules like transferrin have been implicated in iron assimilation in a halotolerant alga, Dunaliella species (27, 28) (Dunaliella tertiolecta accession no. AAF72064). With the objective of defining the molecular components of iron assimilation and homeostasis in *Chlamydomonas*, which is an excellent model for the investigation of metal metabolism, we searched the EST database for candidate homologues of known components of iron metabolism in plants, animals, and fungi. Sequences representing four molecules were analyzed in this work: ferritin (Fer1), an iron permease (Ftr1), an MCO (Fox1), and a copper chaperone (Atx1). ESTs representing a copper transporter that probably interacts with the chaperone also were identified, but these have not yet been analyzed. Homologues of the *Saccharomyces* FRE genes or the *Arabidopsis* FRO genes were not identified (February 2002); instead, ESTs representing at least two different genes for cytochrome b₅-reductase type proteins were identified (3e⁻³⁶ and 3e⁻⁴⁰) that are related to maize NADM-dependent Fe³⁺-chelate reductase, which are implicated in iron metabolism (8). As for the candidate copper transporter, the relevance of the NADM-dependent Fe³⁺-chelate reductase-like sequences in iron metabolism needs to be tested by expression analyses.

**Ferritin.** The ESTs representing a plastid-targeted ferritin appeared to represent a single gene. The discovery of ferritin was not surprising, because algal ferritin has been described previously (50, 87). Ferritin has a storage function and is a key enzyme in maintaining iron homeostasis. Plant ferritin is localized in the plastids in the stroma (10, 102). It is found primarily in roots and leaves of young plants, with much lower levels occurring in mature plants (10). Incorrect expression of ferritin in mature plants results in iron-deficiency chlorosis, confirming that ferritin sequesters iron and supporting the model that ferritin serves as an iron reservoir for iron supply to the photosynthetic apparatus in developing leaves (108). Since ferritins probably function also for transient iron storage, for example, during senescence or other situations where iron proteins are degraded (10), this might explain the anomalous
finding that mRNAs encoding ferritin are up-regulated during iron deficiency (9, 84); in iron-deficiency-induced chlorosis, iron might be released as photosystem I is degraded. In Chlamydomonas, ferritin mRNA is increased about fourfold in iron deficiency relative to total RNA (Fig. 1). This iron-deficiency increase in the mRNA may be part of a mechanism to anticipate iron overload. Two models are offered to explain C. reinhardtii Fer1 gene induction under iron deficiency. First, increased ferritin might allow the cell to handle transient overload when iron is resupplied to the deficient cells following the induction of the uptake pathway, and this is consistent with the observation that the increased mRNA does not result in increased protein (M. Hippler, personal communication). A second possibility is that ferritin may serve to buffer iron as it is released from degrading photosystem I and recycled to other compartments (such as the mitochondrion). This model is supported by the finding that iron deficiency impacts the chloroplast more severely than it does respiration. It also is compat-

