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Dynamic interplay between two copper-titrating components in the transcriptional regulation of cyt *c6*

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The algal plastidic cytochrome *c* (cyt *c6*) is a biochemical equivalent of the copper-containing protein plastocyanin in photosynthetic electron transfer. But generally, cyt *c6* accumulates and functions only under conditions (e.g. Cu-deficiency) where holoplastocyanin cannot be synthesized. In studying the regulation of *Chlamydomonas reinhardtii* cyt *c6* expression by Cu we have determined that repression of cyt *c6* accumulation occurs at the transcriptional level, and specifically in response to Cu as the metal ion regulator. Complete and sustained repression of cyt *c6* transcription requires ~9 × 10^6 Cu ions in the medium/cell. Based on the estimated plastocyanin content of algal cells (8 × 10^6 molecules/cell) and the observation that lower ratios of Cu per cell result in only transient repression of cyt *c6* transcription, we propose that Cu-dependent transcriptional repression of the gene encoding cyt *c6* requires a Cu-binding factor which is titrated by Cu only after the alternate electron transfer catalyst, plastocyanin, has accumulated to the stoichiometry required for photosynthesis. The precise and highly metal-specific, autoregulatory control of cyt *c6* levels—directly by Cu, and indirectly by holoplastocyanin—is in keeping with the functional role of cyt *c6* as an alternate, although perhaps less preferred, electron transfer catalyst.

Key words: Chlamydomonas reinhardtii/copper/cyt *c6*/metal ion regulated gene expression/plastocyanin

Introduction

Metal ions as regulators of gene expression have come into prominence through recent studies in which genes responding to a variety of metals—Hg, Fe, Mo, Cd and Cu—have been identified in both prokaryotic and eukaryotic organisms (Stuart *et al.*, 1984; Munger *et al.*, 1985; Jacobson *et al.*, 1986; Rao *et al.*, 1986; Thiele and Hamer, 1986; Bagg and Neilands, 1987; Laddaga *et al.*, 1987; Merchant and Bogorad, 1987b; O'Halloran and Walsh, 1987; Owen and Kuhn, 1987; Theil, 1987). The metal ions can act through regulatory proteins at either transcriptional or post-transcriptional levels via sequences at either the 5′ or 3′ end of the protein coding region of the gene (e.g. Stuart *et al.*, 1984; Rao *et al.*, 1986; Owen and Kuhn, 1987; Seguin and Hamer, 1987; Casey *et al.*, 1988, 1989; White *et al.*, 1988; Huibregtse *et al.*, 1989; and reviewed by Hamer, 1986; Theil, 1987; O'Halloran, 1989). The functions of the products of these metal-responsive genes can be quite diverse; they might be involved in metal uptake and metabolism (e.g. siderophores, ferritin and the transferrin receptor), metal detoxification (e.g. metallothionein or mercuric reductase) or as metalloprotein catalysts in electron transfer reactions (e.g. cyt *c6* or nitrogenase) (reviewed by Hamer, 1986; Theil, 1987; O'Halloran, 1989). Whereas most of the research efforts to date have concentrated on either metal (primarily Fe) uptake and metabolism or on cellular mechanisms for heavy metal (Hg, Cd, Cu and Ag) resistance, relatively little attention has been paid to the role of metal ions in the biosynthesis of essential metalloproteins. Full understanding of metal ion homeostasis requires the concomitant study and appreciation of the role of essential metal ions, both as functional catalysts in metalloproteins and as gene regulators. In this work, we describe the first example of a metal(Cu)-regulated gene for a metalloprotein which functions in an essential biochemical pathway (photosynthesis); we demonstrate that the response is highly specific for and sensitive to Cu as the regulator, and we show that a putative Cu-dependent transcriptional regulator of that gene competes with a chloroplast metalloprotein (plastocyanin) for available Cu.

Copper is an essential micronutrient for most forms of life primarily owing to its function in the catalysis of respiration (by cytochrome oxidase), oxygen transport (by hemocyanin) or photosynthesis (by plastocyanin) (Boulter *et al.*, 1977). In vascular plants, 80% of leaf Cu is found in plastocyanin, a photosynthetic electron transfer catalyst (Boulter *et al.*, 1977). Terrestrial plants grown in either natural or artificial Cu-deficient habitats contain reduced levels of plastocyanin and show symptoms of Cu deficiency. Aquatic algae and cyanobacteria on the other hand are photosynthetically competent under Cu-deficient growth conditions owing to functional replacement of the Cu redox center by a heme center in a soluble *c*-type cytochrome (Wood, 1978; Bohner *et al.*, 1980; Sandmann *et al.*, 1983; Ho and Krogmann, 1984). The soluble *c*-type cytochromes are ubiquitously distributed heme proteins which function as electron carriers in the energy transducing electron transfer chains of mitochondria, respiring and photosynthetic bacteria, and some chloroplasts (Salemme, 1977; Dickerson, 1980; Meyer and Kamen, 1982; Mathews, 1985). The major metabolic function of these soluble cytochromes *c* is identical to that of plastocyanin in photosynthesis; *viz.* the catalysis of quinol oxidation via a membrane associated cyt *bc* (bc_1, b_6f) complex (Hauska *et al.*, 1983).

