JOURNAL OF BACTERIOLOGY, Jan. 1997, p. 514–521 Vol. 179, No. 2
0021-9193/97/$04.00+0

The *Caulobacter* Heat Shock Sigma Factor Gene *rpoH* Is Positively Autoregulated from a σ^{32}-Dependent Promoter

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Received 5 August 1996/Accepted 30 October 1996

Sigma factor σ^{32}, encoded by *rpoH*, is required for the recognition of heat shock genes during normal growth conditions and in response to heat shock and other stresses. Unlike the well-studied *Escherichia coli rpoH* gene, which is transcribed from four promoters recognized by either a σ^{70} (σ^{D})- or σ^{24} (σ^{E})-containing RNA polymerase, the *Caulobacter crescentus rpoH* gene is transcribed from two promoters, P1 and P2. In this study, we have examined the structure and expression of these promoters and shown that the *rpoH* P2 promoter is σ^{32} dependent. We present evidence here that P2 is specifically recognized and transcribed by the reconstituted *C. crescentus* Eσ^{32} RNA polymerase holoenzyme. We show that site-directed mutations within either the −10 or the −35 regions of P2 have substantial effects on the levels of transcription by the Eσ^{32} polymerase predicted from the σ^{32} promoter consensus sequence. The mutations have similar effects in vivo as assayed with *rpoH-lacZ* transcription fusions. Analysis of the *rpoH* P1 promoter provided evidence that it is σ^{70} dependent. S1 nuclease protection assays of *rpoH* P1- and P2-specific expression after heat shock at 42 or 50°C and during synchronous cell division cycles under normal growth conditions showed that the two promoters are differentially regulated. Mutations within the *rpoH* P2 promoter consensus sequences abolished the response to heat shock induction in *C. crescentus*. We conclude from these results that, unlike *rpoH* genes studied previously in other bacteria, the major transcriptional response of the *C. crescentus rpoH* gene to heat shock depends on positive autoregulation of the σ^{32}-dependent promoter.

All organisms respond to heat shock and other environmental stresses by the rapid and transient acceleration in the rate of synthesis of many heat shock proteins, including the molecular chaperons and proteases. Stress-induced gene regulation has been particularly well documented in the microorganisms *Escherichia coli* and *Saccharomyces cerevisiae* (for a review, see reference 18). When *E. coli* cells are transferred to higher temperatures or exposed to ethanol, complex changes in cellular structure, cell composition, and patterns of gene expression occur. The most dramatic change is the increased synthesis of heat shock proteins under these conditions (23). This heat shock gene response is mainly due to increased levels of the σ^{32} protein, which result from the translational and transcriptional regulation of *rpoH*, as well as the enhanced stability of σ^{32} protein (for reviews, see references 5, 15, and 36).

In *E. coli*, the levels of σ^{32} protein increase 15- to 20-fold during heat shock induction and then fall rapidly within 15 min after the temperature shift due to rapid protein turnover (17, 31). At temperatures below 44°C, the heat shock response is superimposed on an essentially normal pattern of gene expression, suggesting that the synthesis of most proteins is not greatly affected by heat shock induction (16, 23). Response is more extreme when cells are shifted to a higher, potentially lethal temperature, e.g., 50°C. Under these conditions, the synthesis of most non-heat shock proteins is turned off, probably because the activity of σ^{70} is reduced (9), while heat shock proteins account for a major fraction of protein synthesis (24, 26, 32).

Four promoters (P1, P3, P4, and P5) have been found upstream of *E. coli rpoH*. Three of them (P1, P4, and P5) are transcribed by RNA polymerase Eσ^{70} (Eσ^{D}) (9, 11, 21), while the fourth (P3) is transcribed by Eσ^{24} (Eσ^{E}) (8, 20, 27, 30, 33). The strong P1 promoter and the P4 promoter, which is slightly induced by heat shock, are responsible for the expression of the *rpoH* gene under normal growth conditions. P5 is a weak promoter that is regulated by catabolites. The most important promoter for heat shock response is P3; although the *rpoH* gene is transcribed under normal growth conditions from three promoters, P1, P3, and P4, after the temperature is shifted to 50°C, both the P1 and P4 promoters are shut off and the synthesis of σ^{32} depends solely on mRNA transcribed from the σ^{E}-dependent P3 promoter (9).

The heat shock response has also been studied in *Caulobacter crescentus*, a gram-negative aquatic bacterium that divides asymmetrically to produce a new differentiated motile swarmer cell and the mother stalked cell at the end of each cell cycle (reviewed in references 4, 13, and 25). The expression of several heat shock genes, including *dnaK* (14), *groESL* (1), and *hcrA*/*grpE* (29), has been examined in this bacterium, and isolation of the heat shock sigma factor gene *rpoH* has recently been described (28, 34). Transcription from the σ^{32}-like promoter of *groESL* is temporally controlled during the cell cycle at the physiological temperature of 30°C (2). By contrast, transcription from the *dnaK* and *hcrA*/*grpE* heat shock promoters displays no cell cycle regulation at 30°C, although both promoters are expressed at low levels (2, 29).

The *C. crescentus* heat shock gene *rpoH* is transcribed from two promoters, P1 and P2 (28, 34). Like the other α subgroup eubacteria including *Agrobacterium tumefaciens* and *Zymononas mobilis* (22), the *C. crescentus rpoH* gene does not contain 5′ sequences similar to the σ^{E}-dependent promoters found in the *rpoH* promoter of the γ subgroup bacteria, which include *E. coli* (8, 20, 33). Although the P1 promoter shows some similarity to the *C. crescentus* σ^{70} promoter consensus, the unusual, and perhaps unique, aspect of the *C. crescentus rpoH* gene is the similarity of its strong P2 promoter to the heat

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shock promoter consensus sequence (6). Thus, although the structure and function of *C. crescentus* σ^{32} protein are similar to those of its *E. coli* counterpart (28, 34), the promoter architecture of *rpoH* gene in these two species is different, suggesting an unusual regulatory mechanism is involved in the control of *rpoH* gene expression in *Caulobacter*.

In the studies reported here we examined the regulation of wild-type and mutant *rpoH* P1 and P2 promoters generated by site-directed mutagenesis. Expression of *rpoH* P2 promoter constructs assayed both in vivo and in vitro demonstrated that the P2 promoter is σ^{32} dependent and that the *rpoH* heat shock response in vivo depends on the P2 promoter. Similar in vivo experiments suggest that the *rpoH* P1 promoter is σ^{70} dependent. The functional difference between the *rpoH* P1 and P2 promoters was demonstrated by their differential regulation during the cell cycle and in response to heat shock. Thus the *C. crescentus rpoH* gene is subject to a novel mechanism of heat shock response which depends on the positive transcriptional autoregulation of the σ^{32}-dependent P2 promoter.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *E. coli* DH5α was used in this study for propagating plasmids as described previously (34). Cells were cultured on ML medium supplemented with ampicillin (100 μg/ml) or tetracycline (10 μg/ml) as necessary. *C. crescentus* CB15 was grown in either peptone-yeast extract (PYE) medium or M2 minimal glucose medium (7). *C. crescentus* strain CB15F, used as a synchronizable strain, was grown in M2 medium in cell cycle experiments. To synchronize this strain, swarmer cells from late-log-phase culture were isolated by Ludox density centrifugation at 4°C (10), resuspended in M2 medium at 30°C, and allowed to progress through the cell cycle. pBluescript (Stratagene Cloning System) was used in this study for subcloning and sequencing experiments.

