Heme, a Plastid-Derived Regulator of Nuclear Gene Expression in Chlamydomonas

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To gain insight into the chloroplast-to-nucleus signaling role of tetrapyrroles, Chlamydomonas reinhardtii mutants in the Mg-chelatase that catalyzes the insertion of magnesium into protoporphyrin IX were isolated and characterized. The four mutants lack chlorophyll and show reduced levels of Mg-tetrapyrroles but increased levels of soluble heme. In the mutants, light induction of HSP70A was preserved, although Mg-protoporphyrin IX has been implicated in this induction. In wild-type cells, a shift from dark to light resulted in a transient reduction in heme levels, while the levels of Mg-protoporphyrin IX, its methyl ester, and protoporphyrin IX increased. Hemin feeding to cultures in the dark activated HSP70A. This induction was mediated by the same plastid response element (PRE) in the HSP70A promoter that has been shown to mediate induction by Mg-protoporphyrin IX and light. Other nuclear genes that harbor a PRE in their promoters also were inducible by hemin feeding. Extended incubation with hemin abrogated the competence to induce HSP70A by light or Mg-protoporphyrin IX, indicating that these signals converge on the same pathway. We propose that Mg-protoporphyrin IX and heme may serve as plastid signals that regulate the expression of nuclear genes.

INTRODUCTION

During evolution of the chloroplast from a cyanobacterial progenitor, most of the genes from the endosymbionts were transferred to the nuclear genomes of their hosts (Martin et al., 2002; Leister, 2003). In the case of Chlamydomonas reinhardtii, the chloroplast retained 72 genes (Maul et al., 2002). However, chloroplasts are estimated to harbor ~3000 proteins (Abdallah et al., 2000), the vast majority being encoded by the nucleus, translated in the cytosol, and subsequently transported into chloroplasts. Some of these proteins regulate essential steps of gene expression within the chloroplast (Goldschmidt-Clermont, 1998; Leon et al., 1998). In the course of evolution, the plastids acquired new properties, such as the ability to import proteins and to generate retrograde signals that in turn regulate the expression of nuclear genes. Research in recent years has provided evidence for multiple retrograde signaling pathways by which the chloroplast regulates a subset of nuclear genes, most of which encode chloroplast-localized proteins involved in photosynthesis. Among the pathways studied in some detail, one is dependent on products of plastid protein synthesis, for another the signal is singlet oxygen, a third employs chloroplast-generated hydrogen peroxide, a fourth is controlled by the redox state of the photosynthetic electron transport chain, and a fifth involves anabolic intermediates and possibly proteins of the tetrapyrrole biosynthesis pathway (reviewed in Rodermel, 2001; Gray et al., 2003; Pfannschmidt, 2003; Beck, 2005; Nott et al., 2006).

One retrograde signaling pathway for which many data have accumulated involves components of tetrapyrrole biosynthesis (reviewed in Strand, 2004; Beck, 2005; Beck and Grimm, 2006; Nott et al., 2006). In all plants, the steps of tetrapyrrole biosynthesis up to protoporphyrinogen IX (Protogen) occur only within the chloroplast but are catalyzed by nuclear-encoded enzymes (Beale, 1999; Papenbrock and Grimm, 2001). In higher plants, a fraction of Protogen leaves the chloroplast by unknown routes and enters the mitochondria where it serves as precursor of heme. Protogen in the chloroplast serves as precursor of both heme and chlorophyll (Papenbrock and Grimm, 2001). In Chlamydomonas, in contrast with higher plants, the enzymes for the conversion of Protogen into protoporphyrin IX (Proto) and the subsequent insertion of Fe2+ into Proto by ferrochelatase have recently been suggested to be exclusively located within the plastids (van Lis et al., 2005). In this alga, only single genes exist for Protogen oxidase and ferrochelatase whose products have been shown by immunolocalization to localize to chloroplasts (van Lis et al., 2005). Thus, in Chlamydomonas, all heme requirements of the cell appear to be met by the chloroplast, implying a constant flux of heme to all cellular compartments.

Insertion of magnesium into Proto forms Mg-protoporphyrin IX (MgProto) and is catalyzed by Mg-chelatase (CHL), a membrane interacting enzyme composed of three different subunits: CHLD, CHLH, and CHLI (Willows et al., 1996). In norflurazon-treated Arabidopsis thaliana, an increased accumulation of MgProto has been shown to lead to the repression of a nuclear light harvesting chlorophyll a/b binding protein (Lhcb) gene (Strand et al., 2003). Also, in cress (Lepidium sativum) and barley (Hordeum vulgare), the accumulation of Mg-tetrapyrroles correlated with a reduced expression of nuclear photosynthetic genes (Oster et al., 1996; La Roca et al., 2001). Furthermore, a genetic screen based on the use of norflurazon has allowed the identification of a series of Arabidopsis genome uncoupled (gun) mutants that are modified
in chloroplast-to-nucleus signaling (Susek et al., 1993). Four of the mutants (gun2-5) were shown to have lesions in genes involved in tetrapyrrole synthesis and/or degradation. In gun5, which harbors a partially functional mutation of the CHLH gene, the steady state level of MgProto in norflurazon-treated plants was reduced relative to the wild type, correlating with a reduced repression of Lhcb (Mochizuki et al., 2001; Strand et al., 2003). The incubation of leaf protoplasts with MgProto, but not with porphobilinogen, Proto, or heme, repressed Lhcb expression, suggesting a role of MgProto in gene repression. Transgenic tobacco (Nicotiana tabacum) plants that exhibit either over- or underexpression of CHLM, the gene encoding MgProto methyl transferase, contained either decreased or increased pool levels of MgProto, and this correlated with either elevated or reduced expression of Lhcb and HemA (glutamyl tRNA reductase [GluTR]), GSA (glutamate-1-semialdehyde aminotransferase), and CHLH (the H subunit of Mg-chelatase) (Alawady and Grimm, 2005). Also, confirming previous findings, a recently described Arabidopsis mutant that accumulates MgProto due to a mutation in CHLM showed repression of Lhcb also in the absence of norflurazon (Pontier et al., 2007).