[a]The general term cytochrome *c6* is used here to refer to the algal plastidic, soluble *c*-type cytochromes that participate in the Z-scheme of electron transfer. In previous work (from this laboratory and others) these cytochromes have been named according to the absorption maximum of the α band of the reduced cytochrome, *c*-552, *c*-553, *c*-554, etc. The use of the general name, cyt *c6*, stresses its functional role in photosynthesis as opposed to its species-specific physical properties.

In organisms that have genetic information for both plastocyanin and a soluble, photosynthetic *c*-type cytochrome (cyt $c6^a$), the choice between accumulation of one or the other can be experimentally manipulated by varying the amount of Cu in the surrounding medium (Wood, 1978; Bohner *et al.*, 1980; Sandmann and Boger, 1980; Merchant and Bogorad, 1986a,b, 1987a,b). Although the biological basis for this physiological response is evident, its molecular basis remains unexplored. Spectroscopic measurements of electron transfer catalysts in the alga *Chlamydomonas reinhardtii* indicate that the plastocyanin content of the cell can be changed over a 70-fold range by manipulating the concentration of Cu in the medium (from <100 nM to 6 μM) (Wood, 1978). This change is accompanied by a concomitant, reciprocal 200-fold change in the cellular cyt *c6* levels. Cu-dependent plastocyanin accumulation is the result of the decreased stability of the apoprotein ($t_{1/2} \approx 16-18$ min) versus the holoprotein ($t_{1/2} >$ several hours) (Merchant and Bogorad, 1986b), whereas levels of the soluble plastidic cyt *c* (cyt *c6*) are maintained by Cu-dependent regulation of mRNA levels (Merchant and Bogorad, 1987). Additionally, it appears that control of cyt *c6* expression results primarily from direct cellular sensing of available Cu, rather than from the perception of a secondary phenomenon occurring as a consequence of decreased plastocyanin or decreased photosynthesis in Cu-deficient cells (Merchant and Bogorad, 1987b).

Results

In order to distinguish between transcriptional and post-transcriptional events as the principal determinants of Cu-dependent repression of the cyt *c6* gene, we have used a nuclear run-on assay to estimate the extent of cyt *c6* transcription *in vivo* (Figure 1). Nuclear preparations from Cu-supplemented cells do not elongate cyt *c6* transcripts whereas those prepared from Cu-deficient cells do. Transcripts that are not Cu-regulated (plastocyanin or β-tubulin) are synthesized to equivalent extents by either preparation. The addition of Cu salts to the assay does not affect the rate or extent of incorporation of isotope into cyt *c6* transcripts (not shown). We deduce from these data that transcription of the cyt *c6* gene *in vivo* is initiated only in Cu-deficient cells.

Kinetic studies of cyt *c6* mRNA levels indicate that the addition of Cu (500 nM$-$10 μM) to Cu-depleted cells results in almost immediate (<20 min) and complete loss of hybridizable cyt *c6* pre-mRNAs, and rapid decay ($t_{1/2} \approx 45-60$ min) of hybridizable (Figure 2, and K.Hill, H.Li, J.Singer and S.Merchant, submitted) and translatable (data not shown) mRNA levels. The regulatory system is highly specific for Cu as the metal ion signal since other transition metals (Ni, Co, Mn and Zn) tested at micromolar concentrations, are ineffective in repressing cellular cyt *c6* levels (not shown). Furthermore, although the yeast ACE1 Cu-binding *trans*-acting regulator of CUP1 transcription is equally sensitive to Ag ions, both *in vivo* and *in vitro* (Furst *et al.*, 1988; Buchman *et al.*, 1989; Evans *et al.*, 1990), the cyt *c6* gene is completely unresponsive to Ag ions *in vivo* (at 500 nM in the medium; Figure 2). Up to 20-fold higher concentrations of Ag ions were tested (1$-$10 μM), and were found to be completely ineffective in specifically influencing the levels of cyt *c6* transcripts (K.Hill, H.Li, J.Singer and S.Merchant, submitted). These levels of Ag ions, are

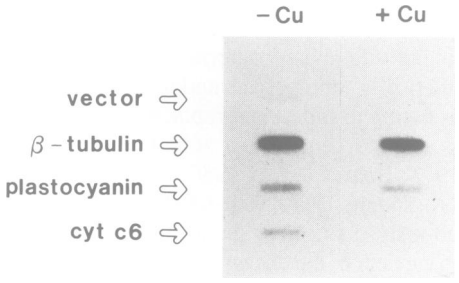

Fig. 1. 'Run-on' transcription assays of *C.reinhardtii* nuclear preparations. Nuclear fractions were prepared from a cell-wall deficient strain, cw-92 (CC-503), obtained from the *Chlamydomonas reinhardtii* Culture Collection at Duke University, Durham, NC. The cells were lysed with a non-ionic detergent (NP-40) and nuclear preparations were assayed for elongation of specific transcripts as described in Materials and methods. $-$Cu, nuclei isolated from Cu-deficient cells; +Cu, nuclei isolated from cells maintained in TAP medium supplemented with 6 μM Cu. The elongated transcripts were hybridized to 2 μg of immobilized DNA sequences as indicated. Vector, pTZ18R; β-tubulin, plastocyanin and cyt *c6* indicate immobilized cloned cDNA sequences containing the entire coding region for β-tubulin, pre-apoplastocyanin and pre-apocyt *c6*, respectively (Youngblom *et al.*, 1984; Merchant and Bogorad, 1987a; Merchant *et al.*, 1990).

