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Chaperonin Genes of the *Synechocystis* PCC 6803 Are Differentially Regulated under Light–Dark Transition during Heat Stress

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Transcriptional startpoints of the two heat inducible chaperonin genes of *Synechocystis* PCC 6803 were mapped within the conservative CIRCE element and proved to be identical irrespective of the temperature treatment. Finding of an ORF encoding for a potential CIRCE binding repressor (HrcA) further suggests that both *groEL*-analogs are regulated in a CIRCE-dependent manner. In contrast to the expectations, the chaperonin twins are differentially expressed under light–dark transition during heat stress. Not the light *per se,* but rather the photosynthetic electron transport appears to be accountable for the regulatory differences. Our findings support the hypothesis that multiple chaperonins play different physiological roles under stress conditions.

Chaperonins—a subclass of molecular chaperones—, belong to the most conserved proteins known (1) and have been shown to facilitate the folding of numerous polypeptides (for a review, see (2)). They could also provide protection against different environmental stresses (3). Given their ability to interact with wide variety of nonnative proteins, the presence of more than one copies of the *groEL*-analog genes described in several bacteria (4-8) is somewhat unexpected. The existence of multiple chaperonins might indicate, that they accomplish different cellular functions.

Transcriptional regulation of the heat shock genes are best characterized in *Escherichia coli,* where an alternative sigma factor, σ^{32}, takes a prominent part in the activation of these genes including the *groESL* operon (9-10). This sigma factor is required for recognition of specific HS promoters by the RNA polymerase (11), thus transcription of the heat shock genes is controlled via the level and activity of σ^{32} (12).

In the past few years a different type of regulation has been emerged in other prokaryotes. Many bacteria contain a highly conserved inverted repeat (designated as CIRCE (13)) in the upstream region of their *groESL* and *dnaK* genes (14). This element may serve as a binding site for the repressor encoded by *hrcA* (also referred to as *orfA*) (15). Disruption of *hrcA* resulted in a high level of constitutive expression of *dnaK* and *groE* operons at physiological temperature in *Bacillus subtilis* (16) and in *Caulobacter crescentus,* where the effect was dependent on the presence of an intact CIRCE element (17). Apparently, this type of transcriptional regulation seems to be more widespread than anticipated and as far as we know CIRCE is the most conserved regulatory sequence found in eubacteria (14).

A rather complex transcriptional regulatory mechanism has been described for *Bradyrhizobium japonicum* containing five homologues of *groESL* genes. The activation of the $groESL_3$ operon was shown to be mediated by the nitrogen fixation regulatory protein, NifA together with the RNA polymerase containing σ^{54} (18). Regulation by the CIRCE element applies to $groESL_4$ and probably to $groESL_5$ whereas the expression of $groESL_1$ seems to be controlled via σ^{32} (8). Further broadening the spectrum of the possible regulatory mechanisms, post-transcriptional regulation of the $groEL_1$ gene was reported for *Streptomyces albus,* containing two *groEL*-homologues (19).

Similarly to higher plants (20), two chaperonin genes have been identified in the widely used photosynthetic model system, *Synechocystis* PCC 6803: a *groESL* operon (21), and an additional *groEL* homologue, *cpn60* (22) without the adjacent *groES*. Both chaperonin genes contain the highly conservative CIRCE element in their upstream regions (23). We have previously documented that different regulatory pathways must be involved in the activation of chaperonin and *dnaK* genes in *Synechocystis* (23). The present report points

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Abbreviations: HS, heat shock; DCMU, 3′,4′-dichlorophenyl-1,1-dimethylurea; nt, nucleotide(s).

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to the specific differences existing in the regulation of the two chaperonin genes in *Synechocystis* and also highlights the complexity of mechanisms involved in their transcriptional control.

MATERIALS AND METHODS

Culture conditions. *Synechocystis* PCC 6803 cells were grown to mid log phase at 30 °C in BG-11 medium at a constant photon flux density of 70 μE $m^{-2}s^{-1}$ as previously described (24). Cells were exposed to heat stress at designated temperatures for 30 min. For dark incubations cultures were wrapped in aluminum foil. Diuron (DCMU) was added to the cells at a concentration of 15 μM 20 min before the incubation at 42 °C.

