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Promoter Specificity of σ^G-Containing RNA Polymerase from Sporulating Cells of *Bacillus subtilis*: Identification of a Group of Forespore-Specific Promoters

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During sporulation in *Bacillus subtilis*, expression of the genes *sspA*, *sspB*, *sspC*, *sspD*, and *sspE*, which encode a family of small, acid-soluble spore proteins, as well as of the *spoVA* and *gdh* operons is transcriptionally activated at stage III of sporulation only in the forespore compartment. Transcription of these genes is mediated by RNA polymerase containing sigma G ($E\sigma^G$), the product of the *sigG* gene, which is itself expressed at stage III in the developing forespore. We have determined the 5′ ends of transcripts generated both in vivo and in vitro by the action of $E\sigma^G$ on various genes of *B. subtilis* and other bacilli. The 5′ ends of the in vivo and in vitro mRNAs were found to coincide and were therefore considered to define the transcription initiation sites for the genes examined. We identified highly homologous DNA sequences centered at 35 and 10 base pairs preceding the transcriptional start sites of the genes examined. Consequently, we propose that these sequences define a class of promoters recognized only by $E\sigma^G$ which allow transcription of genes expressed uniquely at stage III in the developing forespore.

Differentiation in *Bacillus subtilis* proceeds through an ordered series of morphological changes leading from a vegetatively growing bacterial cell to a dormant endospore. This morphological progression is recognized to result from the sequential expression of a set of genes designated *spo*, as mutations in *spo* genes result in arrest of the developmental sequence at characteristic intermediate morphological stages. Although *spo* genes have been well characterized genetically, the mechanisms by which they exert their effect has until recently remained obscure (for reviews, see references 30 and 43).

In parallel with the genetic findings, biochemical evidence has accumulated which indicates that the temporal program of gene expression during *B. subtilis* sporulation is determined at least in part by the sequential action of distinct forms of RNA polymerase containing different sigma subunits that share substantial amino acid sequence homology (10, 25, 47) but recognize unique promoter sequences (29, 30). Perhaps not surprisingly, recent studies have demonstrated that several sporulation-associated sigma factors are themselves the products of previously identified *spo* genes. To date, it has been determined that the *spo* genes *spo0H* (9), *spoIIAC* (49), *spoIIG* (54), and *spoIIIG* (25) encode the minor sigma factors σ^H (formerly σ^{30}), σ^F, σ^E (formerly σ^{29}), and σ^G, respectively.

An intermediate stage in the *B. subtilis* developmental sequence, stage III, is characterized morphologically by the subdivision of the sporulating cell into two physically separate compartments, a forespore surrounded by a mother cell. Each of these compartments carries a complete genome, but the patterns of gene expression in the two compartments differ markedly. The *spoIIIG* (*sigG*) gene, which encodes σ^G, is expressed during stage III of sporulation exclusively in the forespore compartment (25). RNA polymerase containing σ^G ($E\sigma^G$) is directly responsible for the temporal and compartment-specific transcriptional activation at stage III of a set of closely related genes designated *ssp* (49), which encode a family of small, acid-soluble spore proteins (SASP). The SASP, in turn, provide a ready source of amino acids to the germinating spore (reviewed in reference 45), and a subset of the SASP are responsible for spore resistance to UV light (34). Two additional sets of genes expressed in parallel with *ssp* genes at stage III in the developing forespore are those of the *gdh* (5, 19, 55) and *spoVA* (6, 11, 33) operons. Both the *gdh* and *spoVA* operons are also directly dependent on $E\sigma^G$ for expression in vivo (49). Thus, it appears that $E\sigma^G$ is responsible for the transcription of a class of genes expressed uniquely at stage III in the forespore.

In this communication, we define the promoter sequence recognized by $E\sigma^G$ by mapping the 5′ ends of in vivo *ssp* mRNAs and by determining the in vitro transcription initiation sites for the *ssp* genes and the *spoVA* operon. Determination of the transcription start site of the *gdh* operon in vivo and in vitro has been presented elsewhere (40a, 44).

MATERIALS AND METHODS

Bacterial strains and plasmids. The *B. subtilis* strains used in this study are all derived from strain 168 and are described in Table 1. *Bacillus megaterium* QMB1551 was originally obtained from H. S. Levinson, U.S. Army Laboratories, Natick, Mass. Plasmids pUC12 (41), pUC18 (41), pT7-1, and pT7-2 (United States Biochemical Corp.) were used for subcloning, preparation of templates for runoff transcription of the *ssp* genes, and preparation of ^{32}P-labeled RNA probes for nuclease protection experiments. Plasmid pPP33, which carries the promoter-proximal portion of the *spoVA* operon (17, 18), was obtained from Patrick Piggot, Temple University, Philadelphia, Pa. Plasmid pDG298, which carries the *sigG* gene under control of the inducible *spac* promoter in plasmid pDG148 (47a, 57), has been described previously (49). *Escherichia coli* JM83 and JM107 (56) were used for plasmid propagation.

Construction of DNA templates for runoff transcription. The plasmids and inserts used for in vitro transcription

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TABLE 1. *B. subtilis* strains used

Strain	Genotype or phenotype	Source or reference
168	*trpC2*	Laboratory stock
PS766	*trpC2* (pDG298 *spac-sigG* Km^r)	pDG298→168[a]
PS614	*trpC2 spoIIIC94 sspE-lacZ* Cm^r	49
MO428 (PS683)	*trpC2 sigGΔ1*	P. Stragier (25)
PS320	*pheA1 trpC2 spo0A12 sspA-lacZ* Cm^r	33
PS749	*pheA1 trpC2 spo0A12 sspA-lacZ* Cm^r (pDG298 *spac-sigG* Km^r)	pDG298→PS320[a]
PS435	*trpC2 sspE-lacZ* Cm^r	33

[a] Arrow represents transformation of plasmid DNA into competent cells (1) with selection for the appropriate antibiotic resistance.

studies are described in Table 2. All plasmids were constructed by standard recombinant DNA techniques (31). For in vitro transcription, plasmids were cleaved by restriction endonuclease digestion at a site either within the coding sequence for the template gene or within the vector polylinker immediately downstream from the insert in order to generate runoff transcripts of between 50 and 300 nucleotides (nt) in length. Whenever possible, cleavage of the template DNA was performed with two or more different restriction endonucleases such that the lengths of the runoff transcripts generated from the cleaved templates differed by known values.

