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Gene Encoding σ^E Is Transcribed from a σ^A-Like Promoter in *Bacillus subtilis*

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Bacillus subtilis **produces several RNA polymerase sigma factors. At least two of these factors are essential for endospore formation, σ^H, which is present in vegetative cells, and σ^E, which is produced exclusively after the start of endospore formation. The structural gene that encodes σ^E is part of the *spoIIG* operon, which is transcribed after the onset of sporulation. We have determined the starting point of transcription and the nucleotide sequence of the *spoIIG* promoter. This promoter contains sequences that are similar to those found at the −10 and −35 regions of promoters that are used by $E\sigma^A$, the primary form of RNA polymerase in vegetative cells. The unusual feature of this promoter is that these putative σ^A contact sites are separated by 22 base pairs, rather than the typical 17 or 18 base pairs. Single-base substitutions in the −10-like sequence reduced utilization of the *spoIIG* promoter in vivo. Furthermore, $E\sigma^A$, but not $E\sigma^H$ and other secondary forms of RNA polymerase, accurately initiated transcription from the *spoIIG* promoter in an in vitro assay; therefore, we suggest that $E\sigma^A$ transcribes the *spoIIG* operon in vivo. A base substitution in the −35-like sequence caused constitutive transcription from the promoter in vegetative cells; therefore, regulation of this sporulation-specific transcription may involve a novel mechanism.**

Endospore formation by *Bacillus subtilis* requires the expression of at least 50 genetic loci, many of which are operons that contain several sporulation-essential genes. These loci are expressed in a specific temporal sequence during the 8 to 10 h required for development of the endospore (for a review, see reference 15). Recently, several RNA polymerase sigma factors have been isolated from *B. subtilis*. Only two of these, however, have been shown to be essential for endospore formation, σ^H, which is found in vegetative and early sporulating cells (1, 4), and σ^E, which is produced exclusively in sporulating cells (7, 19). Extrapolating from their observations on transcription during lytic growth of bacteriophage SPO1 and the discovery of σ^E, Losick and his colleagues proposed that the sequential production of sigma factors during sporulation controls the temporal pattern of transcription (7, 14). In the case of SPO1 development, the relentless "cascade of sigmas" is activated by injection of phage DNA that encodes the sigma factors. It is not known how production of the first sporulation-specific sigma factor is activated or, in fact, which sigma, if any, begins the cascade.

σ^E is produced about 2 h after the start of endospore formation (7). It is proteolytically processed from an inactive precursor known as P31, which is synthesized about 1 h after the initiation of sporulation (13). The structural gene that encodes σ^E, *sigE*, is part of the *spoIIG* operon, which consists of two genes that are essential for sporulation (10). An adjacent gene downstream from the *spoIIG* operon is also essential for sporulation (11), but its transcription is at least partly independent of the *spoIIG* promoter, since a polar mutation at the promoter-distal end of *sigE* reduced but did not prevent sporulation (10). Recently, we used integrational plasmids to introduce a series of polar mutations into the *spoIIG* operon (10). The effects of these mutations and the results obtained by cloning various fragments of the *spoIIG* operon into a promoter probe plasmid allowed us to identify a 217-base-pair (bp) DNA fragment that contained the promoter for the *spoIIG* operon (10). When *B. subtilis* was lysogenized with a specialized transducing phage that carried a transcriptional fusion of the *spoIIG* promoter and a promoterless derivative of *lacZ* from *Escherichia coli*, β-galactosidase was produced only after the initiation of sporulation (10). Evidently, the *spoIIG* promoter is activated about 30 min after the onset of spore formation (10).

The *spoVG* promoter is also activated at the beginning of sporulation and presently is the most thoroughly studied promoter of a sporulation gene (1, 17, 23, 24). Transcription of *spoVG* is dependent upon a sigma factor that is present in vegetative and early sporulation cells, σ^H. RNA polymerase containing σ^H, $E\sigma^H$, uses this promoter in vitro (1, 4), and allele-specific suppression of an *spoVG* promoter mutation by a second mutation in the σ^H structural gene provides compelling evidence that σ^H directly interacts with the *spoVG* promoter in vivo (P. Zuber, J. Healy, H. L. Carter III, S. Cutting, C. P. Moran, Jr., and R. Losick, manuscript in preparation).

Mutants that fail to express σ^H also fail to activate transcription from the *spoIIG* promoter (10). Is this the beginning of the cascade of sigmas? Does the sporulation-essential sigma factor, σ^H, direct transcription of the gene that encodes σ^E? Here we report on the structure and function of the *spoIIG* promoter. The effects of mutations in the promoter and results of in vitro transcription experiments lead us to conclude that σ^H does not interact with the *spoIIG* promoter. Rather, we suggest that σ^A RNA polymerase, formerly σ^{43}, the primary form in vegetative cells, transcribes the *spoIIG* operon. Moreover, regulation of this sporulation-specific transcription may involve a novel mechanism.

MATERIALS AND METHODS

Bacterial strains. *B. subtilis* strains and phages are shown in Tables 1 and 2.

