[18] Copper-Responsive Gene Expression during Adaptation to Copper Deficiency

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Introduction

Copper is an essential micronutrient for all organisms because of its role as a redox active cofactor in many essential enzymes and electron transfer catalysts.[1] However, because excess copper is toxic, organisms

[1] M. C. Linder, *in* "Biochemistry of Copper." Plenum Press, New York, 1991.

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maintain copper homeostasis by regulating copper uptake and utilization. The unicellular green alga *Chlamydomonas reinhardtii* has proved to be a useful model system for studying one aspect of copper metabolism, specifically, adaptations to copper deficiency. Like other green algae, *C. reinhardtii* does not appear to contain copper enzymes such as copper/zinc superoxide dismutase or polyphenol oxidase that are abundant in vascular plants. The major copper proteins of Chlamydomonas are plastocyanin, utilized in the photosynthetic apparatus where it functions to transfer electrons from the cytochrome b_6f complex to Photosystem I and cytochrome (cyt) oxidase, which is required for respiration in the mitochondrion. In *C. reinhardtii*, plastocyanin is estimated to be at least 10-fold more abundant than cytochrome oxidase, which makes it the major metabolic sink for copper.

When copper is limiting, certain green algae and cyanobacteria synthesize cytochrome c_6 (a heme-containing protein), which can substitute functionally for plastocyanin. The accumulation of one or the other protein is reciprocally dependent on the presence of copper in the growth medium. In *C. reinhardtii*, this is effected by enhanced degradation of plastocyanin in copper-deficient cells[2] and activation of transcription of the *Cyc6* gene (encoding cyt c_6).[3] The extent of transcription of the *Cyc6* gene is directly proportional to the perceived copper deficiency.[4] Thus, measurement of *Cyc6* expression is generally a good assay for copper deficiency. Other changes in gene expression include induction of coprogen oxidase synthesis (encoded by the *Cpx1* gene[5]), which is required to meet the increased demand for heme in copper-deficient cells. Copper deficiency also induces a cell surface cupric reductase and a high-affinity copper transporter; these are likely to be components of an assimilatory uptake system.[6] The regulatory system described is not only a valuable system for the study of copper-responsive gene expression but it may also be useful for the study of mRNA decay mechanisms: the transcription of genes controlled by the copper-responsive elements of the *Cyc6* gene can be selectively and completely turned off simply by the addition of copper to the growth medium. The selectivity of the *Cyc6* promoter can be exploited also to control gene expression in transgenic organisms.

This chapter describes the preparation and use of copper-deficient media to study copper-responsive gene expression and copper metabolism with emphasis on *C. reinhardtii* as an experimental system. The principles

[2] H. H. Li and S. Merchant, *J. Biol. Chem.* **270,** 23504 (1995).
[3] J. M. Quinn and S. Merchant, *Plant Cell* **7,** 623 (1995).
[4] K. L. Hill and S. Merchant, *Plant Physiol.* **100,** 319 (1992).
[5] K. L. Hill and S. Merchant, *EMBO J.* **14,** 857 (1995).
[6] K. L. Hill, R. Hassett, D. Kosman, and S. Merchant, *Plant Physiol.* **112,** 697 (1996).

discussed in this chapter can also be applied to the study of other trace metal responsive processes.[7] General methods for growth and maintenance of Chlamydomonas strains are described in *The Chlamydomonas Sourcebook.*[8] Methods for the measurement of copper metabolism (e.g., uptake of ^{64}Cu, cupric reduction[6]) are not described in this article, because they may not be generally applicable. Although the literature contains a substantial body of work on copper-responsive gene expression in cyanobacteria[9-12] and other algae,[13] we have limited our descriptions to methods that we have experience with.

Growth Media

For growing cells under copper-deficient conditions, it is necessary to use several precautions.

1. All glassware, plasticware (with the exception of colorless disposable plastic pipettes), and stirbars must be washed with 6 *N* hydrochloric acid to displace any residual copper ions. All surfaces that will contact chemicals and solutions are washed with 6 *N* hydrochloric acid, followed by seven washes with distilled water and three washes with MilliQ-purified (Millipore Corp., Bedford, MA) water. Used hydrochloric acid should be treated as hazardous chemical waste and disposed of appropriately.

2. High-purity chemicals should be used for making "copper-free" stock solutions for media preparation. It is recommended that these chemicals be kept separate from other laboratory chemicals to avoid metal contamination from use of metal spatulas and to avoid use of these higher priced chemicals in less stringent applications. Aldrich gold label chemicals (Aldrich Chemical Company, Milwaukee, WI) or other special trace metal grade chemicals (Fisher Scientific, Tustin, CA; Sigma Chemical Company, St. Louis, MO) are recommended. A trace element composition analysis is generally provided with the chemical. The source of chemicals we use are indicated below.

