Induction of Coproporphyrinogen Oxidase in Chlamydomonas Chloroplasts Occurs via Transcriptional Regulation of Cpx1 Mediated by Copper Response Elements and Increased Translation from a Copper Deficiency-specific Form of the Transcript*  

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Coproporphyrinogen III oxidase, encoded by a single nuclear gene in Chlamydomonas reinhardtii, produces three distinct transcripts. One of these transcripts is greatly induced in copper-deficient cells by transcriptional activation, whereas the other forms are induced in copper-deficient cells. The sequence GTAC, which forms the core of a copper response element associated with the Cyc6 gene, is also essential for induction of the Cpx1 gene, suggesting that both are targets of the same signal transduction pathway. The constitutive and induced Cpx1 transcripts have the same half-lives in vivo, and all encode the same polypeptide with a chloroplast-targeting transit sequence, but the shortest one representing the induced form is a 2-4-fold better template for translation than are either of the constitutive forms. The enzyme remains localized to a soluble compartment in the chloroplast even in induced cells, and its abundance is not affected when the tetrapyrrole pathway is manipulated either genetically or by gabcultine treatment.

Chlamydomonas reinhardtii exhibits multiple adaptations to copper deficiency, making it an excellent model system for the study of metal-responsive gene expression. One well characterized metal-responsive pathway in many green algae and cyanobacteria is the reciprocal accumulation of plastocyanin and cytochrome c₆ encoding (Cyc6) gene, which is known to be transcriptionally regulated in copper-deficient cells. The sequence GTAC, which forms the core of a copper response element associated with the Cyc6 gene, is also essential for induction of the Cpx1 gene, suggesting that both are targets of the same signal transduction pathway. The constitutive and induced Cpx1 transcripts have the same half-lives in vivo, and all encode the same polypeptide with a chloroplast-targeting transit sequence, but the shortest one representing the induced form is a 2-4-fold better template for translation than are either of the constitutive forms. The enzyme remains localized to a soluble compartment in the chloroplast even in induced cells, and its abundance is not affected when the tetrapyrrole pathway is manipulated either genetically or by gabcultine treatment.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) AF133671 (for the Chlamydomonas reinhardtii Cpx1 gene) and AF133672 (for the Chlamydomonas reinhardtii Cpx1 cDNA).  
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The abbreviations used are: cyt, cytochrome; coprop, coproporphyrinogen III; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; UTR, untranslated region; TAP, Tris acetate-phosphate; psiII, Bluescript II; CuRE, copper response element; +Cu, copper-supplemented; −Cu, copper-deficient.

This paper is available on line at http://www.jbc.org
The discovery that coprogen oxidase activity was so highly induced in copper-deficient cells raised several questions concerning both the copper-responsive signal transduction pathway and the operation of the tetrapyrrole pathway. Specifically, was the Cpx1 gene regulated by the same mechanism as the Cyc6 gene? Does the observed regulation occur in direct response to copper deficiency or indirectly to heme depletion? Does all of the enzyme remain plastid-localized in copper-deficient cells or is it redistributed to the mitochondrion? Is the subsequent enzyme in the pathway, protoporphyrinogen oxidase, induced? To address these questions, we isolated full-length cDNAs corresponding to transcripts from copper-supplemented and copper-deficient cells, characterized induced and constitutive forms of Cpx1 transcripts with respect to half-lives and translatability in vivo, tested for mitochondrial localization of the enzyme, and analyzed cloned genomic DNA for its ability to confer copper responsiveness to a reporter gene. The possibility that Ppx1 transcripts (encoding the chloroplast form of protoporphyrinogen oxidase) exhibited copper-responsive regulation was also tested.

EXPERIMENTAL PROCEDURES

Strains and Cell Culture—Cultures of C. reinhardtii wild type strains CC124, CC125, 2137, and strain CC849 (cw15) and strains CC124, CC125, and 2137, were maintained under either constant illumination or constant darkness in darkness (25). Cultures grown in the dark and making serial one-half dilutions of cells into fresh medium. Cells took 7–12 days (average, 10 days) to completely de-green.

Isolation of Coprogen Oxidase-encoding Genomic and cDNA Clones—Genomic sequences encoding coprogen oxidase were identified from a C. reinhardtii λ-EMBL3 genomic DNA library (26) by plaque hybridization to cpx440 DNA (18). An ~3.8-kilobase pair SalI fragment was subcloned in both orientations into pBSIIKS(−) (Stratagene) to generate pCpx1a and c. An overlapping 5.8-kilobase pair NotI-SalI fragment from a different λ clone containing ~4.9-kilobase pairs of additional 3′ untranslated sequence was also subcloned and an additional ~800 base pairs of 3′ flanking sequence obtained (Fig. 1, A and B). Two cDNA libraries were screened using cpx440 as a probe. Both libraries were generated from RNA isolated from copper-deficient cultures of either C. reinhardtii strain CC124 (ZipLox library) or 2137 (Δgt11 library) (27). From the Δgt11 library, an ~2.0-kilobase pair fragment that contained the entire 1098 base pairs of coding sequence and 770 base pairs of 3′ UTR was identified. From the ZipLox library, an overlapping ~1.1-kilobase pair fragment that contained an additional 159 base pairs of 5′ untranslated sequence was identified and recovered in plasmid pZL1 following the manufacturer’s excision protocol. The plasmid was named pCPX1.1. The EcoRI fragment from the Δgt11 clone was subcloned by standard techniques into pBSIIKS(−) to generate pCPX2.0.