FIG. 11. Sequence comparison between C. reinhardtii ESTs and copper ATPases. The predicted amino acid sequences encoded by C. reinhardtii ESTs (GenBank accession no. BE761354 and BG844651) were aligned with the relevant regions of copper-transporting ATPases from other organisms. The alignment was generated by using the ClustalW algorithm and BioEdit software (34). The numbers indicate the positions of the amino acids in each sequence. The first sequence in the alignment represents the amino acids encoded by the two C. reinhardtii ESTs, which align with different regions of the copper ATPases but are derived from a single gene. The alignment of the ESTs was based on BLAST output. Residues that are similar or identical in a majority (five) of sequences are shaded gray and black, respectively. A line above the alignment indicates the transmembrane regions. GenBank accession numbers: Synechococcus PacS, P37279; Synechococcus CtaA, AAB82020; A. thaliana RAN1, AF082565; S. cerevisiae Ccc2p, L36317; E. hirae CopA, L13292; H. sapiens MNK, NM_000052; H. sapiens WND, NM_000053.
The discovery of a multicopper ferroxidase in metabolism in plants although plants contain several abundant MCOs. MCOs are the copper-containing ferroxidases. MCOs occur in a wide variety of organisms and have a range of cellular functions, which is reflected in the variety of organic substrates that can be oxidized (77). A distinguishing feature of these proteins is the presence of copper ions that are classified according to their spectroscopic properties and are referred to as type I or “blue,” type II or “normal,” and type III or “binuclear” copper ions, the latter comprising a pair of copper ions (77). The type I copper is bound as a mononuclear species, while the copper ions of the type II and type III sites form a trinuclear cluster. With the exception of ceruloplasmin, which has three type I sites, in general there is one type I site associated with a trinuclear cluster. Typical type I sites are formed by a set of four copper-binding ligands, His, Cys, His, and Met, although one of the three type I sites in ceruloplasmin and the type I sites of fungal laccase and Fet3p have Leu in place of the Met. The trinuclear cluster has eight histidine ligands arranged as four His-Xaa-His sequences along the polypeptide chain (77).

We suggest that Fox1 may be a type I, C-terminally anchored, membrane glycoprotein by analogy to Fet3p (99) and hephaestin (111). Its amino-terminal domain would be extracellular or within an extracytosolic compartment, and only four putative N-glycosylation sites that precede the transmembrane domain would be glycosylated. In sequence, length, and conservation of type I, II, and III copper binding sites, Fox1 was most similar to the mammalian ferroxidases. Till the recent discovery of hephaestin, a peculiarity of ceruloplasmin was the presence of three type I copper sites, whereas other MCOs have only one. With three potential type I copper sites, Fox1 represents the third member of the ceruloplasmin-hephaestin family. This observation together with its three-domain structure makes Fox1 very similar to ceruloplasmin. Given that the MCOs function in specific partnerships with individual iron transporters (7), it seemed unlikely that the Chlamydomonas protein would rescue a fet3 mutant, and hence this experiment was not attempted. If the topological prediction of Fox1 were correct, then one of the three type I sites would have an intracellular location. This raises the possibility for intracellular recognition of the occupancy state of the copper binding sites and perhaps subsequent activation of a degradation pathway. The other MCO sites would occur extracellularly, where they could function in iron uptake together with Ftr1. Another novelty of Fox1 is the position of the type I sites relative to the type II and type III sites. In the other MCOs the HxH motifs precede the HxHxH and HCHxHxH motifs, whereas in Fox1 the latter two motifs precede the two HxH motifs. In addition, these four motifs occur within the first half of Fox1, while in the other MCOs, the HxH motifs are close to the N terminus and the HxHxH and HCHxHxH motifs are close to the C terminus (Fig. 7A). The functional significance of these features of Fox1 can be tested with respect to its structure, copper binding properties, and regulation by copper nutrition.

**Ftr1.** The Ftr1-like permease had features consistent with a role in the transmembrane transport of iron. If Ftr1 functions in a complex with Fox1 as in *S. cerevisiae* (100), then it is predicted to be located in the plasma membrane, where Fox1 is located (39). In this case it would function in assimilation rather than intracellular distribution. The pattern of expression of *Ftr1* argues in favor of a function in iron assimilation. The activation of Ftr1 and Fox1 mRNA accumulation occurs at nutritionally relevant concentrations and precedes the appearance of iron-deficiency phenotypes such as chlorosis (compare Fig. 5 and Fig. 12). Ftr1 contains two copies of the highly conserved RExxE motif found in ferritin and Ftr1p. In ferritin the glutamates interact with iron (64, 107), and in Ftr1p they...
are necessary for iron transport (100). As with the other Ftr1 homologues, one of the two REXXE motifs occurs within the putative signal sequence of C. reinhardtii Ftr1, which raises the possibility of a function in iron-dependent trafficking. A further possibility is that the N-terminal REXXE motif be involved in the maturation of the protein and iron-dependent posttranslational control of the level of functional protein in the membrane, in a manner somewhat analogous to the copper-dependent posttranslational control of S. cerevisiae Ctrlp (82). Alternatively, since this motif is also embedded within a hydrophobic region, it may simply serve as an additional binding site for iron as it traverses the membrane. Two ExxE motifs that were proposed to be involved in iron binding in Ftr1p of S. cerevisiae also were found in the Chlamydomonas sequence. We can propose two topological models for C. reinhardtii Ftr1. If the N-terminal hydrophobic region serves as a signal sequence and is cleaved, then the N and C termini would be extracellular, whereas cleavage of the N terminus would yield a protein with a cytoplasmic N terminus and a C terminus that is extracellular.

