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rpoD Operon Promoter Used by σ^H-RNA Polymerase in *Bacillus subtilis*

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Three promoters direct transcription of the *sigA* (*rpoD*) operon in *Bacillus subtilis*. Promoters P1 and P2 are used during the exponential growth phase, whereas P3 is used only during the stationary phase. We examined the use of these promoters in promoter-probe plasmids and found that expression from P3 was prevented by a mutation in *spo0H*, which encodes the secondary RNA polymerase sigma factor σ^H. Moreover, we found that σ^H-containing RNA polymerase efficiently and accurately used the P3 promoter in vitro. Evidently, this operon, which is essential for exponential growth, is transcribed during the early phase of sporulation by this secondary form of RNA polymerase. Comparison of the nucleotide sequences of the P3 promoter and the *spoVG* promoter, which also is used by σ^H-RNA polymerase, revealed sequences at the −10 and −35 regions of these promoters that may signal recognition of promoters by σ^H-RNA polymerase.

The operons that include the structural genes for the primary RNA polymerase sigma factors in *Escherichia coli* and *Bacillus subtilis* are similar. In *E. coli* the promoter-proximal gene of the operon, *rpsU*, encodes the ribosomal protein S21, the second gene, *dnaG*, encodes the DNA primase, and, finally, *rpoD* encodes the primary RNA polymerase sigma factor σ^{70} (2, 12). In the *B. subtilis* operon, the second and third genes, *dnaE* and *rpoD*, which encode DNA primase and the primary sigma factor, σ^{43} (σ^A), respectively, are homologous to *dnaG* (18) and *rpoD* (6), respectively, of *E. coli*. However, the first gene, P23 in the *B. subtilis* operon, is not homologous to *rpsU*, and the function of P23 is unknown (15).

Recently, Wang and Doi (17) used P23-*lacZ* gene fusions to demonstrate that three translational initiation sites in the same reading frame of P23 function in *B. subtilis*. They used the nucleotide sequence of this region to predict that initiation of translation from these sites produces proteins with molecular weights of 23,000 (P23), 19,000 (P19), and 9,000 (P9). Their most remarkable observation was that the P23 and P19 initiation sites are used mainly in exponentially growing cells, whereas the P9 initiation site is used only after the cells enter stationary phase.

The differential translation of P23 appears to be the result of a novel promoter-switching mechanism. In another study, Wang and Doi (16) used S1 nuclease mapping and promoter-probe plasmids to identify three promoters that direct transcription of the *rpoD* operon. Promoters P1 and P2 are located upstream from the P23 translation initiation site. These promoters are used only during the exponential growth phase (16), probably by the σ^A-containing RNA polymerase, since these promoters are used in vitro by Eσ^A (L.-F. Wang and R. H. Doi, unpublished data). The third promoter, P3, is located within P23 between the P19 and P9 translation initiation sites. Therefore, transcripts initiated at P3 contain only the P9 translation start site. Moreover, P3, unlike P1 and P2, is used exclusively during the stationary phase. Evidently, the exclusive use of the P3 promoter in stationary-phase bacteria results in the exclusive production of P9.

It is not known how the function of any promoter is activated at the beginning of the stationary phase, but in two cases there is compelling evidence that secondary RNA polymerase sigma factors play direct and essential roles. The *ctc* promoter is used in vivo by σ^B (formerly σ^{37})-containing RNA polymerase (Eσ^B) (9), whereas the *spoVG* promoter is used by σ^H (formerly σ^{30})-containing RNA polymerase (Eσ^H) (3, 4, 22). We have examined expression from the P3 promoter in mutants that failed to produce σ^B or σ^H and found that utilization of the P3 promoter was dependent on σ^H but not σ^B. Moreover, we found that the P3 promoter was used in vitro efficiently and accurately by Eσ^H. From these results, we conclude that Eσ^H uses the P3 promoter in vivo. Comparison of the nucleotide sequence of the P3 promoter with the sequence of the *spoVG* promoter and mutant derivatives of *spoVG* has revealed sequences that may signal the utilization of promoters by Eσ^H.

MATERIALS AND METHODS

Strains and media. Sporulation medium, 2× SG (11), was used for all the expression studies. *B. subtilis* ML1 (*trpC2 siqB*::*cat*) was provided by R. Losick (1, 9). *B. subtilis* IS233 (*trpC2 pheA1 spo0HΔHin*dIII) was provided by I. Smith (19). *B. subtilis* DB2 (*trpC2*) was from the laboratory stock of R. Doi.

