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A Compartmentalized Regulator of Developmental Gene Expression in *Bacillus subtilis*

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We have identified a new *Bacillus subtilis* gene, *spoVT*, whose gene product is homologous to the transcriptional regulator AbrB and serves as a regulator of $E\sigma^G$-controlled gene expression. SpoVT acts both positively and negatively in controlling σ^G-dependent gene expression, providing an additional level of refinement to forespore gene regulation and feedback control of *spoIIIG* expression.

Spore formation in *Bacillus subtilis* depends upon an ordered program of gene expression to drive the physiological and morphological changes that result in the formation of a dormant endospore (8). Orchestrating this program of differential gene expression are five sporulation-specific sigma factors (σ^H, σ^F, σ^E, σ^G, and σ^K) that enable RNA polymerase to direct temporal and spatial control during development. About 1 h after the onset of sporulation, an asymmetric septum divides the cell into two compartments termed the prespore and mother cell. An hour later through a program of membrane invagination and engulfment, the smaller compartment becomes a distinct cell termed the forespore, within the mother cell. The forespore is destined to become the mature endospore. In the mother cell, RNA polymerase associated with σ^E ($E\sigma^E$) and σ^K ($E\sigma^K$) acts sequentially to drive the synthesis of gene products required in that chamber (44). Two DNA-binding proteins, SpoIIID and GerE, provide additional regulatory controls to the transcriptional machinery, and a number of σ^E- and σ^K-dependent genes are either negatively or positively regulated by the action of these proteins defining distinct temporal subclasses of mother cell gene expression (20, 55, 56). Additional control is provided by checkpoints that coordinate activation of σ^E and σ^K to the attainment of some feature of the developing cell (24).

In the forespore, at least 18 developmental genes are selectively expressed by the action of $E\sigma^F$ and, later, $E\sigma^G$ (8, 41). σ^F is synthesized (under σ^H control) prior to the division of the sporulating cell into two chambers (mother cell and prespore), yet is active only in the smaller prespore chamber (25). However, *spoIIIG*, the gene for σ^G, is not transcribed by σ^F until engulfment is complete (T_2), despite the fact that σ^F is functional prior to engulfment, and this transcription requires active σ^E in the mother cell (32). Moreover, σ^G is initially synthesized in an inactive form and only becomes active some 30 min after engulfment. Activation of σ^G appears to depend upon the products of a number of mother cell (σ^E-dependent) genes (41). Thus, activation of σ^G represents a checkpoint control (32, 41), requiring completion of an earlier event (prespore engulfment) to facilitate commencement of later events (σ^G-directed gene expression). As soon as the active form of σ^G becomes available, it directs transcription of its own gene, providing a rapid burst of σ^G-directed gene expression and displacing σ^F as the dominant forespore transcription factor (15, 32). In the transitional period between exclusive σ^F or σ^G control, both transcription factors are present within the forespore (σ^F active and σ^G inactive).

To date, only two genes, *spoIIR* and *csfE*, that are exclusively transcribed by $E\sigma^F$ have been identified (11, 16, 23), and a number of genes (*gpr*, *dacF*, and *bofC*) (12, 32, 52, 53) are transcribed first by $E\sigma^F$ and then by $E\sigma^G$. In contrast, $E\sigma^G$ directs the transcription of a large arsenal of genes whose products are required for the later stages of forespore development (e.g., six *ssp* genes encode the SASP proteins that both provide a pool of amino acids following spore germination and protect the forespore genome from UV irradiation [38]). It seems likely that other transcriptional regulators further modulate and refine forespore gene expression. This could provide fine-tuning of the synthesis and assembly of macromolecules during the 5-h period when σ^G is present in the forespore. To date, no such auxiliary transcriptional regulators acting in the forespore compartment have been reported.

In this work, we have identified a new σ^G-controlled gene, *spoVT*, which encodes a protein with substantial homology to the transition-state regulator, AbrB. We show here that the *spoVT* gene product either positively or negatively regulates the expression of σ^G-dependent genes.

MATERIALS AND METHODS

General methods. General *B. subtilis* methods were as described previously (13). Sporulation experiments were made with the resuspension technique of Sterlini and Mandelstam (30, 40). For gene expression studies, 1.0-ml samples of sporulating cells were harvested and assayed for β-galactosidase activity as described by Nicholson and Setlow (30) with the exception that lysozyme (20 μg/ml) was used for cell permeabilization. For weakly expressed genes (i.e., *gerA*, *gerB*, and *gerD*), the MUG (4-methylumbelliferyl-β-D-galactopyranoside) assay (30) was used with the fluorescent substrate MUG. Preparation of mRNA and primer extension analysis were carried out as described previously (5, 6). Spectinomycin was used in media at a final concentration of 100 μg/ml.

Strains. All strains used in this study were congenic derivatives of the prototrophic wild-type strain PY79. Reporter *lacZ* fusions used in this work are shown in Table 1.

Isolation of *spoVT*. A library (obtained from P. Zuber) of partially digested (*Sau*3AI) *B. subtilis* chromosomal DNA fused to the *lacZ* gene of *Escherichia coli* and contained in the specialized transducing phage SPβ was used to infect strain VA5 (*spoIIIGΔ1 spoIVCB23* pDG298). Strain VA5 contains both a deletion in the structural gene, *spoIIIG*, that encodes σ^G (*spoIIIGΔ1*) and an autonomously replicating plasmid, pDG298, which carries the *spoIIIG* gene under the control of the IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible *spac* promoter (51). To prevent the simultaneous induction of σ^K-controlled genes (since σ^G-directed gene expression is required for activation of σ^K [4]), strain VA5 also contained the *spoIVCB23* mutation (*spoIVCB* forms part of the composite gene, *sigK*, that encodes σ^K [43]). To identify σ^G-dependent loci, transductants were transferred in duplicate to sporulation agar plates containing the β-galactosidase substrate X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). After a

