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Regulation of *spo0H*, a Gene Coding for the *Bacillus subtilis* σ^H Factor

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The *Bacillus spo0H* gene codes for σ^H, which, as part of the RNA polymerase holoenzyme $E\sigma^H$, is responsible for the transcription of several genes which are expressed at the beginning of the sporulation process. In this communication, we examined the regulation of the *spo0H* gene of *Bacillus subtilis* by using *lacZ* reporter gene assays, quantitative RNA determinations, and Western immunoassay. The expression of the *spo0H* gene increases as the culture enters the mid-logarithmic stage of growth. This increased expression requires the genes *spo0A*, *spo0B*, *spo0E*, and *spo0F*, and the requirement for at least *spo0A* and *spo0B* can be bypassed when the *abrB* gene is mutated. The expression of the *spo0H* gene is constitutive in the presence of the *abrB* mutation, being expressed at higher levels during vegetative growth. In addition, the *sof-1* mutation, in the *spo0A* structural gene, can bypass the need for *spo0F* in *spo0H* expression. The transcriptional start site of *spo0H* was determined by using RNA made in vivo as well as in vitro. These studies indicate that *spo0H* is transcribed by the major vegetative RNA polymerase, $E\sigma^A$. *spo0H* RNA and σ^H levels during growth are not identical to each other or to the pattern of expression of *spoVG*, a gene transcribed by $E\sigma^H$. This suggests that *spo0H* is regulated posttranscriptionally and also that factors in addition to σ^H levels are involved in the expression of genes of the $E\sigma^H$ regulon.

Minor species of RNA polymerases in general either are present at low levels or are not active under conditions in which they are not necessary for the growth or survival of the cell. Various mechanisms exist to prevent the inappropriate presence or activity of these polymerases. The *rpoH* gene of *Escherichia coli*, which codes for σ^{32}, is transcribed at much higher levels after a heat shock (14, 43). In addition, σ^{32} is a very unstable protein (14). Although the *rpoN* gene product, σ^N, of *E. coli* and *Klebsiella* spp. is not increased by nitrogen deprivation (5, 7), and is stable (19), its activity requires a phosphorylated form of the NtrC activator protein, and the extent of phosphorylation of NtrC depends upon the availability of nitrogen (31).

Sporulation in *Bacillus subtilis* depends upon the synthesis and activation of a series of new σ factors (28). The *sigE* gene product, σ^E, is not made until T_2 (defined as 2 h after T_0, the time at which cells reach stationary phase) and then only as an inactive precursor (25, 44). σ^G is produced only in the forespore (41), and the gene for σ^K is transcribed after a chromosomal rearrangement which occurs only in the mother cell (39). It has also been demonstrated that a 14-kDa protein changes the transcriptional specificity of $E\sigma^K$ (24). Thus, the functioning of minor species of RNA polymerase containing these σ factors can be regulated by processing, by localization within the bacterial cell, and by transcriptional activators.

$E\sigma^H$, the RNA polymerase containing σ^H, which is encoded by *spo0H* (9, 51), is required for the in vivo and in vitro transcription of promoters $spoVGp_1$ (3, 51), $rpoDp_3$ (4), and $citGp_2$ (10, 42). These promoters are turned on just when the culture reaches the stationary phase of growth (T_0) in rich medium. Therefore, the activity of this minor species of RNA polymerase can be detected in vivo at the earliest stage of sporulation but only at very low levels during the logarithmic stage of growth (3). This higher level of activity could result from the increased expression of the *spo0H* gene, increased stability of the gene product, the synthesis of transcriptional activators, removal of repressors, or a combination of the above. In addition, $E\sigma^H$ transcribes *spoIIA* (42), a gene which shows much later expression than the previously described early sporulation genes. The reasons for the delay in expression of this $E\sigma^H$-dependent gene are not known at present.

We have shown (8) that the expression of the *spo0H* gene from *Bacillus licheniformis* is growth regulated, that its full expression requires the *spo0A* gene, and that a double mutation in *spo0A* and *abrB* results in constitutive expression of *spo0H*. However, the homology on the DNA level between *B. licheniformis* and *B. subtilis* is insufficient to allow chromosomal integration, and the *B. licheniformis spo0H-lacZ* fusions had to be integrated at a chromosomal site distant from the *spo0H* gene. These constructs contained only 40 bp upstream from the promoter region, and since it was possible that important upstream regulatory sequences were missing, we decided to study the expression of the *B. subtilis spo0H* gene by using a *spo0H-lacZ* translational fusion integrated in the homologous chromosomal site. In addition, the amount of *spo0H* RNA present at various stages of growth and in different genetic backgrounds was measured. Western immunoassays were also used to ascertain σ^H protein levels during growth.

MATERIALS AND METHODS

Strains. The bacterial strains used for these experiments are listed in Table 1.

