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Identification of a *Caulobacter crescentus* Operon Encoding *hrcA*, Involved in Negatively Regulating Heat-Inducible Transcription, and the Chaperone Gene *grpE*

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In response to elevated temperature, both prokaryotic and eukaryotic cells increase expression of a small family of chaperones. The regulatory network that functions to control the transcription of the heat shock genes in bacteria includes unique structural motifs in the promoter region of these genes and the expression of alternate sigma factors. One of the conserved structural motifs, the inverted repeat CIRCE element, is found in the 5′ region of many heat shock operons, including the *Caulobacter crescentus groESL* operon. We report the identification of another *C. crescentus* heat shock operon containing two genes, *hrcA* (*hrc* for heat shock regulation at CIRCE elements) and a *grpE* homolog. Disruption of the *hrcA* gene, homologs of which are also found upstream of *grpE* in other bacteria, increased transcription of the *groESL* operon, and this effect was dependent on the presence of an intact CIRCE element. This suggests a role for HrcA in negative regulation of heat shock gene expression. We identified a major promoter transcribing both *hrcA* and *grpE* and a minor promoter located within the *hrcA* coding sequence just upstream of *grpE*. Both promoters were heat shock inducible, with maximal expression 10 to 20 min after heat shock. Both promoters were also expressed constitutively throughout the cell cycle under physiological conditions. *C. crescentus* GrpE, shown to be essential for viability at low and high temperatures, complemented an *Escherichia coli* Δ*grpE* strain in spite of significant differences in the N- and C-terminal regions of these two proteins, demonstrating functional conservation of this important stress protein.

One of the more highly conserved systems in all organisms from bacteria to humans is the cellular response to environmental stress, in which a small number of genes (known generally as heat shock genes) are induced and expressed at higher levels in order to help the cell survive (reviewed in references 35 and 42). Many stress-induced proteins are also required during growth under normal conditions and are constitutively present at lower levels. The genes encoding many of these proteins, including *grpE*, are essential for viability (2). In *Escherichia coli*, GrpE interacts with two other heat shock proteins, encoded by *dnaK* and *dnaJ*, to function in a multiprotein complex as molecular chaperones to assist in proper protein folding and stabilization (24, 29, 30). Denatured proteins are recognized and bound either directly by DnaK (in its ATP-associated form) or by a DnaK-DnaJ complex, in which hydrolysis of the bound ATP followed by GrpE-mediated exchange of the ADP for ATP allows the folded protein or peptide to be released (59). GrpE also plays a role in chaperone functions involved in DNA unwinding (65) and protein monomerization (57). In addition, GrpE, along with DnaK and DnaJ, plays a central role in heat shock response regulation by modulating the level of the heat shock-specific σ^{32} (10, 23).

Homologs of *grpE* have been found in a number of organisms, ranging phylogenetically from members of the domain *Archaea* (13) to the mitochondria of higher eukaryotes (41). In many bacteria, *grpE* is found as part of an operon with other heat shock genes, most notably *dnaK* and *dnaJ* (see Fig. 1A). In a variety of bacteria, the *grpE* gene is also located downstream of a conserved open reading frame, most commonly labeled *orfA* (see Fig. 1A). This gene is also suspected to be heat shock inducible, on the basis of its proposed cotranscription with *grpE* (40, 44, 62). Organisms with *orfA* homologs, including *Caulobacter crescentus*, also have a conserved inverted repeat (a CIRCE element) preceeding at least some of the heat shock-inducible genes. This element has been demonstrated to play a role in the regulation of the heat shock response (67, 69). Disruption of the *Bacillus subtilis* operon containing an *orfA* homolog has indirectly suggested that this gene may be involved in regulation of operons preceeded by CIRCE elements (55).

C. crescentus has the unusual property of producing two distinct cell types at each division. These two cell types differ both in their ability to replicate DNA and in their programs of gene expression (reviewed in references 8 and 25). It has been demonstrated that under physiological conditions, several heat shock homologs are synthesized at discrete times during the *C. crescentus* cell cycle (27, 28) and that their products are distributed asymmetrically upon cell division (49). The *dnaKJ* operon was shown to be transcribed from a complex set of promoters that control its response to cell cycle timing cues as well as stress cues (5, 27). In this report, we identify the gene encoding a GrpE homolog, the third component of the functional DnaK-DnaJ chaperone complex, and show that it is not linked with the *dnaKJ* operon, unlike the case in many other organisms (see Fig. 1A). We demonstrate that its transcription responds to heat stress but not to cell cycle control. In *C. crescentus*, GrpE is encoded by an operon composed of an *orfA* homolog, here named *hrcA* (*hrc* for heat shock regulation at CIRCE elements), and *grpE*. The *grpE* gene is cotranscribed

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TABLE 1. Plasmids used

Name	Relevant characteristic(s)	Source or reference
pBluescript SK(+)	Cloning vector	Stratagene
pRKlac290	Cloning vector for transcriptional fusions to *lacZ*	1
pBGST18	pBGS18 (58) with RK2 *oriT* inserted	M. R. K. Alley
pIC20R-*sacB*	Source of *sacB* gene	M. R. K. Alley
pHP45Ω	Source of Spc^{r}Ω cassette	47
pMR20	Mini-RK2 cloning vector; carries RK2 replication and stabilization functions	R. Roberts and C. Mohr
pRKlac290-delI	*dnaKJ* promoter fused to *lacZ* in pRKlac290	5
pJY400L	*fliLM* promoter fused to *lacZ* in pRKlac290	66
pWZ162	*fliQR* promoter fused to *lacZ* in pRKlac290	68
pGO.7	*flbG* promoter fused to *lacZ* in pRKlac290	26
p*fljL-lacZ*	*fliL* promoter fused to *lacZ* in pRKlac290	63
p290-Δ	*ccrM* promoter fused to *lacZ* in pRKlac290	G. Zweiger
pCS141	*mcpA* promoter fused to *lacZ* in pRKlac290	C. Stephens
pAR31	*rpoH* promoter fused to *lacZ* in pRSZ5	A. Reisenauer
pMA11	*groESL* promoter fused to *lacZ* in pRKlac290	4
pRB19	*groESL* promoter region with mutated CIRCE element fused to *lacZ* in pRKlac290	This work
pRTG9-2ΔBss	316 bp with 3′ end of *hrcA*, from *Bss*HII to *Sma*I, in the pRKlac290 polylinker	This work
pRTOΔBss	270 bp with the region upstream of *hrcA*, from *Bss*HII to *Xho*I, in the pRKlac290 polylinker	This work
pRTO6	pBGST18 carrying *sacB* and the Ω cassette disrupting *hrcA* with ~2 kb flanking sequence on either side	This work
pRTG6	pBGST18 carrying *sacB* and the Ω cassette disrupting *grpE* with ~2 kb flanking sequence on either side	This work
pRR312-1	pMR20 carrying the *hrcA-grpE* operon (*Sph*I to *Hin*dIII)	This work
pRR312-2	pMR20 carrying *grpE* only (*Bst*XI to *Hin*dIII)	This work
pRR312-3	pMR20 carrying *hrcA* only (*Sph*I to *Nco*I)	This work
pRR316-1	pBluescript SK(+) carrying *hrcA-grpE* operon, from *Sph*I to *Hin*dIII	This work
pRR316-2	pBluescript SK(+) carrying *grpE* only, from *Bst*XI to *Hin*dIII	This work
pRR316-3	pBluescript SK(+) carrying *hrcA* only, from *Sph*I to *Nco*I	This work

from the *hrcA* heat shock promoter or from a second heat shock promoter within the C-terminal coding sequence of the *hrcA* gene. We show that *grpE* is essential for viability and that the *hrcA* gene can be disrupted with no apparent physiological phenotype. However, the expression of the *groESL* operon, which contains a consensus CIRCE element in addition to a σ^{32}-dependent promoter (4), is elevated in the *hrcA*-disrupted strain only if the CIRCE sequence is intact, demonstrating a negative role of HrcA in regulating expression of CIRCE-containing operons. This, in combination with similar interpretations suggested by disruption of the *B. subtilis hrcA* operon (55), provides evidence that the *hrcA* homologs found upstream of *grpE* encode proteins that are involved in regulation of a class of heat shock-inducible genes.

MATERIALS AND METHODS

Materials. Oligonucleotides were obtained from the Protein, Amino Acid, and Nucleic Acid facility at Stanford University. DNA-modifying enzymes were obtained from Boehringer Mannheim or New England Biolabs and used according to the manufacturer's specifications. Exonuclease III deletions were performed with a kit from Stratagene. Reverse transcriptase and single-stranded RNA standards were obtained from Life Technologies, Inc. [^{35}S]methionine Trans label was obtained from ICN, and α-^{35}S-dATP, [α-^{32}P]dCTP, and [γ-^{32}P]ATP were obtained from Amersham. Sequencing was performed with a Taquence kit from U.S. Biochemicals. Other reagents were obtained from Sigma Chemical Co.