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TABLE 1. *lacZ* fusions used in this study

Locus	Fusion[a]	Reference
spoIIR	TRN[b]	16
spoIIIG	TXN	49
gprR	TXN[b]	50
bofC	TRN[b]	12
dacF	TRN[b]	37
gdh	TRN	35
sspA	TXN	26
sspB	TXN	26
sspD	TXN	26
sspE	TXN	26
gerA	TRN	9
gerB	TRN	3
gerD	TRN	17
spoIVB	TRN	4
spoVA	TRN	27
spoVT	TRN/TRN[b]/TXN	This work

[a] TRN, transcriptional; TXN, translational.
[b] These fusions are located at the *amyE* locus.

short period of growth at 37°C (~8 h), one set of plates was sprayed with a solution of IPTG (200 mM) and incubation continued overnight (NB: incorporation of IPTG in plates leads to overproduction of σ^G during vegetative cell growth and is toxic, inhibiting cell growth). The chromosomal inserts from 10 isolates which were Lac^+ only in the presence of inducer were transferred to the *B. subtilis* plasmid pTV8 (54). One clone, pVA43, carried an 1,880-bp *Sau*3AI insert which contained the 5′ end of a gene fused to *lacZ* and which corresponded to a previously sequenced open reading frame (ORF) on the *B. subtilis* chromosome. This gene was identified as part of the *Bacillus* genome sequencing project and designated *orf178* (GenBank accession no. D26185; nucleotides 95990 to 96523 [31]). We designate this gene henceforth as *spoVT*.

Construction of a *spoVT-lacZ* transcriptional fusion. To construct a *spoVT-lacZ* transcriptional fusion, we used PCR to amplify a 229-bp segment of *spoVT* (Fig. 1) and cloned it into the transcriptional fusion vector pDG268 (42) so that the 5′ region of *spoVT* was placed upstream of, and in the same direction as, the *lacZ* ORF contained in pDG268. pDG268 contains a promoterless *E. coli lacZ* gene and the ribosome binding site of the *B. subtilis spoVG* gene. In addition, flanking the *lacZ* gene, pDG268 carries the 5′ and 3′ ends of the *amyE* gene, allowing insertion of the *lacZ* fusion at the *amyE* locus by a double-crossover marker replacement. The resulting plasmid (pDG8) was linearized (digestion with *Bgl*II) and transformed into wild-type cells (PY79) with selection for chloramphenicol resistance (pDG268 contains the *cat* gene). One Cm^r $AmyE^-$ transformant (strain IB5) arising from integration of pDG8 at *amyE* was isolated, and chromosomal DNA prepared from this strain was used to introduce *spoVT-lacZ* into subsequent mutant strains. To introduce the *spoVT-lacZ* transcriptional fusion at the *spoVT* locus, we transferred the *Eco*RI-*Eco*RV fragment of pDG8 containing the *spoVT* promoter to the integrative plasmid pTK*lac* (18) cleaved with *Eco*RI and *Eco*RV. Following introduction of the resulting plasmid (pIB209) into wild-type cells (strain PY79) by DNA-mediated transformation, *spoVT-lacZ* would integrate at the *spoVT* locus by a single-crossover event.

Construction of a *spoVT-lacZ* translational fusion. We used PCR to amplify a 162-bp fragment of *spoVT*, containing the promoter, untranslated leader sequence, and the first eight codons of *spoVT*. The following oligonucleotides were used as primers: 5′-GGAATTCAATTTTGTTACTCTCTGGTG-3′ and 5′-CG GGATCCCGACGTACGATACCGG-3′. The PCR product was cut with *Eco*RI and *Bam*HI and cloned in the translational fusion vector pJF751 (10), in frame, with the *lacZ* coding region.

Construction of *spoVTΔ::spc*. Two oligonucleotide primers corresponding to sequences at the 5′ and 3′ ends of the putative *spoVT* locus were used to generate, by PCR, a 1,785-bp DNA product containing the entire *spoVT* gene (Fig. 1). The amplification product was cloned into plasmid pSGMU2 (pSGMU2 contains the *cat* gene, encoding Cm^r [33]) to produce pSG4. Next, pSG4 was digested with *Bsi*WI and *Eco*RI, which released a 373-bp fragment internal to the *spoVT* ORF (Fig. 1), and was replaced with a 1.1-kb spectinomycin cassette (from pJL74 [21]), producing plasmid pSG4-1. Finally, pSG4-1 was linearized (*Hin*dIII) and transformed into wild-type cells (PY79) with selection for Sp^r. A Sp^r transformant (strain IB1) was checked further. First, introduction of *spoVT*::*spc* by marker replacement was confirmed by screening Sp^r transformants for sensitivity to chloramphenicol (Cm^r would arise because of a Campbell-type integration of nonlinear DNA). Second, transformation linkage of the Sp^r marker to markers lying near the *spoVT* ORF was verified.

***trans* complementation.** Two primers, IB60 (5′-CCCAAGCTTAATTTTGTT ACTCTCTGGTG) and IB61 (5′-CGGGATCCAGGTTATTATACCACATC CC) were used to amplify by PCR a DNA product containing the entire putative *spoVT* transcription unit (Fig. 1), cloned into the vector pDG364 to create pIB79, and introduced into the *amyE* locus of strain IB1 (*spoVTΔ::spc*) as described previously (7).

Electron microscopic procedures. Sporulation of wild-type and mutant cells (25 ml) was induced by exhaustion in DSM medium (30). Samples were fixed directly in culture with a final volume of 1% (vol/vol) gluteraldehyde, after which they were harvested by filtration and taken up into agar blocks as described previously (14). Samples were dehydrated in ethanol at room temperature: 50%, 10 min; 70%, 10 min; 90%, 10 min; 100%, 2 times for 15 min. Infiltration of the samples was with LR White resin (hard grade; London Resin Co., Basingstoke, United Kingdom): (1:1 pure LR White, 100% ethanol, 15 min; pure LR White, 4 times for 30 min, and polymerization was by the cold chemical catalytic method at 0°C (29). Sections were cut on an LKB Ultramicrotome III and stained with aqueous 4% uranyl acetate for 5 min and Millonig's lead acetate (1:5 dilution) for 30 s. The sections were viewed in a Philips CM12 transmission electron microscope operating at 80 kV.