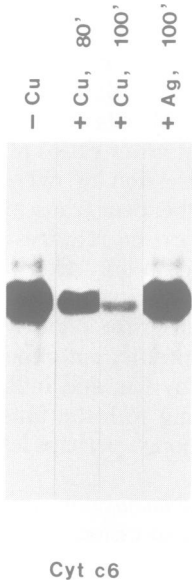

Fig. 2. Northern analysis of total RNA for cyt *c6* transcripts. Total RNA was purified (Merchant and Bogorad, 1986b) from Cu-deficient *C.reinhardtii* cells ($-$Cu) or cells supplemented with 500 nM $CuSO_4$ (+Cu) or $AgNO_3$ (+Ag) and 10 μg of the isolated RNA was analyzed by hybridization to radiolabeled cyt *c6* specific sequences. Cells were lysed prior to RNA isolation at the indicated times (80 min or 100 min after the addition of the metal ions). The faint band with lower electrophoretic mobility in the lane marked '$-$Cu' (compared with the intense band seen for the cyt *c6* mRNA) results from hybridization of the probe to a partially processed precursor to the mRNA. This conclusion is supported by the following observations: (i) the RNA also hybridizes to an intron-specific probe prepared from a genomic clone for cyt *c6*, (ii) the signal is not observed in polyadenylated mRNA preparations and finally, (iii) kinetic studies indicate that its disappearance precedes that of the stable mRNA pool. The pre-mRNA is undetectable $\sim 20-30$ min after Cu-supplementation of the medium (consistent with the observation that nuclear preparations from cells collected 30 min after Cu-supplementation do not synthesize cyt *c6* transcripts) whereas signal corresponding to the mRNA is completely absent only after 180 min with an estimated half-life of 45$-$60 min (K.Hill, H.Li, J.Singer and S.Merchant, submitted).

however, sufficient to induce a heavy metal toxic response (and associated phytochelatin-peptide synthesis) in *C.reinhardtii* (G.Howe and S.Merchant, submitted), indicating that Ag in the medium is indeed accessible to cellular proteins.

To determine the sensory range of a putative Cu-responsive cellular factor to Cu in the medium, the abundance of precursor and mature cyt *c6* transcripts was analyzed by Northern hybridization of total RNA isolated from Cu-deficient cells supplemented with nanomolar (1−10 nM) medium concentrations of Cu salts (Figure 3). When analyzed an hour later, any concentration >2.5 nM (3×10^5 Cu ions/cell) was sufficient to repress transcription as gauged by the absence of detectable pre-mRNAs and the decrease in levels of accumulated mature transcripts. However, when the same cells were sampled later (5.5 h after supplementation with $CuSO_4$), cyt *c6* precursor and mature transcripts in those cells supplemented with 1−10 nM $CuSO_4$ were found to have returned to the high levels maintained in Cu-deficient populations. The transient repression of transcription in these cells cannot be accounted for by intracellular dilution or titration of Cu ions owing simply to cell division (compare lanes 3 and 4, top panel with lanes 4 and 5, bottom panel), and suggests that Cu ions in the medium, although immediately accessible to a Cu-dependent transcriptional regulator, are sequestered with time so that Cu is no longer available to this effector molecule.

To estimate the level of medium Cu ions required for the sustained repression of cyt *c6* expression, soluble proteins extracted from cells maintained in medium containing various concentrations of $CuSO_4$ were analyzed for plastocyanin and cyt *c6* levels (Figure 4). At very low cell densities (top panel), 'Cu-free' medium (estimated to contain <3 nM Cu) can support plastocyanin accumulation to the extent observed in fully Cu-supplemented cells, and cyt *c6* is not induced. In more dense cultures (middle and bottom panels), the Cu content of the 'Cu-free' medium is no longer adequate, and cyt *c6* accumulates. For example, at 4×10^6 cells/ml, >30 nM $CuSO_4$ is required to achieve normal levels of plastocyanin and to inhibit cyt *c6* accumulation completely, and at 2×10^7 cells/ml, even higher levels of medium $CuSO_4$ (between 100 and 300 nM) are required. The same results are obtained if cyt *c6* message levels are examined as a function of cell density and medium Cu concentration (Figure 5). At low cell density (6×10^5 cells/ml), the cyt