Materials. Restriction enzymes were purchased from either New England Biolabs or Boehringer-Mannheim. S1 nuclease, T4 DNA ligase, T4 DNA polymerase, and RNase-free DNase were obtained from Boehringer-Mannheim. [α-^{32}P]dATP and [α-^{32}P]UTP were purchased from Amersham. Oligonucleotides were synthesized by Princeton Syn/Seq facility.

RNA polymerase purification. The identification and isolation of *C. crescentus* RNA polymerase (RNAP) core enzyme were performed as described elsewhere (35). The *Caulobacter* σ^{32} protein was overexpressed and purified from *E. coli*, as described previously (34).

In vitro transcription assays. The formation of open complexes at the σ^{32}-dependent promoters from *C. crescentus rpoH* gene was measured in single-cycle runoff transcription assays. The Eσ^{32} holoenzyme was reconstituted by addition of the purified *C. crescentus* σ^{32} protein to the purified *C. crescentus* RNAP core enzyme (final concentration at 1 μM) and incubation for 10 min at 4°C. Transcription-competent open complexes were formed at 37°C for 15 min in 50 μl of transcription buffer (50 mM Tris-HCl [pH 7.8], 100 mM NaCl, 1 mM dithiothreitol, 200 μg of bovine serum albumin per ml, 0.1 mM EDTA, 10 mM $MgCl_2$) containing 100 μM each of ATP, CTP, and GTP and 5 nM linear DNA template. Elongation of initiated transcript was performed by adding 5 μCi of [α-^{32}P]UTP, 10 μM UTP, and 0.5 mg of heparin per ml and incubating the mixture at 37°C for 10 min. The reactions were stopped by the addition of an equal volume of stop solution, and the transcripts were precipitated with ethanol. The samples were dissolved in 10 μl of sample buffer and run on 7 M urea–polyacrylamide gels with end-labeled *Sau*3A fragments of pUC18 as DNA size markers.

S1 nuclease protection analysis of *rpoH* mRNA during heat shock and cell cycle. The *Xba*I/*Hin*dIII fragment from plasmid pJW222 (34) was subcloned into the *Xba*I and *Hin*dIII sites of M13mp18. Single-strand DNA made from this plasmid was used as a template for extension of an oligonucleotide primer, 5′-CGAGGCGCAGGTGGCTGG-3′, by T4 DNA polymerase. The extended duplex DNA containing the labeled strand was digested with *Xba*I and the 650-bp single-strand probe was purified by electrophoresis through a 7 M urea–polyacrylamide gel. The corresponding fragment was electroeluted from the gel and used as the probe in S1 nuclease mapping (34). For heat shock induction, *C. crescentus* cells were grown at 30°C and then shifted to 42 or 50°C. Total mRNA was isolated at the times indicated. For cell cycle experiment, total mRNAs were isolated at different times from a culture of purified swarmer cells that were allowed to proceed through the cell cycle. The total mRNAs were hybridized to the labeled probe and treated with 400 U of S1 nuclease. The protected DNA fragments were subjected to electrophoresis on a sequencing gel containing 7 M urea, and the radioactive fragment was visualized by autoradiography.

Mutagenesis of the *rpoH* promoters and construction of *rpoH-lacZ* transcriptional fusions. A *Xba*I-*Hin*dIII DNA fragment from plasmid pJW240 was subcloned into mutagenesis vector pALTER-1 (Promega), creating plasmid pJW242. Single-stranded DNA made from pJW242 was used as a template and a synthesized oligonucleotide pHM5 was used as a primer to generate plasmid pJW247, containing a new *Hin*dIII site located between the *rpoH* P1 and P2 promoters (see Fig. 2). The mutated *Xba*I-*Hin*dIII fragment from pJW247 was then subcloned into pALTER-1 to create plasmid pJW248. Single-stranded DNA made from pJW248 was used as a template and four oligonucleotides were used as primers to generate plasmids pJW301, pJW302, pJW303, and pJW304 containing four different mutations, M6, M7, M8, and M9, respectively. The *Xba*I-*Hin*dIII fragments from plasmids pJW247, pJW301, pJW302, pJW303, and pJW304 were subcloned upstream of the promoterless reporter gene *lacZ* to create promoter-*lacZ* fusion plasmids pJW267, pJW305, pJW306, pJW307, and pJW308, respectively (see Fig. 2).

Single-stranded DNA made from plasmid pJW242 was used as a template and four oligonucleotides were used as primers to generate four *rpoH* P2 promoter mutations in plasmids pJW243, pJW244, pJW245, and pJW246, respectively. The *Xba*I-*Hin*dIII fragments from pJW242, pJW243, pJW244, pJW245, and pJW246 were subcloned into *Xba*I and *Hin*dIII sites of the *lacZ* fusion vector to generate transcriptional fusions to the promoterless *lacZ* reporter gene, yielding plasmids pJW262, pJW263, pJW264, pJW265, and pJW266, respectively (Fig. 1A). The *Xba*I-*Eco*RI fragments from plasmids pJW242, pJW243, pJW244, pJW245, and pJW246 were used as templates, $P2_{WT}$, $P2_{M1}$, $P2_{M2}$, $P2_{M3}$, and $P2_{M4}$, respectively, for the in vitro transcription assays (Fig. 1B).

RESULTS

***C. crescentus rpoH* P2 promoter is specifically recognized in vitro by a reconstituted Eσ^{32} RNA polymerase.** S1 nuclease protection assays have been used to identify the *rpoH* transcriptional start sites and to show that the gene is transcribed from two distinct promoters, P1 and P2, under normal growth conditions (28, 34). The similarity of the P2 promoter sequences to the heat shock promoter consensus sequence (Table 1) prompted us to examine whether it can be recognized in vitro by Eσ^{32} RNA polymerase reconstituted from purified *C. crescentus* components. Our previous studies demonstrated that the reconstituted Eσ^{32} RNAP specifically recognizes and initiates transcription from the *dnaK* heat shock promoter (*dnaK* P1) of *C. crescentus* (34). The results of runoff transcription assays using the *rpoH* DNA template pJW262 (Fig. 1A) showed that transcription initiation is dependent on the presence of purified RNA polymerase core enzyme and σ^{32} (Fig. 1B, lanes 1 and 2). The RNA product migrated at ca. 70 nucleotides (nt), the predicted size of transcript initiated from the *rpoH* P2 promoter (Fig. 1A), which we have previously mapped by in vivo S1 nuclease protection assays (34). In none of these experiments was a transcript of 138 nt detected, which would be expected from the P1 promoter. These results support the conclusion that P2 is a σ^{32}-dependent promoter while P1 is not.