RESULTS

Identification of the σ^G-controlled gene *spoVT*. *spoVT* was identified as part of an effort to isolate additional σ^G-dependent genes. Using a strategy successfully employed to identify σ^E-dependent promoters (see reference 2 and Materials and Methods), we found one gene, now designated *spoVT* (based on its sporulation phenotype described below), which had been previously identified as a functional ORF (*orf178*) in the *Bacillus* genome sequencing project (31) and positioned at 4° on the chromosome. The nucleotide sequence of the *spoVT* locus predicted a monocistronic operon which could encode a 178-amino-acid polypeptide of 19.7 kDa. To verify that the *spoVT* locus was indeed monocistronic, we cloned a segment of DNA containing the entire putative *spoVT* transcription unit into the vector pDG364 (pIB79, shown in Fig. 1). This vector allows insertion of cloned DNA at the *amyE* locus. Integration of *spoVT*$^+$ at *amyE* in a strain carrying a Δ*spoVT*::*spc* insertion-deletion mutation at the *spoVT* locus (strain IB1, described below) gave cells that were phenotypically Spo^+, demonstrating in *trans* complementation. This result demonstrates that the *spoVT* mutation is not polar on any gene essential for sporulation.

The SpoVT polypeptide had substantial similarity to the *B. subtilis* transcriptional regulator AbrB (94 amino acids [34]) and its close homolog Abh (92 amino acids [19]). This homology was most striking at the N-terminal region (residues 1 to

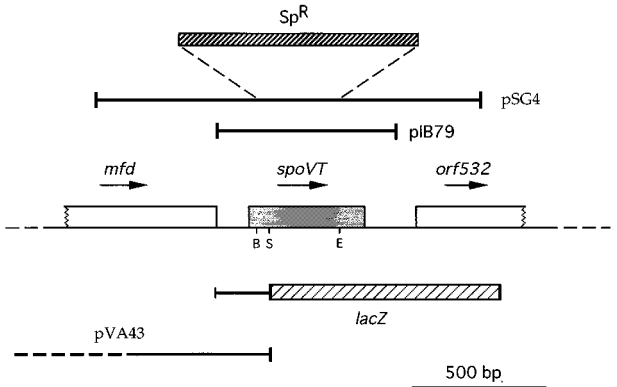

FIG. 1. Physical map of the *spoVT* locus. The *spoVT* ORF is boxed (dark shading), and the direction of transcription is indicated. Also shown are (i) the 3′ end of the *Sau*3AI fragment identified in clone pVA43, (ii) a schematic representation of the *spoVT-lacZ* transcriptional fusion showing the 229-bp segment of DNA cloned upstream of *lacZ* in the vector pDG268, (iii) the 1,785-bp fragment of DNA contained in pSG4 and used for construction of the *spoVTΔ::spc* mutant, and (iv) the cloned insert in pIB79 used for in *trans* complementation analysis. The two ORFs on either side of *spoVT*, *mfd* and *orf532*, are separate loci. Relevant restriction sites used in this study are shown: B, *Bsi*WI; S, *Sau*3AI; E, *Eco*RI.

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SpoVT   1   MKATGIVRRIDDLGRVVIPKEIRRTLRIREGDP  33
AbrB    1   MKSTGIVRKVDELGRVVIPIELRRTLGIAEKDA  33
Abh     1   MKSIGVVRKVDELGRIVMPIELRRALDIAIKDS  33

SpoVT   34  LEIFVDRDGEVILKKYSPISELGDFAKEYADAL  66
AbrB    34  LEIYVDDEKIILKKYKPNMTCQVTGEVSDDNLK  66
Abh     34  IEFFVDGDKIILKKYKPHGVCLMTGEITSENKE  66

SpoVT   67  YDSLGHSVLICDRDVYIAVSGSSKKDYLNKSIS  99
AbrB    67  LAGGKLVLSKEGAEQIISEIQNQLQNLK       94
Abh     67  YGNGKITLSPEGAQLLLEEIQAALKE         92

SpoVT   100 EMLERTMDQRSSVLESDAKSVQLVNGIDEDMNS 132

SpoVT   133 YTVGPIVANGDPIGAVVIFSKDQTMGEVEHKAV 165

SpoVT   166 ETAAGFLARQMEQ
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FIG. 2. Amino acid sequence similarity among SpoVT, AbrB, and Abh. Identical residues and conserved substitutions are boxed. The SpoVT sequence was identified in the *B. subtilis* genome sequencing project (31) (GenBank accession no. D26185). The AbrB sequence is from the work of Perego et al. (34), and the Abh sequence is from the work of Kobayashi et al. (19).

51) where there was almost 60% identity (Fig. 2). The well-characterized AbrB protein is involved in the regulation of gene expression at the transition from exponential growth phase to stationary phase and sporulation (34). The *abh* gene product has not been extensively studied but appears to play a regulatory role in the stationary phase of cell growth (45).