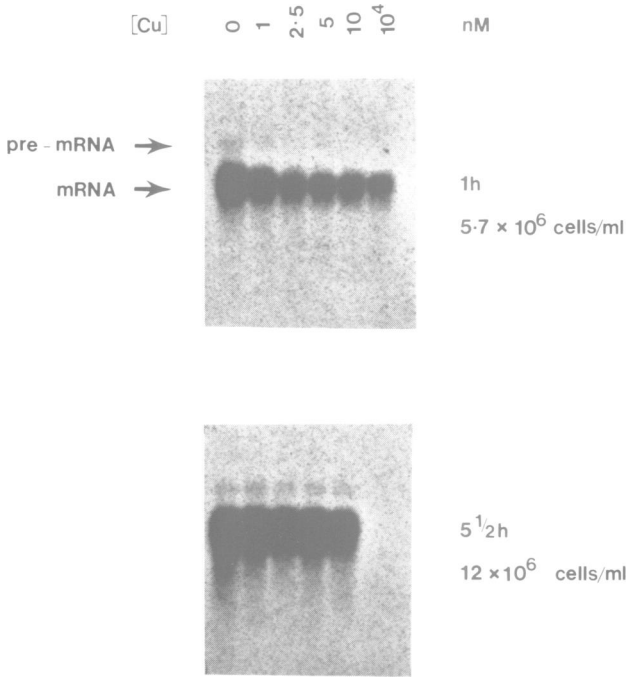

Fig. 3. Northern analysis of the levels of cyt *c6* pre-mRNA after titration with 'sub-saturating' levels of Cu. Total RNA was isolated from Cu-deficient (strain 2137, CC-1021) cells (0) or cells supplemented with various concentrations of $CuSO_4$ (1, 2.5, 5, 10 and 10^4 nM) for the times indicated. Levels of pre-mRNA and mature transcripts were detected by Northern hybridization of 3 μg immobilized, fractionated RNA to a radiolabeled cDNA fragment containing the entire coding sequence for pre-apocyt *c6*. The density of the culture (cells/ml) was determined (Harris, 1989) at the time the cells were sampled for purification of RNA, and is indicated.

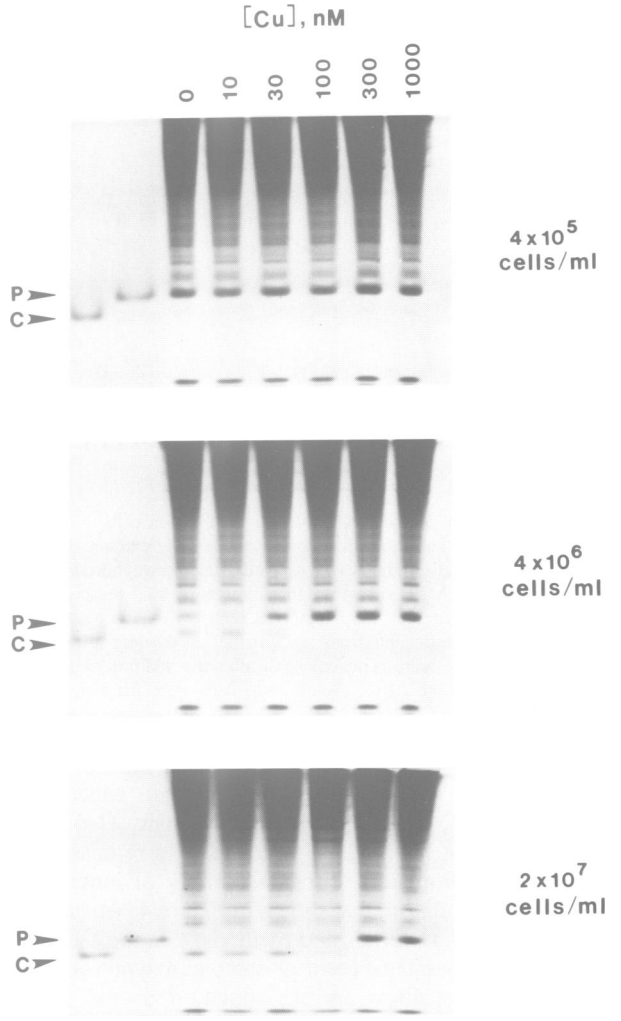

Fig. 4. Quantification of plastocyanin and cyt *c6* as a function of medium Cu concentration. Total soluble proteins from *C.reinhardtii* (strain 137c, CC-124) were analyzed after electrophoretic separation (Davis, 1964) by staining with Coomassie Blue. The arrows marked P and C indicate the positions of migration of purified plastocyanin and cyt *c6*, respectively. The cells were maintained at various concentrations of supplemental $CuSO_4$ (0, 10, 30, 100, 300 and 1000 nM) and sampled for analysis at the indicated densities. Plastocyanin and cyt *c6* fractionate entirely with soluble proteins and are not detected in membrane fractions (by Western blot analysis).

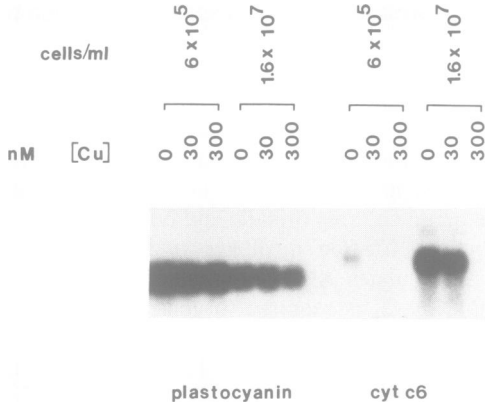

Fig. 5. Northern analysis of plastocyanin and cyt *c6* transcripts as a function of cell density and medium Cu ion concentration. Immobilized, fractionated RNA (7 μg) was analyzed by hybridization to a radiolabeled cDNA fragment encoding pre-apocyt *c6* (right half). The cells were maintained at the specified concentrations of supplemental Cu ions (0, 30 and 300 nM) and total RNA was prepared when cultures reached the indicated cell density. Hybridization of equivalent RNA samples to a radiolabeled plastocyanin-encoding cDNA was performed as an internal control (left half).