3. MilliQ-purified water (or the equivalent analytical grade water, <0.02 ppb Cu[14]) should be used for preparation of media and all stock solutions

[7] C. D. Cox, *Methods Enzymol.* **235,** 315 (1994).
[8] E. H. Harris, *in* "The *Chlamydomonas* Sourcebook: A Comprehensive Guide to Biology and Laboratory Use," p. 25. Academic Press, San Diego, California, 1988.
[9] L. M. Briggs, V. L. Pecoraro, and L. McIntosh, *Plant Mol. Biol.* **15,** 633 (1990).
[10] A. Bovy, G. deVrieze, M. Borrias, and P. Weisbeek, *Mol. Microbiol.* **6,** 1507 (1992).
[11] L. Zhang, B. McSpadden, H. B. Pakrasi, and J. Whitmarsh, *J. Biol. Chem.* **267,** 19054 (1992).
[12] M. Ghassemian, B. Wong, F. Ferriera, J. L. Markley, and N. A. Straus, *Microbiol.* **140,** 1151 (1994).
[13] M. Nakamura, M. Yamagachi, F. Yoshizaki, and Y. Sugimura, *J. Biochem. (Tokyo)* **111,** 219 (1992).
[14] J. Mather, F. Kaczarowski, R. Gabler, and F. Wilkins, *Biotechniques* **4,** 56 (1986).

to minimize copper contamination from the water source (especially if the house water is supplied via copper pipes).

Source and Preparation of Reagents

The standard recipes for *C. reinhardtii* growth medium are described by Harris.[8] Below we list special sources or special preparation methods.

Beijerinck's Solution (1 liter)

8 g Ammonium chloride, 99.998% (Aldrich)
1 g Magnesium sulfate, 99.99+% (Aldrich)
1 ml 1 g/ml Calcium chloride, 99.99+% (Aldrich)
(The gold label $CaCl_2$ is deliquescent and is difficult to weigh. A 1 g/ml solution is prepared and stored at 4°.)

Tris-Acetate (1 liter)

242.2 g Tris base (Fisher Scientific, or other biotechnology grade)
100 ml Glacial acetic acid (Fisher Scientific, trace metal grade)

Phosphate Solution (1 liter)

18.5 g Potassium phosphate, monobasic, 99.99% (Aldrich)
Dissolve 18.5 g potassium phosphate in ~800 ml MilliQ water. Adjust the pH of the solution to 7.1 using a 3 *N* copper-free solution of potassium hydroxide, prepared by dissolving potassium hydroxide pellets (Aldrich, 99.99%) in water. Bring to a final volume of 1 liter. Store at 4°.

Trace Element Solution (1 liter)

50 g Ethylenediaminetetraacetic acid (EDTA) (Fisher Scientific or other ACS grade)
22 g Zinc sulfate, heptahydrate, 99%, ACS reagent (Aldrich)
11.4 g Boric acid, 99.99% (Aldrich)
5.1 g Manganese chloride, tetrahydrate, 99.99% (Aldrich)
5 g Ferrous sulfate, heptahydrate, 99+%, ACS reagent (Aldrich)
1.6 g Cobalt chloride, hexahydrate, 98%, ACS reagent (Aldrich)
1.1 g Ammonium molybdate, tetrahydrate, 99.98% (Aldrich)
The solution should be prepared as detailed by Harris[8] except that the color of the solution should turn from orangy red to burgundy red. The orange precipitate is removed by filtration through a scintered glass filter (washed with 6*N* HCl as described earlier). The solution is stable for at least 1 year at 4°.

To make stock solutions for copper-supplemented media, the same recipes are followed for Beijerinck's, Tris-acetate, and phosphate solutions with the usual ACS grade laboratory chemicals, and 1.6 g of copper chloride

pentahydrate is added to the trace element recipe. Copper-supplemented trace elements will turn from olive green to purple on standing.

For radiolabeling experiments, the sulfate content of the medium in which the cells are grown can be reduced (if 35S-sulfate is used as the source of label) by substituting 1.63 g of $MgCl_2 \cdot 6H_2O$ (Aldrich, 99.995%) for 1 g of $MgSO_4$ in Beijerinck's solution. For the actual labeling procedure, the cells are resuspended in sulfate-free medium (prepared by using sulfate-free Beijerinck's solution and omitting the trace elements).

The estimated copper ion concentration of copper-deficient media prepared with the above specified chemicals is less than 3 n*M*.

Solid Medium

For preparation of copper-deficient agar plates, the agar must be washed. Twenty grams of TC agar (JRH Biosciences, Lenexa, KS) is added to an acid-washed 2-liter flask or fleaker (on which the 1-liter level has been marked) containing a stir bar. MilliQ-purified water and EDTA (from a 0.5 *M*, pH 8.0, stock) are added to a final volume of 2 liters and a final EDTA concentration of 20 m*M*. The agar is stirred gently at 4°. Stirring should be just fast enough to maintain suspension of agar. If the agar is stirred too vigorously, the particles will break up and they will not settle well between washes, which leads to unacceptably high losses during the decanting. After stirring for 8–16 hr, the agar is allowed to settle for 1–2 hr and the liquid is decanted carefully, retaining as much agar as possible. The EDTA wash is repeated three more times, followed by four washes performed in the same manner with MilliQ-purified water. This serves to remove any residual EDTA which inhibits growth. The final settled agar is used to make Tris-acetate phosphate (TAP) or minimal media in the same flask using the 1-liter mark to bring the solution to volume. For any comparative studies of cell growth, enzyme expression, etc., between copper-supplemented and copper-deficient cells it is recommended that the agar used for copper-supplemented plates also be prepared by washing with EDTA because this treatment undoubtedly removes many other (potentially inhibitory) compounds from the agar in addition to divalent metal ions.

