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Sigma H-Directed Transcription of *citG* in *Bacillus subtilis*

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The RNA polymerase sigma factor σ^H is essential for the onset of endospore formation in *Bacillus subtilis*. σ^H also is required for several additional stationary-phase-specific responses, including the normal expression of several genes that are required for the development of competence for DNA uptake. It is necessary to identify the genes that are transcribed by σ^H RNA polymerase ($E\sigma^H$) in order to understand the role of this sigma factor during the transition from exponential growth to stationary phase. Feavers et al. (Mol. Gen. Genet. 211:465–471, 1988) proposed that *citG*, the structural gene for fumarase, is transcribed from two promoters, one of which (*citGp2* [P2]) may be used by $E\sigma^H$. It is likely that the *citGp2* promoter is used by $E\sigma^H$ because we found that this promoter was used accurately in vitro by $E\sigma^H$ and directed expression of *xylE* in vivo. This *xylE* expression was dependent on *spo0H*, the structural gene for σ^H, and was independent of the *citGp1* promoter. Comparison of the nucleotide sequences of several σ^H-dependent promoters showed that these sequences were similar at two regions approximately 10 and 35 base pairs upstream from the start points of transcription. These sequences may signal recognition of these promoters by $E\sigma^H$. Primer extension analyses were used to examine transcription from three σ^H-dependent promoters during growth and sporulation. The *citGp2* promoter appeared to be active during the middle and late stages of exponential growth, whereas activation of the *spoIIA* promoter was delayed until after the end of exponential growth. Evidently, promoters used by $E\sigma^H$ can display different temporal patterns of expression.

Sigma H (σ^H) is one of at least nine different RNA polymerase sigma factors produced by the bacterium *Bacillus subtilis* (14, 15). Association of each sigma factor with the core subunits of RNA polymerase directs the holoenzyme to use a different class of transcriptional promoters. Since mutations in *spo0H*, the structural gene for σ^H (6), prevent the onset of sporulation, it is likely that RNA polymerase containing σ^H ($E\sigma^H$) transcribes at least one gene that is necessary for the onset of sporulation. Mutations in *spo0H* also reduce the development of competence for DNA uptake (1) and the production of stationary-phase-specific extracellular enzymes (8). Evidently, $E\sigma^H$ transcribes a diverse group of genes that play important roles in the bacterium's responses to nutrient depletion.

The genes that are transcribed by $E\sigma^H$ and necessary for the stationary-phase-specific responses are unknown. $E\sigma^H$ has been shown to use two promoters, *spoVG* (22) and *rpoDp3* (4), but transcription from these two promoters is not necessary for the onset of sporulation or the other stationary-phase-specific responses (18; R. H. Doi, personal communication). Moreover, recent results show that σ^A, the primary sigma factor in growing cells, directs the transcription of several sporulation-essential genes at the onset of endospore formation (9, 11; T. J. Kenney, K. York, P. Youngman, and C. P. Moran, Jr., unpublished results). It is not known why some genes are transcribed by $E\sigma^A$ at the onset of sporulation while other genes are transcribed by $E\sigma^H$. Furthermore, transcription from the *spoVG* and *rpoDp3* promoters by $E\sigma^H$ is activated near the end of the exponential growth phase, but $E\sigma^H$ is also present in exponentially growing cells (3). Zuber and Losick (23) suggested that the product of *abrB*, a negative regulatory factor, may directly affect *spoVG* promoter activity. These observations raise the possibility that expression from some $E\sigma^H$-dependent promoters may be regulated by ancillary factors, resulting in different temporal patterns of expression.