RNase protection experiments. The 223-bp *Hin*dIII-*Aha*III DNA fragment from pTK11 (10) that contained the *spoIIG*

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TABLE 1. *B. subtilis* strains

Strain	Relevant genotype and derivation[a]
EU8701	Δ*sigE*::*erm* (10)
EU8747	EU8701 SPβ::*IIG217-lacZ*
EU8748	EU8701 SPβ::*IIG6-lacZ*
EU8749	EU8701 SPβ::*IIG11-lacZ*
EU8750	EU8701 SPβ::*IIGΔ105-lacZ*
EU8751	EU8701 SPβ::*IIG60-lacZ*
EU8752	EU8701 SPβ::*IIG45-lacZ*
EU8753	EU8701 SPβ::*IIΔ97-lacZ*
EU8754	EU8701 SPβ::*IIG38-lacZ*

[a] If a reference is not given, the strain was isolated as a derivative of the strain indicated (e.g., strain EU8747 is a SPβ::*IIG217-lacZ* lysogen of EU8701.

promoter was cloned between the *Hin*dIII and *Sma*I sites of plasmid pT7T3-18 (Bethesda Research Laboratories, Inc.) so that T7 RNA polymerase could be used to generate a radiolabeled antisense RNA. After this template was cleaved with *Hin*dIII, ^{32}P-labeled RNA was synthesized and purified as described in the Promega catalog. This ^{32}P-labeled RNA was mixed with 50 μg of RNA that was isolated from vegetative or sporulating cells as described previously (10), and then this mixture was incubated in hybridization buffer (80% formamide, 40 mM PIPES [piperazine-*N*,*N*'-bis(2-ethanesulfonic acid)] [pH 6.7], 0.4 M NaCl, 1 mM EDTA) for 5 min at 85°C and then overnight at 40°C. This hybridization mixture was incubated for an additional 60 min at 32°C after the addition of 2 μg of RNase T_1 per ml and 40 μg of RNase A per ml. The remaining RNA in these samples was purified as previously described (16) and subjected to electrophoresis in a 8.3 M urea slab gel containing 8% (wt/vol) polyacrylamide.

Primer extension experiments. RNA was isolated from vegetative and sporulating *B. subtilis* EU8747, a derivative of EU8701 (10) that is a lysogen of SPβ*IIG217-lacZ*. SPβ*IIG217-lacZ* is identical to the SPβ*IIG-lacZ* previously described (10), except that only the 217-bp *Taq*I-*Aha*III region of the *spoIIG* promoter is fused to *lacZ*. This RNA was used in primer extension experiments as previously described (9). The sequence of the oligonucleotide that was labeled at its 5' end and used to prime the reactions was 5' GTTGGGTAACGCCAGGG 3', which is complementary to part of *lacZ* in SPβ*IIG217-lacZ*.

TABLE 2. Phages

Phage	*spoIIG* promoter[a]
SPβ *c2 del2*::Tn*917*	
SPβ::*IIG217-lacZ*	Wild-type *spoIIG* promoter
SPβ::*IIG6-lacZ*	Transversion at position −6
SPβ::*IIG11-lacZ*	Transversion at position −11
SPβ::*IIGΔ105-lacZ*	Sequences downstream from +13 deleted from *spoIIG217*
SPβ::*IIG60-lacZ*	Transversion at position +6
SPβ::*IIG45-lacZ*	Transversions at positions +4 and +5
SPβ::*IIGΔ97-lacZ*	Sequences downstream from +5 deleted from *spoIIG45*
SPβ::*IIG38-lacZ*	Transition at position −38

[a] The phages are derivatives of SPβ *c2 del2*::Tn*917* (8) that were isolated after recombination with a plasmid that contains a derivative of the *spoIIG* promoter fused to *lacZ* as described previously (10). The *spoIIG* promoter carried by each phage is indicated (e.g., SPβ::*IIG6-lacZ* is the same as SPβ::*IIG217-lacZ*, except for a single-base substitution at position −6 of the *spoIIG* promoter.

In vitro transcription reactions. Transcription reactions were done as described previously (1), except that the DNA templates were small linear DNA fragments (less than 300 bp). After these DNA fragments were electroeluted from polyacrylamide slab gels, they were eluted through an Elutip-d column as recommended by the manufacturer (Schleicher & Schuell, Inc.). The template DNA used in the transcription reactions shown in Fig. 3 was the 255-bp *Hin*dIII-*Eco*RI fragment from pTK16. pTK16 was constructed by cloning the 223-bp *Hin*dIII-*Aha*III DNA fragment containing the *spoIIG* promoter from pTK11 (10) between the *Hin*dIII-*Hin*cII sites of pUC19 (20). We also used the 228-bp *Hin*dIII-*Xba*I fragment and the 234-bp *Hin*dIII-*Bam*HI fragment, both from pTK16. The lengths of the runoff transcripts generated from the *spoIIG* promoter on these fragments were 156, 129, and 135 nucleotides, respectively.

Mutagenesis of the *spoIIG* promoter. The sequences of the five mutagenic oligonucleotides were as follows: oligonucleotide 1, 5' GCTTGCTTGATACTTAT 3'; oligonucleotide 2, 5' CTTTATACGTATGAAGC 3'; oligonucleotide 3, 5' ACT TATGAAGCTTGAAGGGGAACA 3'; oligonucleotide 4, 5' TGAAGCAACAAGGGGAA 3'; oligonucleotide 5, 5' ACA TTAATCGACAGACT 3'. Oligonucleotides 1, 2, and 3 were used to mutagenize the 223-bp *Hin*dIII-*Aha*III DNA fragment from pTK11 that contained the *spoIIG* promoter (10), which was cloned between the *Hin*dIII and *Hin*cII sites of M13mp18 DNA, following the procedures of Zoller and Smith (22). These mutant promoters were designated *spoIIG11*, *spoIIG6*, and *spoIIG45*, respectively. Mutagenesis with oligonucleotides 4 and 5 was done the same way, except the procedure was modified as described by Kunkel (12). These promoters were designated *spoIIG60* and *spoIIG38*, respectively. The wild-type and mutagenized *spoIIG* promoters were cut from the M13mp18 vector and cloned next to a promoterless derivative of *lacZ* from *E. coli* as described previously (10). The promoter-*lacZ* fusion was transferred to an SPβ specialized transducing phage as previously described (10).