RNA isolation. Total RNA was purified by guanidine thiocyanate method using reagents of the RNAgents kit (Promega) excluding TE and DS solutions. After different treatments 30 ml of cells were cooled below 4°C in liquid nitrogen and collected by centrifugation (8000 × g, 4 °C). Cells were resuspended in ice cold TE (10 mM TRIS-HCl, 1mM EDTA, pH 8) and transferred to Eppendorf tubes. After centrifugation (10 000 × g, 4 °C, 2 min) 400 μl chloroform and 400 μl of glass beads (0.1 mm, Braun) were added in DS (0.1% SDS, 0.125% Triton X-100, 0.25% sarcosyl). Following vortexing for 1 min, 400 μl of denaturing solution was added and cells were disrupted by additional vortexing for 2×1 min. After centrifugation (see above) total RNA was extracted according to the manufacturer's (Promega) directions.

Northern blot analysis. Five micrograms of RNA were run on 1% agarose gel containing 6% formaldehyde and blotted onto nylon membrane (ZetaProbe, BioRad). DNA probes specific for *groESL* (ranging from position 1053 to 1396; accession number D12677) and *cpn60* (1800-2495; J05707) genes were generated as described previously (21). Fragments were radiolabeled using Multiprime DNA labeling kit (Amersham). Hybridization, washing and stripping were performed as recommended by the manufacturer of ZetaProbe. Hybridizations with the 0.7 kb HindIII fragment of plasmid pAN4 (kindly provided by M. Sugiura) containing the ribosomal RNA genes of *Anacystis nidulans* served as loading control (25).

Primer extension. Synthetic oligonucleotides C_1 (5′AGTAGAAACATTAATGGAAAT3′) and C_2 (5′TGGATTCATCCTTAAAGGAA3′) were used to determine the transcriptional startpoint of the *groESL* (C_1) and *cpn60* (C_2) genes. Primers were labeled with T_4 polynucleotide kinase (USB) as recommended by the manufacturer. Five pmol of labeled oligonucleotides were coprecipitated with 25μg of total RNA. After denaturation at 95°C for 2 min, annealing were carried out at room temperature for 20 min. Extension reactions were performed at 42°C in a total volume of 15 μl containing 50mM Tris-HCl pH 8.3, 8mM $MgCl_2$, 50 mM NaCl, 5mM DTT, 1mM each of dNTPs, 20U RNasin (Promega) and 12U AMV reverse transcriptase (USB). Reactions were stopped after 30 min, and loaded onto a standard sequencing gel. Plasmids pESL (pUC18 containing the XmnI fragment of the *groESL* operon) and pCPN (pUC18 carrying the BclI-KpnI fragment of the *cpn60* gene) served for generating sequencing ladders using primers C_1 and C_2, respectively.

RESULTS

Heat shock regulation and determination of the 5′ end of the chaperonin genes. Total RNA of *Synechocystis* cells cultured at 30 °C and exposed to different temperatures was extracted and subjected to Northern blot analysis (Fig. 1) Hybridization with *groES*-specific probe revealed that the *groESL* mRNA level increased gradually up to 44 °C (Fig. 1A). At 46 °C, shorter hybridizable bands appeared whereas at 48 °C the level of the *groESL* transcript decreased dramatically. The expression pattern of the *cpn60* gene proved to be very similar to that of *groESL* except that the amount of the intact *cpn60* transcript was significantly reduced at 46 °C and disappeared at 48 °C. (Fig. 1B). The presence of shorter mRNA species can be explained by either degradation or early termination. However, rehybridizations with *rRNA* probe indicates, that no "general" RNA degradation occurred at higher temperatures.