Strains, growth conditions, and plasmids. *E. coli* cells were cultured in Luria-Bertani broth at 37°C (53), and *C. crescentus* cells were grown in PYE medium (45) or M2G minimal medium (19) at 30°C. For *C. crescentus*, ampicillin, spectinomycin, and nalidixic acid were used at a concentration of 20 μg/ml; kanamycin was used at 5 μg/ml; and tetracycline was used at 1 μg/ml. For *E. coli*, ampicillin, kanamycin, and spectinomycin were used at 50 μg/ml and tetracycline was used at 10 μg/ml. The *E. coli* strains used were TG1 (12) and S17-1 (56), with the latter employed for conjugal transfer of plasmids into *C. crescentus* cells. Also used were the *E. coli grpE* deletion strain DA259 and its isogenic parent, DA258 (2). The *C. crescentus* strain used was NA1000 (formerly known as CB15N) (21); this strain with integrated plasmid pRTO6 was called LS2292 and with integrated plasmid pRTG6 was called LS2294. The strain with pRTO6 vector sequences excised, resulting in an internal disruption of the chromosomal *hrcA* gene, was called LS2293.

Plasmids used in this study are listed in Table 1. Sequencing of the region upstream of *dnaN* encoding *grpE*, *hrcA*, and *rph* was based on subclones generated from the cosmid pGF1 (50), using mapped restriction sites and exonuclease III-generated deletions. All the sequences obtained were aligned and analyzed to identify putative open reading frames and potential homologous genes from other organisms (in the PIR and SWISSPROT databases), with the Genetics Computer Group sequence analysis program package (16).

The regions upstream of *hrcA* and *grpE* were each analyzed for promoter activity by using deletions cloned into the pRKlac290 polylinker immediately upstream of the *lacZ* gene (1), to create transcriptional fusions (see Fig. 5 for diagrams of these deletions).

To disrupt the chromosomal *hrcA* or *grpE* gene, first plasmids that carried these genes with approximately 2 kb of surrounding sequence on either side were constructed. The Ω cartridge from pHP45Ω encoding *aad1* (conferring resistance to spectinomycin) flanked by both transcriptional and translational terminators was inserted at blunted unique sites within either *hrcA* or *grpE* (*Aat*II or *Nco*I sites, respectively; see Fig. 1B). These disrupted regions were transferred to the polylinker of the narrow-host-range plasmid pBGST18, carrying *nptI* (encoding resistance to kanamycin), the RK2 *oriT* for conjugal transfer, and the *sacB* gene of *B. subtilis* from pIC20R-*sacB*, to create the plasmids pRTO6 and pRTG6, for disruption of *hrcA* or *grpE*, respectively.

Complementation of the *hrcA* or *grpE* disruption strains was tested with plasmids carrying these genes in *trans*. Such plasmids included either the *hrcA-grpE* operon, the *grpE* gene alone, or the *hrcA* gene alone, in the vector pBluescript SK(+). These regions were then transferred into the polylinker of the mini-RK2 broad-host-range replicon pMR20.

The *groESL* promoter region and associated CIRCE element, from positions −318 to +3 relative to the translational start codon, were amplified by PCR and subcloned to the *lacZ* transcriptional reporter plasmid pRKlac290 to create pMA11. A derivative of this plasmid, pRB19, had the distal CIRCE repeat altered by site-directed mutagenesis as previously described (33). The CIRCE repeat sequence 5′-GTTGGCACTCG-n7-CGACTGCTAAC-3′ was altered to 5′-GTTGTTTCTCG-n7-CGACTGCTAAC-3′, to disrupt the inverted repeat (underlined bases are the sites of mutagenesis).

Disruption of *hrcA* and *grpE* genes in the *C. crescentus* chromosome. The *hrcA* and *grpE* genes were disrupted by double homologous recombination of the plasmids pRTO6 and pRTG6, by using sucrose resistance as selection of the second recombination event as previously described (6). Integration of these plasmids generated strains which were diploid for the targeted genes, with one copy wild type and the other copy disrupted. Such strains, with or without an

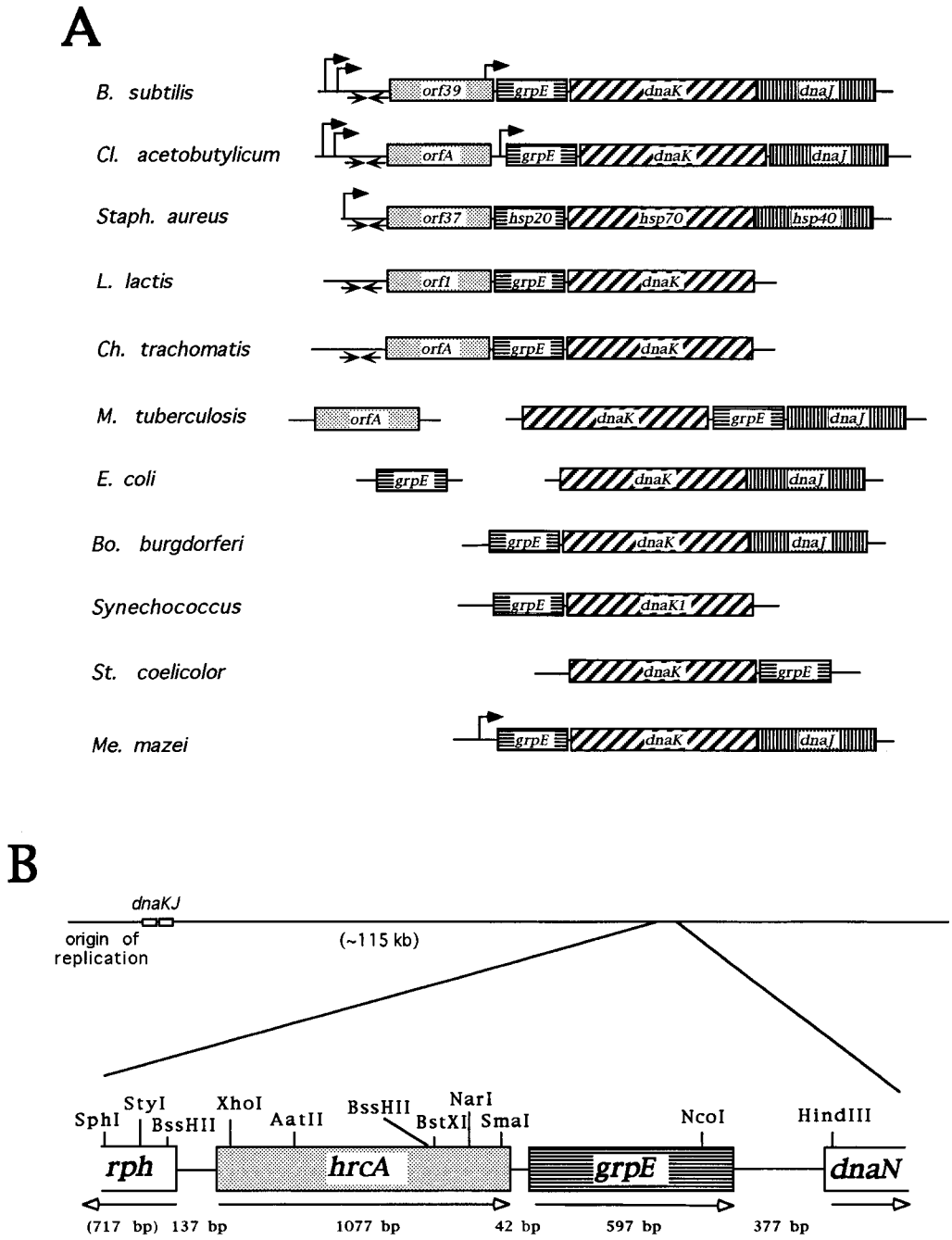

FIG. 1. Identified *grpE* regions from diverse bacteria. (A) Genetic diagrams of the genes surrounding *grpE* in a variety of bacteria, including *B. subtilis* (62), *C. acetobutylicum* (40), *Staphylococcus aureus* (44), *L. lactis* (17), *C. trachomatis* (20, 54), *M. tuberculosis* (arrangement deduced from homologous genes identified in GenBank), *E. coli* (36, 52), *B. burgdorferi* (60), *Synechococcus* sp. (43), *S. coelicolor* (9), and *M. mazei* (13). Stippled boxes represent *hrcA* homologs, and other homologs are as marked. Bent arrows represent identified promoters; in all cases heat-inducible transcription initiates at the same site as constitutive transcription. Paired inverted arrows represent the location of the consensus CIRCE regulatory inverted repeat sequence. The *dnaK* region is not linked to *orfA* in *M. tuberculosis* or to *grpE* in *E. coli*. (B) Genetic diagram of the *C. crescentus grpE* region, indicating the locations of the *rph*, *hrcA*, *grpE*, and *dnaN* genes, in relation to the *dnaKJ* operon and origin of replication. Significant restriction sites are indicated above the diagram, and lengths of open reading frames and intergenic spaces are indicated below. Open arrows indicate the direction of transcription.

added plasmid in *trans* to supply the gene product of the targeted disruption, were plated on medium containing 3% sucrose. This selected for cells in which a second homologous recombination had excised the integrated plasmid, thus leaving behind either the native or the disrupted gene. Determination of antibiotic resistance profiles allowed these alternatives to be distinguished. Chromosomal disruption of the *hrcA* gene was confirmed by Southern blot analysis (53).