The *spoVT* gene product is required for the terminal stages of spore development. The Δ*spoVT*::*spc* mutant developed to morphological stage V, as judged by phase-contrast microscopy, resulting in the formation of refractile spores (although these were clearly abnormal compared with wild-type spores). Electron microscopic analysis showed that the cortical layers of mature *spoVT* spores were normal but that the spore coat was defective in structure (Fig. 3). The electron-dense outer coat layer, in particular, was less defined than in wild-type spores and appeared more diffuse, most noticeably at the two poles of the spore. In addition, we noticed that in many cells the outer spore coat layer was aberrantly assembled and either was not correctly attached to the inner coat layer or formed swirls of

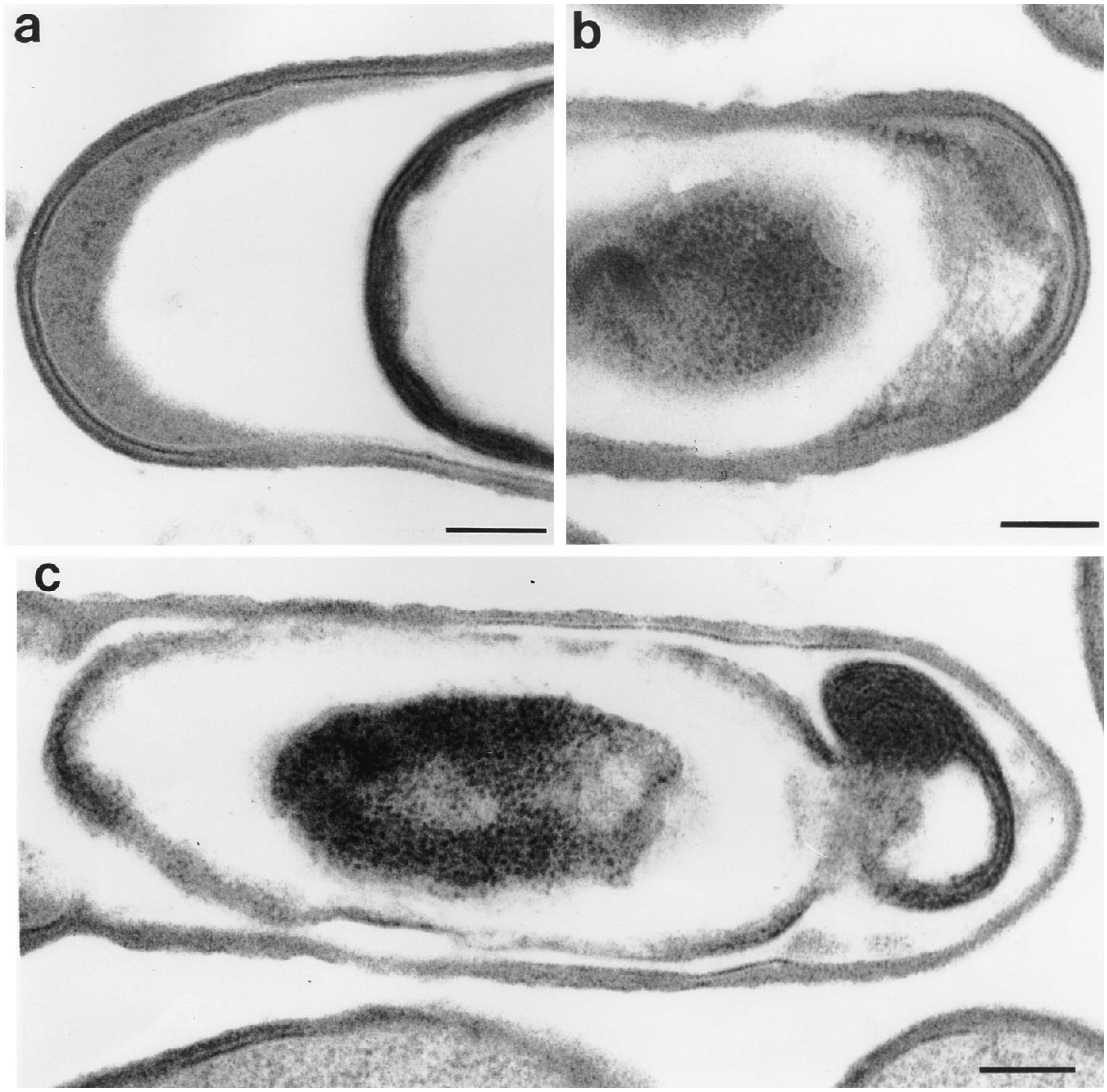

FIG. 3. Electron micrograph of a *spoVT* mutant sporangium. Comparison of the cortex and coat layers of *spo*$^+$ (a) (strain PY79) and *spoVT*Δ::*spc* (b) (strain IB1) mature spores (prepared at the 20th hour [T_{20}] following the onset of spore formation). Note the discrete appearance of the electron-dense outer coat layer in the wild type (a) and how the spore coat has misassembled in the mutant (b), usually observed at either of the poles of the developed spores. On occasion, this misassembly resulted in swirls of the proteinaceous coat material being formed at one of the spore poles of the mutant (c). Bar, 0.2 μm.

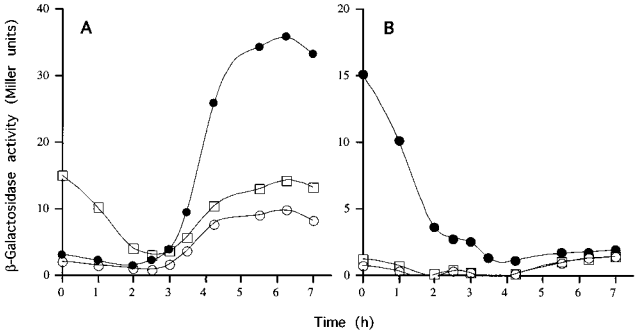

FIG. 4. Developmental expression of *spoVT*. (A) Shown is the pattern of β-galactosidase synthesis obtained in a wild-type strain containing a *spoVT-lacZ* transcriptional fusion integrated at the *amyE* locus (○), a *spoVT-lacZ* transcriptional fusion integrated at the *spoVT* locus (□), or a *spoVT-lacZ* translational fusion integrated at the *spoVT* locus (●). (B) Shown is the pattern of β-galactosidase synthesis obtained in a *spoIIIG* mutant strain containing a *spoVT-lacZ* transcriptional fusion at the *amyE* locus (□), a *spoVT-lacZ* transcriptional fusion at the *spoVT* locus (●), or a *spoVT-lacZ* translational fusion at the *spoVT* locus (○). Background levels of enzyme activity obtained in cells containing no fusion have been subtracted.

coat material in the mother cell chamber while still partially attached to the spore. Such coat defects have been reported in *spoIVA* and *spoVM* mutants and are indicative of the misassembly of the spore coat proteins onto the developing forespore (22, 36). *spoVT* mutant spores showed a measurable reduction, compared with wild-type spores, in their resistance to chloroform, heat, and lysozyme (2 to 30% of the total CFU per milliliter). We purified wild-type and mutant spores and measured their ability to reduce the colorless tetrazolium dye to red formazan in the presence of germinants (30). Our results showed that Δ*spoVT*::*spc* spores are germination defective, which can be attributed, in part, to the incorrect assembly and function of the spore coat.

