

The CIRCE Element and Its Putative Repressor Control Cell Cycle Expression of the *Caulobacter crescentus* *groESL* Operon

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The *groESL* operon is under complex regulation in *Caulobacter crescentus*. In addition to strong induction after exposure to heat shock, under physiological growth conditions, its expression is subject to cell cycle control. Transcription and translation of the *groE* genes occur primarily in predivisional cells, with very low levels of expression in stalked cells. The regulatory region of *groESL* contains both a σ^{32} -like promoter and a CIRCE element. Overexpression of *C. crescentus* σ^{32} gives rise to higher levels of GroEL and increased levels of the *groESL* transcript coming from the σ^{32} -like promoter. Site-directed mutagenesis in CIRCE has indicated a negative role for this *cis*-acting element in the expression of *groESL* only at normal growth temperatures, with a minor effect on heat shock induction. Furthermore, *groESL-lacZ* transcription fusions carrying mutations in CIRCE are no longer cell cycle regulated. Analysis of an *hrcA* null strain, carrying a disruption in the gene encoding the putative repressor that binds to the CIRCE element, shows constitutive synthesis of GroEL throughout the *Caulobacter* cell cycle. These results indicate a negative role for the *hrcA* gene product and the CIRCE element in the temporal control of the *groESL* operon.

The heat shock response is a universal phenomenon by which all living cells, when exposed to temperatures higher than their normal physiological temperatures, induce the synthesis of a group of proteins generally known as the heat shock proteins (Hsps).

In prokaryotes, the best-studied mechanism of heat shock induction is that of the gram-negative bacterium *Escherichia coli*. In this organism, the heat shock response is positively regulated at the level of transcription by the alternate sigma factor σ^{32} , encoded by the *rpoH* gene, whose levels increase drastically (about 20-fold) during the first few minutes after heat shock due to derepression of its translation and to a transient increase in its half-life (for a review, see reference 32). The level and activity of σ^{32} are negatively regulated in *E. coli* by the products of the heat shock genes *dnaK*, *dnaJ*, and *grpE*. DnaK, DnaJ, and GrpE cooperate to present σ^{32} to the FtsH protease, which degrades the heat shock sigma factor (14, 26), whereas DnaK and DnaJ work together to inhibit σ^{32} activity in heat shock gene transcription by preventing its binding to the RNA polymerase core and GrpE partially reverses this inhibition (9).

In contrast to *E. coli*, no evidence for involvement of σ^{32} -like factors in heat shock gene expression was found in gram-positive bacteria, such as *Bacillus subtilis* (13) and *Clostridium acetobutylicum* (19). However, a highly conserved inverted repeat (IR) sequence was detected in front of some of the major heat shock genes (*dnaK* and *groE*), which was proposed to substitute for the σ^{32} regulation of the heat shock response in gram-positive bacteria. It soon became apparent that this inverted repeat sequence, also known as the CIRCE element (named CIRCE for controlling inverted repeat of chaperone expression), was more widespread than initially imagined, being identified in front of the *groESL* operons of several gram-

negative bacteria, including those presenting σ^{32} -like promoters (2, 24).

The role of the CIRCE element has been investigated, and evidence indicates that it functions as an operator site to which a repressor binds (24, 31, 33). The protein coded by *orf39*, the first gene in the *dnaK* operon of *B. subtilis*, with homologs found in several other bacteria and recently renamed *hrcA* (22, 23), was found to bind the IR at the DNA level and to serve as the repressor in *B. subtilis* (31).

In the gram-negative bacterium *Caulobacter crescentus*, several heat shock-inducible genes have been characterized and they all present σ^{32} -like promoters (2, 11, 22). The gene encoding the σ^{32} homolog of *C. crescentus* has been isolated, and one of its promoters (P2) aligns with the σ^{32} consensus sequence, with transcription from this promoter increasing dramatically during heat shock (21, 29). Recently, in vitro transcription assays using *C. crescentus* E σ^{32} RNA polymerase holoenzyme and in vivo studies using *rpoH-lacZ* transcription fusions have confirmed the identity of P2 as a σ^{32} -dependent promoter (30). The levels of σ^{32} increase transiently during heat shock in *C. crescentus*, and the increased transcription of *rpoH* seems to account for the induction of σ^{32} levels (21, 30). This mode of regulation for *C. crescentus* σ^{32} differs from that of its *E. coli* counterpart, whose complex regulatory region does not include a σ^{32} -dependent promoter (7).

The *C. crescentus* *groESL* operon has been characterized and shown to be subject to a dual type of control (2). Besides being heat shock inducible, its expression is cell cycle regulated during growth at normal temperatures. The results of primer extension analysis suggested the presence of two putative promoters regulating the expression of *groESL*, with only one (P2) presenting characteristics of a σ^{32} -transcribed promoter and being induced by heat shock. Furthermore, a CIRCE element was found downstream of P2, suggesting that both a σ^{32} promoter and the IR element regulate the expression of *groESL* in *Caulobacter* (2).

In this report, we confirm the σ^{32} -dependent expression of *groESL* by overexpressing the *C. crescentus* heat shock sigma factor using a multicopy plasmid and showing that the increase

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in σ^{32} levels results in an increase in the amount of GroEL and an increase in the amount of the transcript coming from the σ^{32} -like promoter. Furthermore, the role of the CIRCE element in the regulation of the *Caulobacter groESL* operon was investigated by using site-directed mutagenesis to obtain mutations in this *cis*-acting element. The *groE* regulatory regions containing each of these mutations were fused to a promoterless *lacZ* gene, and expression of β -galactosidase was analyzed in *C. crescentus* cells harboring these transcription fusions after heat shock and throughout the cell cycle at normal temperatures. A *C. crescentus* strain with a disruption in the *hrcA* gene (22) was also investigated for GroEL expression. Data obtained indicated that HrcA and the CIRCE element are involved in cell cycle control of the *groESL* operon.

MATERIALS AND METHODS

Bacterial strains and plasmids. The synchronizable *C. crescentus* NA1000 (8) and LS2293 (*hrcA*) (22) strains were grown in PYE (5) medium or in modified minimal M2 glucose medium (15). Cultures were synchronized by centrifugation through a Ludox gradient using a modified version (6) of the procedure of Evinger and Agabian (8). *E. coli* TG-1 was used for phage propagation and cloning, and S17-1 was used as the donor strain in conjugations with *C. crescentus*. Plasmid *placZ/290* (10) contains a promoterless *lacZ* gene and was used for transcriptional fusions with the *C. crescentus groESL* regulatory region. Plasmids pTrc-His B (Invitrogen) and pPROEX-1 (Gibco-BRL) were used for overexpressing *C. crescentus* GroEL and DnaK proteins, respectively, in *E. coli*. Plasmid pAR33 (21) is a derivative of the multicopy plasmid pBBR1 (1) carrying the *C. crescentus rpoH* gene coding for σ^{32} and its promoter region. Plasmid pCS225 contains a *lacZ* transcription fusion with the promoter of the *xytX* gene (17), and cells carrying this plasmid were grown in PYE medium containing 0.1% xylose.

