

Coordinate Copper- and Oxygen-responsive *Cyc6* and *Cpx1* Expression in *Chlamydomonas* Is Mediated by the Same Element*

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***Chlamydomonas reinhardtii* activates the transcription of the *Cyc6* and the *Cpx1* genes (encoding cytochrome *c*₆ and coprogen oxidase) in response to copper deficiency. Mutational analysis of promoter regions of the *Cyc6* and *Cpx1* genes revealed a four nucleotide sequence, GTAC, which was absolutely essential for copper responsiveness. The *Cyc6* promoter contains two copper response elements, each with a functionally important GTAC sequence, whereas the *Cpx1* promoter contains only one. This may contribute to the stronger and more tightly regulated expression of the *Cyc6* gene. Mutation or deletion of sequences flanking the GTACs implicates additional nucleotides contributing to copper-responsive expression, but none are absolutely essential. Metal ion selectivity of *Cpx1* expression is identical to that described previously for *Cyc6* and is restricted to the copper deficiency-induced *Cpx1* transcript. The *Cyc6* and *Cpx1* genes are also induced by oxygen deficiency. Reporter gene constructs indicate that the induction occurs at the level of transcription and requires the same GTAC sequence that is critical for copper responsiveness. We suggest that components of the copper-responsive signal transduction pathway are used for some of the changes in gene expression in hypoxic cells.**

Copper functions in biology as a cofactor in proteins that catalyze electron transfer reactions or reactions involving oxygen chemistry. For most organisms, especially respiring eukaryotes, copper is an essential micronutrient owing to its function in various enzymes including particularly cytochrome oxidase, ceruloplasmin, and superoxide dismutase. Symptoms of acquired or inherited copper deficiency have been documented in most organisms, humans, plants, yeast, and fungi, but is studied at the molecular level mainly in *Saccharomyces cerevisiae* (1, 2), where related transcriptional activators Ace1p and Mac1p (Ref. 3 and reviewed in Ref. 4) are responsible for maintaining copper homeostasis by regulation of transport, distribution, and chelation in response to variations in nutritional supply (5). For example, copper-deficient cells induce uptake mechanisms involving cupric reductases and a family of plasma membrane permeases, whereas cells treated with ex-

cess copper synthesize metallothioneins like Cup1p and Crs5p to sequester the copper and prevent potentially deleterious Fenton chemistry. Copper is a positive effector of Ace1p (6), whose function is critical for tolerance to toxic amounts of copper (7, 8), and a negative effector of Mac1p (9), whose function is critical for adaptation to copper deficiency (3). Mac1p-dependent adaptation to copper deficiency involves the coordinate expression of genes, *CTR1*, *CTR3*, *FRE1*, and *FRE7*, encoding assimilatory components, through copper-response elements associated with each of these genes (10–12). A consensus copper-response element TTTGC(T/G)C(A/G) (12) is a binding site for Mac1p.

Much less is known about copper metabolism in photosynthetic eukaryotes. In plants, some well known copper enzymes include plastocyanin, an electron transfer protein in the thylakoid lumen, plastid CuZn-superoxide dismutase, polyphenol oxidase, also in the thylakoid lumen, mitochondrial cytochrome oxidase, the ethylene receptor in the plasma membrane, extracellular laccase involved in lignification, and several oxidases. Copper is an essential micronutrient for plants (13). Copper deficiency prevents growth, inhibits flowering, and leads to necrosis. One of the primary targets of copper deficiency in plants is plastocyanin, which can account for most of the copper in photosynthetic tissue (14, 15). Many green algae and cyanobacteria can tolerate severe copper deficiency in nature and also in the laboratory because they have specific genetic mechanisms for adaptation (16–18). The unicellular green alga *Chlamydomonas reinhardtii* is one of these (19, 20). The green algae contain fewer copper enzymes than plants. They lack both CuZn-superoxide dismutase and polyphenol oxidase, which are abundant enzymes in plant chloroplasts, and probably also lack laccase and the ethylene receptor. Accordingly, plastocyanin and cytochrome oxidase are, metabolically, the most important copper enzymes in *Chlamydomonas*. The occurrence of a well defined adaptive mechanism and the dearth of copper enzymes in the green algae relative to plants makes *Chlamydomonas* an excellent model for the study of copper metabolism in the context of deficiency.

Chlamydomonas exhibits multiple adaptations to conditions of copper deficiency; the best understood of these is the replacement of plastocyanin function by a heme-containing cytochrome (19, 20). Copper-deficient cells cannot support holoplastocyanin formation. Therefore, they degrade apoplastocyanin, which requires a copper-repressible protease, and induce the synthesis of cytochrome (cyt)¹ *c*₆ by transcriptional activation of the *Cyc6* gene (21–24). Besides apoplastocyanin degradation and *Cyc6* transcription, copper-deficient *Chlamydomonas* cells display several other responses including transcriptional acti-

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¹ The abbreviations used are: cyt, cytochrome; CuRE, copper-response element; coprogen, coproporphyrinogen III; TAP, Tris acetate-phosphate; Arg⁺, arginine prototrophs; +Cu, copper supplemented; -Cu, copper deficient; WT, wild type.

vation of the *Cpx1* gene encoding coproporphyrinogen oxidase and enhanced ability to assimilate copper (25, 26). Each of these responses appears to occur coordinately. For instance, the amount of copper required to turn off the induced processes is the same and it occurs with similar kinetics. A wild-type *Chlamydomonas* cell maintains plastocyanin at an abundance of 8×10^6 molecules/cell (24). If copper is available at or greater than this concentration, the cells are in a copper-sufficient state; when copper concentrations fall below this level, the cells perceive a deficiency and induce the adaptive mechanisms to the extent required to compensate for this deficiency (24). Therefore, if the copper content in the medium corresponds to 4×10^6 /cell, a plastocyanin deficiency of 50% is anticipated and the *Cyc6* gene is induced to 50% of maximal levels (27). The *Cpx1* gene and the copper assimilation pathway likewise show the same behavior (25, 26). These results suggest that the adaptive processes (*Cyc6* and *Cpx1* transcription, plastocyanin degradation, and copper transport) are targets of the same signal transduction pathway as is the case for *S. cerevisiae* (see above).

In previous work, we showed that the *Cyc6* gene contains at least two copper-response elements (CuREs) (28). These elements reside within an 80-base pair fragment, which can be separated into two segments, corresponding to positions -129 to -110 and -110 to -50 nucleotides upstream of the transcription start site, each with CuRE activity. Each functions as a target for an activator in copper-deficient cells. The *Cpx1* gene also has an associated copper response element that lies between positions -197 and +1 (29). If the two genes are targets of the same signal transduction pathway, it might be possible to identify a copper-response element by comparison of the three CuRE-containing regions (two from the *Cyc6* gene and one from the *Cpx1* gene), and if the *Chlamydomonas* pathway were related to the one in *Saccharomyces*, then it might be possible to identify a binding site for a Mac1p-type protein. Nevertheless, this is not the case. A CuRE could not be identified by inspection, and the CuRE-containing sequences do not show any similarity to the *Saccharomyces* Mac1p binding site. The latter is not surprising, because the metal selectivity of the *Chlamydomonas* copper sensor is distinct from that noted for Mac1p, suggesting a Cu(II) binding site in the protein from *Chlamydomonas* rather than a Cu(I) binding site as in Ace1p and Mac1p (24, 30–33). Therefore, we undertook a mutagenesis study to define a *Chlamydomonas* CuRE.