The intact cortex, defective coat layers, and impaired germination properties allowed the classification of this mutant as being blocked at stage V.

Developmental expression of *spoVT*. Since *spoVT* was identified as a σ^G-dependent gene, we predicted that the regulation of *spoVT* expression during sporulation would be similar to that of other σ^G-controlled genes. However, it was conceivable that expression of this gene was controlled, in part, by σ^F-RNA polymerase ($E\sigma^F$). We constructed a *spoVT-lacZ* transcriptional fusion (Fig. 1), integrated it at the *amyE* locus of the wild-type strain PY79 and mutant strains blocked at different stages of development, and examined *spoVT* expression during sporulation. Our results (Fig. 4 and Table 2) showed, first, that expression in wild-type cells began 2.5 h after the initiation of sporulation and had a profile similar to those of genes controlled exclusively by σ^G. We saw no evidence of early expression, at a time (T_1) when σ^F would be active. Second, the requirements for *spoVT* expression were similar to those of other σ^G-dependent genes. Mutations in either *spoIIAC* or *spoIIIG*, the genes which encode the forespore transcription factors σ^F and σ^G, respectively, prevented expression of the *spoVT-lacZ* fusion. Similarly, mutations in mother-cell-expressed genes (*spoIIG* and *spoIID*) required for activation of σ^G in the forespore compartment (in the σ^G checkpoint [32, 41]) also impaired *spoVT-lacZ* expression. In addition, a mutation in the mother cell *spoIIID* gene caused a partial reduction (25 to 40%) in *spoVT-lacZ* expression. This partial dependence of σ^G-directed gene expression on the *spoIIID* gene product is not yet understood but is diagnostic of, and specific for, σ^G-dependent gene expression (4). A mutation in *spoIVCB*, a gene required for synthesis of σ^K in the mother cell, caused no impairment of *spoVT* expression.

TABLE 2. Genetic dependence of *spoVT* expression

Strain	Relevant allele[a]	Synthesis of β-galactosidase[b]
SC1159	*spoIIAC1*	−
SC137	*spoIIGB*::Tn*917ΩHU325*	−
SC138	*spoIID*::Tn*917ΩHU298*	−
SC500	*spoIIIGΔ1*	−
BK541	*spoIIIDΔ*::*erm*	(+)
BK556	*spoIVCB23*	+

[a] Isogenic strains containing the indicated allele were transformed with chromosomal DNA containing the *spoVT-lacZ* transcriptional fusion at the *amyE* locus (from strain IB5).

[b] *spoVT*-directed β-galactosidase synthesis in the above strains was compared with that in a congenic *spo*$^+$ strain (IB5) at the time of maximal enzyme activity (4 to 5 h after the initiation of sporulation). Activity was defined as follows: −, less than 15% of wild-type activity; (+), 50 to 75% of wild type; +, wild type. Sporulation experiments were repeated at least once.

Lastly, we asked whether, in vegetatively growing cells of a strain defective in σ^G synthesis (SC500 *spoIIIGΔ1*) and engineered to produce σ^F, we could induce expression of *spoVT*. A plasmid, pSDA4 (39), containing the structural gene for σ^F (*spoIIAC*) fused downstream from, and under the control of, the IPTG-inducible *spac* promoter failed to elicit expression of the *spoVT-lacZ* fusion during vegetative growth. In contrast, vegetative cells containing plasmid pDG298 (51) carrying *Pspac-spoIIIG* (encoding σ^G) efficiently expressed *spoVT-lacZ* following addition of IPTG (data not shown).

We determined the start point of *spoVT* transcription in vivo by primer extension analysis with end-labeled primers and mRNA isolated from vegetative cells of a strain engineered to produce σ^G by IPTG induction (with plasmid pDG298 [see above]). We identified an extension product present only in RNA prepared from IPTG-induced cells, the size of which suggested that *spoVT* transcription initiated at one of two A residues 82 to 83 bp upstream of the *spoVT* ORF (Fig. 5 and 6). Centered 9 and 36 bp upstream from the apparent *spoVT* transcription start site are sequences (Fig. 6) of high similarity to the −10 and −35 sequences, respectively, for σ^G-controlled promoters (28). We also identified the same apparent transcriptional start point in RNA prepared from sporulating cells (PY79 *spo*$^+$) extracted at the fifth hour (T_5) after the initiation of sporulation and the absence of any extension product in T_0 samples (results not shown). No extension product was found when we used RNA prepared from vegetative cells engineered to produce σ^F by IPTG induction, and we infer that *spoVT* transcription is controlled exclusively by $E\sigma^G$ (NB: by using the same RNA, we could identify the 5′ terminus for the $E\sigma^F$-σ^G-controlled gene *bofC* [12]).

On the *B. subtilis* chromosome, *spoVT* is located immediately downstream of the *mfd* gene, which encodes a transcription-repair coupling factor (1). There is no apparent transcriptional terminator following the *mfd* coding region, suggesting the possibility of read-through transcription of *spoVT* from the *mfd* gene. To address this, we integrated the *spoVT-lacZ* transcriptional fusion at the *spoVT* locus and analyzed its expression during vegetative growth and sporulation (Fig. 4). *spoVT* was efficiently expressed during vegetative growth; its expression gradually declined as the cells entered the sporulation pathway but recovered during a second phase of expression commencing 2.5 h after the onset of sporulation (corresponding to the beginning of σ^G-dependent expression [Fig. 4A]).