The identification of the genes that are transcribed by $E\sigma^H$ may aid our understanding of the role of σ^H and the mechanisms that operate to regulate the activity of promoters that are used by $E\sigma^H$. Recently, Feavers et al. (7) discovered two transcripts of *citG*, the structural gene for fumarase. The longer *citG* transcript was initiated from the P1 promoter and accumulated in a *spo0H* mutant. In contrast, the shorter transcript was not present in a *spo0H* mutant. Since the nucleotide sequence of the DNA upstream from the 5′ end of the *spo0H*-dependent transcript was found to be similar to that of other promoters that are used by $E\sigma^H$, Feavers et al. (7) proposed that $E\sigma^H$ may transcribe *citG* from this promoter, which they called P2. We have examined the possibility that *citG* is transcribed by $E\sigma^H$. We found that $E\sigma^H$ efficiently and accurately used the *citGp2* (P2) promoter in vitro. Furthermore, the P2 promoter, even in the absence of P1, directed transcription of *xylE* in a wild-type but not a *spo0H* (σ^H deficient) strain. We also examined the temporal pattern of expression from several promoters that are used by $E\sigma^H$ during growth and sporulation.

MATERIALS AND METHODS

Bacterial strains and media. *Escherichia coli* DH5α was obtained from Betheseda Research Laboratories. *B. subtilis* IS233 (*trpC2 pheA1 spo0H* Δ*Hin*dIII) was provided by I. Smith. *B. subtilis* JH642 (*trpC2 pheA1*) was provided by J. Hoch. DS medium (19) was used as the sporulation medium for *B. subtilis*.

Plasmids. pAAM91 (7) contained the promoter region of *citG*, both P1 and P2. To study the function of the *citGp2* promoter in the absence of P1, plasmid pKT89 was constructed by cloning the *Rsa*I-*Dra*I fragment of plasmid pAAM91 (nucleotides 234 to 538) (7) into the *Hpa*I site of plasmid pLC4 (17) so that transcription from the *citGp2*

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promoter was directed through a promoterless derivative of *xylE*, the structural gene for catechol-2,3-dioxygenase ($CatO_2ase$), as described previously (17). The orientation of the insert containing the promoter was determined by restriction site analysis and verified by determining the nucleotide sequence of the insert. pKT89 was introduced into *E. coli*, and transformants were selected on LB medium containing 60 μg of ampicillin per ml as described previously (17). Plasmid pKT89 was purified by an alkaline lysis procedure, followed by centrifugation in cesium chloride-ethidium bromide density gradients (2), and introduced into competent *B. subtilis* JH642 and IS233.

$CatO_2ase$ assay. DS medium containing 5 μg of chloramphenicol per ml was inoculated with cells to an OD_{550} of 0.05. These cultures were incubated at 37°C with vigorous shaking until the cultures reached an OD_{550} of 0.3, at which time the cultures were diluted fivefold in DS medium, and incubation was continued. A 10-ml amount of these cultures was collected at selected intervals during growth and sporulation, as illustrated in Fig. 3. Assays for $CatO_2ase$ were done as previously described (17) with the following modifications. $CatO_2ase$ activities were determined spectrophotometrically at 30°C in a total volume of 3 ml that contained 1 ml of cell extract, 2 ml of 100 mM potassium phosphate (pH 7.5), and 0.07 M catechol. The specific activity was calculated as units of activity per OD_{550} unit of cells.

RNA isolation, in vitro transcription, and primer extension analysis. *B. subtilis* JH642 and IS233 were grown in DS medium at 37°C with vigorous shaking until the cultures reached an OD_{550} of 0.3, at which time the culture was diluted fivefold in DS medium, and incubation was continued. Samples (100 ml) were harvested by centrifugation at selected intervals during growth and sporulation, as illustrated in Fig. 3 and 5. RNA was isolated as previously described (10).

σ^H RNA polymerase was isolated from *B. subtilis* CR101 (*sigB*) as described previously (3). RNA transcripts were generated in vitro from a linearized plasmid template, pAAM91 (7), that had been cleaved with *Dra*I, and the transcripts were analyzed by electrophoresis on urea-polyacrylamide gels as described previously (3).

The in vitro transcripts for primer extensions were generated from pAAM91 that had been cleaved with *Dra*I, which cleaves 177 base pairs (bp) downstream from the *citGp2* promoter. The procedure for primer extension analysis has been described previously (11). The sequence of the oligonucleotide that was used to prime the DNA synthesis was 5′-GCAGGAACTTTTACTTC-3′ for *citG*, 5′-GGTGATCG AGTTCGCCTG-3′ for *spoIIA*, and 5′-GTGATCCAGCGT GATGGAT-3′ for *spoVG*. The size standards were generated with the oligonucleotide for *citG* with pAAM91 as a template in dideoxy sequencing reactions with T7 DNA polymerase.

