Identification of a plastid response element that acts as an enhancer within the *Chlamydomonas HSP70A* promoter

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**ABSTRACT**

Chloroplast-derived signals control a subset of nuclear genes in higher plants and eukaryotic algae. Among the types of signals identified are intermediates of chlorophyll biosynthesis such as Mg-protoporphyrin IX (MgProto). In *Chlamydomonas reinhardtii*, it was suggested that this tetrapyrrole mediates the light induction of chaperone gene *HSP70A*. Here we have analyzed *cis* elements involved in the regulation of *HSP70A* by MgProto and light. We identified two promoters and between their transcription start sites two regulatory regions that each may confer inducibility by MgProto and light to both *HSP70A* promoters. These regulatory regions, when cloned in front of basal non-light inducible heterologous promoters, conferred inducibility by MgProto and light. The orientation and distance independent function of these *cis*-regulatory sequences qualifies them as enhancers that mediate the response of nuclear genes to a chloroplast signal. Mutational analysis of one of these regulatory regions and an alignment with promoters of other MgProto-inducible genes revealed the sequence motif (G/C)CGA(C/T)N(A/G)N15 (T/C/A)(A/T/G) which, as shown for *HSP70A*, may confer MgProto responsiveness. This *cis*-acting sequence element is employed for induction of *HSP70A* by both MgProto and light, lending support to the model that light induction of this gene is mediated via MgProto.

**INTRODUCTION**

The existence of three DNA-containing organelles in eukaryotic algae and higher plants requires a coordination of gene expression in these compartments. The nucleus is the major contributor of gene products that find their way into chloroplasts and mitochondria. It also exerts a tight control over gene expression in these organelles [reviewed in (1,2)]. On the other hand, intact, functional organelles are a prerequisite for the expression of a subset of genes in the nucleus [reviewed in (3–5)]. These observations support the concept of a bi-directional exchange between the DNA-containing organelles.

For the retrograde communication of plastids with the nucleus, experimental data until now have provided evidence for five different signaling pathways that may coordinate the expression of nuclear genes with the requirements of chloroplasts. One of these pathways is dependent on product(s) of plastid protein synthesis since mutants lacking plastid ribosomes or the application of plastid translation inhibitors caused a decrease in the expression of a set of nuclear genes [(4,6–8) and references therein]. For another retrograde pathway the signal is chloroplast-generated singlet oxygen, shown in *Arabidopsis* to result in the specific upregulation of 70 nuclear genes and the downregulation of 9 nuclear genes (9). Mutation in gene *EXECUTER1* abrogated the response to singlet oxygen, suggesting that the gene product either perceives the signal or is involved in its transduction (10). A third pathway employs chloroplast-generated hydrogen peroxide which was shown to specifically activate the nuclear gene for ascorbate peroxidase (*APX2*) in *Arabidopsis* (11,12). A fourth pathway by which plastoplasts exert control on the expression of nuclear genes is triggered by the redox state of photosynthetic electron transport components (13–15). This redox signaling has been proposed to play a role in the adaptation of nuclear gene expression to changes in light intensity providing a feedback response loop in which the expression of photosynthesis genes is coupled to the function of the photosynthetic process [reviewed in (16)]. For all of these chloroplast-to-nucleus signaling pathways, the downstream components, short of *Executor1* are not yet known, nor is it known if the signals converge at a single element in the promoters of responsive genes.

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A fifth pathway for chloroplast-nucleus communication involves tetrapyrrole biosynthesis intermediates. In plants, plastids harbor the entire tetrapyrrole biosynthetic pathway to heme and to chlorophylls (17). Indirect evidence for a role of tetrapyrroles in the light regulation of a nuclear LHCB gene was derived from mutant and inhibitor studies using synchronized cultures of the green alga Chlamydomonas reinhardtii (18,19). In higher plants an accumulation of Mg-porphyrins, caused by treatment with inhibitors of chlorophyll or carotenoid biosynthesis, was correlated with lower levels of LHCB and RBCS transcripts, suggesting a role of these tetrapyrroles in the control of nuclear transcriptional activities (20,21).

We found that the chlorophyll precursors Mg-protoporphyrin IX (MgProto) or Mg-protoporphyrin IX monomethyl ester (MgProtoMe) may induce nuclear heat-shock genes HSP70A and HSP70B of C.reinhardtii, mimicking the normal light induction for these genes (22). These chaperone genes are also induced in higher plants by a shift from dark to light, but an involvement of chlorophyll precursors has not yet been tested (23). A mutant defective in the Mg2+ insertion into protoporphyrin IX (Proto) and thus devoid of Mg-porphyrins (24) showed no light induction of HSP70A and HSP70B (22). Thus, MgProto and MgProtoMe are candidates for a plastid signal in this response pathway (3,25,26).

A role for Mg-porphyrins as plastid signals is supported by the characterization of Arabidopsis gun (genome uncoupled) mutants in which expression of an LHCB gene is uncoupled from plastidal development (27). In these mutants, LHCB gene expression is not diminished when chloroplasts are rendered otherwise non-functional by norflurazon inhibition of carotenoid biosynthesis. Thus, the expression of a subset of nuclear genes became uncoupled from the physiological state of the plastid. The identified genes of four out of five of these gun mutants encode proteins involved in tetrapyrrole synthesis. The gun2 and gun3 mutations affect the heme branch of the pathway, and are presumed to affect flux through the chlorophyll branch as a result of feedback regulation (28,29). The GUN4 protein has been postulated to modulate nuclear gene expression by regulating MgProto synthesis or trafficking (30). The gun5 mutation affects the gene encoding the H-subunit of Mg-chelatase (31). The enhanced accumulation of MgProto upon norflurazon treatment observed in wild type but not in the gun5 mutant, in addition to MgProto feeding experiments to leaf protoplasts, provided evidence for a role of this plastid compound in the downregulation of the LHCB gene studied (28).