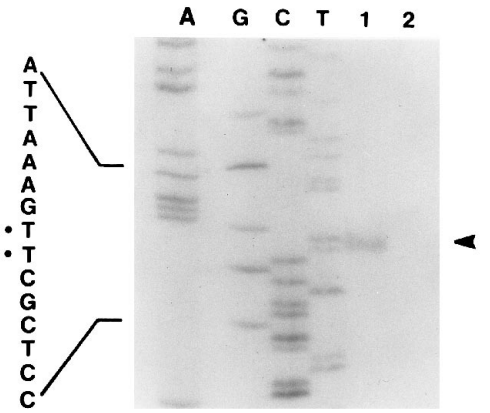

FIG. 5. Primer extension analysis of the 5′ terminus of *spoVT* mRNA. An oligonucleotide (Pr1) complementary to the 5′ end of the nontranscribed strand of the *spoVT* ORF (Fig. 6) was used to direct cDNA synthesis of total RNA purified from vegetatively growing cultures of *B. subtilis* engineered to produce σ^G. Lanes 1 and 2 show the extension products (arrowhead) produced from RNA purified from mid-log-phase cultures of a strain (SC500 *spoIIIGΔ1*) containing the plasmid pDG298, grown in Luria-Bertani medium in the presence (lane 1) or absence (lane 2) of IPTG (1 mM). pDG298 contains the *spoIIIG* gene (encoding σ^G) under the control of the IPTG-inducible *spac* promoter. The transcription start site corresponded to one of two A residues (dots at left) (see Fig. 6). To produce a sizing template, the same oligonucleotide was used in parallel to prime sequencing reactions (lanes A, G, C, and T) from a plasmid template containing the 5′ region of *spoVT*. In parallel experiments using a different oligonucleotide primer (Pr2), we obtained the same 5′ terminus for *spoVT* mRNA (not shown).

These results suggest that there are two phases of *spoVT* transcription, an early phase due to read-through transcription from the *mfd* gene and a late phase derived from the σ^G-dependent promoter. The strain containing the *spoVT-lacZ* fusion at the *spoVT* locus produced wild-type spores, even though in this case integration of the *spoVT* fusion plasmid separated the *spoVT* gene from the *mfd* promoter by 7 kb. Similarly, our complementation studies described above indicated that the *spoVT* transcriptional unit containing only the σ^G-dependent promoter of *spoVT* complements in *trans* the *spoVT* mutant phenotype, implying that the early phase of *spoVT* transcription derived from *mfd* is dispensable for sporulation at least under our laboratory conditions. Since *spoVT* might be involved in some other nondevelopmental function, we asked whether SpoVT was translated from the early transcript. We constructed a *spoVT-lacZ* translational fusion, integrated it at the *spoVT* locus, and analyzed expression during vegetative growth and sporulation. No significant β-galactosidase activity was observed during vegetative cell growth; the activity only commenced 2.5 h after the initiation of sporulation. This result indicates that only the transcript derived from the σ^G-dependent promoter is translated. The transcript derived by read-through from *mfd* is, for unknown reasons, not translated.

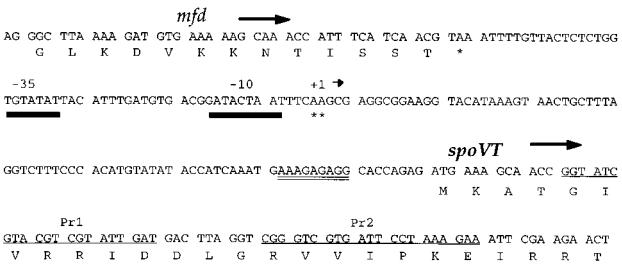

FIG. 6. 5′ region of *spoVT*. Shown is the nucleotide sequence of the 5′ region of the nontranscribed strand of *spoVT*. The start point (+1) and orientation of transcription is shown by an arrow and corresponded to one of two A residues. The −10 and −35 regions of the *spoVT* promoter are underlined, and the ribosome binding site is underlined twice. The Pr1 and Pr2 oligonucleotides used for primer extension analysis are also shown.

TABLE 3. Effect of the *spoVT* mutation on forespore-specific gene expression

Gene[a]	Transcription[b]	Effect of *spoVT* mutation[c]
spoIIR	$E\sigma^F$	No effect
gpr	$E\sigma^F/E\sigma^G$	++ (σ^G)
dacF	$E\sigma^F/E\sigma^G$	++ (σ^G)
bofC	$E\sigma^F/E\sigma^G$	++ (σ^G)
spoIIIG	$E\sigma^F/E\sigma^G$	++ (σ^G)
gdh	$E\sigma^G$	++
spoVT	$E\sigma^G$	++
sspE	$E\sigma^G$	++
gerA	$E\sigma^G$	++
gerB	$E\sigma^G$	++
sspA	$E\sigma^G$	−
sspB	$E\sigma^G$	−
sspD	$E\sigma^G$	−
spoIVB	$E\sigma^G$	−
gerD	$E\sigma^G$	−
spoVA	$E\sigma^G$	−−

[a] Forespore-expressed genes not tested were *csfE*, *sspC*, and *sspF*.
[b] Transcription of these genes was controlled by either RNA polymerase associated with σ^F, or with σ^F and σ^G, or by σ^G alone. In the case of *gerA*, *sspE*, and *spoIVB*, very low levels of σ^F-dependent transcription have been reported but it is unclear whether this σ^F control is of any functional significance (12, 50).
[c] *lacZ* fusions to the indicated genes were introduced into wild-type and *spoVT*Δ::*spc* cells, and sporulation-specific gene expression was measured. ++, overexpression in the *spoVT* mutant; −−, dramatic reduction of expression in the *spoVT* mutant; −, reduced rate of expression in the *spoVT* mutant.