Sequencing—Genomic and cDNA clones were sequenced on both strands at the sequencing facility at UCLA using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer). Sequences were edited and assembled using the ABI PRISM Sequencing Analysis and Autoassembler programs (Perkin-Elmer), and further analyzed using DNA Strider, version 1.2 (28). Site-directed mutations were verified by sequencing (T7 Sequenase, version 2.0, sequencing kit, Amersham Pharmacia Biotech).

PCR Amplification of Fpx1 cDNA Clone—Primers for amplification of C. reinhardtii Ppx1 cDNA (encoding protoporphyrinogen IX oxidase I) were designed based on the partial genomic DNA sequence (29), and were used to amplify a 435-base pair fragment. This fragment was digested with SacII and PstI and the resulting 196-base pair fragment was cloned into pBSIIKS(−), yielding pPpx196.

Isolation and Analysis of Nucleic Acids—Total DNA and total RNA from C. reinhardtii cells was isolated and analyzed by DNA or RNA blot hybridization as described previously (30–32). The following cDNAs were used as probes: the cpx440 fragment (18), the 710-base pair insert from pTZ18Cv552–7A (27), the ~7 × 10^6-base pair insert from pM1

![Fig. 1. Map of plasmids (A), the genomic DNA region encoding coprogen oxidase and the sequencing strategy (B), and a schematic diagram of the Cpx1 cDNA (C).](http://www.jbc.org/)
Cu-responsive Regulation of Coprogen Oxidase Accumulation

Table I

<table>
<thead>
<tr>
<th>Primer</th>
<th>Position in genomic sequence</th>
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<td>TGCGGGGACAAATAGGCCG</td>
</tr>
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</tr>
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<td>-52 to -55</td>
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</table>

* Underlined nucleotides indicate mutations.

† Primer 15 is designed to amplify the cDNA and hence the sequence is interrupted by the first intron. Primers 15 and 18 are designed with BamHI restriction sites at the 5’ ends to facilitate cloning in-frame into the thioredoxin fusion expression vector pTrxFus.

(33), the 11 × 10^4-base pair BamHI fragment from pJD27 (34), the 577-base pair insert of pT72R:CrP (38) and the 16 × 10^4-base pair insert of pSKBluescript:GSAT (36). The 6 × 10^4-base pair insert of pScp2p: Hema (37), and the 196-base pair site of pPp4p166 (described above). The specific activities of the probes ranged from 0.9 × 10^4 to 4 × 10^8

**condition** conditions were 95 °C for 5 min prior to Taq polymerase addition, G4 cycles of 94 °C for 1 min, 53 °C for 1 min, and 72 °C for 2 min followed by 26 cycles of ΔTm for 1 min, 58 °C for 45 s, and 72 °C for 1 min, with a final 15-min extension at 72 °C. The resulting product was gel-purified, digested with BamHI and cloned in-frame to the carboxyl terminus of the thioredoxin-encoding sequence of the expression vector pTrxFus (Invitrogen Corp., San Diego, CA), and introduced into Escherichia coli host strain G1T24 for tryptophan-inducible expression.

**Preparation** of enriched inclusion bodies was solubilized (44) and used directly for antiseraum production. Polyclonal antibodies were raised in rabbits by Cocalico Biologicals Inc. (Reamstown, PA) by polyclonal lymph node injection of the purified antigen (0.25 mg) followed by three intramuscular boosts (0.15 mg). The resulting antiseraum was designated anti-cpx-trx.

The cDNA fragment cpx440 (18) was cloned into the glutathione S-transferase fusion vector pGEX 4T-1 (Amersham Pharmacia Biotech). The resulting overexpressed fusion protein localized to inclusion bodies, which were isolated (44), and the fusion protein was purified by SDS gel electrophoresis (45). Protein bands were visualized with ice cold 0.25 sodium chloride and gel slices containing the band of interest were sent to Cocalico Biologicals for antiseraum production (anti-cpx-glutathione S-transferase production).

**Radio labeling** of cells and Immunoprecipitation—CC425 or CC124 cells were grown in copper-free low sulfate TAP medium to mid-log phase. Cultures were divided into fresh acid-washed flasks, and CuCl2-EDTA (to 6 μm), and/or gabaculine (3-amino-2,3-dihydrobenzoic acid; Sigma) (to 2 μm) was added to cells. After incubation (6.8–19 h), cells were collected by centrifugation, and resuspended to 1 × 10^8 cells/ml in either copper-supplemented or copper-free, sulfate-free TAP medium, and allowed to recover for 2 h in a tissue culture wheel at 25 °C under constant illumination (~50 μmol m^-2 s^-1) before radiolabeling. Just prior to radiolabeling, a 1-ml aliquot was removed for preparation of total RNA, and a 0.5-ml aliquot was removed for extraction of soluble protein to allow for quantitation of the abundance of Cpx1 mRNA and coprogen oxidase. Radiolabeling and immunoprecipitation were carried out with the remaining part of the culture using anti-cpx-trx antiseraum (46).