In the original work describing the induction of cytochrome c_6 in copper-deficient cells, Wood (16) noted that the protein (identified primarily by spectrophotometric characterization) could also be induced in copper-supplemented medium when the cells were kept suspended by slow basal stirring rather than by vigorous agitation. The increase in cyt c_6 correlated with a decrease in oxygen in the culture and occurred even when a huge excess of copper ($>1000 \times 10^6$ /cell) was provided (16). These results raised several questions. Is the *Cyc6* gene induced by oxygen deprivation? Is the *Cpx1* gene also induced by oxygen deprivation? Is the mechanism of oxygen-responsive gene expression related to the mechanism of copper-responsive expression? Do the signal transduction pathways share components? To address these questions we analyzed *Cyc6* and *Cpx1* expression in oxygen-depleted cultures of *Chlamydomonas* and used a reporter gene construct (*Cpx1-Ars2*) to test whether the regulation occurred by transcriptional activation and whether CuRE(s) were involved.

EXPERIMENTAL PROCEDURES

Strains and Growth Conditions—*C. reinhardtii* strain CC425 (*arg2*, *cw15*) was the recipient strain used for all transformations. CC425 was cultured in copper-supplemented or copper-deficient TAP liquid me-

dium (34) supplemented with $200 \mu\text{g ml}^{-1}$ arginine. Transformants of strain CC425 were grown on +Cu or -Cu TAP liquid medium at 200 rpm or on agar plates without arginine at 22–25 °C and $\sim 100 \mu\text{mol m}^{-2} \text{ s}^{-1}$ light intensity. Strain CC125 was cultured under the same conditions in +Cu or -Cu TAP medium without added arginine.

Transformation and Analysis of Transformants—The glass bead method was used for transformation (35). Each construct (4 μg) was cotransformed with 4 μg of plasmid pArg7.8 (encoding the argininosuccinate lyase gene) (36) into strain CC425, arginine prototrophs were selected on copper-supplemented TAP medium, and individual isolates were spotted on +Cu versus -Cu TAP plates. Arg+ transformants expressing the cotransformed reporter were identified by treatment of the replica plates with 5-bromo-4-chloro-3-indolyl sulfate (Sigma), and single colony isolates of these were analyzed for gene expression by RNA blot analysis and by quantitative enzyme assay on cell suspensions as described (28). In some experiments, arylsulfatase activity in the medium was measured after removal of the cells by centrifugation. For CC425-derived strains, either method of assay (cell suspensions versus medium alone) gave the same result. The cotransformation frequency for each construct was determined by polymerase chain reaction amplification of genomic DNA, isolated as described below, from 16 randomly chosen Arg+ transformants. A vector-specific primer was used in conjunction with an *Ars2* specific primer to amplify introduced constructs. Amplification conditions were 95 °C for 5 min prior to *Taq* polymerase addition, 4 cycles of 94 °C for 1 min, 40 °C for 1 min, 72 °C for 2 min followed by 26 cycles of 94 °C for 1 min, 45 °C for 45 s, 72 °C for 1 min, with a final 15 min extension at 72 °C.

Preparation of Genomic DNA—Five ml cultures of Arg+ transformants in +Cu TAP medium were grown on a tissue culture wheel at 25 °C and $\sim 50 \mu\text{mol m}^{-2} \text{ s}^{-1}$ light intensity until late log to early stationary phase. All centrifugation steps were carried out at room temperature in a microfuge at 14,000 rpm (16,000 $\times g$). Cells (1.5 ml) were harvested by centrifugation for 5 min, resuspended in 20 μl of water, and 50 μl of lysis buffer (100 μM Tris-Cl, pH 8.0, 40 μM EDTA, 400 μM NaCl, 2% SDS, 100 $\mu\text{g ml}^{-1}$ RNase A) was added. Samples were heated at 50 °C for 15 min, and then 480 μl of 6 M NaI was added. Lysates were mixed by inversion, and clarified by centrifugation for 5 min. Supernatants were removed (avoiding the green film floating at the top) to fresh tubes containing 10- μl silica gel suspension (37), incubated at room temperature for 5 min, with occasional (once/minute) mixing by inversion to keep the silica gel suspended. The silica gel was collected by centrifugation for 10 s, washed three times with 120 μl of ice-cold New Wash (Bio 101, Vista, CA), resuspended in 25 μl of water, and incubated at 55 °C for 5 min. The silica gel was removed by centrifugation for 1 min, the supernatants were removed to fresh tubes, and 8 μl were used for polymerase chain reaction analysis.

Analysis of RNA—Total RNA was isolated and analyzed by RNA blotting as described by Hill *et al.* (38) for *Cpx1* and *Cyc6*, and as described by Quinn and Merchant (28) for *Ars2*. The following cDNAs were used as probes: the *cpx440* fragment (25) for detection of *Cpx1* RNA, the 710-base pair insert from pTZ18Cr552-7A (39) for *Cyc6* transcripts, the $\sim 7 \times 10^2$ -base pair insert from pM1 (40) for transcripts encoding the small subunit of ribulose-bisphosphate carboxylase/oxygenase (*RbcS2*), the 11×10^2 -base pair *Bam*HI fragment from pJD27 (41) to detect *Ars2* transcripts, and the 577-base pair insert of pTZ18R:CrPC6-2 (42) to detect *Pcy1* transcripts. Specific activities of probes ranged from 3 to 5×10^8 cpm μg^{-1} DNA.

Chimeric Constructs—The reporter gene vectors pJD54 and pJD100 (43, 44), containing the promoterless arylsulfatase-encoding gene *Ars2* or *Ars2* driven by a minimal β -tubulin promoter sequence (*Tub2-Ars2*), respectively, were used to generate all *Cyc6-Ars2* and *Cpx1-Ars2* constructs. Mutations in the *Cyc6* promoter sequences were generated by amplification of *Cyc6* genomic DNA (38) using one primer containing the target mutation and a second primer outside the CuRE containing region (-127 to -110 or -110 to -50 relative to the 5'-end of the *Cyc6* transcript (28)). Mutations in the *Cpx1* promoter were generated by overlap extension polymerase chain reaction (45). Mutated fragments were cloned into the *Kpn*I site of pJD54 or pJD100 or into the *Eco*RI site of *Eco*RI-pJD100 (in which the *Kpn*I site mutated to an *Eco*RI site). The presence of the desired mutation was confirmed by sequencing.