We also examined expression of the *spoVT-lacZ* transcriptional and translational fusions in a *spoIIIG* mutant (Fig. 4B). The transcriptional fusion was expressed during vegetative growth and declined upon the onset of sporulation. However, the second, σ^G-dependent, phase of *spoVT* transcription did not occur in the *spoIIIG* mutant. No expression of the *spoVT-lacZ* translational fusion was observed in the *spoIIIG* mutant.

Effect of the *spoVT* mutation on forespore-specific gene expression. The striking homology between the SpoVT and AbrB polypeptides suggested that SpoVT might, like AbrB, function as a transcriptional regulator. Since expression of *spoVT* is under σ^G control, we predicted that SpoVT's action would be confined to σ^G-controlled genes and possibly also to σ^F-controlled genes (since active σ^F is also present within the forespore, albeit briefly). To this end, we introduced *lacZ* fusions of various forespore-expressed genes (Table 1) into the Δ*spoVT*::*spc* mutant. Sporulation was induced in these cells, and β-galactosidase activity was compared with that in the isogenic *spo*$^+$ parent strain containing the same fusion. Our results, summarized in Table 3 (and representative examples shown in Fig. 7), showed that (i) the *spoVT* mutation had both positive and negative regulatory effects on forespore gene expression and (ii) these effects were restricted to σ^G-directed gene expression.

Expression of the *spoIIIG-lacZ* fusion was substantially prolonged in the *spoVT* mutant, suggesting that SpoVT may normally shut off *spoIIIG* transcription (Fig. 7A). In the *spoVT* mutant, overexpression was first detectable about 1 h ($T_{3.5}$)

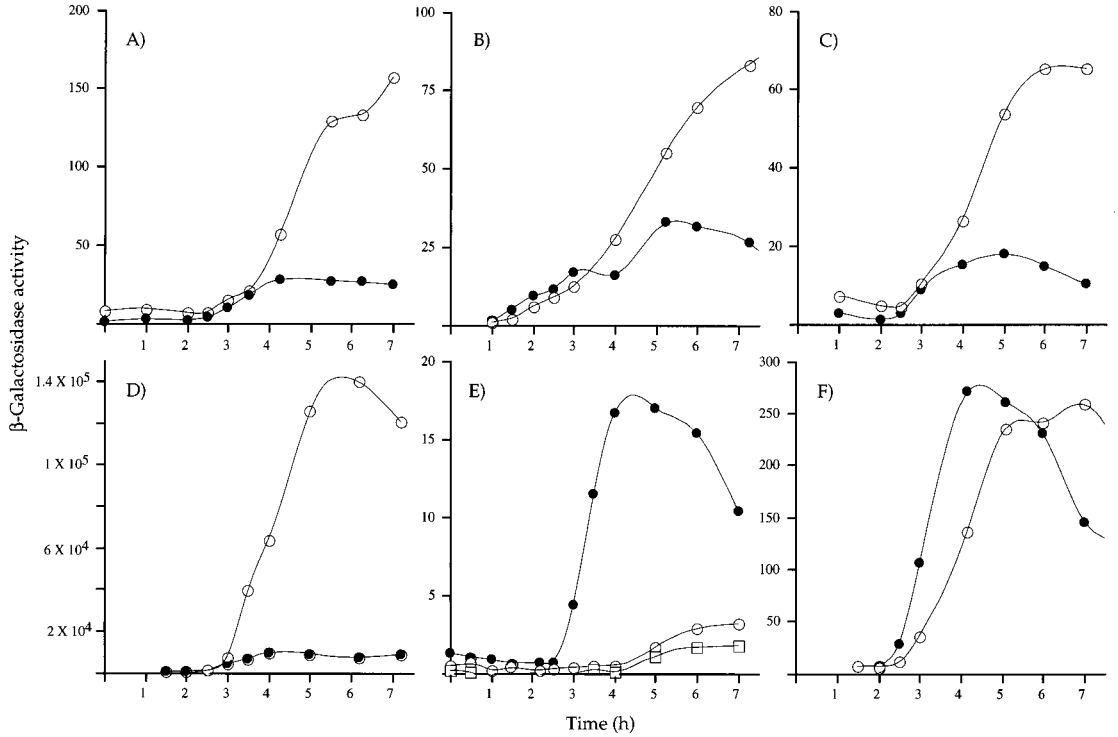

FIG. 7. Effect of the *spoVT* mutation on σ^G-controlled genes. The specific activity of β-galactosidase in sporulating cells containing a *lacZ* fusion to either *spoIIIG* (A), *gpr* (B), *spoVT* (C), *gerA* (D), *spoVA* (E), or *sspA* (F) was determined at the indicated times after the initiation of sporulation (T_0) in *spo*$^+$ cells (●; PY79), *spoVT*Δ::*spc* mutant cells (○; IB1), or *spoIIIG* mutant cells (□). NB: in the case of *gerA-lacZ* expression, we used MUG to analyze β-galactosidase activity and values are given in MUG units (30). Background levels of enzyme activity obtained in cells containing no fusion have been subtracted.

after the onset of *spoIIIG* transcription ($T_{2.5}$). We also observed substantially prolonged expression of other σ^G-dependent genes (*gpr*, *dacF*, *bofC*, *spoVT*, *gdh*, *sspE*, *gerA*, and *gerB* [Table 3]). Some of these genes were under dual σ^F-σ^G transcriptional control, but only the σ^G-dependent phase of expression was affected by the *spoVT* mutation (e.g., Fig. 7B shows *gpr* expression).