Site-directed mutagenesis of the *groESL* regulatory regions and construction of *lacZ* transcription fusions. Site-directed mutagenesis was performed by the method of Kunkel et al. (16), using a DNA fragment containing the regulatory region of the *groESL* operon (2) cloned in M13mp19 as the template. The synthetic oligonucleotides ML3 (5'-CCAGCGTCGTTGTTTCTCGAATGGAGC-3'), LRC (5'-GAATGGAGCGACAATAACAGTCCCGC-3'), LDR (5'-G AATGGAGCGACAACAGTCCCGC-3'), MH10 (5'-CGAGGGCCGCGGGGCGGACACCAGCG-3'), GP135 (5'-GAAGCGCTCCCGCGAGCGCCGAAA-3'), and GP136 (5'-GAAGCTCCCGCGCCGCGCCGAA-3') were used to obtain the site-directed mutations. The underlined bases correspond to the changes introduced in the wild-type sequence. The DNA fragments containing the mutations introduced by using ML3, LRC, LDR, MH10, GP135, and GP136 were then ligated to *placZ/290*, giving rise to transcription fusions pRB19, pRB20, pRB22, pRB23, pRB35, and pRB36, respectively (for the location of each mutation, see Fig. 3). Transcription fusion pRB21 was obtained by doing a second round of site-directed mutagenesis, using construct pRB19 as the template and the synthetic oligonucleotide LRC. Plasmids containing these transcription fusions were introduced into *C. crescentus* by conjugation with an *E. coli* donor (S17-1). β -Galactosidase activity in mid-log-phase cultures was measured by the method of Miller (18).

Western blots. Aliquots of *C. crescentus* cultures at 30°C or after heat shock at 42°C for different lengths of time were collected, and total protein was extracted as previously described (2). The protein extracts were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and the proteins were transferred to nitrocellulose membranes by the method of Towbin et al. (27). Analysis of the membranes was performed as previously described (2), except for the use of specific polyclonal antisera anti-GroEL or anti-DnaK and an enhanced chemiluminescence kit (Amersham). The anti-GroEL antiserum was obtained from a rabbit immunized with a fusion protein overexpressed in *E. coli*, corresponding to about 36 kDa of the carboxy-terminal portion of *C. crescentus* GroEL containing an N-terminal histidine tag from the expression vector pTrc-His B (Invitrogen). The anti-DnaK antiserum was obtained essentially the same way, but using expression vector pPROEX-1 (Gibco-BRL) and a DNA fragment encoding 65 kDa of the carboxy-terminal portion of *C. crescentus* DnaK.

Immunoprecipitation. The temporal control of *groE-lacZ* transcription fusions and GroEL synthesis was investigated in synchronized cultures by pulse-labelling aliquots of cells at different times of the cycle with [³⁵S]methionine for 5 min and immunoprecipitation of the labelled proteins using either anti- β -galactosidase (Sigma) or anti-GroEL antiserum, as previously described (12). As a control of synchronization, antisera against *C. crescentus* flagellins were also used. Similarly, the time course of heat shock induction of β -galactosidase synthesis in *groE-lacZ* transcription fusions was investigated by pulse-labelling mid-log-phase cultures with [³⁵S]methionine for 2 min and immunoprecipitation of the labelled proteins using anti- β -galactosidase antiserum.

Primer extension assay. Mapping of the transcriptional start sites was carried out by a primer extension assay. A synthetic oligonucleotide (18-mer) complementary to nucleotides -15 to +3 (see Fig. 2B) was 5' end labelled with [γ -³²P]ATP and polynucleotide kinase and hybridized to 50 μ g of total RNA

isolated from *C. crescentus* cells grown at 30°C or after a 15-min exposure to 42°C. Annealing was carried out at 50°C for at least 4 h in 25 μ l of 100 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES) buffer (pH 7.0) containing 1 M NaCl and 5 mM EDTA. The nucleic acids were ethanol precipitated and resuspended in 50 μ l of 25 mM Tris-HCl (pH 8.3) containing 3 mM MgCl₂, 75 mM KCl, 1 mM dithiothreitol, 40 U of RNase inhibitor, and 1 mM (each) dATP, dCTP, dGTP, and dTTP. The annealed primer was extended at 37°C for 90 min using 300 U of Moloney murine leukemia virus reverse transcriptase (Amersham). RNA was digested for 30 min at 37°C by the addition of 1 μ g of RNase A, and the extended products were analyzed by electrophoresis on denaturing sequencing gels followed by autoradiography.

RESULTS

***C. crescentus groESL* operon expression is controlled by σ^{32} .** To demonstrate that the *C. crescentus* σ^{32} homolog regulates expression of the *groESL* operon, we analyzed a *C. crescentus* strain carrying a high-copy-number plasmid (pAR33) containing the σ^{32} gene and its promoter region (21). For cells grown at 30°C, the levels of σ^{32} in NA1000 cells harboring pAR33 were investigated by Western blotting using antiserum against the *E. coli* σ^{32} and shown to be about eightfold higher than those in NA1000 cells lacking the plasmid (Fig. 1). After heat shock, σ^{32} levels were twofold higher in the strain carrying pAR33 than in the control strain (Fig. 1). Since *in vitro* transcription studies have shown that the purified *C. crescentus* σ^{32} protein in the presence of RNA polymerase core enzyme specifically recognizes the heat shock-regulated promoter P1 of the *C. crescentus dnaK* gene (29), we investigated the levels of DnaK protein in the σ^{32} -overexpressing strain. As shown in Fig. 1, the amount of DnaK in this strain was about fivefold higher in cells growing at 30°C than in the wild-type strain. During the first 30 min after heat shock at 42°C, DnaK protein levels were also higher in the σ^{32} -overexpressing strain (about twofold). After that, the amount of DnaK became equal to or lower than that in the control strain subjected to heat shock, probably reflecting the fact that the levels of σ^{32} decrease faster in the overexpressing strain under these conditions (Fig. 1).

The amount of GroEL was similarly investigated in the σ^{32} -overexpressing strain, and as shown in Fig. 1, for cultures grown at 30°C, GroEL levels were sixfold higher in this strain than in the control. After heat shock, GroEL levels were higher (two- to fivefold) in the strain harboring pAR33 up to 45 min of exposure to 42°C. For longer periods of heat shock, as observed for DnaK, the amount of GroEL was determined to be lower in the σ^{32} -overexpressing strain.

To investigate if the increase in the amount of GroEL was due to increased transcription of the *groESL* operon, primer extension experiments were performed by using an 18-nucleotide synthetic oligomer complementary to nucleotides -15 to +3 (Fig. 2) and RNA was isolated from cells of NA1000 strain carrying or not carrying the multicopy plasmid pAR33, grown at 30°C or after 15 min at 42°C, as described in Materials and Methods. As shown in Fig. 2, the amounts of transcript initiating at the σ^{32} -like promoter were about 10-fold higher in σ^{32} -overexpressing cells growing at 30°C and about 7-fold higher when the cells were subjected to a 15-min heat shock at 42°C compared to cells with normal levels of σ^{32} , as determined by densitometry scanning of the autoradiogram (not shown).