Plasmid construction. To study the effects of *B. subtilis* mutants containing null alleles of *sigB* and *spo0H* on the expression of the *rpoD* operon promoters, the *Sau*3A fragment (nucleotides 610 to 820) (15, 16) containing P1 and P2 was subcloned into the *Bam*HI site in front of the promoterless β-lactamase gene in pSL-4 (20), resulting in pSL-P43. In a similar way, pSL-P30 was constructed by subcloning the adjacent *Sau*3A fragment (nucleotides 821 to 1136) (15, 16) containing P3 in the same site of pSL-4 as for pSL-P43. The correct orientation of these promoter-containing inserts in the pSL plasmids was confirmed by high-level expression of β-lactamase in DB2 and by restriction site analysis after being introduced into ML1 and IS233 by transformation. Plasmid pUC18-74R was constructed by cloning the 335-

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base-pair *Eco*RI fragment containing P3 from pSB-74 (15) into the *Eco*RI site of pUC18.

β-Lactamase assay. β-Lactamase present in the culture supernatant at designated times was assayed by using 7-(thienyl-2-acetamido)-3-[2-(4-*N*,*N*-dimethylaminophenylazo) pyridinium methyl]-3-cephem-4-carboxylic acid (PADAC; Calbiochem-Behring) as substrate. A 3-mg portion of PADAC was dissolved in 3 ml of methanol and mixed with 97 ml of 50 mM sodium phosphate buffer (pH 7.0) to form the PADAC assay solution, which gave an A_{570} of around 0.95 to 1.0. To start the reaction, 5 to 20 μl of enzyme solution (the supernatant) was mixed with 780 to 795 μl of PADAC assay solution prewarmed to 37°C in cuvettes which were placed in the measuring chamber of a Gilford 2400 spectrophotometer with a constant chamber temperature of 37°C. The decrease in A_{570} was recorded on a chart recorder, and the rate of A_{570} change in the linear range was used to calculate the enzyme activity present in the assay mixture. Enzyme specific activity was expressed as 10^3 international units per Klett unit per milliliter.

In vitro transcription reactions. σ^H RNA polymerase was isolated from *B. subtilis* CR101 (*sigB*) (14) as described previously (3). Runoff transcripts were generated from linearized plasmid templates and assayed by electrophoresis in urea-polyacrylamide gels as described previously (3), except that two 500-μl reaction mixtures were used to generate the transcripts that were used in the primer extension experiments. The transcripts for the primer extension experiments were generated from pUC18-74R that had been cleaved with *Pvu*II within the *lacZ* coding region, which was downstream from the P3 promoter. The transcription reactions were stopped by addition of 10 μg of DNase (Promega Biotec) and 80 μg of RNasin (Promega Biotec). After incubation at 37°C for 30 min, the solution was extracted with phenol, and the RNA was precipitated by the addition of ethanol. This RNA was used in primer extension experiments as described previously (10). The sequence of the oligonucleotide primer that was labeled at its 5′ end and used to prime the reactions was 5′-GTTGGGTAACGCCAGGG-3′, which is complementary to the region that encodes amino acids 19 to 25 of *lacZ* in pUC18-74R.

RESULTS

Deletion of the σ^H structural gene prevents transcription from P3. Since the nucleotide sequence of the P3 promoter is similar to that of promoters used by $E\sigma^B$, Wang and Doi (16) speculated that this promoter may be used by this secondary RNA polymerase in vivo. Recently, however, genetic evidence was used to show that $E\sigma^B$ does not use the *spoVG* promoter in vivo (9), even though the sequence of this promoter allowed its use in vitro by $E\sigma^B$, albeit inefficiently. Furthermore another secondary sigma factor, σ^H, was discovered and found to be encoded by *spo0H*, a gene that is essential for transcription of *spoVG* in vivo (3, 4). To determine whether these secondary sigma factors are essential for utilization of the P3 promoter in vivo, we examined the activity of P3 in mutants that have null alleles of σ^B and σ^H structural genes *sigB* and *spo0H*, respectively.