To furnish additional support for this conclusion, we examined the activities of promoter mutations generated by site-directed mutagenesis within either the −10 or −35 region of the *rpoH* P2 promoter (Fig. 1A). Two of the mutations generate sequences that conform more closely to the σ^{32}-dependent promoter consensus, while the other two promoter mutations generate sequences away from the consensus. The four mutant promoters were first assayed by the in vitro transcription assay with reconstituted Eσ^{32} RNAP. As expected, no P2-specific mRNAs were detected in the absence of σ^{32}, regardless of the template used (Fig. 1B, lanes 1, 3, 5, and 7). In the presence of σ^{32}, both two-base substitutions away from consensus either abolished transcription from the P2 promoter ($P2_{M2}$; AT to GG in the −10 region; Fig. 1B, lane 6) or reduced transcription substantially ($P2_{M4}$; TT to GG in the −35 region; Fig. 1B, lane 10). More interestingly, a one-base substitution toward consensus in the −35 region ($P2_{M3}$; C to G) increased the activity of P2 promoter substantially (Fig. 1B, lane 8). Another two-base substitution toward consensus in the −10 region ($P2_{M1}$; AA to CC), to our surprise, did not increase or decrease promoter activity (Fig. 1B, lane 4), which may suggest that these bases are not critical for Eσ^{32} recognition of the *rpoH* P2 promoter.

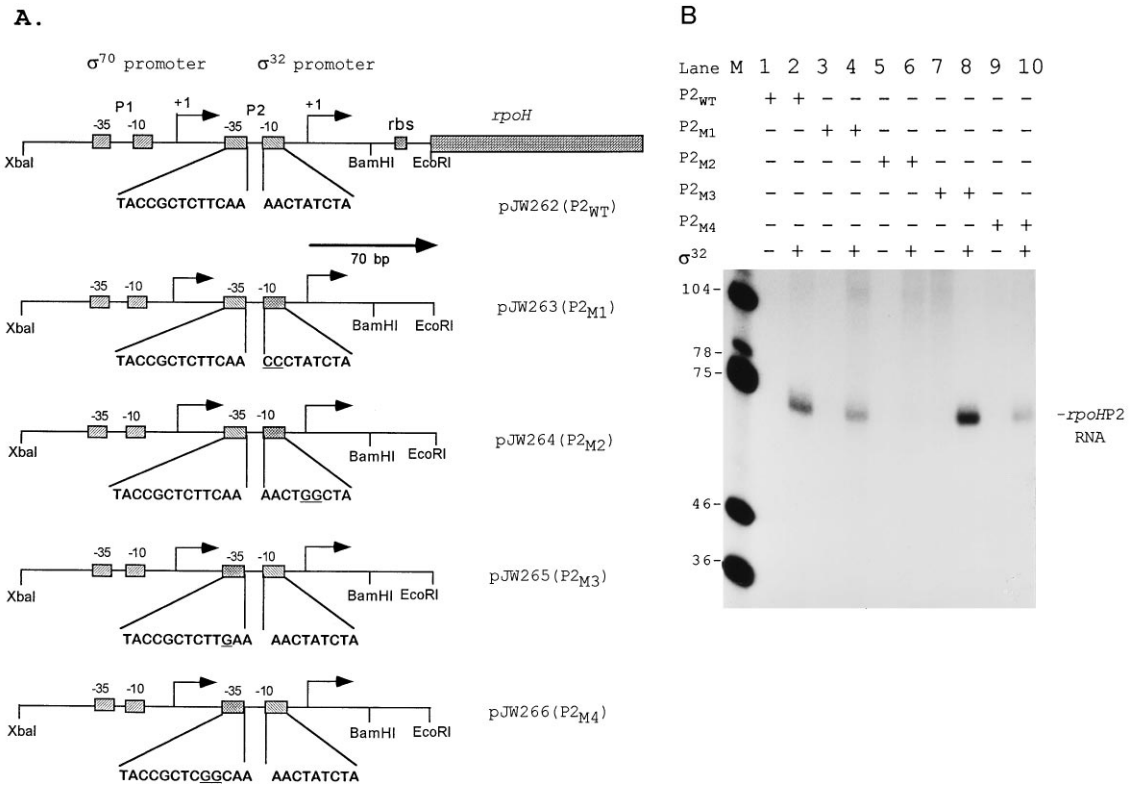

FIG. 1. Effect of promoter mutations on transcription from the *rpoH* P2 promoter by reconstituted *C. crescentus* Eσ^{32} RNA polymerase. (A) Diagram of promoters from *rpoH* gene and four *rpoH* P2 promoter mutations. The organization of *rpoH* gene upstream regulatory region is indicated at the top. Plasmid pJW263 contains a P2 promoter with a mutation (AA to CC) in the −10 region toward consensus (Table 1). pJW264 contains a mutation in P2 promoter in which two highly conserved bases (AT) in −10 region have been changed to GG. A single-base mutation (C to G) in plasmid pJW265 directs the −35 sequence of P2 promoter towards the consensus sequence. pJW266 contains a mutation in which two highly conserved bases (TT) in the −35 region have been changed to GG. The altered bases are underlined. (B) Transcription activity of the wild-type and mutant P2 promoter determined by in vitro transcription assays. In vitro runoff transcription assays were carried out by using 1 μM *C. crescentus* RNAP core enzyme alone (lanes 1, 3, 5, 7, and 9) or Eσ^{32} RNAP holoenzyme reconstituted with 1 μM core enzyme and 5 μM purified *C. crescentus* σ^{32} protein (lanes 2, 4, 6, 8, and 10). The *Xba*I-*Eco*RI fragment from pJW262 was used as wild-type template DNA (lanes 1 and 2). The *Xba*I-*Eco*RI fragments from pJW263 (lanes 3 and 4), pJW264 (lanes 5 and 6), pJW265 (lanes 7 and 8), and pJW266 (lanes 9 and 10) were used as the mutant template DNA.

Effects of promoter mutations on transcription from the *rpoH* P2 in vivo. We also examined the activities of mutant P2 promoters in vivo by fusing them transcriptionally to the promoterless *lacZ* report gene and measuring levels of β-galactosidase activity. Results presented in Table 2 show that the β-galactosidase activities from the two promoter down-mutants, P2$_{M2}$-*lacZ* and P2$_{M4}$-*lacZ*, were reduced about 10- and 5-fold, respectively. The β-galactosidase activity from the P2$_{M3}$-*lacZ* fusion in which the mutant promoter is closer to consensus was ca. 2.5-fold higher than from wild-type fusion, while the relative level of P2$_{M1}$-*lacZ* fusion expression was the same as that of P2$_{WT}$-*lacZ* fusion. These results are consistent with those from in vitro transcription assays of the mutant promoters (Fig. 1B) and lead us to conclude that the P2 promoter of *C. crescentus rpoH* is in fact a σ^{32}-dependent promoter that is specifically recognized by Eσ^{32} RNA polymerase both in vitro and in vivo.