The mutation created with oligonucleotide 3 created a *Hin*dIII site at position +3 of the *spoIIG45* promoter. A deletion derivative of this promoter was created by cleavage with *Hin*dIII before it was fused to *lacZ* and used to create *spoIIGΔ97-lacZ*. A second deletion derivative of the *spoIIG217* promoter (*spoIIGΔ105*) that had lost the sequence downstream from +13 was created by partial *Mnl*I digestion of the 223-bp *Taq*I-*Aha*III DNA fragment containing the wild-type promoter from pTK11 (10). These deleted derivatives were fused to *lacZ* as described above, and β-galactosidase produced by lysogens that harbored each promoter-*lacZ* fusion was assayed as previously described (10).

We also deleted the sequences between −88 and −62 (promoter *spoIIGΔ62*), between −88 and −51 (promoter *spoIIGΔ51*), between −88 and −39 (promoter *spoIIGΔ39*), and between −88 and −37 (promoter *spoIIGΔ37*). These deletions were generated by oligonucleotide mutagenesis of the *spoIIG217* promoter in M13mp18. The sequences of the oligonucleotides used were as follows: *spoIIGΔ62*, 5' TGC CAAGCTTATCGATTGTATTTTCCTCTC 3'; *spoIIGΔ51*, 5' TGCCAAGCTTATCGATCTCAACATTAATTG 3'; *spoIIGΔ39*, 5' TGCCAAGCTTATCGATTGACAGACTT TCCC 3'; and *spoIIGΔ37*, 5' TGCCAAGCTTATCGAG ACAGACTTTCCCAC 3'. The deleted promoters were fused to *lacZ* as described above, and β-galactosidase produced by lysogens that harbored each promoter-*lacZ* fusion was assayed as previously described (10).

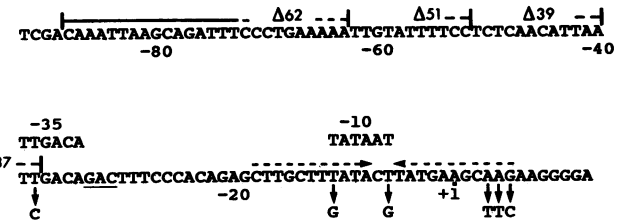

FIG. 1. Nucleotide sequence of the *spoIIG* promoter. Shown is the nucleotide sequence (nontranscribed strand) of the *spoIIG* promoter (10). Transcription occurs from left to right and is initiated at the position indicated as +1. The canonical sequences found at the −10 and −35 regions of promoters used by $E\sigma^A$ (18) are indicated above the promoter sequence. Base substitutions are indicated below the arrows. A region of dyad symmetry is indicated by the dotted overline, and a 3-base sequence similar to a −35-type sequence is underlined. The endpoints of the deletions in promoters *spoIIGΔ62*, *spoIIGΔ51*, *spoIIGΔ39*, and *spoIIGΔ37* are indicated above the sequence.

RESULTS

Structure of the *spoIIG* promoter. We previously reported the nucleotide sequence of a 217-bp *Taq*I-*Aha*III DNA fragment that contains the *spoIIG* promoter (10) (Fig. 1). We used RNase protection experiments to confirm that transcription is initiated from this region during sporulation. A radiolabeled antisense RNA, produced from this 217-bp *Taq*I-*Aha*III DNA fragment, was incubated in hybridization buffer with RNA that was isolated from wild-type vegetative and sporulating cells. We found that RNA from sporulating cells, but not RNA from vegetative cells, was able to protect a region of this antisense RNA from digestion with RNase T_1 and RNase A (Fig. 2A).

Primer extension experiments were used to map the 5′ end of the *spoIIG* transcript more precisely. RNA was extracted from vegetative and sporulating *B. subtilis* EU8747, which is a lysogen of SPβ*IIG217-lacZ*. An oligonucleotide that was complementary to a region in *lacZ* was allowed to hybridize to the mRNA, and this primer was extended with reverse transcriptase. The size of the DNA copy of the mRNA was determined by electrophoresis into a DNA sequencing gel and compared with the sizes of the dideoxy-terminated products of a similar DNA template (Fig. 2B). Again, the *spoIIG* transcript was found only in sporulating cells (Fig. 2B, lane f). These results were consistent with our previous Northern (RNA) blot analysis and transcriptional fusion data, which indicated that the *spoIIG* promoter is located in this DNA fragment and is activated after the start of sporulation (10). The location of the 5′ end of the *spoIIG* transcript, which is indicated in Fig. 1, was also supported by the results of the dinucleotide priming experiments (Fig. 2c). These results are discussed below.

Use of the *spoIIG* promoter by $E\sigma^A$ in vitro. Several forms of RNA polymerase are present in *B. subtilis* at the earliest stage of sporulation (see reference 1 and references therein). To determine which of these may use the *spoIIG* promoter, we reconstructed transcription from this promoter in vitro, a strategy that has previously led to the discovery of several of the secondary forms of RNA polymerase (1, 7). Total RNA polymerase was isolated from *B. subtilis* within 1 h after the start of sporulation and fractionated by elution from a DNA-cellulose column as described previously (1). Each fraction was tested for its ability to generate a runoff transcript from a DNA fragment that contained the *spoIIG* promoter. Several fractions, which eluted before $E\sigma^H$, produced a runoff transcript from the *spoIIG* promoter (data not shown). The fractions that contained this transcribing activity were applied to a heparin-agarose column and eluted with a gradient of KCl. When these fractions were tested in the runoff transcription assay, we found that the transcribing activity eluted in fractions 7, 9, and 11 (Fig. 3B, lanes e, f, and g, respectively). The proteins in all of the fractions were analyzed by electrophoresis into a sodium dodecyl sulfate-polyacrylamide slab gel (Fig. 3A). We found that the fractions that could produce the runoff transcript from the *spoIIG* promoter contained predominantly the core subunits of RNA polymerase and σ^A, the primary sigma factor in vegetative cells (Fig. 3A). Moreover, the polymerase in fractions 7, 9, and 11 was shown to utilize the *tms* promoter (18), which is used by $E\sigma^A$, in the in vitro transcription assay, whereas the polymerase in fractions 1, 3, and 5 was shown to use promoter 28-1 (5), which is used by $E\sigma^D$ (data not shown). The amount of σ^A in each fraction directly correlated with the amount of runoff transcript generated from the *spoIIG* promoter.