**Quantitation of Coprogen Oxidase—Soluble** extracts were prepared and analyzed by immunoblotting (47) using anti-cpx-trx antiseraum. Antigen-antibody complexes were detected using [125I]-labeled protein A, and signals quantitated using the PhosphorImager and Image QuanNT software.

**Nuclear Run-on Assays—** Nuclear material was prepared, stored, and assayed as described previously (47). Hybridization signals were quantitated using the PhosphorImager.

**Chloroplast and Mitochondria Purification and Immunoblot Analysis—** Mitochondrial preparations were made as described (48) from strain CC849. Proteins were separated on 12% SDS-polyacrylamide gels and analyzed by immunoblotting (47, 49), except that nonfat dry milk was used instead of calf serum as the blocking reagent for detection of carbonic anhydrase. Anti-cpx-glutathione S-transferase antiseraum was used at a dilution of 1:3000, anti-carbonic anhydrase antiseraum was used at a dilution of 1:2000, and anti-Oee1 antiseraum was used at a dilution of 1:3000. Bound primary antiseraum was detected with an alkaline phosphatase-conjugated secondary antiseraum and chromogenic substrate.
RESULTS

Different Cpx1 Transcripts from a Single Gene—When copper is added to −Cu cells, the abundance of Cpx1 transcripts decreases dramatically within 60 min, reminiscent of the decay of Cyc6 transcripts (32). But in contrast to the situation with Cyc6, in which the transcripts continue to decay over a period of 180 min until they are completely gone, Cpx1 transcripts dropped to a minimal level and then reaccumulated to reach a new steady state comparable to that in cells maintained constantly under copper replete conditions (Fig. 2). We noted a small but highly reproducible shift in the mobility of the Cpx1 hybridizing band during establishment of the new steady state. The Cpx1 transcript from copper-supplemented cells appeared slightly larger than the form in −Cu cells (compare 60- and 100- or 120-min time points in Fig. 2). This suggested that there were at least two types of Cpx1 mRNAs. The different species might represent alternative transcripts from one gene, or they might represent products of two different genes.

Southern analysis of total DNA from C. reinhardtii cut with four different restriction enzymes revealed a single hybridizing band in each case (Fig. 3), and its size matched the size predicted from the sequence of the Cpx1 gene described in this work (Figs. 1 and 4). The same hybridization pattern was observed even under low stringency conditions (hybridization temperature, 50 °C; data not shown). Therefore, we conclude that coprogen oxidase is encoded by a single gene in C. reinhardtii: the different sized transcripts must result then from differential processing of a precursor RNA or from initiation of transcription at different sites.

The nucleotide sequence of overlapping genomic clones (Fig. 1B; see also under “Experimental Procedures”) corresponding to all six exons and five introns plus 5′ and 3′ flanking DNA was determined on both strands (Fig. 4). The sequence of the cDNA (which was prepared from RNA of −Cu cells) is in complete agreement with the genomic sequence (Fig. 4).

The cDNA encodes a protein of 365 amino acids, with a predicted molecular mass of 41.4 kDa. The amino-terminal sequence of the purified protein (18) was determined by Edman degradation to be ATAIEAENTVKQAPQ, which matches the translated sequence of the cDNA clone starting at codon 32 of the open reading frame. The molecular mass of the mature enzyme (codons 32–365) is calculated to be 37.9 kDa. The 31-residue amino-terminal extension presumably corresponds to the transit sequence, which would function to direct the translation product to the plastid. In fact, when anti-coprogen oxidase-reactive species are immunoprecipitated from radiolabeled cells, two species are observed initially: a major form (mass, 35 kDa), corresponding to the mature protein, and a minor form (mass, ~39 kDa) (see Fig. 8). The latter corresponds to the primary translation product and exhibits the characteristics of a precursor form: it comigrates with the single species immunoprecipitated from in vitro translated poly(A)+ RNA, and it is observed only in briefly labeled cells but not when the samples are chased for several minutes. The ~4-kDa size difference between the two bands is consistent with the calculated size of the putative 31 residue chloroplast targeting presequence (3.5 kDa).

To distinguish the physical basis for the different migration of Cpx1 transcripts in copper-replete versus copper-deficient cells, we analyzed portions of the transcripts by amplification of first strand cDNA (reverse transcription-PCR) using primer pairs 1+2, 3+4, 3+6, or 3+RACE1 (Fig. 1C). Comparison of the products derived from total RNA from copper-supplemented versus copper-deficient cells revealed no size differences (data not shown). We concluded that the region from the start codon through the poly(A) tail of the cDNA clone did not contribute to the apparent size difference and deduced that the difference might lie in the 5′ untranslated region.