Analysis of transcriptional fusions containing mutations in the CIRCE element. In order to investigate the role of the CIRCE element in expression of the *C. crescentus groESL* operon, mutations in either one or both arms of the inverted repeat were constructed by site-directed mutagenesis and the mutated *groESL* regulatory regions were subcloned into the transcription reporter vector *placZ/290*. As can be observed in

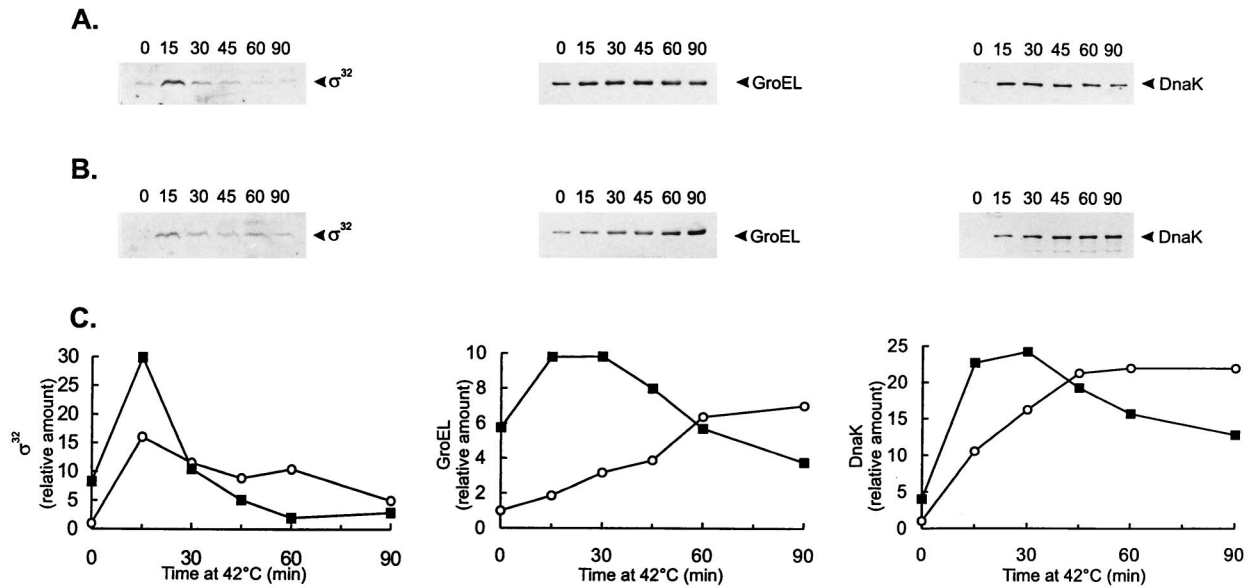


FIG. 1. (A and B) Western blot analyses of GroEL and DnaK in *C. crescentus* strains containing different levels of σ^{32} . NA1000 cells carrying (A) or not carrying (B) plasmid pAR33 (high-copy-number plasmid containing the *C. crescentus rpoH* gene) were grown to mid-log phase at 30°C or shifted to 42°C for the indicated times (minutes). Protein extracts were prepared and separated by SDS-polyacrylamide gel electrophoresis. The proteins were transferred to nitrocellulose membranes and probed with antibody to *C. crescentus* GroEL or *C. crescentus* DnaK and antibody against *E. coli* σ^{32} , as described in Materials and Methods. Bound antigen was visualized by chemiluminescence and recorded on X-ray film. Equal amounts of protein were applied to each lane. (C) The relative levels of σ^{32} , GroEL, and DnaK, determined by densitometry scanning of the films, for strains NA1000 (○) and NA1000 carrying pAR33 (■) are shown.

Fig. 3, when three bases were changed on either the left arm or the right arm of the inverted repeat, as in transcription fusions pRB19 and pRB20, higher levels of β -galactosidase activity were observed for cells grown at 30°C. These levels were about 50% higher than those observed in the wild-type construct

pMA11. In both cases, however, the levels of β -galactosidase activity still increased after exposure of the cells to 40°C for 1 h. When both arms were mutated, reconstructing the dyad symmetry of the inverted repeat but with a different nucleotide sequence, as in pRB21, the levels of β -galactosidase activity

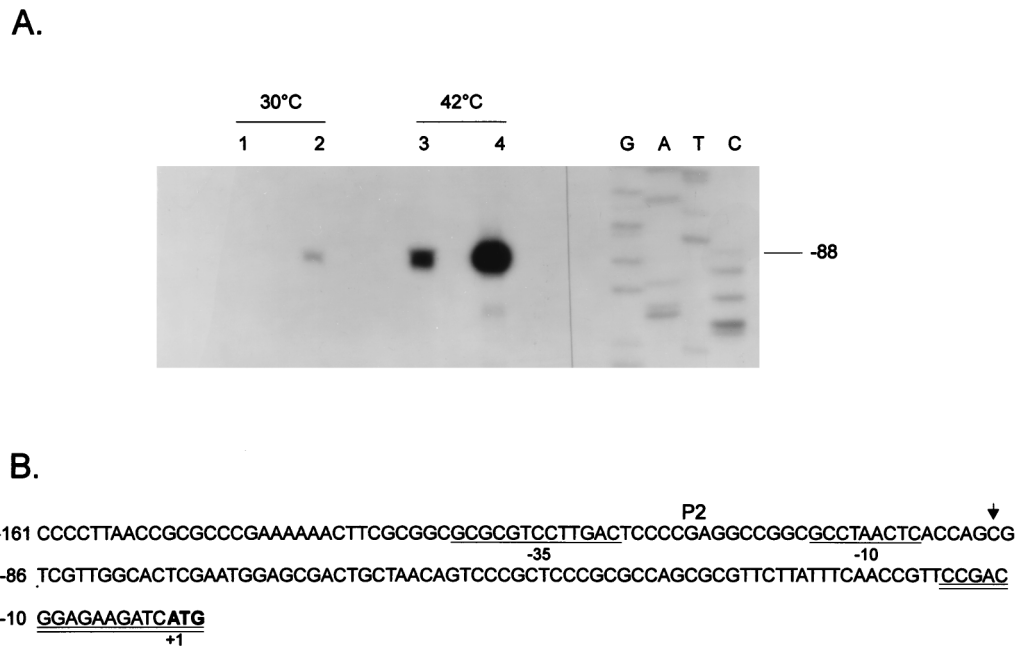


FIG. 2. Increased *groESL* mRNA levels in a σ^{32} -overexpressing *C. crescentus* strain. (A) An 18-nucleotide primer complementary to nucleotides -15 to +3 of the 5' region of the *groESL* operon was 5' end labelled and hybridized to 50 μ g of total RNA isolated from NA1000 cells carrying (lanes 2 and 4) or not carrying (lanes 1 and 3) the plasmid pAR33 (high-copy-number plasmid containing *C. crescentus rpoH* gene) grown at 30°C or exposed to 42°C for 15 min. The hybrids were then extended by using reverse transcriptase, and the extension products were resolved by denaturing gel electrophoresis and autoradiography. (B) Sequence of the 5' region of the *groESL* operon. The transcription start site is shown by an arrow over the nucleotide sequence. The putative -10 and -35 regions of the P2 promoter are underlined. The ATG of the initiator methionine is shown in bold type. The oligonucleotide used in primer extension experiments is complementary to the sequence underlined twice.