Northern blot (RNA) analysis. Levels of the *groESL* and *dnaKJ* transcripts were determined at 30°C or after heat shock at 42°C for 10 min, by Northern blotting with DNA probes specific to these regions. Equivalent amounts of RNA isolated as previously described (53) were electrophoresed in a formaldehyde-alkaline agarose gel and Northern blotted as previously described (53). The amount of RNA was quantitated by densitometry. The size of the transcripts from the *hrcA-grpE* region was also determined by Northern blotting. Aliquots (10 μg each) of RNA extracted from *C. crescentus* cells grown at 30 or 42°C for 5, 10, 15, or 20 min were electrophoresed, along with RNA size standards. The

A

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B. subtilis        1 ..........  ......mltn RqllILqvII ndFiksaqPV GSrtLskkde itfSSATIRN  44
C. crescentus      1 mtqlfpqpiv rtpglaelda RardIFrrVV esYletqePV GSrtis.kqg valSpAsIRN  59
CONSENSUS            ----------  ---------- R---IL--IV --Y-----PV GS--L-----  ---SSATIRN

B. subtilis       45 EMadLEeLGF IektHsSSGR vPsekGYRyY VDhLlspvkL tksDldqIhs .ifkekiF..  101
C. crescentus     60 tMqdLaqLGL ldapHtSaGR mPthaGLRmF VDqFlevqdv aeqEkraIea rlavkqr...  116
CONSENSUS           EM--LE-LGY I---H-SSGR -P---GYR-Y VD-L-----L ---E---I--  -------Y--

B. subtilis      102 elektvqksa qiLSdLtnyt siVlgPklse nylkqIqiIp iqpdmavaIl VtntGhVenk  161
C. crescentus    117 sfeeaLaeAs siLSqLaqqa qiVvtP.vre qqVkhVeFVp lqqqqVlaVm VfedGqVenr  175
CONSENSUS            -----L--A- --LS-L----  --V--P----  --V--I-LI-  -----V--V-  V---G-V---

B. subtilis      162 tinfptkmdl sdieklvNiL ndrLsGvpmd elnerifkEv vmylrqhikn ydnildalrs  221
C. crescentus    176 lmrqapqvtp saLqeasNFL narLrGrtlt eartemqqEl daarrqlnet aarlvedgla  235
CONSENSUS            ----------  --L----NFL ---L-G----  ----------  ----------  ----------

B. subtilis      222 tF..hstnhv EklFfgGkin Mlnqpefhdi trvrslLsli EkeqdvlkLv ..qsphtgIs  277
C. crescentus    236 aWsqqeqdar slivrqqanl Ladararedi drvrqlFddl EqkqqliqLl ddvrdaeqVr  295
CONSENSUS            -L--------  E--Y--G---  L---------  ------L---  E-------L-  --------V-

B. subtilis      278 .IkIGkEndy eemencS..l ItasYsvd.q kqiGsiaIIG PtrMnYsrVV sllqhVtsdl  333
C. crescentus    296 .IyIGaEtrl fslsqsS..V IaapYmtgrq kvLGaigVIG ParLnYarVI plvdvtarvl  352
CONSENSUS            -V-IG-E---  ------S--V  I---Y-----  --LG---VIG  P--M-Y--VI  -----V----

B. subtilis      334 skaLtslyde  343
C. crescentus    353 grmMdq     358
CONSENSUS            ---L------
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FIG. 2. Alignment of proteins encoded by the *C. crescentus grpE* region. Open reading frames in the DNA sequence upstream of the *C. crescentus dnaN* gene were identified, and then the predicted proteins from these open reading frames were aligned by using the Pileup feature of the Genetics Computer Group package (16). (A) Comparison of the *C. crescentus* HrcA amino acid sequence (underlined) with the homolog from *B. subtilis* (28.6% identity with *C. crescentus*; GenBank accession no. P25499). The consensus sequence shown represents the presence of identical or similar residues in five of the seven sequences calculated with the HrcA proteins from *C. crescentus*, *B. subtilis*, *C. acetobutylicum* (25.6% identity; P30727), *S. aureus* (26.8% identity; D14715), *M. tuberculosis* (29.8% identity; U00016), *L. lactis* (22.1% identity; X76642), and *C. trachomatis* (25.4% identity; L25105). Residues that match the consensus are shown in uppercase letters. (B) Comparison of the *C. crescentus* GrpE amino acid sequence (underlined) with homologs from other bacterial species, including *M. tuberculosis* (29.4% identity with *C. crescentus*; X58406), *S. coelicolor* (24.2% identity; L08201), *C. trachomatis* (28.5% identity; P23575), *B. burgdorferi* (31.6% identity; P28609), *M. mazei* (27.3% identity; X74353), *B. subtilis* (32.0% identity; P15874), *S. aureus* (34.5% identity; D14715), *L. lactis* (35.0% identity; X76642), *C. acetobutylicum* (31.0% identity; P30726), *E. coli* (32.7% identity; P09372), and *S. cerevisiae* (30.9% identity; X78350) (numbers given are GenBank accession numbers). The consensus sequence shown represents the presence of identical or similar residues in 9 of 12 sequences; residues that match the consensus are shown in uppercase letters. The conserved domains identified by Wu et al. (64) are boxed and numbered I through V. Only the N-terminal sequence for *C. crescentus* is shown; listing of the other sequences begins at the residues indicated at the start of the first line. Asterisks indicate that the C-terminal sequences are not shown for *M. tuberculosis* (24 amino acids), *S. coelicolor* (19 amino acids), and *C. crescentus* (14 amino acids).

probes used included DNA fragments from either *hrcA* or *grpE*, end labeled as previously described (53).

Promoter localization. DNA fragments from regions upstream of either *hrcA* or *grpE*, transcriptionally fused to the reporter gene *lacZ* in pRKlac290, were established in *C. crescentus* cells via biparental conjugation. Promoter activity was determined with the standard assay for the *lacZ* gene product β-galactosidase (39). Results, reported in Miller units, are the average of at least three independent assays per construct, performed with mid-log-phase cultures grown at a temperature of 30°C.

Primer extension. For primer extension reactions, oligonucleotide primers with sequences complementary to the predicted RNA sequence at the 5′ end of the *hrcA* and *grpE* genes (5′-CTGGGAAGAGCTGCGTCATGTC-3′ and 5′-CCATCATCCGCCCGAGCACC-3′, respectively) were first radioactively labeled as previously described (53). Approximately 0.5 pmol of labeled primer was then annealed either to 40 μg of yeast tRNA (included as a control) or to 40 μg of *C. crescentus* cellular RNA that was isolated via the standard protocol (53) from cells grown at 30°C or at 42°C for 5, 15, or 20 min. These oligonucleotides were then used as primers for the enzyme reverse transcriptase. DNA sequencing products obtained with these same primers were used as standards to identify the positions of the transcriptional start sites (3).

Promoter expression during heat shock. *C. crescentus* cells containing *lacZ* transcriptional fusions representing the minimal *hrcA* or *grpE* promoter regions as well as *lacZ* fusions to a variety of other *C. crescentus* promoters were grown to mid-log phase in minimal M2G medium at 30°C and then shifted to 42°C to begin the heat shock assay. At 5, 10, 15, 20, 30, 45, and 60 min, 1 ml of cells was removed and pulse labeled with 10 μCi of [^{35}S]methionine Trans label for 2 min to radioactively label newly synthesized proteins. Protein labeling was also done at 30°C as representational of proteins present under non-heat shock conditions. After labeling, the cells were pelleted and lysed by boiling and then immunoprecipitated with anti-β-galactosidase antibody mixed with equivalent numbers of counts (typically 2×10^6 cpm) for each sample, as described previously (32).

Complementation of an *E. coli* Δ*grpE* mutant. The ability of *grpE* from *C. crescentus* to functionally substitute for the *E. coli* gene was tested by introducing plasmids expressing *C. crescentus hrcA*, *grpE*, or both into the Δ*grpE E. coli* strain DA259 or its isogenic parent, DA258. Growth of these strains was assessed at 42°C while maintaining selection for the plasmids, and capacity to replicate bacteriophage λ was determined by infecting approximately 10^7 cells grown under selection for the complementing plasmid with 10^2 PFU of λ clear phage (a gift from Dan Wall). The cells and phage were overlaid in top agar onto Luria-Bertani plates, incubated at 30°C, and monitored for the formation of plaques.