Use of Chelators

An alternative or additional method for preparing metal-deficient solutions (such as the Tris-acetate or phosphate stock, that do not contain other metal ions) when high-purity chemicals or solvents are not available is to treat the solution with dithizone (1,5-diphenylthiocarbazone).[15] In a chemi-

[15] T. Matsubara, K. Frunzke, and W. G. Zumft, *J. Bacteriol.* **149,** 816 (1982).

cal fume hood, 5 ml of a 0.05% solution of dithizone in chloroform is added per liter of solution in an acid-washed 1-liter separatory funnel with a glass or Teflon stopcock. The phases are mixed thoroughly. The chloroform phase settles to the bottom from where it is removed. The aqueous phase will be pink from residual dithizone, which is removed by repeated extractions with 5-ml aliquots of chloroform until the aqueous and organic phases are colorless. After two additional extractions, the final aqueous phase is left in an open container in the hood to allow the residual chloroform to evaporate. If required, dithizone-extracted solutions can be supplemented with other metal ions from high-purity sources.

Other methods for removing ions can also be used. Chelex resin (Bio-Rad, Richmond, CA) exhibits high selectivity for divalent versus monovalent ions (5000:1). The solution to be deionized should be mixed gently for >1 hr with the resin. The resin can be removed by filtration, and salts (of high purity) can be added back to the solution as desired. In our system, Chelex treatment of the medium does not enhance the expression of the *Cyc6* gene compared to medium prepared with "gold label" chemicals (see above); nevertheless, the treatment may be useful for demetalation of solutions when suitably pure ingredients are not available. In theory, it should also be possible to use soluble copper-selective chelators, e.g., *o*-phenanthroline or CDTA, to deplete the medium of available copper. In this case, the concentration of chelator should be calculated from the stability constants of the chelator–metal complexes (Data for Biochemical Research) such that copper is chelated but other essential cations (e.g., Zn^{2+}) are not. We have tested bathocuproinedisulfonate in copper-supplemented TAP medium and found it to be ineffective at preventing copper utilization in that medium. However, we have not tested *o*-phenanthroline or CDTA. Because *C. reinhardtii* can utilize copper from a copper–EDTA complex, the utility of the chelators needs to be experimentally determined.

Culturing

To generate copper-deficient cultures, cells in the pretransfer culture are grown to mid to late log phase (10^6–10^7 cells per ml) and diluted 1/100 to 1/200 into fresh copper-deficient medium. One transfer can be sufficient, but copper carryover (e.g., from internal stores or from cell-wall bound forms) can vary from strain to strain. Therefore, three sequential transfers (from 10^6–10^7 cells/ml into fresh medium) are recommended to ensure that the residual copper in the copper-deficient culture is reduced to <3 n*M*. After the third transfer, the culture is generally copper deficient. Nevertheless, it is advisable to confirm this (see below). On solid medium, cells appear (on the basis of plastocyanin and cyt c_6 accumulation) to

become copper deficient after a single transfer.[16] Note that many photosynthetic mutants grow poorly in copper-deficient medium: copper-deficient cultures of such mutants may never exceed 10^6 cells/ml. To obtain sufficient quantities of cells for analyses, it may be necessary to grow larger cultures that can be harvested at lower densities. For certain cell-wall deficient strains we have noted during collection of cells that the cell pellet from copper-supplemented cultures is more tightly packed in the bottom of the microcentrifuge tube than the pellet from copper-deficient cells (which tends to smear against the wall of the tube). The reason for this difference is not known to us, but the phenomenon does not affect any of the assays described later.

Even when media are prepared with scrupulous attention to sources of potential metal contamination, fresh copper-deficient medium generally contains some amount of residual copper ions (see below for measurement by spectroscopy). At low cell densities, this amount is sufficient to satisfy the plastocyanin biosynthetic pathway.[17] The low level of residual copper is also sufficient to cause transient changes in gene expression when cells are transferred to fresh medium (e.g., prior to radiolabeling experiments), and if this is a consideration, the experiment should be designed to avoid transfer to fresh medium.

Scenedesmus obliquus is another green alga that is closely related to *C. reinhardtii.* This organism also responds to copper-deficient growth conditions with alteration in the accumulation of cyt c_6 and plastocyanin. However, in *S. obliquus*, copper-responsive regulation of plastocyanin expression occurs via mechanisms that affect protein and also messenger RNA levels.[18] In the copper-supplemented TAP medium described earlier, copper ions are chelated by EDTA but this chelated form is available to *C. reinhardtii* for plastocyanin synthesis. However, this chelated form of copper appears to be invisible to *S. obliquus* cells. Specifically, *S. obliquus* cells grown in the usual TAP medium behave as if they were copper deficient (no plastocyanin, high levels of cyt c_6). To grow *S. obliquus* in fully copper-supplemented medium, copper salts (20 μM copper sulfate or chloride) must be added. This difference between *C. reinhardtii* and *S. obliquus* with respect to their ability to utilize chelated copper suggests that it is important to test for appropriate copper-supplemented versus copper-deficient growth conditions when initiating studies on other organisms.