The runoff transcript generated by $E\sigma^A$ was shown to initiate from the *spoIIG* promoter by using three different-sized DNA templates that produced different-sized runoff transcripts (156, 135, and 129 bases) in the in vitro assay with $E\sigma^A$ (data not shown). Furthermore, 16 different dinucleotides were used to prime transcription by $E\sigma^A$ in the presence of low concentrations of ribonucleoside triphosphates. The dinucleotides ApA and ApG worked most efficiently and the dinucleotide GpA generated a detectable level of transcription in several experiments. The transcript that was initiated with ApA appeared to be longer than the one initiated with ApG (Fig. 2C). These results are consistent with that expected from the sequence around the start site of the *spoIIG* promoter (Fig. 1). From these results, we concluded that $E\sigma^A$ accurately uses the *spoIIG* promoter in vitro. Moreover, highly purified $E\sigma^H$, $E\sigma^E$, $E\sigma^C$, $E\sigma^B$, and $E\sigma^D$ failed to use this promoter in vitro (data not shown).

Effects of mutations in the *spoIIG* promoter. The nucleotide sequence, centered 10 bp upstream from the start point of transcription at the *spoIIG* promoter, is similar to the canonical sequence found at this region of promoters that are used efficiently by $E\sigma^A$ (18). It contains only a single-base mismatch with the canonical sequence, and this mismatch is at one of the least conserved positions (Fig. 1). Another sequence is identified in Fig. 1 as being identical to the canonical sequence found at the −35 region of promoters that are used efficiently by $E\sigma^A$. This sequence, however, is separated by 22 bp from the −10 region of the *spoIIG* promoter, rather than the typical 17 or 18 bp. This unusual spacing may be a sufficient explanation of our observation that this promoter is used relatively inefficiently by $E\sigma^A$ in vitro.

To determine if these sequences in the *spoIIG* promoter play a role in utilization of this promoter in vivo, we used oligonucleotide-directed mutagenesis to construct two single-base-pair substitutions that change the two most highly conserved positions of the −10 region (positions −6 and −11) and one single-base-pair substitution at a highly conserved position of the −35-like sequence (position −38). To assay the effects of these mutations on promoter function, we fused the mutant promoters to a promoterless derivative of *lacZ* and used an SPβ specialized transducing phage to carry these constructs into the chromosome of *B. subtilis*. The two single-base substitutions in the −10 region severely reduced the amount of β-galactosidase synthesized from the promoter (Fig. 4A); therefore, we concluded that these