Three different methods were used to determine the 5′ ends and the lengths of the 5′ untranslated regions of the Cpx1 transcripts. Primer extension analysis using primer 10 gave a strong product of 166 nucleotides (corresponding to an end at position +64 on the genomic sequence shown in Fig. 4A) and a weaker product of 199 nucleotides (corresponding to position +31) when RNA from copper-deficient cells was used as the template, but a product was not detected when RNA isolated from copper-supplemented cells was used as the template (data not shown). Complementary DNAs corresponding to the 5′ ends were generated instead by 5′-RACE and cloned. Several cloned products generated from RNA derived from copper-supplemented and copper-deficient cells were sequenced. The majority of clones derived from copper-deficient cells revealed the same 5′ end as did primer extension analysis (position +64, Fig. 4A), whereas the majority of clones derived from copper-supplemented cells had 5′ ends at +1 (data not shown). These results suggested that a subset of Cpx1 transcripts from copper-deficient cells was significantly shorter than those from copper-supplemented cells, consistent with the mobility difference observed in RNA gel blots.

To verify that distinct 5′ ends are present in copper-deficient versus copper-supplemented cells and to rule out the possibility that the different 5′ ends resulted from premature termination caused by stable RNA secondary structure, S1 nuclease (Fig. 5A) and RNase protection assays (Fig. 5B) were also employed. Both assays revealed that fragments corresponding in size to 5′ ends at positions +1, +31, and +64 were found in RNA from copper-deficient cells, whereas in copper-supplemented cells,
only forms with ends at +1 and +31 were detected (Fig. 5). The +1 and +31 forms (designated Cpx1-A and Cpx1-A', respectively) are present in both +Cu and −Cu cells, whereas the +64 form (Cpx1-B) is present exclusively under −Cu conditions. The abundance of the Cpx1-B transcript in −Cu cells in this experiment was about 12-fold higher than the sum of the Cpx1-A and Cpx1-A'9 transcripts, and this can account for the 15-fold increase in Cpx1-mRNA levels in copper-deficient cells, as noted in the RNA blot analysis.

The Cpx1-B Transcript Is Generated by Transcriptional Regulation—What mechanism(s) accounts for the presence of the Cpx1-B transcript exclusively in −Cu cells? Two possibilities were considered: greatly increased half-life of Cpx1-B in copper-deficient relative to copper-sufficient cells or transcriptional activation in copper deficiency (as described for the Cyc6 gene). To determine whether transcriptional regulation contributes to copper-responsive Cpx1 expression, the activity of the Cpx1 gene in vivo in copper-deficient versus copper-sufficient cells was assessed initially by nuclear run-on experiments (Table II). Indeed, nuclear preparations from −Cu cells synthesized more Cpx1 transcripts than did those from +Cu cells,
Cu-responsive Regulation of Coprogen Oxidase Accumulation

and the increase is comparable to that measured for the Cyc6 gene (data not shown).

In a more definitive experiment, reporter gene constructs consisting of 5′ upstream sequences from the Cpx1 gene fused to the promoterless Ars2 gene (41) were tested for copper-responsive accumulation of Ars2 mRNAs and arylsulfatase activity (Table III). Constructs pCpxArs1 and pCpxArs2, containing Cpx1 5′ upstream sequences from −1049 to +207 and −197 to +207, respectively, each exhibited copper-responsive expression of the reporter gene (Table III). The average increases in arylsulfatase expression in −Cu cells were 17- and 5-fold for pCpxArs1 and pCpxArs2, respectively (Table III). For any given construct, the overall level of expression was different in each transformant (Fig. 6A). This is attributed to the fact that the construct is integrated at a different position in each transformant. Nevertheless, for both constructs, pCpxArs1 and pCpxArs2, each of the transformants showed increased expression of arylsulfatase in −Cu versus +Cu (see Fig. 6A, for example). Furthermore, the pattern of expression of the reporter gene faithfully mimicked expression of the endogenous Cpx1 gene when chimeric Cpx1-Ars2 transcripts were analyzed with respect to the time course of their copper-dependent loss (Fig. 6, B-D) and the metal ion selectivity of the response (data not shown). Each construct also produces the constitutive forms, which accumulate at low abundance in both copper-sufficient and copper-deficient cells (labeled Cpx1-Ars2-A and A′), and a shorter form (labeled Cpx1-Ars2-B), which accumulates to high levels only in copper-deficient cells (Fig. 6E). The 5′ ends of the chimeric transcripts (Cpx1-Ars2-A, A′, and B) correspond exactly to the 5′ ends of the endogenous Cpx1 gene (Cpx1-A, A′, and B).

To assess whether mechanisms affecting mRNA stability are superimposed upon transcriptional regulation, we compared the half-life of Cpx1 mRNA in cells grown with or without copper in the presence of actinomycin D, an inhibitor of RNA polymerase II-dependent transcription (Fig. 7). The decay rate of Cpx1 transcripts was identical in +Cu versus −Cu cells and also very similar to the rate of loss of Cpx1 transcripts when copper is provided to −Cu cells in the absence of actinomycin D. In the absence of actinomycin D, transcription appears to reinitiate, bringing Cpx1 mRNA levels back to the +Cu level as noted already in Fig. 2. We conclude, therefore, that the half-lives of the Cpx1 transcripts are not altered in copper-deficient relative to copper-supplemented cells and that transcriptional regulation is a key feature contributing to increased accumulation of Cpx1 mRNA in −Cu cells.