Nucleotide sequence accession number. The DNA sequence data described in this work have been deposited in GenBank with accession number U33324.

RESULTS

DNA sequence determination of the region upstream of *dnaN*. Previous work has shown that the *C. crescentus dnaN* gene is located on the identified cosmid pGF1 (50). In the process of sequencing the *dnaN* gene (51), we determined that, unlike other bacteria in which *dnaA*, *rpmH*, and/or *rnpA* genes are located upstream of *dnaN* (11), a *Caulobacter* homolog of *grpE* was located at this position (Fig. 1B). Furthermore, we identified another open reading frame, separated from *grpE* by 42 bp (Fig. 1B), with significant homology to an open reading frame found upstream of *grpE* in several other bacteria, including *B. subtilis* (62), *Clostridium acetobutylicum* (40), *Lactococcus lactis* (17), *Staphylococcus aureus* (44), and *Chlamydia trachomatis* (54). This *C. crescentus* open reading frame was designated *hrcA* (*hrc* for heat shock regulation at CIRCE elements) on the basis of data suggesting its function (see below). Another open reading frame was identified upstream of *hrcA*,

B

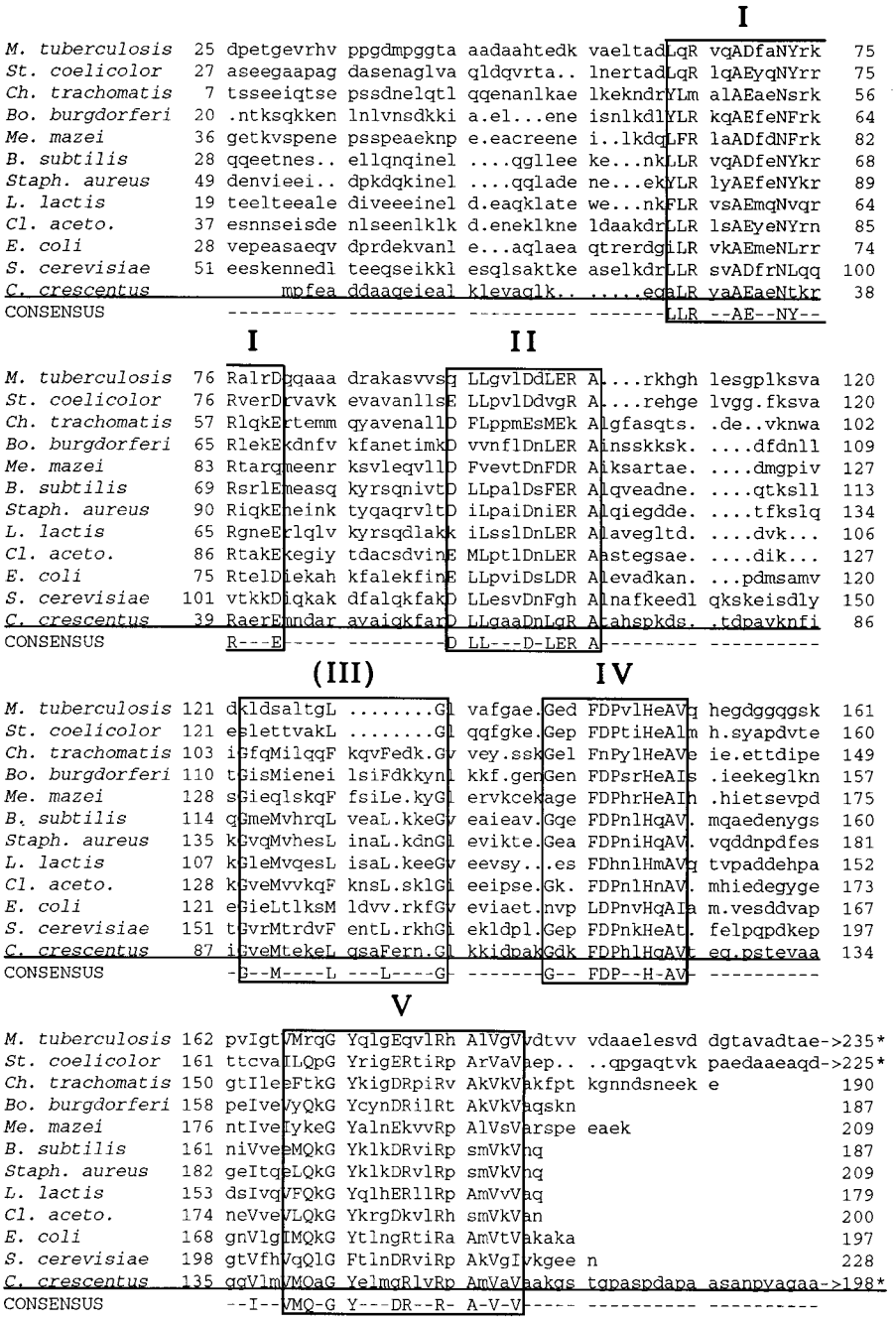

FIG. 2—*Continued.*

in the opposite orientation, with strong homology (55.3% overall identity at the amino acid level) to the *rph* gene from *E. coli* encoding the RNase PH protein. The role of RNase PH in bacterial growth is not clear, since in *E. coli* and *B. subtilis* this gene can be disrupted with no apparent adverse effect on the cells (15, 46). The divergent transcription of *rph* demonstrates that *hrcA* and *grpE* constitute an independent transcriptional region and are not part of an operon extending upstream of *hrcA*. Alignments of the predicted protein sequences with their homologs in other organisms are shown in Fig. 2A (*hrcA*) and B (*grpE*).

Effect of chromosomal disruptions of *grpE* or *hrcA*. It has been reported that disruptions of the *grpE* gene are lethal in *E. coli* except in certain genetic backgrounds (2). Attempts to independently disrupt the *C. crescentus grpE* and *hrcA* genes were made. The *grpE* gene appeared to be essential, since no disrupted strains could be obtained at either 30 or 18°C (Table 2). Disrupted strains were readily generated, however, if a plasmid supplying either *grpE* alone or both *hrcA* and *grpE* was present in *trans* but not if the plasmid supplied only *hrcA* or

TABLE 2. Insertional inactivation of *C. crescentus* *hrcA* and *grpE* genes

Gene disrupted	Plasmid in *trans*	Gene(s) in *trans*	Resolution at[a]: 30°C Wild type[b]	30°C Disrupted[c]	18°C Wild type[b]	18°C Disrupted[c]
hrcA	None	None	161	101	ND[d]	ND
grpE	None	None	457	0	546	0
grpE	pMR20	(Vector)	496	0	389	0
grpE	pRR312-1	*hrcA*, *grpE*	43	47	ND	ND
grpE	pRR312-2	*grpE*	53	36	ND	ND
grpE	pRR312-3	*hrcA*	497	0	383	0

[a] Numbers represent the number of colonies displaying each phenotype.
[b] Resolution of LS2292 or LS2294 by a second homologous recombination, screened at either 18 or 30°C, to delete vector sequences and the disrupted copy of the gene, leaving the wild-type gene in the chromosome.
[c] Resolution of LS2292 or LS2294 by a second homologous recombination, screened at either 18 or 30°C, to delete vector sequences and the wild-type copy of the gene, leaving the disrupted gene in the chromosome.
[d] ND, not determined.

none of the genes (vector only), confirming that *grpE* is essential for the viability of *C. crescentus* (Table 2).

For *hrcA*, a chromosomal insertional disruption could easily be obtained at 30°C, showing that this gene is not essential (Table 2). To assess the function of *hrcA*, we compared the phenotypes of isogenic NA1000 (wild type) and LS2293 (disrupted *hrcA*) cells. No detectable phenotype was observed upon analysis of a variety of parameters, including growth rates at 30, 37, or 42°C; viability at elevated temperatures; establishment of thermotolerance; DNA synthesis rates at 30 or 42°C; or susceptibility to other stress inducers, including ethanol, the electron transport inhibitor sodium azide, or the heavy metal sodium arsenite. Further, disruption of *hrcA* had no marked effect at either 30 or 40°C on expression from the heat shock promoters transcribing *rpoH*, *dnaK*, *hrcA*, or *grpE* (Table 3). These promoters lack all CIRCE element consensus sequences shown to be important in the regulation of chaperone gene operons in other *hrcA* homolog-carrying organisms. The *C. crescentus groESL* operon, however, is preceded by a consensus CIRCE element (4), and disruption of *hrcA* substantially increased expression from the *groESL* promoter at physiological temperature (although not at elevated temperature), as measured by Northern blotting (Fig. 3) or by transcriptional fusion (Table 3). It should be noted that P_{groESL} was not fully derepressed at 30°C by disruption of *hrcA*, suggesting that another level of heat shock control exists, perhaps through the heat shock sigma factor σ^{32}. When the CIRCE element was inactivated by changing three nucleotides in the left half of the inverted repeat, P_{groESL}-mediated expression increased to the level seen for the Δ*hrcA* strain, and subsequent disruption of *hrcA* had no further effect (Table 3). This suggests that insertional inactivation of *hrcA* eliminates the factor responsible for CIRCE-mediated negative regulation, either directly or indirectly.