Copper metabolism. Molecules that might function in the biosynthesis of Fox1, such as homologues of the Ccc2p transporter and the metallochaperone Atx1p, also are found in Chlamydomonas. The latter occurs as a single-copy gene (data not shown) and is induced under iron-deficient conditions, albeit not as strongly as Fox1 and Ftr1, supporting its assignment in copper delivery to the secretory pathway. The accumulation of Cox17 mRNA (encoding a different copper chaperone involved in copper supply to the mitochondrion) is not affected by iron, suggesting that the effect of iron deficiency on Atx1 mRNA is specific and significant. Ccc2 and Atx1 homologues probably function to load a variety of copper-containing enzymes in the secretory pathway in Chlamydomonas besides Fox1, which would account for the smaller fold difference in regulation. For instance, in plants, RAN1, a Ccc2p homologue, is required for loading copper into the active site of the ethylene receptor (46). In Synecocystis strain PCC 6803 a copper metallochaperone, also designated Atx1, was identified and shown to interact with the CtaA copper importer to acquire and then deliver copper to the PacS copper ATPase which in turn provides copper for proteins involved in photosynthetic and respiratory electron transport within the thylakoid membranes (106).

Functional assignment of C. reinhardtii Atx1 is also supported by its ability to complement S. cerevisiae atx1Δ and sod1Δ mutant strains, the former reflecting its ability to interact with the copper binding domains of Ccc2p and the latter reflecting its ability to bind copper. Atx1 has the conserved metal binding motif MxCxxC present in all metallochaperones identified to date and in the copper-transporting ATPases. The Thr residue immediately adjacent to the Met is conserved among the yeast, C. elegans, and mammalian Atx1 homologues as well as the copper ATPases and is conservatively replaced by Ser in the plant sequences. Based on structural studies of Atx1 and HAH1, a suggested function for this residue is to modulate, via hydrogen bonding, the interaction between the chaperone and its copper ATPase partner (51). The significance of the Met residue at the equivalent position in the C. reinhardtii sequence is unclear. Several lysine residues form a positively charged surface on the ATX1 molecule and were hypothesized previously to be involved in electrostatic interactions with a corresponding acidic face of the copper ATPase target protein (85, 94). Three of these (corresponding to S. cerevisiae Lys28, Lys62, and Lys65) are conserved in all of the Atx1 homologues, including C. reinhardtii Atx1. Lys65 is required for the copper-dependent interaction and possibly metal transfer between ATX1 and Ccc2p and for the antioxidant role of ATX1 and HAH1 (51, 52, 85, 94), whereas Lys28 and Lys62 as well as Lys24 and Lys61 of ATX1 are required only for copper delivery to Ccc2p (85).

C. reinhardtii Atx1 had two features in common with the mammalian homologues. One of these was the aspartic acid residue immediately preceding the methionine of the MxCxxC motif instead of the glycine residue that is conserved in the copper ATPases. A. thaliana CCH, rice ATX1, soybean CCH, and C. elegans CUC-1. Structural studies will clarify the significance of this substitution. The other was the absence of the C-terminal extension that is present in the plant homologues. In A. thaliana this region was postulated to interact with other molecules (45). A. thaliana CCH is down-regulated by copper and up-regulated during leaf senescence, possibly for copper sequestration during this process (45). In contrast to A. thaliana CCH but similar to S. cerevisiae ATX1, C. reinhardtii Atx1 is not regulated by copper but is regulated by iron and therefore is likely to share more structural and functional similarities with the mammalian and yeast homologues than with the plant homologues.