A GTAC Sequence Is Part of the Copper Response Element (CuRE) Associated with the Cpx1 Gene—An additional construct pCpxArs3, containing 5′ Cpx1 sequence from −197 to +1, was generated in order to localize the CuRE-containing region. This construct also exhibited copper-responsive arylsulfatase expression (Table III). We conclude that the region from −197 to +1 is necessary and sufficient for copper-responsive expression of the Cpx1gene. Furthermore, because these constructs lack the sequence from +1 to +64 (present in the A-forms of the Cpx1 mRNA), we can confirm that the 5′ UTR is not necessary for copper-responsive accumulation of Cpx1 mRNA.

Mutational analysis of the copper-responsive element containing region of the Cyc6 gene identified two GTAC sequences that are absolutely critical for the copper-responsive expression. Alteration of the sequence at any one of the four positions destroys the ability of that DNA to function as an activator in copper-deficient cells. Analysis of the region from −197 to +2 nucleotides in the Cpx1 genomic sequence revealed two GTAC sequences: a proximal one at −2 to +2 and a distal one at −40 to −37. Mutational analysis of each of these revealed that only the distal GTAC sequence is required for copper-responsive expression (Table III, construct pCpxArs5), whereas mutation of the proximal GTAC sequence (pCpxArs4) has no effect on copper-responsive expression (Table III). We conclude that the CuRE associated with the Cpx1 gene has at its core a GTAC sequence, as do the CuREs associated with the Cyc6 gene.

Coordinate Accumulation of Cpx1 and Cyc6 Transcripts—Previously, Hill and Merchant (18) used radiolabeling methods to show that the synthesis of the 35-kDa polypeptide (determined later to be coprogen oxidase) was coordinated with the synthesis of cyt c6. Because we now know that both genes are transcriptionally regulated by copper through related CuREs, we compared the time course of the responses at the level of RNA abundance. The rate of mRNA loss might be unique for each message if transcriptional regulation is the key control point. RNA hybridization analysis (Fig. 6, B and C) shows that Cpx1 transcripts decay rapidly upon addition of copper to copper-deficient cells, with a half-life (in this strain) of ~40 min. This half-life is significantly shorter than that observed for Cyc6 (half-life ~80 min). The half-lives of Cyc6 and Cpx1 transcripts are strain-dependent (see Fig. 7 for comparison), but the half-life of Cpx1 transcripts is always shorter than that of Cyc6 transcripts in the same strain. Unlike Cyc6 transcripts, which continue to decay and are almost completely gone within 4 h of copper addition (Fig. 6, B and C) (32), Cpx1 transcripts reached a minimal level 90 min after copper addition and then slowly rose, reaching the level observed in plus copper-adapted cells within 4 h. RNase protection analysis (Fig. 6D) of RNA from the time course experiment revealed that the Cpx1-B transcript behaved like Cyc6 in that it is completely turned off in copper-supplemented cells, whereas it is the Cpx1-A message whose transcription is increased upon copper addition accounting for the eventual slow increase in mRNA abundance starting 90 min after copper addition to establish a new steady state (Fig. 6C).

The Tetrapyrrole Pathway—The induction of coprogen oxidase has been rationalized on the basis of an increased demand for heme during synthesis of cyt c6. Quite naturally, this raises the question of whether other enzymes in the pathway might be copper-responsive as well. Because δ-aminolevulinic acid is a key intermediate in the tetrapyrrole pathway, genes encoding enzymes involved in δ-aminolevulinic acid metabolism were examined. In addition, Ppx1 (encoding the chloroplast form of protoporphyrinogen oxidase) was of interest, because it encodes the enzyme that metabolizes the product of coprogen oxidase. Nevertheless, the effect of copper appears to be specific for Cpx1 induction. The abundance of Gsa, Gar, Alad, and Ppx1 transcripts (encoding glutamate-1-semialdehyde transferase (36), glutamyl-tRNA reductase (37), δ-aminolevulinic acid de-
Cu-responsive Regulation of Coprogen Oxidase Accumulation

TABLE III
Copper-responsive arylsulfatase expression of CpxArs reporter gene constructs

<table>
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<tr>
<th>Construct</th>
<th>Arylsulfatase activity&lt;sup&gt;b&lt;/sup&gt;</th>
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<td>3</td>
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<sup>a</sup> Shaded boxes, Cpx1 sequences; open boxes, Ars2 coding sequence; closed box, Tub2 minimal promoter sequence.
<sup>b</sup> Activity is expressed as nmol of p-nitrophenol/min/10<sup>9</sup> cells.
<sup>c</sup> No arylsulfatase activity was detected. This is attributed to mutation of the +1 nucleotide corresponding to the 5' end of the constitutive form Cpx1-Ae.
<sup>e</sup> Symbols open oval (pCpxArs4) and filled oval (pCpxArs5) represent mutations of GTAC to AATT at positions 2 to 37, 40 to 51, and 9. The mutation at 40 to 37 results in conversion of the KpnI site (K) to an EcoRI site (E). S, Sall site.

To test whether the Cpx1 gene might be sensitive to the operation of the tetrapyrrole pathway, we studied its expression in gabaculine-treated cells and in light versus dark grown cells. Depletion of cellular heme by gabaculine treatment (2 mM) did not affect coprogen oxidase or Cpx1 mRNA abundance after a short treatment (6.8 h). A 6.8-h treatment is sufficient to decrease in protein levels was noted even after 60 min of chase (data not shown).