[16] Z. Xie, D. Culler, B. W. Dreyfuss, R. Kuras, F.-A. Wollman, J. Girard-Bascou, and S. Merchant, *Genetics* **148,** 681 (1998).
[17] S. Merchant, K. Hill, and G. Howe, *EMBO J.* **10,** 1383 (1991).
[18] H. H. Li and S. Merchant, *J. Biol. Chem.* **257,** 9368 (1992).

Sample Preparation

Chlamydomonas reinhardtii

A soluble protein fraction containing plastocyanin and cyt c_6 can be prepared as described by Howe and Merchant.[19] Special precautions to maintain the cells in a copper-free state are not necessary for preparation of protein fractions because the proteins are stable for hours. The supernatants may be analyzed as described below.

For the preparation of RNA from copper-deficient *C. reinhardtii* cultures, it is important to remember that the $t_{1/2}$ of the mRNA encoding cytochrome c_6 can be equivalent to the RNA preparation time (~45 min), and that of the copper-deficient form of the *Cpx1* RNA appears to be even shorter. Therefore, in order to avoid loss of the RNA of interest, it is essential to maintain "copper-free" conditions while collecting the cells and also during the lysis step. Concentrations as low as 2–5 n*M* Cu ions result in changes in the abundance of *Cyc6* and *Cpx1* transcripts.[5,17] Therefore, solutions are prepared with gold label chemicals, where possible, and all glassware and other material used for preparation of the solutions and collection of cells are acid washed.

For RNA blot analysis of the *Cyc6* and *Cpx1* mRNAs, we have used the hybridization conditions described by Church and Gilbert[20] and hybridization and wash temperatures of 65°. With oligo-labeled probes[21] of specific activity in the range of 1.5 to 3×10^8 cpm/mg and 5–10 μg of total RNA per lane, an excellent signal is obtained for the *Cyc6* and *Cpx1* mRNAs with an overnight exposure at −80° to Kodak XAR-5 film (two intensifying screens) or a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) screen.

Nuclei can be isolated from copper-supplemented and copper-deficient cultures of cell-wall deficient strains of *C. reinhardtii* for analysis of transcription of genes of interest (e.g., *Cyc6* or *Pcy1*) by nuclear run-on assays.[17,22] All solutions and materials used during the preparation of nuclei should be "copper free" to minimize copper contamination (see RNA isolation just discussed). Transcription of *Cyc6* is not affected by the addition of copper to isolated nuclei; thus, it is not necessary to maintain copper-free conditions during the assay of the nuclei.

Scenedesmus Obliquus

Scenedesmus obliquus cells are somewhat hardier than *C. reinhardtii* cells; thus, the procedures described above must be modified slightly. For

[19] G. Howe and S. Merchant, *EMBO J.* **11,** 2789 (1992).
[20] G. M. Church and W. Gilbert, *Proc. Natl. Acad. Sci. U.S.A.* **81,** 1991 (1984).
[21] A. P. Feinberg and B. Vogelstein, *Anal. Biochem.* **137,** 266 (1984).

preparation of soluble proteins, the cells are broken by sonication in a microcentrifuge tube (microtip probe, 60% intensity, 5 min, Sonic Dismembrator, Fisher Scientific).[18] Other methods for breaking *S. obliquus* cells involve the use of glass beads in a Vibrogen (Buhler, Germany) cell mill[23-25] or a Bead Beater[26] (Biospec Products, Bartlesville, OK). We have used the Bead Beater to lyse *S. obliquus* cells for large-scale purification of plastocyanin.

For preparation of RNA, the cell pellet is resuspended in water, transferred to a mortar containing liquid nitrogen and ground to a fine powder under liquid nitrogen.[18,27] The paste, on thawing, is transferred immediately to a disposable centrifuge tube, diluted with one volume of 2× lysis buffer, and the preparation continued as for *C. reinhardtii* cells.

Testing for Copper Deficiency in the Culture

Copper deficiency in the context of the photosynthetic apparatus should be assessed by measuring plastocyanin and/or cyt c_6 abundance in the culture of interest. Several different methods have been employed; the sensitivity of the method must be balanced with the simplicity and speed of the procedure. The extent of expression of the cyt c_6-encoding gene is directly proportional to the copper content in the medium on a per cell basis (up to 10^7 Cu ions per cell; see Fig. 1).[11,17] Thus, measurement of cyt c^6 content of cells is a good measure of the copper deficiency. Likewise, holoplastocyanin formation is directly proportional to the amount of copper available to the cell; measurement of plastocyanin content relative to a fully copper-supplemented culture can give a good indication of the copper status of the cell.[28] Of the methods described later, heme and Coomassie staining are the easiest and use common laboratory reagents. Although immunoblots have the potential to provide greater sensitivity, appropriate antisera are required. The spectrophotometric method is the cheapest (assuming a suitable instrument is available) but much larger amounts of biological material are required.