Construction of a translational *spo0H-lacZ* fusion. The 1.1-kb *Hin*dIII fragment from pIS139 contains about 600 bp upstream from the *B. subtilis spo0H* promoter as well as

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TABLE 1. Strains

Strain	Relevant genotype	Source or reference
IS230	*trpC2 pheA1*	J. Hoch, JH642
IS233	*trpC2 pheA1 spo0HΔHin*dIII	45
IS402	*trpC2 pheA1 spo0AΔ204 abrB703*	P. Zuber, ZB369
IS403	*trpC2 pheA1 abrB703*	P. Zuber, ZB449
IS414	*trpC2 pheA1*::pZL207[a]	P. Zuber, ZB212
IS442	*trpC2 pheA1 spo0AΔ204*	P. Zuber, ZB515
IS478	*trpC2 pheA1*::pIS162	This work
IS481	*trpC2 pheA1 spo0AΔ204*::pIS162	This work
IS482	*trpC2 pheA1 spo0AΔ204 abrB703*::pIS162	This work
IS483	*trpC2 spo0BΔPst*[b]::pIS162	This work
IS492	*trpC2 pheA1 spo0E11*::pIS162	This work
IS493	*trpC2 pheA1 spo0F221*::pIS162	This work
IS495	*trpC2 pheA1 abrB703*::pIS162	This work
IS520	*trpC2 spo0BΔPst*[b] *abrB703*::pIS162	This work
IS521	*trpC2 spo0F221 sof-1*::pIS162	This work
IS591	*metB4 leuA8 hisA1 spo0H*Δpromoter[c]::pBD97[d]	This work
IS719	*trpC2 pheA1 abrB*::pJM5154	J. Hoch, JH12586

[a] pZL207 is a *spoVG-lacZ* transcriptional fusion construct integrated into the *B. subtilis* chromosome by reciprocal recombination at the homologous site (48).
[b] This deletion was made by *Pst*I restriction of a clone carrying the *spo0B* gene, followed by making the ends blunt and religation. It was integrated into the chromosome by gene conversion (unpublished results).
[c] This deletion was made by *Hin*dIII restriction of pIS139 and self-ligation. The deletion starts about 600 bp upstream from the *spo0H* promoter and ends within the structural gene of *spo0H* (Fig. 1). It was integrated into the *B. subtilis* chromosome by gene conversion (unpublished results).
[d] Integration of pBD97 (6).

about one-third of the structural gene (9). This fragment was cloned into the *Eco*RI cloning site of pIS112 (27), after making all the ends blunt by filling in with the Klenow fragment of DNA polymerase. The junction between the filled-in *Eco*RI and *Hin*dIII sites was verified by DNA sequencing. This construct, pIS162, (Fig. 1), contains part of the structural gene of *spo0H* in frame with the *lacZ* gene. It

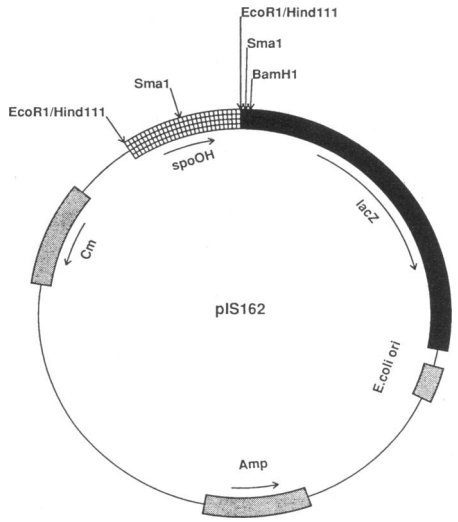

FIG. 1. Construction of a *spo0H-lacZ* translational fusion. pIS112 (27) was cut at the unique *Eco*RI site, and pIS139 (9) was cut with *Hin*dIII to release a 1-kb fragment containing about 500 bp upstream of the *spo0H* promoter and about one-third of the *spo0H* structural gene. All the ends were made blunt by the fill-in reaction of the Klenow fragment of DNA polymerase, followed by ligation. The resulting plasmid, pIS162, confers resistance to ampicillin and chloramphenicol in *E. coli*. The plasmid cannot replicate in *B. subtilis* (27), but it can integrate in the *B. subtilis* chromosome by a Campbell-like mechanism at the *spo0H* locus, expressing chloramphenicol resistance.

can replicate only in *E. coli* but can integrate into the *B. subtilis* chromosome in the *spo0H* allelic site by a Campbell-like mechanism of homologous recombination, conferring resistance to chloramphenicol. The integrated form of pIS162, at the *spo0H* locus, was moved by transformation into a set of strains which carried various mutations in *spo0* genes but were otherwise isogenic (Table 1). Southern blot analysis showed that pIS162 integrated at the region of chromosomal homology, in single copy (data not shown).

Media and β-galactosidase determinations. Cells were grown in Schaeffer's nutrient sporulation medium (NSM) as described previously (8), and samples were removed at intervals to assay β-galactosidase activity. The assays were performed with toluenized cells, as described previously (8). Activity is expressed as units (1 U = 1 nmol of *o*-nitrophenyl hydrolyzed per min) per mg of protein. Protein determinations were made with the Bio-Rad protein assay.