Transcription from the *hrcA-grpE* region. Northern blot analyses with probes derived from either *hrcA* or *grpE* were done to determine whether these genes constitute an operon, as their homologs do in *B. subtilis* (62) and *C. acetobutylicum* (40). A transcript of approximately 1.6 kb was detected in blots with either the *hrcA* probe (Fig. 4) or the *grpE* probe (data not shown). This corresponds to the expected size for a transcript encoding both genes; no other transcripts were detected in the blots with either probe. Examination of mRNA abundance after heat shock revealed a dramatic increase in the 1.6-kb mRNA, demonstrating that transcription of this operon is induced by heat shock.

To identify functional promoters, subclones of the *hrcA-grpE* operon were transcriptionally fused to *lacZ* and assayed for β-galactosidase activity in *C. crescentus* cells grown at 30°C (Fig. 5). Weak promoter (P_{hrcA}) activity transcribing into *hrcA* (~1,000 Miller units) was localized to the region between the divergent *hrcA* and *rph* genes, between the *Bss*HI and *Xho*I sites (Fig. 5A). As shown in Fig. 5B, promoter activity (P_{grpE}) was also detected in the region between the *Xho*I and *Nco*I sites, encoding most of *hrcA* and *grpE* (pRTG7-1). Further deletions localized promoter activity to a 316-bp region located entirely within the C-terminal *hrcA* coding sequence, between the *Bss*HII and *Sma*I restriction sites (Fig. 5B). The reduction from ~700 to ~400 Miller units is likely to be due to differences in the 3′ ends and/or the position of cloning of these fusions within the reporter vector, factors known to alter expression levels up to twofold when this vector is used (data not shown). Testing of further deletions constructed with the internal *Nar*I site revealed activity for both upstream and downstream fragments. This appears to be the result of bisection of the −10 and −35 promoter regions by digestion at the *Nar*I site (see Fig. 7) and fusion of fortuitous vector sequences that reconstructed functional promoters (data not shown). Promoter activity of these deletions was also monitored after heat shock at 40°C; all showed small but reproducible increases in activity (data not shown), suggesting that the elements required for heat induction are intrinsic to the promoters.

Transcriptional start sites within the *hrcA-grpE* region. On the basis of the genetic mapping of the promoters, primer extension was employed to precisely identify the transcriptional start sites. A single start site for *hrcA* was found 78 bp upstream of the proposed translational start codon (Fig. 6A).

TABLE 3. HrcA acts to negatively regulate expression of a CIRCE-controlled operon

Growth temp and plasmid	Promoter from[a]:	Activity in[b]: NA1000 cells	Activity in[b]: LS2293 cells	*hrcA*/wt ratio[c]
30°C				
pRKlac290	–[d]	89	76	0.85
pAR31	*rpoH*	2,978	2,480	0.83
pRKlac290-Del1	*dnaK*	3,245	2,829	0.87
pRTOΔBss	*hrcA*	672	500	0.74
pRTG9-2ΔBss	*grpE*	297	251	0.85
pMA11	*groE*	1,805	3,191	1.77
pRB19	*groE*mutCIRCE	3,011	2,819	0.94
40°C				
pRKlac290	–	54	57	1.06
pAR31	*rpoH*	4,172	4,200	1.01
pRKlac290-Del1	*dnaK*	4,662	4,512	0.97
pRTOΔBss	*hrcA*	867	766	0.89
pRTG9-2ΔBss	*grpE*	512	518	1.01
pMA11	*groE*	4,161	4,155	1.00
pRB19	*groE*mutCIRCE	3,905	3,701	0.95

[a] The promoter indicated was transcriptionally fused to the *lacZ* gene in the plasmid pRKlac290, and then this plasmid was established in the indicated isogenic strains.
[b] Activity of promoters was assayed by measuring the level of β-galactosidase in the cells grown to mid-log phase at 30°C or after shift to 40°C for 1 h. The results are reported in Miller units and represent the average of triplicate assays.
[c] wt, wild type.
[d] –, no promoter.

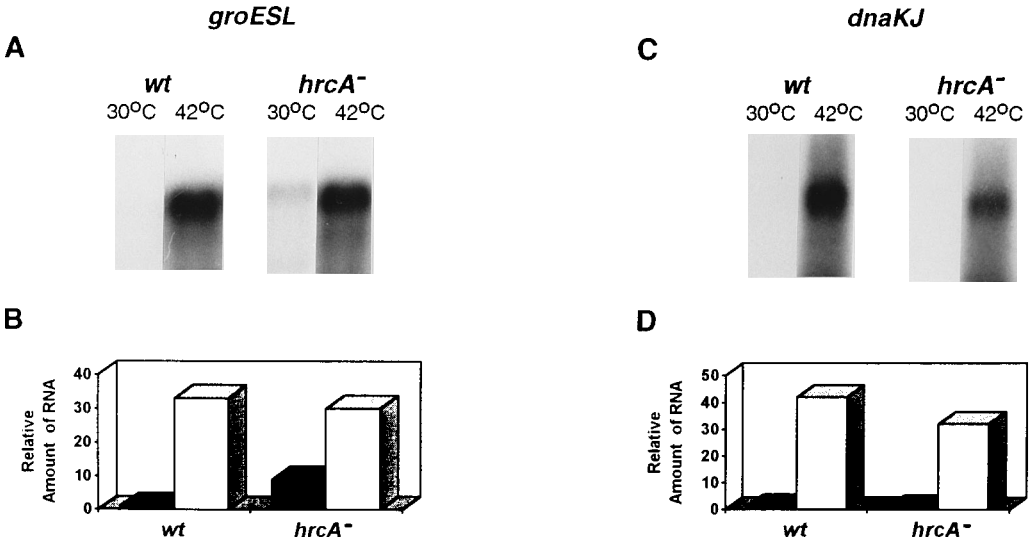

FIG. 3. Northern analysis of *groESL* and *dnaKJ* transcription in wild-type and *hrcA* disruption strains. (A and C) Northern blot analysis of the levels of *groESL* and *dnaKJ* transcripts, respectively, at 30°C and after shift to 42°C for 10 min. (B and D) Quantitation of the mRNA levels observed in panels A and C, respectively, with the amount of each type of mRNA present in wild-type (wt) NA1000 cells at 30°C set at 1.0. ■, 30°C; □, 42°C.

For *grpE*, one transcriptional start site was identified, 158 bp from the second candidate start codon (Fig. 6B), which aligned better with the *C. crescentus* Shine-Dalgarno consensus sequence. The *hrcA* and *grpE* promoter regions upstream of these start sites exhibited some homology to the consensus for σ^{32} promoters in *E. coli* as well as to other *C. crescentus* heat shock promoters (Fig. 7).

Transcriptional patterns of P_{hrcA} and P_{grpE} during the *C. crescentus* cell cycle. Rates of transcription of the minimal *hrcA* and *grpE* promoter fusions to *lacZ* (pRTOΔBss and pRTG9-2ΔBss, respectively) were measured in synchronous cultures at 30°C as previously described (21). The β-galactosidase reporter protein failed to show a substantial, reproducible change in its level of expression for either *hrcA* or *grpE* during the cell cycle (data not shown), demonstrating that transcription from both P_{hrcA} and P_{grpE} at 30°C is constitutive.