Studies with tobacco plants that harbor transgenes in antisense or sense orientation for Mg-chelatase subunits CHL H and CHL I as well as for MgProto monomethyl transferase CHL M indicate a more complex role of tetrapyrrole biosynthesis and intermediates in signaling at least in higher plants. In all transformants, reduced levels of Proto correlated with lower transcript levels of nuclear genes HEMA, GSA and ALAD, encoding glutamyl-tRNA reductase, glutamate-1-semi-aldehyde aminotransferase and 5-aminolevulinic acid dehydratase, respectively (32–34).

In Chlamydomonas, a limited number of genes that are regulated by MgProto have so far been identified (35). These comprise genes HSP70A and HSP70E, both encoding cytosolic chaperones, HSP70B encoding a plastidal chaperone and HEMA, encoding glutamyl tRNA reductase, the first enzyme specific for porphyrin biosynthesis. These four genes are induced by the feeding of MgProto in dark; they also are induced by light.

For HSP70A evidence has accumulated that MgProto controls expression of this gene in response to irradiation; this signaling pathway being different from the pathway that controls the stress response. Upon shift from dark to light both Mg-porphyrin levels and HSP70A mRNA increase transiently (36,37). The light-induced accumulation of Mg-porphyrins was shown to be essential for induction since inhibitors of cytoplasmic protein synthesis, which abolished MgProto accumulation, were shown to prevent the induction by light but not that by heat shock. Also, gametic cells shown to lack a light-induced accumulation of MgProto did not respond to light but responded to heat shock (36,38). In both cases, the addition of MgProto to cell cultures in the dark resulted in induction of HSP70A, indicating that this Mg-porphyrin could substitute for the light signal (36).

Here we report on the identification of two HSP70A promoters and on two sequence elements between these promoters that appear to act as enhancers in response to the plastid signal MgProto.

**MATERIALS AND METHODS**

**Algal strains and culture conditions**

C.reinhardtii strain 325 (cw15, arg7-8, mt+) used throughout, was kindly provided by R. Matagne (University of Liège, Belgium). Cultures were grown photomixotrophically on TAP medium (39) on a rotary shaker at 23°C under continuos irradiation with white light (60 µEm−2 s−1) provided by fluorescent tubes (Osram L36W/25). TAP medium was supplemented with 100 mg l−1 of arginine when required. Light induction and heat shock were performed according to (38) and (37), respectively.

**Nuclear transformation of C.reinhardtii**

C.reinhardtii nuclear transformation was performed using the glass beads method (40), modified as described by (38). Before transformation, the constructs (all containing the ARG7 gene) were linearized by restriction either upstream from HSP70A promoter sequences or downstream from the reporter gene. For the transformation of 1 × 108 cells, 100 ng of plasmid DNA was sufficient to routinely generate at least 100–200 transformants. 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 µEm−2 s−1) and transformants were collected and analyzed after 1 week.

**Sources of porphyrins**

MgProto was prepared as described previously (22). Its purity was checked by HPLC. PROTO was purchased from Sigma-Aldrich (Taufkirchen, Germany).

**RNA gel blot analysis**

Total RNA was isolated from 10 ml cultures grown to 2–4 × 106 cells per ml. The procedures employed for RNA
extraction, separation on agarose gels, blotting and hybridization were the same as described previously (37) but for the nylon membranes used that were Hybond N (Amersham, Braunschweig, Germany). Blots were probed with the 213 bp TAG sequence for detection of the HSP70A, HSP70B and CYC6 reporter gene mRNAs or with CBSP (41) for a loading control. Northern blots were screened by exposing membranes to BAS-MP imaging plates (Fuji). They were evaluated by a phosphoimager (Bio-Rad Laboratories GmbH, Munich, Germany). 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

Total RNA (10 μg) of each sample was applied to Hybond N nylon membranes using a dot blot apparatus. For hybridization, washing of the membranes and their quantitative evaluation we used the same conditions/programs as for RNA gel blot analyses.

Nucleic acid manipulations

As the basic plasmid used for all promoter activity tests we employed pCB412 which is pARG7.8cos (42) with a polylinker inserted into the unique NruI site. All constructs tested were located on this vector.

The conversion of HSP70A and HSP70B into reporter genes by insertion of a TAG sequence has been described previously (38,43). The \( P_{RBC52}^{HSP70B-TAG} \) construct harbored RBC52 promoter sequences from −183 to +15 (with respect to the transcription start site) inserted into the HSP70B 5'-untranslated region (5'-UTR) 18 bp upstream from the translation start codon (44). A 1.5 kb Ncol/XhoI fragment of genomic DNA harboring the CYC6 gene (45), after blunt-ending, was inserted into the SmaI site of pBlue- script SKII+ (Stratagene, Amsterdam, The Netherlands). This version of CYC6 harbored 259 bp of sequence upstream from the transcription start site. CYC6 in this vector was converted into a reporter by the blunt-end insertion of the TAG sequence (38) into the unique NheI site (after a fill-in reaction) located within the CYC6 3’-UTR.

Various promoter deletion constructs in HSP70A were generated by PCR with primers that at the upstream side had a BamHI site and a BgIII site at the downstream side. For the latter primer, sequences around the unique BgIII site located within the 5' end of the HSP70A coding region were used. The PCR fragments were cloned into a variant of pBluescript SK+ that in its polylinker contained a BgIII site. The 3’ end of HSP70A was added to the upstream regions as BgIII, KpnI fragment. The complete HSP70A constructs were excised as BamHI, EcoRI fragments and inserted into BamHI, EcoRI opened vector pCB412.

 Constructs that had HSP70A promoter fragments in front of HSP70B-TAG, \( P_{RBC52}^{HSP70B-TAG} \) or CYC6-TAG were generated in two steps. First the HSP70A promoters (flanked by BamHI, NheI sites) were produced by PCR and inserted into pCB412 opened with BamHI, NheI. Second the cassettes of the reporter genes HSP70B-TAG and \( P_{RBC52}^{HSP70B-TAG} \) were inserted as SpeI, EcoRI, and in the case of CYC6-TAG as SpeI, EcoRV fragments into the NheI, EcoRI/EcoRV opened plasmids generating constructs with HSP70A promoter fragments in front of the reporter genes.