Primer extension analysis. To analyze the transcriptional start site of *spo0H*, the primer 5′-ATGACCTGCTC GTCCTC-3′, which is complementary to a region of the RNA starting 80 bases from the purported +1 start site of transcription, was 5′-end labeled with [γ-^{32}P] ATP by using polynucleotide kinase. RNA (75 μg), prepared as described previously (26), in 7 μl, was added to 1 μl of 10× buffer (500 mM Tris [pH 8], 500 mM KCl, 80 mM $MgCl_2$, 10 mM dithiothreitol [DTT]). To this were added 1 μl of Inhibitace (an inhibitor of RNase, purchased from 5′-3′ Inc.) and 1 μl of γ-^{32}P-labeled primer (about 7 ng). The mixture was heated at 62°C for 10 min and then slowly cooled to room temperature. Then 8 μl of a 2.5 mM mixture of deoxynucleotide triphosphates and 1 μl of avian myeloblastosis virus reverse transcriptase (AMV Super RT; Molecular Genetic Resources) were added to the annealed samples, which were incubated for 30 min at 42°C, and the reaction was stopped with 2 μl of 0.5 M EDTA. Samples were precipitated by adding 6 μl of 8 M ammonium acetate and 60 μl of ethanol. After centrifugation, the pellets were dried in a Speed-Vac centrifuge and resuspended in 5 μl of H_2O, and 1 μl of RNase was added. Loading buffer (4 μl; 90% deionized formamide, 0.02%

bromophenol blue, 0.02% xylene cyanol) was then added, and the samples were heated at 90°C for 3 min before being loaded on a sequencing gel. The same primer was used in sequencing reactions. The template was double-stranded DNA from plasmid pIS139, containing the intact region of the *spo0H* gene (9). The protocol used was from Hattori and Sakaki (15) as described before (44a). Sequenase was from U.S. Biochemical Corp. In vitro RNA was prepared as described below.

RNA polymerase transcription assays. PolydAT-dependent synthesis and run-off transcriptions, utilizing prebinding of RNA polymerase to the template and heparin addition to ensure single-round synthesis, were performed as described previously (30, 38). The template was pIS139 restricted with *Rsa*I or a purified *Rsa*I fragment containing the *spo0H* promoter. A run-off transcript of 150 nucleotides was expected. For in vitro primer extension, a four-times-larger transcription reaction mixture was incubated, but with nonradioactive ribonucleoside triphosphates and supercoiled plasmid. After 10 min at 37°C, the reaction was stopped by the addition of pancreatic DNase, the sample was treated with phenol, and the resulting aqueous layer was precipitated with ethanol. The RNA pellet was then resuspended and used for primer extension as described for the in vivo RNA (see above).

Purification of RNA polymerase. Ten grams of IS230, grown in NSM medium to the mid-log stage of exponential growth, was washed, disrupted in a French pressure cell, and purified through the phase partition step, as described by Shorenstein and Losick (38). The final ammonium sulfate pellet from the phase treatment was resuspended in 1.5 ml of buffer A (10 mM Tris [pH 8.4], 1 mM EDTA, 10 mM $MgCl_2$, 5% phenylmethylsulfonyl fluoride, 10% glycerol, 0.3 mM DTT), and it was dialyzed against the same buffer. The next steps were done by fast protein liquid chromatography (FPLC), modifying a previously published technique which was worked out for *Streptomyces* RNA polymerases (2). The dialyzed sample was clarified by a low-speed centrifugation step (20,000 × *g* for 10 min), and the supernatant fraction was fractionated by Suberose 6 exclusion chromatography on a Pharmacia FPLC apparatus. Buffer A was used for the elution, and peak fractions of RNA polymerase activity, as measured by poly(dA-dT)-dependent transcription, were immediately loaded onto a Mono Q anion-exchange column, using the same FPLC apparatus. Elution was carried out with a linear KCl gradient, 0.1 to 0.45 M, in buffer A. Peak fractions of activity, assayed as above, were stored at −20°C after overnight dialysis against storage buffer (10 mM Tris [pH 8.0], 10 mM $MgCl_2$, 0.1 mM EDTA, 100 mM KCl, 0.3 mM DTT, 50% glycerol). The fraction with the greatest $E\sigma^A$ activity, as measured by activity with the $sinp_3$ promoter (13), and which was estimated to be at least 80% pure by gel electrophoresis was used for the in vitro transcription assays.

Quantitative estimation of RNA by primer extension and S1 nuclease analyses. To compare levels of *spo0H* RNA in different genetic backgrounds, S1 nuclease analyses were performed. RNA was prepared from cultures growing in NSM as described before (26). The restriction fragment used for S1 nuclease analysis was an 800-bp *Eco*RV-*Eco*RI *spo0H* promoter-containing fragment, end labeled at the *Eco*RI site, which is within the *spo0H* structural gene. It was isolated by gel purification from pIS139 (9). Detailed procedures for S1 nuclease analysis were described previously (26). Quantitation of RNA levels during growth by primer extension was performed as described previously (22) with a probe complementary to the RNA transcript from the +123 to +93 region of the *spo0H* gene and with various amounts of RNA. The use of a 30-mer oligomer primer in these studies gave much cleaner backgrounds than the 17-mer oligomer primer used for determining the transcriptional start site (compare Fig. 4 and 6). The amount of RNA was estimated by the orcinol reaction (6). The quantity of labeled DNA protected from S1 nuclease digestion was estimated by densitometry of the relevant bands observed in autoradiograms with a Bio-Rad video scanning densitometer (model 620) on line with an IBM PC.

Immunological estimation of σ^H levels. Crude extracts from cells grown in NSM were separated on 12.5% polyacrylamide gels by electrophoresis. The protein samples were electrophoretically transferred to nitrocellulose filters, and the filters were then incubated with rabbit antibodies prepared against a *spo0H-lacZ* protein fusion. Antibody was detected by using alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin antibodies. Details of these procedures and a more complete description of the *spo0H* antibodies have been described previously (9).