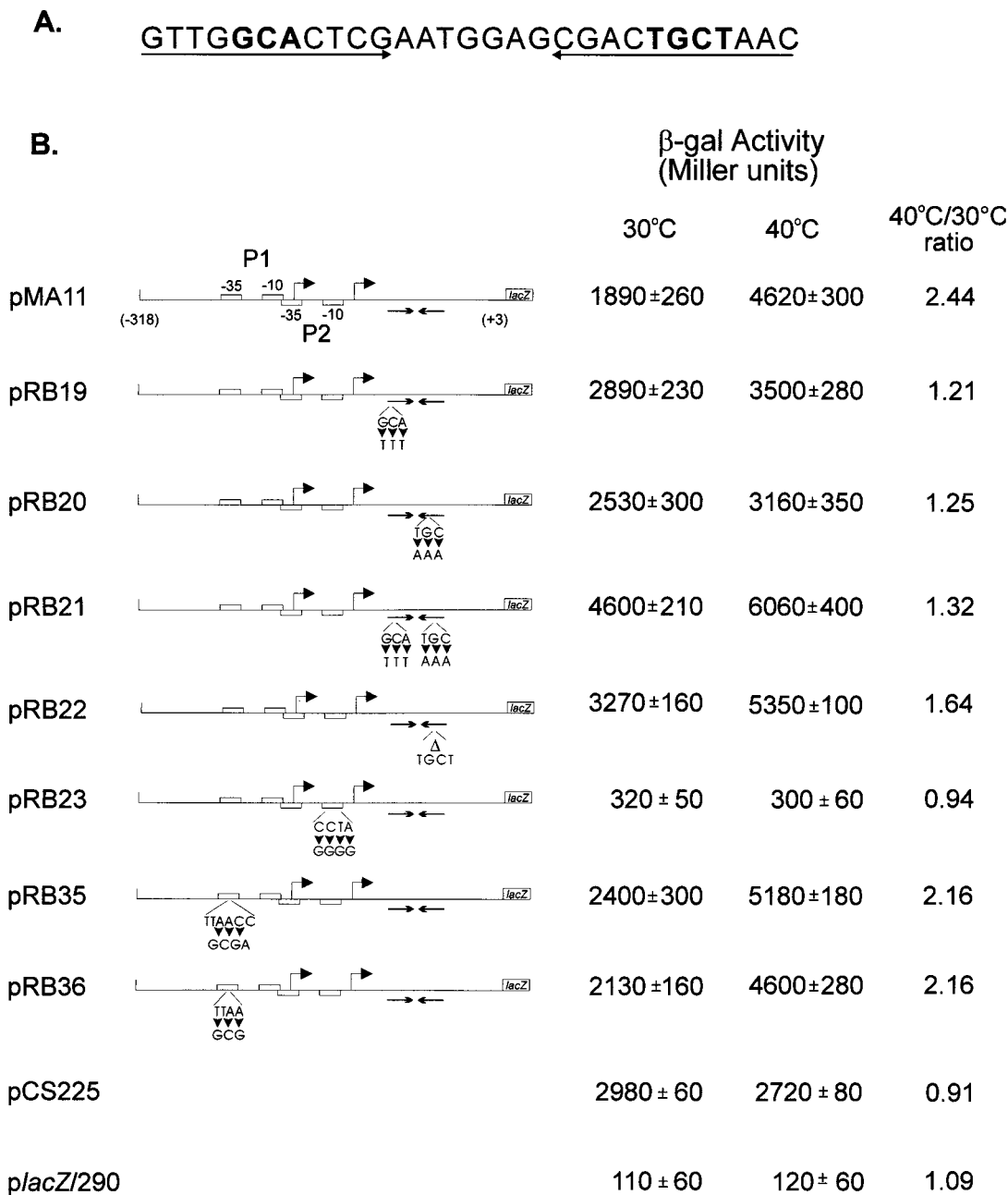


FIG. 3. Changes in transcriptional activity due to mutations in the *groESL* regulatory region. (A) Nucleotide sequence of the inverted repeat (CIRCE element) located downstream of heat shock promoter P2. Nucleotides shown in bold type have been mutated. (B) Schematic representations of the fragments cloned into the transcription reporter vector *placZ/290* and the corresponding β-galactosidase (β-gal) activity of each construct. Site-directed mutations were carried out by the method of Kunkel et al. (16), with M13mp19 containing the *groESL* regulatory region, as described in Materials and Methods. The arrowheads indicate base changes, and the triangle indicates a four-base deletion. All mutant constructs and the control plasmids were assayed for β-galactosidase activity (18) in mid-log-phase, plasmid-bearing *C. crescentus* NA1000 cultures either grown at 30°C or after 1 h of incubation at 40°C. pCS225 is a transcription fusion containing the promoter region of the *xyfX* gene, required for growth on xylose, fused to the *lacZ* gene in *placZ/290* (17). The values of β-galactosidase activity and the corresponding standard deviations represent the averages of six independent assays.

were even higher at 30°C, reaching 240% of the value obtained for the wild-type construct pMA11, and here also β-galactosidase levels increased still further after exposure of the cells to 40°C, reaching levels 30% higher than those observed after heat shock with the wild-type transcription fusion pMA11. Similar results were obtained when four bases were deleted in the right arm of the inverted repeat, as in transcription fusion pRB22. These results indicate that it is not the formation of a

putative stem-loop structure that regulates expression of *groESL*, since in all four mutants, such a structure could still be formed, as determined by using the FOLDRNA program of the Genetics Computer Group package (not shown). Instead, the data suggest that a protein or proteins probably bind to the inverted repeat in a sequence-specific manner, negatively regulating expression of *groESL* at normal temperatures.

Figure 3 also shows that nucleotide changes in the putative

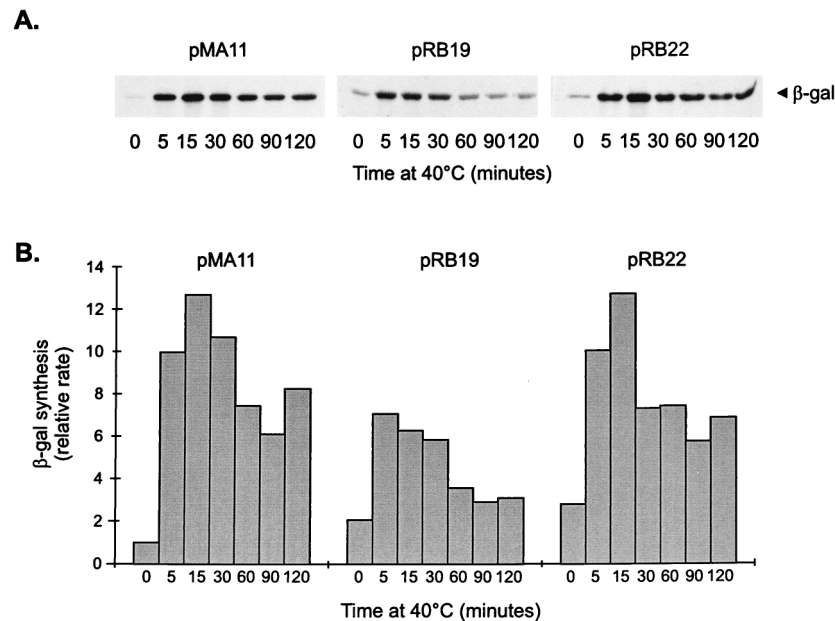


FIG. 4. Time course of heat shock induction of β -galactosidase (β -gal) synthesis in different *groESL-lacZ* transcription fusions. (A) *C. crescentus* NA1000 cells harboring transcription fusion pMA11, pRB19, or pRB22 were subjected to heat shock at 40°C, and aliquots of cells were pulse-labelled with [35 S]methionine for 2 min at the indicated times. Cells were then harvested by centrifugation, and protein extracts were immunoprecipitated with anti- β -galactosidase antibody to determine the rate of β -galactosidase synthesis before and during heat shock. (B) Relative rates of β -galactosidase synthesis determined by densitometry scanning of the autoradiograms.