For the generation of either deletion or point mutations in regulatory region I, PCR that employed partially overlapping primers or synthetic double-stranded oligonucleotides were used. The mutated fragments, flanked by BamHI (upstream side) and NheI (downstream side) sites, were inserted into pCB412. Their fusion to the reporter genes was done as described above. Upstream of wild-type or mutated regulatory regions I or II, either a 100 bp sequence derived from the coding region of gene CFE1 (wild type and mutations 1, 2, 3, 4, 5, 7 in Table 1) (46) or a 82 bp sequence (a Stul, HindIII fragment) from the multiple cloning site of pFASTBAC1 (Invitrogen, Karlsruhe, Germany) (mutations 6, 8 in Table 1) was inserted. Before transformation, all plasmids were linearized at the upstream side of these spacer sequences. In all constructs, the promoter regions were controlled by sequencing before transformation.

DNA sequencing

Sequencing was carried out by the dideoxyribonucleotide chain termination method (47) using the ALF DNA Analysing system (Amersham Biosciences Europe, GmbH).

Primer extension experiments

We employed the method described by (48). The 3’ end of the HSP70A-specific primer 5'-AGTCAACCTGACCGTGTTG-CCGAGTCATACCGATAGCG-3’ started 105 bp downstream from transcription start site \( T_{S_1} \). The 3’ end of the HSP70B-specific primer 5’-CTTGTGGGTGTCGATGAGCGCCGAGCCA-3’ used to determine the start sites of HSP70B and the HSP70A-RBC52 fusion promoters in front of HSP70B started 73 bp downstream from \( T_{S_R} \) and 150 bp downstream from \( T_{S_{II}} \). The 5’ ends of the primers were radioactively labeled using \( [\gamma-^{32}P]ATP \) and polynucleotide kinase (both from Amersham). The sequencing gels were from Stratagene (Cast Away Precast Sequencing Gel) as per manufacturer’s instructions.

RESULTS

Induction of HSP70A by Mg-protoporphyrin IX and light is achieved by two promoters

Previous studies have suggested that MgProto mediates the light induction of HSP70A (22). Here we set out to define the promoter elements involved in MgProto- and light-mediated induction of HSP70A. To identify such elements in their own sequence context, we converted HSP70A into a reporter gene by the insertion of a 199 bp DNA fragment from Rhodopseudomonas palustris into its 3’-UTR (TAG) (38). This and all subsequent constructs were inserted into a vector (pCB740) that harbors the ARG7 gene of \( C.reinhardtii \) for the selection of transformants in arg7 mutants (44). Transformants were grown in the dark and exposed to light or MgProto or were heat-shocked in the light. Total RNA from these transformants was analyzed by RNA gel blot. About 50% of Arg+ transformants with constructs that harbored 285 bp of sequence upstream from the HSP70A translation start site—including three putative
heat-shock elements (HSE)—were inducible by MgProto, light and heat shock (Figure 1). The degree of induction observed upon MgProto feeding or dark-to-light shift was similar. A deletion construct that lacked the three HSEs (Δ-149) was no longer inducible by heat shock but induction by MgProto and light was preserved. Surprisingly, even deletion of sequences upstream from and including the previously defined transcription start site at position −89 (TS_{A1}) (49) exhibited MgProto and light inducibility (Δ-81), suggesting that an additional promoter was located further downstream. In transformants with this deletion construct the degree of mRNA accumulation upon MgProto/ light treatment was similar to that observed with the longer constructs. Since in transformants containing a construct harbored only 20 bp upstream from the translation start site (Δ-20) TAG-specific mRNA was not detected (Figure 1) the additional promoter is assumed to be located between positions −81 and −20.

Consistent with the deletion data, primer extension experiments revealed two HSP70A transcription start sites. As judged from the increase in signal intensity observed with RNA from heat-shocked cells the upstream start site at position −89 (TS_{A1}) is used upon heat shock (49) (Figure 2a). A second transcription start site (TS_{A2}) could be defined at position −23 (Figure 2). Upon dark-to-light shift signals of increased intensity were detected not only at TS_{A2} but also at TS_{A1}. This was also observed after MgProto treatment in the dark. A second promoter of HSP70A thus appears to be located downstream from TS_{A1}. This promoter was named P_{A2}; the one further upstream P_{A1}. Comparison of sequences directly upstream from the two transcription start sites did not reveal any obvious homology other than a TATA motif (Figure 2b). This, however, in the case of P_{A2} is only located 12 bp upstream from the transcription start site.

In order to confirm and to extend these data we generated constructs that harbored HSP70A upstream sequences of various lengths in front of a different reporter gene; here we used the promoter-less HSP70B gene. This gene, which contained a residual 5′-UTR of 18 bp, has been converted into a reporter gene by insertion of the TAG sequence described above into its 3′-UTR (44). Transformants harboring the longest HSP70A promoter construct (from −23 to −285 relative to the translation start site) exhibited induction of the reporter gene by MgProto, light and heat shock (Figure 3). Transformants that only contained the reporter gene showed at most weak expression but no induction by light or MgProto (Figure 3). Deletion of sequences −23 to −80, resulting in a loss of P_{A2} and upstream sequences, abolished the inducibility by both MgProto and light. In these transformants, heat-shock induction was inhibited, indicating that promoter P_{A1} was not affected. Presence of HSP70A sequences downstream from the heat shock-inducible promoter and including promoter P_{A2} (sequence between −23 and −81) conferred inducibility by MgProto and light on the reporter gene (Figure 3). These results indicate that promoter P_{A2} also functions outside the context of the HSP70A gene. Transformants with a construct lacking sequences from −23 to −55 including P_{A2}, but harboring sequences from −55 to −81, showed no expression of the reporter gene in response to either MgProto or light. However, the presence of this same sequence (from −55 to −81) downstream from promoter P_{A1} conferred inducibility by MgProto and light to transformants with this construct (Figure 3, third construct from top). These data imply that (i) HSP70A promoter P_{A1} also may be activated by MgProto and light and (ii) sequence elements between positions −55 to −81 may confer this inducibility. Thus, HSP70A has two promoters: P_{A1} is used for heat-shock activated transcription mediated by upstream HSEs (38); for an activation by MgProto and light, both promoters, P_{A1} and P_{A2}, may be employed.