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Functional Analysis of Constitutive versus Induced Transcripts—The occurrence of an mRNA with a distinct 5' end and a shorter 5' UTR in copper-deficient cells raises the question of whether this has functional significance. Specifically, is the short form translated or metabolized differently? Although differential degradation of the total Cpx1 mRNA population does not contribute to the different level of accumulation of Cpx1 transcripts in -Cu versus +Cu cells, it is possible that each form might have a distinct half-life. Therefore, the half-life of each form was measured in actinomycin D-treated copper-deficient cells. RNA was isolated at various times after actinomycin D treatment and analyzed by RNase protection assay, and each species was quantitated by PhosphorImager processing.

Analysis of the sequence of the longer transcript did not suggest the possibility of different translation initiation sites, indicating that the different forms of mRNA should yield the same protein product. Indeed, when coprogen oxidase was immunoprecipitated from radiolabeled cells, the size of the primary translation product (corresponding to the precursor form) was the same in copper-deficient compared with copper-supplemented cells (Fig. 8, less intense band marked M in the samples labeled for 10<sup>9</sup>). The more intensely labeled species (marked M') corresponds to the mature protein, which is very stable. No decrease in protein levels was noted even after 60 min of chase (data not shown).

To test whether the different 5' UTRs might influence translation, we looked at the rate of synthesis of coprogen oxidase in vivo. Copper-deficient and copper-supplemented cultures were labeled with <sup>35</sup>SOA<sub>4</sub>, and newly synthesized coprogen oxidase was immunoprecipitated from whole cell extracts. To determine whether the amount of newly synthesized coprogen oxidase was proportional to the amount of Cpx1 mRNA, total RNA was isolated from the same cells at the same time and Cpx1 transcripts were quantitated by RNA blot analysis. If each form of the transcript is equally effective as a template, then the ratio of newly synthesized coprogen oxidase in -Cu cells relative to +Cu cells should be comparable to the ratio of RNA template. But, as shown in Table IV, in four separate experiments we noted that the amount of newly synthesized coprogen...
oxidase in copper-deficient cells was 2–4-fold greater than could be accounted for by the increase in Cpx1 mRNA in the same –Cu cells. These data indicate that the shorter (Cpx1-B) transcript is a better template for translation.

Localization of Coprogen Oxidase to the Chloroplast—The presence of a presequence with features similar to those found in other stroma-targeted nucleus-encoded proteins suggested that coprogen oxidase should be targeted to and localized in the chloroplast. Chlamydomonas cells were fractionated into mitochondrial and chloroplast fractions, and these fractions were analyzed by immunoblotting. Coprogen oxidase was readily detected in chloroplast preparations from both copper-supplemented and copper-deficient cells (Fig. 6B–D), 10 μg of RNA from duplicate time points was analyzed by hybridization. Signals were visualized after 2 days of exposure. C, the RNA blots were exposed to a PhosphorImager screen, and the relative band intensities were quantified and normalized to RbcS2 hybridization signals. Data points are the average of duplicate samples ± S.E. Cpx1, closed circles; Ars2, open circles; Cyc6, closed squares. D, 5 μg of RNA from each time point was analyzed by RNase protection assay. The bands labeled Cpx1-Ars2-A and Cpx1-Ars2-B correspond to transcripts from the reporter gene construct, which start at +1 and +64, respectively. E, total RNA was isolated from representative strains containing either construct 1 or 2 (Table III) grown in either +Cu or –Cu media and analyzed by RNase protection assay. The sequencing ladder is as described in the legend to Fig. 5. The doublet band immediately above Cpx-Ars-B corresponds to form Cpx1-A’, and the doublet band immediately above Cpx1-A corresponds to Cpx1-Ars2-A’.

FIG. 6. Expression of reporter gene constructs. A, arylsulfatase assay data on four individual transformants containing construct pCpxArs1 (Table III) grown in +Cu or –Cu TAP media. Each bar is the average of two determinations. Units of arylsulfatase activity are nmol of p-nitrophenol/min/10^9 cells. Cpx1, closed circles; Ars2, open circles; cyc6, closed squares. B, –Cu; Cu, –Cu; Cu. B–D, time course of copper-responsive loss of Cpx1 and reporter gene mRNA analyzed by RNA blot analysis (B), PhosphorImager quantitation (C), and RNase protection assay (D). A representative transformant containing construct 1 (Table III) in strain CC425 was grown under copper-deficient conditions. Copper chloride (10 μM) was added to the culture and total RNA isolated at the indicated times. B, 10 μg of RNA from duplicate time points was analyzed by hybridization. Signals were visualized after 2 days of exposure. C, the RNA blots were exposed to a PhosphorImager screen, and the relative band intensities were quantified and normalized to RbcS2 hybridization signals. Data points are the average of duplicate samples ± S.E. Cpx1, closed circles; Ars2, open circles; Cyc6, closed squares. D, 5 μg of RNA from each time point was analyzed by RNase protection assay. The bands labeled Cpx1-Ars2-A and Cpx1-Ars2-B correspond to transcripts from the reporter gene construct, which start at +1 and +64, respectively. E, total RNA was isolated from representative strains containing either construct 1 or 2 (Table III) grown in either +Cu or –Cu media and analyzed by RNase protection assay. The sequencing ladder is as described in the legend to Fig. 5. The doublet band immediately below Cpx-Ars-B corresponds to form Cpx1-A’, and the doublet band immediately above Cpx1-A corresponds to Cpx1-Ars2-A’.