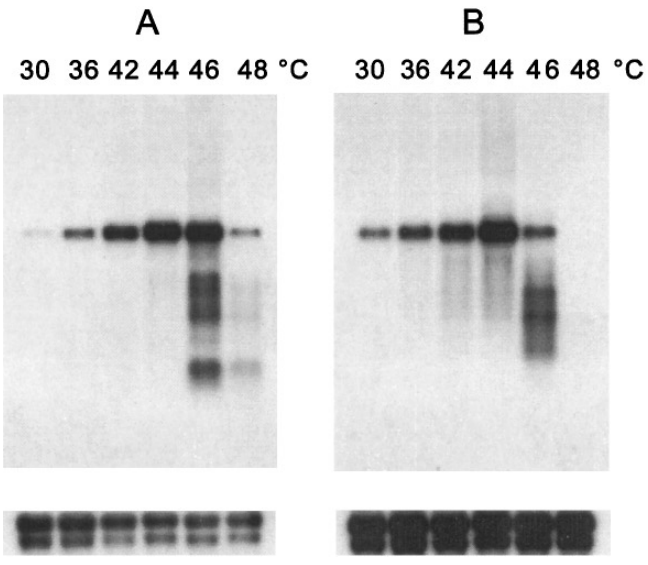

FIG. 1. Effect of heat stress on the level of chaperonin mRNAs. Cells were incubated at temperatures indicated above and the extracted total RNA were subjected to Northern analysis using *groESL* (A) and *cpn60* (B) specific probes (upper panels). Hybridizations with *rRNA* were used as loading controls (lower panels).

Primer extension experiments were carried out to identify the 5′ end of both chaperonin transcripts. (Fig. 2). A single startsite for *groESL* was found 74 nucleotides (nt) upstream of the proposed translational start codon tested upon normal and heat shock conditions, as well. (Fig. 2A). Similarly, the transcriptional startpoint of the *cpn60* gene was localized at position −73 irrespective of the temperature treatment (Fig. 2B). The above findings strongly suggest, that no additional heat shock promoters are present in the upstream regions of the chaperonin genes. The 5′ end of both *groESL* and *cpn60* transcripts is localized within the CIRCE element (Fig. 2), as found for the *groESL* in *Synechococcus* PCC 7942 (26). If CIRCE really functions as an operator, one might predict existence of the corresponding *hrcA* analog in the genome of *Synechocystis*. We performed a similarity search with the conserved SSATIRN motif (17) of the HrcA proteins in

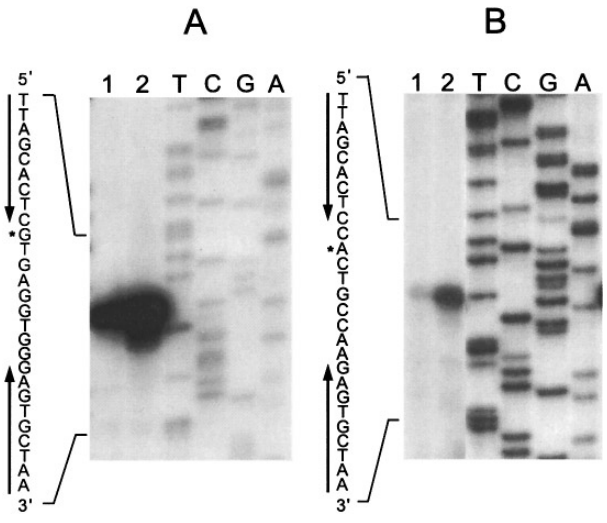

FIG. 2. Primer extension analysis of the *groESL* (A) and *cpn60* (B) transcripts under normal (30°C, lanes 1) and heat shock (42 °C, lanes 2) conditions. The extension products were analyzed on a sequencing gel. T, C, G, and A are products of the sequencing reactions obtained by using the same oligonucleotides as primers. The depicted sequences represent the noncoding strands. The converging arrows mark CIRCE elements. Potential transcriptional startpoints are indicated by asterisks.

Cyanobase (27) and explored that the predicted amino acid sequence of the ORF named as *sll1670* displays homology to other eubacterial *HrcA* proteins (Fig. 3). Analysis of the aligned sequences verified the existence of regions of strong conservation both at the N-terminal and a short stretch near the C-terminal end. From the results obtained we conclude that CIRCE is involved in the regulation of both chaperonin genes in *Synechocystis* PCC 6803.