RESULTS

***spo0H*-directed β-galactosidase activity.** *B. subtilis* strains carrying various *spo0* mutations as well as the integrated *spo0H-lacZ* fusion pIS162 (construction and chromosomal integration are described in Materials and Methods and Fig. 1) were grown in NSM, and samples were collected at various times and assayed for β-galactosidase activity (Fig. 2 and 3). The results show that *spo0H-lacZ* expression began to increase gradually during the mid-log stage of growth and reached a peak at or near T_0 when growth was monitored past this stage (Fig. 3). Less expression was observed in the presence of mutations in *spo0A*, *spo0B*, *spo0F*, and *spo0E*, although there was activity, and this residual activity still showed a rise over time. The *spo0F* requirement for full expression was completely bypassed in a *sof-1 spo0F* double mutant. When the experiment was done in IS233, a strain lacking an intact copy of *spo0H* (in the *spo0HΔHin*dIII null mutant background, described by Weir et al. [45]) (Table 1), the results were the same as with the spo^+ strain (data not shown).

The *spo0A* and *spo0B* requirement for *spo0H-lacZ* activity could be bypassed in the presence of the *abrB703* mutation (Fig. 3). In fact, expression of the *spo0H* gene was constitutive in all *abrB* mutant strains tested, showing much higher levels during vegetative growth. The effects described here of *spo0A* and *spo0A abrB* double mutations on *spo0H* expression are similar to those reported previously by Zuber et al. (50). However, in the previous work, *spo0B* lesions had no effect on *spo0H-lacZ* expression. We have no explanation for the difference in these results, but those presented here are in agreement with direct measurements of *spo0H* RNA levels in *spo0* mutant strains (Table 2). In our experiment, a null mutation in *spo0B*, constructed by inserting a chloramphenicol resistance gene into the *spo0B* open reading frame, was used. The *spo0B136* mutation used by Zuber et al. has not been sequenced, and it is possible that this mutation allows partial function. However, we have not tested the effect of *spo0B136* on *spo0H* expression.

spo0H-lacZ activity was measured after treatment with the inhibitor of GTP biosynthesis decoyinine, and in contrast with previously reported induction of the *B. licheniformis* gene (8), no induction was observed with the *B. subtilis* gene (data not shown) (16). An extensive analysis of *spo0H-lacZ*

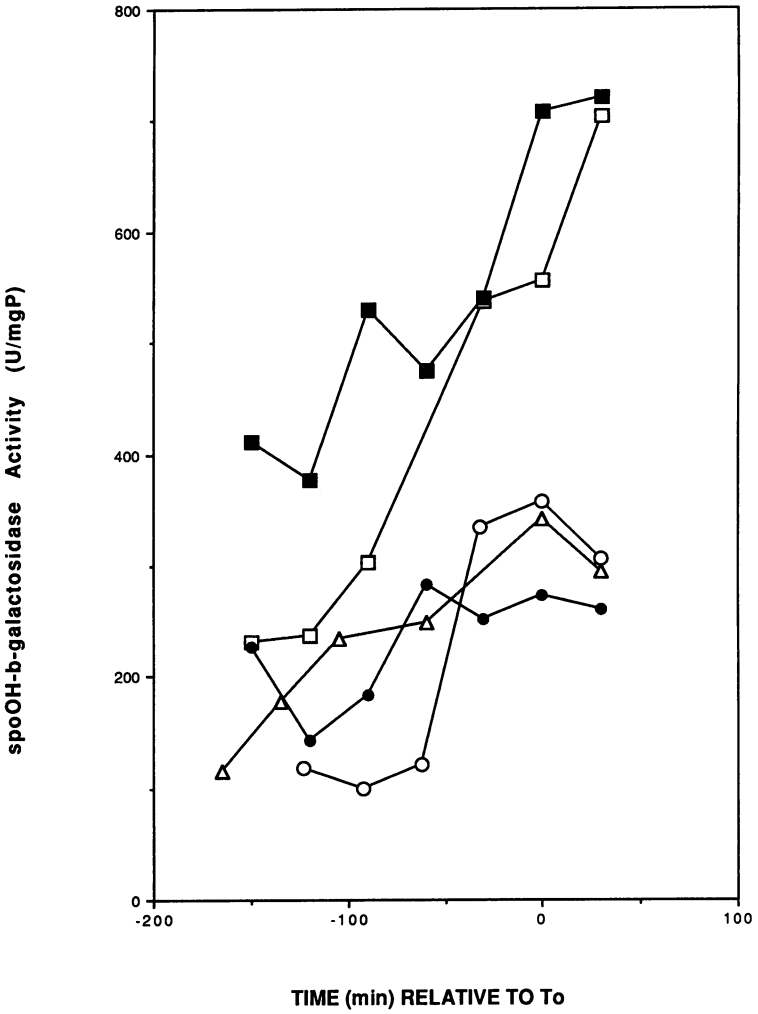

FIG. 2. *spo0H-lacZ*-directed β-galactosidase activity. pIS162 was integrated, by a Campbell-type event, into the *B. subtilis* chromosome at the *spo0H* locus of a Spo$^+$ strain. DNA from this strain (IS473) was used to transform various *spo0* mutants and an isogenic Spo$^+$ strain. Cultures were grown in NSM, and samples were removed at various times to assay for β-galactosidase activity. Time is expressed in minutes before or after T_0, and the β-galactosidase activity is expressed as defined in the text. The strains used, each containing a *spo0H-lacZ* translational fusion, were IS478 (*spo*$^+$) (□), IS521 (*spo0F sof-1*) (■), IS483 (*spo0B*) (●), IS493 (*spo0F*) (△), and IS492 (*spo0E*) (○).