Characterization of the *rpoH* P1 promoter. The *rpoH* P1 promoter is similar to the *Caulobacter* σ^{70}-dependent promoter consensus (19, 34), although the alignment is better at the −10 region with 6 out of 8 bases matching consensus (Table 1). To analyze the P1 promoter sequence, we introduced four mutations into this promoter by site-directed mutagenesis. The effects of the mutations on P1 promoter activity were measured in vivo by fusing the *lacZ* reporter gene transcriptionally to the wild-type or mutant *rpoH* P1 promoter at a *Hin*dIII site, which was introduced to eliminate the σ^{32}-dependent P2 promoter (Fig. 2). Promoters P1$_{M6}$ and P1$_{M8}$ contain altered sequences closer to the *C. crescentus* σ^{70}-dependent promoter consensus (Table 1; Fig. 2) and they resulted in a 3- and 5-fold increase in transcription over the wild-type level,

TABLE 1. Promoter sequences from *C. crescentus* heat shock genes[a]

Promoter	−35 sequence	Spacing (bp)	−10 sequence	Reference
σ^{70}-like				
rpoH P1	**TCCAAAC**	15	**ACAATATC**	34
dnaK P2	**TTGACG**A	13	CGC**ACAAC**	14
groE P1	**TTAACCG**	11	A**CTTC**GCG	1
Consensus	**TTGACGS**	11–15	**GCTANAWC**	19
σ^{32}-like				
rpoH P2	**TACCGC**T**CTTCAA**	13	AA**C**T**ATCTA**	34
dnaK P1	**TTATGGCCTTG**CG	14	**CCCCATAT**C	14
groE P2	G**CGCG**T**CCTTGA**C	15	G**CC**T**AACTC**	1
hrcA	**GGGGA**GAT**TTGAA**	13	**CCGCTTATC**	29
grpE	C**TTT**CGGG**TT**CCT	14	**CCC**T**ATATG**	29
Consensus	**TNNCNCCCTTGAA**	13–15	**CCCCATNTA**	6

[a] Bases in each promoter that conform to the consensus sequence are shown in boldfaced type.

TABLE 2. Effect of P2 promoter mutations on the expression of *rpoH-lacZ* fusion genes

Strain	*lacZ* fusion	β-Gal activity	
		U	mut/wt
PC1602	$P2_{WT}$-*lacZ*	1,133.4	1.00
PC1603	$P2_{M1}$-*lacZ*	1,142.1	1.01
PC1604	$P2_{M2}$-*lacZ*	109.4	0.10
PC1605	$P2_{M3}$-*lacZ*	2,817.4	2.50
PC1606	$P2_{M4}$-*lacZ*	215.6	0.19

[a] Ratio of activity from mutant promoter to that from wild-type promoter.

respectively, as measured by β-galactosidase activity (Fig. 2). The third mutation, $P1_{M7}$, which contains two alternate bases, one of which results in a change towards consensus (A to C) and the other away from consensus, had no effect on promoter activity. The fourth mutation examined in this study, $P1_{M9}$, contains altered sequences away from the consensus and does not affect transcription from P1 as measured by β-galactosidase activity (see Discussion). These results support the conclusion that the *rpoH* P1 promoter is recognized in vivo by $E\sigma^{70}$ RNA polymerase.

Cell cycle regulation of *rpoH* expression. To examine the pattern of *rpoH* transcription from the P1 and P2 promoters during the cell division cycle, we carried out S1 nuclease protection assays using mRNA isolated from synchronous swarmer cells grown at 30°C. As shown in Fig. 3, *rpoH* P1-specific mRNA was not detectable at the beginning of the cell cycle (0 min), when the culture contained approximately 95% swarmer cells. P1-specific mRNA was first detected at 80 min, when swarmer cells differentiated into stalked cells. The level of P1 mRNA then increased to a maximum at 100 min and remained at a constant low level throughout the remainder of the cell cycle. These results suggest that the *rpoH* P1 promoter is temporally regulated during the cell division cycle in *Caulobacter*.

The highest level of *rpoH* P2-specific RNA was detected in the newly collected swarmer cells (0 min; Fig. 3). The amount of P2 mRNA rapidly decreased to a lower but still detectable level after 80 min and then increased three- to fivefold during the remainder of the cell cycle. The level of P2 transcript in swarmer cells at the beginning of the experiment (0 min) was much higher than at the end of the cell cycle (200 min) when the culture contained an equivalent number of new swarmer cells. This observation suggests that manipulations required for the preparation of synchronous swarmer cells (Materials and Methods), possibly the incubation at 4°C during fractionation, may induce a stress response that contributes to the elevated level of *rpoH* P2-specific mRNA detected at 0 min. To test the possibility, we isolated the two cell types from a synchronous culture that had just completed division. The swarmer and stalked cells were fractionated and harvested, and total RNA was isolated at 4°C from the two cell types, as well as from a sample of the unfractionated cell culture, in all cases without exposure of cells to 30°C. The level of P2-specific mRNA in the isolated swarmer cells was higher than in the separated stalked cells but almost the same as in an unfractionated population of the divided swarmer and stalked cells (Fig. 4). These results suggest that swarmer cells are probably more susceptible than stalked cells to cold shock effect on levels of messenger RNA from the *rpoH* P2 promoter. Thus, the major effect on regulation of P2-specific mRNA in the newly isolated swarmer cells (Fig. 3) probably results from the effect of recovery from cold shock during the first 60 min of the cell cycle.