Heat shock inducibility of *hrcA* and *grpE* promoters. Primer extension analysis with RNA from heat-shocked *C. crescentus* cells indicated an increase in *grpE* and *hrcA* transcripts throughout the 20-min period of heat shock, initiating in both cases from the same transcriptional start site as that used during normal growth at 30°C (Fig. 6). Heat shock inducibility of gene expression from these promoters was also assessed by shifting *C. crescentus* cultures carrying the minimal *hrcA*::*lacZ* and *grpE*::*lacZ* reporter constructs (pRTOΔBss and pRTG9-2ΔBss, respectively) to 42°C. Fusion expression was assayed by pulse labeling and immunoprecipitation of β-galactosidase; expression from both P_{hrcA} and P_{grpE} increased only moderately during the first 5 to 10 min after the start of heat shock and then decreased during the remainder of the assay (Fig. 8A). This apparent discrepancy between mRNA level and protein expression upon heat shock may indicate that the reporter protein β-galactosidase is not synthesized as efficiently at elevated temperatures. Similar patterns were seen with larger subcloned fusions (pRTOΔSty and pRTG9-2), indicating that the expression patterns were not an artifact caused by the deletion of an important sequence within the promoter regions (data not shown). Expression of the *dnaK* heat shock promoter region (5, 27) exhibited the same general kinetics as that of the *hrcA* and *grpE* promoters (although with greater magnitude of induction), peaking during the first 10 min and decreasing over the remainder of the assay (Fig. 8A). This pattern suggests the importance of these genes in mounting the initial response to elevated temperature. Such patterns are in contrast to expression of other *C. crescentus* promoters fused to *lacZ*, which showed a 10- to 15-fold repression of transcription after 5 to 10 min of exposure to high temperature. All of the flagellar and chemotaxis gene promoters assayed, including P_{fliLM}, P_{flgE}, and P_{fljL} (Fig. 8B), as well as P_{fliQR}, P_{cheA}, and the related promoter P_{ccrM} (data not shown), were repressed with no recovery even after an hour at 42°C. Lack of expression at this temperature

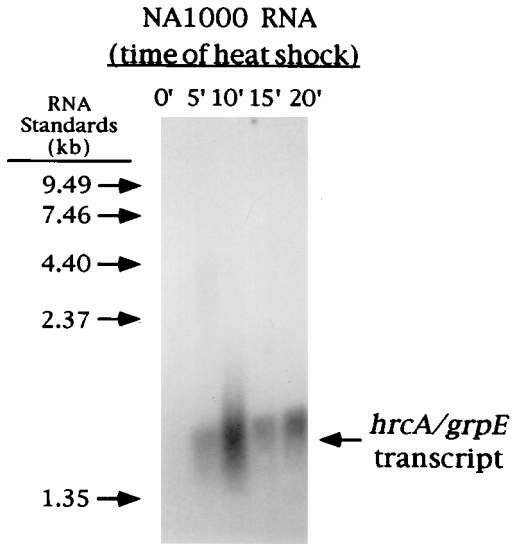

FIG. 4. Northern analysis of transcripts from the *hrcA-grpE* region. Cotranscription of the *hrcA* and *grpE* genes was assessed by Northern blotting of RNA from *C. crescentus* NA1000 cells grown at 30°C (0′) or at 42°C for 5, 10, 15, or 20 min (5′, 10′, 15′, and 20′, respectively). The blot was probed with sequences from the *hrcA* gene, and the hybridizing band was compared with single-stranded RNA standards of the indicated sizes.

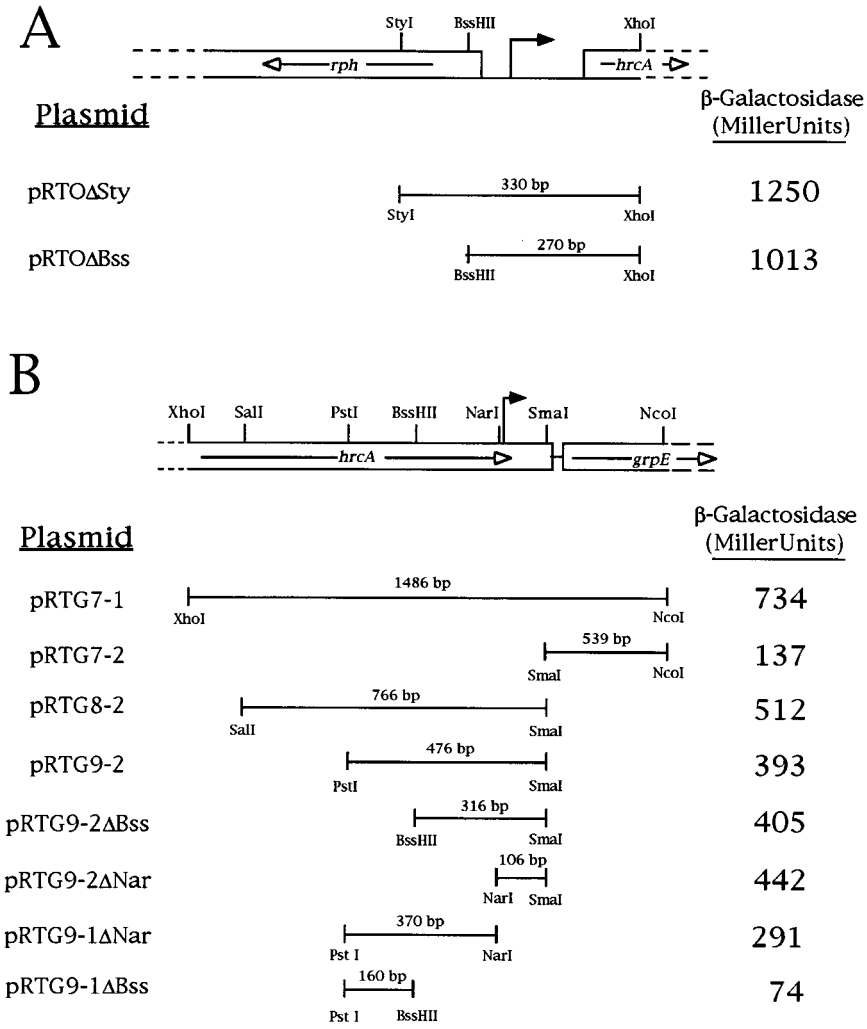

FIG. 5. Genetic localization of *hrcA* and *grpE* promoters. Relative promoter activities of various fragments of both the *hrcA* (A) and *grpE* (B) upstream regions, transcriptionally fused to the reporter gene *lacZ* in the plasmid pRKlac290 and assayed at the physiological temperature of 30°C. The positions of the different restriction sites are shown in each region, and the lines indicate the fragments assayed for promoter activity. Bent arrows indicate the approximate locations of promoter activity, based on the results obtained. The background activity for the pRKlac290 vector was 121 Miller units; this was not subtracted from the values shown.

correlates well with the observed loss of motility of *C. crescentus* cells at 42°C (28). Fusions to the *xyl* promoter (38) (Fig. 8B) and also P_{dnaN}, P_{dnaX}, and P_{gyrB} (data not shown) demonstrated a similar repression during the first 10 min of the assay, but promoter activity was restored at later time points, suggesting a general adaptation to the elevated temperature. The kinetics of P_{hrcA} and P_{grpE} induction compared with that of the other promoters tested further supports their assignment as heat shock promoters.

Complementation of the *E. coli* Δ*grpE* mutant. In *E. coli*, a disruption of the *grpE* gene can be made in the presence of uncharacterized extragenic suppressors of *dnaK* mutations; however, such a strain exhibits thermal sensitivity at 42°C and cannot support DNA replication of bacteriophage λ (2). The *C. crescentus grpE* gene was tested for functional complementation of these phenotypes. Plasmids containing the *C. crescentus hrcA* and/or *grpE* genes expressed from a P_{lac} promoter in the vector were introduced into the *E. coli* Δ*grpE* strain DA259 or its isogenic parent, DA258. The resulting strains were examined for temperature-sensitive growth and the ability to support replication and plaque formation by bacteriophage λ. As expected, the wild-type parent DA258 showed no detectable phenotypic differences in the presence of any of the plasmids. DA259, carrying the vector alone or a plasmid supplying only *hrcA*, was temperature sensitive and resistant to λ infection, demonstrating no complementation. However, when either the *hrcA-grpE* operon or *grpE* alone was supplied in *trans*, the strain was able to grow at 42°C and support λ infection, demonstrating functional complementation in *E. coli* by the *C. crescentus grpE* homolog (Table 4).

DISCUSSION

The *C. crescentus grpE* gene is not linked to *dnaK* and *dnaJ* but rather is flanked upstream by *rph* and *hrcA* and downstream by *dnaN* (Fig. 1B). The *hrcA* and *grpE* genes constitute an operon, as shown by cotranscription demonstrated by Northern analysis (Fig. 4). The genes are separated most likely by 42 bp (to the second potential AUG start codon, which aligns with a better Shine-Dalgarno consensus sequence [18]). This genetic arrangement of *hrcA* and *grpE* is similar to that seen in many other bacteria (Fig. 1A) (17, 40, 44, 54, 62). The