RESULTS

Primer extension analysis of *citG* transcription. Primer extension analysis was used to characterize *citG* transcripts in *B. subtilis* and a *spo0H* mutant during growth and sporulation as described in Materials and Methods. Two transcripts (labeled P1 in Fig. 1) were present in both wild-type cells and the *spo0H* mutant. In contrast to this transcript, a prominent transcript, labeled P2 in Fig. 1, was present in the wild-type strain (lanes e to j) but not in the *spo0H* mutant (lanes k to p). The P2 transcript was most abundant near the end of exponential growth (lane g) and began to disappear after the start of stationary phase (lanes h to j).

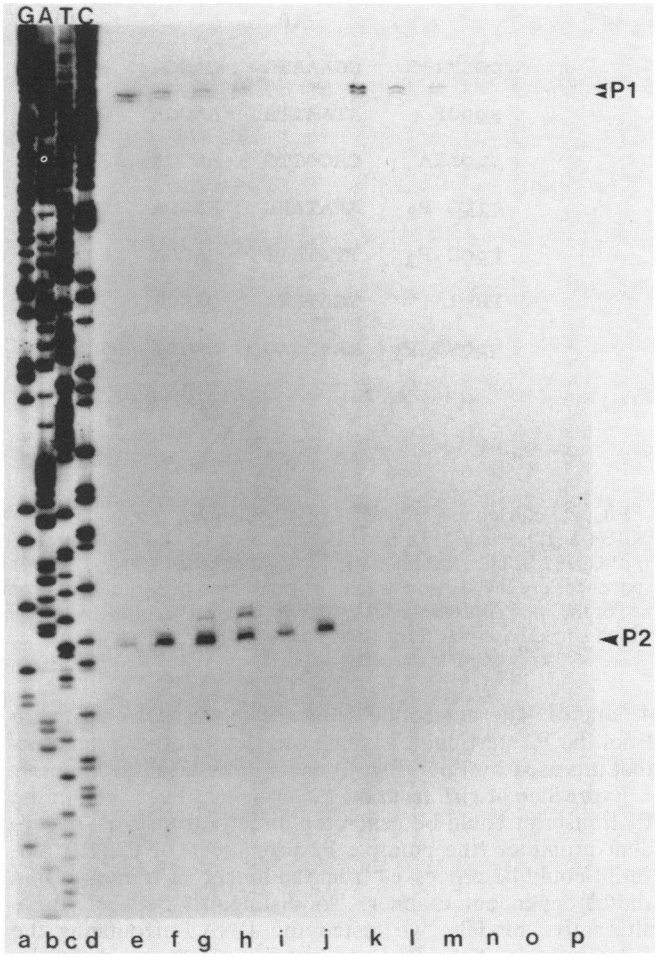

FIG. 1. Primer extension analysis of *citG* transcripts. RNA was isolated from *B. subtilis* JH642 (lanes e to j) and from a *spo0H* mutant, IS233 (lanes k to p), at intervals during growth and sporulation as described in Materials and Methods. The times at which the cells were harvested during growth is indicated on the growth curve shown in Fig. 3. For example, RNA used in lanes e and k was isolated from cells in the early stage of exponential growth, whereas the RNA used in lanes j and p was isolated from cells 4 h after the end of exponential growth. A radiolabeled oligonucleotide that was complementary to *citG* RNA was used to prime DNA synthesis from the RNA templates as described in Materials and Methods (lanes e to p). A 10-μg amount of RNA was used in each reaction mix, and similar patterns were observed when 40 μg of RNA was used. The same oligonucleotide was used in dideoxy sequencing reactions with a DNA template that contained *citG* (pAAM91) (lanes a to d). The letters above these lanes indicate the dideoxynucleotide used to terminate each reaction. Shown in the figure is an autoradiograph produced after electrophoresis of the reaction products into an 8% polyacrylamide gel that contained 8.3 M urea.