activity in minimal medium with and without decoyinine has not been undertaken. However, in a wild-type strain, IS478, the *spo0H* gene was constitutive in S6C, the minimal growth medium used for decoyinine induction (with the average activity of *spo0H-lacZ* in the range of 300 to 400 U/mg of protein). Essentially the same levels of β-galactosidase activity were observed in IS481 and IS482, isogenic *spo0AΔ204* and *spo0AΔ204 abrB703* strains, respectively grown in the same medium (data not shown). The results with the *spo0H* gene from *B. licheniformis* (8) were also somewhat different in that we did not observe a dependence on *spo0B* or *spo0F* and we observed a much higher level of activity in a strain lacking an intact copy of *spo0H*. The reasons for these contradictory results are unknown, but the *B. licheniformis spo0H-lacZ* fusion used had only 40 bp upstream from the promoter region, and the construct was integrated into the chromosome at a nonhomologous site. The *B. subtilis* fusion had approximately 500 bp upstream from the transcriptional start site and was integrated at the region of *spo0H* homology. Integration of the *B. subtilis spo0H* fusion at the heterologous site used for the *B. licheniformis* fusion or the use of translational fusions with different lengths of the *B. subtilis spo0H* open reading frame and *lacZ* did not alter the decoyinine effect on *spo0H* gene expression (data not shown).

Analysis of the *spo0H* transcriptional start site. RNA prepared from a *spo*$^+$ strain as well as from *spo0A* and *spo0A abrB* mutants was analyzed in primer extension experiments to determine the in vivo start site for *spo0H* transcription (Fig. 4). The same analysis was performed with RNA transcribed in vitro by Eσ^A from pIS139, a plasmid containing the *B. subtilis spo0H* gene (9). The start site was the same regardless of which RNA was used for primer extension, and it is clear that the +1 start site which had been postulated from the sequence of the *spo0H* gene (9) was utilized in both in vivo and in vitro transcription. We conclude that the *spo0H* promoter is transcribed both in vivo and in vitro by RNA polymerase containing σ^A. Figure 5 shows the 150-nucleotide run-off transcript synthesized by Eσ^A from the pIS139 plasmid linearized within the *spo0H* gene.

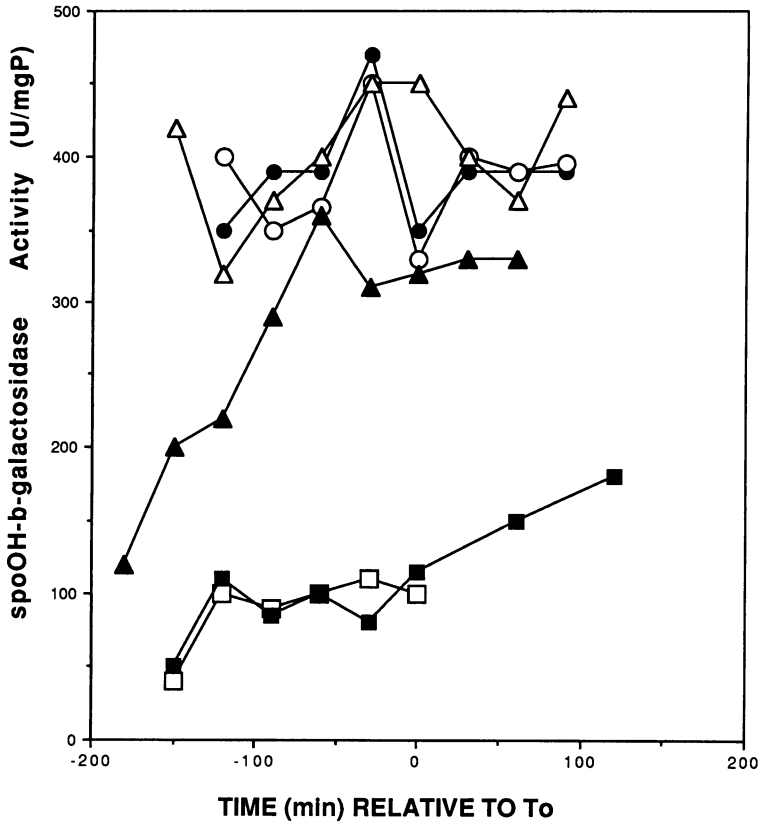

FIG. 3. Effect of the *abrB* mutation on *spo0H-lacZ* activity. The experiments were done and data are expressed as described in the legend to Fig. 2. The strains used were IS478 (*spo*$^+$) (▲), IS481 (*spo0A*) (□), IS482 (*spo0A abrB*) (●), IS483 (*spo0B*) (■), IS520 (*spo0B abrB*) (○), and IS495 (*abrB*) (△).