Analysis of Oxygen-responsive Expression—CC125 and representative *Cpx1-Ars2* transformants grown in copper-supplemented liquid TAP medium were inoculated at a starting density of $1-2 \times 10^6$ cells ml^{-1} in 200 ml of +Cu TAP in 250-ml flasks. Cultures were allowed to grow at room temperature (21–23 °C) with shaking at 200 rpm under dim light (12 $\mu\text{mol m}^{-2} \text{ sec}^{-1}$). Cultures were bubbled with various gas mixtures consisting of either 0 or 1% air, 0 or 2% CO_2 , and 97 or 100% N_2 . The concentration of dissolved oxygen in the cultures was monitored with an

TABLE I
Mutagenesis of region I, the -129 to -110 nucleotide CuRE-containing fragment

Construct	Copper-responsive expression?	Arylsulfatase activity ^a					
		x 1			x 2		
		-Cu	+Cu	(n)	-Cu	+Cu	(n)
WT	✓ ^b	130	10	(8)	55	1	(6)
1	✓ ^d	360	36	(6)	140	23	(6)
2	X	36	16	(1)	c		
3	X	c			c		
4	✓	390	6	(8)	1160	7	(7)
5	✓	480	82	(4)	1030	28	(5)
6	✓	56	28	(1)	770	0	(8)
7	✓	130	22	(5)	1270	7	(21)
8	✓	170	31	(1)	1360	16	(13)
9	✓	150	36	(5)	340	3	(15)
10	X	c			c		
11	X	39	39	(5)	38	32	(2)
12	X	19	28	(2)	8	9	(3)
13	X	c			c		
14	X	c			51	32	(1)
15	X	98	58	(5)	49	29	(6)
16	X	20	36	(1)	c		
17	X	30	17	(1)	c		
18	X	150	100	(1)	c		
19	X	c			10	10	(1)
20	X/✓	140	98	(4)	210	41	(4)
21	X	17	10	(4)	6	11	(1)
22	✓	240	120	(3)	280	2	(10)
23	✓	210	31	(7)	220	8	(8)
24	✓	38	14	(7)	53	0	(8)

^a Nanomoles *p*-nitrophenol/min/10⁹ cells. The indicated activities represent the average of (*n*) transformants analyzed.

^b ✓, copper-responsive arylsulfatase expression; X, copper-insensitive arylsulfatase expression.

^c No arylsulfatase expressing arg⁺ transformants were detected of 128 screened. The cotransformation frequency for these constructs was within the same range as that determined for constructs for which expressers were readily detected.

^d Dashes indicate the wild-type sequence.

Orion model 810 DO meter and a model 081010 electrode. Total soluble proteins were extracted and analyzed by immunoblotting with antiserum to plastocyanin, cyt *c*₆, or coprogen oxidase (29, 34). Total RNA was isolated and analyzed by hybridization as described above.

RESULTS

GTAC Forms the "Core" of a CuRE—In previous work, we found that the regulatory region of the *Cyc6* gene could be separated into 2 fragments, from -129 to -110 and -110 to -50 nucleotides (designated region I and region II, respectively), each of which could separately confer copper-responsive expression on the *Ars2* reporter gene (28). To identify a copper-response element within these fragments, we undertook a scanning mutagenesis experiment, initially with region I because of its smaller size. The "wild-type" construct contained region I (-129 to -110) fused to a reporter gene (*Ars2*) driven by a minimal promoter from the *Chlamydomonas Tub2* gene. In the test constructs (Table I, lines 1–5), groups of 3–6 nucleotides were transversally mutated to the noncomplementary nucleotides. Each construct was introduced into *Chlamy-*

domonas in co-transformation experiments as described previously. Because integration of reporter gene constructs into the *Chlamydomonas* nuclear genome does not usually occur via homologous recombination, the position of the reporter gene construct in the genome of each transformant will have an effect on expression. Expression can be affected also by the number of copies of the construct present at each integration site or in each transformant and by gene silencing. Therefore, for each test construct we analyzed multiple independent transformants² for copper-responsive reporter gene expression

² For several of the constructs analyzed in this work (e.g. 3, 10, 13, and 45 in Tables I and III) no (or very few) arylsulfatase-expressing cotransformants were recovered. To verify that the lack of arylsulfatase expression was due to the mutation in the construct and not due to low cotransformation frequency, genomic DNA from 16 randomly chosen Arg⁺ transformants was amplified to estimate the cotransformation frequency. Cotransformation frequencies ranged from 50 to 87% for all constructs, and there was no correlation found between the cotransformation frequencies and arylsulfatase expression (data not shown).

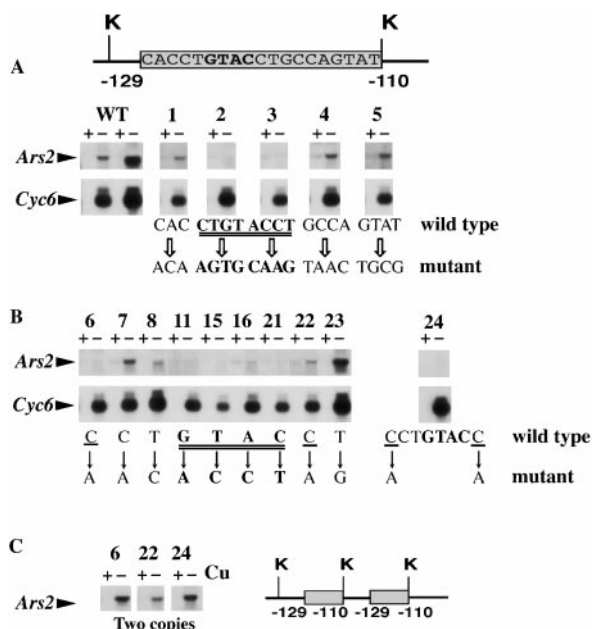


FIG. 1. RNA blot analysis of representative strains containing mutant constructs derived from the -129 to -110 *Cyc6* promoter fragment. Total RNA isolated from copper-supplemented and copper-deficient cultures of representative transformants of the mutants described in Table I was analyzed by RNA blotting as described under "Experimental Procedures." Due to position effects, the level of RNA is not compared between transformants, but rather the level of *Ars2* mRNA in +Cu versus -Cu cells of the same transformant is compared to determine if the mutated sequence can still confer copper-responsive expression. Blots were probed for expression of the reporter gene (*Ars2*) and also for the endogenous *Cyc6* gene to ensure that the cultures were copper-deficient. **A**, analysis of transformants containing constructs that have one copy each of the indicated WT or mutated (panels 1-5) -129 to -110 *Cyc6* promoter fragment. The group of nucleotides mutated is indicated beneath each panel (top line), and the nucleotides substituted for the wild-type sequence are indicated on the second line. *Double underlined* sequences indicate those nucleotides which, when mutated, lose the ability to confer copper-responsive expression. **B**, analysis of representative transformants containing constructs that have one copy each of the indicated single nucleotide mutation, except for construct 24 in which two nucleotides are mutated. The *double underlined* sequence indicates those mutations that abolish copper-responsive expression. The *single underline* indicates mutations that attenuate copper-responsive expression. **C**, analysis of representative transformants containing constructs 6, 22, and 24, which have tandem copies of mutated *Cyc6* promoter fragments.