The 5′ ends of the *citG* transcripts were determined by comparing the mobility of the reverse transcripts with the extension products of the dideoxy sequencing reactions that were copied from a *citG* DNA template. The 5′ end of the major P2 transcript is indicated on the sequence in Fig. 2 and is in close agreement with that determined by Feavers et al. (7). A minor transcript that migrated slightly slower than the major P2 transcript was visible in lanes g and h of Fig. 1, but this may be an artifact of the primer extension since this transcript was not present in all RNA preparations (e.g., Fig.

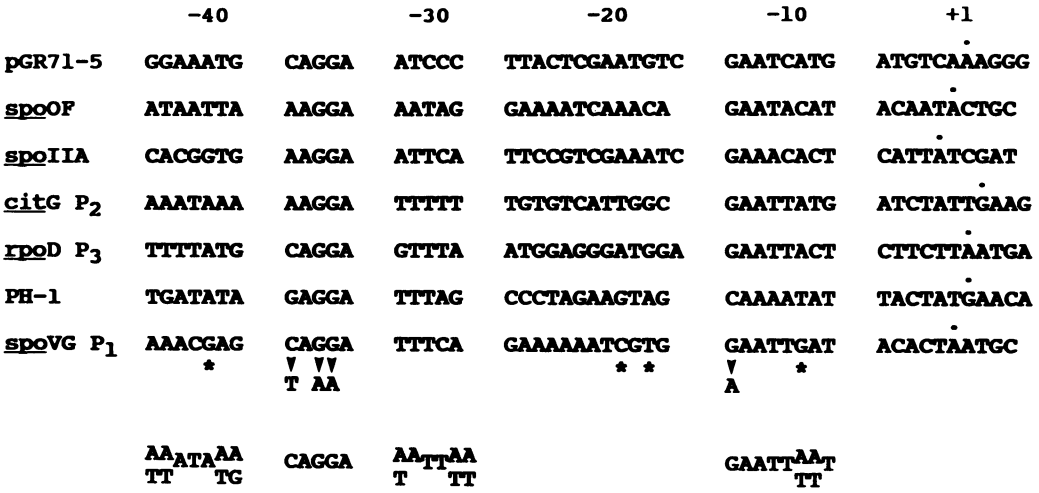

FIG. 2. Nucleotide sequences of σ^H-dependent promoters. Shown is the nucleotide sequence (nontranscribed strand) of the promoters pGR71-5 (12), *spo0F* (13), *spoIIA* (21), *citGp2* (7), *rpoDp3* (4), *pH1* (H. L. Carter, K. M. Tatti, and C. P. Moran, Jr., unpublished data), and *spoVGp1* (22). The start point of transcription is indicated as +1 (●), and transcription proceeds from left to right. Shown below the promoter sequences are the sequences that are conserved between these promoters. The arrowheads indicate base pair substitutions that decrease the use of the *spoVG* promoter in vivo to less than 25% of the wild-type level, and the asterisks indicate the positions in the *spoVG* promoter at which single-base-pair transitions had less-severe effects (22).

4, lane c). The nucleotide sequence of the DNA upstream from the P2 start point is similar to that in other promoters that are used by $E\sigma^H$ (Fig. 2) and will be discussed later.

Expression of *xylE* from the P2 promoter. The 5′ end of the P2 transcript could be generated directly from a σ^H-dependent promoter (the putative P2 promoter), or the P2 transcript could be processed from the longer P1 transcript in a *spo0H*-dependent manner. To distinguish between these alternative models, we tested the DNA surrounding the putative start of the P2 transcript for promoter activity by fusing a 305-bp *Rsa*I-*Dra*I DNA fragment containing this region to a promoterless derivative of *xylE*, the structural gene for $CatO_2$ase. This 305-bp DNA fragment did not contain the upstream promoters that generated the P1 transcripts. *xylE* expression was monitored during growth of strains containing the *citGp2-xylE* fusion on plasmid pKT89. $CatO_2$ase accumulated at the beginning of stationary phase (Fig. 3). The accumulation of the $CatO_2$ase appeared to be somewhat delayed when compared with the accumulation of the P2 transcript (Fig. 1). This discrepancy most likely resulted because the *citGp2-xylE* fusion is carried on a multicopy plasmid, since no delay was observed when *citGp2* activity was examined by fusion to *lacZ* in the *B. subtilis* chromosome (16) (data not shown). This accumulation was dependent on σ^H, since it did not occur in a *spo0H* mutant (Fig. 3). Expression of *xylE* was not detected when the DNA fragment was cloned in the opposite orientation (data not shown). These results clearly demonstrate that the 305-bp DNA fragment contained a σ^H-dependent promoter.