Transcriptional regulation of groEL-type genes in response to light–dark transitions under normal and heat shock conditions. As it was pointed out before, proteins encoded by multiple chaperonin genes in *Synechocystis* might fulfill physiologically distinct roles. If this hypothesis is valid, one might predict, that *groESL* and *cpn60* display altered expression pattern under different circumstances. Under natural condition changes in ambient temperature usually occur in combination with alterations in light quality and irradiation, so we decided to analyze the expression of the chaperonin genes under various light and thermal conditions. *Synechocystis* cells were preincubated for 30 min at 30°C in light or dark then heat shocked at 42 °C or left at 30°C either with or without illumination for an additional 30 min. After each treatments the expression of *groESL* (Fig. 4A) and *cpn60* (Fig. 4B) was followed by Northern blot analysis. Both chaperonin genes were transcribed at 30°C when cells were continuously illuminated (lane 1). In contrast neither *cpn60* nor *groESL* mRNA could be detected at this temperature in cells kept in darkness (lane 2). Sublethal temperature stress strongly enhanced the accumulation of both chaperonin transcripts in light (lane 3), but no heat activation could be observed without illumination (lane 4 vs. 3). This specific condition disclosed, however, an important difference in the level of transcript accumulation of chaperonin twins. Despite exposing cells to thermal stress the amount of *groESL* mRNA detected remained comparable to that of observed at 30°C in light (lane 1), whereas the *cpn60* transcript was only barely detectable. Obviously, 30 min duration of dark treatment seemed to repress both chaperonin genes at growth temperature (lane 5) to a similar degree as found after 1 hour darkness (lane 2). Pretreatment at 30°C without illumination (lane 6) caused no alteration in the mRNA levels of *groEL* analogs when compared to full light conditions (lane 1). The activation of the chaperonin genes was remarkably different, when *Synechocystis* cells were preincubated in light, then heat shocked in darkness (lane 7). While the level of *cpn60* mRNA declined, the *groESL* transcript showed significant accumulation in the same sample (lane 7 vs. lane 1). It is noted however, that both chaperonin genes produced elevated mRNA levels if compared to dark preincubated samples (lane 7 vs. lane 4). Dark pretreatment did not affect the heat induction of chaperonin genes in cells subjected to thermal stress under illumination (lane 8).

Effect of photosynthetic electron transport inhibitor DCMU on transcript levels of chaperonins. To test whether the decline in the chaperonin mRNA levels observed upon dark shift is causally linked to the operation of intact photosynthesis the effect of DCMU has been tested. For this purpose *Synechocytis* cells were heat shocked at 42 °C in the presence of 15 μM DCMU in light. At the concentration applied diuron fully blocked photosynthetic electron transport, assayed as O_2 evolution (data not shown). Northern blot analysis was in line with our previous findings which demonstrated characteristic transcriptional differences for the chaperonin analogs under dark conditions. Whereas DCMU resulted in only a slight decline in the heat induced transcript level of *groESL* (Fig. 5A), hindrance of electron flow between PSII complex and the plastoquinone pool abolished the *cpn60* activation (Fig. 5B).

DISCUSSION

Northern blot analysis revealed that the two chaperonin genes are strongly heat inducible in *Synechocystis* PCC 6803. Both mRNA levels increased gradually parallel with the temperature shift up to 44 °C but started to decline at higher temperatures. It is noted, that other cellular processes like PS II activity and *de novo* protein synthesis were shown to be impaired at identical temperature range (28).