by comparing arylsulfatase activity (Table I, lines 1-5) or *Ars2* mRNA abundance (Fig. 1A) in copper-supplemented versus copper-deficient cells of the same transformant. Thus we are assaying only the response to copper rather than the absolute level of gene expression from each construct. On this basis we concluded that mutations encompassing the sequence from -126 to -119, 5'-CTGTACCT-3', affected copper-responsive expression. The analysis was refined by changing each of these eight nucleotides individually (Table I, lines 7-23; Fig. 1B) and testing for copper-responsive expression. Mutations at four positions, corresponding to the sequence from -124 to -121, 5'-GTAC-3', abolished copper-responsive expression. The effect is generally independent of the particular nucleotide substitution (for example, lines 10-12, 13-15, and 16-18). A few other mutations (e.g. -127C → A and -120C → A; lines 6 and 22) also affected copper responsiveness (only 2-fold activation in -Cu conditions) but apparently not as strongly as the ones altering the GTAC sequence. We concluded, therefore, that the sequence GTAC was a critical determinant of a CuRE. Mutation of any one of these nucleotides must affect drastically the affinity of a putative CuRE-binding protein for this sequence.

We sought to assess the relative contribution of each of these

nucleotides to the affinity of the CuRE/CuRE-binding protein interaction by testing each mutation in the context of a tandem duplication of the CuRE (Table I, column on right side), the rationale being that the native *Cyc6* promoter contains at least two CuREs, and in general DNA-binding proteins exhibit cooperative interactions when multiple binding sites are available. Mutations within the GTAC sequence were just as drastic in the two copy context as in the one copy context, except for the -121C → G mutation (Table I, line 20), where copper responsiveness is retained in the two-copy context. When the mutations with a milder phenotype were tested in the two copy context, copper responsiveness was retained (compare Fig. 1, B and C; constructs 6 and 22). It would appear that the Cs at position -127 and -120 contribute less to sequence-specific binding than does the GTAC sequence. When the mutations in constructs 6 and 22 are combined (Table I, Fig. 1, B and C, construct 24), the effect is not significantly different from that seen in constructs carrying single mutations.

Examination of region II (-110 to -50) (Table II and Fig. 2) showed that it contained two GTAC sequences; both were part of the *KpnI* restriction sites defining the ends of the fragment. Mutation of the distal *KpnI* site (GGTACC) at position -110 to an *EcoRI* site (GAATTC) did not affect copper responsiveness (construct 25), but if the proximal site was also mutated (construct 38), copper responsiveness was abolished, suggesting that the proximal site might be part of a CuRE. To confirm this and to determine whether the distal *KpnI* site might independently be important for CuRE activity, each site was mutated individually in the context of the native *Cyc6* promoter sequence (from -127 to -7). The GTAC in region I was destroyed by mutation so that the effect of the test mutations could be discerned. Comparison of constructs 41 (wild-type region II), 42 (distal *KpnI* site mutated), and 43 (proximal *KpnI* site mutated) confirmed that mutation of the GTAC in the proximal *KpnI* site destroyed the copper responsiveness of region II and showed that mutation of the GTAC in the distal *KpnI* site had no effect (Table III). In addition, as noted previously, only one CuRE is necessary and sufficient to confer copper responsiveness (Table III, see constructs 40 and 41).

To locate other sequences besides the proximal GTAC, which might form part of a CuRE in region II, we scanned the region by generating constructs 26-32 and 35-37 (Table II and Fig. 2) carrying sets of transversional mutations. Construct 32 in which the wild-type hexanucleotide sequence 5'-CTGCCA-3' at position -73 to -68 was mutated did not show copper-responsive *Ars2* expression when region II was present in single copy. Transitional mutation of this sequence to TCATTG (Table II, construct 33, and Table IIIB, construct 44) resulted in normal copper-responsive arylsulfatase expression, whereas transversional mutation to the complementary sequence GACGGT (constructs 34 and 45) abolished copper-responsive expression. In constructs containing two copies of region II, the mutations had much less effect (Table II, right column). Therefore, as for region I, there are additional sequences, which are probably important for sequence-specific DNA binding *in vivo*, but their contribution to binding affinity is much less.

RNA blot analysis of representative strains containing constructs 1-45 (Figs. 1 and 2 and Table III) showed that *Ars2* mRNA expression parallels arylsulfatase enzyme activity such that all constructs that confer copper-responsive arylsulfatase activity also show copper-responsive transcript accumulation, and those constructs in which copper-dependent arylsulfatase activity is lost also do not show induced accumulation of *Ars2* transcripts in -Cu condition. We conclude that copper-responsive transcriptional regulation of *Cyc6* requires CuREs with a critically important GTAC core sequence, and that other nu-

TABLE II
Mutagenesis of region II, the -110 to -50 nucleotide CuRE-containing fragment

Construct	Copper-responsive expression?	Arylsulfatase activity ^a					
		x 1			x 2		
		-Cu	+Cu	(n)	-Cu	+Cu	(n)
-110	-50						
WT GGTACCGCGCGCTACCGATGCGTGTAGTAGAGCTTGCTGCCATACAGTAACTCTGGTACC	✓	1600	110	(4)	710	30	(7)
25 -AATT-----	✓	75	10	(11)	not done		
26 -----TTATAT-----	✓	110	33	(7)	92	2	(6)
27 -----AGCAAT-----	✓	500	85	(11)	1560	60	(7)
28 -----CGTAT-----	✓	330	19	(5)	110	0	(7)
29 -----GTGC-----	✓	69	18	(14)	680	10	(12)
30 -----TACTCT-----	✓	100	33	(9)	310	1	(5)
31 -----AGGT-----	✓	1260	100	(3)	140	2	(12)
32 -----AGTAAC-----	X/✓	320	240	(4)	87	1	(5)
33 -----TCATTG-----	✓	310	91	(7)	193	1	(11)
34 -----GACGGT-----	X	32	51	(4)	35	5	(2)
35 -----GCAC-----	✓	1350	500	(3)	2320	410	(5)
36 -----TGCC-----	✓	830	190	(7)	1310	170	(10)
37 -----AGAG-----	✓	350	120	(13)	250	46	(7)
38 -AATT-----AATT-	X	35	58	(5)	not done		

^a Nanomoles *p*-nitrophenol/min/10⁹ cells. The indicated activities represent the average of (n) transformants analyzed.

^b Dashes indicate the wild-type sequence.