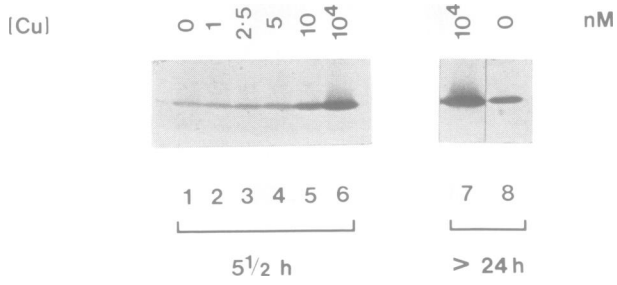

Fig. 6. Western blot analysis of plastocyanin levels in cells supplemented with 'sub-saturating' levels of Cu. The amount of immunoreactive plastocyanin was estimated after electrophoretic separation (SDS−PAGE) of equivalent amounts of total soluble protein extracts prepared from cells supplemented with Cu ions at a cell density and to final concentrations as described in the legend to Figure 3. Lanes 1−6, cells were sampled 5.5 h after the addition of the indicated concentrations of Cu; lanes 7−8, cells were maintained in Cu-deficient or fully Cu-supplemented medium and sampled so as to indicate the normal level of plastocyanin in Cu-supplemented medium.

c6 gene is >95% repressed with no Cu salts added to the culture medium, whereas at high cell density (1.6×10^7 cells/ml) at least 300 nM Cu is required to repress cyt *c6* transcription completely. From a number of independent experiments, similar to that shown in Figure 4, we calculate (as described in Materials and methods) that the biological response, i.e. maintenance of plastocyanin synthesis at the rate required to allow its accumulation to the desired stoichiometry in the electron transfer chain, and the complete suppression of cyt *c6* levels requires a fixed ratio of $\sim 9 \times 10^6$ medium Cu ions/cell. This ratio is of the same magnitude as the ratio of plastocyanin molecules per *C.reinhardtii* cell, i.e. 8×10^6/cell, indicating that the amount of Cu ions required to repress cyt *c6* expression is exactly matched by that required for holoplastocyanin formation.

The re-initiation of transcription from the cyt *c6* gene in cells supplied with low (1−10 nM) concentrations of Cu ions can therefore be accounted for by the titration of intracellular Cu with newly synthesized plastocyanin. This suggestion is supported by the demonstration that plastocyanin levels in cells supplemented with nanomolar (1−10 nM) concentrations of Cu ions indeed exhibit a Cu-dependent increase in 5.5 h (Figure 6). However, the amount of holoplastocyanin accumulated by these cells during this period is insufficient for maintenance of an adequate amount of plastocyanin for photosynthesis (compare lanes 5 and 7). Hence, in order to remain photosynthetically competent, the cells re-initiate synthesis of the alternate catalyst, cyt *c6*.

Discussion

Our results provide evidence for competition between plastocyanin and a putative Cu-dependent cellular factor (possibly a transcriptional regulator) for intracellular Cu (Figure 7). Although these results might suggest that holoplastocyanin itself functions directly as a regulator of cyt *c6* transcription, we have previously shown that a plastocyanin-less mutant (completely lacking plastocyanin message and protein) contains a normal Cu-repressible cyt *c6* gene; implying that the cellular signal affecting cyt *c6* transcription does not respond primarily to holoplastocyanin levels (Merchant and Bogorad, 1987b). Further, phenotypically-suppressed strains were capable of photoautotrophic growth in Cu-supplemented medium owing to a higher sensory threshold for Cu-dependent cyt *c6* suppression. These suppressed phenotypes were found also to have a higher tolerance threshold for Cu/Ag toxicity. This suggests that the regulatory factor controlling cyt *c6* expression responds directly to available Cu ion levels. The phenotypically-suppressed strains were demonstrated to repress cyt *c6* accumulation in a titratable response that was effective only in the micromolar range (Merchant and Bogorad, 1987b). Thus, the Cu-titratable repression of cyt *c6* in both wild-type (Figures 4 and 5) and plastocyanin-less strains (Merchant and Bogorad, 1987b) points towards a distinct Cu-binding protein that either directly interacts with sequences controlling the transcription of the cyt *c6* gene, or does so indirectly through other macromolecules. The kinetics of the response (e.g. Figure 3) in wild-type cells suggest that this factor is likely to be pre-existing. This Cu-binding factor may mediate control of cyt *c6* transcription either by functioning as an activator in the apo-form or as a repressor when it is complexed with Cu (Figure 7). The specificity of the regulatory response for Cu, with respect to both stabilization of plastocyanin and repression of the cyt *c6* gene, is biologically reasonable given the function of the regulatory pathway. It would be interesting to determine whether structurally similar Cu-binding sites function in plastocyanin and the transcriptional regulator.