Isolation of $E\sigma^G$ RNA polymerase. *B. subtilis* PS614 was grown in the liquid sporulation medium 2×SG (27). Appearance of in vivo $E\sigma^G$ activity was monitored by assaying β-galactosidase synthesized from the *sspE-lacZ* gene fusion present in PS614 as described previously (33, 49). When the β-galactosidase activity reached ~20% of the maximum value, cells were harvested, washed, and frozen as previously described (49). RNA polymerase was isolated from strain PS614 essentially as described previously (49), with the following modifications. Frozen cells (100 g) were suspended in ice-cold buffer B (49) and disrupted by homogenization in the presence of 0.1-mm-diameter glass beads (Bead Beater homogenizer; Biospec Products, Bartlesville, Okla.). RNA polymerase was purified from the high-speed supernatant by heparin-agarose column chromatography, followed by DNA-cellulose chromatography and glycerol gradient ultracentrifugation. Fractions collected from column chromatography or from glycerol gradients were assayed for RNA polymerase activity on poly(dA-dT) as described previously (49). Fractions that contained significant levels of poly(dA-dT) activity were then assayed for $E\sigma^G$ activity by mixed-template runoff transcription assays, using both the *sspA* and the *sspE* templates, under either the in vitro transcription assay conditions described previously (49) or the in vitro transcription conditions described by LeGrice et al. (26). The two assays gave comparable results.

RNA polymerase isolated from PS614 as described above contains both the $E\sigma^F$ and the $E\sigma^G$ holoenzymes (49). RNA polymerase lacking both $E\sigma^F$ and $E\sigma^G$ was prepared by essentially the same procedure from strain 168 harvested during the exponential growth phase in 2×YT medium (38), which does not support sporulation. RNA polymerase containing $E\sigma^F$ but not $E\sigma^G$ was isolated as described above from sporulating cells of strain PS683, which harbors a deletion in the *sigG* gene. RNA polymerase containing $E\sigma^G$ but not $E\sigma^F$ was isolated from vegetative-phase cells of *B. subtilis* or from *E. coli* JM107, each carrying plasmid pDG298, as follows. Cells were grown to early exponential growth phase in 2×YT medium containing kanamycin (10 μg/ml); isopropyl-β-D-thiogalactopyranoside (IPTG) was then added to the culture to a final concentration of 1 mM to induce synthesis of σ^G. Two hours after induction, cells were harvested and RNA polymerase was isolated through the DNA-cellulose column step as described previously (49). *E. coli* RNA polymerase lacking σ^G was isolated from strain JM107 harboring the vector plasmid pDG148.

In vitro transcription. Runoff transcription reactions were performed as described previously (49) or as described by LeGrice et al. (26), with the following modifications. [α-^{32}P] UTP or [α-^{32}P] CTP (2 to 4 μCi; 800 Ci/mmol Dupont, NEN Research Products, Boston, Mass.) and 2 μg (0.4 to 0.8 pmol) of template DNA were used, and all reactions were started by addition of RNA polymerase. For dinucleotide priming reactions (39), all nucleoside triphosphates were at 3 μM, and the appropriate dinucleotide was at 250 μM.

TABLE 2. Construction of templates for in vitro transcription

Gene	Fragment cloned	Resulting plasmid
B. subtilis		
sspA	450-bp *Eco*RI fragment isolated from pUB-A (33) cloned into pUC18	pPS706
sspB	800-bp *Eco*RI fragment A (3) cloned into pUC12	pPS771
sspC	End-filled 270-bp *Bgl*II-*Hha*I fragment (4) cloned into *Hinc*II site of pT7-1	pPS532
sspD	350-bp *Pst*I-*Dra*I fragment (3) cloned into *Pst*I-*Hinc*II site of pUC18	pPS807
sspE	800-bp *Eco*RI-*Hin*dIII fragment (22) cloned into pUC12 (49)	pPS591
spoVA	230-bp *Pst*I-*Pvu*II fragment from pPP33 (17, 18) cloned into *Pst*I-*Hinc*II site of pUC18	pPS808
B. megaterium		
SASP-A (*sspA* analog; 50)	290-bp *Cla*I-*Eco*RI fragment (13) cloned into *Acc*I-*Eco*RI site of pT7-2	pPS530
SASP-C (*sspB* analog; 50)	1.5-kbp *Xba*I-*Hae*III fragment (13) cloned into *Xba*I-*Hinc*II site of pT7-1	pPS536
SASP-B (*sspE* analog; 50)	0.5-kbp *Eco*RI fragment (21) cloned into pT7-1	pPS506
B. cereus		
SASP-B (*sspE* analog)	1.8-kbp *Xba*I fragment cloned in pUC12 (48)	pPS456
B. stearothermophilus		
SASP-B (*sspE* analog)	0.5-kbp *Dra*I-*Hin*dIII fragment cloned in pUC12 (48)	pPS413

Transcription reactions using RNA polymerase isolated from sporulating cells of *B. subtilis* were labeled for 10 min at 37°C and then chased for a further 5 min at 37°C with 250 μM (final concentration) unlabeled UTP or CTP. Alternatively, transcription reactions using either RNA polymerase isolated from sporulating *B. subtilis* or $E\sigma^G$-containing RNA polymerase isolated from vegetative *B. subtilis* or *E. coli* carrying pDG298 were carried out as follows. Reactions were incubated at 37°C for 1 or 2 min; then 500 μg of heparin per ml (final concentration) was added to prevent reinitiation and to inhibit endogenous nuclease activity. Incubation with heparin for 2 or 4 min was followed by a 2-min chase with unlabeled UTP or CTP as described above, and reactions were terminated by ethanol precipitation. Air-dried samples were dissolved in gel loading buffer, heated to 90°C for 1 min, chilled quickly on ice, and analyzed by autoradiography after electrophoresis through 6% polyacrylamide–8 M urea sequencing gels (36). Lengths of runoff transcripts were determined by comparison of their mobilities with those of transcripts originating from the bacteriophage T7 promoter of plasmid pT7-1 carrying inserts of known length.

RNA isolation and analysis. The 5′ ends of in vivo mRNAs from *ssp* genes were determined by RNase protection essentially as described previously for the *sspE* gene (49). Total RNA was isolated as described previously (7, 32) from sporulating cells of *B. subtilis* PS435 or from *B. megaterium* QMB1551, each containing high levels of *ssp* mRNA. Total RNA was hybridized with a ^{32}P-labeled antisense RNA probe prepared from the appropriate *ssp* gene. The labeled probes were prepared by phage T7 RNA polymerase-mediated in vitro transcription of the appropriate *ssp* gene cloned in the antisense orientation in either plasmid pT7-1 or plasmid T7-2 (32), using the conditions described by the manufacturer. RNase protection reactions and size determinations of the protected probes were performed as previously described for the in vivo *sspE* transcript (49, 58).