For these studies the promoters P1-P2 and P3 of the *sigA* operon were placed in front of a promoterless β-lactamase gene to form expression plasmids pSL-P43 and pSL-P30 (see Materials and Methods for details of the constructions). These plasmids were used to transform *B. subtilis* DB2, IS233, and ML1 to test for promoter activity in wild-type cells and in null mutants of *sigB* and of *sigH* (*spo0H*), respectively. The results in Fig. 1 show the expression pattern of β-lactamase as controlled by promoters P1-P2 and P3. Promoters P1-P2 are expressed in all three strains during the log phase and are switched off in all strains at about T_2 to T_3. This pattern is similar to that reported previously for these *sigA* enzyme-controlled promoters (16). The expression of promoter P3 in the wild-type strain and *sigB* mutant was identical during the stationary phase, and these results indicated that the transcription from P3 was not affected by the null mutation in *sigB*. We conclude that σ^B is not directly involved in P3 utilization, but since P3 was carried on a multicopy plasmid, an indirect role for *sigB* in P3 utilization

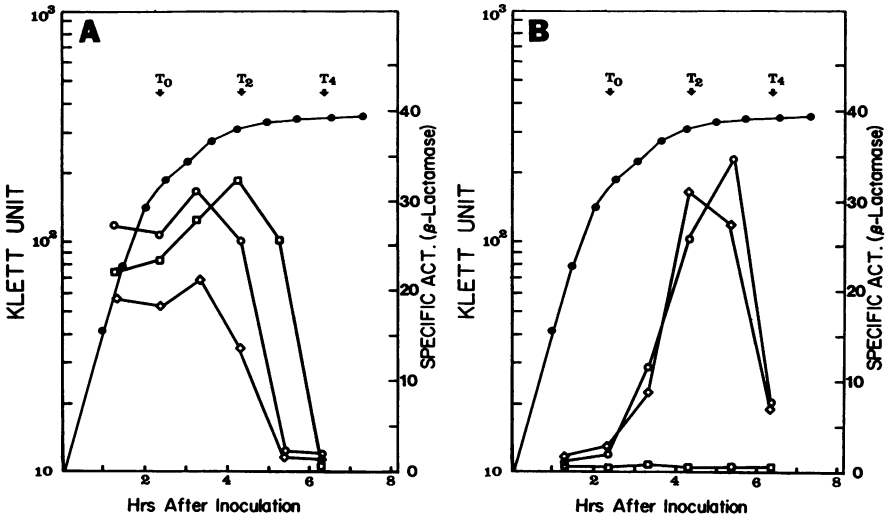

FIG. 1. Expression of *rpoD* operon promoters in mutants containing null alleles of *sigB* and *spo0H*. (A) Expression curves obtained from different strains containing plasmid pSL-P43, which reflect the expression patterns of the tandem σ^A promoters P1 and P2 of the *rpoD* operon; (B) expression curves for pSL-P30, which reflect the expression pattern of the putative σ^H promoter P3. Cell growth in sporulation medium 2× SG containing 5 μg of kanamycin per ml was monitored with a Klett-Summerson colorimeter (●). The subscripts of T indicate the time in hours after the initiation of sporulation. The β-lactamase activities, and hence the promoter activities, in *B. subtilis* DB2 (*trpC2*) (○), IS233 (*trpC2 pheA1 spo0HΔHind*III) (□), and ML1 (*trpC2 sigB*::*cat*) (◇) are shown.

may not have been detected. On the other hand, transcription from P3 in the *sigH* (*spo0H*) strain was dramatically inhibited.

Use of P3 by Eσ^H in vitro. The observation that σ^H is necessary for utilization of the P3 promoter in vivo raises the possibility that σ^H-containing RNA polymerase (Eσ^H) utilizes the P3 promoter. To test this hypothesis, we used Eσ^H to transcribe a DNA template (pLW9) containing the P3 promoter in an in vitro assay. In the first experiments, pLW9 that had been linearized with either *Eco*RI or *Pvu*II was transcribed with Eσ^H. In each case a single runoff transcript was generated, 220 nucleotides from the *Pvu*II-cleaved template and 160 nucleotides from the *Eco*RI-cleaved template. The size of the runoff transcripts was approximately the size expected for transcripts initiated at the P3 promoter (data not shown).

We used a primer extension analysis to determine more precisely whether Eσ^H generated the transcript from the P3 promoter (Fig. 2). A DNA template (pUC18-74R digested with *Pvu*II) that contained the P3 promoter was transcribed by Eσ^H in the presence of nonradiolabeled nucleoside triphosphates as described in Materials and Methods. The 5′ terminus of the resulting RNA transcript was determined by the use of reverse transcriptase to extend a radiolabeled primer that was complementary to a region of transcript downstream from the polylinker region in pUC18-74R. The size of the extended primer was determined by electrophoresis into a DNA sequencing gel (Fig. 2, lane e) next to the dideoxy sequencing products of pUC18-74R that had been sequenced with the same primer (Fig. 2, lanes a to d). This primer extension experiment demonstrated that Eσ^H initiated transcription from P3 at the same nucleotide as that located at the 5′ terminus of the P3 transcript that was identified in vivo by Wang and Doi (16) (see Fig. 4).