The regulation of nuclear genes by chloroplast-derived signals has been examined by studying the induction of the nuclear gene for the chaperone HSP70 in the green alga Chlamydomonas. Feeding of MgProto or its methylester (MgProtoMe) to Chlamydomonas induces HSP70A (Kropat et al., 1997). Recently, a cis-acting plastid response element (PRE) with qualities of an enhancer was identified in the HSP70A promoter. PRE mediates MgProto induction of HSP70A; other genes that harbor PRE in their promoters were likewise induced by MgProto feeding (Vasileuskaya et al., 2004; von Gromoff et al., 2006). In Chlamydomonas, MgProto appears to mediate the light induction of HSP70A seen after a shift of cultures from dark to light. Thus, a CHLH mutant, which does not produce MgProto due to a mutation in the gene for the H-subunit of Mg-chelatase prevented the light induction of HSP70A, as did conditions abrogating the accumulation of MgProto in wild-type strains, such as the addition of cycloheximide or cells that are partially differentiated toward gametes (pregametes). However, in all cases, feeding of MgProto to cultures growing in the dark resulted in HSP70A induction, supporting a role of MgProto as a messenger in the signaling pathway by which light induces the HSP70A chaperone gene (Kropat et al., 1997, 2000). For this induction, the Mg-tetrapyrroles may be made available to the cytosol and the nucleus in a light-dependent manner (Kropat et al., 2000; Beck, 2005). MgProto in the cytosol after Norflurazon treatment has recently been observed in Arabidopsis using confocal laser scanning spectroscopy (Ankele et al., 2007). The observed light induction of HSP70A via tetrapyrroles is supported by an analysis of mutations in PRE: mutant PREs abolished induction of a fused reporter gene by light and MgProto (von Gromoff et al., 2006). However, a unified view on the regulatory role of tetrapyrrole biosynthesis is not yet in sight. An alternative hypothesis has been proposed that variations in Mg-porphyrin levels are sensed at the sites of Mg-porphyrin synthesis by an enzyme complex and subsequently transmitted to the cytosol/nucleus (Alawady and Grimm, 2005; Beck and Grimm, 2006).

A new set of mutants affecting genes of CHL subunits and thus causing diminished formation of MgProto allowed us to take a new look at the role of tetrapyrroles in chloroplast-to-nucleus communication. These mutants accumulate heme, which could be shown to control the same set of genes previously identified as inducible by MgProto. The signaling pathways of both tetrapyrroles converge at the same cis-acting element.

### Identification of Mutants Defective in CHL Subunits D and H

To examine the role of MgProto in chloroplast-to-nucleus communication, we first isolated mutants in the subunits of CHL. To isolate mutants, we screened UV-mutagenized lines for chlorophyll deficiency. Four mutants, which were generated by UV mutagenesis, lacked chlorophyll and showed the formation of yellow-brown colonies. Based on the initial determination of Proto levels, these mutants were selected as potentially defective in CHL activity. CHL is a heterotrimeric complex composed of subunits named D, H, and I (reviewed in Walker and Willows, 1997). Analysis of the Chlamydomonas genome sequence revealed a single gene for subunit D, one gene and a presumed pseudogene for subunit H, and two genes for subunit I (Lohr et al., 2005). To identify the mutant loci, we first identified BAC clones containing the functional genes for the various CHL subunits using gene-specific probes. DNA of these BAC clones was employed in complementation assays: the BAC DNA was used to transform each mutant line; clones that could complement a mutant produced colonies capable of autotrophic growth. Two of the four mutants were complemented by BAC clones containing the CHLD gene (mutants named chlD-1 and chlD-2), and two mutants were complemented by BAC clones with CHLH (mutants named chlH-1 and chlH-2) (Table 1). These data from complementation tests with BAC clones were subsequently

### Table 1. Complementation of Chlorophyll-Deficient Mutants with BAC Clones That Harbor the CHL Genes Indicated

<table>
<thead>
<tr>
<th>Mutants Tested</th>
<th>CHLH</th>
<th>CHLI1</th>
<th>CHLI2</th>
<th>CHLD</th>
<th>No DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>CF194 chlH-1</td>
<td>16</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>CF215 chlD-1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>CF225 chlH-2</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>134</td>
<td>2</td>
</tr>
<tr>
<td>CF227 chlH-1</td>
<td>14</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Transformations were performed as described in Methods.*
confirmed by transformation with the subcloned genes (data not shown).

Characterization of Defects in Mutants chlD-1 and chlD-2

Chlamydomonas mutants defective in CHLH have previously been characterized (Chekounova et al., 2001). We therefore focused our mutant characterization on chlD-1 and chlD-2. The structure of the Chlamydomonas CHLD gene, as deduced from combined genomic and EST sequence data (http://genome.jgi-psf.org/Chlre3/Chlre3.home.html), is presented in Figure 1A. The mutations in the gene were identified by sequencing of PCR fragments generated with genomic DNA of the mutants. In the chlD-2 mutant, a transversion mutation was discovered in exon 12 (a C-to-A change), which resulted in the generation of a stop codon (Figure 1A). Analysis of mutant chlD-1 revealed a G-to-T transversion at a highly conserved position at the 5’ intron side of the border between exon 11 and intron 11 (Figure 1A). To elucidate the effect of this mutation, the mRNA in this mutant was analyzed by RT-PCR employing primers homologous to exon 10 and exon 12. RT-PCR of mRNA from chlD-1 yielded two products: one slightly smaller than the wild-type product (610 versus 630 bp) and the other distinctly larger (798 bp).

The sequence of the short product revealed that removal of intron 11 had taken place. But, in addition, 20 nucleotides from the 3’ end of exon 11 were missing (Figure 1B). The splicing machinery apparently recognized an alternative 5’ splice site in the sequence GGT//GTA located within exon 11, 20 nucleotides upstream from the wild-type splice site. Consensus sequences for 5’ and 3’ splice sites in Chlamydomonas are (C/A)/(A/C)GA//GT(G/A) (Silflow, 1998). At the exon side, specificity appears to be relaxed since all nucleotides have been observed at these three positions (Silflow, 1998). Splicing at this alternative position results in a shift of the reading frame that ends, after codons for 40 amino acids, with a stop codon. In the case of the longer RT-PCR product, intron 11 was not removed. In this case, a stop codon occurs after 12 intron-derived codons (Figure 1B).

Proto and Heme Accumulate in CHL Mutants

While chlorophyll in all four mutants was very low or not detectable, the concentration of noncovalently bound heme in the mutants was elevated twofold to fivefold compared with the wild type, and the substrate of CHL, Proto, accumulated to levels more than ninefold higher than in the wild type (Table 2). The levels of MgProto were distinctly lower in all mutants. Mg-protoporphyrin IX monomethyl-ester (MgProtoMe) levels also were reduced (Table 2). The elevated heme levels suggest that the impairment of chlorophyll synthesis in CHL-defective mutants, resulting in an accumulation of Proto, enhances heme synthesis.