In vitro transcription of *citG* with $E\sigma^H$. The σ^H-dependent transcription from the P2 promoter raises the possibility that this promoter is used directly by $E\sigma^H$. To test this hypothesis, we used $E\sigma^H$ to transcribe a DNA template (pAAM91) containing the *citGp2* promoter in an in vitro assay. In the first experiments, pAAM91 that had been linearized by cleavage with *Dra*I was transcribed with $E\sigma^H$, and a single run-off transcript was generated. The size of this transcript (~177 nucleotides) was approximately the size expected for a transcript initiated from the P2 promoter (data not shown). We used a primer extension analysis to determine more precisely whether $E\sigma^H$ generated the transcript from the P2 promoter. The DNA template that contained the *citGp2* promoter (pAAM91 linearized with *Dra*I) was transcribed by $E\sigma^H$ in the presence of nonradiolabeled nucleoside triphosphates as described in Materials and Methods. The location of the 5′ terminus of the resulting RNA transcript was determined by using reverse transcriptase to extend a primer that was complementary to a region 80 nucleotides downstream from the P2 promoter (Fig. 4, lanes a and e). The size of the cDNA extended from the primer was determined by electrophoresis into a DNA sequencing gel next to the dideoxy-sequencing products of pAAM91 that had been generated from the same oligonucleotide primer (Fig. 4,

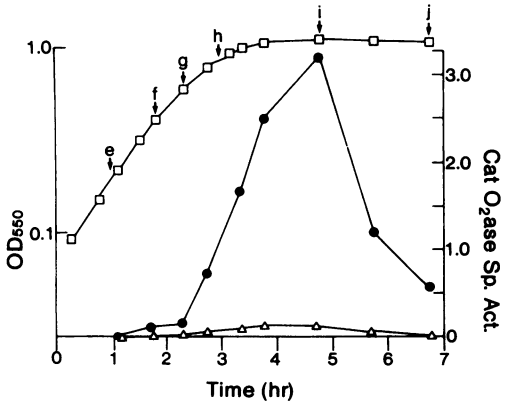

FIG. 3. Time course of *citGp2-xylE* expression. Shown are the specific activities of $CatO_2$ase from JH642 containing pKT89 (●) and the *spo0H* mutant IS233 containing pKT89 (△). The cells were grown in DS medium, and growth was measured by OD_{550}. Shown is a typical growth curve for the JH642 derivative strains (□). The growth of IS233 derivatives followed a similar pattern. The specific activity of $CatO_2$ase was determined as described in Materials and Methods. The letters e to j above the growth curve indicate the points at which JH642 and IS233 cells were harvested for extractions of the RNA used in the primer extension analysis shown in Fig. 1, lanes e to j and k to p, respectively.

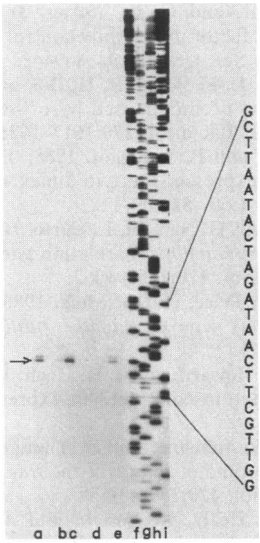

FIG. 4. Primer extension analysis of the *citG* transcript produced in vitro by $E\sigma^H$. Primer extension analysis was done as described in the legend to Fig. 1 except that the RNA used was produced by transcription of a *citG* template in vitro with EσH (lanes a and e). RNA isolated from *B. subtilis* JH642 during exponential growth (lane c) and 2 h after the end of exponential growth (lane b) was also analyzed by primer extension. Shown in lanes f to i are the dideoxy sequencing products of the *citG* DNA template terminated with dideoxycytosine, dideoxythymine, dideoxyadenosine, and dideoxyguanosine, respectively.