DISCUSSION
Mechanism of Coprogen Oxidase Induction in Copper Deficiency—Increased coprogen oxidase synthesis in copper-deficient cells occurs through transcriptional regulation as demonstrated by nuclear run-on assays and analysis of reporter gene constructs. Unlike the co-regulated Cyc6 gene, Cpx1 is also expressed constitutively (with respect to cellular copper status) because the product functions in what might be considered a “housekeeping” metabolic pathway. Analysis of the Cpx1-A, Cpx1-B, and Cpx1-C transcripts produced in copper-supplemented versus copper-deficient cells revealed three distinct transcripts. The shortest transcript Cpx1-B was observed only in copper-deficient cells. The B form appears coordinately with Cyc6 mRNA, and its appearance accounts completely for the increase in Cpx1 mRNA abundance. The two longest transcripts, Cpx1-A and A’, represent constitutive forms with respect to copper ion availability. Whereas the levels of Cpx1-A and A’ transcripts appear to rise during a time course of copper addition to copper-deficient cells (Fig. 6D), comparison of Cpx1-A and A’ levels in fully copper-supplemented versus copper-deficient cells in several independent experiments showed no significant differences in the abundance of these two transcripts relative to a control mRNA (data not shown). It is possible that the copper-dependent repression of Cpx1-B synthesis transiently affects synthesis of the other two transcript forms.

The sequences mediating copper-responsive expression of...
Cu-responsive Regulation of Coprogen Oxidase Accumulation

**FIG. 7.** Persistence of Cpx1 (A) and Pcy1 transcripts (B). A copper-deficient culture of CC125 was subdivided into nine 98-ml cultures (a–i). Thirty milliliters of cell was removed (t = 0) for preparation of RNA. To cultures a–c, actinomycin D was added to 20 μg/ml, to d–f, actinomycin D and copper (to 6 μM) were added, and to cultures g–i, only copper was added. Samples were removed at the indicated times for RNA preparation. Ten micrograms of RNA from each sample was analyzed by hybridization to cpx440. Each time point is the average from triplicate cultures ± S.E. A. Cpx1: +Cu and +actinomycin D, closed circles; -Cu and +actinomycin D, open circles; +Cu and -actinomycin D, closed squares. B. Pcy1: +Cu and -actinomycin D, open squares; +Cu and +actinomycin D, closed triangles.

**FIG. 8.** Synthesis and maturation of pre-coprogen oxidase in vivo in copper-supplemented versus copper-deficient cells. CC425 cells were labeled at 18° C for 10 min with 1 μCi/ml Na235SO4. The labeled cells were either extracted immediately into acetone (P, pulse) or 20 min after addition of unlabeled Na2SO4 to 30 mM and cycloheximide to 60 μg/ml (C, chase). Coprogen oxidase was immunoprecipitated from total resuspended acetone precipitates (copper-supplemented cultures) or from 1/10 of the total precipitate (copper-deficient cultures). Poly(A)+ RNA (0.8 μg) isolated from copper-deficient wild type cells was translated in vitro in the presence of radiolabeled methionine in a rabbit reticulocyte lysate system (Promega Corp., Madison, WI). A single polypeptide that was immunoprecipitated by anti-coprogen oxidase antiserum was analyzed on the same gel with the immunoprecipitates from the pulse-chase samples. Quantitative immunoprecipitation was verified by re-immunoprecipitating the supernatants from the first round of immunoprecipitation.

**FIG. 9.** Localization of coprogen oxidase. Enriched mitochondria or total soluble protein preparations isolated from copper-supplemented or copper-deficient CC849 cells were analyzed by immunoblotting using antiserum generated to coprogen oxidase, mitochondrial carbonic anhydrase, or Oee1. mt, mitochondrial.

Cpx1 were localized to a 5’ flanking region through analysis of reporter gene constructs (Table III). In other work, we have identified a tetranucleotide sequence, GTAC, which forms part of the CuRE and is essential for the copper response activity of the 5’ region flanking the Cyc6 gene.2 Mutagenesis of the Cpx1-Ars2 reporter gene constructs confirmed that one of two GTAC sequences in the 5’ flanking region of Cpx1 was necessary for its copper response activity (Table III). We predict that the Cpx1 gene is the target of the same DNA-binding protein and signal transduction pathway as is the Cyc6 gene, and this is supported by the coordinate expression of the Cpx1-B transcript and the Cyc6 transcript (Fig. 6B).

Mutation of the second GTAC found at position -2 to +2 in the Cpx1 genomic sequence did not prevent its increased expression in copper-deficient cells; however, it did affect the expression of the construct in copper-supplemented cells (Table III). The reason for this might be that the mutation (GTAC to AATT), which alters the first two 5’ nucleotides for the Cpx1-A transcript, affects the transcription start site for the constitutive forms. It is likely that the A and B forms represent the products of two transcription initiations: initiation at +1, representing copper-independent expression of Cpx1, and initiation at +64, representing a new transcription start site resulting from the superimposition of a copper response to the existing pattern of gene expression.