Regulatory Properties of the CHL Mutants

Since subunits of CHL have been suggested to participate in the regulation of nuclear genes (Papenbrock et al., 2000a, 2000b; Mochizuki et al., 2001), we first tested whether the light regulation of all four functional CHL genes was affected in the chlD and chlH mutants. In the parental strain, all four genes exhibited a light-induced accumulation of their transcripts (Figure 2). This induction was dependent on cytoplasmic protein synthesis since presence of the inhibitor cycloheximide prevented this light-induced mRNA accumulation (see Supplemental Figure 1 online). By contrast, inhibition of plastid protein synthesis by chloramphenicol had no effect (see Supplemental Figure 1 online). The chlD and chlH mutants showed accumulation of their mRNAs that were similar to those of the wild type (Figure 2). Analysis of the expression patterns of the various CHL genes in the chlD and chlH mutants revealed no significant changes in light-induced mRNA accumulation (Figure 2). Although the kinetics of mRNA accumulation exhibited some strain-dependent variations, an increase in mRNA levels was observed for all mutants in independent experiments, indicating that light induction is not blocked by the accumulation of Proto or reduced levels of Mg-tetrapyrroles in the first 4 h of low-level irradiation.
We analyzed the expression of HSP70A in response to light and observed an induction in the wild-type strain and in the four mutants defective in CHL (Figure 3). A reduction of approximately twofold in induced HSP70A mRNA levels compared with the wild type was observed in the chlD mutants. Also, progeny of chlD mutants from crosses with the wild type showed this reduction, indicating that this reduction is apparently not due to second-site mutations in the original mutants. We included a previously characterized CHLH-defective mutant, brs-1 (though a clone of different origin than the one originally used by Kropat et al., 1997), and observed light induction of the heat shock gene. Using the four newly isolated mutants, these data document that a block in the synthesis of Mg-tetrapyrroles does not prevent the induction of HSP70A by light.

A Role for Heme as an Inducer of HSP70A

We previously postulated an essential role for MgProto and/or MgProtoMe in the light induction of HSP70A (Kropat et al., 1997, 2000; Beck, 2005). Such a model was supported by the discovery of sequence elements within the HSP70A promoter called PREs that mediate HSP70A induction in response to MgProto feeding (von Gromoff et al., 2006). Since in the CHL mutants heme was shown to accumulate (Table 2) and heme previously has been identified as a regulator of the nuclear gene HEMA (Vasileuskaya et al., 2005), we speculated that heme also plays a role in HSP70A expression. This was tested by feeding of hemin (which harbors a Fe$^{3+}$ atom, conferring higher stability in solution than the Fe$^{2+}$ contained in heme) to cultures in the dark. The data shown in Figure 4A reveal that hemin feeding in the dark induced HSP70A mRNA accumulation. The kinetics of mRNA accumulation upon hemin feeding were delayed compared with MgProto feeding, but the degree of induction with 9 μM hemin was similar to that seen after MgProto feeding (9 μM) or a shift from dark to light (DLS). We conclude that heme, just like MgProto, may activate HSP70A, while Proto and protochlorophyllide, as shown previously (Kropat et al., 1997), have no inducing effect.

To verify and extend these studies, we analyzed the induction of HSP70A by various concentrations of hemin. As shown in Figure 4B, HSP70A mRNA levels rose with an increase in the hemin concentration applied to cell cultures in the dark. Maximal induction was observed upon feeding of 16 μM hemin. In subsequent experiments, a final concentration of 9 μM hemin was employed.

Changes in Tetrapyrrole Levels after a Shift from Dark to Light

Exposure of dark-adapted wild-type cells to light resulted in an immediate rise (within <60 min) in Proto, MgProto, and MgProtoMe pool levels (Figure 5). While the pool levels of Proto rose approximately fourfold within 60 min, the increase in MgProto and MgProtoMe pools exhibited a rise with fluctuations. A rapid accumulation 10 min after start of illumination was followed by a...
drop in pool levels in the 20 min samples, followed by a second increase. Such fluctuations, also observed by others (Pöpppel et al., 1998), may reflect a fine-tuning process among the enzymatic activities of CHL and downstream enzymes affecting the regulatory equilibrium after a sudden shift of cultures from dark to light. Upon extended exposure of cultures to light (i.e., >60 min), the increase of tetrapyrroles stops and reduced pool levels were observed for Proto and MgProto (Figure 5). A similar rapid and transient, light-induced increase in pool sizes of MgProto and MgProtoMe has also been observed in higher plants grown in light/dark cycles (Pöpppel et al., 1998; Papenbrock et al., 1999).

By contrast, levels of total noncovalently bound heme, determined from the same cell cultures, exhibited an approximately twofold transient reduction in pool levels after a delay of 10 min (Figure 5). This decrease may possibly be accounted for by an enhanced demand of heme for assembly into proteins. Alternatively, the decrease in heme levels may reflect a channeling of Proto into the Mg-branch for chlorophyll biosynthesis. A preferential channeling may be explained by an activation of CHL under these conditions. Indeed, an increase in CHL activity upon shift of cells from dark to light was observed (Figure 6). This increase showed some fluctuations (Figure 6). An elevated CHL activity seen 5 min after start of illumination was followed by a decrease seen at 10 min, and this again was followed by an increase. These initial fluctuations in CHL activity are mirrored by changes in MgProto and MgProtoMe pool levels right after start of illumination (Figure 5). In the latter, an initial peak at 10 min was followed by lowered pool levels at 20 min. However, whether there is a strict interdependence between the fluctuations at the level of enzyme activity and pool levels remains to be investigated. The rapid change in CHL activity precludes an enhanced de novo synthesis of the enzyme as an explanation for the increase in activity. Rather, the data support a light activation of the enzyme at the posttranslational level, possibly via thioredoxins, which have been shown to interact with CHL. A stimulation of the enzyme by reduction was observed in isolated chloroplasts (Balmer et al., 2003; Ikegami et al., 2007).

We also analyzed the tetrapyrrole pools of the mutants upon shift from dark to light. No significant changes were observed (i.e., heme and Proto pools remained high; Table 2), while the Mg-tetrapyrrole pools remained at a low level (see Supplemental Figure 2 online).

Is the Same cis Sequence Element Involved in the Induction of HSP70A by Heme and MgProto?