Spectroscopic Analysis of Soluble Extracts

Plastocyanin and cytochrome c_6 display unique visible spectra; thus, difference spectroscopy can be applied to estimate the amount of the two

[22] L. R. Keller, J. L. Schloss, C. D. Silflow, and J. L. Rosenbaum, *J. Cell Biol.* **98,** 1138 (1984).
[23] H. Bohner and P. Boger, *FEBS Lett.* **85,** 337 (1978).
[24] V. Breu and D. Dornemann, *Biochim. Biophys. Acta* **967,** 135 (1988).
[25] J. Schnackenberg, R. Schulz, and H. Senger, *FEBS Lett.* **327,** 21 (1993).
[26] B. A. Diner, D. F. Ries, B. N. Cohen, and J. G. Metz, *J. Biol. Chem.* **263,** 8972 (1988).
[27] D. Hermsmeier, R. Schulz, and H. Senger, *Planta* **193,** 406 (1994).
[28] P. M. Wood, *Eur. J. Biochem.* **87,** 9 (1978).

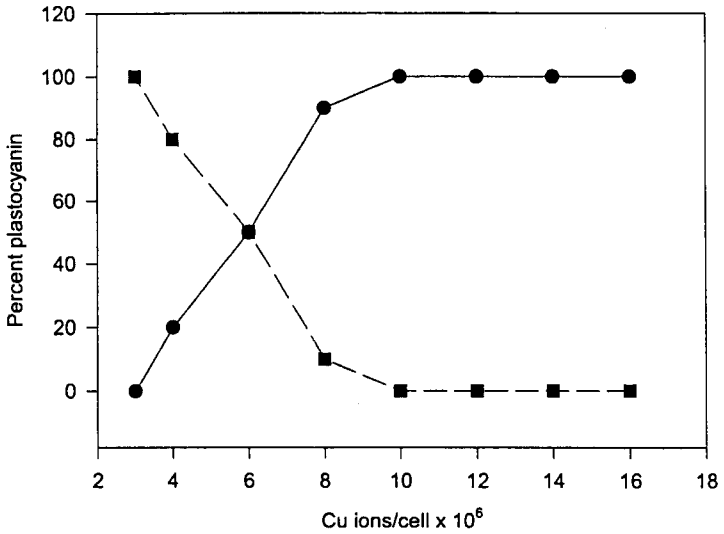

FIG. 1. Reciprocal expression of cyt c_6 and plastocyanin in response to copper ions in the medium.

proteins in cell extracts. Soluble protein extracts (from cells equivalent to 1 mg/ml chlorophyll) are reduced with sodium ascorbate (to 1 m*M*) or oxidized with potassium ferricyanide (1 m*M*). The samples are scanned from 500 to 650 nm and the signal difference is recorded. For *C. reinhardtii* cytochrome c_6, a $\Delta\varepsilon$ (reduced–oxidized) at 552.5 nm of 20 mM^{-1} is used and for plastocyanin, a $\Delta\varepsilon$ (oxidized–reduced) at 597 nm of 4.5 mM^{-1} is used. Although spectroscopic analysis is simple and rapid and does not require specialized reagents, it is several orders of magnitude less sensitive than the chemical or immunochemical methods described later. For instance, for cyt c_6, an extract from fully copper-deficient wild-type cells equivalent to 1 mg/ml chlorophyll yields a maximum absorbance of only 4×10^{-2} at 552.5 nm. The lower detection limit for cyt c_6 corresponds to an amount equivalent to 10% of the amount in a fully copper-deficient wild-type cell owing to the presence of mitochondrial cyt *c* (λ_{max} = 550 nm).[19] The lower detection limit for plastocyanin is reported to be 2% of wild-type levels[29] but it should be noted that the samples need to be at least 5- to 10-fold more concentrated for a useful measurement owing to the lower extinction coefficient for plastocyanin. Spectroscopic analyses have been used to estimate cyt c_6 and plastocyanin content in several other

[29] D. S. Gorman and R. P. Levine, *Plant Physiol.* **41,** 1648 (1966).

organisms. Bohner and Boger[23] used an extinction coefficient of 17.3 mM^{-1} cm^{-1} at 553 nm for cyt c_6 (determined for the *S. obliquus* protein) and a $\Delta\varepsilon$ of 4.9 mM^{-1} cm^{-1} for plastocyanin (determined for the spinach protein). Sandmann *et al.*[30] used the same values to evaluate cultures of numerous different algae, and Zhang *et al.*[11] used the same value for cyt c_6 for studies with *Synechocystis*. On the basis of direct measurement, Kong and Whitmarsh[31] suggest a value of 19.5 ± 0.5 mM^{-1} cm^{-1}.

Immunoblot Analysis for Estimation of Plastocyanin and Cytochrome c_6 Content[19]

Plastocyanin and cyt c_6 are small proteins (10.3 and 9.7 kDa, respectively) and are therefore most conveniently separated by electrophoresis through gels polymerized from higher concentrations of acrylamide (12–15%). For immunoblot analysis, denaturing gel electrophoresis is recommended.