**Sequences located between transcription start sites TS_{A1} and TS_{A2} may mediate activation of heterologous promoters by MgProto and light**

Fusion of the RBCS2 minimal promoter (comprising sequences from +15 to −183 relative to the transcription start site) (P_{R}) in front of reporter gene HSP70B-TAG (resulting in a P_{R}-HSP70B-TAG construct) only very rarely resulted in transformants that expressed the reporter gene at elevated levels (<1 per 700 transformants assayed) (C. F. Beck, unpublished data and (44)). In these rare cases we assume that the reporter gene became fused to an active gene within the C. reinhardtii genome. Insertion of the HSP70A promoter region from −23 to −285 into this construct upstream from P_{R} resulted in the frequent appearance of transformants that expressed the reporter gene in response to heat shock, MgProto and a dark-to-light shift (Figure 4a). The degree of induction by light and MgProto was similar to that seen with the HSP70A reporter gene (Figure 1). We observed that most but not all of the heat-shock-inducible clones also could be activated by light. Most but not all of those responded to MgProto.

Primer extension experiments were performed to elucidate the transcription start site(s) used after induction by light and heat shock. The experiments revealed that after light induction, transcription started at a site TS_{D} defined previously as the start site of the RBCS2 promoter (50) (Figure 4b and c). The absence of transcripts from P_{A1} or P_{A2} after MgProto or light induction was confirmed by RNA blot experiments in which P_{R} upstream sequences were used as a probe (data not shown). Confirming and extending previous data (44), we showed that transcripts generated in response to heat shock used promoter P_{A1}. In these experiments, we also observed two transcription start sites for HSP70B (TS_{B1} and TS_{B2}); TS_{B1} being one bp upstream from the site defined previously by the 5′ ends of cDNA clones (51). As deduced from the increase in signal intensity, this start site appears to be employed preferentially after the application of heat shock while TS_{B2} may be used for constitutive expression (Figure 4b).

From these results we conclude that HSP70A promoter regulatory sequences which confer inducibility by MgProto and light do not activate their native promoters when the heterologous P_{R} promoter is located downstream. To gain information on the HSP70A sequence elements involved in P_{R} activation we generated a construct that in front of P_{R} lacked HSP70A sequences between positions −23 and −80. Transformants that harbored this construct exhibited no induction by MgProto and light (Figure 4a). As expected from the intactness of the HSEs and promoter P_{A1}, these transformants were inducible by heat shock. Presence of the
sequence between the start sites of HSP70A promoters PA1 and PA2 (−23 to −81) in front of P_R-HSP70B-TAG conferred inducibility by MgProto and light to transformants with this construct (Figure 4a).

Since the promoter context has been shown to affect the results from an analysis of light-responsive elements in Arabidopsis (52), we tested whether a different C.reinhardtii promoter may also gain inducibility by MgProto and light when HSP70A promoter sequence elements are present. The promoter of the cytochrome c6 gene (CYC6) under normal growth conditions is essentially silent; but it has been shown to be strongly induced by copper deficiency (45). In order to ensure the specificity of signals detected in transformants, the CYC6 gene was converted into a reporter by insertion of the TAG sequence into its 3'UTR. This gene contained the CYC6 promoter (PC) up to position −259 from its transcription start site and thus all elements needed for induction by Cu²⁺ deprivation (45). The CYC6 gene alone in a transgene setting was not inducible by light or MgProto (Figure 5a). Presence of HSP70A promoter sequences from −23 to −285 in front of PC conferred inducibility by MgProto, light and heat shock to transformants harboring this construct (Figure 5a). Upon heat shock, the PA1 promoter apparently is used as judged from the bigger size of the transcript (Figure 5b). The enhanced expression seen after MgProto feeding or dark-to-light shift lead to a smaller transcript that appears to originate from PC. Cloning of HSP70A promoter sequences from −23 to −81 in front of PC made this promoter responsive to MgProto and light (Figure 5a). The activation of PC and not of PA1 or PA2 by MgProto and light in these transformants was confirmed by the absence

Figure 1. Analysis of HSP70A promoter (PA) deletion constructs. Northern blot hybridization of total RNA (10 μg per lane for CD, DLS, MP, 2 μg for HS) isolated from transformants harboring the HSP70A promoter deletion constructs shown upstream from the tagged HSP70A gene. The transformants were either kept in the dark (CD) for 16 to 20 h, exposed to light for 1 h after the dark period (DLS), treated with MgProto (9 μM) in the dark (MP) or heat-shocked for 40 min at 40°C in continuous light (HS). The HSP70A reporter gene transcripts were detected with the 199 bp TAG probe. For each construct, 30 individual transformants were assayed. The number of transformants showing induction by the various treatments is indicated. The average levels of mRNA accumulation relative to the dark control (corrected for differences in loading) ± SEM are given below these numbers. TS_A designates the transcription start site used upon heat shock. HSE stands for heat shock elements. The gray shaded bar represents the HSP70A gene converted into a reporter by insertion of the TAG sequence. For a loading control, blots were stripped and rehybridized with a probe for the CBLP gene (41).