Levels of *spo0H* RNA and σ^H. The observations on the regulation of *spo0H-lacZ* expression were made with a translational *lacZ* fusion and therefore could be the result of transcriptional and/or translational regulation. In addition, the use of *lacZ* fusions necessitates the measurement of β-galactosidase levels. While this is relatively easy, one is actually studying the accumulation of β-galactosidase activity. This parameter is determined by the rate of synthesis and degradation of a fusion protein which may or may not

TABLE 2. Levels of *spo0H* RNA in *spo0* mutant strains[a]

Strain	Genotype	*spo0H* RNA at T_0 (% of control)
IS478 (control)	*spo*$^+$	100
IS481	*spo0A*	16
IS483	*spo0B*	9
IS493	*spo0F*	3
IS492	*spo0E*	ND[b]

[a] RNA prepared from different strains at the T_0 stage of growth was hybridized with an 800-bp fragment containing the *spo0H* promoter and part of the structural gene. The fragment was 5′-end labeled at the *Eco*RI site, which is internal to the *spo0H* gene and is 366 nucleotides from the transcriptional start site. The DNA fragment annealed to the RNA samples (50 μg) was treated with S1 nuclease, and the protected fragment was analyzed by polyacrylamide gel electrophoresis. The values are from densitometer tracings of the autoradiograms. The differences between the various *spo0* mutants were not considered significant.

[b] ND, None detected.

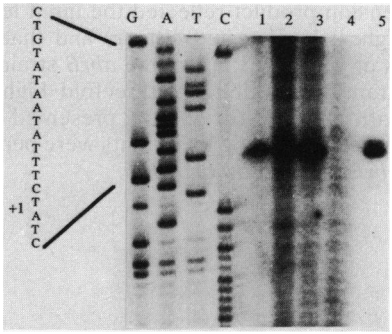

FIG. 4. Transcriptional start site of the *spo0H* gene. The start site was determined by primer extension on in vivo RNA prepared from (lane 1) IS478 (*spo*$^+$), (lane 2) IS481 (*spo0A*), (lane 3) IS482 (*spo0A abrB*), (lane 4) IS591 (*spo0H* promoter deletion), and (lane 5) RNA made by in vitro transcription from supercoiled plasmid pIS139 with purified RNA polymerase Eσ^A. The in vivo RNA was prepared from cultures grown in NSM at T_0, and the in vitro RNA was prepared as described in the text. Since the levels of RNA used for primer extension were not determined quantitatively in these experiments, it is impossible to compare levels of product obtained. After the ^{32}P-labeled primer was annealed with the RNA, reverse transcriptase was used to extend the primer in the presence of a mixture of deoxynucleotide triphosphates. The DNA sequence (the G, A, T, and C lanes) was performed on double-stranded DNA with plasmid pIS139. The dideoxy method of sequencing was used, with the same primer used for the primer extension in the other lanes. (The sequence illustrated is the complement of that read from the gel.)

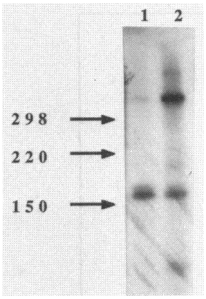

FIG. 5. In vitro transcription of the *spo0H* gene by $E\sigma^A$. FPLC-purified $E\sigma^A$ was incubated for 1 min at 37°C with 1 μg of an *Rsa*I fragment from pIS139 containing the *spo0H* promoter region (lane 1) or 2 μg of *Rsa*I-restricted pIS139 (lane 2). Transcription was initiated by the addition of a ribonucleotide triphosphate mix, including [α-^{32}P]CTP. Heparin was added after 1 min at 37°C, followed by a nonradioactive nucleotide chase for 10 min. The radioactive transcripts were analyzed by acrylamide gel electrophoresis (9% acrylamide–urea), followed by autoradiography. Size standards were ^{32}P-end-labeled *Hin*fI restriction fragments of pBR322 (not shown). The approx. 350-nucleotide transcript in lane 2 is an unidentified band which presumably comes from the vector containing the *spo0H* gene. Its absence from lane 1 indicates that it does not arise from the *spo0H* promoter-containing fragment.

reflect similar values of σ^H, the *spo0H* product. In order to define the regulation of *spo0H* more precisely, we measured the amount of *spo0H* RNA present at different stages of growth and in various *spo0* mutants.

To first test the reliability of the primer extension method for determining levels of RNA, early-log-phase RNA (2 h before T_0) was prepared from two strains, a wild type and an *abrB* null mutant, and increasing levels of RNA were used for the primer extension analysis with an oligonucleotide primer complementary to the +123 to +93 region of the *spo0H* gene (Fig. 6). The primer extension analysis indicated that the extension product reflected the input level of RNA up to the highest level tested (10 μg) and that there were higher levels of *spo0H* mRNA in the *abrB* strain than in the wild-type strain (approximately threefold higher). This is consistent with the *lacZ* fusion data presented earlier (Fig. 3), even though the former experiments were performed with a different *abrB* mutation (*abrB703*).

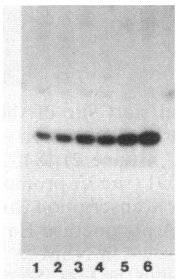

FIG. 6. Primer extension analysis of *spo0H* RNA. RNA was isolated from early-log-phase cultures of IS414 (*spo*$^+$) and IS719 (*abrB*::pJM5154). Increasing levels of RNA were used for the primer extensions, using as primer an oligonucleotide complementary to the *spo0H* RNA sequence +123 to +93. The single band in each lane represents the 123-base primer extension product, and no other transcripts were observed. Lanes 1, 2, and 3 correspond to transcripts obtained with IS414 samples at 2.5, 5, and 10 μg of RNA, respectively. Lanes 4, 5, and 6 correspond to transcripts obtained with 2.5, 5, and 10 μg of RNA from IS719, respectively.