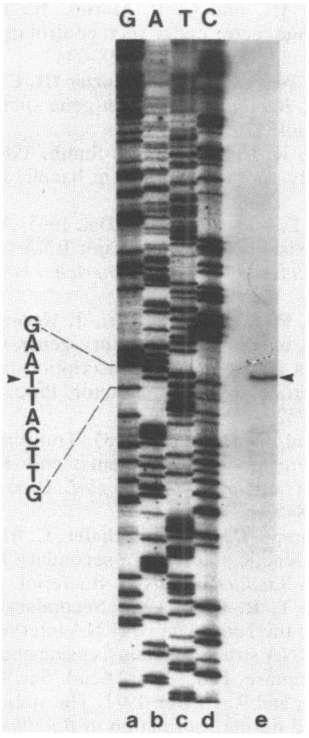

FIG. 2. Primer extension analysis of in vitro transcript. A radiolabeled oligonucleotide primer that was complementary to a region downstream from the P3 promoter in pUC18-74R was allowed to hybridize to RNA produced by transcription of pUC18-74R with Eσ^H. After extension with reverse transcriptase, this mixture was subjected to electrophoresis in an 8 M urea–8% (wt/vol) polyacrylamide gel (lane e). The dideoxy-terminated products produced with T7 DNA polymerase after annealing of the same oligonucleotide to pUC18-74R DNA were used as molecular mass markers (lanes a to d). The letters above lanes a to d indicate which dideoxy nucleotide was used to terminate the reaction. The arrowheads indicate the position of the primer extension product in the autoradiograph of the gel (lane e) and on the sequence.

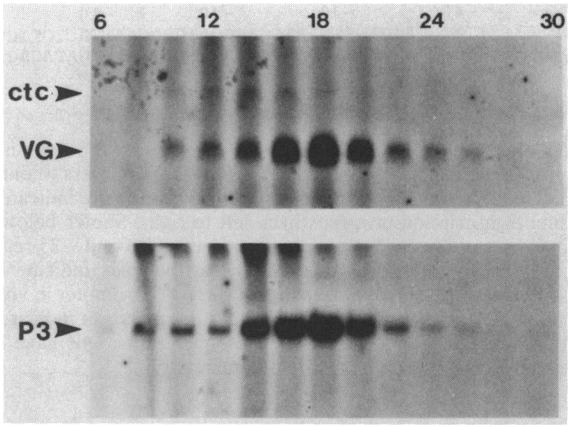

FIG. 3. Gradient elution of RNA polymerase from DNA-cellulose. RNA polymerase purified through Sephacryl-300 as described previously (3) was eluted from a DNA-cellulose column with a linear gradient of KCl as described previously (3). Each even-numbered fraction (2 μl) was used in in vitro transcription reactions that included a linear template containing the P3 promoter or two linear templates containing the *ctc* or *spoVG* promoter. ^{32}P-labeled runoff transcripts were visualized by autoradiography after electrophoresis into a 7 M urea slab gel containing 9% (wt/vol) polyacrylamide. The positions of the runoff transcripts generated from each promoter are indicated by arrowheads. The numbers above the lanes indicate the fractions of RNA polymerase used in the two transcription reactions.

To strengthen support for the model that Eσ^H uses the P3 promoter and that the accurate transcription from the P3 promoter in vitro was not due to the presence of an undetected and previously unidentified sigma factor in the preparation of Eσ^H, we showed that the P3-transcribing activity eluted simultaneously with Eσ^H from a DNA-cellulose column. During the purification of the Eσ^H, RNA polymerase was fractionated by elution from DNA-cellulose with a gradient of KCl. Each fraction was tested in runoff transcription assays with templates containing the P3 promoter or the *ctc* promoter and the *spoVG* promoter. The fraction containing the peak of P3-transcribing activity (Fig. 3, fraction 18) was the same as the fraction containing the peak of *spoVG*-transcribing activity (i.e., Eσ^H) (Fig. 3). The weak *ctc*-transcribing activity, which peaks in fraction 14 (Fig. 3), was due to Eσ^C, which eluted in this fraction. Eσ^B, which actively transcribes *ctc*, was not present in this mutant strain. Taken together, these data support the model that Eσ^H accurately and efficiently uses the P3 promoter in vitro.