Figure 2. Determination of HSP70A transcription start sites. (a) Primer extension experiments were performed as described in Materials and Methods using total RNA (10 μg) from cells incubated either with continuous irradiation (CL), exposed to 40°C for 40 min in the light (HS), or exposed to light (60 μEm⁻² s⁻¹) after 16 to 20 h in the dark (DLS) or treated with MgProto (9 μM) in the dark (MP). Next to the probes from the primer extension experiments, a sequence ladder from the corresponding part of the HSP70A promoter region is shown. (b) The arrangement of two HSP70A promoters PA1 and PA2 and their transcription start sites TSA1 and TSA2 as deduced from the primer extension experiments. Underlined are putative TATA motifs.
of hybridization signals in RNA blots when \( P_c \) sequences were used as a probe (Figure 5b). The absence of \( P_c \)-specific mRNA showed that in this setting, similar to the one observed with \( P_R \) (Figure 4a), neither \( P_{A1} \) nor \( P_{A2} \) were activated by treatment with MgProto or light. Rather, promoter \( P_c \) appears to get activated. We draw the conclusion that \( HSP70A \) regulatory regions between transcription start sites \( TS_{A1} \) and \( TS_{A2} \) may confer MgProto and light inducibility to the heterologous promoters \( P_R \) and \( P_C \).

**Definition of two regulatory regions with enhancer activity**

Inspection of the \( HSP70A \) sequence between positions \(-23\) and \(-81\) revealed two 24 bp sequences that share 16 common nucleotides (shaded in Figure 6a). These sequences are located upstream from the \( HSP70A \) translation start site at positions \(-31\) to \(-54\) and \(-56\) to \(-79\) i.e. between the two transcription start sites \( TS_{A1} \) and \( TS_{A2} \) defined previously (Figure 2). We tested both sequences individually for their potential enhancer function in front of the \( P_R \)-reporter construct. Transformants that harbored constructs with nt \(-31\) to \(-54\) in front of \( P_R \) activated the reporter gene in response to MgProto and dark-to-light shift (Figure 6a). The same was observed with constructs that harbored the more upstream sequence from \(-56\) to \(-79\). These results indicate that two regulatory regions (named I and II referring to the upstream and downstream regions, respectively) between the \( HSP70A \) transcription start sites may activate transcription when positioned upstream of the heterologous \( P_R \) promoter (Figure 6a). Both regions thus may harbor sequence motifs that possibly qualify as enhancers. We also tested transformants with these constructs for an effect of heat shock; the absence of a response indicates that sequence elements that mediate induction by heat shock are not present within the sequences tested.

Two hallmarks used for the definition of enhancers are their functionality irrespective of orientation and, within limits, their independence from the distance of the promoter they may activate (53). To test the effect of orientation, we generated constructs that harbored regulatory region I in inverse orientation in front of the \( P_R \) reporter gene. Transformants harboring this construct exhibited induction of the reporter gene in response to light and MgProto (Figure 6a).

To test the effect of distance from the activated promoter transformants harboring constructs with spacer sequences between regulatory regions I and II and the \( P_R \) promoter were generated. Spacer sequences of 100 or 200 bp [derived from the coding region of \( Chlamydomonas \) gene \( CGE1 \) (46)] by themselves in front of promoter \( P_R \) only very rarely resulted in transformants that showed enhanced expression of the reporter gene in response to a dark-to-light shift. The insertion of either regulatory region I or II into these constructs upstream from the spacer sequences conferred inducibility by light and MgProto to the reporter gene (Figure 6b). In the active clones we confirmed that transcription started at \( P_R \) by RNA blot analyses in which the spacer was used as a probe (data not shown). Since the spacer elements apparently lack enhancer as well as promoter activity, we conclude that regulatory regions I and II, also over an increased distance, may activate promoter \( P_R \) in response to light and MgProto. Both regions thus appear to harbor sequence motifs that qualify as enhancers.

**Characterization of a sequence motif that causes enhancement of transcription in response to MgProto and light**

To gain information on the sequence motifs that in regulatory regions I and II are responsible for the enhancing effect, we introduced mutations into region I located upstream from the \( P_R \) promoter fused to the \( HSP70B \) reporter gene (Table 1). Before transformation, all of these constructs were linearized at the upstream side of a spacer placed upstream from region I. For spacers we employed either a 100 bp sequence derived from the coding region of \( CGE1 \) (46) or a 82 bp sequence from the multiple cloning site of vector.
pFASTBAC1 (Materials and Methods). Total RNA of 30–60 transformants that were grown in the dark and exposed to light or MgProto was analyzed by RNA dot blot. Transformants expressing the reporter gene (only 50–60% of the Arg\textsuperscript{+} transformants harbored an intact reporter gene) were evaluated quantitatively (Table 1). Constructs that lacked region I yielded transformants that did not respond to MgProto or light. The analysis of transformants with deletions that entered region I from the upstream side showed that absence of the GCGAC motif (mutation 1) essentially abolished activation of \( P_R \) (Table 1). Deletions from the downstream side that removed a TATACATA motif present in both regulatory regions (mutations 3 and 4) drastically reduced inducibility by both MgProto and light. When the 11 nt located between the GCGAC and TATACATA motifs were removed, again MgProto and light activation of \( P_R \) was abolished (mutation 5). However, a smaller deletion of 5 bp from the center of region I (mutation 6) resulted in constructs that retained inducibility of \( P_R \) by MgProto and light. These results pointed to a functionally important role of nucleotides located in the upstream as well as downstream parts but not within the central segment of region I.

We next analyzed point mutations introduced into the two flanking sequences that are shared by regulatory regions I and II. A transversion mutation converted GCGAC into GATAC (mutation 7) which, when cloned in front of \( P_R \), resulted in transformants that showed a distinctly reduced activation by light and MgProto. The same mutation 7 in front of \( P_C \) yielded transformants that did not respond to MgProto or light (Table 1). It appears that, depending on the target promoter employed, the stringency of sequence requirements for promoter activation may differ. A transversional mutation within the TATACATA sequence shared by regions I and II to TAGCAATA did not significantly affect the MgProto and light activation of \( P_R \); a result substantiated with 60 transformants that harbored this mutation in front of \( P_C \) (Table 1).