Copper versus iron nutritional status. Previously, we had argued that iron metabolism in Chlamydomonas was independent of copper because copper-deficient cells did not display any symptoms of iron deficiency, such as chlorosis. Also, the pattern of gene expression in –Cu versus –Fe cells was distinct (42). The analysis of iron-deficiency-induced transcripts in this study confirms the previous work (Fig. 5). Transcripts that increase up to several-hundred fold in iron-deficient cells are not affected by copper deficiency. The discovery of a ceruloplasmin-hephaestin-like MCO in Chlamydomonas was unexpected (Fig. 6 to 8). Its involvement in iron metabolism and, more specifically, iron assimilation is supported on the basis of its coordinated expression with Ftr1, a putative iron permease, in iron-deficient cells (Fig. 5); its membrane localization (Fig. 8); and its extracellular ferroxidase activity (39) (http://lin2.biologie.hu-berlin.de/~botanik/deutsch/ffp.html). The lack of impact of copper nutritional status on iron metabolism presented a conundrum.

We considered two possibilities: first, that ferroxidase function was not compromised in copper-deficient cells because of preferential allocation of residual copper to iron assimilation versus photosynthesis, and second, that the effect of copper nutritional status would be apparent only in iron-limited cells. To address these hypotheses, ferroxidase abundance was monitored as a function of copper concentration (Fig. 8). Regardless of cellular iron status, copper deficiency (this work) or copper removal (39) had a drastic impact on ferroxidase accumulation or activity, respectively. We suggest that assembly of the holoprotein is defective when copper is limiting, leading perhaps to degradation of the apoprotein. No change in the abundance of lower- or higher-molecular-weight immunoreactive bands, indicative of enhanced protease susceptibility or ubiquitination, was noted, but this does not argue against deg-
radiation, because such intermediates may be short lived. An effect of copper on translation also cannot be ruled out. Interestingly, ferroxidase abundance does increase in iron-deficient cells compared to iron-replete cells even without copper in the medium. This may result from increased allocation of intracellular copper to the ferroxidase biosynthetic pathway in iron-deficient versus iron-sufficient cells through increased Atx1 expression (Fig. 5). Alternatively, it may merely reflect a new steady state resulting from increased synthesis as a consequence of an increase in the Fox1 mRNA pool. The second hypothesis, that copper deficiency might impact only iron-limited cells, was tested by examining the effect of copper nutrition on marker gene expression (Fig. 5) and chlorophyll accumulation and growth (Fig. 12) at a range of medium iron concentrations. Clearly copper nutritional status does not affect the response to iron nutrition despite its impact on ferroxidase accumulation.

A third hypothesis that can be tested genetically is that there may be a copper-independent pathway for iron assimilation. We propose that the expression of such a pathway would be controlled tightly. It would serve only as a backup for the Fox1-dependent pathway, just as cytochrome c₆ serves as a backup for plastocyanin (71, 74).

The similarity in catalytic ability between copper and iron enzymes prompts speculation that the replacement of a copper enzyme with a backup iron version might represent a general metabolic adaptation to copper deficiency. There are several examples where copper- and iron-containing proteins represent alternative alternatives that carry out the same function, and these include plastocyanin/cytochrome c₆ cytochrome oxidase/alternative oxidase, and CuZn-SOD/Fe-SOD, among others. In Chlamydomonas, clearly there must exist a copper-independent pathway for iron uptake, and whether there are components of such a pathway that serve as true backup proteins for the copper proteins involved in copper-dependent iron uptake remains to be determined. A genetic screen for C. reinhardtii iron metabolism mutants will help to identify such components.

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