The data presented above suggest that heme and the magnesium containing porphyrins act through a similar or even identical signaling pathway for the induction of HSP70A. To study this issue in more detail, we tested whether heme induction is possibly mediated via the same regulatory regions within the HSP70A promoter. For these analyses, we used constructs in which the HSP70A promoter (P$_A$), fused to the RBCS2 promoter (P$_R$), was located upstream of a reporter gene (HSP70B-TAG) (Figure 7A). In transformants with such constructs, induction of the reporter gene by light or MgProto has previously been shown to occur via activation of the P$_R$ promoter by enhancer elements within P$_A$ (von Gromoff et al., 2006). Thirty transformants with each of the constructs shown in Figure 7A were analyzed for expression of the reporter gene after shift from dark to light (DLS) or after feeding (in the dark) of MgProto or hemin (9 μM final concentration each). In agreement with previous data, MgProto activation

Figure 3. Light Induction of HSP70A in the Wild Type and Mutants Defective in the D and H Subunits of CHL.

Cultures grown in the dark at time 0 were shifted into dim light (10 μE m$^{-2}$ s$^{-1}$), and samples for RNA isolation were taken at the time points indicated (hours). RNA gel blot hybridizations were performed using probes specific for HSP70A and CBLP, the latter serving as a loading control. The quantitative evaluation of the RNA gel blots in the case of the wild type and mutants chlH-1, chlH-2, brs-1, comprised four independent experiments. In the case of chlD-1 and chlD-2, the data from three independent experiments with the original mutant strains and of four mutant progenies from crosses with the wild type were combined. The average levels of mRNA accumulation relative to the dark control (corrected for differences in loading) ± SE are given.
of $P_n$ was seen in transformants even with the shortest $HSP70A$ promoter fragment (Figure 7A). This fragment, named regulatory region I, has been shown to harbor an enhancer element that may confer inducibility by MgProto to heterologous promoters (von Gromoff et al., 2006). Clearly, heme activation also is mediated via regulatory region I (Figure 7A).

We next tested whether the element that mediates induction by MgProto within regulatory region I also is involved in the activation by heme. Within regulatory region I, the PRE sequence motif GCGACNAN$_{15}$TA was shown to confer MgProto responsiveness (von Gromoff et al., 2006). Two mutant constructs that lacked sequences either from the 5’ or the 3’ ends of regulatory region I (mutations 1 and 2 in Figure 7B), and thus were defective in PRE, were tested for transcriptional activation of the reporter gene. Transformants with these constructs did not show a response to either heme or MgProto. By contrast, transformants with the wild-type construct or a deletion construct lacking five internal base pairs but maintaining the PRE motif (mutation 3) were inducible by both tetrapyrroles (Figure 7B). From these results we conclude that heme induction appears to be mediated via PREs, suggesting also that it may employ the same or similar protein components as MgProto to activate $HSP70A$.

**Hemin Activates Other MgProto-Responsive Genes**

We have previously identified a group of nuclear genes that were inducible by MgProto feeding (Vasileuskaya et al., 2004; von Gromoff et al., 2006). All of these genes harbor in their promoter regions the cis-acting PRE motif (von Gromoff et al., 2006). The response of these genes to MgProto in wild-type strain CC-124 was confirmed (Figure 8). All five genes also responded to hemin feeding, although the kinetics and the degree of mRNA accumulation showed some variation compared with MgProto feeding, suggesting the involvement of additional factors in the expression of these genes. The effect of the cell wall on the kinetics of hemin induction was assessed using the cell wall–deficient mutant CW15. The response of the genes in this mutant to hemin appears to be more rapid for all genes (Figure 8), suggesting that the cell wall represents some barrier for hemin uptake. These results show that in *Chlamydomonas* a number of genes exist that responds to two different tetrapyrroles, heme, and MgProto. These data support the concept of an overlapping role for heme and MgProto that, as organelle-derived molecules, appear to play a regulatory role in transcription within the nucleus.

**Assay of Other Metalated Porphyrins**

To test whether only specific tetrapyrroles play a role in gene regulation, we tested the effect of three additional metalated porphyrins. In contrast with the Mg- and Fe-derivatives, neither Zn, Co, nor Mn-protoporphyrin IX, when fed to cultures in the dark, resulted in induction of $HSP70A$ or HEMA (Figure 9A). Also, Proto applied at higher concentrations had no effect on the expression of the genes tested (Figure 9B). We previously showed that Proto fed to cultures in the dark results in the accumulation of MgProto. This MgProto, produced by chloroplast-localized enzymes, apparently is not accessible to cytosol/nucleus in dark-grown cultures (Kropat et al., 2000). These results suggest a high degree of
specificity in the interaction of Mg- and Fe-porphyrins with down-
stream signaling components.

Extended Incubation with Hemin Results in Turning off the
Downstream Tetrapyrrole Signaling Pathway

The incubation of dark-grown cells with hemin resulted in a
transient \( HSP70A \) mRNA accumulation. Twelve hours after the
addition of hemin, \( HSP70A \) mRNA levels were about the same as
prior to treatment (Figure 10), although hemin was still present at
\( \sim 50\% \) of the initial concentration (data not shown). When such a
hemin-treated culture was exposed to light (DLS), at most a very
weak accumulation of \( HSP70A \) mRNA was observed (Figure 10).

For an explanation we considered the possibility that hemin still
present in the culture medium may have prevented a light-
induced accumulation of the Mg-tetrapyrroles (e.g., via feedback
inhibition of GluTR, the first enzyme specific for tetrapyrrole bio-
synthesis). Such an accumulation of Mg-tetrapyrroles has pre-
viously been shown to be essential for the \( HSP70A \) activation
that follows a shift from dark to light (Kropat et al., 2000). An
increase in Proto, MgProto, and MgProtoMe pool levels indeed
was not observed when cultures treated with hemin for 12 h in the
dark were exposed to light (see Supplemental Figure 3 online).
The defect in light induction in these cells appears to involve
additional factors. To test whether downstream signaling com-
ponents may have been affected by hemin treatment, the cul-
tures were fed with MgProto after 12 h with hemin. No induction

Figure 6. Changes in CHL Activity after a Shift of Cultures from Dark to
Light.

Activity of CHL was determined with cultures of wild-type strain 4A+
grown in TAP medium to a density of 3 to 5 \( \times 10^6 \) cells/mL. Exponentially
growing cultures after an incubation in dark for 16 to 18 h were
transferred into light (50 \( \mu \)E m\(^{-2}\) s\(^{-1}\)) at time 0. The cells were harvested
at the time points indicated and disrupted by sonication at 4°C. The
enzyme assays were performed as described in Methods. Each time
point represents the average of data from two independent experiments.