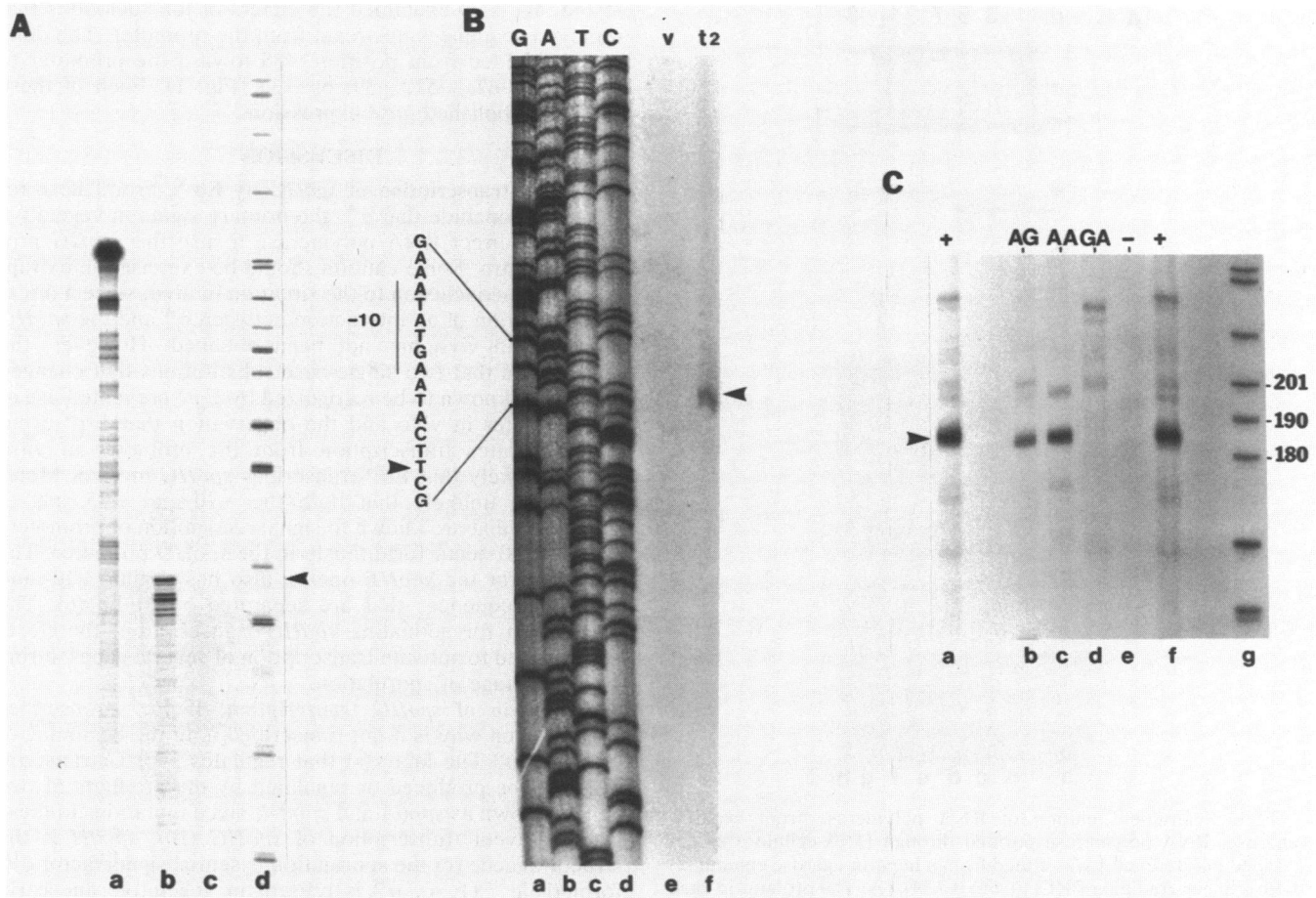

FIG. 2. Analysis of transcripts from the *spoIIG* promoter. (A) RNase protection experiment. The radiolabeled antisense RNA copy of the 217-bp *Taq*I-*Aha*III DNA fragment that contained the *spoIIG* promoter was allowed to hybridize with RNA isolated from *B. subtilis* during the second hour of sporulation (lane b) or during exponential growth (lane c). After digestion of these mixtures with RNases T_1 and A, they were subjected to electrophoresis and autoradiography. Lane a shows the antisense RNA that was not treated with RNase. Radiolabeled *Hpa*II fragments of pBR322 were used as molecular mass standards (lane d). The position of the 125-base fragment of the antisense RNA that was protected from RNase digestion by the RNA from sporulating cells is indicated with an arrowhead. (B) Primer extension experiment. A radiolabeled oligonucleotide primer that was complementary to a region downstream from the *spoIIG* promoter was allowed to hybridize with *B. subtilis* RNA that was isolated during exponential growth (lane e) (vegetative cells) or during the second hour of sporulation (lane f). After extension of this primer with reverse transcriptase, the mixtures were subjected to electrophoresis as described previously (9). The dideoxy-terminated products that were produced with T7 DNA polymerase after annealing the same oligonucleotide to plasmid DNA that contained a *spoIIG* promoter were used as molecular mass markers (lanes a through d). The letters above lanes a through d indicate which dideoxynucleotide was used to terminate the reaction. The arrowheads indicate the position of the primer extension product in the gel (lane f) and on the sequence. (C) Dinucleotide-primed transcription. Radioactive RNA was transcribed in vitro from the 255-bp *Hin*dIII-*Eco*RI fragment from pTK16 by $E\sigma^A$ as described in Materials and Methods except in the presence of only 2 μM ribonucleotide triphosphates. Shown is an autoradiograph of the transcripts after electrophoresis into an 8.3 M urea gel containing 8% (wt/vol) polyacrylamide. Transcription was primed without additional nucleotide triphosphate (indicated by − above lane e), or by the addition of 120 μM each ATP, GTP, and UTP (indicated by + above lanes a and f), or by a 150 μM concentration of each dinucleotide as indicated above each lane (lanes b through d). The results with the 13 other dinucleotides that did not efficiently prime transcription are not shown on this figure. The arrowhead indicates the position of the 156-nucleotide transcript. Radiolabeled pBR322 that had been digested with *Hpa*II was used as molecular mass markers. The 201-, 190-, and 180-nucleotide fragments are indicated.

sequences are essential for promoter utilization in vivo. The base substitution in the −35-like sequence at position −38 caused the promoter to be active in vegetative cells (Fig. 4C). Note the change in scale of panel C in Fig. 4. The activity of promoter *spoIIG38* appeared greater in exponential-phase cells than that of the wild-type promoter in sporulating cells. The *spoIIG38* promoter remained active during sporulation (Fig. 4C), and primer extension analysis showed that the start point of the transcript in vegetative and sporulating cells was the same as that from the wild-type promoter (data not shown). The implications of the effect of this mutation will be discussed below.

The sequence of nucleotides between positions −18 and +5, inclusive, of the *spoIIG* promoter exhibits a dyad symmetry similar to the target sequences for several site-specific DNA-binding proteins (Fig. 1). To test the role of this sequence, we made a single base substitution at position +6 and in a second case made base substitutions at both positions +4 and +5 (promoters *spoIIG60* and *spoIIG45*, respectively). Again, these mutant promoters were fused to a promoterless *lacZ* in SPβ. Lysogens containing these two mutant *spoIIG* promoters accumulated β-galactosidase at rates that were slightly lower than that produced by the wild-type promoter (Fig. 4). We cannot make any strong