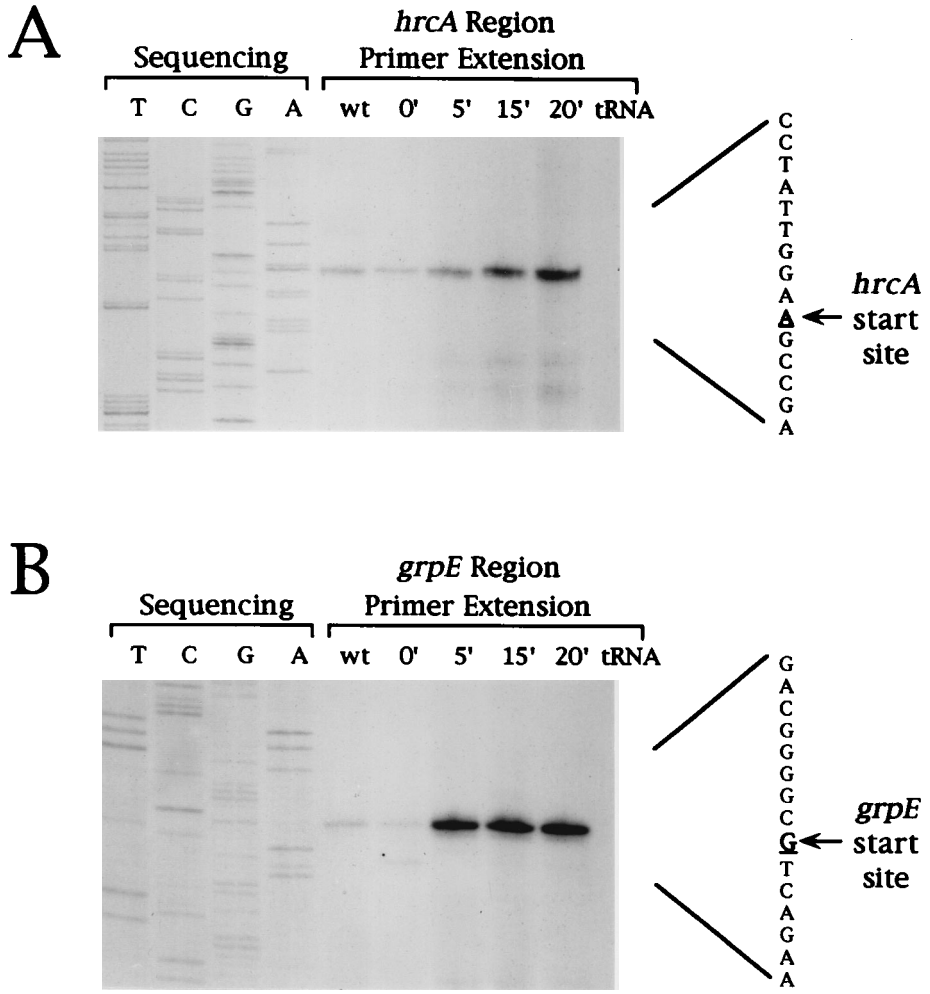

FIG. 6. Transcriptional start site mapping by primer extension. Primer extension reactions were performed with oligonucleotide primers and RNA isolated from *C. crescentus* NA1000 cells grown at 30°C (wt) or at 42°C for 0, 5, 15, or 20 min (0′, 5′, 15′, and 20′, respectively). Reactions with yeast tRNA as template were included as negative controls. Products of the reactions were electrophoresed along with DNA sequence ladders generated with the same primers. Analyses of the regions upstream of *hrcA* (A) and *grpE* (B) are shown, with the DNA sequence surrounding the start site indicated. The identified transcriptional start sites are indicated by boldfaced underlined nucleotides in the sequences. Exposure times for panels A and B were different; thus, no comparison of relative transcript abundance may be made between them.

only bacterium in which homologs of the *hrcA* and *grpE* genes appear to be genetically separated is *Mycobacterium tuberculosis*, for which the identification of *hrcA* reported here is based on sequence homology with an uncharacterized cosmid (number B1937), and this organism's *grpE* gene is flanked instead by *dnaK* and *dnaJ* (Fig. 1A). No *hrcA* homolog has been detected in *E. coli* (40, 62); this may be significant, since *E. coli* chaperone genes also lack CIRCE elements, strengthening the genetic correlation between the presence of CIRCE elements and the *hrcA* gene.

Analysis of the GrpE homolog of *C. crescentus* revealed amino acid identities ranging from 24.5 to 35.0% (Fig. 2B). Closer analysis showed complete conservation within all of the five blocks of amino acid similarity proposed by Wu et al. (64); these regions (with the exception of region III, which is not strongly conserved in *M. tuberculosis* or *Streptomyces coelicolor*) are strongly conserved among GrpE homologs from *Archaeobacteria* (*Methanosarcina mazei* [13]) to the lower eukaryotes (*Saccharomyces cerevisiae* [7, 31, 34, 61]). GrpE homologs have also recently been identified in mammalian mitochondria on the basis of their interaction with *E. coli* DnaK (41), suggesting a very broad range of functional conservation of the *grpE* gene. It is interesting to note that while the identified blocks of sequence are strongly conserved, and at least in *E. coli* have been shown to be important for function (64), the spacing between blocks II and III is quite variable, as are the lengths and sequences of N- and C-terminal tails. It is likely that these regions are not significant in the functioning of *grpE*, since it was found that, like the *Borrelia burgdorferi grpE* gene (60), the *C. crescentus* gene was able to complement an *E. coli* Δ*grpE* mutant strain for growth of bacteriophage λ and temperature sensitivity (Table 4), in spite of the substantial differences between *E. coli* and *C. crescentus* GrpE sequences in the N- and C-terminal regions.

Homology among HrcA family members, ranging from 22.1 to 29.8% amino acid identity (49.8 to 54.5% similarity), was found. While weak, this homology was significant in comparison with alignment of these sequences after randomization, which yielded identities of only 14.4 to 18.9%. Analysis of the aligned sequences revealed regions of strong conservation both at the N-terminal end and in a short stretch near the C terminus. Members of the HrcA protein family do not contain any

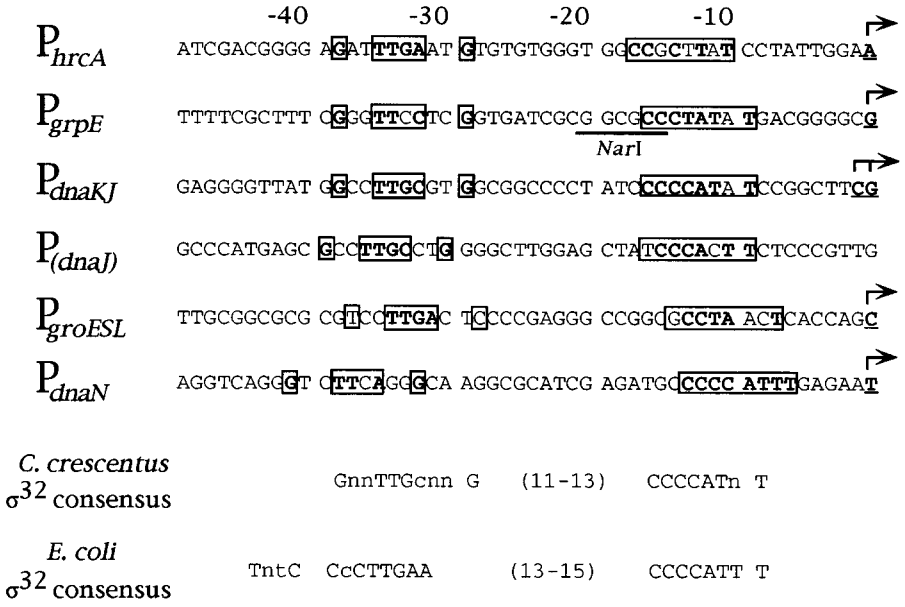

FIG. 7. Comparison of *C. crescentus* heat shock promoter sequences. An alignment of the *C. crescentus* heat shock promoters identified to date is shown, with boxes indicating conserved sequence elements. Boldface nucleotides within the boxes show conservation with the indicated putative consensus sequence. The promoters shown include those for *hrcA* and *grpE* (this work), *dnaK* and *dnaJ* (27), *groESL* (4), and a heat shock promoter upstream of *dnaN* (51). Heat shock-inducible transcriptional start sites (shown by boldfaced, underlined bases) have been mapped for all of these promoters except *dnaJ*, which is based only on sequence comparison, as indicated by the parentheses surrounding the gene name. Also shown is the *E. coli* heat shock σ^{32} consensus sequence (14). The underlined sequence in P_{grpE} is the *Nar*I restriction site shown in Fig. 5.

strongly hydrophobic regions, nor are their predicted secondary structures strikingly similar (results not shown).

Transcription of *hrcA* and *grpE* in *C. crescentus*, assessed by monitoring mRNA levels, was shown to be strongly induced by heat shock. This correlates well with the large increase in synthesis rates of the proteins DnaK and GroEL upon heat shock (28, 49). Additionally, the expression rate of a reporter gene transcriptionally fused to either the *hrcA* or *grpE* promoter increased during the initial 10 min of heat shock, although to a much smaller degree. This observed difference in the magnitude of the heat shock response is not due to instability of the β-galactosidase protein (which has a half-life of more than 90 min at either 30 or 42°C [data not shown]) but rather may reflect a general inhibition in translational efficiency of this protein. This is supported by the finding that β-galactosidase expression from non-heat shock promoters decreased more than 10-fold upon heat shock, although nonflagellar promoters did regain activity later, perhaps in adaptation to the elevated temperature.