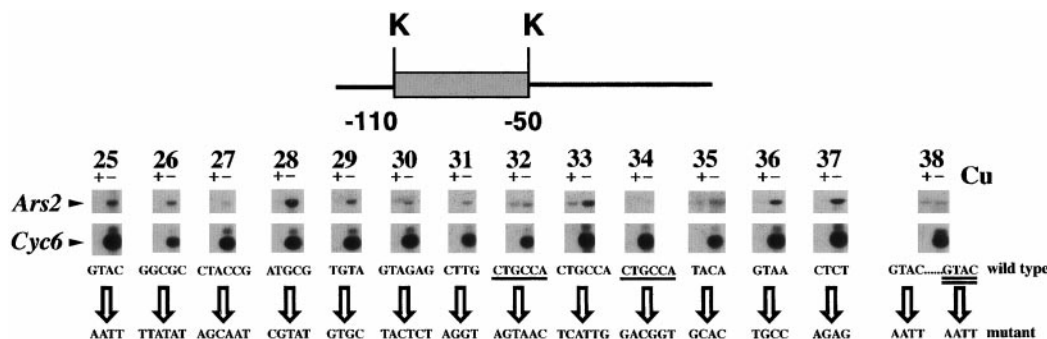


FIG. 2. RNA blot analysis of representative strains containing mutant constructs derived from the -110 to -50 *Cyc6* promoter fragment. Representative transformants containing constructs with single copies of the indicated mutated *Cyc6* promoter fragment (Table II) were analyzed by RNA blot hybridization as described in Fig. 1. The double underlined sequence indicates a GTAC sequence required for copper-responsive expression. Single underlined sequences indicate different mutations of the nucleotide sequence from -72 to -68, which attenuate copper-responsive expression.

cleotides -127C, -120C, and -73 to -68, must contribute to sequence-specific binding *in vivo*.

A GTAC Sequence Is also Part of a Single CuRE in the Cpx1 Promoter—Because the *Cpx1* gene is regulated coordinately with *Cyc6*, we wondered whether it might be a target of the same copper-responsive signal transduction pathway. In this case, we would expect to identify a CuRE, which would be related to those in the *Cyc6* promoter, and the mechanism of regulation would be the same. Indeed this is the case. The sequence GTAC within a *KpnI* restriction site is also essential for copper responsiveness of the *Cpx1* gene (Table IV and Fig. 3) (29). Construct C displays copper responsiveness, whereas construct E in which the *KpnI* site is mutated to an *EcoRI* site has lost copper responsiveness. Another GTAC very close to the

KpnI restriction site is not part of a CuRE because a mutation to AATT does not change copper responsiveness of that fragment (Table IV, construct F).³ Thus, as for the *Cyc6* gene, there must be nucleotides beyond the GTAC that define a CuRE. Whereas the *Cyc6* gene has two separable CuREs, the *Cpx1*

³ The mutation in construct F does appear to affect the magnitude of expression. The reduced expression in construct F relative to C may be attributed to the fact that the test mutation (at positions -2 to +2) also changes the +1 position corresponding to the 5'-end of the constitutive form of the *Cpx1* transcript, and this may interfere with transcription (29). The abundance of transcripts derived from construct F relative to A and C is also decreased (Fig. 3), which is consistent with the interpretation that reduced arylsulfatase expression is due to reduced transcription.

TABLE III
Dissection of the CuRE-containing region II

Construct	Copper-responsive expression?	Arylsulfatase activity ^a			c
		-Cu	+Cu	(n)	
A WT ^b -129 K K -7	✓	340	10	(4)	+ - Cu
39 -129 E K -7	✓	240	10	(6)	
40 -129 K E -7	✓	270	65	(4)	
41 -129 X K K -7	✓	120	0	(9)	
42 -129 X E K -7	✓	140	3	(7)	
43 -129 X K E -7	X	14	14	(6)	
B 44 -129 X TCATTG K K -7	✓	201	4	(4)	
45 -129 X K GACGGT K -7	X	0	0	(1)	

^a Nanomoles *p*-nitrophenol/min/10⁹ cells. The indicated activities represent the average of (*n*) transformants analyzed.

^b Data from Ref. 28.

^c RNA blot analysis of representative transformants. The *Ars2* cDNA was used as the probe.

gene has only one. Mutation of the GTAC destroys all copper-response activity associated with the fragment from -197 to +207 (Table IV, compare constructs C and E), and the sequence up to 1 kilobase upstream does not appear to have any copper-response activity (Table IV, construct B) although the increased constitutive expression from the *Tub2-Ars2*-derived construct suggests that the upstream DNA may well contain other enhancers that affect expression independently of copper.

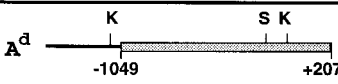
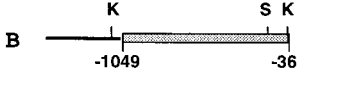
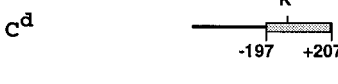




The CuREs associated with the *Cyc6* gene function primarily as targets for an activator in copper-deficient cells rather than a repressor in copper-supplemented cells (28). To determine if this is the case also for the *Cpx1* gene, its CuRE-containing region was tested not only in the context of a promoterless reporter gene (*Ars2*) but also in the context of a reporter gene driven by a basal promoter from the *Tub2* gene (*Tub2-Ars2*). The latter reporter displays constitutive low level arylsulfatase activity (construct G, Table IV). When a CuRE from the *Cpx1* gene is fused to *Tub2-Ars2*, expression is activated over the basal level (compare constructs A, C, and F to G).

Similar Metal Ion Selectivity of *Cpx1* and *Cyc6* Expression—In previous work, we found that the *Cpx1* and *Cyc6* genes were coordinately deactivated (as a function of time) when copper-deficient cells were supplied with copper (29). Mutational analysis of the *Cyc6* and *Cpx1* regulatory regions shows that the two genes are transcriptionally regulated through related CuREs, which is consistent with the model that they are targets of the same response regulator. To confirm that the two genes also respond to the same sensor, we compared the

metal selectivity of the responses. Copper ions are the most effective in turning off *Cyc6* expression with concentrations as low as 500 nM being effective for sustained deactivation (38). Mercuric ions are also effective, but concentrations as high as 10 μM are required for the same response, whereas silver ions are ineffective (38). RNA blot analysis shows that the endogenous *Cpx1* gene also responds to copper and mercuric ions but not to silver, as does the reporter gene driven by *Cpx1* sequences (Fig. 4A). Three transcripts are produced from the *Cpx1* gene (29). The two longer forms (A and A') represent the constitutive forms, whereas the shorter form (B) is induced in copper deficiency. The reporter gene likewise generates three transcripts, A and A' corresponding to the copper-independent forms, and B corresponding to the induced form (29). RNase protection assay (Fig. 4B) revealed that mercury treatment resulted in the specific reduction of the *Cpx1*-B and *Ars2*-B transcripts, whereas the A forms persist. Silver treatment affected the accumulation of constitutive and copper deficiency-specific transcripts to equivalent extents and this is attributed to the toxicity of silver rather than to a specific deactivation of the copper deficiency response (46). These results are consistent with a model in which the *Cyc6* and *Cpx1* gene are targets of a common sensor.