DISCUSSION

Eσ^H and promoter switching. Examination of the effects of null alleles of *sigB* and *spo0H* on use of the P3 promoter shows that σ^H is essential for use of this promoter in vivo. *spo0H* probably is essential for P3 expression because Eσ^H

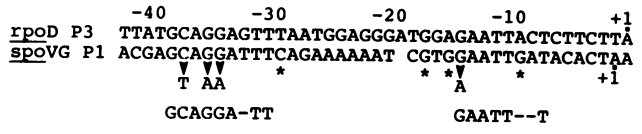

FIG. 4. Nucleotide sequence of the P3 promoter. Shown is the nucleotide sequence of the P3 and *spoVG* promoters (nontranscribed strands). The starting points of transcription are indicated as +1, and transcription proceeds from left to right. Shown below the promoter sequences are the sequences at the −10 and −35 regions that are similar in *spoVG* and P3. The arrowheads indicate base 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 *spoVG* at which single-base-pair transitions have less-severe effects (Losick, personal communication).

uses the P3 promoter and not because of an indirect effect of σ^H on the P3 promoter, since $E\sigma^H$ was shown to use the P3 promoter in vitro.

Although expression from several promoters has been shown to be at least indirectly dependent on σ^H, P3 is only the second promoter to be identified as being used directly by $E\sigma^H$. These two promoters, *spoVG* (22) and P3, are activated at the end of exponential growth, but it is not known how transcription is activated from them. $E\sigma^H$ is present in exponentially growing cells (3, 5), and the rate of transcription of the σ^H structural gene does not increase dramatically at the onset of stationary phase (5). We have not conclusively disproved models in which σ^H is modified or the amount of σ^H increases significantly, but it is possible that other auxiliary factors regulate the use of the *spoVG* and P3 promoters by $E\sigma^H$. An analogous situation may be the regulation of *ntrA*-dependent promoters by *ntrC* in enteric bacteria (7, 8). Since σ^H is present in exponentially growing cells, we may yet find promoters that are used by $E\sigma^H$ during exponential growth, and these promoters may be regulated by different factors.

The role of *sigH* encompasses the expression not only of sporulation-specific functions such as *spoVG*, but also of genes (*sigA* operon) that have a major function during growth and sporulation. The reduced activity of $E\sigma^A$ on the expression of the *sigA* operon during early sporulation (14) is compensated for by promoter switching and the activity of $E\sigma^H$. It will be of interest to see whether other genes and operons that are required during growth and sporulation also use a similar type of regulatory mechanism. Transcription of *citG*, the structural gene for fumerase, evidently is transcribed from three promoters, one of which is dependent on *spo0H* (13). This may be another example of promoter switching, but the effects of different growth conditions on the use of these promoters and use of the *spo0H*-dependent promoter by $E\sigma^H$ in vitro has not been reported.

Nucleotide sequences that signal recognition by $E\sigma^H$. Now that two promoters that are used by $E\sigma^H$ are known, we can begin to ask what sequences signal the utilization of a promoter by $E\sigma^H$. Comparison of the nucleotide sequences of the *spoVG* and P3 promoters reveals only two regions of extensive similarity. These are centered near the −10 and −35 regions of these promoters (10 and 35 base pairs, respectively, upstream from the start of transcription). Losick and co-workers have examined the effects of several base substitutions in the *spoVG* promoter (21; R. Losick, personal communication). Base substitutions that reduced the utilization of the *spoVG* promoter in vivo altered several base pairs that are conserved in P3, and four single-base substitutions that did not dramatically affect the use of *spoVG* changed the base pairs to the ones found in P3 (Fig. 4). An A+T-rich region upstream from the *spoVG* promoter is necessary for efficient use of that promoter (22). The sequence of this region of P3 is not similar, but mixed-template reactions indicate that P3 is used as efficiently as the *spoVG* promoter in vitro (data not shown). As our current working model, we suggest that utilization of promoters by $E\sigma^H$ is signaled by the sequences at the −10 and −35 regions that are similar in *spoVG* and P3 promoters (Fig. 4). This model must be tested by mutagenesis of these promoters and the characterization of additional promoters that are used by $E\sigma^H$.

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