More precise mapping of the 5′ ends of in vivo *sspA*, *sspB*, and *sspE* mRNAs from *B. subtilis* was performed by primer extension analysis (24, 37). ^{32}P-end-labeled sense-strand DNA primers were prepared from the following restriction fragments: (i) a 76-nt *Dde*I-*Eco*RI fragment from within the *sspA* coding sequence (3), (ii) a 124-nt fragment extending from the *Eco*RI site within the *ssp*B coding sequence to the upstream *Hpa*I site (3), and (iii) a 59-nt *Dde*I-*Eco*RI fragment from within the *ssp*E coding sequence (22). The end-labeled primers were hybridized with total RNA extract from *B. subtilis* cells and were extended by using reverse transcriptase (24, 37). The primer extension products were analyzed by autoradiography after electrophoresis through a 6% sequencing gel adjacent to a DNA sequencing ladder. Primer extension analysis was also performed on *B. subtilis sspB* mRNA synthesized in vitro as follows. Plasmid pPS771 linearized with *Bam*HI was transcribed in vitro by using RNA polymerase isolated from sporulating cells of strain PS614. To isolate sufficient quantities of in vitro *sspB* mRNA for primer extension analysis, the following modifications of the standard in vitro transcription reaction were used: (i) the reaction mixture was scaled up 10-fold, (ii) the final concentration of dithiothreitol was increased from 0.2 to 3.0 mM, (iii) all four unlabeled ribonucleoside triphosphates were at a final concentration of 150 μM, and (iv) 200 U of RNasin RNase inhibitor (Promega Biotec) was included in the reaction mixture. After in vitro transcription, the sample was treated with 115 U of RNase-free DNase I (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) for 15 min at 37°C before extraction with buffer-saturated phenol-chloroform and precipitation with ethanol. The resulting RNA was hybridized to the ^{32}P-end-labeled *ssp*B-specific DNA primer and was subjected to primer extension analysis as described above. Quantitation of specific *sspB* reverse transcripts was accomplished by excising the bands of interest from the gel and counting them in a scintillation counter.

RESULTS

Nucleotide sequences preceding the transcriptional start sites of stage III, forespore-specific genes. Figure 1 shows the nucleotide sequences of promoters recognized by $E\sigma^G$ RNA polymerase and summarizes the results of experiments concerned with mapping the 5′ ends of both in vivo and in vitro mRNA. Mapping of the 5′ end of *B. subtilis sspA* mRNA is described in detail below; the 5′ ends of mRNAs from the other genes were determined in a manner essentially identical to that used for *sspA*; the results of these experiments are summarized in Fig. 1 and Table 3. Mapping of the 5′ ends of the *B. megaterium* and *B. subtilis sspB* mRNAs is discussed separately (see below).

5′ end of in vivo *sspA* mRNA. A ^{32}P-labeled antisense RNA probe complementary to *B. subtilis sspA* mRNA (Fig. 2A) was prepared as described in Materials and Methods, hybridized to total *B. subtilis* RNA isolated from sporulating cells, and subjected to digestion with a combination of RNase A and RNase T_1. The length of the RNase-resistant RNA probe was estimated to be 113 nt by comparison of its mobility with that of RNA markers of known length (Fig. 2B). The size of the RNase-protected probe was consistent with the 5′ end of in vivo *ssp*A mRNA mapping at the position indicated by the underlined cytidine residue in Fig. 1. The 5′ ends of mRNAs from other *B. subtilis ssp* genes, as well as their cognates from *B. megaterium*, were determined in a manner directly analogous to that described above for *sspA* mRNA (data not shown) and are shown in Fig. 1A. In addition, the in vivo 5′ ends of the *B. subtilis sspA* and *sspE* mRNAs were localized by high-resolution primer extension mapping within 1 nt of their respective positions determined by RNase protection (Fig. 1A; data not shown). Also shown in Fig. 1A is the in vivo transcription start site for the *gdh* operon, which was determined by primer extension analysis (40a, 44). The 5′ end of *spoVA* mRNA in vivo has not yet been determined.

In vitro start site of *sspA* transcription. In an earlier communication, it was reported that the *B. subtilis sspE* gene was transcribed from the same start site both in vivo and in vitro by $E\sigma^G$ and that the expression of other stage III, forespore-specific genes is $E\sigma^G$ dependent in vivo (49). To define the promoter sequences shared by this class of genes, we mapped the 5′ ends of RNA produced in vitro by the action of $E\sigma^G$ on various templates (Table 2). The results of these experiments are summarized in Table 3.

The in vitro start site of *sspA* transcription was determined by runoff transcription of plasmid pPS706 linearized by digestion with *Pst*I, *Xba*I, or *Bam*HI (Fig. 3A), using RNA polymerase isolated from sporulating cells of strain PS614 (Fig. 3B). Transcription of pPS706 linearized with *Pst*I, *Bam*HI, or *Xba*I yielded runoff transcripts of 148, 133, and 139 nt, respectively (Fig. 3). Similar results were obtained with $E\sigma^G$-containing RNA polymerase isolated from vegetative cells of strain PS749 after induction with 1 mM IPTG (data not shown).

The in vitro transcription start site of *sspA* was localized more precisely by testing the ability of various dinucleotides to prime in vitro transcription of *sspA* by $E\sigma^G$ in the