Although we have been unable to note any difference in the photoautotrophic growth rate of *C.reinhardtii* cells in Cu-supplemented versus Cu-deficient medium (at various light intensities, 15−120 $\mu E/m^2/s$), the degree and level of regulation of these interchangeable electron transfer catalysts intimates that plastocyanin is preferred. The cell continues to synthesize, target and process apoplastocyanin whether or not Cu is available for plastocyanin function (Merchant and Bogorad, 1986b). If Cu is not available, apoplastocyanin is degraded with a half-life of 16−18 min and cyt *c6*

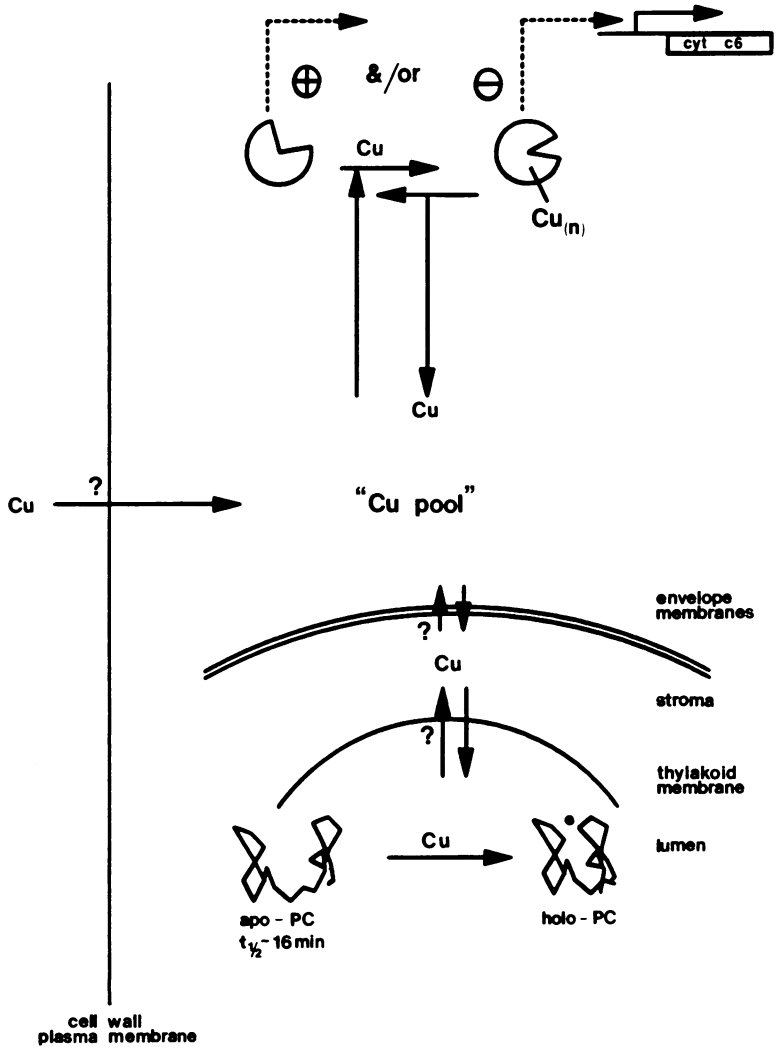

Fig. 7. Model demonstrating competition between a putative transcriptional regulator and plastocyanin for intracellular Cu. Medium Cu ions are rapidly internalized and are accessible to a Cu-binding factor that functions either as an activator of cyt *c6* transcription in its apo-form, or as a repressor when it is complexed with Cu. Although the number of regulatory Cu-binding sites is unknown, we would predict multiple, possibly co-operative, sites so as to allow precise sensing of available intracellular Cu. Since plastocyanin synthesis and targeting occurs constitutively, the intracellular 'Cu pool' is ultimately sequestered in the lumen as holoplastocyanin is synthesized. If the available Cu is insufficient to maintain the desired plastocyanin to P700 ratio, the transcriptional regulator allows cyt *c6* synthesis to make up the deficiency. However, if sufficient Cu is available, the Cu in excess of that required for plastocyanin synthesis can be used for the formation of the regulator−Cu complex and subsequent repression of cyt *c6* synthesis. As yet unknown Cu-transporting systems are indicated with a question mark. The + sign indicates positive regulatory control, or stimulation of transcription and the − sign indicates a negative effect on transcription, or repression.