–10 region of the heat shock promoter P2 lead to very low levels of β -galactosidase activity, similar to the values obtained with the vector alone. Mutations in the –35 region of the P1 promoter, however, do not result in lower levels of transcriptional activity, since the values of β -galactosidase activity determined for pRB35 and pRB36 were found to be higher than that of the wild-type construct either at 30°C or after 1 h at 40°C. These results seem to indicate that if P1 is a real promoter, it does not contribute significantly to transcription of the *groESL* operon, either at 30°C or during heat shock. For a control, we used the *xylX-lacZ* transcription fusion where the promoter region of the *xylX* gene, which is required for growth on xylose and is not heat shock inducible, was subcloned into *placZ*/290, giving rise to plasmid pCS225 (17). We observed that β -galactosidase activity in this case does not increase after heat shock (Fig. 3).

To confirm that the promoter constructs containing mutations in the CIRCE element are still heat shock inducible, the rate of synthesis of β -galactosidase was determined in cells harboring pMA11, pRB19, or pRB22, after different lengths of exposure to 40°C. As shown in Fig. 4, the rate of β -galactosidase synthesis increases significantly after heat shock in cells harboring each of the three constructs. In the case of pRB22, for instance, the pattern of heat shock induction of β -galactosidase synthesis is similar to that observed for the wild-type construct pMA11. In cells harboring pRB19, the rate of β -galactosidase synthesis presents a lower but significant level of induction (3.5-fold) after heat shock. The fact that the amount of induction observed with pRB19 does not reach wild-type levels could be due to a decrease in the half-life of the mRNA produced during heat shock, which is suggested by an apparently faster turnover of the response in this case.

Role of the CIRCE element in cell cycle control of *groESL* expression. We had previously shown that sequences upstream of the heat shock promoter (P2) were not necessary for cell cycle control of the *groESL* operon (2). Since the σ^{32} protein

was shown to be synthesized constitutively throughout the cell cycle at 30°C (21), we decided to investigate the role of the CIRCE element in *groESL* temporal control. Synthesis of β -galactosidase directed by the *groESL* regulatory region carrying mutations in the CIRCE element was analyzed throughout the *C. crescentus* cell cycle. To measure the rate of β -galactosidase synthesis, samples of synchronized NA1000 cultures harboring the *groESL-lacZ* transcription fusion pMA11, pRB19, pRB20, or pRB21 were pulse-labelled with [35 S]methionine at different times during the cell cycle, and extracts of these cells were immunoprecipitated with anti- β -galactosidase antibody.

Figure 5 shows that the rate of β -galactosidase synthesis, which is temporally controlled in the wild-type construct (pMA11), becomes constant throughout the cell cycle when the CIRCE element carries a mutation in the left arm (pRB19), the right arm (pRB20), or in both arms of the inverted repeat (pRB21). As an internal control for cell synchrony, the rate of synthesis of the flagellins was determined in all experiments. In Fig. 5, we present the experiment done with cells harboring the pRB21 construct, showing the normal pattern of flagellin synthesis. These results indicate that *groESL-lacZ* transcription fusions carrying mutations in the CIRCE element do not present cell cycle control of β -galactosidase synthesis.

Disruption of the *C. crescentus hrcA* gene leads to constitutive expression of *groESL* at physiological temperatures. The *C. crescentus hrcA* gene encodes a protein with homologs reported for several other bacteria (22, 25) and was shown both in *Caulobacter* and in *B. subtilis* to be involved in the regulation of heat shock operons containing the CIRCE element (22, 31, 33). We have previously shown that in *C. crescentus* disruption of the *hrcA* gene increased transcription of the *groESL* operon ninefold at physiological temperatures with no significant effect on transcription after heat shock (22). This effect on transcription was not observed in the *dnaKJ* operon of *Caulobacter*,

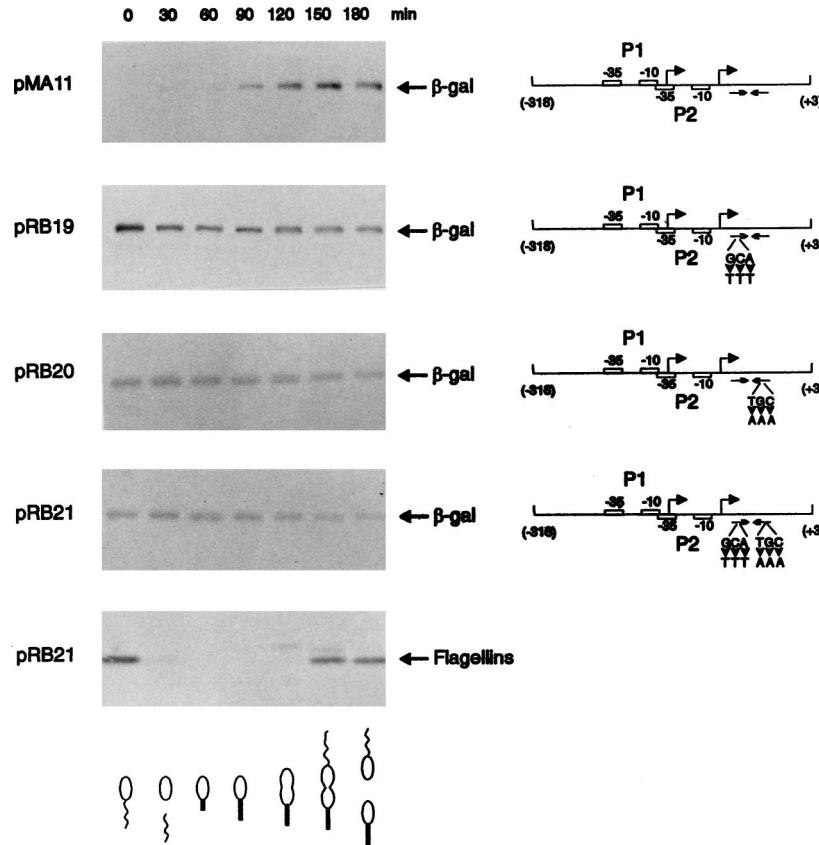


FIG. 5. Transcription fusions containing mutations in the CIRCE element lose cell cycle control. Synchronized cultures of *C. crescentus* NA1000 harboring different transcription fusions were pulse-labelled with [³⁵S]methionine at the indicated times during the cell cycle. Extracts of these cells were then immunoprecipitated with anti-β-galactosidase antibody, as described in Materials and Methods, to analyze the rate of β-galactosidase synthesis as a function of the cell cycle. A schematic representation of each transcription fusion is shown on the right. pMA11 is the wild-type construct; pRB19 and pRB20 carry mutations in the left arm and the right arm of the IR, respectively; pRB21 has mutations in both arms of the IR. As an internal control for cell synchrony, flagellin synthesis was determined throughout the cycle with the pRB21 construct. The drawings at the bottom of the figure are schematic representations of the *C. crescentus* cell cycle corresponding to the time indicated in each lane.