To measure *spo0H* RNA levels during growth, RNA was prepared at different stages of growth from an NSM liquid culture of a *spo*$^+$ strain (IS414) containing an integrated *spoVG-lac* fusion (48). The activity of the *spoVG* gene was monitored in all the samples as a measure of in vivo activity of the $E\sigma^H$, and levels of RNA were determined by the primer extension method.

As Fig. 7A shows, levels of *spo0H* mRNA increased gradually during growth, showing a maximum at about 40 min before the cessation of growth, T_0. The activity of the *spoVG* gene, which is transcribed by $E\sigma^H$ (3), peaked slightly later than the levels of *spo0H* RNA (Fig. 7C), in this case showing maximum expression at T_0. This is in agreement with previously determined levels of *spoVG* RNA, which showed a peak at T_0 (42). The levels of σ^H, determined by Western immunoanalysis (in a separate culture), showed a markedly different pattern, the highest levels being observed between T_1 and T_2 (Fig. 7B). These latter data were in agreement with other immunological determinations of σ^H levels during growth in NSM (16).

RNA prepared from strains with various *spo0* mutations was subjected to the same type of analysis but with S1 nuclease protection, and the results (Table 2) show that at T_0, less *spo0H* RNA was present in strains with *spo0A*, *spo0B*, *spo0F*, and *spo0E* mutations than in the *spo*$^+$ strain, which is consistent with the *lacZ* fusion data (Fig. 2).

DISCUSSION

The data presented in this communication indicate that the expression of the *spo0H* gene of *B. subtilis* increases as the cells enter the mid-logarithmic stage of growth in NSM and that this expression is dependent upon the genes *spo0A*, *spo0B*, *spo0E*, and *spo0F*.

When the *abrB* mutation is introduced into *spo*$^+$, *spo0A*, or *spo0B* strains, the expression of the *spo0H* gene, as measured by expression of *lacZ* fusions, is constitutive. Levels of *spo0H* mRNA are also higher in early vegetative growth when the *abrB* gene is mutated. The simplest explanation for these results is that the *abr* gene product is a major, if not the only, repressor of *spo0H* gene expression and that this repression is gradually lifted as the cells enter the mid-logarithmic growth stage. The *abrB* protein seems to be the sole repressor of several other growth-regulated genes, namely *tycA* (29) and *spo0E* (32), and it has been shown that this protein binds to the promoter regions of the genes *spo0E*, *aprE*, and *abrB* (40), as well as *spoVG* and *tycA* (35). However, there must be other controls acting on *spoVG* and *aprE*, since an *abrB* mutation does not affect the temporal regulation of these genes (11, 49).

One of the functions of Spo0A is the downregulation of the *abrB* gene (33). Since the removal of the *abrB* gene product is sufficient to derepress transcription of the *spo0H* gene and the gene products of *spo0A*, *spo0B*, *spo0E*, and *spo0F* are all required for the full induction of *spo0H*, it would seem that the *spo0A* gene product must be activated in order to downregulate the *abrB* gene. However, the increase in *spo0A* and *spo0F* expression at T_0, which is dependent upon *spo0H* (47), is not necessary for *spo0H* expression, because there was no effect on *spo0H-lacZ* expression in a strain carrying a *spo0H* null mutation.

It has been postulated that the gene products of *spo0B*, *spo0F*, and *spo0E* are all required to "activate" the product of the *spo0A* gene, based upon the fact that certain mutations in the *spo0A* structural gene (*sof-1*, *SurOB20*) bypass the requirements for *spo0B*, *spo0F*, and *spo0E* genes (18, 37).