Oxygen-responsive Expression of *Cyc6* and *Cpx1*—Wood (16) had noted that copper-sufficient *Chlamydomonas* cultures could accumulate cyt *c*₆ if the cells were grown in "well filled flasks" with "slow basal stirring" instead of by vigorous agitation. He noted also that such cultures were depleted of oxygen.

TABLE IV
 Copper-responsive regions of *Cpx1* promoter

Construct	Copper-responsive expression?	Arylsulfatase activity ^a					
		Ars2			Tub2-Ars2		
		-Cu	+Cu	(n)	-Cu	+Cu	(n)
 A ^d	✓	300	17	(4)	550	40	(1)
 B	X	f			160	204	(3)
 C ^d	✓	220	47	(5)	120	29	(9)
 D	X	f			50	36	(2)
 E ^{b, d}	X	4	3	(5)	99	76	(9)
 F ^{c, d}	✓	49	0	(8)	83	20	(11)
 G ^e	X	f			40	40	(7)

^a Nanomoles *p*-nitrophenol/min/10⁹ cells. The indicated activities represent the average of (*n*) transformants analyzed.

^b The *KpnI* site at position -41 is mutated to an *EcoRI* site (GGTACC to GAATTC), indicated by an E.

^c Nucleotides -2 to +2 are mutated from GTAC to AATT, indicated by an X.

^d Data for the constructs in the *Ars2* context from Quinn et al. 1999. The solid line indicates vector sequence.

^e Data from Ref. 28.

^f No arylsulfatase expressing arg⁺ transformants were detected of 128 screened. The cotransformation frequency for these constructs was within the same range as that determined for other constructs (where expressers were detected readily).

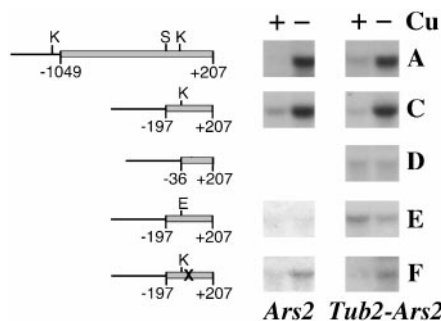


FIG. 3. RNA blot analysis of representative strains containing *Cpx1-Ars2* constructs. RNA was isolated from transformants containing the indicated *Cpx1-Ars2* (promoterless *Ars2* construct) or the *Cpx1-Tub2-Ars2* constructs described in Table IV and analyzed by hybridization.

Because Wood (16) had not identified the protein beyond spectrophotometric characterization, we sought to 1) confirm the identity of the induced protein and 2) determine whether the induction resulted from transcriptional regulation. We have rationalized the induction of *Cpx1* in copper-deficient cells on the basis of an increased demand for heme in cells producing cyt *c*₆, and therefore, we 3) tested whether *Cpx1* was induced with *Cyc6* under these conditions.

Erlenmeyer flasks (250 ml) were filled with 200 ml of copper-supplemented TAP medium (>3600 × 10⁶ Cu cell⁻¹) and inoculated with CC125 cells to a density of 1 × 10⁶ cells ml⁻¹. After 1–2 days of growth with stirring on a magnetic stir plate set at the lowest possible speed, cells were collected for preparation of soluble extracts (for immunoblot analysis of cyt *c*₆, coprogen

oxidase, and plastocyanin abundance) and RNA (for accumulation of *Cyc6* transcripts). We found that plastocyanin abundance in these cultures was not affected, indicating that the cells were not copper-depleted, yet the cells accumulated cyt *c*₆ (data not shown). Also, coprogen oxidase was induced under these growth conditions (data not shown). We concluded that the conditions of growth (poor aeration) allowed activation of the *Cyc6* and *Cpx1* genes despite the presence of more than enough copper for plastocyanin biosynthesis (>3600 × 10⁶ Cu cell⁻¹, corresponds to a 400-fold excess). Although the growth conditions resulted in oxygen depletion, a causal relationship between oxygen deprivation and *Cyc6* and *Cpx1* expression had not been established. We varied oxygen supply to standard cultures (100 ml of TAP medium in a 250-ml flask, 200 rpm agitation on a shaker) and tested for *Cyc6* and *Cpx1* expression. Induction of both transcripts occurred rapidly within 1–2 h of transfer to oxygen-free conditions (Fig. 5A). When the cultures were bubbled instead with 100% air, neither *Cyc6* nor *Cpx1* was induced. The level of CO₂ does not affect *Cyc6* or *Cpx1* expression in these experiments because there was no difference in cultures bubbled without CO₂, air levels of CO₂, or 2% CO₂ (not shown).

Does oxygen-responsive expression of *Cyc6* and *Cpx1* require the same components as does the copper-responsive pathway? Or is it an independent response to a different signal? To distinguish between these two possibilities, we analyzed the oxygen-responsive expression of representative transformants containing various *Cpx1-Ars2* reporter gene constructs by measuring the accumulation of reporter gene RNA (Fig. 5B) and enzyme activity (Table V). In all cases, transformants that display increased arylsulfatase activity in copper-deficient cells

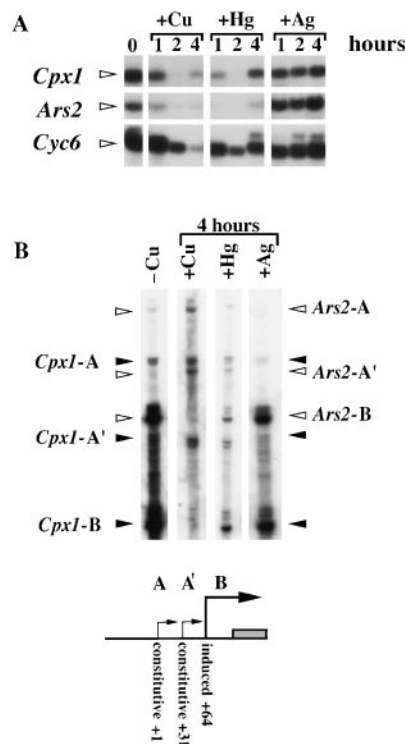


FIG. 4. **Metal ion selectivity of *Cpx1* expression.** A, total RNA was isolated from a representative strain transformed with construct A in the promoterless *Ars2* reporter gene context (Table IV). The strain was cultured in copper-deficient medium and supplemented with $10 \mu\text{M}$ CuSO_4 (+Cu), AgNO_3 (+Ag) or HgCl_2 (+Hg) for 0–4 h. RNA was analyzed by gel blot hybridization. Ten micrograms of RNA was loaded/lane, and hybridization signals were visualized after 21 h of exposure. The blot was probed with radiolabeled cDNAs corresponding to *Cpx1*, *Ars2*, or *Cyc6*. B, five μg of RNA from the 4-h time point was analyzed by RNase protection assay. The bands labeled *Cpx1*-A, *Cpx1*-A', *Ars2*-A, *Ars2*-A', *Cpx1*-B, and *Ars2*-B correspond to transcripts from either the endogenous gene (filled arrowheads) or the reporter gene construct (open arrowheads), which start at +1 (A, full-length constitutive form), +32 (A', intermediate constitutive form), and +64 (B, induced form).

also show increased enzyme activity in hypoxic cells (constructs A and C), whereas those that have lost copper-responsive expression (construct E, GTAC mutated to AATT) also do not show oxygen-responsive expression (Table V). The level of induction of arylsulfatase activity is comparable between copper-deficient and oxygen-deficient cells. The increase in arylsulfatase activity correlates with increased accumulation of *Ars2* transcripts (Fig. 5B). We conclude that oxygen-responsive expression of *Cpx1* requires the same GTAC sequence required for the copper-response pathway.