In each of these experiments, every time point represents a kinetic
analysis of MgProto formation from Proto (for details, see Methods).
was observed (Figure 10), suggesting that either the downstream signaling pathway has been turned off or, potentially, hemin treatment had activated nucleases that rapidly degrade HSP70A mRNA. To address this latter point, heat shock treatment was performed with the cells incubated with hemin for 12 h. This treatment elicited an initial HSP70A mRNA accumulation similar to that observed upon the application of heat shock to hemin-free cultures (von Gromoff et al., 1989). We conclude that hemin treatment apparently had not impaired the potential of cells to accumulate HSP70A mRNA (Figure 10).
DISCUSSION

Here, we report on experiments that allow a new look at the role of tetrapyrroles in chloroplast to nucleus signaling. They are based on four newly identified mutants with defects in CHL, the first enzyme specific for the chlorophyll biosynthetic branch. Two of the mutants were shown to be defective in the H subunit of the enzyme since they could be complemented by a wild-type CHLH gene (Table 1). Two other mutants are defective in the D subunit. These four mutants exhibited either no or only residual levels of chlorophyll (Table 2), indicating that the mutations blocked chlorophyll synthesis. All four mutants accumulated Proto (Table 2). In contrast with *Chlamydomonas*, an accumulation of Proto has not been observed in higher plants with deficient CHL activity (Papenbrock et al., 2000b). However, accumulation of Proto was seen in other *Chlamydomonas* mutants defective in CHLH (Wang et al., 1974; Kropat et al., 2000; Chekounova et al., 2001).

Analysis of the expression of nuclear genes upon shift of cultures from dark to light revealed that the four mutants did not exhibit significant differences in the light-induced expression of the CHL genes (Figure 2), HEMA, ALAD, or several LHC genes. Also, basal level expression of these genes appeared not to be affected (L. Meinecke and C.F. Beck, unpublished data).

The observation that *HSP70A* was light inducible in the mutants (Figure 3) at first glance was surprising, since previously a mutant defective in CHLH (brs-1) did not show light induction of *HSP70A* (Kropat et al., 1997). A strain with this mutation, although a different subclone, was included in our studies and here showed light induction of *HSP70A* (Figure 3). The lack of light induction in previous experiments may be explained by a second-site mutation in the brs-1 mutant clone originally tested. Indeed, we discovered second-site mutations that caused altered regulation of nuclear genes with high frequency in a screen of mutants defective in chlorophyll synthesis. Thus, five out of 13

Figure 8. Test of MgProto-Inducible Genes for Induction by Hemin.
Wild-type cells and cells of the cell wall–deficient mutant CW15 were incubated in the dark for 16 h and then treated with hemin (9 μM) or MgProto (9 μM). Samples for RNA isolation were taken at the times indicated. RNA gel blot hybridizations were performed as described in Methods using the gene-specific probes described either in Methods or by Vasileuskaya et al. (2004). CBLP (von Kampen et al., 1994) served as a loading control.

Figure 9. Test for Inducing Potential of Various Metalated Porphyrins and of Proto at Different Concentrations.
Cells of the wild type grown in TAP medium were incubated in the dark for 16 to 18 h. At time 0, the various porphyrins were added. Samples for RNA isolation were taken at the times indicated. RNA gel blot hybridizations were performed using the probes described in Methods. CBLP served as a loading control.

(A) The metalated porphyrins listed were added to a final concentration of 9 μM.
(B) The concentrations of Proto indicated were tested for inducing potential.
Cultures grown in the dark for 16 h were treated with 9 μM hemin. To another aliquot, MgProto (MP) was added to a final concentration of 9 μM, but residual hemin was present from the previous incubation) and incubation was continued for 2 h (third panel from top). To a third aliquot, hemin (H) was added (the newly added H corresponded to a concentration of 9 μM, but residual hemin was present from the previous incubation) and incubation was continued for 2 h in the dark (fourth panel from top). In another aliquot, the response of cells to heat shock after 12 h incubation with hemin was tested. For this purpose the culture was shifted from 23 to 40°C in the dark for up to 2 h (fifth panel from top). The disappearance of HSP70A mRNA and 18s,25s rRNA 2 h after heat shock in cultures treated with hemin has been observed in independent experiments, suggesting a destabilization of RNA after prolonged incubation at elevated temperature. A dark-to-light shift (DLS) was performed with a culture not treated with hemin to illustrate the responsiveness of HSP70A to light (sixth panel from top). In all cases, samples for RNA isolation were taken at the times indicated. RNA gel blot hybridizations were performed using specific probes for HSP70A mRNA and 18s rRNA, the latter serving as a loading control. In these experiments, CBLP could not be used since the mRNA of this gene disappeared upon extended hemin treatment (>6 h).

In view of the accumulated evidence for a crucial role of tetrapyrroles in mediating the light induction of HSP70A (Kropat et al., 1997, 2000; von Gromoff et al., 2006), we considered an involvement of other tetrapyrroles besides the Mg-tetrapyrroles in this induction. Heme was an attractive candidate since this tetrapyrrole activates HEMA, encoding GluTR, the first enzyme specific for tetrapyrrole biosynthesis (Vasileuskaya et al., 2004). Indeed, feeding experiments with hemin in the dark induced HSP70A mRNA accumulation (Figure 4). This suggested that heme may play a similar role to MgProto in signaling.

A crucial aspect of an understanding of tetrapyrrole gene regulation was the definition of a sequence motif within the HSP70A promoter that may activate the gene upon hemin feeding. The previously identified motif (named PRE) that mediates HSP70A induction by MgProto (von Gromoff et al., 2006) was shown also to confer heme-responsive activation on HSP70A since mutations in PRE that abolished induction by MgProto also abolished activation by hemin (Figure 7). These data provide solid evidence for a specific role of heme in the regulation of nuclear genes. Indeed, other genes that harbor a PRE were also induced by the feeding in the dark of MgProto or hemin (Figure 8).

In the two chlD mutants, a reproducible reduction in HSP70A mRNA accumulation (Figure 4) point to a complex role of individual CHL subunits in signaling, a view supported by the observation that Arabidopsis mutants with lesions in the CHL1 gene do not exhibit a gun phenotype upon norflurazon treatment (Mochizuki et al., 2001).

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Salient features of our previous model were two prerequisites for HSP70A light activation: first, a light-induced transient accumulation of Mg-tetrapyrroles and, second, a light-activated export of tetrapyrroles from the chloroplast (Kropat et al., 2000; Beck, 2005; Beck and Grimm, 2006). Here, in view of our new results, we want to modify and extend this model (Figure 11).