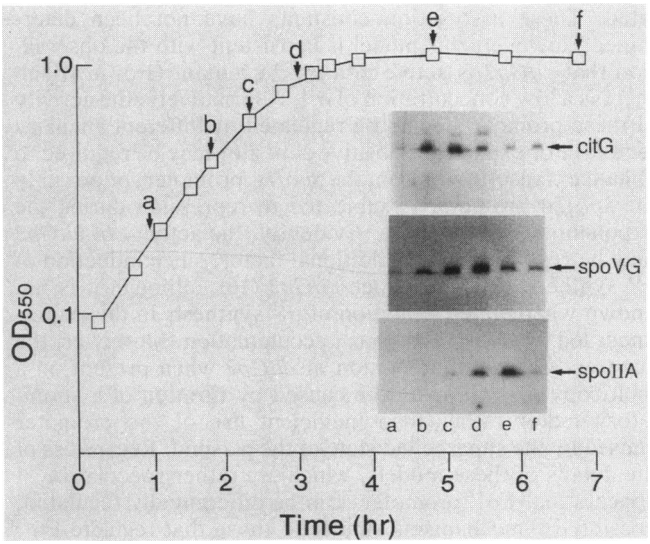

FIG. 5. Primer extension analysis of σ^H-dependent transcripts. Primer extension analysis was done as described in the legend to Fig. 1 except that oligonucleotides that were complementary to *spoVG* and *spoIIA* RNA were also used. The products of each reaction were subjected to electrophoresis and autoradiography as described in the legend to Fig. 1, but only that part of the autoradiograph that contains the σ^H-dependent transcript is shown in this figure. The letters a to f above the growth curve indicate the times at which RNA was isolated and analyzed in lanes a to f, respectively. These are the same RNA samples used in Fig. 1. The *spoIIA* and *spoVG* transcripts were not detected in RNA preparations from the *spo0H* mutant (data not shown).

lanes f to j). Reverse transcriptase and the same primer were used to copy *citG* RNA isolated from *B. subtilis* (Fig. 4, lanes b and c). This experiment demonstrated that $E\sigma^H$ initiated transcription from the P2 promoter at the same nucleotide as that located at the 5′ terminus of the P2 transcript that was identified in vivo. Moreover, we found that the P2-transcribing activity eluted simultaneously with $E\sigma^H$ from a DNA-cellulose column during purification of $E\sigma^H$ (data not shown). Therefore, it is unlikely that P2-transcribing activity is due to the presence of an undetected and previously unidentified sigma factor in the $E\sigma^H$ preparation.

Regulation of σ^H-dependent promoters. RNA was isolated from *B. subtilis* at intervals during growth and sporulation in DS medium. We used primer extension analyses to examine the temporal patterns of expression from several σ^H-dependent promoters. The *citGp2* transcript had accumulated to its maximum level by the time that the cells had completed the exponential growth phase (Fig. 5, lane c). In contrast, the *spoIIA* transcript was most abundant 2 h after the start of stationary phase (Fig. 5, lane e). Accumulation of the *spoVG* transcript appeared to reach its maximum level within 1 h after the end of the exponential growth phase (Fig. 5, lane d). The rate of accumulation of these transcripts was dependent on their rates of synthesis and degradation. The relative stability of these transcripts is unknown, although the *spoVG* transcript is more stable than most RNA species (20). Therefore, the apparent difference between the times at which the *citGp2* and *spoVG* transcripts accumulated to maximum levels may not reflect differences in promoter utilization. However, if the half-lives of the *spoIIA* and *citGp2* transcripts are similar, these results indicate that the *citGp2* promoter is activated earlier during growth than is the *spoIIA* promoter.