DISCUSSION

GTAC Forms the Core of a CuRE—In this work we have dissected the upstream regulatory regions associated with the *Cyc6* and *Cpx1* genes to identify CuREs. Mutagenesis reveals that a 4-nucleotide sequence, GTAC, is necessary for CuRE activity (Tables I–IV). Several DNA-binding proteins, for example, helix-turn-helix proteins, represented by the homeodomain transcription factors and the Ets domain of transcription factor PU.1, show specificity for a 4-nucleotide core within their binding sites with flanking nucleotides making more minor contributions to specificity and affinity (47–49). Two lines of evidence indicate that additional nucleotide sequences are involved in copper-responsive gene expression *in vivo*. First, there are several GTACs in the upstream regulatory regions, but for the *Cyc6* gene only two, and for the *Cpx1* gene only one, are essential for CuRE activity. Mutation of the GTAC at –109 to –106 in the *Cyc6* promoter (Table II, construct 25) or the GTAC at –2 to +2 of the

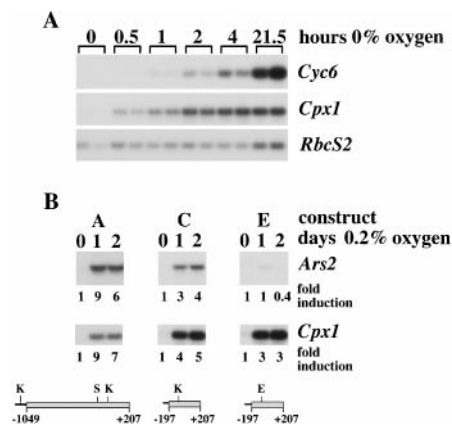


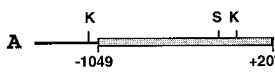
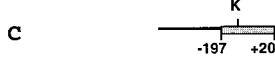
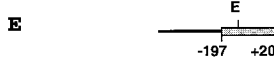
FIG. 5. **Oxygen-responsive expression of *Cyc6*, *Cpx1*, and reporter gene constructs.** RNA blot analysis of total RNA. A, RNA isolated from duplicate cultures of copper-supplemented CC125 cells after transfer to oxygen-deficient conditions (2% CO_2 in N_2) for the indicated times. Exposure times were 23 h at -80°C for *Cyc6*, 24 h at room temperature for *Cpx1*, and 2 h at room temperature for *RbcS2*. B, total RNA was isolated from representative strains containing the indicated *Cpx1* reporter gene constructs (Table IV) grown in +Cu TAP and transferred to hypoxic conditions (1% air, 2% CO_2 in N_2) for the indicated times. Exposure time was 24 h at -80°C . Hybridization signals were normalized to *RbcS2*, and the fold induction of *Ars2* or *Cpx1* transcripts at 1 and 2 days relative to the basal expression at day 0 (which has been designated as 1) is indicated.

Cpx1 promoter (Table IV, construct F), for example, does not affect copper-responsive expression of the reporter gene. Second, mutagenesis of flanking nucleotides (e.g. –127C, –120C) (constructs 6 and 22 in Table I) and –73 to –68 (Table III constructs 32–34 and Table III, construct 45) for the *Cyc6* CuREs suggests some, albeit weaker or less specific, contribution at certain positions. These mutations have an effect in the context of a single CuRE, but the effect is masked when two copies of the CuRE are present (compare *right* and *left* columns in Tables I and II). Alignment of the DNA sequences flanking the functionally important GTACs (Fig. 6) shows that a 3'-C is conserved in all cases, but recognition of a consensus CuRE is not possible. Specificity for flanking nucleotides could be degenerate with a preference, for example, only for a purine *versus* a pyrimidine base. This is apparent for the set of mutations in constructs 32–34 (Table II), where the construct with a set of transitional mutations retains CuRE function, but the one with a set of transversional mutations is nonfunctional as a CuRE. The purification of a CuRE-binding protein and the characterization of its interactions with the *Cyc6* and *Cpx1* regulatory regions are prerequisites for further definition of CuREs.

Relation of *Chlamydomonas* CuREs to Those in Yeasts—Copper-responsive gene expression in the eukaryotes *S. cerevisiae* and *Candida glabrata* has been studied extensively, initially in the context of copper-dependent activation of metallothionein genes during response to high copper. For both organisms, the consensus sequence of the *cis*-element mediating Ace1p- or Amt1p-dependent activation of metallothionein and superoxide dismutase gene expression in copper-treated cells is NNNNTNNGCTGNNN, with the first conserved T always found in a 4–6 nucleotide A-T-rich region (50). Clearly, the *Chlamydomonas* CuRE is distinct from this sequence; this is not unexpected because Ace1p and Amt1p are required for adaptation to toxic levels of copper, whereas a system for adaptation to copper deficiency is involved in this work.

In *Saccharomyces*, adaptation to copper deficiency requires an Ace1p/Amt1p-related protein called Mac1p. Amt1p, Ace1p, and Mac1p show significant sequence similarity to one another within their highly conserved amino-terminal copper/DNA binding domains (sequence identity 48–53%) (3).

TABLE V
Oxygen-responsive expression of *Cpx1* constructs

	Arylsulfatase activity ^a						
		Ars2			Tub2-Ars2		
		+Cu 20% O ₂	-Cu 20% O ₂	+Cu 0.2% O ₂	+Cu 20% O ₂	-Cu 20% O ₂	+Cu 0.2% O ₂
A		103	2,520	2,400	not done		
C		63 157	842 1,304	833 1,600	70 74	701 1,234	643 666
E		4 0	4 0	0 0	38 104	42 123	52 126

^a Nanomoles *p*-nitrophenol/min/10⁹ cells. The indicated activities are for one transformant analyzed (construct A) or two independent transformants analyzed (constructs C and E).