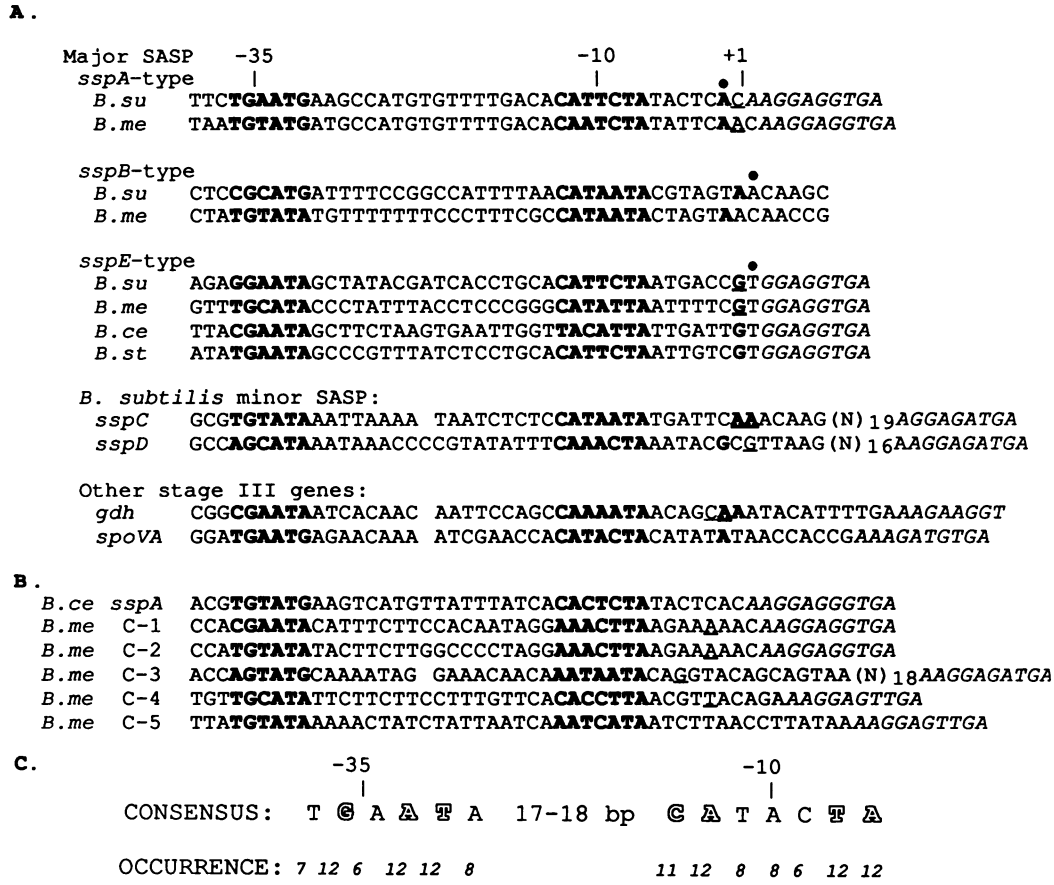

FIG. 1. Nucleotide sequences of the promoter-proximal region of stage III, forespore-specific genes. The figure depicts the nucleotide sequence of the nontranscribed strand of each gene and summarizes the evidence for their transcriptional start points. Sequence data are taken from references 3, 4, 13 to 17, 21, 22, 28, 40a, 44, and 48. The underlined bases represent the transcription start sites in vivo as determined by nuclease protection (A), by size determination of mRNA by Northern blot analysis (*B. megaterium* SASP C-1, C-2, C-3, and C-4 genes; panel B and references 13 and 16), or by homology (*B. megaterium* SASP C-5 and *B. cereus sspA* genes; panel B and references 13 and 28). In panel A, bases representing in vivo transcription start sites determined by primer extension analysis versus DNA sequencing ladders (●), and bases located at or adjacent to +1 that are in vitro transcription start points determined by runoff transcription and dinucleotide priming analysis (bold face) are indicated. In panels A and B, conserved sequences at −35 and −10 are in boldface; ribosome-binding sites are italicized. (C) Consensus $E\sigma^G$ promoter sequence and summary of the frequency of base occurrence at each position, taken from the sequences listed in panel A. Outlined bases are most highly conserved. Abbreviations: *B. su*, *B. subtilis*; *B. me*, *B. megaterium*; *B. ce*, *B. cereus*; *B st*, *B. stearothermophilus*; (N), sequence not listed to conserve space.

presence of a low concentration of all four ribonucleoside triphosphates. The results of the dinucleotide priming experiments showed that the dinucleotides CpA and ApC were most efficient at priming *sspA* transcription, that the dinucleotides UpC and ApA were weakly active, but that ApG was ineffective (Fig. 4). The lengths of the *ssp*A runoff transcripts and the dinucleotide preference are consistent with placement of the boldface adenine residue as the in vitro transcription start site of the *sspA* gene (Fig. 1A and 4B). The results of similar analyses performed on other stage III, forespore-specific genes are presented in Table 3 and depicted schematically in Fig. 1A.

Consensus promoter sequence for stage III, forespore-specific genes. The results of experiments mapping the in vivo and in vitro transcription start sites of various stage III, forespore-specific genes are summarized in Fig. 1. With the exception of the *B. subtilis* and *B. megaterium sspB* genes, which are discussed below, the 5′ ends of in vivo mRNAs were located within 1 to 2 nt of their respective $E\sigma^G$-dependent in vitro transcription start sites (Fig. 1A). Since expression of these genes is $E\sigma^G$ dependent in vivo (49), these data suggest that the 5′ ends of the in vivo mRNAs also represent the in vivo transcription initiation sites of these genes. When the nucleotide sequences preceding these stage III, forespore-specific genes were aligned at their transcriptional start sites (Fig. 1A), striking homologies were observed in the regions centered approximately 35 and 10 nt preceding the transcriptional start sites (Fig. 1A). The consensus sequence derived from this alignment, TGAATA−17 to 18 base pairs (bp)−CATACTA (Fig. 1C), differs substantially from *B. subtilis* promoter sequences recognized by RNA polymerase containing the sigma factor σ^A, (40), σ^B (51), σ^C (23), σ^D (20), σ^E (52), or σ^H (2). In the 12 promoters examined in detail (Fig. 1A), bases at 7 of 13 positions were found to be invariant or nearly so (Fig. 1C). In addition, close matches to the consensus $E\sigma^G$ promoter sequences were observed in the *Bacillus cereus* analog of the *B. subtilis sspA* gene and in the genes coding for five minor SASP from *B. megaterium* (Fig. 1B).

Examination of the nucleotide sequences surrounding the −35 and −10 regions of the promoters listed in Fig. 1 failed to uncover any additional conserved sequences, which im-

TABLE 3. Summary of in vitro transcription experiments[a]

Plasmid	Promoter	Digest	Transcript length (nt)	Relative efficiency of in vitro transcription primed by[b]: AA	AC	AG	AU	CA	CC	CG	CU	GA	GC	GG	GU	UA	UC	UG	UU
B. subtilis																			
pPS706	*sspA*	*Xba*I	139	±	+	−		+									±		
		*Bam*HI	133																
		*Pst*I	148																
pPS771	*sspB*	*Bam*HI	218	+	±	+		−		±					+	+			
		*Xba*I	222																
		*Pst*I	230																
pPS532	*sspC*	*Pst*I	165	+	±	−	−	+				−	−				+	−	±
pPS807	*sspD*	*Eco*RI	131																
		*Bam*HI	110																
		*Xba*I	106																
pPS591	*sspE*	*Eco*RI	195	−	±	−	−		+	+					+			−	
pPS808	*spoVA*	*Eco*RI	162																
		*Bam*HI	143																
		*Xba*I	136																
B. megaterium																			
pPS530	SASP-A (*sspA*)[c]	*Eco*RI	101	+	±	−	±	+								±	±		−
pPS536	SASP-C (*sspB*)	*Bgl*II	57	+	+	−		+	−	−	−	−			+	±			
		*Cla*I	63																
pPS506	SASP-B (*sspE*)	*Eco*RI	271	−		−	−			+					+		±	−	−
B. cereus (pPS456)	SASP-B (*sspE*)	*Hin*dIII	168			−	−								+			+	±
B. stearothermophilus (pPS413)	SASP-B (*sspE*)	*Eco*RI	188	−		−	−			+					+	±	+	−	−

[a] Plasmids were constructed as described in Table 2, digested with the indicated restriction endonucleases, and subjected to in vitro transcription, using $E\sigma^G$ RNA polymerase as described in Materials and Methods.

[b] +, Active; ±, weakly active; −, inactive at priming in vitro transcription.

[c] The *B. subtilis* analog is given in parentheses.

plied that the properly spaced −35 and −10 regions are themselves sufficient to define the functional limits of $E\sigma^G$-type promoters. In experiments published elsewhere, direct evidence in support of this suggestion has been obtained by both in vitro and in vivo analyses of the promoter efficiencies of in vitro-generated mutations in the *gdh* promoter region (40a, 44).