The reduction in heme levels that follows a shift of cultures from dark to light (Figure 5) provides new information on chlH mutants from the initial screen for chlorophyll-deficient mutants exhibited deregulation of HSP70A. In each case, crosses revealed a segregation of the phenotypes of chlorophyll deficiency and deregulated gene expression. For our studies, we chose two of the chlH mutants that exhibited normal regulation of HSP70A. In the two chlD mutants, a reproducible reduction in HSP70A mRNA accumulation by a factor of about two was observed upon dark-to-light shift compared with the wild type or other CHL mutants (Figure 3). In this case, second-site mutations being responsible for the reduced induction with high probability could be ruled out since all mutant progenies from crosses of the two strains with the wild type revealed a similar reduction (data not shown). These data point to a complex role of individual CHL subunits in signaling, a view supported by the observation that Arabidopsis mutants with lesions in the CHL1 gene do not exhibit a gun phenotype upon norflurazon treatment (Mochizuki et al., 2001).
intraplastidal regulatory events that result in the transient accumulation of Mg-tetrapyrroles upon dark-to-light shift. Three different regulatory events may account for these changes. (1) In *Chlamydomonas* cultures, a shift from dark to light not only results in a transient increase in Proto and MgProto/MgProtoMe pool levels but also in a decrease in noncovalently bound heme (Figure 5). The lowering of heme levels in the chloroplasts after shift to light can be expected to correlate with an at least partial release of GluTR from feedback inhibition by heme (Vothknecht et al., 1996), resulting in a higher flux through the tetrapyrrole biosynthetic pathway and thus the accumulation of Proto, MgProto, and MgProtoMe (pathway 1 in Figure 11). The decrease in heme levels in the presence of increasing Proto levels indicates a preferential channeling of Proto into chlorophyll biosynthesis, which may be explained by the observed light activation of CHL (Figure 6). (2) A negative regulator of tetrapyrrole biosynthesis that affects the Mg branch of tetrapyrrole biosynthesis is the Flu protein. Mutants of *Arabidopsis* that carry a lesion in the FLU gene are no longer able to downregulate δ-amino levulinic acid synthesis and thus accumulate protochlorophyllide when grown in the dark (Meskauskiene et al., 2001). The Flu protein has subsequently been shown to interact with GluTR (Meskauskiene and Apel, 2002). A shift from dark to light enables catalysis of light-dependent protochlorophyllide reduction by protochlorophyllide oxidoreductase, and this, in the case of higher plants (Meskauskiene et al., 2001) and *Chlamydomonas* (Pöppel, 1996), has been shown to result in a reduction in protochlorophyllide levels. In light, GluTR may be relieved from a negative control by the Flu protein, resulting in an enhanced synthesis of tetrapyrroles (pathway 2 in Figure 11). (3) There remains the possibility that early steps of tetrapyrrole biosynthesis are subject to direct light activation (e.g., via post-translational modification) (pathway 3 in Figure 11). Which of these routes or combination of routes results in the increased synthesis of tetrapyrroles observed upon illumination remains to be elucidated.

In light of our model on the involvement of tetrapyrroles in chloroplast retrograde signaling in *Chlamydomonas*, we can now address the differences between green algae and higher plants. The recent identification of the *Arabidopsis* GUN1 gene and the analysis of mutants defective in this gene resulted in a model in which a chloroplast-localized protein with DNA/RNA binding activity (GUN1) generates a signal that coordinates nuclear gene expression in response to various alterations within the plastids (i.e., the accumulation of MgProto, inhibition of plastid gene expression, and the redox state of the photosynthetic electron...
transfer chain) (Koussevitzky et al., 2007). Our data obtained with the green alga *Chlamydomonas* reveal a number of differences: (1) The sequence motifs that mediate the response to elevated MgProto levels in higher plants and *Chlamydomonas* are not related (Strand et al., 2003; von Gromoff et al., 2006; Koussevitzky et al., 2007). In Arabidopsis, the ABI4 transcription factor mediates the response to a signal from GUN1, in *Chlamydomonas*, the factor that binds PRE is still elusive. (2) Heme in *Chlamydomonas* has a regulatory function in gene regulation; in Arabidopsis, no evidence was observed for a role of heme similar to that of MgProto (i.e., in the repression of Lhcb) (Strand et al., 2003). (3) Within the *Chlamydomonas* HSP70A promoter, three different regions have been identified that mediate activation of the gene in response to tetrapyrroles, hydrogen peroxide, and singlet oxygen (Shao et al., 2007). This indicates that in *Chlamydomonas* different plastid signals appear not to converge in a common pathway. (4) A gene with homology to GUN1 (i.e., encoding a pentatricopeptide repeat protein with a mutS-related domain) was not detected in the *Chlamydomonas* genome sequence (http://genome.jgi-psf.org/Chlre3/Chlre3.home.html).

How can the light induction of HSP70A, previously postulated to be mediated by MgProto, be explained in view of the reduced MgProto levels in the four CHL mutants? Here, we propose that, besides MgProto, heme may also mediate light induction. However, this function of heme may be restricted to a special set of conditions (e.g., when Mg-tetrapyrrole levels are low and heme levels are elevated). The heme levels in the mutants are twofold to fivefold higher than in the wild type (Table 2), and they do not decrease upon shift of mutant cultures from dark to light (see Supplemental Figure 2 online). Since the mutants retain HSP70A activation in response to light, we postulate that heme in a light-dependent step is made available to a downstream signaling pathway in the cytosol/nucleus (see below) (Figure 11).

The presence of a signaling pathway for heme, including a defined promoter element by which heme may modulate gene expression, is viewed as a strong indicator for a regulatory role of heme in *Chlamydomonas*. We envision that, besides a role in light induction of PRE–harboring genes, heme may have regulatory functions under physiological conditions when heme levels are elevated but MgProto is not available or strongly reduced. Such conditions in plants exist in non-green tissue but also have been documented for tobacco grown under a 12-h-light/12-h-dark cycle where pool levels of various tetrapyrroles were shown to change in opposite directions. Toward the end of the light period, pools of Proto, MgProto, and MgProtoMe were shown to drop approximately fivefold, while heme levels increased ~1.5-fold (Papenbrock et al., 1999).

In *Chlamydomonas*, the chloroplast has been suggested to be the only site of heme synthesis (van Lis et al., 2005), and heme, also in the dark, will constantly be required to serve as prosthetic group of proteins in the cytosol and the mitochondria. Thus, a low level of heme must constantly exit from the chloroplast. Heme efflux from chloroplasts has indeed been observed (Thomas and Weinstein, 1990). We can envision that the *K_0* of the heme binding factor(s) involved in signaling in the cytosol is significantly higher than that of reactions involved in the insertion of heme as a prosthetic group. The signaling factors thus only may become activated when the heme concentration in the cytosol/nucleus is raised (e.g., after an active, light-driven export of heme from the chloroplast). This hypothesis may be tested once heme binding factors have been identified.