Function of Coprogen Oxidase Induction in Copper Deficiency—Why are coprogen oxidase levels up-regulated to such a great extent (up to 50-fold at the level of protein synthesis) in copper-deficient cells? The synthesis of cyt c6 in response to copper deficiency probably results in a relatively sudden and large increase in the cellular demand for heme. Certainly coprogen oxidase is up-regulated in other systems when there is a large increase in the cellular demand for heme. Certainly coprogen oxidase synthesis in response to anaerobic conditions, chlorophyll rather than heme is the major end product of the tetrapyrrole pathway, and if coprogen oxidase is rate-limiting, the resulting demand for a large amount of heme: for instance, when hemo- globin is synthesized during red blood cell development (52) or leghemoglobin during nodule development (53). Nevertheless, in C. reinhardtii, chlorophyll rather than heme is the major end product of the tetrapyrrole pathway, and one might wonder whether the de novo synthesis of a single (albeit abundant) cytochrome would result in a big draw on tetrapyrrole pathway intermediates. On the other hand, it should be noted that synthesis of cyt c6 occurs in green cells, which would be in “maintenance” mode with respect to chlorophyll accumulation. Indeed, in vascular plants, the flux through the tetrapyrrole pathway is dramatically reduced in fully expanded leaves compared with newly emerging leaves with developing chloroplasts (54). The de novo synthesis of cyt c6 and the resulting demand for heme could then require an increase in the flux through the tetrapyrrole pathway, and if coprogen oxidase is rate-limiting, increased expression would be necessary. In Saccharomyces cerevisiae, coprogen oxidase is induced under anaerobic conditions in wild type cells. Mutants that are unable to induce coprogen oxidase synthesis in response to anaerobic conditions.
Cu-responsive Regulation of Coprogen Oxidase Accumulation

A significant improvement in copper deficiency levels during copper deficiency raised the question of whether any coprogen oxidase might be redistributed to the mitochondrion in Cu cells. This question became especially intriguing in light of recent discoveries describing specific chloroplast and mitochondrial targeted forms of the two enzymes downstream of coprogen oxidase in the heme biosynthetic pathway (21–24). We wondered whether increased production of coprogen oxidase in copper-deficient cells might speak to a metabolic branch point by compartmentation, and we therefore tested whether the enzyme might be targeted to mitochondria in copper-deficient cells. But this is not the case. Nor is it the case that the enzyme redistributes from a soluble compartment into a membrane fraction. Immunoblot analysis of supernatant versus pellet fractions of +Cu and −Cu cells shows that less than 1% of the total coprogen oxidase in either +Cu or −Cu cells is associated with the pellet fraction (data not shown). The little coprogen oxidase that is detected can be removed by washing the pellets, indicating that it is simply trapped in the volume of the pellet rather than being specifically associated with a pellet component (such as membranes).

Function of the Shorter Copper Deficiency-specific Form of Cpx1 mRNA—Why is the synthesis of a shorter transcript induced in copper-deficient cells? When a single gene produces two or more transcripts that differ in the length of their 5′ UTRs, the transcripts often contain alternate exons (57–62) or more than one in-frame AUG codon (63), yielding different sized proteins with distinct amino-terminal sequences. The resulting proteins can be targeted to different subcellular locations (61, 63), exhibit different tissue expression patterns (57–60), or have different functions (58, 60). In the case of coprogen oxidase in C. reinhardtii, the different transcripts produce the same size protein product. Analysis of the Cpx1 sequence between +1 and +214 did not reveal any potential exons, and only two protein products of the predicted sizes for pre-coprogen oxidase and mature coprogen oxidase were detected when anti-coprogen oxidase-reactive species were immunoprecipitated from in vivo radiolabeled cells, regardless of whether the cells were copper-deficient or copper-supplemented. The different transcripts also did not differ in their half-lives. However, the ratio of newly synthesized protein in copper-deficient to copper-supplemented cells was 2–4-fold greater than the ratio of mRNA template in the same cells. Because the Cpx1-A form accounts for at least 80% of Cpx1 mRNAs in copper-depleted cells and 0% in copper-replete cells, this suggests that the shorter Cpx1-B transcript is a better template for translation.

Despite the more efficient translation of Cpx1 mRNA in −Cu cells, the abundance of the protein in −Cu relative to +Cu cells parallels almost exactly the relative abundance of the mRNA (data not shown). This implies that the enzyme must have a shorter half-life in −Cu cells. We could not measure this directly because the protein is very stable in both +Cu and −Cu conditions (see lanes marked C in Fig. 8) and must have a half-life comparable to the doubling time of the cells. Nevertheless, it is possible that copper-deficient cells lack (an as yet undiscovered) repair or antioxidant activity, and this might affect the half-life of certain enzymes.

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Cu-responsive Regulation of Coprogen Oxidase Accumulation

229–239
Induction of Coproporphyrinogen Oxidase in \textit{Chlamydomonas} Chloroplasts Occurs via Transcriptional Regulation of \textit{Cpx1} Mediated by Copper Response Elements and Increased Translation from a Copper Deficiency-specific Form of the Transcript

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