Apparent discrepancy between the in vivo and in vitro *sspB* transcription start site. As mentioned above, the $E\sigma^G$-dependent in vitro transcription start sites for stage III, forespore-specific genes generally agreed well with the 5′ ends of the corresponding in vivo mRNAs. However, large differences were observed between the $E\sigma^G$-dependent in vitro transcription start sites and the 5′ ends of the corresponding in vivo mRNAs from the *sspB* genes of *B. subtilis* and *B. megaterium*. The 5′ ends of the in vivo mRNAs from the two genes were located by nuclease protection at positions +22 (*B. subtilis sspB*) and +33 (*B. megaterium* SASP-C, the analog of *sspB*; 50) with respect to the $E\sigma^G$-dependent in vitro transcription start sites (Fig. 5A). This large discrepancy was surprising, as the in vitro transcription start sites of these *sspB* genes were in close proximity to DNA sequences exhibiting good adherence to the canonical $E\sigma^G$-type promoter sequence (Fig. 1; Table 3).

In an attempt to resolve this apparent discrepancy, we isolated RNA either from sporulating cells of *B. subtilis* or from vegetative cells after IPTG induction of σ^G-synthesis, two conditions under which cells produce high levels of *sspB* mRNA. In addition, we isolated *sspB* mRNA produced in vitro by $E\sigma^G$-mediated transcription of plasmid pPS771 linearized with *Bam*HI (see Materials and Methods). Each RNA preparation was hybridized with a ^{32}P-end-labeled 124-nt DNA primer originating from within the *sspB* gene, and the primer was extended with reverse transcriptase. It was predicted that extension of the primer to the in vivo +1 site (as defined by nuclease protection mapping) would result in a 182-nt reverse transcript, whereas extension of the primer to the in vitro +1 site would result in a reverse transcript of 203 nt.

Strikingly, *sspB* mRNA either isolated from cells of *B. subtilis* or produced by in vitro transcription of the cloned *sspB* gene consisted of a population of two RNAs. The primer extension products produced from these two RNAs corresponded very closely in length to that expected if transcription were beginning at either the in vivo (186 nt, as opposed to the 182 nt predicted from the nuclease protection experiment) or the in vitro (203 nt) transcription start point. The lengths of these reverse transcripts were further confirmed by high-resolution electrophoresis in parallel with a DNA sequencing ladder (Fig. 5A; data not shown). However, the relative proportions of long (203 nt) and short (186 nt) reverse transcripts were very different in the in vivo and in vitro RNA preparations (Fig. 5B). Quantitation of the relative amounts of the long and short reverse transcripts, by excising the appropriate radioactive bands from the sequencing gel and counting them in a scintillation counter, showed that RNA isolated either from sporulating cells or from vegetative cells containing $E\sigma^G$ each contained predominantly (>95%) the shorter, in vivo *sspB* transcript, although some of the longer in vitro transcript was present. In contrast, the *sspB* mRNA produced in vitro was predominantly (80%) in the longer, in vitro form, although a substantial amount (20%) of the shorter, in vivo species was also present. Although the possibility exists that the presence of the two transcripts both in vivo and in vitro represents utilization of two different promoters, independent lines of

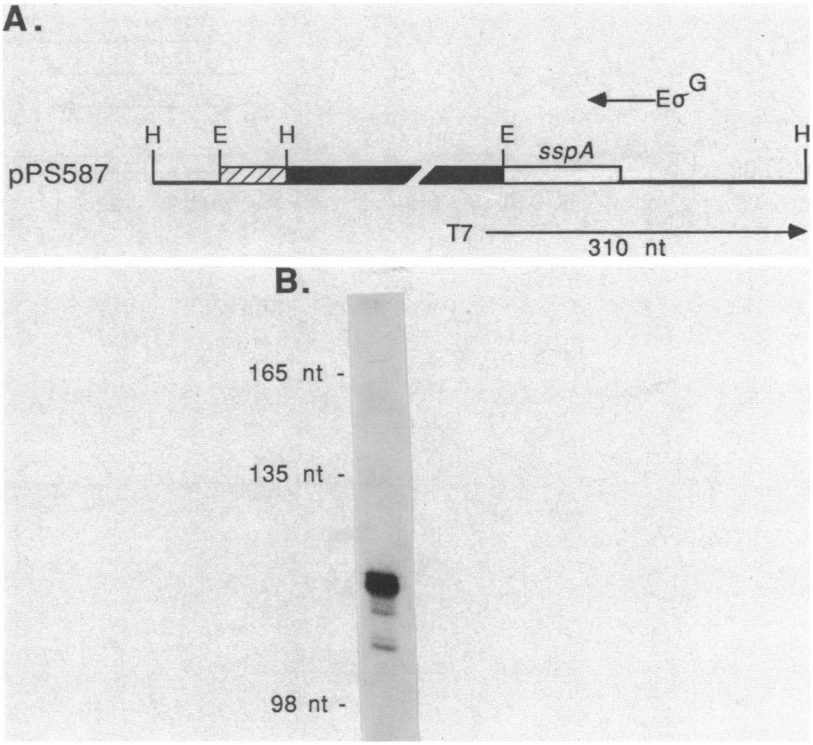

FIG. 2. Determination of the 5′ end of in vivo *sspA* mRNA by RNase protection. (A) Schematic representation of plasmid pPS587, a vector for producing antisense *sspA* RNA, after linearization with *Hin*dIII. Symbols and abbreviations: ▬, plasmid pT7-1; ▭, *sspA* coding sequence; ——, *B. subtilis* DNA preceding *sspA*; ▨, pT7-1 polylinker; E, *Eco*RI; H, *Hin*dIII. Transcription from the phage T7 promoter by T7 RNA polymerase produces a 310-nt antisense transcript (→); ←, direction of *sspA* transcription by $E\sigma^G$ in *B. subtilis*. (B) Autoradiogram of the RNase-resistant *sspA* antisense probe on a 6% sequencing gel. Markers were runoff RNA transcripts produced from the T7 promoter of plasmid pT7-1 carrying inserts of known lengths.

evidence lead us to favor an alternative explanation whereby the longer *sspB* transcript is processed into the shorter form (see Discussion).

Both $E\sigma^G$ and $E\sigma^F$ utilize $E\sigma^G$-type promoters in vitro. Although expression of stage III, forespore-specific genes depends only on the presence of $E\sigma^G$ in vivo, it has been found (49) that RNA polymerase preparations isolated from sporulating cells of *B. subtilis* PS614 at stage III actually contain two distinct forms of RNA polymerase capable of transcribing the *sspE* gene accurately in vitro. One of these RNA polymerase forms contained σ^F, the product of the *spoIIAC* (or *sigF*) gene, and the other contained σ^G (49).

By manipulation of *B. subtilis* strains and growth conditions, it was possible to isolate preparations of RNA polymerase that contained either $E\sigma^F$ or $E\sigma^G$, neither $E\sigma^F$ nor $E\sigma^G$, or both $E\sigma^F$ and $E\sigma^G$ (see Materials and Methods; Fig. 6B). As was found previously by using an *sspE* template (49), either $E\sigma^F$ or $E\sigma^G$ was capable of transcribing the *sspA* gene in vitro, with each form of RNA polymerase utilizing the same in vitro start site (Fig. 6A). Essentially identical results were obtained with in vitro transcription assays using these RNA polymerase preparations to transcribe the *ssp*B, *ssp*E, and *gdh* templates (data not shown). Even RNA polymerase isolated after induction of *sigG* expression in *E. coli* JM107 carrying plasmid pDG298 was capable of accurately transcribing either *sspA* or *sspE* templates in vitro by using the same start sites as *B. subtilis* $E\sigma^G$ (data not shown). This activity, which we assume to be due to *E. coli* core RNA polymerase containing *B. subtilis* σ^G, was absent from RNA polymerase preparations isolated from *E. coli* JM107 harboring only the vector plasmid pDG148 (data not shown).