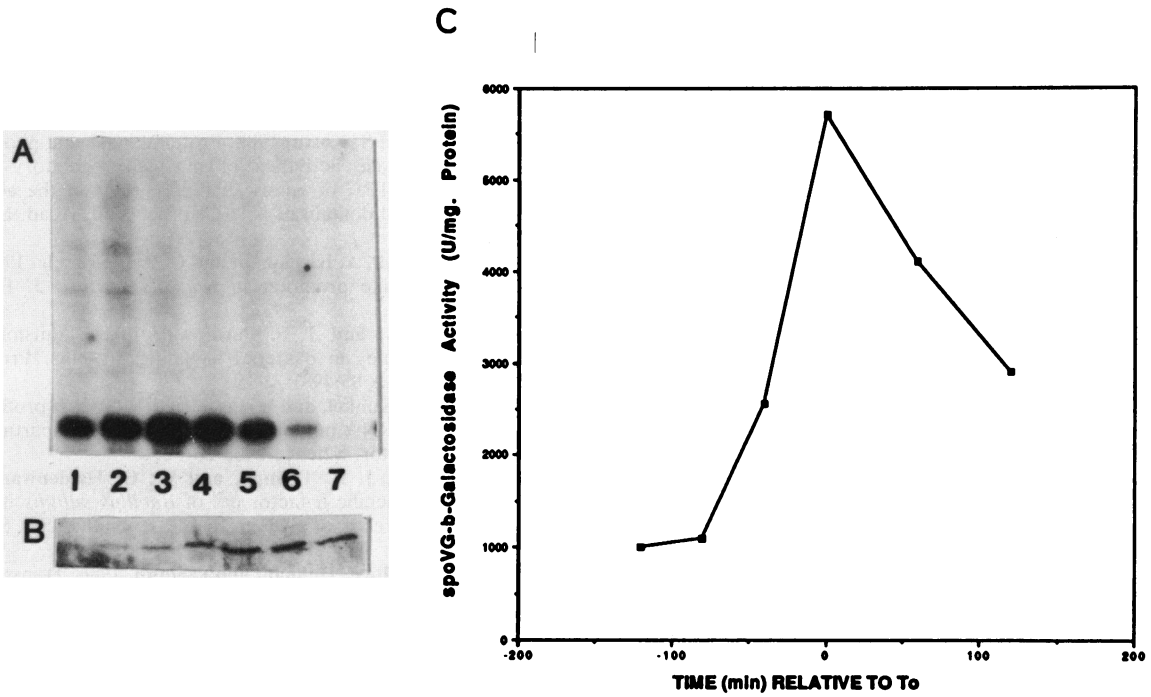

FIG. 7. *spo0H* RNA and σ^H levels during growth. (A) RNA isolated from IS414, a *spo*$^+$ strain containing a *spoVG-lacZ* fusion, at different stages of growth in NSM was used for primer extension assays. RNA (10 μg) was annealed to the primer described in the legend to Fig. 6. Lanes 1 to 6 correspond to −120 min, −80 min, −40 min, T_0, T_1, and T_2, respectively. Lane 7 contained 10 μg of RNA isolated from IS591 (an isogenic *B. subtilis* strain with a deletion of the entire *spo0H* gene). (B) Crude extracts from IS414 were prepared at different stages of growth in NSM. This was a different culture than that used in panels A and C. However, growth conditions were identical. A 50-μg amount of protein was added to each lane, and after electrophoresis (12.5% acrylamide gels) the proteins were transferred to a nitrocellulose filter, which was reacted sequentially with rabbit anti-*spo0H* antibodies and then goat anti-rabbit immunoglobulin antibody coupled to alkaline phosphatase. Lanes 1 to 6 refer to the same time points as in panel A, and lane 7 contained 50 μg of an extract of IS1223 (a *spo0H* deletion strain, IS233, carrying pIS1a, a multicopy plasmid containing the *spo0H* gene). IS233 alone did not show the 30-kDa protein observed in the other lanes (data not shown). (C) Aliquots of the IS414 culture used for the RNA determinations in panel A were also used to determine levels of *spoVG*-driven β-galactosidase activity.

The mutant form of Spo0A is thought to be in either a permanently "active" form or one that is more easily activatable. This activation presumably occurs via phosphorylation of the protein, given the fact that Spo0A shows great similarity to the response regulator type of protein in bacterial signal transduction systems (12), which is usually phosphorylated by a histidine kinase protein (17, 20, 21, 23, 36, 46). SpoIIJ, which is related to the histidine kinase class of protein (1, 34) and can phosphorylate *spo0A* and *spo0F* in vitro (34), may play a role in this activation.

It seems likely that the activated Spo0A represses the synthesis of the sporulation repressor *abrB*, thus lifting repression of the *spo0H* gene. Although it is not yet known whether $E\sigma^H$ transcribes *spoIIJ*, *spo0A*, and *spo0F*, *spo0H* is required for the expression of all three genes (1, 47), and the promoter sequences of these genes, as determined by primer extension with in vivo RNA, are of the $E\sigma^H$ type (34a). As *spo0H* is derepressed, more σ^H is produced, which ultimately causes increased transcription of the *spo0F*, *spo0A*, and *spoIIJ* genes, all of which are part of the signal transduction system. The higher levels of the Spo0A, Spo0F, and SpoIIJ proteins are presumably essential for later stages of sporulation. Therefore, the early states of sporulation can be pictured as a gradual crescendo of mutually dependent gene activation which follows signal transduction events that lead to the activation of the *spo0A* protein. This hypothesis requires that basal levels of the signal transduction proteins and $E\sigma^H$ be present before the sporulation signal is received. Thus, the system will be poised for the postulated cascade discussed above.

Another level of complexity in the regulation of $E\sigma^H$-dependent genes is introduced by the finding that σ^H levels have a different pattern throughout growth in rich medium compared with *spo0H* mRNA and *spoVG-lacZ* expression, showing a maximum at T_1 to T_2 (16) (Fig. 7). In addition, other genes transcribed by $E\sigma^H$, e.g., *citG* and *spoIIA*, show completely different patterns of expression, *citG* transcript being more numerous in mid-log-phase cultures, while *spoIIA* expression is maximal at T_2 (42). Thus, it appears that *spo0H* expression is regulated posttranscriptionally. The data presented in this communication do not allow us to distinguish between enhanced translation of the *spo0H* mRNA and increased stability of σ^H after T_0 but other experiments have shown that increased protein stability plays a major role in the regulation of σ^H levels (16). The lack of correlation between absolute σ^H levels and the expression of $E\sigma^H$-dependent genes suggests that ancillary factors, i.e., repressors or activators, may play an important role in the transcriptional control of the $E\sigma^H$ regulon.

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