DISCUSSION

Sequences recognized by $E\sigma^H$. There are seven promoters known that may be used by $E\sigma^H$. Of these seven promoters, shown in Fig. 2, all except the *spo0F* promoter and the promoter on pGR71-5 have been shown to be used in vitro by $E\sigma^H$ (3, 4; H. L. Carter, J. Wu, P. Piggot, K. M. Tatti, and C. P. Moran, Jr., unpublished results). Expression from all seven promoters is eliminated by mutations in *spo0H*, and therefore their activities depend on σ^H (4, 7, 12, 13, 21, 22; H. L. Carter, K. M. Tatti, and C. P. Moran, Jr., unpublished data). Comparison of the nucleotide sequences of these promoters reveals that these sequences are similar at two regions centered approximately 10 and 35 bp upstream from the start points of transcription (Fig. 2). These conserved sequences are probably important for promoter activity, since several single-base-pair substitutions of these nucleotides in the *spoVG* promoter were found to reduce its activity (Fig. 2) (22). Obviously, examination of additional mutations in the other σ^H-dependent promoters will be needed to test the role of these conserved sequences.

Regulation of σ^H-dependent promoters. It appears that the *citGp2* promoter is activated during exponential growth in DS medium, whereas the *spoIIA* promoter appears to be activated after the end of exponential growth (Fig. 5). Two models can account for the different temporal patterns of expression from these promoters. The concentration of σ^H increases about fivefold between the early stages of exponential growth and stationary phase in DS medium (J. Healy and R. Losick, personal communication; I. Smith, personal communication). If the binding constant for the association of $E\sigma^H$ with the *spoIIA* promoter is lower than that for the *citGp2* promoter, then the *spoIIA* promoter may be used only after the maximum concentration of σ^H has accumu-

lated. These association constants have not been determined; however, this model is consistent with the observation that *citGp2* is active in a *spo0A* mutant (16), in which there is a low concentration of σ^H. Alternatively, the activity of these promoters could be regulated by different ancillary factors. For example, a positive activator may be required to enhance transcription from the *spoIIA* promoter, or possibly the *spoIIA* promoter is subjected to repression during the exponential growth phase. Evidently, the activity of *citGp2* may be regulated by an additional factor, since induction of σ^H synthesis did not induce *citGp2* (16), although it is not known whether the induction of σ^H synthesis in this experiment led to an increase in σ^H accumulation. Moreover, the apparently delayed activation of *citGp2* when present on a multicopy plasmid could be caused by titration of a stimulatory factor rather than inefficient use of the promoter caused by the supercoiled state of the plasmid. Regardless of the details of these models, which are rather speculative, it appears that $E\sigma^H$ promoters can be differentially regulated, possibly by mechanisms similar to those that regulate $E\sigma^A$ promoters.

The role of the σ^H regulon. It is not obvious why *B. subtilis* uses σ^H to direct transcription, since $E\sigma^A$ evidently transcribes some genes during the early stages of stationary phase (9, 11; T. J. Kenney, P. Youngman, and C. P. Moran, Jr., unpublished data), and σ^H promoters may be regulated by mechanisms analogous to those that modulate expression from σ^A promoters. Many of the genes transcribed by $E\sigma^H$ are also transcribed by other polymerases. *citG* (7, 16) and *rpoD* (4) are transcribed from promoters upstream from P2 and P3, respectively, that are probably used by $E\sigma^A$. The region downstream from *pH1* is read from a second upstream promoter at late stages of endospore formation (K. M. Tatti, unpublished results) as is the *spoIIA* operon (5). Jaacks and co-workers (10) found that transcription of several genes that can be induced by production of σ^H was not entirely dependent on σ^H; therefore, these genes may also be read by more than one form of RNA polymerase. Since σ^A can direct transcription of some genes during the transition from growth to stationary phase, the role of σ^H may be to ensure efficient transcription of specific genes during this period of stress. In this scenario, σ^H is acting to reserve a fraction of RNA polymerase for use on promoters that must remain very active under poor growth conditions. The role of the σ^H should become evident after additional genes that are transcribed by $E\sigma^H$ are identified.

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