DISCUSSION

In this communication, we describe the promoter sequence utilized by the $E\sigma^G$ form of RNA polymerase, which transcribes a class of sporulation-specific genes expressed uniquely during stage III of sporulation and exclusively in the developing forespore compartment. The transcription start sites of these genes mapped by in vitro transcription using $E\sigma^G$ were found to closely match the 5′ ends of the corresponding in vivo mRNAs (Fig. 1), thus supporting the contention that $E\sigma^G$ transcribes these genes in vivo. Examination of the DNA sequences preceding the in vivo and the in vitro transcription start sites of these genes (summarized in Fig. 1) shows that the hexamer sequence TGAATA, centered at approximately −35, and the heptamer sequence CATACTA, centered at approximately −10, not only are highly conserved among stage III, forespore-specific genes of *B. subtilis* but are also found in *ssp* cognate genes cloned from the related sporeforming species *B. megaterium*, *B. cereus*, and *B. stearothermophilus* (Fig. 1). Such cross-species sequence conservation among *ssp* promoters implies that the analog of σ^G itself is strongly conserved among the bacilli. Similar cross-species conservation of sporulation sigma factors has been observed in the case of *sigH*, the gene encoding σ^H, which was isolated first from *Bacillus licheniformis* on the basis of its ability to complement a *B. subtilis* *sigH* (i.e., *spo0H*) mutation (8).

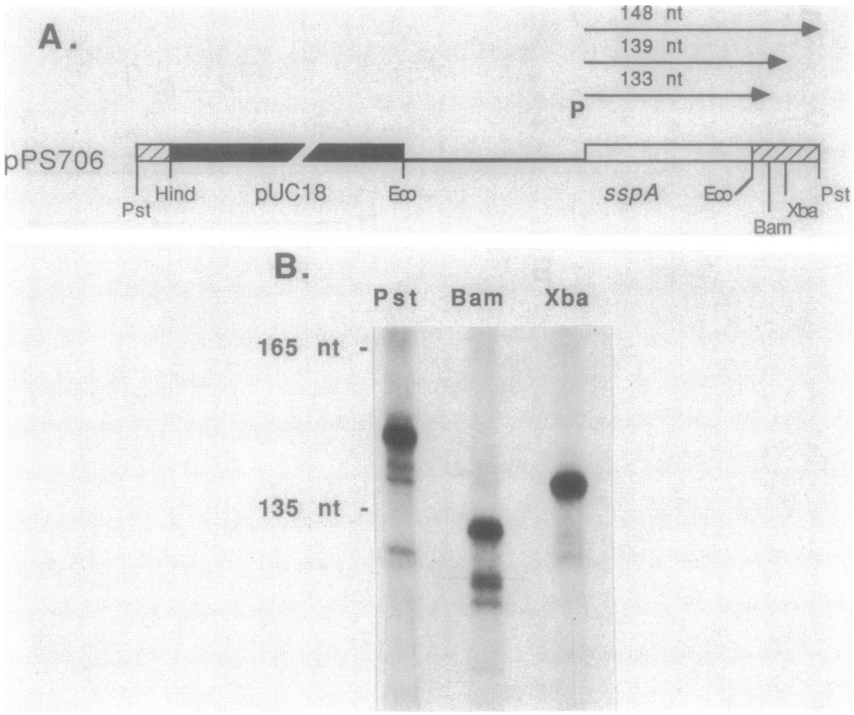

FIG. 3. In vitro transcription of the *B. subtilis sspA* gene by $E\sigma^G$. (A) Schematic representation of plasmid pPS706 linearized with *Pst*I. Symbols: ■, pUC18 vector DNA; ——, *B. subtilis* DNA preceding *sspA*; ▭, *sspA* DNA; ▨, pUC18 polylinker DNA. Sizes of transcripts from in vitro runoff transcription to the *Bam*HI, *Xba*I, or *Pst*I sites from the *sspA* promoter (P) are indicated (→). (B) Autoradiogram of in vitro runoff transcripts generated by $E\sigma^G$ action on plasmid pPS706 linearized with *Pst*I, *Bam*HI, *Xba*I. $E\sigma^G$ was isolated from sporulating cells of strain PS614. Markers are as described in the legend to Fig. 2.

The transcription start sites defined by in vitro transcription using $E\sigma^G$ generally coincided very closely to the 5′ end of the corresponding in vivo mRNA (Fig. 1). In the cases of the *sspB*-type genes from *B. subtilis* and *B. megaterium*, however, large differences were observed between the in vivo and the in vitro start sites of transcription (Fig. 5). Primer extension mapping revealed that *B. subtilis sspB* mRNA produced either in vivo or in vitro exhibited both types of 5′ ends, although in different relative abundances (Fig. 5). The reason for the presence of these two different-sized transcripts is at present unclear. Although it is formally possible that the presence of the two *sspB* transcripts reflects transcription arising from two distinct promoters, additional evidence leads us to suggest rather that the appearance of the shorter *sspB* transcript results from posttranscriptional processing of the longer form. First, despite the fact that *sspB* transcription in vivo is entirely dependent on $E\sigma^G$ (49), no $E\sigma^G$-type promoter was observed in the −35 and −10 regions preceding the putative downstream transcription initiation site (Fig. 1 and 5). Second, Northern (RNA) hybridization experiments have revealed that both the long and the short forms of full-length *sspB* mRNA can be detected in vivo and that the long *sspB* transcript appears to rapidly decay into the short form after treatment of cells with rifampin (P. Fajardo-Cavazos and P. Setlow, unpublished observations).