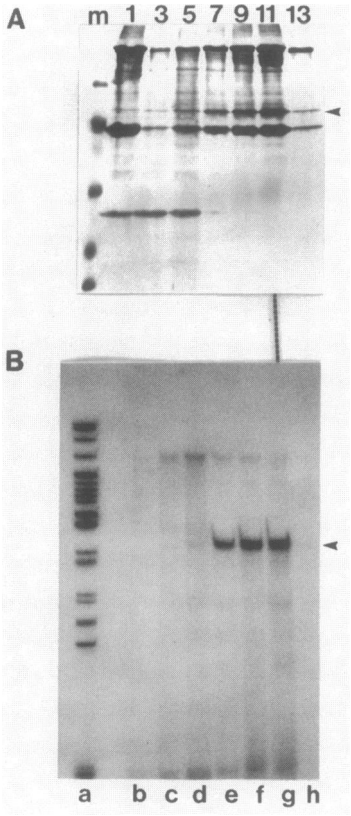

FIG. 3. Gradient elution of RNA polymerase from heparin-agarose. RNA polymerase purified through DNA-cellulose as described in reference 1 was eluted from a heparin-agarose column (3) with a linear gradient of KCl (0.4 to 0.7 M). (A) The proteins in each odd-numbered fraction were visualized by staining with Coomassie blue after electrophoresis of 200 μl into a sodium dodecyl sulfate slab gel containing 14% (wt/vol) polyacrylamide. The number above each lane indicates the fraction number. The arrowhead indicates the position of σ^A. The molecular mass markers in lane m are bovine plasma albumin (66 kilodaltons [kDa]), egg ovalbumin (45 kDa), pepsin (34.7 kDa), trypsinogen (24 kDa), and β-lactoglobulin (18 kDa). (B) Each odd-numbered fraction (2 μl) was used in an in vitro transcription reaction that included the 255-bp *Hin*dIII-*Eco*RI DNA fragment that contains the *spoIIG* promoter. ^{32}P-labeled runoff transcripts were visualized by autoradiography after electrophoresis into a 7 M urea slab gel containing 9% polyacrylamide. Lanes b through h, runoff transcripts generated by RNA polymerase from the odd-numbered fractions from 1 to 13, respectively; a, molecular mass markers from radiolabeled pBR322 that had been cut with *Hpa*II. The arrowhead indicates the position of the runoff transcript from the *spoIIG* promoter.

conclusion about the role of the dyad sequence based on the modest effects of these mutations, since these mutations could also affect the stability of the mRNA.

We also deleted sequences downstream from the *spoIIG* promoter and assayed the effects by fusion of these mutant promoters to *lacZ* as described above. As expected, deletion of the sequences downstream from position +13 had no effect on promoter activity (Fig. 4B, *spoIIGΔ105*). However, deletion of the sequences downstream from +3 completely inactivated the promoter (Fig. 4B, *spoIIGΔ97*). From these data, we conclude that sequences downstream from +13 are not necessary for promoter activity. Sequences between +3 and +13 may be important for promoter activity or for mRNA stability. These two possibilities have not yet been tested. We also examined the effects of four deletions that removed sequences upstream from the promoter. The deletions extended from position −88 toward the promoter to positions −62, −51, −39, or −37 (Fig. 1). Each of these deletions abolished *lacZ* expression.

DISCUSSION

Possible transcription of *spoIIG* by Eσ^A. From these results, we conclude that σ^A, the primary sigma in vegetative cells, can direct RNA polymerase to use the *spoIIG* promoter in vitro. Some caution should be exercised in extrapolating this conclusion to the situation in vivo, since a direct demonstration of an interaction between σ^A and the *spoIIG* promoter in vivo has not been obtained. However, the observation that two single-base substitutions that changed sequences known to be recognized by Eσ^A prevented use of the promoter in vivo and the observation that Eσ^A accurately initiates transcription from the promoter in vitro makes it likely that Eσ^A transcribes *spoIIG* in vivo. Moreover, it is unlikely that both the −10 and −35 sets of sequences that are known to signal recognition of promoters by Eσ^A (18) occur fortuitously in the *spoIIG* promoter. The promoter for the *spoIIE* operon also has similar −10- and −35-like sequences that are separated by 21 bp (6). The mechanism for activating *spoIIG* transcription, therefore, may be used to activate transcription of several genes during the early stage of sporulation.

Regulation of *spoIIG* transcription. If Eσ^A transcribes *spoIIG*, then why is it not transcribed until the beginning of sporulation? The factor(s) that regulates *spoIIG* transcription may be produced or regulated by the products of two genes, known as *spo0A* and *spo0H*, since mutations in these genes prevent transcription of *spoIIG* (10). *spo0H* is the structural gene for the sporulation-essential sigma factor σ^H, formerly σ^{30} (1, 4). σ^H is present in vegetative and early sporulating cells. It is essential for the expression of many genes that are expressed during sporulation and directly transcribes at least two genes that are expressed at the beginning of sporulation (1, 2, 4). Since RNA polymerase containing this sigma, Eσ^H, did not use the *spoIIG* promoter in vitro, it is likely that Eσ^H transcribes another gene, whose product is necessary for activation of *spoIIG*. The effect of *spo0A* could be direct or, alternatively, the effect of Spo0A on *spoIIG* promoter function could simply reflect the level of *spo0H* expression, since Spo0A is necessary for full expression of *spo0H* (1; I. Smith, personal communication). This is unlikely because a mutation at *abrB* that restores *spo0H* expression in a *spo0A* background does not restore *spoIIG* promoter activity in a *spo0A* background (10). It will be interesting to identify the factor(s) that is produced by the concerted action of *spo0H* and *spo0A* to activate the *spoIIG* promoter.