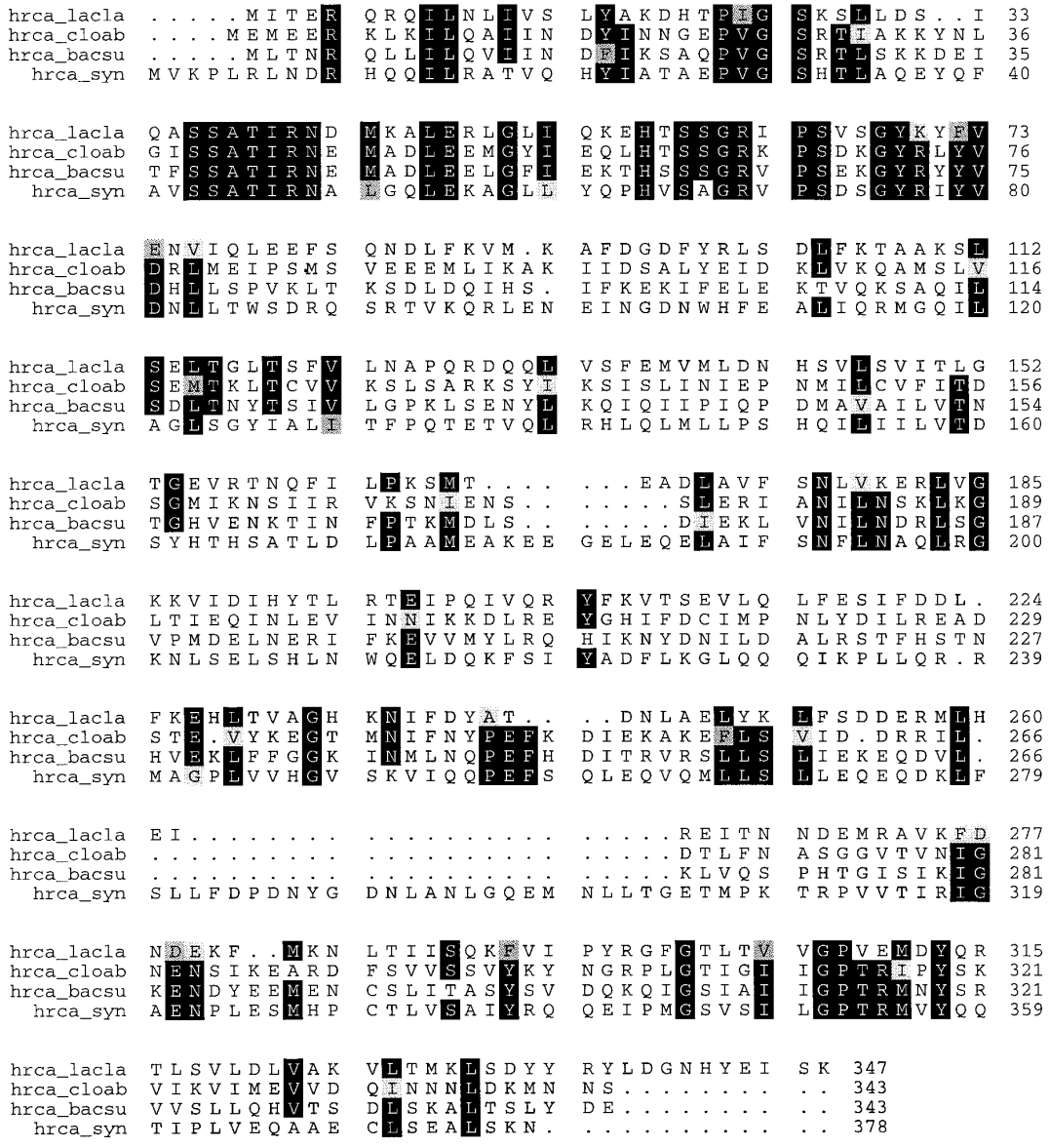

FIG. 3. Comparison of HrcA proteins. Sequences are presented with one-letter amino acid code and identified by species codes. hrca_lacla: *Lactococcus lactis* P4230; hrca_cloab: *Clostridium acetobutylicum* P30727; hrca_bacsu: *Bacillus subtilis,* P25499; hrca_syn: *Synechocystis* PCC 6803). Sequences were aligned by using the GCG program package (Genetic Computer Group Inc., Wisconsin Package, Version 8, 1994).

In order to explore the mechanism of their heat induced activation, 5′ end mapping of the *cpn60* and *groESL* mRNAs were carried out. Primer extension experiments revealed, that the transcriptional startpoints of both genes are identical irrespective of the temperature treatment (Fig. 2). These results clearly suggest that -in contrast to *E. coli*-, the same promoters direct the transcription of the chaperonin genes in *Synechocystis* under normal and heat shock conditions. In addition, the first nucleotide of the mRNAs is located within the highly conservative CIRCE element, similarly as it was shown for *Synechococcus* PCC 7942 (26). Recent studies demonstrated that CIRCE might serve as a binding site for the repressor protein HrcA and could be involved in negatively regulating the heat-inducible transcription (15,17). Providing CIRCE plays similar role in the regulation of chaperonin genes, it would be expected, that an *hrcA* homologue is present in the genome of *Synechocystis* PCC 6803. In fact, database (27) searching uncovered the existence of a potential CIRCE binding protein displaying remarkable homology to other HrcA analogues (Fig. 3) strongly suggesting, that CIRCE is implicated in the regulation of both chaperonin genes.