The *sspB*-type genes are unique among *ssp* genes in that they contain an approximately 100-nt untranslated region between their promoters and ribosome-binding sites (3, 14). The RNA in this untranslated region has the potential of forming a very stable secondary structure in both *B. subtilis*

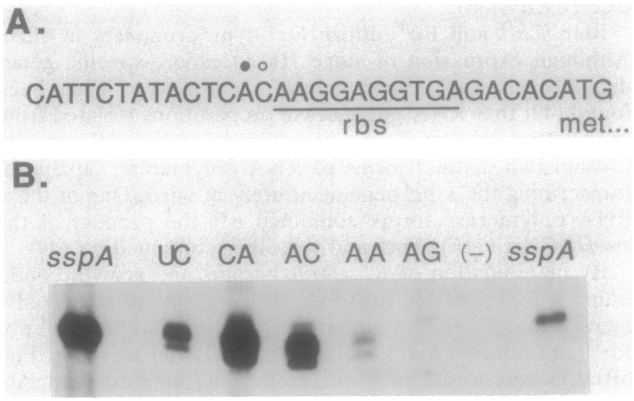

FIG. 4. Determination of the in vitro transcription start site of *sspA* by dinucleotide priming. (A) Nucleotide sequence (3) surrounding the *sspA* transcriptional start site as determined from the in vivo data presented in Fig. 2 and the in vitro data presented in Fig. 3. The assigned in vitro (●) and in vivo (defined by nuclease protection [○] or by primer extension [●]) transcriptional start sites of *sspA* are indicated. Underlined bases represent the *sspA* ribosome-binding site (rbs); met, *sspA* translation initiation codon. (B) Autoradiogram of in vitro transcription of plasmid pPS706 linearized with *Xba*I, using $E\sigma^G$ isolated from sporulating cells of strain PS614 and primed by the dinucleotide listed above each lane. Other lanes: *sspA*, runoff transcripts of the same template in the presence of excess ribonucleoside triphosphates; (−), dinucleotide priming control reaction performed without added dinucleotide.

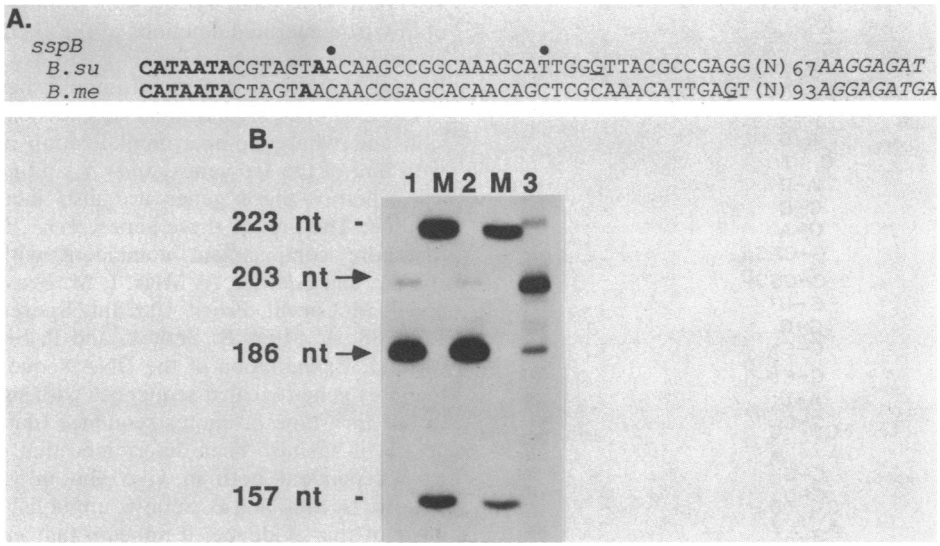

FIG. 5. Primer extension mapping of the 5′ ends of *sspB* mRNA produced in vivo or in vitro. (A) Sequences of the *B. subtilis* (*B. su*) *sspB* gene and the *B. megaterium* (*B. me*) SASP-C (*sspB* analog) gene aligned relative to their −10 regions (boldface; see Fig. 1). The single boldface adenine residues represent the in vitro transcription start sites as determined from the sizes of runoff transcripts and by dinucleotide priming (Fig. 1; Table 3). The underlined guanine residues represent the 5′ ends of in vivo mRNAs determined by nuclease protection. The 5′ ends of in vivo *B. subtilis sspB* mRNA, determined by primer extension analysis versus a DNA sequencing ladder, are indicated (●). (N), Sequences omitted to conserve space. Ribosome-binding sites are italicized. (B) Autoradiogram of primer extension products from various RNAs run on a 6% sequencing gel. Lanes: 1, RNA isolated from sporulating cells of strain PS435; 2, RNA isolated from vegetative cells of strain PS766 2 h after induction of σ^G synthesis with 1 mM IPTG; 3, RNA isolated after in vitro transcription of plasmid pPS771 with $E\sigma^G$. Markers (lanes M) consisted of ^{32}P-labeled *Hin*fI fragments of plasmid pBR322 end labeled with Klenow fragment.

(Fig. 7) and *B. megaterium sspB* (data not shown) mRNAs. The in vivo 5′ end of *sspB* mRNA, as determined by primer extension mapping, is located at the very base of this potential stem-loop structure (Fig. 7; data not shown). The relative abundances of the shorter versus the longer *ssp*B transcript in in vivo RNA preparations (Fig. 5) and the relatively short in vivo half-life of the long form (Fajardo-Cavazos and Setlow, unpublished data) suggest that in vivo the longer transcript rapidly becomes processed to the shorter form. Presumably, the 5′ untranslated region of the

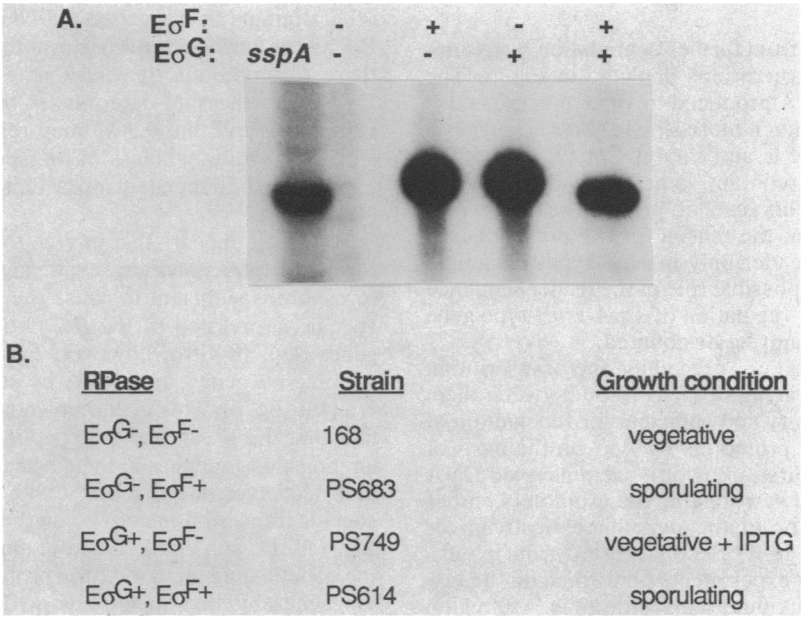

FIG. 6. In vitro transcription of *sspA* by $E\sigma^F$ and $E\sigma^G$. (A) Autoradiogram of in vitro runoff transcripts obtained by using the RNA polymerase preparation listed and plasmid pPS706 linearized with *Xba*I. Lane *sspA* is a runoff transcript of *Xba*I-cleaved pPS706 with RNA polymerase isolated from sporulating cells of strain PS614. (B) Origins of the RNA polymerase (RPase) preparations used in panel A (see Materials and Methods).