There are several candidates for genes that encode this factor. One such gene is *spoIIN*, a gene identified as temperature-sensitive allele *spo279*, a mutation that blocks sporulation at stage II (21). This mutation does not map in the *spoIIG* operon, but it prevents transcription from the *spoIIG* promoter (our unpublished data). Mutations in *spoIIL* (our unpublished data) and *spoIIF* (P. Stragier, personal communication) also prevent transcription from the *spoIIG* promoter. Among several possibilities, one of these genes may encode a factor that binds to the *spoIIG* promoter or to Eσ^A or may encode a new sigma factor that has not been detected in our in vitro transcription assay.

Constitutive expression from promoter *spoIIG38*. Recently, the observations that the deleterious effects of mutations at

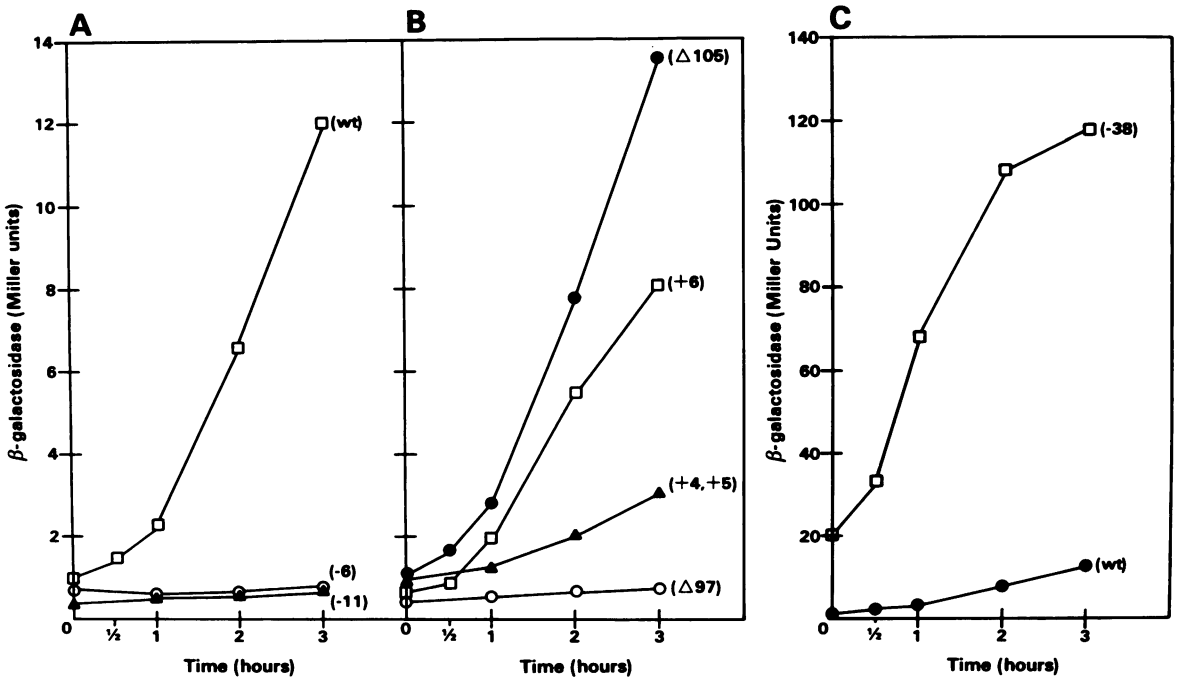

FIG. 4. Effects of mutations on *spoIIG-lacZ* expression. Mid-exponential cultures of *B. subtilis* strains that contained different SPβ::*spoIIG-lacZ* prophages were induced to sporulate by the addition of decoyinine (time zero), and β-galactosidase was monitored at the intervals indicated on each graph. The mutation in the *spoIIG* promoter is indicated in parentheses. Symbols for panel A: □, EU8747 (SPβ::*IIG217-lacZ*); ○, EU8748 (SPβ::*IIG6-lacZ*); ▲, EU8749 (SPβ::*IIG11-lacZ*). Symbols for panel B: ●, EU8750 (SPβ::*IIGΔ105-lacZ*); □, EU8751 (SPβ::*IIG60-lacZ*); ▲, EU8752 (SPβ::*IIG45-lacZ*); ○, EU8753 (SPβ::*IIGΔ97-lacZ*). Symbols for panel C: ●, EU8747 (SPβ::*IIG217-lacZ*); □, EU8754 (SPβ::*IIG38-lacZ*).

the −10 region and the −35 regions of promoters can be suppressed by allele-specific mutations in genes encoding sigma factors have been used to argue that the sigma subunit of RNA polymerase makes sequence-specific contacts at the −10 and −35 regions of most promoters (Zuber et al., in preparation; M. Susskind, personal communication). It is likely that a novel mechanism is used by the activator of the *spoIIG* promoter to enable $E\sigma^A$ to use this promoter in which the sigma contact sites on the promoter are separated by an unusually large distance. Several models for the activation of *spoIIG* transcription probably can be eliminated by the effect of the base substitution in the −35-like sequences. In one model, *spoIIF*, *spoIIL*, *spoIIN*, or another gene encodes a new sigma factor that utilizes the same −10- and −35-like sequences that are used by σ^A, but the new sigma is able to use these contact sites that are separated by 22 bp. In contrast to the observed effect, this model predicts that the base substitution at −38 would reduce utilization of the promoter. In a second model, $E\sigma^A$ uses the *spoIIG* promoter after a helix-distorting or σ^A-distorting factor compensates for the aberrant spacing between −10 and −35 contact sites. In its simplest form, this model also predicts that the transition at −38 would reduce promoter activity.