When the actual sequences of the various point mutations and deletions of region I located in front of the heterologous promoters \( P_B \) and \( P_C \) were compared, it became evident that 8 nt (shadowed in Table 1) were present in all
constructs that resulted in an activation by MgProto and light >75% of the wild-type control. The arrangement of these nucleotides reveals a striking feature since 15 nt which did not appear to play an essential role separate a TA motif at the downstream side from the upstream motif. The importance of this TA motif (or T or A individually) for the activation of PR by both MgProto and light is supported by the lack of induction in three constructs without TA represented by mutations 3, 4 and 5, generated by deletion of either downstream or central sequences (Table 1). Inspection of the

**Figure 5.** Analysis of HSP70A promoter fragments in front of the CYC6 gene. For RNA blot analyses 2 µg of total RNA was used after heat shock versus 10 µg for the other treatments. (a) The Chlamydomonas CYC6 gene including its endogenous promoter (Pc) and the TAG sequence within its 3'-UTR were cloned behind various HSP70A promoter fragments. A total of 60 transformants generated with each construct were analyzed for induction by light (DLS), MgProto (MP) in the dark and heat shock (HS) as described in the legend of Figure 1. (b) RNA blot analysis of transformants generated with the first and second construct shown in (a). The filter was probed with a fragment of the CYC6 promoter (PC) (sequence from /C0 to /C0 relative to the transcription start site) and, after stripping, with the TAG probe. Hybridization with a probe for the CBLP gene served as a loading control.

**Figure 6.** Test of short HSP70A promoter sequences for enhancer activity. (a) Regulatory regions I and II were cloned in front of the HSP70B-TAG reporter gene with the RBCS2 promoter Pr. Region I was cloned in two orientations. The inducibility of transformants with these constructs by MgProto in the dark, by light and for control by heat shock was assayed as described in the legend of Figure 1. For RNA blots 10 µg of total RNA were employed. Nucleotides shared between regions I and II are shadowed. (b) The distance of regulatory regions I and II to the PR promoter was varied by the insertion of spacer sequences (striped bars) of 100 or 200 bp, derived from gene CGE1 (46). A total of 60 transformants with each construct were analyzed as described in the legend of Figure 1.

Data (Table 1) revealed that all mutations that affected the light activation also affected the activation by MgProto. Although the role of individual nucleotides in promoter activation among the 8 nt conserved in all active regions I
Table 1. Effect of mutations in region I, the −81 to −55 nt PRE-containing fragment, on the activation of $P_R$ and $P_C$

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Strategy</th>
<th>Sequence in front of reporter</th>
<th>relative increase in reporter gene expression$^d$</th>
<th>$P_C$-reporter</th>
<th>$P_R$-reporter</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>DLS</td>
<td>MP</td>
</tr>
<tr>
<td>Region I (positions −81 to −55)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td></td>
<td>GCGACAAAAACGGACCTTGATATATA</td>
<td>4.4 ± 0.7</td>
<td>2.0 ± 0.4</td>
<td>35 (21/60)</td>
</tr>
<tr>
<td>1</td>
<td>−Δ−-</td>
<td>GCGACAAAAACGGACCTTGATATATA</td>
<td>1.3 ± 0.3</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>−−−−−Δ−-</td>
<td>TACGACCTGCTATATATATA</td>
<td>5.9 ± 2.6</td>
<td>7 (2/30)</td>
<td>—</td>
</tr>
<tr>
<td>3</td>
<td>GCGACAAACCGACCT−−−−−Δ−-</td>
<td>GATCACCGTCTATATATATA</td>
<td>1.3 ± 0.3</td>
<td>33 (10/30)</td>
<td>—</td>
</tr>
<tr>
<td>4</td>
<td>GCGAC−−−−−−−−−−Δ−−−−−−−−−−</td>
<td>GATCACCGTCTATATATATA</td>
<td>1.1 ± 0.1</td>
<td>10 (3/30)</td>
<td>—</td>
</tr>
<tr>
<td>5</td>
<td>GCGAC−−−−−−−−−−Δ−−−−−−−−−−</td>
<td>GATCACCGTCTATATATATA</td>
<td>1.0 ± 0.2</td>
<td>37 (11/30)</td>
<td>—</td>
</tr>
<tr>
<td>6</td>
<td>GCGACAA−−−−Δ−−−−ACTATATATA</td>
<td>GATCACCGTCTATATATATA</td>
<td>3.0 ± 1.0</td>
<td>21 (6/30)</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>7</td>
<td>GATCACACAA−−−−−Δ−−−−ACTATA</td>
<td>GATCACCGTCTATATATATA</td>
<td>1.7 ± 0.3</td>
<td>38 (23/60)</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>8</td>
<td>GCGACGGAAAAACTCCTATAGAATA</td>
<td>GATCACCGTCTATATATATA</td>
<td>3.4 ± 0.8</td>
<td>35 (21/60)</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.8 ± 0.1</td>
<td>27 (8/30)</td>
<td>1.0 ± 0.4</td>
</tr>
<tr>
<td>Region II (positions −54 to −31)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td></td>
<td>GCGACTATTTTGCTGCTATATATA</td>
<td>3.6 ± 0.6</td>
<td>23 ± 0.3</td>
<td>33 (10/30)</td>
</tr>
<tr>
<td>Consensus</td>
<td></td>
<td>GCGACNAN15TA</td>
<td>3.6 ± 0.6</td>
<td>23 ± 0.3</td>
<td>33 (10/30)</td>
</tr>
</tbody>
</table>

$^a$RNA dot blot analysis of transformants with mutated region I in front of the $P_C$- and $P_R$-reporter constructs presented in Figures 4a and 5a, respectively. Reporter gene transcripts were detected with the TAG probe.

$^b$Deletion (Δ) or point mutations (underlined lower case letters) introduced into region I.

$^c$The actual nucleotide sequence located in front of $P_R$ or $P_C$. Nucleotides conserved in regions I and II, as well as in various region I mutants were shadowed in grey.