Information about the downstream signaling pathway was gained by experiments in which cultures were incubated with hemin for 12 h (Figure 10). This pathway was shown to share a genuine property of most signaling pathways (i.e., it is inactivated upon prolonged stimulation). In cells treated with hemin for 12 h, HSP70A light induction was essentially abolished (Figure 10). Since the addition of MgProto in the dark also did not elicit an induction of HSP70A, we may conclude that shared components of the downstream signaling pathway are blocked. The lack of HSP70A induction by either MgProto or hemin feeding suggests that either the receptor(s) for these tetrapyrroles or downstream components got inactivated. Indeed, the employment of the same *cis*-acting PRE element of the HSP70A promoter for the activation by both MgProto and heme (Figure 7) may point to a sharing of transcription factors by the two tetrapyrroles.

These studies provide evidence for a regulatory role of heme in gene expression in *Chlamydomonas*. Whether this tetrapyrrole also is active in gene regulation in higher plants remains to be elucidated. In yeast and animal systems, a role of heme in regulation of gene expression is well established (reviewed in Mense and Zhang, 2006). Thus, in mammals, heme has recently been demonstrated to be an amplifier of the inflammatory response via specific activation of toll-like receptor 4 (Figureiredo et al., 2007). The details of the signaling pathways in *Chlamydomonas* can now be addressed, and this may provide clues to a regulatory role of heme in higher plants.

### METHODS

#### Algal Strains and Culture Conditions

*Chlamydomonas reinhardtii* strain 325 (cw15, arg7-8, mt-1) was kindly provided by R. Matagne (University of Liège, Belgium). Wild-type strain CC-124 (mt-1) and the cell wall–deficient mutant CW15 were obtained from the Chlamydomonas Culture Collection at Duke University. Wild-type strain 4A× (mt-1) was provided by J.-D. Rochaix (University of Geneva). The mutants were generated by M. Kobayashi in the 4A× strain by UV mutagenesis applying between 30 and 60 mJ cm⁻². After mutagenesis, the cells were plated and incubated in the dark. Mutant clones were recognized by their altered pigmentation. If not otherwise noted, strains were grown photomixotrophically in Tris-acetate phosphate (TAP) medium (Harris, 1989) on a rotatory shaker at 23°C under continuous irradiation with white light (~60 µE m⁻² s⁻¹) provided by fluorescent tubes (Osram L36W/25). Mutant stocks were maintained on TAP agar medium in the dark. Light induction and heat shock were performed according to Kropat et al. (1995) and von Gromoff et al. (1989), respectively. For Arg-requiring strains, the medium was supplemented with Arg (final concentration 100 µg/ml).

#### Nuclear Transformation of *Chlamydomonas*

*Chlamydomonas* nuclear transformation was performed using the glass bead method (Kindle, 1990), modified as described previously (Kropat et al., 2000). For the transformation of 10⁶ cells, 100 ng of plasmid DNA or 1 µg of BAC DNA were used. Immediately after vortexing with glass beads, cells were spread onto freshly prepared TAP-agar (1%) plates. Plates were incubated at 23°C in the light (~60 µE m⁻² s⁻¹), and...
transformants were collected and analyzed after 1 week. For promoter activity tests, strain 325 was transformed as described by von Gromoff et al. (2006). The constructs (all containing the ARG7 gene) were linearized by restriction either upstream from HSP70A promoter sequences or downstream from the reporter gene.

RNA Gel Blot Analysis
Total RNA was isolated from 10 mL cultures grown to 2 to 4 × 10⁶ cells per mL. The procedures employed for RNA extraction, separation on agarose gels, blotting, and hybridization were as described previously (von Gromoff et al., 1989) except that the nylon membranes used were Hybrid-N (Amersham). The probes used for hybridization were as follows: HSP70A (von Gromoff et al., 1989), CHLH1 (Chekounova et al., 2001), CHLI (a Spel-Xhol cDNA fragment of ~1.5 kb from EST clone AV843220), CHLD (a Spel-Xhol fragment of ~1.8 kb from EST clone AV623288), CHLD (a 680-bp cDNA fragment), and HAM (a 560-bp cDNA fragment), kindly provided by S. Beale. The EST clones were obtained from the Japanese collection (Asamizu et al., 1999). Either CBLP, which encodes a G-like polypeptide (von Kampen et al., 1994), or a clone (Ba235) harboring the 18s and 25s RNA genes (Marco and Rochaix, 1980) was used as loading controls. Probes were labeled with 50 μCi [α-32P]dCTP (Amersham) according to the random priming protocol (Feinberg and Vogelstein, 1983). Hybridizations were performed as described previously (Wegener and Beck, 1991). After hybridization, the membranes were washed twice in 2× SSC for 5 min at room temperature, once in 2× SSC, 1% SDS for 30 min at 65°C, and once in 0.1× SSC, 1% SDS for 30 min at 65°C. RNA gel blots were screened by exposure to BAS-MP imaging plates (Fuji). They were evaluated by a phosphor imager (Bio-Rad Laboratories). For the quantitative determination of changes in mRNA concentrations, all clones that exhibited a response were evaluated using the Quantity One 4, 5, 1 program from Bio-Rad Laboratories.

RNA Dot Blot Analysis
Ten micrograms of total RNA from each sample was applied to Hybond-N nylon membranes using a dot blot apparatus. The membranes were dried by baking for 2 h at 80°C. After hybridization, washing of the membranes, and their quantitative evaluation, we used the same conditions/programs as for RNA gel blot analyses.

Isolation of Genes for CHL
BAC clones containing genes CHLH, CHLD, CHLI, and CHLD were identified by screening the membrane-immobilized DNA of a Chlamydomonas BAC library (LeFebvre and Silflow, 1999) obtained from Clemson University Genomics Institute (http://www.genome.clemson.edu/) using the same probes as described for RNA detection. Hybridization of the membranes was performed using the protocol provided by the Clemson University Genomics Institute. Positive BAC clones that contained the complete genes (17G11-CHLH1, 26C5-CHLD, 35P13-CHLI1, and 5J15-CHLD2) were obtained from Clemson University Genomics Institute. DNA was isolated using the protocol of the BAC clone providers (http://www.genome.clemson.edu/protocols.shtml).