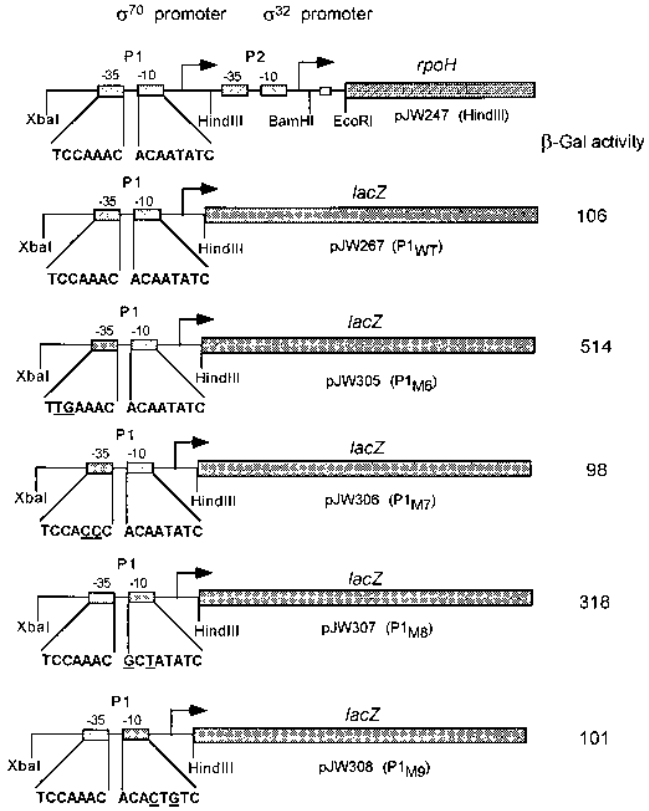

FIG. 2. Effect of *rpoH* P1 promoter mutations on transcription activity of *rpoH* P1-*lacZ* fusion genes. A diagram of *rpoH* gene and its upstream regulatory regions is at the top. A *Hin*dIII site was introduced into the *rpoH* regulatory region between P1 and P2 promoters in plasmid pJW247 by site-directed mutagenesis. Plasmid pJW305 contains a mutation (CC to TG) in the −35 region of *rpoH* P1 promoter toward the *C. crescentus* σ^{70}-dependent promoter consensus (19). A two-base change (AA to CC) in pJW306 is a change away from the consensus in the −35 region. In pJW307, two single-base mutations (A to G and A to T) in the −10 region of *rpoH* P1 promoter resulted in a change towards consensus. Two other single-base mutations (A to C and A to G) in the −10 region of pJW308 resulted in a change in P1 away from consensus. The *Xba*I-*Hin*dIII fragments containing only the *rpoH* P1 promoter (wild type or mutant) were subcloned upstream of the reporter gene *lacZ* to yield transcriptional fusions. The promoter activity was determined by measuring the levels of β-galactosidase. The numbers at right indicate β-galactosidase activity in Miller units.

The *rpoH* P2 promoter is primarily responsible for heat shock induction. To investigate the response of the *rpoH* P1 and P2 promoters to heat shock induction, we used S1 nuclease protection assays on total mRNAs isolated from *C. crescentus* cells shifted from 30 to either 42 or 50°C (Fig. 5). In cells grown at 30°C, two protected bands corresponding to the P1- and P2-specific mRNA were observed (lane 1). When the temperature was shifted from 30 to 42°C for 5, 10, and 15 min (lanes 2, 3, and 4, respectively), the levels of P1-specific mRNA did not change, but there was a dramatic increase in the levels of P2 RNA at 42°C (lanes 2, 3, and 4). At 20 and 25 min the levels of P1 mRNA increased slightly and the level of P2 mRNA decreased somewhat (lanes 5 and 6), although there was still 10- to 15-fold more P2 mRNA at these times than at 30°C before the heat shock (lane 1). More interestingly, the activity of P1 promoter was shut down completely at 50°C, while the P2 promoter was still active after 20 min (lane 7). These results clearly demonstrate that the heat shock induction of the *C. crescentus rpoH* transcription is primarily from the σ^{32}-dependent P2 promoter and that the *rpoH* P2 promoter and σ^{70}-

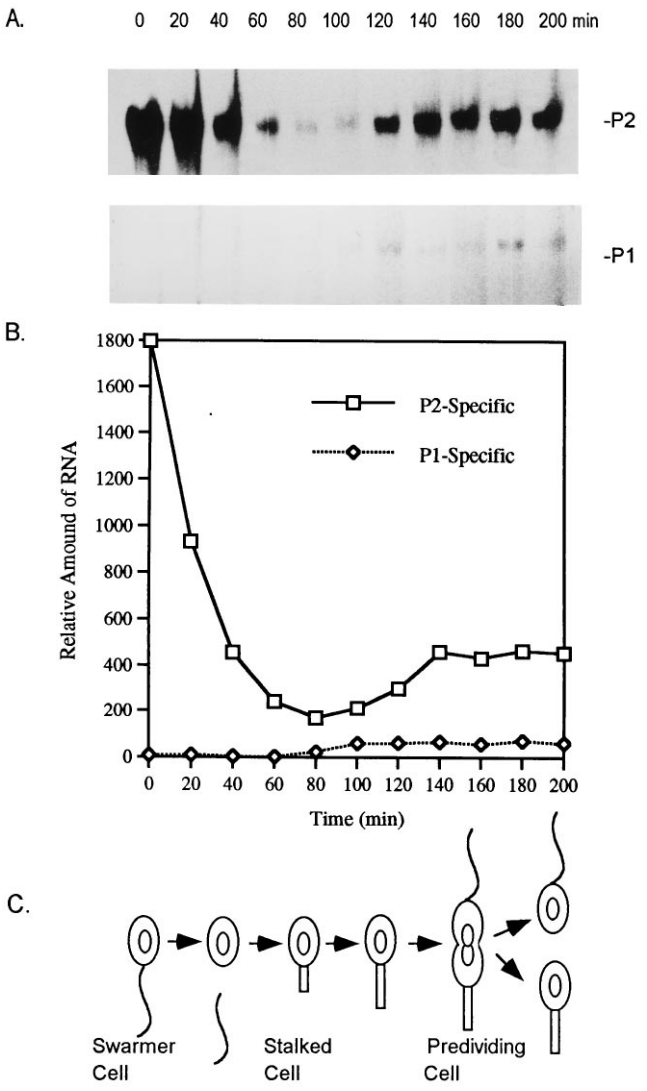

FIG. 3. Cell cycle regulation of transcription from *rpoH* P1 and P2 promoters. The levels of *rpoH* gene transcription during *C. crescentus* cell cycle were determined by S1 nuclease protection assays. Swarmer cells were isolated by Ludox density centrifugation at 4°C and allowed to progress through the cell cycle in minimal medium (M2) at 30°C. The quality of the synchrony and duration of the cell cycle were determined by light microscopy and by FACS (fluorescence-activated cell sorter) analysis. Cell division occurred at approximately 200 min. Total mRNA was isolated from cells at different stages of the cell cycle and used as template for S1 protection assays. (A) Autoradiographs of P1- and P2-specific mRNAs determined by S1 nuclease protection assays during the *C. crescentus* cell cycle. (B) Quantification of *rpoH* P1- and P2-specific transcription by Molecular Dynamics PhosphoImager. (C) Diagram of the *C. crescentus* cell cycle.