Transcriptional fusions and primer extension analysis demonstrated that transcription of the *hrcA-grpE* operon in *C. crescentus* was dependent on both a primary promoter located upstream of *hrcA* and a weaker promoter potentially transcribing the *grpE* gene alone. This organization is somewhat reminiscent of the *C. crescentus dnaKJ* operon, which carries a σ^{32} recognition promoter in front of *dnaK* and another proposed σ^{32} promoter upstream of *dnaJ*, whose function has not yet been addressed (27). Although mRNA initiating from P_{grpE} was observed by primer extension, no transcript from P_{grpE} could be detected by the Northern blotting technique, possibly because of its weaker expression or the sensitivity of Northern blotting to sample degradation. The in vivo role of P_{grpE} in expression of *grpE* is still not clear. Both promoters were examined for similarity to other *C. crescentus* heat shock-induced promoters. The derived consensus sequence is GnnTTG(C/A)nnG at −35 bp, 11 to 13 bp, and CCC(C/T) ATnT at −10 bp, relative to the start of transcription. This consensus is distinct from the *C. crescentus* σ^{70} consensus (37) but does show some similarity to the σ^{32} consensus sequence for *E. coli* (14). The differences among the *C. crescentus* heat shock promoters may explain the variable extent of heat shock induction or may indicate that multiple sigma factors are involved in recognition of these promoters.

Like *E. coli grpE* (2), the *C. crescentus grpE* homolog is an essential gene. In contrast, *hrcA* is not essential, and its inactivation caused no discernable effect on physiological growth or on the stress response parameters tested. Transcription of most *C. crescentus* heat shock-inducible operons was also not affected by disruption of *hrcA*. The only exception was expression from the *groESL* promoter region, which was elevated at vegetative temperature in the *hrcA* disruption mutant when either mRNA levels or expression of a transcriptional fusion was assessed. The *groESL* operon is the only *C. crescentus* operon thus far shown to contain the conserved CIRCE element important in regulating chaperone expression in other bacteria (69). This suggests a CIRCE-dependent negative regulatory role either for HrcA or, if the *hrcA* disruption causes polar effects, for GrpE. On the basis of the viability of the mutant *hrcA* strain, expression of the essential *grpE* gene in this strain is likely to be close to that in the wild type, by virtue of both the minor promoter within *hrcA* transcribing *grpE* and transcriptional readthrough from the Ω cassette itself (the transcriptional terminators flanking this cassette are not efficiently used in *C. crescentus* [1a]). This leaves HrcA as the most likely candidate for the negative regulatory factor.

A model in which transcription of HrcA-regulated genes is repressed at vegetative temperature by association of HrcA (or a factor controlled by HrcA) with the CIRCE element to block transcription may be proposed. Expression of these genes would be induced at elevated temperature by removal of HrcA

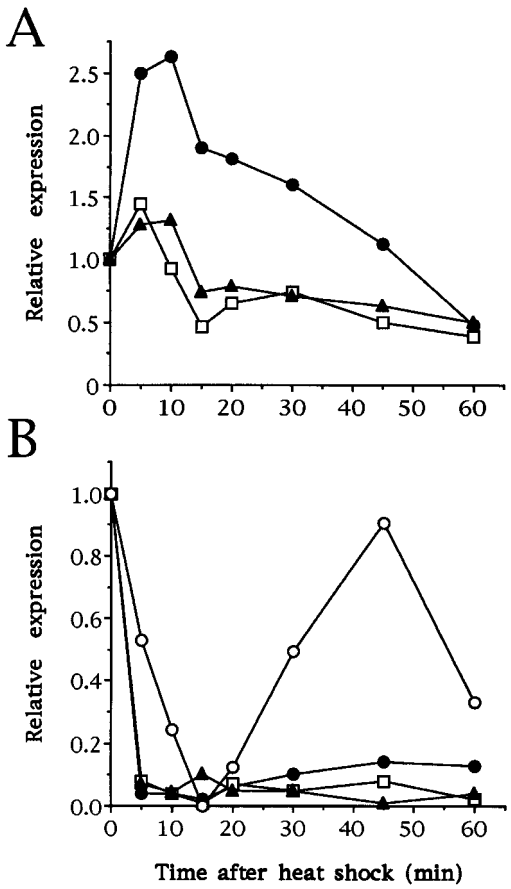

FIG. 8. Expression of *C. crescentus* promoters following heat shock. The levels of expression of various *C. crescentus* promoters during 60 min of exposure to 42°C, as determined by monitoring transcriptional fusions of the promoters to *lacZ* by immunoprecipitation of the β-galactosidase reporter, are shown. The levels of expression of these fusions were evaluated relative to expression of each gene at 30°C (normalized to 1.0). Time zero in each case represents the time of shift from 30 to 42°C. (A) Closed circles represent P_{dnaK} (pRKlac290-delI), squares represent the minimal P_{hrcA} (pRTOΔBss), and triangles represent the minimal P_{grpE} (pRTG9-2ΔBss). (B) Closed circles represent P_{fliLM} (pJY400L), squares represent P_{flgE} (pGO.7), triangles represent P_{fljL} (p*fljL-lacZ*), and open circles represent P_{xylt}

control. Indeed, inactivation of the CIRCE element blocked the effect of *hrcA* disruption (Fig. 3 and Table 3), suggesting that the negative regulation of HrcA is mediated through the CIRCE element. The model is further supported by the lack of an effect of the *hrcA* disruption mutation when expression was assayed at 42°C, at which temperature HrcA should no longer have an effect. These observations are consistent with the CIRCE-mediated negative regulatory role suggested by Schultz et al. for the *B. subtilis hrcA* homolog (*orf39*) (55). It is still not clear whether HrcA interacts directly with the CIRCE elements or exerts its repressive effect indirectly. It is also apparent that the *C. crescentus groESL* operon is regulated by more than just the HrcA-CIRCE system, since mRNA levels still show heat shock inducibility in the *hrcA* disruption strain (Fig. 3). This is consistent with the importance of the σ^{32}-like promoter in heat shock expression (4); the combination of the CIRCE element and a σ^{32}-like promoter to regulate heat shock-inducible transcription has been reported in only one other instance: the *groESL* operon of *Bordetella pertussis* (22).

While the *C. crescentus dnaKJ* operon lacks a CIRCE consensus, substantial secondary structure in the 5′ untranslated leader of the *dnaK* mRNAs has been shown to attenuate expression of this operon (5). This attenuation is not dependent on the presence of HrcA. Thus, an additional mechanism of heat shock gene regulation at a posttranscriptional level acting to regulate expression of the *dnaKJ* mRNA is likely to exist in *C. crescentus*.

Recently, a homolog of *rpoH* encoding the heat shock σ^{32} protein has been identified in *C. crescentus* (48); direct interaction of this sigma factor with the proposed σ^{32} promoter sequences may now be tested in vitro. With the identification of these additional components of the heat shock response, a more comprehensive picture of heat shock gene expression and function under physiological conditions or stress in *C. crescentus* is emerging. This bacterium appears to regulate its heat shock genes by multiple mechanisms, including the use of the heat shock sigma factor σ^{32} (for *dnaKJ*, *hrcA*, *grpE*, and *groESL*), CIRCE element-HrcA-mediated repression (*groESL*), and another mechanism(s) dependent on mRNA secondary structure (*dnaKJ*). Additionally, the *dnaKJ* and *groESL* operons are transcribed by σ^{70}-dependent promoters in a cell cycle-dependent manner, suggesting a function for these chaperones during the cell cycle.

TABLE 4. Complementation of *E. coli* Δ*grpE* with the *C. crescentus grpE* gene

Strain	Plasmid in *trans*	Gene(s) in *trans*	Growth at 42°C[a]	Replication of λ clear[b]
DA258	—[c]	—	+	+
DA259	None	None	−	−
DA259	Vector	(None)	−	−
DA259	pRR316-1	*hrcA*, *grpE*	+	+
DA259	pRR316-2	*grpE*	+	+
DA259	pRR316-3	*hrcA*	−	−

[a] Temperature sensitivity of the strains was monitored by growth on Luria-Bertani plates containing ampicillin (to maintain selection for the plasmid in *trans*) at 30 and 42°C; all strains grew at 30°C. +, growth; −, no growth at 42°C.

[b] Capacity to support phage λ replication was assayed by mixing the indicated strains (in mid-log-phase growth) with λ clear phage, plating in top agar, and then monitoring for the appearance of plaques. +, ability of λ to replicate and form plaques; −, no plaque-forming ability.

[c] —, all of the DA258 strains, regardless of the absence or presence of any plasmid in *trans*, behaved indistinguishably from DA258 alone in temperature sensitivity and λ replication assays.

ACKNOWLEDGMENTS

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ADDENDUM IN PROOF

After submission of this work, it was reported that the *Bacillus subtilis hrcA* homolog also plays a role in the negative regulation of heat-inducible transcription of genes preceded by CIRCE elements (by G. Yuan and S.-L. Wong [J. Bacteriol. **177:**6462–6468, 1995] and A. Schulz and W. Schumann [J. Bacteriol. **178:**1088–1093, 1996).

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