synthesis continues; any Cu that is available is rapidly chelated by apoplastocyanin to form stable (and functional) plastocyanin (Merchant and Bogorad, 1986b), creating (as it continues to be synthesized and stabilized) a pool of bound or otherwise inaccessible Cu in the lumen of the thylakoid membrane. Only when the available Cu is in excess of the level required to support holoplastocyanin accumulation at the stoichiometry maintained in the thylakoid membranes of fully Cu-supplemented cells does Cu become available to the transcriptional regulator so that cyt *c6* synthesis may be repressed (Figure 7). In fully Cu-supplemented cells, plastocyanin levels attain a steady state and do not increase beyond the amounts required for maintenance of the stoichiometry of electron transfer catalysts (Merchant and Bogorad, 1986a); and, cyt *c6* synthesis is very tightly regulated so that neither message nor protein is detectable (Merchant and Bogorad, 1986a, 1987a). The observed competition between plastocyanin and the factor controlling transcription of cyt *c6* can be explained either by higher affinity of apoplastocyanin for Cu, or by sequestration owing to unidirectional compartmentation of Cu ions in the lumen (Figure 7). This autoregulatory circuit is analogous to the feedback control of Cu-metallothionein in yeast (Wright *et al.*, 1988) with the interesting distinction that in our system, competition for the ligand is proposed to occur between the putative transcriptional regulator of the cyt *c6* gene and the functional counterpart of the gene product, i.e. plastocyanin (Figure 7), rather than between the transcriptional regulator (ACE1) of the gene (CUP1) and its own gene product, i.e. metallothionein. In addition, our results are unique in that we have been able to quantify precisely the ligand sensing range of the transcriptional response and, with the knowledge of the stoichiometry of photosynthetic electron transfer components, we can propose a biological basis for the sensory range of the response.

We are currently pursuing the identification of copper-

regulated elements on the cyt *c6* gene, and of Cu-responsive DNA binding proteins interacting with these elements. Quite aside from their intrinsic interest as a general, and broadly distributed class of genetic regulators, metal ion-responsive promoters are of potential practical value in biotechnology (Butt and Ecker, 1987) as molecular switches since they can be activated for the controlled expression of foreign genes in genetically engineered organisms by the simple manipulation of metal concentration in the medium. It is for this reason, in part, that the basic functional aspects and the practical applications of prokaryotic, fungal and mammalian metal ion-regulated promoters are currently being investigated in a number of laboratories. Our work contributes the first example of a metal ion-responsive promoter in a plant system and one with several notable and unprecedented features: unique metal specificity, very high sensitivity and the reciprocal regulation of synthesis of two different (but functionally equivalent) proteins, with the metal playing a dual role as gene regulator and as an essential structural and catalytic cofactor. Thus the Cu-dependent, reciprocal synthesis of plastocyanin and cyt *c6* offers an excellent model for exploring the molecular and mechanistic aspects of metal-responsive gene expression, and for studying the interplay between the regulatory and structural/catalytic roles of trace metals.

Materials and methods

'Run-on' transcription assays

A cell-wall deficient strain of *C.reinhardtii* (CC-503, cw92) was grown in Cu-deficient medium as previously described (Wood, 1978; Merchant and Bogorad, 1986a). Where appropriate and possible, 'Gold label' chemicals from Aldrich Chemical Co., Milwaukee, WI and acid washed glassware or plasticware were used in the preparation of solutions in order to prevent exposure of the cells to Cu ions and to reduce the level of Cu ions in the biochemical preparations. Nuclear material was prepared, stored and assayed essentially as described by Keller *et al.* (1984). In brief, cells at a density of 6×10^6/ml were collected by centrifugation (650 *g*, 5 min), washed and lysed by the addition of Nonidet P-40 (to 0.5%) at a density of 1×10^8 cells/ml. The cells were monitored by phase contrast microscopy for complete lysis. Nuclei were pelleted, washed, resuspended to a density of $\sim 10^9$ nuclei/ml, frozen in liquid nitrogen, and stored at −80°C. Nuclei were assayed for elongation of specific transcripts during a 60 min reaction as described by Keller *et al.* (1984) with the exception that [α-^{32}P]CTP and GTP, at final concentrations of 1 μM, 0.8 μCi/μl, were used for radiolabeling the transcripts instead of UTP. The reaction was deproteinized after the addition of a 'stop solution' by three extractions with phenol:chloroform: isoamylalcohol (50:48:2), followed by one extraction with chloroform. Elongation of specific transcripts was determined by hybridization of the reaction to cloned DNA sequences corresponding to the gene of interest. Denatured plasmid DNA molecules were immobilized to Genescreen membranes (Dupont/New England Nuclear, Wilmington, DE) by UV-irradiation and hybridized according to the procedure of Church and Gilbert (1984) with the exception that the hybridization and pre-hybridization solutions contained in addition, EDTA (final concentration, 1 mM), yeast tRNA (100 μg/ml) and polyriboadenylic acid (20 μg/ml). The membranes were exposed to XAR-5 film for 2 days at −80°C with two enhancing screens (Lightning Plus, Dupont/New England Nuclear, Wilmington, DE).

Northern analysis of cyt c6 specific transcripts

Total RNA was isolated and analyzed as previously described (Merchant and Bogorad, 1987a,b). The RNA molecules were denatured with formaldehyde and separated on a denaturing gel containing 1.5% agarose, transferred to Genescreen (method according to instruction manual from Dupont/New England Nuclear), and hybridized to a ^{32}P-Klenow-labelled (Feinberg and Vogelstein, 1984), gel-purified (Maniatis *et al.*, 1982) restriction fragment containing DNA sequences corresponding to the coding region of cyt *c6*. The specific activity of the radiolabeled fragments ranged from 8×10^8 to 15×10^8 c.p.m./μg DNA. The membrane was exposed to Kodak XRP-1 or XAR-5 film for ~8–24 h at −80°C with two intensifying screens.