Traditional denaturing electrophoresis[32] (running gel = 12% acrylamide, 0.33% bis) in the MiniProtein II gel system (BioRad, Richmond, CA) is the routine method in our laboratory. The samples are separated by electrophoresis until the tracking dye (bromophenol blue) reaches the bottom. The separated proteins may be transferred to 0.1-μm nitrocellulose or polyvinylidene fluoride (PVDF) membranes (50 V, 2 hr, 4°) and analyzed by heme staining or immunedecoration. For effective transfer of plastocyanin and cyt c_6, it is important to omit sodium dodecyl sulfate (SDS) from the standard Tris-glycine transfer buffer and to include methanol (to 20%). Several different anti-plastocyanin antisera preparations from our laboratory have shown weak cross-reactivity with cytochrome c_6 (despite high purity of the immunizing antigen), but not vice versa. To avoid visualization of the cross-reactive signal, a lower sensitivity assay (nitrocellulose membrane, HRP-conjugated secondary antibody) may be used. When greater sensitivity is required, PVDF membranes and AP-conjugated secondary are preferred.

Typically, we load soluble extract equivalent to 20 μg of chlorophyll per lane, and obtain a suitable signal on PVDF membranes with an alkaline-phosphatase conjugated secondary antibody within 1–2 min of exposure to the developing solution, or with a horseradish peroxidase conjugated secondary antibody within 5–10 min. The use of chemiluminescent reagents in place of chromogenic substrates will increase the sensitivity. For instance, after treatment of the membrane with the luminol reagents for the peroxi-

[30] G. Sandmann, H. Reck, E. Kessler, and P. Boger, *Arch. Microbiol.* **134,** 23 (1983).
[31] Y. Kong and J. Whitmarsh, personal communication (1997).
[32] U. K. Laemmli, *Nature (Lond.) New Biol.* **227,** 680 (1970).

dase assay, a 60-sec exposure to film is sufficient to detect signals from extracts equivalent to 0.2 μg of chlorophyll.

Visualization of Cytochrome c_6 on the Basis of Its Heme-Dependent Peroxidase Activity and Coomassie Strain Analysis of Plastocyanin and Cytochrome c_6

A sensitive and quantitative method for estimating holocytochrome c_6 exploits the peroxidase activity of the heme cofactor. In this case, the samples (from cells equivalent to 20 μg of chlorophyll) are separated by electrophoresis in a nondenaturing gel (see below). Alternatively, if denaturing gels are used, 2-mercaptoethanol should not be added and the samples should not be heated. Heme staining can either be performed directly on the gel after electrophoresis with TMBZ (3,3′,5,5′-tetramethylbenzidine) as the substrate,[33] and this traditional method is the fastest; or for a more sensitive assay that is also amenable to quantitation, the heme stain can be performed after transfer to a PVDF membrane with a chemiluminescent substrate.[34,35] In either case, plastocyanin content of the sample can also be assessed after heme staining by either staining the gel with Coomassie Blue or by immunedecoration of the PVDF membrane (Fig. 2).

For the TMBZ heme stain, the gel is soaked (in the dark at room temperature) for 1–3 hr immediately following electrophoresis in a solution containing 0.045% w/v TMBZ in 0.175 *M* sodium acetate, pH 5.0, 30% methanol. Hydrogen peroxide is added to 30 m*M* to initiate staining; blue-green bands corresponding to heme proteins appear in 5–10 min. The gel can be photographed (Polaroid Type 667, shutter speed 1/125, *f*-stop 11–16) under tungsten lamp illumination for a permanent record. In samples from copper-deficient cells, the signal corresponding to cyt c_6 is the strongest; other cytochromes give weaker reactions. Following photography, the gel can be stained with Coomassie Blue as usual. The detection limit corresponds to 1% of wild-type levels (extract equivalent to 0.2 μg of chlorophyll).

For the chemiluminescent heme stain, the proteins are transferred to PVDF as described earlier, the membrane is rinsed in TBS and wetted rapidly in chemiluminescent reagent (described below) (~0.1 ml/cm^2). The same preparation of reagent can be used immediately for several separate membranes. The reagent is distributed by vigorous agitation for ~30 sec. The excess liquid is blotted off the membrane with laboratory wipes, and the membrane is wrapped in plastic wrap and exposed to film (5 min to 1

[33] P. E. Thomas, D. Ryan, and W. Levin, *Anal. Biochem.* **75,** 168 (1976).
[34] D. W. Dorward, *Anal. Biochem.* **209,** 219 (1993).
[35] C. Vargas, A. G. McEwan, and J. A. Downie, *Anal. Biochem.* **209,** 323 (1993).