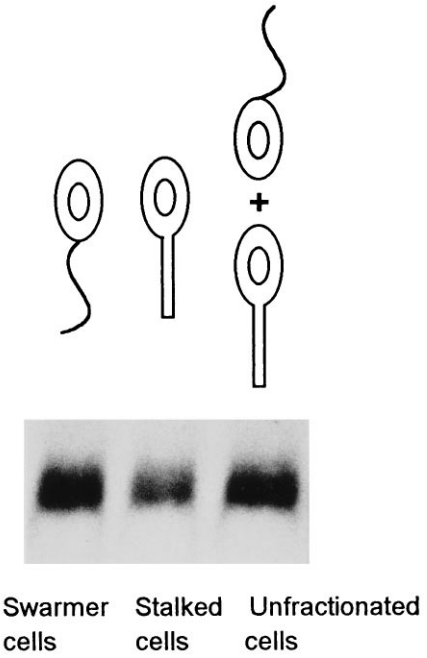

FIG. 4. S1 analysis of *rpoH* P2 transcript in fractionated swarmer and stalked cells. Swarmer cells and stalked cells were separated from a synchrony cell culture immediately after division by Ludox density centrifugation at 4°C. Total mRNA was isolated from these cells at 4°C and used as a template for S1 nuclease protection assays. As a control, mRNA was also isolated from a sample of unfractionated swarmer and stalked cells from the same synchrony culture.

dependent P1 promoter are differentially regulated, as indicated by the patterns of mRNA accumulation, in response to heat shock at both 42 and 50°C.

We also examined the requirement of the P2 promoter for the heat shock response in vivo by comparing expression from transcriptional fusions containing either the wild-type promoter (*rpoH*$P2_{WT}$-*lacZ*; strain PC1602) or a mutant *rpoH* P2 promoter (*rpoH*$P2_{M2}$-*lacZ*; strain PC1604). Cultures were shifted from 30 to 42°C for various periods of time, and the cells were pulse-labeled with [^{35}S]methionine for 3 min and the incorporation of label into β-galactosidase was measured by immunoprecipitation (Fig. 6), as described in Materials and Methods. The rate of transcription from the wild-type *rpoH* P2 promoter increased by ca. fivefold during the first 15 min after the temperature shift to 42°C and then decreased between 15 and 30 min (Fig. 6), which is consistent with previous results (28). However, in the strain carrying the *rpoH* P2 promoter mutation (*rpoH*$P2_{M2}$-*lacZ* fusion), the rate of synthesis measured from the reporter gene was lower at 0 min after the temperature shift and remained at this low level during the course of the experiment (Fig. 6). These results confirm that the heat shock response of *rpoH* measured in these experiments requires the σ^{32}-dependent P2 promoter, and they support the conclusion that transcription of *rpoH* in response to heat shock is positively autoregulated from this promoter.

DISCUSSION

We have used biochemical and genetic approaches to investigate the regulation of the promoters *rpoH* P1 and *rpoH* P2 of *C. crescentus* heat shock gene *rpoH*. The effects of site-directed mutations on promoter activity measured in vivo and in vitro lead us to conclude that the P1 promoter is a σ^{70}-dependent promoter, while the P2 promoter is a σ^{32}-dependent promoter recognized by RNA polymerase containing the σ^{32} heat shock factor. Heat shock induction of *rpoH* transcription is primarily from the P2 promoter, and disruption of the σ^{32}-dependent P2 promoter eliminates this transcriptional response to heat shock in vivo. Thus transcription from the *rpoH* P2 promoter is positively autoregulated in response to this stress condition. Although the structure and function of the *Caulobacter* σ^{32} protein is similar to its *E. coli* counterpart, the mechanism of *Caulobacter rpoH* gene regulation is different from that reported for *rpoH* genes from other bacteria, but as discussed below (Fig. 7), it is very similar to that of the second *E. coli* heat shock regulatory gene *rpoE*.

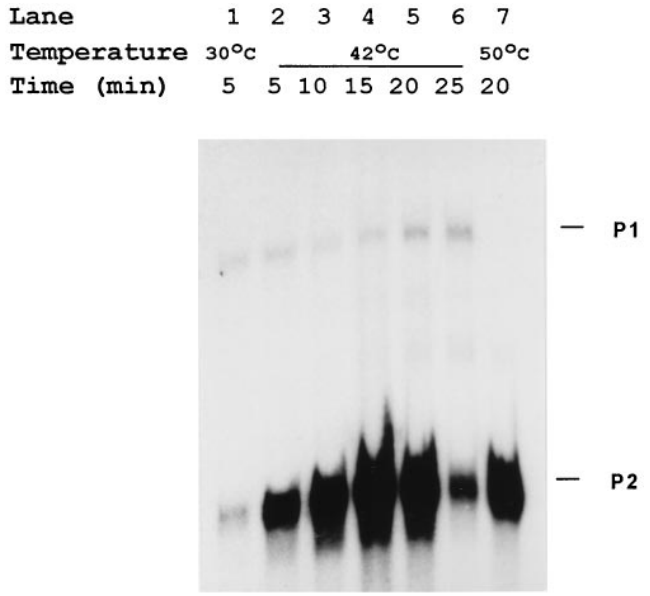

FIG. 5. Heat shock response of *C. crescentus rpoH* P1 and P2 promoters. The levels of transcription from *rpoH* P1 and P2 promoters at different temperatures were measured by S1 nuclease protection assays. Cells of *C. crescentus* strain CB15 were grown in M2 medium at 30°C and then shifted to 30°C for 5 min (lane 1), 42°C for 5 to 25 min (lanes 2 to 6), or 50°C for 20 min (lane 7). Total mRNA was isolated from equal numbers of cells and used as templates for S1 assays. P1 and P2 indicate probe fragments (see Materials and Methods) protected by *rpoH* P1- and P2-specific transcripts, respectively.