Estimation of plastocyanin and cyt c6 levels

Cells grown at 120 μE/m^2/s in Cu-deficient or Cu-supplemented Tris−acetate−phosphate medium (Wood, 1978; Merchant and Bogorad, 1986a; Harris, 1989) were collected at the indicated cell densities by centrifugation, washed with a solution containing 10 mM sodium phosphate, pH 7.0, and resuspended in the same solution to a density equivalent to 1 mg chlorophyll/ml. Soluble proteins were quantitatively released after two slow freeze−thaw cycles (−80°C to room temperature) and collected by centrifugation at 12 000 *g* for 15 min at 4°C. Aliquots (20 μl) of the supernatant fraction were prepared for non-denaturing electrophoresis by the addition of 5 μl of a 5× sample buffer (Davis, 1964). The extracted, soluble proteins were separated by electrophoresis for 90 min at 100 V through a 5% acrylamide stacking gel (8 × 1.5 mm) and a 15% acrylamide separating gel (52 × 1.5 mm) as described by Davis (1964), and visualized by staining with Coomassie Blue R-250. Western blot analysis of plastocyanin and cyt *c6* levels has been previously described (Merchant and Bogorad, 1986a, 1987b). In brief, soluble cell extracts were separated by SDS−PAGE (5% stacking gel, 8 × 1.5 mm; 15% separating gel, 52 × 1.5 mm) and the separated proteins were transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA) at 50 V for 2 h. Proteins of interest were localized by the use of specific antibodies (Merchant and Bogorad, 1986a) followed by detection of bound antibody with a horseradish peroxidase second antibody (BioRad Laboratories, Richmond, CA) and visualization by horseradish peroxidase activity on 4-chloro-1-naphthol.

Estimation of Cu content, cell densities and plastocyanin levels in cultures

The Cu content of our 'Cu-deficient' medium is estimated to be <3 nM from calculations based on the known heavy metal content of 'Gold Label' inorganic salts purchased from Aldrich Chemical Co., Milwaukee, WI, for use in the medium used to culture *C.reinhardtii*, and on Cu analysis of some stock solutions used in preparation of media (by atomic absorption). Owing to very strong interference from the high concentration of (10^3–10^5 excess over Cu) of other metals present in 'Hutner's trace element solution' (Harris, 1989), we have been unable to determine precisely the amount of Cu taken up by *C.reinhardtii* cells. We have, however, determined that Cu-deficient cells are capable of rapid uptake of supplied Cu, and, as evidenced by experiments of the type shown in Figure 3, Cu-deficient cells can demonstrate a rapid, physiologically-relevant, biochemical response to as little as 1 nM medium Cu ions. This implies that the cells have access to sub-nanomolar levels of medium Cu ions and the assumption that the amount of supplied medium Cu ions is representative of cellular Cu content in cultures that are Cu-deficient (or barely Cu-sufficient) is therefore very reasonable.

The calculation of the amount of medium Cu required to sustain plastocyanin synthesis and inhibit cyt *c6* accumulation relies on analysis of three experiments of the type shown in Figure 4. By comparing the ratios of medium Cu per cell in samples known to synthesize and accumulate both plastocyanin and cyt *c6* with those in samples known to synthesize only plastocyanin, we can put a lower limit of 7.7×10^6 medium Cu/cell and an upper limit of 9×10^6 medium Cu ions/cell on our reported value for the amount of Cu ions required to suppress cyt *c6* expression.

Cell densities were determined by counting (average of two determinations) in a hemocytometer after immmobilization as described (Harris, 1989). The chlorophyll content of whole cells was determined spectrophotometrically after extraction into 80:20 acetone:methanol from the extinction coefficients calculated by Arnon (1949). Based on a chl *a*/*b* ratio of 2.6:1 for strain 137c determined by Owens *et al.* (1989), a weighted average molecular weight of 897 was used in the calculation of the amount of chlorophyll per cell. The average chlorophyll content (from 14 determinations) of *C.reinhardtii* cells (for the experiment shown in Figure 4) was $1.95 \pm 0.3 \times 10^9$ molecules per cell, although the values ranged from 1.5×10^9 to 2×10^9 molecules/cell in independent experiments and cultures. These measurements agree very well with the measurements made by other laboratories (Harris, 1989). The ratio of chlorophyll *a* + chlorophyll *b* to P700 was determined by Owens *et al.*, cited above, to be 478 ± 48 for the strain used in the experiment shown in Figure 4 and this value was used in our calculation of the amount of P700/cell. The ratio of plastocyanin to P700 was assumed to be ~2, based on the reported ratio determined by spectroscopic measurements of soluble extracts of *C.reinhardtii* cells after quantitative release of plastocyanin (Wood, 1978), and on the range suggested by Graan and Ort (1984). We thus calculate a plastocyanin content of 8.2×10^6 per *C.reinhardtii* cell under our laboratory growth conditions.

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