-110 to -50 in vector context GGTACCGCGCGCTACCGATGCGTGTAGTAGAGCTTGCTGCCATACAGTAACTCTGGTACCggggccccc

-129 to -110 in vector context ccctcgacctcgaccACTGTACTGCCAGTAT

-110 to -50 in *Cyc6* promoter context GGTACCGCGCGCTACCGATGCGTGTAGTAGAGCTTGCTGCCATACAGTAACTCTGGTACCCCGCCAC

-129 to -110 in *Cyc6* promoter context AGGCAGGAAGCGGCTGCCAAGCCCGCTTTCACTGAAGACTGGGATGAGCGCACTGTACTGCCAGTAT

Cpx1 promoter AGTGGCGGCGCCAGGGACTGCAATCTGGGGCATCGAGATCGGGACTGGGAATGAGGCTACCAACATACA

FIG. 6. Comparison of sequences containing functionally essential GTACs associated with CuRE activity. The GTAC sequence is boxed, and nucleotides are identified by site-directed mutagenesis as potentially contributing to copper-responsive expression are double underlined. The uppercase sequence is from *Cyc6* or *Cpx1* gene, and the lowercase sequence is from vector.

TABLE VI
Expression from multiple CuREs

Construct	Spacing ^b	Arylsulfatase activity ^a				(n)
		Average		Range		
		-Cu	+Cu	-Cu	+Cu	
Region I × 1		130	10	35–520	5–20	(8)
Region I × 2	38	55	1	19–93	0–4	(6)
Region I × 4	38	2032	3	1161–2560	0–5	(4)
Region II × 1		1600	110	40–4700	0–370	(4)
Region II × 2	51	710	30	37–3134	0–197	(7)
Region I + II (native)	62	2470	61	144–7625	7–111	(5)

^a Nanomoles *p*-nitrophenol/min/10⁹ cells. The indicated activities are the average of (n) transformants analyzed.

^b Spacing equals the number of nucleotides between GTACs.

Mac1p-dependent activation of genes involved in copper uptake requires a copper-response sequence, TTTGC(T/G)C(A/G), which is similar to the Ace1p/Amt1p binding site (2, 11, 12, 51). The regulatory system described here is related functionally to the Mac1p-dependent system of *Saccharomyces* in that both involve adaptation to nutritional copper deficiency, and it would be reasonable to suspect that a similar system operates in *Chlamydomonas*. This is preceded by the finding of a Mac1p ortholog, *GRISEA*, in *Podospora anserina* (52) but is, nevertheless, not the case. The *Chlamydomonas* components involved in adaptation to copper deficiency are undoubtedly unique. Besides the difference in the copper-response elements in the two systems, we have described previously a difference also in the metal selectivity of the sensor. The Ace1p/Amt1p/Mac1p factors bind Cu(I) and hence Ag(I) is an effective substitute *in vitro* and *in vivo* (6, 11, 30–33, 53). In the *Chlamydomonas* system, Hg(II) rather than Ag(I) can replace copper as a repressor of *Cyc6* and *Cpx1* expression (Fig. 4 and Ref. 24). We argue against a nonspecific effect of Hg(II) because the constitutive forms of the *Cpx1* transcript are maintained in Hg(II)-treated cells. Only

the B form, induced by copper-deficiency, is lost. Because the *Cyc6* gene is completely silent in copper-supplemented cells, in previous work on *Cyc6* expression we could not distinguish between a specific effect of Hg(II) in mimicking copper *versus* a nonspecific effect where Hg(II) might simply inactivate a critical thiol in a transcription factor. Although the *Chlamydomonas* CuRE-binding protein has not yet been identified, we predict that it will bind Cu(II) rather than Cu(I), suggesting that it should be a novel copper sensor.

Multiple CuREs Enhance the Magnitude of Copper Responsiveness—Nuclear transformation in *C. reinhardtii* does not occur by homologous recombination to any significant extent (54); therefore, the expression of a reporter gene construct in any given strain will depend on where the exogenous DNA has inserted into the genome. This does not affect our ability to ascertain if a particular construct retains copper-responsive expression, because we can compare the level of reporter gene activity in -Cu *versus* +Cu cells of the same transformant. However, it does make it difficult to discern whether particular mutations might affect the magnitude of gene expression in -Cu cells, unless very large numbers of transformants are

analyzed for each construct. We have, in essence, done this. Examination of the data in Tables I and II suggests that two copies of a CuRE-containing fragment tend to show either tighter regulation (fold activation in $-Cu$ versus $+Cu$ conditions) or simply higher expression in $-Cu$ conditions. Furthermore, some mutations show a phenotype in the single copy context but show much more significant copper responsiveness when the fragment is present in a tandem duplication (see constructs 6, 20, 22, 24, Table I, and Fig. 1C). To test this idea directly, we generated constructs with either two or four tandem copies of CuRE-containing region I from the *Cyc6* gene (Table VI). Two copies of region I give tighter regulation over a single copy (55-fold versus 13-fold) by suppressing the basal level expression in $+Cu$ cells (compare WT constructs in Table I). In the construct with four copies of region I, the tight regulation is retained, very little expression is noted in $+Cu$ cells, and the magnitude of expression in $-Cu$ cells is also increased dramatically. In fact, it approaches the expression we see with the native promoter region from the *Cyc6* gene, which contains two CuREs (Table VI). Two copies of region II also give slightly tighter regulation compared with a single copy (23-fold versus 14-fold), but the magnitude of expression in $-Cu$ cells is decreased as was the case for the two copy version of region I. Constructs containing the *Cpx1* regulatory region (Table IV) have only one CuRE, and in general their expression is similar to the *Cyc6* constructs containing only one copy of either region I (Table I). This interpretation would be consistent with the observation that *Cpx1* transcripts in $-Cu$ cells appear to be less abundant than *Cyc6* transcripts (e.g. Fig. 5).

Mechanism of Oxygen-responsive Expression of *Cyc6* and *Cpx1*—Wood (16) had suggested that cytochrome c_6 was induced in oxygen-deficient conditions even in copper-supplemented cells because he noted that oxygen-depleted cultures accumulated cytochrome c_6 . We have now shown that cytochrome c_6 accumulation does occur in response to oxygen deprivation (Fig. 5). It occurs by transcriptional regulation and is accompanied also by increased coprogen oxidase accumulation through transcriptional activation of the *Cpx1* gene. The maintenance of plastocyanin confirms that the oxygen-depleted cultures are not copper-limited. Surprisingly, the regulatory sequences required for oxygen responsive regulation are the same as those important for CuRE activity (Table V). A simple model suggests that oxygen-depleted cells might perceive a deficiency in Cu(II) because of an alteration in the intracellular redox poise. In this case, provision of excess copper should suppress the anaerobic effect but it does not (data not shown and Ref. 16). An alternate model would be one in which the sensor responds to the ratio of Cu(II) to Cu(I), which would be affected by the supply of oxygen to the cell. Copper deficiency in nature can result from precipitation of copper as insoluble sulfides in an anaerobic or microaerobic environment (as might be created by an algal bloom) (55). Therefore, the response to low oxygen might be a way for the organism to anticipate copper deficiency. In this case, it is appealing to consider a model in which the oxygen-response pathway uses components of the nutritional copper-response pathway so that the same set of target genes can be activated.

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