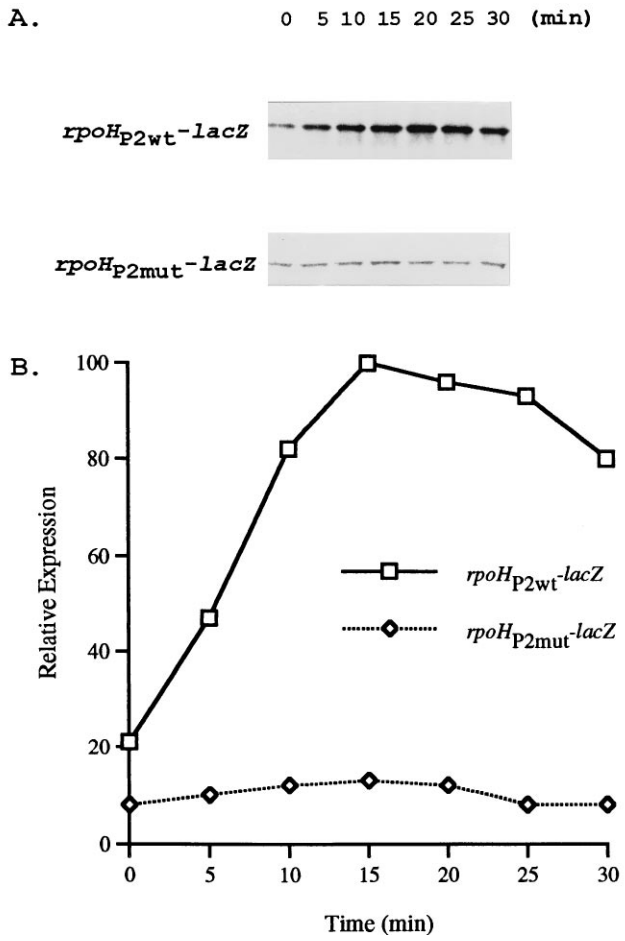

FIG. 6. Effect of heat shock on expression of β-galactosidase transcribed from *rpoH-lacZ* transcriptional fusions. (A) The transcription of *rpoH-lacZ* fusion was measured in strain PC1602 carrying the *rpoH*$P2_{WT}$-*lacZ* fusion or in strain PC1604 carrying the *rpoH*$P2_{M2}$-*lacZ* fusion (Table 2). β-Galactosidase synthesis was determined at 5 min intervals after shifting the temperature from 30 to 42°C by pulse-labeling cells with [^{35}S]methionine for 3 min. Labeled β-galactosidase was immunoprecipitated from cell extracts and visualized by autoradiography as described in Materials and Methods. Protein extracts from equal cell culture volumes were applied to each reaction. (B) The graph shows β-galactosidase protein levels relative to their highest level (normalized to 100) observed throughout the heat shock response determined by Molecular Dynamics PhosphoImager.

***rpoH* P1 and P2 promoter structure and organization.** The *rpoH* P2 promoter is similar in sequence to the heat shock promoter consensus (6) with 6 of 9 bp identical to the −10 sequence and 11 of 13 bp identical to the −35 sequence (Table 1). We have used an in vitro transcription assay system to demonstrate that the *rpoH* P2 promoter is recognized by a reconstituted *C. crescentus* Eσ^{32} RNA polymerase (Fig. 1B). This promoter specificity was confirmed by the effect of P2 promoter mutations in both the −10 and −35 sequences on transcription both in vitro (Fig. 1B) and in vivo (Table 2).

DNA sequence alignments show the similarity of the *rpoH* P1 promoter to *C. crescentus* σ^{70} promoters (Table 1). Mutational analysis supports the possibility that *rpoH* P1 may be σ^{70} dependent: changes toward the σ^{70} consensus at −10 and −35 enhanced promoter activity by three- to fivefold. Failure of mutations away from consensus to substantially affect transcription may indicate that bases at the positions chosen for mutagenesis are not critical for polymerase recognition. In addition, *rpoH* P1 contains only 3 of 7 consensus residues at −35 (Table 1), and therefore it is a weak σ^{70} promoter. From assays on the *rpoH-lacZ* fusion constructs (Fig. 2; Table 2), we estimate that the P1 promoter is ca. 10-fold less active than the P2 promoter at 30°C.

Several lines of evidence support the conclusion that the promoters P1 and P2 are differentially regulated and that P1 is not a σ^{32}-dependent promoter. (i) During heat shock at 42°C, the level of P1-specific mRNA remained relatively constant for 15 min after the temperature shift, while P2-messenger accumulated to its maximum level (Fig. 5, lanes 1 to 4). Although Reisenauer et al. (28) have reported a heat shock response from the *rpoH* P1 promoter after 5 min at 42°C, we observed a small increase (<1.5-fold) in P1-dependent expression and then only after 20 to 25 min at 42°C (Fig. 5). (ii) Transcription from the P1 promoter, unlike that from the P2 promoter, was shut down completely at 50°C (Fig. 5, lane 7). (iii) The two promoters are differentially regulated in the cell cycle (Fig. 3). (iv) The P2 promoter, but not the P1 promoter, is recognized by the reconstituted Eσ^{32} RNA polymerase (Fig. 1B).

An *rpoH* gene containing a σ^{32}-dependent and a σ^{70}-dependent promoter has not been reported previously. This promoter arrangement is, however, similar to that in other known *Caulobacter* heat shock genes, including *dnaKJ* (14), *groESL* (1), and *hrcA/grpE* (29). This promoter structure could reflect a common mechanism controlling heat shock gene expression in response to both stress and cell cycle regulation in *C. crescentus*, thus making *rpoH* regulation an interesting system for the study of heat shock induction and developmental gene expression in this bacterium. More interestingly, *Caulobacter* heat shock sigma factor σ^{32} not only is responsible for the regulation of other heat shock genes, including chaperone genes *dnaK* and *groE*, but also is required to autoregulate its own expression during heat shock, as shown by the effect of