which does not present a CIRCE element in its regulatory region (2, 11, 22). Higher levels of *groESL* mRNA observed in cells of the *hrcA* mutant strain growing at normal temperatures are accompanied by an increase in the levels of GroEL protein, which were observed in Western blots using anti-GroEL antibody. As shown in Fig. 6, *hrcA* null mutant cells growing at 30°C present increased levels of GroEL (about fourfold) compared to those of the wild-type strain at the same temperature. The amount of GroEL still increased after heat shock, reaching similar levels in both mutant and wild-type cells after 45 min at 42°C, compatible with the data on transcription. Consistent with the fact that no increase in *dnaKJ* transcription is observed in *hrcA* mutant cells growing at 30°C compared to wild-type cells (22), no increase in the levels of DnaK was observed when the same Western blot was analyzed with anti-DnaK antibody (Fig. 6).

To confirm that GroEL synthesis is induced after heat shock in the *hrcA* null strain, wild-type and mutant cells were pulse-labelled with [³⁵S]methionine after different times of exposure to 42°C, and extracts of these cells were immunoprecipitated with anti-GroEL antibody. As shown in Fig. 7, the rates of GroEL synthesis increase to similar extents in the *hrcA* null strain (fivefold) and the wild-type strain (sevenfold) during heat shock. As a control, the rate of DnaK synthesis was also investigated by immunoprecipitating the same cell extracts

with the anti-DnaK antibody (Fig. 7). The increase in the rate of DnaK synthesis during heat shock was about ninefold in both the wild type and the *hrcA* null strain. These results indicate that *C. crescentus* HrcA negatively regulates expression of the *groESL* operon at normal temperatures, with no significant effect on expression during heat shock.

To investigate whether *hrcA* disruption affected temporal expression of the *groESL* operon, cultures of strain LS2293 were synchronized and synthesis of GroEL was analyzed throughout the cell cycle. Aliquots of cells at various stages of the cycle were pulse-labelled with [³⁵S]methionine, cell extracts were prepared and the proteins were immunoprecipitated with anti-GroEL antiserum. As shown in Fig. 8, the rate of GroEL synthesis in the *hrcA* mutant strain is not controlled by the cell cycle, in contrast to the differential synthesis of GroEL observed in the *hrcA*⁺ strain NA1000. As an internal control of cell synchrony, the flagellins were also immunoprecipitated by adding anti-flagellin antisera to the same samples, as described in Materials and Methods. As apparent in Fig. 8, there is no difference in the pattern of flagellin synthesis for the *hrcA* and *hrcA*⁺ strains, indicating that loss of temporal control is specific for the *groESL* operon. Furthermore, cell synchrony, as observed through light microscopy, was exactly as in the wild-type cells.

To confirm that the effect of *hrcA* disruption on GroEL

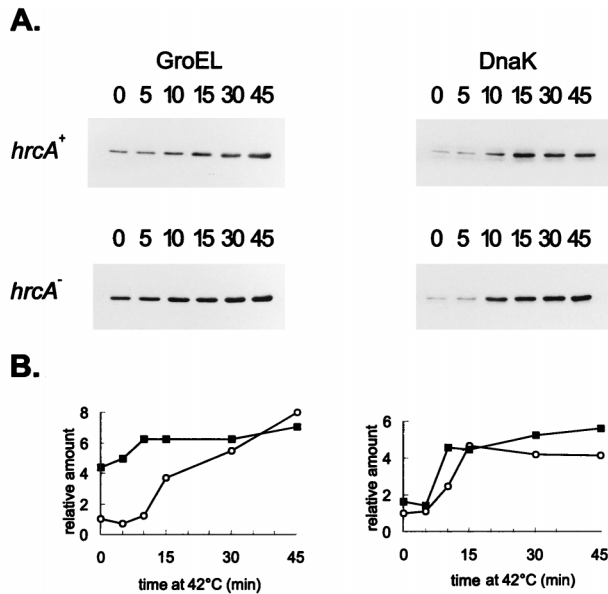


FIG. 6. Effect of HrcA on the level of GroEL. Cells of *C. crescentus* NA1000 (*hrcA*⁺) or LS2293, which presents a disruption of the *hrcA* gene, were grown to mid-log phase at 30°C or shifted to 42°C for the times (minutes) indicated. Protein extracts were prepared and separated by SDS-polyacrylamide gel electrophoresis. After transfer of the proteins to nitrocellulose membranes, the blots were probed with antibody to GroEL or to DnaK, as described in Materials and Methods. Equal amounts of protein were applied to all lanes, and *hrcA*⁺ and *hrcA*⁻ samples were loaded on the same gel and processed together. The relative levels of GroEL and DnaK, determined by densitometry scanning of the films, are shown below the blots; *hrcA*⁺ (○) and *hrcA*⁻ (■).

expression is at the level of transcription, we looked at β -galactosidase synthesis in synchronized cultures of strain LS2293 harboring the wild-type transcription fusion pMA11. As shown in Fig. 9, the rate of β -galactosidase synthesis is no longer cell

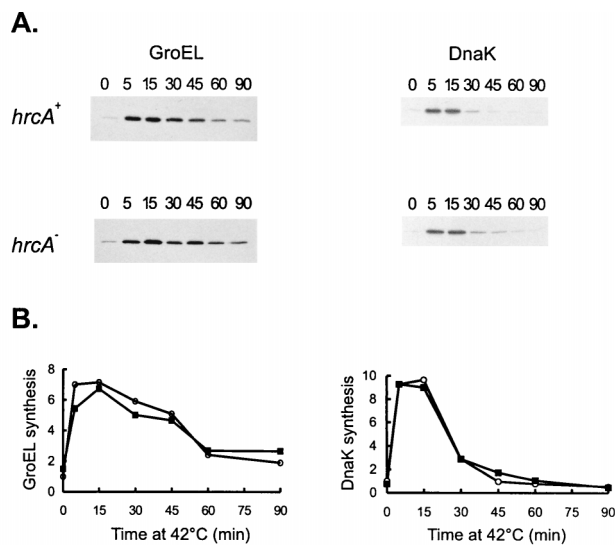


FIG. 7. Effects of HrcA on GroEL and DnaK syntheses during heat shock. Cells of *C. crescentus* NA1000 (*hrcA*⁺) or LS2293 (*hrcA*⁻) were subjected to heat shock at 40°C, and aliquots of cells were pulse-labelled with [³⁵S]methionine for 2 min at the indicated times (in minutes). Cells were then harvested by centrifugation, and protein extracts were immunoprecipitated with anti-GroEL or anti-DnaK antibodies. The relative rates of GroEL and DnaK syntheses were determined by densitometry scanning of the autoradiograms. Symbols: ○, *hrcA*⁺; ■, *hrcA*⁻.

cycle controlled in the *hrcA* null strain, indicating that transcription directed by the *groESL* regulatory region becomes constitutive in the absence of HrcA protein.