$^d$Dot blot signals resulting from hybridization with the TAG probe from individual transformants either incubated in the dark, or exposed to light for 1 h after incubation in the dark for 18 h (DLS) or to 9 μM MgProto for 1 h in the dark (MP) were compared. The change in average signal intensity as compared to cultures kept in the dark ± SEM is given. Rare transformants that after dark-to-light shift exhibited a 10-fold increase in signal intensity when compared to the dark-grown culture were omitted. Such transformants may have resulted from the integration of the reporter gene in the vicinity of a light-activated enhancer.

$^e$The percentage of transformants that exhibited a signal by dot blot analysis as well as the absolute numbers are given. Data of a small percentage of transformants that exhibited high signal intensity after DLS (<10-fold) but not after MgProto feeding was omitted.

The search for sequence motifs that serve as response elements for MgProto in the upstream region of the $HSP70A$ gene revealed the existence of two promoters: the upstream one ($P_{A2}$) is used for the response to heat shock mediated via $HSE$s (Figure 1). The second promoter, $P_{A1}$, located downstream from $P_{A2}$, was found to be activated by MgProto and light (Figures 1 and 3). MgProto and light also may activate $P_{A1}$ when sequences downstream from its transcription start site ($T_{S_{A1}}$) are present (Figure 3).

In the region between the transcription start sites of the two promoters, two related 24 bp sequences designated as regulatory region I and II for the upstream and downstream regions, respectively, were localized. Both regulatory regions share 16 identical nucleotides (indicated in Figure 6a). When cloned in front of basal/silent heterologous Chlamydomonas promoters $P_R$ or $P_C$, they were shown to confer inducibility by MgProto and light to these promoters (Figures 4a and 5a). Since variations in the distance between the regulatory regions and the promoters as well as an opposite orientation (tested for region I) still conferred light and MgProto inducibility on a basal $P_R$ promoter (Figure 6), we concluded that regions I and II harbor sequence motifs that may qualify as enhancers (53). Their response to MgProto, a compound exclusively synthesized in plastids, led to their designation as plastid response elements ($PRE$s).

A mutational dissection of regulatory region I provided information about essential nucleotides that make up the $PRE$. Nucleotides that in regulatory regions I and II may be important for $PRE$ function became apparent when the actual DNA sequences of various mutated and wild type regulatory regions cloned in front of $P_R$ or $P_C$ were aligned (Table 1). Such an alignment revealed a motif GCGACNAN15TA that is present in both wild type and all mutant regulatory regions which exhibited enhancer activity. We noted that the TA at position 15. However, this does not rule out a partic-
at the first position, C and A also may occur. At the second position, an A is present in 4 out of 8 PREs; here T and G were also observed. However, we noticed that in all cases studied, either T is present at the first position or A at the second position. We considered that sequences flanking the putative PREs may contribute to their functionality. However, the heterogeneity in flanking nucleotides (at least three different ones at each position) (Table 2) suggests this to be unlikely. Although additional nucleotides may well play a role in the interaction of PRE with regulatory protein(s) a consensus sequence of PRE derived from mutant studies and an alignment with putative PREs of other MgProto-regulated genes may be predicted: (G/C)CGA(C/T)N (A/ G)N15 (T/C/A) (A/T/G). The relative frequency of individual nucleotides at each position may be deducted from the sequence logo (Table 2). This element clearly is distinct from the G-box (CUGI) element (CAGTA) that in the promoter of the Arabidopsis LHCBI gene has been implicated in its regulation by MgProto (28) (see below).

Table 2. PRE in Mg-Proto-inducible promoter sequences

<table>
<thead>
<tr>
<th>Promotors analyzed</th>
<th>PRE-containing sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSP70A</td>
<td>TCA GCAGA CAGCTCCTTATCA CAC GGC -56</td>
</tr>
<tr>
<td>HSP70B</td>
<td>TCA GCAGA CAGCTCCTTATCA CAC GGC -56</td>
</tr>
<tr>
<td>HSP70B</td>
<td>TCA GCAGA CAGCTCCTTATCA CAC GGC -56</td>
</tr>
<tr>
<td>HSP70E</td>
<td>TCA GCAGA CAGCTCCTTATCA CAC GGC -56</td>
</tr>
<tr>
<td>HMA</td>
<td>TCA GCAGA CAGCTCCTTATCA CAC GGC -56</td>
</tr>
<tr>
<td>HMA inverted</td>
<td>TCA GCAGA CAGCTCCTTATCA CAC GGC -56</td>
</tr>
<tr>
<td>ALAD</td>
<td>TCA GCAGA CAGCTCCTTATCA CAC GGC -56</td>
</tr>
<tr>
<td>CCE1 inverted</td>
<td>TCA GCAGA CAGCTCCTTATCA CAC GGC -56</td>
</tr>
</tbody>
</table>

*MgProto inducibility of genes HSP70B, HSP70E and HEMA has been demonstrated by Vasilievskaya et al. (66). Genes ALAD and CGE1 which in this study showed no response to MgProto (owing to the genetic background of the strain used) in later studies were inducible by MgProto (data not shown). Promoter sequences of HSP70B (51), HEMA (67) and CGE1 (46) have been published previously. Upstream sequences of HSP70E and ALAD were from gene models C_50001 and C_680014, respectively, from the Chlamydomonas draft genome (http://genome.jgi-psf.org/chlre2/chlre.home.html). The nucleotides conserved in active PREs (Table 1) are boxed. Reference points are the translation start sites (A of ATG is set +1).†PRE logo as generated by using the program published by Crooks et al. (67). For this presentation, in addition to the sequences from the PREs presented, sequence data of mutations 6 and 8 in Table 1 were also used.‡*}

Transformants that exhibited induction by light but not by MgProto may be explained by chromatin structure imposed by sequences flanking the insertion [since this number was reduced when freshly transformed clones were analyzed (data not shown)]. This suggested that both regulatory regions harbor sequence motifs that are targets for MgProto- and light-activated transcription factors. Mutant analyses of regulatory region I showed that a mutational reduction in MgProto activation always was accompanied by a comparable lowering in reporter gene activation by light (Table 1). These data support a model in which the PRE mediates the induction by both light and MgProto. The definition of the PRE as common target for plastid- and light-activated signaling pathways is in agreement with the concept that plastid-derived MgProto is an intermediate in the pathway by which light activates HSP70A (3,22,36). Whether the PRE is activated by transcription factor(s) that directly interact(s) with MgProto as shown in yeast for transcription factor HAP1 that is activated by mitochondria-derived heme (54,55), or indirectly via a signaling cascade, remains to be elucidated.