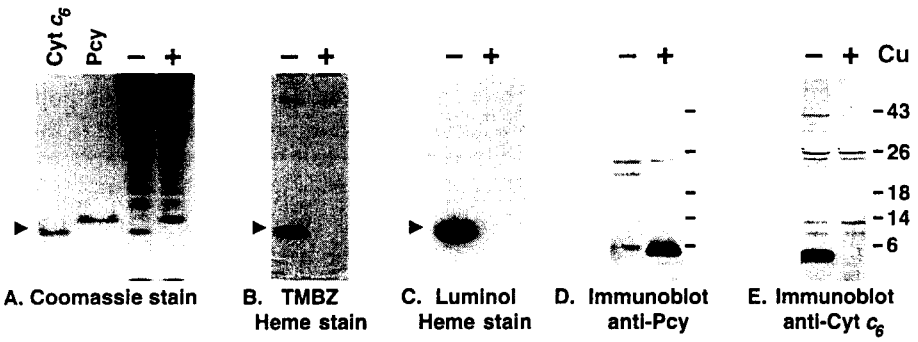

FIG. 2. Visualization of plastocyanin and cyt c_6 in extracts of soluble proteins from *C. reinhardtii*. Extracts equivalent to 20 (A, B, D, and E) or 5 (C) μg of chlorophyll were prepared from copper-supplemented (+) or copper-deficient (−) cultures and separated by electrophoresis under nondenaturing conditions (A–C) or denaturing conditions (D and E). (A) Purified plastocyanin (Pcy) and cytochrome (Cyt) c_6 were used as markers in the left-hand lanes. (B) The gel was stained with TMBZ as described in the text. Note the presence of other heme proteins in both samples. These can serve as a loading control. (C) The proteins were transferred to PVDF membranes and the membrane was treated with chemiluminescence reagents for 30 sec as described in the text, and immediately exposed to NEN/Dupont-Reflection film for 5 min. (D and E) The proteins were transferred to PVDF membranes. The membranes were decorated with antibodies raised against plastocyanin (1 : 500 dilution) or cyt c_6 (1 : 500 dilution) as indicated. An alkaline phosphatase-conjugated secondary antibody and chromogenic substrates were used for visualization. Plastocyanin (molecular weight 10,300) migrates with the 6000 molecular weight marker and cyt c_6 (9800) migrates a little faster.

hr). The peak light output occurs within the first 5 min and decays thereafter. To quantitate cyt c_6, a dilution series of purified cyt c_6 can be analyzed in parallel under exactly the same conditions. With a 5-min exposure, cyt c_6 can be detected in copper-deficient, wild-type extracts corresponding to 0.05 μg of chlorophyll. In principle, it should be possible to increase the sensitivity by one or two orders of magnitude by increasing exposure time and by increasing the amount of sample analyzed. Several different exposures of the membrane may be necessary to give a signal in the linear range of the film. Several companies market chemiluminescent reagents, including Pierce Chemical Company, Rockford, IL (Supersignal CL-HRP substrate) and Dupont–NEN, Boston, MA (Renaissance Western blot chemiluminescent reagent). The reagent can also be made in the laboratory with luminol (3-aminophthalhydrazide) and *p*-coumaric acid.[36] The staining procedure cannot be applied twice to the same membrane.

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Coomassie Stain Analysis of Plastocyanin and Cytochrome c_6 in Native Polyacrylamide Gels

When extracts of soluble protein are separated in nondenaturing polyacrylamide gels[37] made with 15% acrylamide monomer, cytochrome c_6 and plastocyanin can be resolved from each other and from most of the other proteins in the extract (owing to their acidic p*I*'s and small size). (Fig. 2A) The samples are separated by electrophoresis (90 V running) on a Bio-Rad MiniProtein II system until 30 min *after* the tracking dye has migrated off the bottom. The gel can be stained with Coomassie Blue or used for transfer.

Reporter Gene Assay

A reporter gene that is controlled by the promoter of the *Cyc6* gene can be introduced into *C. reinhardtii* cells to provide an alternate method for assay of copper content in the medium, or can be used for studies of copper-responsive gene expression. The gene encoding arylsulfatase is the reporter gene used in our laboratory. It was described by Davies *et al.*[38] The assays performed in our laboratory reproduce exactly the methods described in that work.[38,39] Arylsulfatase is not normally expressed in cells grown in standard laboratory media (TAP or minimal) and is therefore very useful for the study of gene expression in *C. reinhardtii*. It has been exploited also for the study of development in *Volvox carterii*.[40]

Two assays are described. The plate assay is useful for screening large numbers of *C. reinhardtii* colonies for transgenic ones that express the reporter gene construct of interest[3,38] or for isolating regulatory mutants.[41] The liquid assay is employed when quantitation is necessary. Both assays are simplified by the fact that the gene product (arylsulfatase) is located in the periplasm and is secreted into the medium in cell-wall deficient strains.[39] Both assays can be performed with both walled and wall-less strains.[39,42–44]

Since the reporter gene integrates into the *C. reinhardtii* genome by nonhomologous recombination, the expression of the reporter gene can vary between independent transgenic strains. Therefore, for studies of cop-

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[40] A. Hallmann and M. Sumper, *Proc. Natl. Acad. Sci. U.S.A.* **91,** 11562 (1994).
[41] J. P. Davies, F. Yildez, and A. Grossman, *Plant Cell* **6,** 53 (1994).
[42] T. Lien and O. Schreiner, *Biochim. Biophys. Acta* **384,** 168 (1975).
[43] F. H. Yildez, J. P. Davies, and A. R. Grossman, *Plant Physiol.* **104,** 981 (1994).
[44] J. P. Davies, personal communication (1997).

per-responsive gene expression, it is more important to measure copper-responsive arylsulfatase activity as the difference between the activity in copper-supplemented versus copper-deficient cells of a single transgenic strain than to make comparisons between strains. It is essential that several independently generated transgenic strains be analyzed to ensure that the result is independent of the site of integration.