Enhanced transcription in CIRCE and *hrcA* mutants is from the σ^{32} promoter. In order to investigate from which promoter the increased transcription directed by the *groESL* regulatory region was occurring in the *hrcA* null strain and in the CIRCE element mutants, primer extension experiments were carried out with an 18-nucleotide synthetic oligomer complementary to nucleotides -15 to +3 (Fig. 10C) and RNA isolated from cells of different mutant strains grown at 30°C or subjected to heat shock at 42°C for 30 min, as described in Materials and Methods. As shown in Fig. 10, a single transcription start site was observed in all mutant strains, which coincides with the initiation site highly induced during heat shock, indicating that in all cases enhanced transcription was occurring from the σ^{32} promoter (P2). The fact that no transcript originating from the putative P1 promoter was observed in these experiments, in conjunction with the data using site-directed mutagenesis (Fig. 3), strongly suggests that P1 might not be a true promoter.

Even though the primer extension product coming from the chromosomal *groESL* operon and from the plasmid-borne fusion have the same size, the data in Fig. 10 clearly show that there is enhanced transcription occurring from the heat-inducible promoter in the CIRCE mutants and the *hrcA* null strain, and no other transcription initiation site is observed in the mutant strains.

DISCUSSION

The initial description of a highly conserved inverted repeat sequence, the CIRCE element, in front of the *dnaKJ* and *groESL* operons of the gram-positive bacteria *B. subtilis* and *C. acetobutylicum*, in association with a vegetative σ^{70} promoter, led to the idea that this inverted repeat was substituting for a σ^{32} promoter and therefore would be found only when σ^{32} was absent. Nevertheless, it soon became clear that the CIRCE element is quite widespread, being found in the *dnaK* and/or *groE* operons of numerous phylogenetically distant bacteria (24), including bacterial species containing σ^{32} -mediated heat shock regulation (2, 20, 25). In particular, this report presents evidence of an operon controlled by a σ^{32} promoter and the CIRCE element combined: the *C. crescentus groESL* operon.

Previous characterization of the *groESL* regulatory region had indicated the existence of two putative promoters, P1 and P2, corresponding to two transcriptional start sites, determined in primer extension experiments (2). Only P2 was highly induced during heat shock and presented -10 and -35 sequences conforming to the consensus sequence of σ^{32} promoters. In the present study, we show that mutations in the putative -35 region of P1 do not affect expression of the *groESL-lacZ* transcription fusion, either in cells growing at 30°C or during heat shock. Furthermore, primer extension experiments under conditions somewhat different from those previously used (2) did not confirm the transcription initiation site corresponding to the P1 promoter. Together these results indicate that P1 might not be a real promoter. We also showed that a mutation in the putative -10 region of the P2 promoter gave rise to very low levels of β -galactosidase activity, which were close to the levels determined for the reporter vector alone, indicating that the heat shock-inducible promoter P2 is essential for transcriptional activity, at physiological temperatures and during heat shock. Furthermore, the observation that cells harboring extra copies of the *C. crescentus* σ^{32} gene presented a significant increase in GroEL levels (sixfold at 30°C),

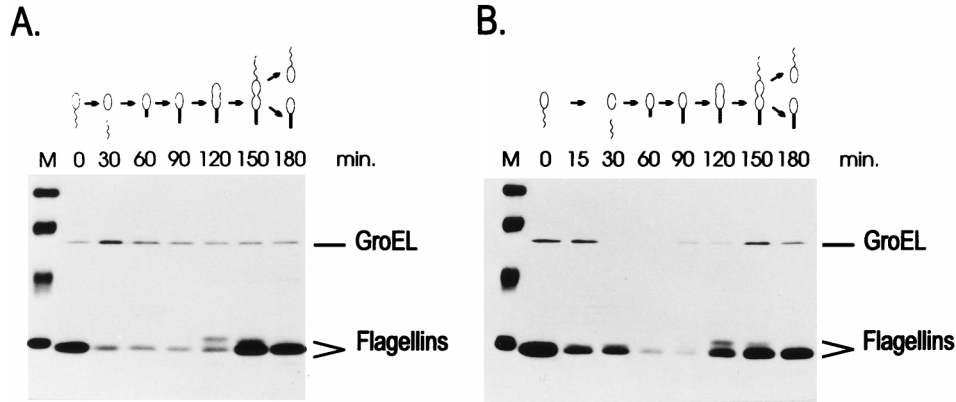


FIG. 8. GroEL synthesis is constitutive in the *hrcA* null strain. Synchronized cultures of *C. crescentus* NA1000 (B) or LS2293 containing a disruption of the *hrcA* gene (A) were pulse-labelled with [³⁵S]methionine at the indicated times during the cell cycle. Extracts of these cells were then immunoprecipitated with antisera against *C. crescentus* GroEL and antisera against *C. crescentus* flagellins, as described in Materials and Methods, to analyze GroEL and flagellin (as a control) synthesis as a function of the cell cycle. The drawings at the top of the figure are schematic representations of the *C. crescentus* cell cycle corresponding to the time indicated in each lane. M lanes contain ¹⁴C-labelled molecular mass markers (Amersham) in descending order: 97,400 Da, 66,000 Da, 46,000 Da, and 30,000 Da.

similar to that observed for the DnaK protein whose gene has been shown by in vitro transcription studies to be σ^{32} dependent (29), and the results of primer extension experiments showing that the amount of the transcript initiating at the σ^{32} -like promoter is increased in the σ^{32} -overexpressing strain corroborate the hypothesis that P2 is a σ^{32} promoter.

Besides the σ^{32} -like promoter, the *groESL* operon has a highly conserved CIRCE element located downstream of P2 (2). In *C. crescentus*, only the *groESL* operon, not the *dnaKJ* operon, contains the CIRCE element, (2, 3). A third heat shock operon was recently isolated in *C. crescentus* (22); this operon encodes the protein GrpE, which works in conjunction with DnaK, DnaJ, and HrcA, which is the putative repressor that binds to the CIRCE element. The *hrcA-grpE* operon regulatory region contains a σ^{32} promoter but no CIRCE element (22).

Consistent with the presence of the CIRCE element in the regulatory region of the *groESL* operon, its expression is al-

tered in a *hrcA* null mutant. Higher *groESL* mRNA levels were observed in *hrcA* mutant cells growing at physiological temperatures than in *hrcA*⁺ cells (22). As expected, no differences were detected in *dnaKJ* mRNA levels in *hrcA* and *hrcA*⁺ cells. In addition, no significant differences were observed in mRNA levels of both operons during heat shock when *hrcA*⁺ and *hrcA* cells were compared, suggesting a role for *hrcA* only at normal growth temperatures.