Several models that have not been tested offer possible explanations of the effect of the −38 mutation. For example, the base pair at position −38 may be part of the binding site of a repressor. Alternatively, $E\sigma^A$ may bind tightly to this −35-like sequence but be unable to reach the −10 region. An activator would prevent binding of $E\sigma^A$ to this −35-like sequence and enable it to productively interact with the −10 sequence. An appealing feature of this model is that this activator may act to repress transcription from typical $E\sigma^A$-type promoters. In this model, the mutation at −38 may decrease binding of $E\sigma^A$ to the −35-like sequence and allow use of a secondary −35-like sequence (3 base match, at positions −31, −32, and −33 [Fig. 1]) that is appropriately separated from the −10 sequence. Finally, the mutation at −38 may create a new promoter that bypasses the requirements for all of the factors that normally interact with the wild-type *spoIIG* promoter. This seems unlikely, since the transcript is initiated at the same nucleotide of the mutant and wild-type promoters. Identification of the *spoIIG* activator will enable us to test these models.

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LITERATURE CITED

1. **Carter, H. L., III, and C. P. Moran, Jr.** 1986. New RNA polymerase sigma factor under *spo0* control in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **83**:9438–9442.
2. **Carter, H. L., III, L.-F. Wang, R. H. Doi, and C. P. Moran, Jr.** 1988. *rpoD* operon promoter used by σ^H-RNA polymerase in *Bacillus subtilis*. J. Bacteriol. **170**:1617–1621.
3. **Davison, B. L., T. Leighton, and J. C. Rabinowitz.** 1979. Purification of *Bacillus subtilis* RNA polymerase with heparin-agarose. J. Biol. Chem. **254**:9220–9226.
4. **Dubnau, E., J. Weir, G. Nair, H. L. Carter III, C. P. Moran, Jr., and I. Smith.** 1988. *Bacillus* sporulation gene *spo0H* codes for σ^{30} (σ^H). J. Bacteriol. **170**:1054–1062.
5. **Gilman, M. Z., J. L. Wiggs, and M. J. Chamberlin.** 1981.

Nucleotide sequences of two *Bacillus subtilis* promoters used by *Bacillus subtilis* sigma-28 RNA polymerase. Nucleic Acids Res. **9**:5991–6000.

6. **Guzmán, P., J. Westpheling, and P. Youngman.** 1988. Characterization of the promoter region of the *Bacillus subtilis spoIIE* operon. J. Bacteriol. **170**:1598–1609.
7. **Haldenwang, W. G., N. Lang, and R. Losick.** 1981. A sporulation-induced sigma-like regulatory protein from *B. subtilis*. Cell **23**:615–624.
8. **Igo, M. M., and R. Losick.** 1986. Regulation of a promoter that is utilized by minor forms of RNA polymerase holoenzyme in *Bacillus subtilis*. J. Mol. Biol. **191**:615–624.
9. **Inoue, T., and T. R. Cech.** 1985. Secondary structure of the circular form of the *Tetrahymena* rRNA intervening sequence: a technique for RNA structure analysis using chemical probes and reverse transcriptase. Proc. Natl. Acad. Sci. USA **82**:648–652.
10. **Kenney, T. J., and C. P. Moran, Jr.** 1987. Organization and regulation of an operon that encodes a sporulation-essential sigma factor in *Bacillus subtilis*. J. Bacteriol. **169**:3329–3339.
11. **Kobayashi, Y.** 1987. The role of sigma factors in transcription regulation. Cell. Technol. **6**:593–604. (In Japanese.)
12. **Kunkel, T. A.** 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA **82**: 488–492.
13. **LaBell, T. L., J. E. Trempy, and W. G. Haldenwang.** 1987. Sporulation-specific σ factor σ^{29} of *Bacillus subtilis* is synthesized from a precursor protein, P31. Proc. Natl. Acad. Sci. USA **84**:1784–1788.
14. **Losick, R., and J. Pero.** 1981. Cascades of sigma factors. Cell **25**:582–584.
15. **Losick, R., P. Youngman, and P. J. Piggot.** 1986. Genetics of endospore formation. Annu. Rev. Genet. **20**:625–669.
16. **Melton, D. A., P. A. Kneg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green.** 1984. Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res. **12**:7035–7056.
17. **Moran, C. P., Jr., N. Lang, C. D. B. Banner, W. G. Haldenwang, and R. Losick.** 1981. Promoter for a developmentally regulated gene in *Bacillus subtilis*. Cell **25**:783–791.
18. **Moran, C. P., Jr., N. Lang, S. F. J. LeGrice, G. Lee, M. Stephens, A. L. Sonnenshein, J. Pero, and R. Losick.** 1982. Nucleotide sequences that signal the initiation of transcription and translation in *Bacillus subtilis*. Mol. Gen. Genet. **186**:339–346.
19. **Trempy, J. E., C. Bonamy, J. Szulmajster, and W. G. Haldenwang.** 1985. *Bacillus subtilis* sigma factor σ^{29} is the product of the sporulation-essential gene *spo*IIG. Proc. Natl. Acad. Sci. USA **82**:4189–4192.
20. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103–119.
21. **Young, M.** 1976. Use of temperature-sensitive mutants to study gene expression during sporulation in *Bacillus subtilis*. J. Bacteriol. **126**:928–936.
22. **Zoller, M. J., and M. Smith.** 1983. Oligonucleotide-directed mutagenesis of DNA fragments cloned into M13 vectors. Methods Enzymol. **100**:468–500.
23. **Zuber, P., and R. Losick.** 1983. Use of a *lac*Z fusion to study the role of *spo*0 genes in developmental regulation in *Bacillus subtilis*. Cell **35**:275–283.
24. **Zuber, P., and R. Losick.** 1987. Role of AbrB in Spo0A- and Spo0B-dependent utilization of a sporulation promoter in *Bacillus subtilis*. J. Bacteriol. **169**:2223–2230.