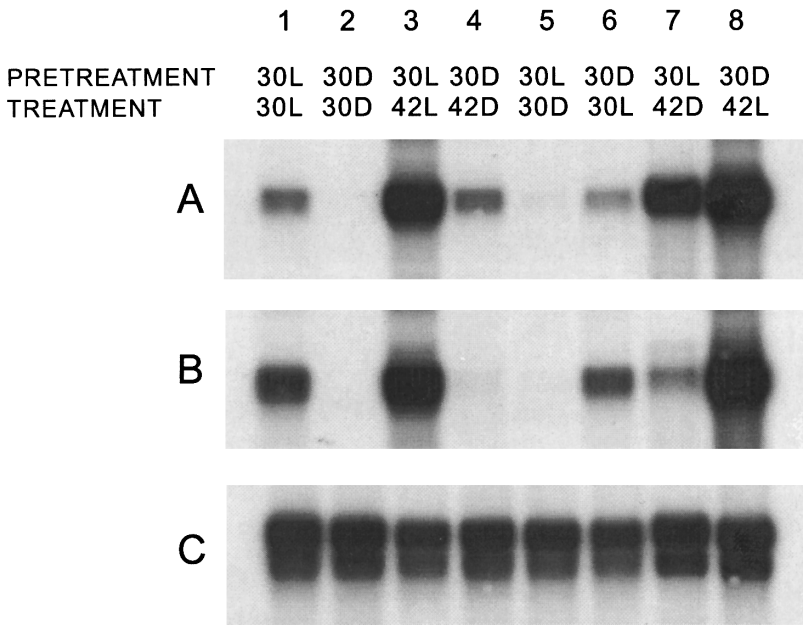

FIG. 4. Expression of the chaperonin genes under various thermal and light conditions. Prior to RNA isolation *Synechocystis* cells were preincubated at 30 °C (30) in light (L) or dark (D) for 30 min, then shifted to 42°C (42) or left at 30°C (30) either in light (L) or in dark (D) for additional 30 min. After hybridization with *groESL* specific probe (panel A), the filter was reprobed with radiolabelled *cpn60* fragment (panel B). The amount of loaded RNA was verified by *rRNA* hybridization (panel C).

In many eubacteria, *hrcA* was found to be a constituent of a *dnaK* or *dnaJ* operon, containing CIRCE element in their upstream regions (14, and references therein). This genetic arrangement should result in the negative autoregulation of the repressor and the *dnaK* family of molecular chaperones (15). Based on data obtained from Cyanobase (27), neither *dnaK* nor other heat shock genes are present in the surroundings of the potential *hrcA* in *Synechocystis* PCC 6803. The nearest open reading frame with the same orientation possibly codes for shikimate kinase (27), and is located more than 300 nt downstream of the *hrcA* gene. Thus, it is highly conceivable that the *hrcA* mRNA is monocistronic (for comparison, the distance between *groES* and *groEL* is 96 nt). Furthermore, no CIRCE sequence can be identified in the upstream region of this gene. Though the functional importance of this genetic arrangement is not yet known, one might speculate that the regulation of *hrcA* (and therefore *groESL* and *cpn60* as well) in *Synechocystis* PCC 6803 is different from that seen in many other bacterial species (14 and refs. therein).

Since the CIRCE element is found in the 5′ region of both *groEL*- type genes, one would propose that their heat shock activation proceeds through an identical mechanism. In contrast, heat shock performed in darkness explored a striking difference in the expression of the two *groEL*-like genes. In algal cells, pretreated and heat stressed in the dark, *cpn60* mRNA was undetectable while a remarkable amount of *groESL* transcript was present. If darkness was imposed on cells only during the heat exposure the *groESL* operon produced elevated mRNA level, but *cpn60* remained to be much less active. The very same result reflecting characteristic differences for the regulation of two *groEL* analogs was obtained using photosynthesis inhibitor, DCMU (Fig. 5). We conclude, that our findings cannot be attributed solely to light regulation, but may also be related to the altered energy status of the cells. Data are available that both the mRNA stability (29) and transcriptional activation (30) can be controlled by the photosynthetic electron transport in *Synechocystis* PCC 6803. Obviously, further studies are required to elucidate the regulatory cascade that -besides CIRCE- modulates the induction and/or mRNA stability of the chaperonin genes in *Synechocystis* PCC 6803.