In the present report, we complemented these studies by looking at expression at the protein level. In agreement with a negative regulatory role for *hrcA* in CIRCE-containing operons, GroEL protein levels were observed to be fourfold higher in *hrcA* mutant cells than in *hrcA*⁺ cells growing at physiological temperatures, whereas no significant differences were observed in DnaK protein levels in both strains. GroEL levels increased further after heat shock, reaching similar levels in *hrcA*⁺ and *hrcA* cells after 30 min of exposure to 42°C. In addition, we showed that the rate of GroEL synthesis increases during heat shock to the same extent in the *hrcA* null mutant (threefold) and in wild-type cells (fourfold). These results are compatible with a repressor role for HrcA in cells growing at physiological temperatures.

Furthermore, we show here that point mutations in the CIRCE element located in the left arm, the right arm, or both arms of the inverted repeat, as well as a four-base deletion in the right arm, give rise to higher levels of expression of *groESL-lacZ* transcription fusions (50 to 240% higher) in cells growing at 30°C, with no significant effect on expression in cells subjected to heat shock. The data presented also indicated that HrcA binds to the CIRCE element in a sequence-specific manner.

The experiments with the *hrcA* null mutant and the site-directed mutations in the inverted repeat suggested a role for CIRCE and HrcA under non-heat shock conditions, and indeed the cell cycle-regulated expression *groESL* observed at physiological temperatures (2) is lost when either the CIRCE element is mutated or when the *hrcA* gene product is not present. Moreover, enhanced transcription observed at physiological temperatures in *hrcA* and CIRCE mutants was shown to occur from the σ^{32} -like promoter.

How the CIRCE element and the product of the *hrcA* gene control temporal expression of the *groESL* operon remains to be determined. The rate of *C. crescentus hrcA* transcription, determined by using a *hrcA-lacZ* transcription fusion, did not

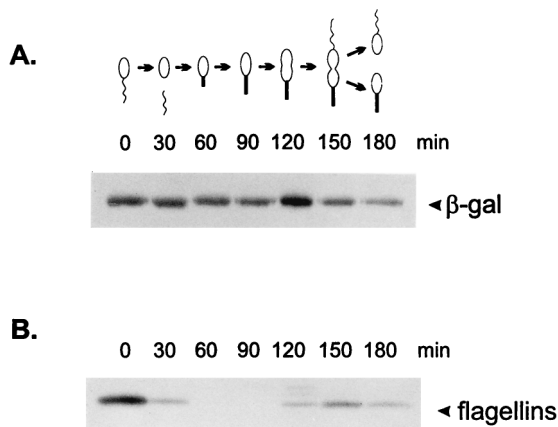


FIG. 9. Transcription directed by *groESL* regulatory region is constitutive in the *hrcA* null strain. Synchronized cultures of *C. crescentus hrcA* null strain (LS2293) harboring the wild-type *groESL-lacZ* transcription fusion pMA11 were pulse-labelled with [³⁵S]methionine at the indicated times during the cell cycle. Extracts of these cells were then immunoprecipitated with anti- β -galactosidase antibody, as described in Materials and Methods, to determine the rate of β -galactosidase (β -gal) synthesis as a function of the cell cycle. As a control of cell synchrony, the rate of flagellin synthesis was determined throughout the cycle.

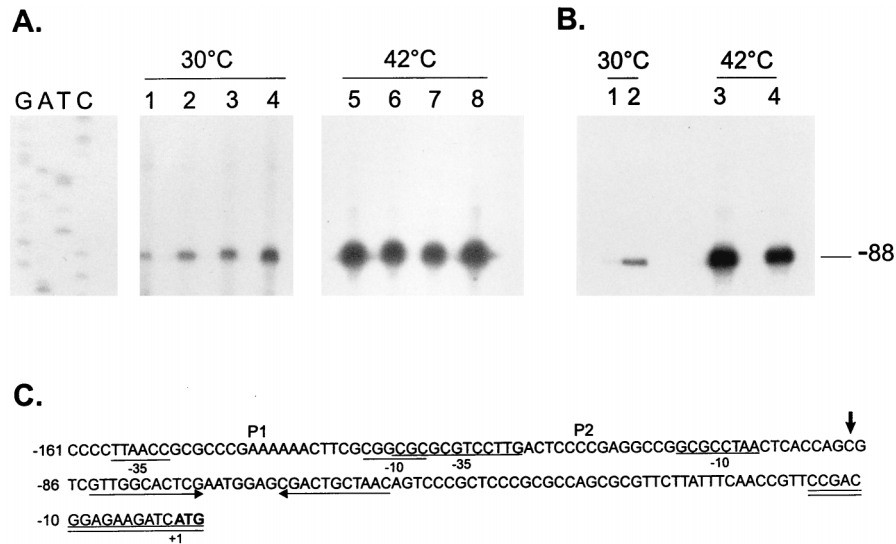


FIG. 10. Primer extension mapping of the transcription start sites in *hrcA* and CIRCE mutants. (A) An 18-nucleotide primer complementary to nucleotides -15 to $+3$ was $5'$ end labelled and hybridized to $50 \mu\text{g}$ of total RNA isolated from NA1000 cells harboring transcription fusion pMA11 (lanes 1 and 5), pRB19 (lanes 2 and 6), pRB20 (lanes 3 and 7), or pRB21 (lanes 4 and 8) grown at 30°C or exposed to 42°C for 30 min. The hybrids were then extended by using reverse transcriptase, and the extension products were resolved by denaturing gel electrophoresis and autoradiography. (B) The same procedure as described above for panel A was performed except that the RNA used was isolated from NA1000 (lanes 1 and 3) or LS2293 cells (lanes 2 and 4) grown at 30°C or after a 15-min exposure to 42°C . The sequencing ladder shown in panel A was generated by using the same 18-residue oligonucleotide as the primer and the plasmid pUC19 containing the *groESL* regulatory region as the template. (C) Sequence of the $5'$ region of the *groESL* operon. The transcription start site is shown by an arrow over the nucleotide sequence. The putative -10 and -35 regions of the P1 and P2 promoters are underlined. The CIRCE element is depicted by arrows under the nucleotide sequence. The oligonucleotide used in primer extension experiments is complementary to the sequence underlined twice. The ATG of the initiator methionine is shown in bold type.

show significant variations during the cell cycle (22). The levels of HrcA protein, however, have not been determined. A possible cell cycle-dependent posttranslational activation or inactivation of HrcA should also be investigated. For instance, the cell cycle-regulated expression of *Caulobacter* late flagellar genes is due to a temporally controlled posttranslational activation of the transcriptional regulator FlbD (28). A similar type of control could be occurring with HrcA.

The reason why *groESL* expression is under cell cycle control in *C. crescentus* is not known. However, the roles of GroEL and GroES in the assembly of multiprotein complexes and the increase in GroEL levels observed in predivisional cells (2), coincident with the time of flagellum biogenesis, suggest a possible explanation for cell cycle control of *groESL* expression. The description of an *E. coli groEL* mutant with a defect in motility strengthens this hypothesis (4). A similar study with the characterization of *C. crescentus groEL* mutants is presently being undertaken to investigate a possible role for GroEL in flagellum assembly.

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