Cloning and Sequencing of CHLD
Genomic sequence data (http://genome.jgi-psf.org/Chla3/Chla3. home.html) provided information on restriction sites used for cloning of CHLD. An 8.2-kb fragment containing the entire CHLD gene was obtained by digestion of DNA of BAC clone 26C5 with Nhel and Hpal. The resulting 8.2-kb fragment after blunt ending was ligated into the EcoRV site of the pBluescript SK+/- vector (Stratagene). The clone containing the correct insert was verified by partial sequencing and restriction analysis. DNA of this clone was tested for its ability to complement mutants defective in chlorophyll synthesis upon transformation. For the identification of the mutations in two mutants that could be complemented by the CHLD gene, DNA was extracted from mutant strains chlD-1 and chlD-2 using the CTAB method (von Gromoff et al., 1989). The sequence of the CHLD mutant alleles was determined after amplification of segments that comprised the entire CHLD coding region, using six primer pairs. The upstream (A) and downstream (B) primers were as follows: 1A, 5’-CACAACGAGGAG-ACTTCA-3’; 1B, 5’-ATCGAGCCCTTGAGTC-3’; 2A, 5’-TCTGAGGA-GAGACTCCA-3’; 2B, 5’-GGGTCGGACAGACTTCT-3’; 3A, 5’-AGGGCAGACGTGTTTTCCA-3’; 3B, 5’-CGATGTCACGTCCTCCA-3’; 4A, 5’-TCTATCGGCTGCTCGGA-3’; 4B, 5’-CTGTCTCGACGACGTCG-3’; 5A, 5’-TCTGTCGAGGAGTTTCTGA-3’; 5B, 5’-ATGCGCTGTACTCAGT-GTCTG-3’; 6A, 5’-CTCATTCACCGAGACGAC-3’; 6B, 5’-ACTTCAGTCA-CTCGCCAGA-3’.

The reactions were performed for 35 cycles of 1 min at 94°C, 1 min at 50 or 55°C, and 2.5 min at 72°C with Phi DNA polymerase (Fermentas), which possesses proofreading activity. Resulting overlapping PCR products were subcloned into the pGEM-T vector system (Promega) and sequenced. Sequencing was performed by the dideoxynucleotide chain termination method (Sanger et al., 1977) using the ALF DNA analyzing system (Amersham Biosciences Europe).

Nucleic Acid Manipulations
As the basic plasmid used for all promoter activity tests, we employed pCB412, which is pARG7.8cos (Purton and Rochaix, 1995) with a polylinker inserted into the unique NruI site. All constructs tested were located on this vector and have been described previously (von Gromoff et al., 2006).

Sources of Porphyrias
The MgProto used came either from Frontier Scientific or was prepared as described previously (Kropp et al., 1997). This latter MgProto was kindly provided by U. Oster. The concentration of MgProto was determined in ether by spectrophotometric measurements using a millimolar extinction coefficient of 308 at 419 nm (Falk, 1964). The concentration of hemin (Sigma-Aldrich) was determined in a solvent consisting of 66.5% ethanol, 17% acetic acid, and 16.5% water (v/v/v) using a millimolar extinction coefficient of 144 at 398 nm (Thomas and Weinstein, 1990). Other metalated porphyrisins were from Frontier Scientific. Their concentrations were determined as specified by the supplier. For tests in vivo, tetrapyrroles were dissolved in dimethyl sulfoxide and used at the final concentration indicated. The final dimethyl sulfoxide concentration in the culture medium in all cases was 0.25%.

Pigment Analyses
Porphyrin steady state levels were determined in cells grown in liquid. Cells were harvested and stored at ~80°C prior to rupture by sonication in an ice bath. Porphyrisins were extracted in three steps with (1) methanol, (2) K-phosphate buffer, and (3) a mixture of acetone: methanol: 0.1 N H₄OH (10:9:1, v/v/v) (Alawady and Grimm, 2005). Aliquots of the supernant were separated by HPLC (model 1100; Agilent) on an RP 18 column (Novapak C18, 4 μm particle size, 3.9 × 150 mm; Waters Chromatography) at a flow rate of 1 mL/min in a methanol/0.1 M ammonium acetate, pH 5.2, gradient and eluted with a linear gradient of solvent B (90% methanol and 0.1 M ammonium acetate, pH 5.2) and solvent A (10% methanol and 0.1 M ammonium acetate, pH 5.2). The eluted samples were monitored by a
fluorescence detector at an excitation wavelength of 405 nm and emission wavelength of 595 nm for Mg-porphyrins. The porphyrins and metalloporphyrins were identified and quantified by comparison with authentic standards purchased from Frontier Scientific. The content of noncovalently bound heme was determined after removal of chlorophyll from *Chlamydomonas* cells by intensive washing with ice-cold alkaline acetone containing 0.1 N NaOH (9:1; v/v) (Weinstein and Beale, 1984). Heme was then extracted with acidic acetone containing 5% HCl, transferred to diethyl ether, concentrated, and washed on a DEAE-Sephadex column. Absorbance was monitored at 398 nm using a millimolar extinction coefficient of 144. The concentration of heme standard solutions was determined spectrophotometrically (Weinstein and Beale, 1983).

**Determination of CHL Activity**

For determination of CHL activity, *Chlamydomonas* cells were grown in 500 mL of liquid TAP medium to a density of 3 to 5 x 10^8 cells per mL, harvested by centrifugation, and resuspended in homogenization buffer (0.5 M sorbitol, 0.1 M Tris-HCl, 1 mM DTT, and 0.1% BSA, pH 7.5). The cells were disrupted by sonication in an ice bath. The cell extracts were centrifuged at 15,000 rpm for 30 min and the supernatants were used for enzyme assays. CHL was assayed as described by Walker and Weinstein (1991). The assay mixture contained 40 mM MgCl₂ and 20 mM ATP. The reaction, performed at 28°C in individual opaque tubes, was started by adding 100 µM Proto and stopped after 5, 10, 15, 30, and 60 min by freezing the tubes in liquid nitrogen. Samples from each enzyme assay were extracted and the products quantified according to methods described above.

**Accession Numbers**

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AJ307055 (CHLH) and AF343974 (CHLI). The other genes may be found as gene models in the *Chlamydomonas* genome sequence (http://genome.jgi-psf.org/Chlre3/Chlre3.home.html) (CHL2, gene model C_119312; CHLD, gene model C_60239).

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure 1.** Test of the Effect of Inhibitors of Cytosolic and Organelar Protein Synthesis on the Light Activation of Mg-Chelatase Genes.

**Supplemental Figure 2.** Assay of Tetrapyrrole Pool Levels in Mg-Chelatase Mutants after a Shift from Dark to Light.

**Supplemental Figure 3.** Assay of Tetrapyrrole Pool Levels in Wild-Type Cells That, before a Dark-to-Light Shift, Were Incubated with Hemin in the Dark for 12 h.

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