Recently, it was suggested that photosynthetic apparatus has a dual function: it is able to transduce light energy and also acts as a primary sensor of environmental change through modulation of chloroplastic redox signal (31). In line of this reasoning, the altered expression of *groESL* and *cpn60* genes under different light conditions might have functional significance. It has been shown, that in many bacteria which are known to have only a single copy of *groESL* operon, the GroEL proteins have a glycine-methionin rich motif at their C-terminal end. It appears, that in other prokaryotes which have two types of GroELs, the one linked with GroES, does not have the conservative GGM motif. This situation has been known in case of

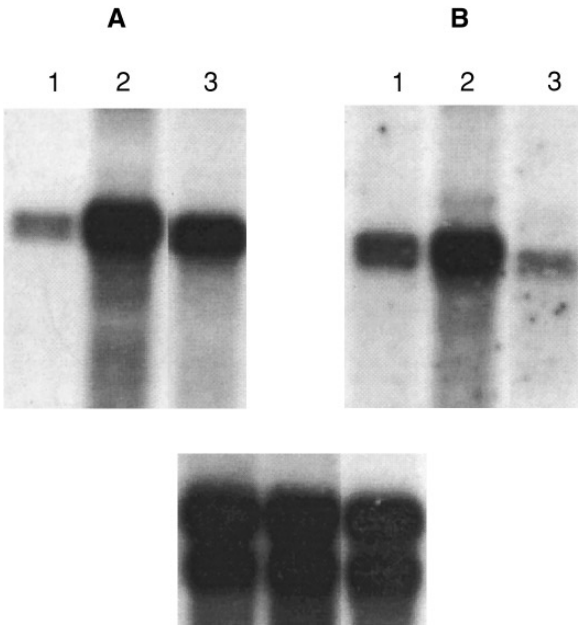

FIG. 5. The combined effect of diuron and heat stress on the transcriptional activity of *groESL* (A) and *cpn60* (B) genes. Cells were incubated at 30 °C (lanes 1) or heat shocked at 42°C (lanes 2) for 30 min. Samples in lanes 3 were treated as described for lanes 2 with the exception, that 15μM diuron were added 20 min before applying thermal stress. Lower panel shows hybridization with the *rRNA* probe.

S. albus (4), *Mycobacterium leprae* (5) and in *Synechocystis* PCC 6803 as well. The glycine-methionine rich tail is implicated in the association of active protein-folding GroESL chaperonins with lipid bilayers (32). In line of a currently proposed model the chaperonin-lipid interaction may act as a rapid and effective adaptive tool to stabilize thermally stressed membranes (32). Heat preadaptation of *Synechocystis* cells accomplished in light conferred protection to the photosynthetic oxygen evolution during a subsequent heat stress. In contrast, preconditioning proceeded in darkness did not result in any protection of thylakoid functions. It is noted, that the profile of chaperonin proteins was significantly altered at different light conditions (Török et al, manuscript in preparation). It is tempting to postulate, that Cpn60 is preferentially required for the assembly of multimeric photosynthetic complexes in light. On the other hand, the previously referred inability of *Synechocystis* cells to develop thermotolerance in dark upon sublethal heat hardening may causally be related to the highly restricted formation of Cpn60. Though presumably less important in the acquirement of thylakoid thermotolerance, GroEL with higher abundance must serve some more fundamental functions and may represent a generally required chaperonin.

In summary, our results demonstrate for the first time that the multiple chaperonins of the *Synechocystis* are differentially regulated under light-dark transition during heat stress. The abundance of the mRNAs is tightly controlled by the photosynthetic electron transport. Complete characterization, including promoter analysis and mutagenesis on the two *Synechocystis groEL*-like genes will be necessary to disclose their exact regulation and different physiological roles they might play within this photosynthetic organism.

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