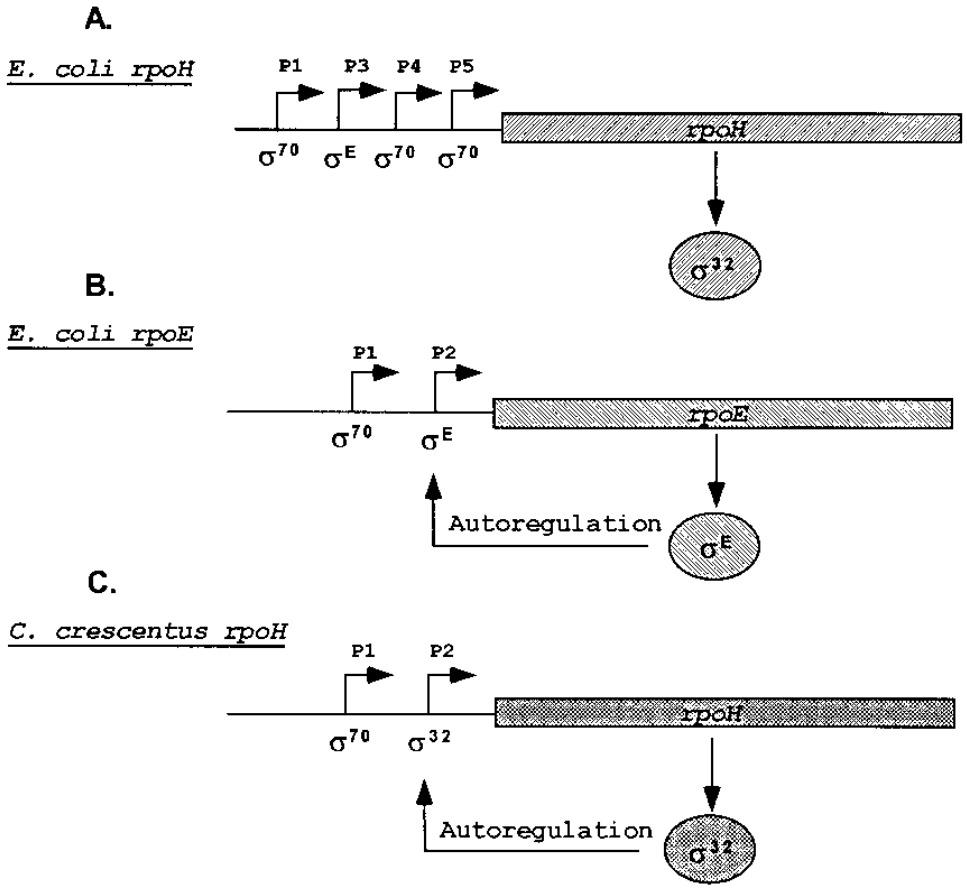

FIG. 7. Regulation of heat shock sigma factor genes in *E. coli* and *C. crescentus*. (A) Transcription of *E. coli rpoH* encoding the heat shock sigma factor σ^{32} is under the control of four different promoters, P1, P3, P4, and P5 (8, 11, 21, 33). P1, P4, and P5 are recognized by $E\sigma^{70}$ RNAP, while P3 is recognized by RNA polymerase containing another heat shock sigma factor σ^E encoded by *rpoE* (27, 30). (B) Transcriptional regulation of *E. coli rpoE* gene involves two promoters, P1 and P2. The *rpoE* P1 may be recognized by a $E\sigma^{70}$ RNAP, while the P2 promoter is recognized by a $E\sigma^E$ RNAP and is primarily responsible for heat shock induction. Therefore, the *rpoE* gene is positively autoregulated by its own product σ^E (27, 30). (C) Promoter structure and proposed autoregulation of *C. crescentus rpoH* gene are different from that of its counterpart *E. coli rpoH* but are very similar to that of the second heat shock sigma factor gene *rpoE* of *E. coli*. *C. crescentus rpoH* contains two promoters, P1 and P2. Our results demonstrated that P1 is a σ^{70}-dependent promoter and P2 is a σ^{32}-dependent P2 promoter which is primarily responsible for heat shock induction and is also recognized and thus positively autoregulated by the $E\sigma^{32}$ RNAP.

mutations in the *rpoH* P2 promoter on the heat shock response (Fig. 6).

Cell cycle regulation of P1 and P2 promoters. The patterns of expression from the P1 and P2 promoters are dramatically different in the cell cycle. P1-specific mRNA was undetectable in swarmer cells, and then it accumulated at a low but easily detectable level during the swarmer to stalked cell transition and the rest of the cell cycle (Fig. 3). In contrast to P1 expression, we detected extremely high levels of P2-specific transcript in swarmer cells at the beginning of the cell cycle. In measurements made by using an *rpoH-lacZ* fusion, Reisenauer et al. (28) also observed a high level of *rpoH* expression. Our results show that this effect can probably be attributed in large part to a stress response from cold shock during cell fractionation (Fig. 4). The rapid decrease in the level of P2 activity to a low level at 80 min when stalk formation occurs (Fig. 3) suggests homeostasis at 30°C. Interestingly, the two- to threefold increase in P2-mRNA during the S phase parallels the pattern of *rpoH* P1-specific expression. We speculate that P1 promoter activity may modulate P2 promoter expression in the cell cycle.

Heat shock regulation of *rpoH* transcription. During heat shock induction, the *rpoH* P2-specific transcript accumulates rapidly to a maximum level at 15 min (Fig. 5, lane 4). After 25 min of induction, synthesis of the P2 mRNA decreases, but it is still ca. fivefold higher than the level before the temperature shift (lane 6). At the more extreme temperature of 50°C, *rpoH* P1 transcript could no longer be detected, while the P2-specific transcript remained at a very high level (Fig. 5, lane 7). This pattern of *rpoH* gene expression is similar to that observed in *E. coli*: in a temperature shift to 50°C the σ^{70}-dependent promoters shut down, while transcription from the σ^E-dependent promoter P3 proceeds at a high level (15). Moreover, the heat shock response of *rpoH* gene depends on the P2 promoter, since a mutation in the P2 promoter abolished response of the *rpoH* gene to heat shock induction (Fig. 6). Thus we conclude that the σ^{32}-dependent P2 promoter in *Caulobacter* is required for *rpoH* transcription in response to heat shock induction and that the P2 promoter may be functionally analogous to the σ^E-dependent *rpoH* P3 promoter in *E. coli* (Fig. 7).

These findings are of interest because none of the previously described *rpoH* genes has been reported to contain a σ^{32}-dependent promoter. In *E. coli*, the promoter of *rpoH* gene responsible for heat shock is P3, a σ^E-dependent promoter (Fig. 7A) (8, 33). σ^E-dependent promoters have also been found upstream of *rpoH* genes from other γ subgroup of eubacteria, including *Pseudomonas aeruginosa* (3), *Citrobacter*

freundii (12), *Enterobacter cloacae*, *Serratia marcescens*, and *Proteus mirabilis*, but not from the α-subgroup eubacteria, including *A. tumefaciens* and *Z. mobilis* (22).

Autoregulation of the *C. crescentus rpoH* and *E. coli rpoE* genes. The similarity between the transcriptional regulation of the *C. crescentus rpoH* gene and the *E. coli rpoE* gene in response to heat shock induction is striking, as diagrammed in Fig. 7B and 7C. *rpoE*, which encodes the second *E. coli* heat shock sigma factor σ^E, contains two major promoters, P1 and P2 (27, 30). Recent analyses have demonstrated that the *rpoE* P2 promoter is a σ^E-like promoter which is recognized by a reconstituted $E\sigma^E$ RNA polymerase in vitro and that it is positively autoregulated by σ^E in vivo (27, 30). The *E. coli rpoE* gene belongs to a family of genes responding to extracytoplasmic stress (8). It is induced by overproduction of outer membrane proteins, suggesting that $E\sigma^E$ responds to a signal generated by events occurring in the extracytoplasmic compartment (20). These observations indicate that *E. coli* contains two distinct heat-sensitive regulons responding to stresses in different cellular compartments: $E\sigma^{32}$ responds to intracellular stress, while $E\sigma^E$ responds to extracytoplasmic stress. It remains to be determined if *C. crescentus* uses only the heat shock sigma factor σ^{32} in responding to both intracellular and extracytoplasmic stress, or if extracellular responses are mediated by another and as yet unidentified mechanism.

In addition to positive autoregulation observed in the stress response of *rpoH*, the gene also appears to be negatively regulated, perhaps at the posttranscriptional and/or translational levels. This may explain the significant decreases in the level of *rpoH* P2-specific RNA as measured by S1 nuclease protection assays at 25 min after heat shock (Fig. 5, lane 1) and after the transfer of synchronous swarmer cells from 4 to 30°C (Fig. 3). The decreased levels of P2-specific transcript could result from the rapid turnover of the *rpoH* messenger RNA or inactivation of the RpoH protein by a posttranslational mechanism which would turn off or down-regulate transcription of *rpoH*. The rapidity of these responses and the typically short half-life of bacterial messenger RNA are most consistent with the latter possibility. It is known that RpoH in *E. coli* is regulated posttranslationally by protein turnover, as well as translationally (for a review, see reference 36). These mechanisms could contribute directly to the autoregulation of the *rpoH* expression in *C. crescentus* in response to stress and in the cell cycle during normal growth conditions.

ACKNOWLEDGMENT

This work was supported by Public Health Service grant GM22299 from the National Institutes of Health to A.N.

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