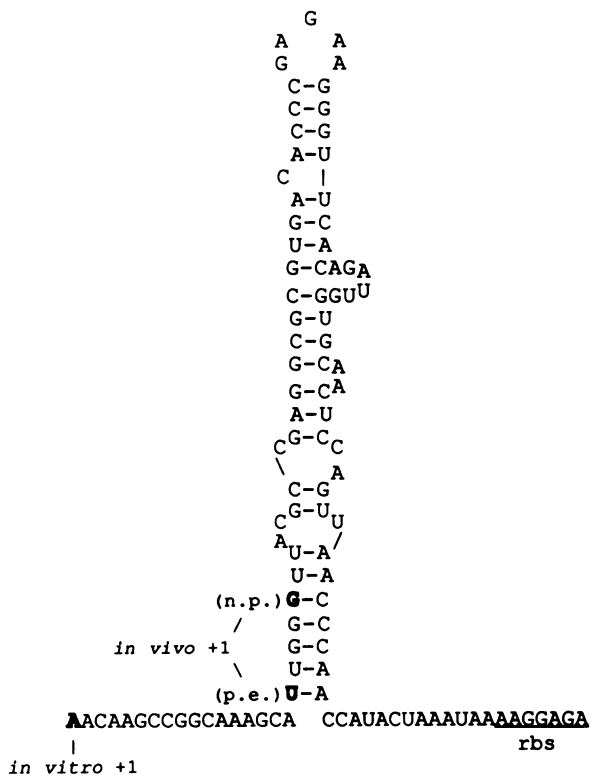

FIG. 7. Schematic diagram of potential secondary structure in the 5′ untranslated region in *B. subtilis sspB* mRNA. According to the base-pairing rules of Tinoco et al. (53), the potential free energy of this structure is −24.1 kcal (ca. 100.8 KJ)/mol. Shown in boldface are the 5′ ends of the message determined on the most predominant in vitro mRNA by primer extension, runoff transcription, and dinucleotide priming analysis and determined on in vivo mRNA by nuclease protection (n.p.) or by primer extension (p.e.) analysis. rbs, Ribosome-binding site.

shorter form is protected from further degradation by assuming a stable stem-loop structure as depicted in Fig. 7. The fact that even *sspB* mRNA produced in vitro undergoes this processing event, albeit much more slowly (Fig. 5), suggests that either the processing is autocatalytic or that our RNA polymerase preparations contain some residual processing activity. Neither possibility can at present be ruled out. Whatever the mechanism, the reason for the presence of a long untranslated RNA leader only in *sspB*-type transcripts remains a mystery, but a possible role of the leader sequence in the observed feedback regulation of *sspA-sspB*-type gene expression (32 to 34) cannot be discounted.

The −35 and −10 regions preceding the transcription initiation sites and the spacing of 17 to 18 bp between them appear to be both necessary and sufficient for recognition of the *ssp*, *spoVA*, and *gdh* promoters by $E\sigma^G$ on the basis of the following evidence. First, no additional conserved DNA sequences were observed surrounding the promoters, either in their spacer regions or in the immediate upstream or downstream sequences (Fig. 1). Second, results from in vitro site-directed mutagenesis experiments performed on the *gdh* promoter showed that only mutations within the −10 region or the −35 region abolished promoter function in vivo and in vitro, whereas mutations in the spacer region or deletions removing DNA upstream from −35 or downstream from −10 had essentially no effect on *gdh* promoter activity in vivo or in vitro (40a, 44). Results from preliminary analyses of in vitro-generated deletions of the *sspE* and *sspB* promoters support this conclusion (P. Fajardo-Cavazos, F. Tovar-Rojo, D. Sun, and P. Setlow, unpublished results).

The existence of additional genes whose timing of expression and whose compartmentalization appear to coincide with that of the *ssp* genes, *spoVA*, and *gdh* prompted us to ask whether these genes are also included in the $E\sigma^G$ regulon. The first of these genes, *gerA*, is expressed in the forespore compartment coincident with the *ssp* genes, *spoVA*, and *gdh* (59; A. Moir, I. M. Feavers, A. R. Zuberi, and J. McCarvill, Progr. 10th Int. Spores Conf., abstr. no. 12, 1988; A. Moir, B. Setlow, and P. Setlow, unpublished results). Examination of the DNA sequence (12) preceding the *gerA* gene revealed sequences with significant homology to the $E\sigma^G$-type promoter sequence (data not shown). Recently, it has also been determined that *gerA* expression is $E\sigma^G$ dependent both in vivo and in vitro (A. Moir, B. Setlow, D. Sun, and P. Setlow, unpublished results). On the basis of this evidence, it appears that *gerA* belongs to the $E\sigma^G$ regulon. The second gene examined is *sigG* itself, whose transcription in the late mode is apparently dependent on its own product, σ^G (25). Examination of the nucleotide sequence immediately preceding the 5′ end of in vivo *sigG* mRNA produced in the late mode (35) revealed a reasonably good $E\sigma^G$-type promoter (10 of 13 matches; data not shown). Moreover, purified $E\sigma^G$ was able to transcribe *sigG* from this promoter sequence in vitro (data not shown). The third of these genes tested, *0.3 kb*, is expressed in the forespore compartment but at a slightly later time than are *ssp* genes, *spoVA*, or *gdh* (42). Examination of the DNA sequence immediately preceding the 5′ end of in vivo *0.3 kb* mRNA (46) revealed a sequence with somewhat weak resemblance to an $E\sigma^G$-type promoter (7 of 13 matches; data not shown) but in which the spacing between the −10 and −35 regions was only 15 bp rather than the preferred 17 to 18 bp. Although *0.3 kb* transcription is dependent on $E\sigma^G$ in vivo (42), attempts to transcribe *0.3 kb* in vitro by using purified $E\sigma^G$ were unsuccessful (data not shown). On the basis of these observations, it seems reasonable to speculate that $E\sigma^G$ is not the sole determinant of forespore-specific gene expression and that *0.3 kb* may represent an example of at least one additional class of forespore-specific genes whose expression is dependent on a factor(s) in addition to $E\sigma^G$ (42).

Finally, it appears that in vivo the appearance of the $E\sigma^G$ form of RNA polymerase at stage III in the developing forespore is sufficient to cause the stage- and compartment-specific expression of the *B. subtilis ssp*, *gdh*, and *spoVA* genes (49). In vitro, however, $E\sigma^F$ is also capable of transcribing *ssp* genes, apparently by utilizing $E\sigma^G$-type promoters (49; Fig. 6). This is perhaps not surprising in light of the fact that the σ^F and σ^G polypeptides share nearly identical amino acid sequences in their −35 and −10 binding domains (25). However, in vitro $E\sigma^G$ exhibits a 20- to 50-fold-higher specific transcription activity on the *sspE* template than does $E\sigma^F$ (49; D. Sun and P. Setlow, unpublished results). Given the genetic evidence (49), it is probable that transcription of *ssp* genes by $E\sigma^F$ in vitro is artifactual; however, it may imply a close resemblance between $E\sigma^F$- and $E\sigma^G$-type promoter sequences (25). Support of this contention must necessarily await the isolation of truly $E\sigma^F$-dependent target genes and characterization of their promoter sequences.

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