Surprisingly, neither of the HSP70A promoters P_A1 nor P_A2 was activated by treatment with MgProto or light when placed upstream of F_R or P_C (Figures 4a, 5 and 6). Rather, P_R or P_C were shown to be activated in the presence of a functional PRE. In contrast, both HSP70A promoters exhibited PRE-dependent activity when cloned upstream of promoterless reporter gene HSP70B (Figure 3). These results support previous observations in which a start of transcription at the most downstream P_R promoter upon light activation was noted even when three RBCS2 promoters were sandwiched between the HSP70A promoter and the reporter gene (44). We envision two alternatives that could account for the lack of PRE-mediated activation of P_A1 or P_A2 when a heterologous promoter is situated downstream. (i) Transcription is restricted to the promoter located proximal to the reporter gene. (ii) Transcripts initiated at the distal promoter(s) were unstable due to the presence of additional 5' sequences. We consider this latter possibility unlikely, since transcripts originating from P_A1 were detected after heat shock (Figures 4a and 5a). To account for the first possibility, we speculate that the formation of transcription initiating complexes promoted by PRE-bound factor(s) at all promoters but the one(s) situated most proximal to the translational start site is prevented. Here, nucleosome positioning and chromatin structure may play an important role. This idea is supported by the observation that activation of transcription by heat shock, which employs promoter P_A1 situated close to HSEs, correlates with a loosened chromatin structure (56).

*Trans-acting regulatory elements have been defined previously within promoter regions of various Chlamydomonas genes. Thus, the multiple presence of a 10 bp tub box in the β2-tubulin promoter was shown to be essential for increased transcription following deflagellation as well as upregulation of the promoter during the cell cycle (57). In the nitrate reductase gene (NIA1) promoter, two elements with 68% sequence identity were shown to be required for induction by nitrate and for repression by ammonium of a fused heterologous minimal β2-tubulin promoter (58). Elements that activate promoters in response to copper and oxygen deficiencies have been defined in the upstream
regions of CYC6 and CPX1 (encoding coproporphyrinogen oxidase). Transcriptional activation occurs through copper responsive elements. These copper responsive elements, two are found within the CYC6 promoter and one within the CPX1 promoter, share a GTAC core (59). Additional nucleotides in the flanking regions were shown to contribute to copper-responsive expression, but none are absolutely essential. Similar to the redundant function of the two PREs of the HSP70A promoter defined here, both copper responsive elements of the CYC6 promoter appear to function independently by activating transcription from a heterologous cis-associated promoter, in this case the β2-tubulin promoter (45). Recently, a transcription factor Cr1 that interacts with the copper responsive elements has been identified previously (60). Mutations in the CRR1 gene were shown not to activate copper response element-containing genes such as CYC6 and CPX1 (61).

In higher plants, multiple attempts have been made to define specific plastid responsive elements. Light responsive promoters of genes that are also subject to control by a plastid signal—in most cases coding for the photosynthetic machinery—were compared and subjected to mutational analyses. A conclusion derived from these studies suggested that light and the plastid signal control expression of nuclear genes via the same cis-acting elements (62,63). In these studies, the plastid signal involved in controlling nuclear gene expression was modulated by the addition of norflurazon that, in the light, leads to photooxidation of the thylakoid membrane.

Subsequently it was shown that regulation of nuclear genes by light and a plastid factor, activated by norflurazon treatment, employs complex cis elements formed by aggregation of cognate sequences of different transcription factors (52,64). A modular array of two motifs—an I-box and a G-box—were shown recently to be sufficient for the regulation of a minimal promoter by light and a norflurazon treatment-derived plastidial signal (65).

In Arabidopsis, the repression of gene LHCBI by norflurazon treatment was shown to correlate with the accumulation of MgProto (28). A role of this tetrapyrrole in LHCBI regulation was strengthened by a repression of LHCBI in leaves incubated with MgProto. The target of the norflurazon/MgProto mediated repression was revealed in transgenic lines harboring fusions of wild type or mutant LHCBI promoters to a reporter gene. A mutated G-box motif within the LHCBI promoter made it refractive against inhibition by norflurazon treatment. Levels of expression were the same in wild type and the gun5 mutant that, due to a defect in the H-subunit of Mg-chelatase was shown to accumulate less MgProto in presence of the herbicide. This lead to the conclusion that the G-box motif (CACGTA) may confer regulation by MgProto on the LHCBI promoter. This G-box motif or the closely related CACGTG motif were observed in 42 out of 70 genes that responded by repression to norflurazon treatment-induced MgProto accumulation (28).

On the basis of our results we propose a model for the MgProto-mediated light activation of HSP70A (Figure 7). A shift of cultures from dark to light induces a transient increase in plastidial Mg-porphyrin levels and the transport of a subfraction of MgProto or its monomethyl ester to the cytosol (36). In the cytosol, MgProto is bound by a regulatory protein, possibly a transcription factor, and modifies its activity and/or translocation to the nucleus. As a consequence, this protein or factor(s) activated through this protein may interact with enhancer PRE, resulting in the activation of upstream promoter PA1 and downstream promoter PA2 (Figure 7a). The PRE may also activate heterologous promoters in response to MgProto or light, provided it is in the vicinity of these promoters (Figure 7b). With a sequence element at hand that is a target for a plastid-derived factor, strategies to identify the protein(s) that, upon MgProto feeding, interact with these elements, can now be devised.

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