Plate Assay. Colonies of interest are transferred to a gridded plate. A grid of about 64 squares on a 100-mm plate is optimal. When the colonies are about 1–2 mm in diameter (4–6 days if grown at 22° under a light intensity of 10–40 μmol/m^2/s), they are transferred, in duplicate, to +Cu and −Cu plates. The colonies are transferred two more times following growth for a total of three serial transfers. Before testing the colonies resulting from the third transfer, a small amount of cells from each colony should be transferred to a fresh plate to create a master because it may not be possible to recover viable cells from the test plate. The test plates are sprayed with a solution of 10 m*M* 5-bromo-4-chloro-3-indolyl sulfate (X-SO_4) in 0.1 *M* Tris-Cl, pH 7.5, and left at room temperature for 16–24 hr. Colonies that express arylsulfatase activity will display blue halos around and beneath the colony. It is advisable to score for expression on the basis of comparison between the −Cu versus +Cu staining to ensure that the assay measures *copper-responsive* expression. Colonies of interest can be recovered from the master and tested by the liquid assay (see below) for a more quantitative assessment of reporter gene activity. The expression of the same construct in independent isolates can be quite varied: some colonies display differential expression after a single transfer to +Cu versus −Cu plates, while other isolates may require up to three transfers through copper-deficient versus copper-supplemented medium to maximize the difference in expression.

Quantitative Assay on Liquid Cultures. Colonies to be tested are adapted to copper-deficient and copper-supplemented growth conditions by serial transfer (three times) in liquid medium (as described previously). Arylsulfatase can be assayed as described by deHostos *et al.*[39]

Direct Measurement of Copper in Cell, Media, and Solutions

Atomic Absorption Spectroscopy

The concentration or amount of copper in samples of cells or solutions can be measured directly by atomic absorption (AA) spectroscopy using either a flame or graphite furnace atomizer. The choice of atomizer depends on the sensitivity required and the amount of sample available. Both procedures can be applied to samples of media (acidified by the addition of nitric

acid to 0.1 *N*) or cells/tissues. We have not tested methods for disrupting *C. reinhardtii* cells for AA spectroscopy but methods have been developed for dissolution of other walled microorganisms including bacteria[45] (where dried cells are resuspended in 10% nitric acid + 10% perchloric acid and heated to 100° for 30 min) and yeast[46] (where the paste is refluxed in 0.1% (w/v) nitric acid at 100°C overnight and the final supernatant analyzed), and these methods should be applicable to photosynthetic microorganisms as well. It is important that digestion be complete and metal contamination avoided for reproducible results. A spectrometer equipped with a flame atomizer has a detection limit for copper of 1 ppb, while for one with a graphite furnace the limit is 0.02 ppb. The graphite furnace offers the additional advantage of requiring as little as 20 μl of liquid sample. To avoid unacceptable background noise in applications that require the higher sensitivity of the graphite furnace, all glassware and plasticware used in the preparation or handling of samples must be pretreated by soaking for 2 days in 2.4 *N* hydrochloric acid followed by another 2 days of soaking in 3.2 *N* nitric acid. The soaked labware must be rinsed thoroughly in MilliQ-purified water and covered to prevent airborne particles from falling within. The standard curve is prepared by diluting a commercially available copper standard in 0.1 *N* nitric acid. For the graphite furnace, the standard should be prepared just before use. With either method, the working concentration range is only two orders of magnitude.

Inductively Coupled Plasma Mass Spectroscopy

Inductively coupled plasma mass spectroscopy (ICP-MS) is the detection method of choice when high precision and accuracy (±5%) are required. ICP-MS analysis can detect as little as 0.07 ppb in a 0.1% nitric acid solution. Unlike AA in which the concentration of only a single element can be determined in a sample, multiple elements can be measured by ICP-MS. This may be advantageous for studies involving multiple metal measurements or when the abundance of another element is used as an internal control for the comparison of copper concentrations in multiple samples. The concentration range for ICP-MS is also wider (three to four orders of magnitude, compared to two for AA). While many institutions have AA instruments on site, the availability of ICP-MS instruments is rare. The University of Wisconsin Soil and Plant Analysis Laboratory, Soil Science Department 5711 Mineral Point Road, Madison, WI 53705, (608)262-4364, has performed our analyses (for a reasonable fee).

[45] A. Odermatt, R. Krapf, and M. Solioz, *Biochem. Biophys. Res. Commun.* **202,** 44 (1994).
[46] J. Goto and E. Gralla, personal communication (1997).

Using the *Cyc6* Promoter to Control Gene Expression

Copper-responsive sequences are located upstream of the *Cyc6* transcription start site and can be used to direct copper-responsive gene expression in transgenic *C. reinhardtii*.[3] A fragment corresponding to *Cyc6* sequences from −852 to −7 can be cloned upstream of the gene of interest. If a promoterless fragment of the gene of interest is used, the resulting construct will show minimal expression in +Cu medium. For convenience, we have also mutated the start site of translation in order to generate a restriction site at that position (*Nde*I). This makes it convenient to construct ATG fusions. In this case, the chimeric construct would contain *Cyc6* sequences from −852 to +80. Similar levels of expression result from either construct. Smaller promoter fragments (from −127 to −110 or from −110 to −55) can also be used but the level of expression appears to be more sensitive to context. Further, it may be necessary to use the small fragments in multiple copies (at least two) in order to obtain reliably high levels of expression.

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