The expression of one σ^G-dependent gene, *spoVA*, was dramatically reduced in the *spoVT* mutant (Fig. 7E). The low level of *spoVA* expression seen in the absence of SpoVT was indistinguishable from that found in a *spoIIIG* mutant, suggesting that SpoVT and σ^G are both needed for maximal *spoVA* transcription. Five other σ^G-dependent genes (*sspA*, *sspB*, *sspD*, *spoIVB*, and *gerD*) were expressed in the *spoVT* mutant at the same levels as in wild-type cells, but the time of expression was delayed by about 2 h (e.g., *sspA* [Fig. 7F]).

DISCUSSION

Characterization of *spoVT* gene expression. There are two phases of *spoVT* transcription: (i) read-through transcription from the upstream *mfd* gene during vegetative growth and (ii) σ^G-dependent transcription from the *spoVT* promoter starting 2.5 h after the onset of stationary phase. Surprisingly, the *spoVT* ORF was not translated from the *mfd-spoVT* transcript but only from the σ^G-dependent transcript. This feature of *spoVT* expression is similar to the case of *spoIIIG*, in which read-through transcription from the upstream σ^A-dependent *spoIIG* gene leads to the accumulation of a *spoIIG-spoIIIG* transcript just after the onset of sporulation. Synthesis of σ^G from this bicistronic mRNA is thought to be prevented by a stem-loop structure which includes the *spoIIIG* ribosome binding site and a motif located between the *spoIIG* and *spoIIIG* ORFs. Translation of *spoIIIG* only follows synthesis of a σ^F-σ^G-specific *spoIIIG* mRNA transcript at hour 2 (41). Interestingly, just downstream of the *mfd* ORF lies a 6-bp stretch (CTCTCT), complementary to *spoVT*'s Shine-Dalgarno sequence, which could introduce the appropriate mRNA secondary structure to prevent translation of *spoVT*.

Although *spoVT* was identified as a σ^G-dependent gene, there was a possibility that *spoVT* is first transcribed by σ^F from the same promoter. In addition to *spoIIIG*, there are three other genes (*gpr*, *dacF*, and *bofC*) which are transcribed first by $E\sigma^F$ and then by $E\sigma^G$, producing a biphasic profile of gene expression (12, 52, 53). However, the profile of *spoVT* expression, its genetic requirements, and primer extension analysis provide compelling evidence that *spoVT* is not transcribed by σ^F.

SpoVT participates in the regulation of σ^G-dependent gene expression. The significant homology between SpoVT and AbrB suggests that SpoVT might be a transcriptional regulator of forespore-specific genes. We found, in fact, that SpoVT was specifically implicated in expression of σ^G-dependent genes. Expression of all σ^G-dependent genes tested was affected in the *spoVT* mutant, and on the basis of these effects, three distinct groups of SpoVT-controlled genes could be identified: class I, genes whose expression was elevated in the *spoVT* mutant (i.e., *spoIIIG*, *gpr*, *dacF*, *bofC*, *gdh*, *sspE*, *gerA*, *gerB*, and *spoVT* itself); class II, consisting of one gene, *spoVA*, whose expression is almost entirely abolished in the *spoVT* mutant; and class III, genes which have a reduced rate of expression in the *spoVT* mutant (i.e., *sspA*, *sspB*, *sspD*, *spoIVB*, and *gerD*).

Two explanations could account for the elevated levels of gene expression found in the absence of SpoVT. First, SpoVT

might negatively regulate *spoIIIG*. In this case, in the absence of SpoVT, overexpression of *spoIIIG* would lead to increased levels of σ^G and increased levels of σ^G-dependent expression. Alternatively, SpoVT might negatively regulate directly a subset of σ^G-controlled genes including *spoIIIG*. By negatively regulating σ^G-dependent expression, SpoVT may provide a mechanism of feedback control that is important for forespore development.

SpoVT also acts directly or indirectly as a positive regulator of *spoVA* expression. In other work, it has been shown that an unidentified *B. subtilis* protein can bind to the upstream region of the *spoVA* gene and is required for efficient *spoVA* expression (27). This protein activity was found in sporulating cells 1 h after the initiation of sporulation and was present in all known stage 0 mutants and also in a *spoIIIG* mutant. This protein cannot be SpoVT since it is present and functional in a *spoIIIG* mutant. The mechanism by which SpoVT stimulates *spoVA* transcription remains mysterious.

Lastly, expression of six σ^G-dependent genes (class III genes) was apparently enhanced by the presence of SpoVT. In the *spoVT* mutant, the accumulation of β-galactosidase was slower or delayed. This effect is more striking taking into account the elevated levels of σ^G found in the *spoVT* mutant and may suggest that this positive requirement for SpoVT is different from that for *spoVA* expression.

Aberrant regulation of forespore gene expression produces a defective spore. The *spoVT* mutant showed a number of spore defects, including reduced resistance properties, misassembled spore coats, and a poor germination response. The germination response is presumably linked to the fidelity to the spore coat layers. The coat is synthesized in the mother cell chamber from a number of polypeptides which undergo their own program of hierarchical synthesis and assembly driven by the σ^E- and σ^K-directed pathway of mother cell gene expression. Although SpoVT controls forespore events, it is clearly also required for mother cell events. In the *spoVT* mutant, the abnormal expression of *spoIVB* may interfere with the intercompartmental signal transduction pathway (in which SpoIVB is the signal [4]) that activates σ^K in the mother cell and promotes the ensuing program of spore coat protein synthesis. This possibility is currently being investigated in our laboratory.

spoVT is the first described gene coding for an auxiliary regulator of forespore-specific gene expression. In contrast, two DNA-binding proteins, SpoIIID and GerE, optimize expression of σ^E- and σ^K-dependent genes, respectively, and function both positively and negatively in regulating gene expression in the mother cell (20, 55, 56). An intriguing feature of SpoVT is its similarity to AbrB. The AbrB protein regulates a wide variety of stationary-phase responses, acting both as a positive and as a negative regulator, and plays an important role in preventing entry into the developmental life cycle (46). AbrB is a DNA-binding protein, but it does not contain any of the classical DNA-binding motifs. It is active as a hexamer of identical subunits and must assume this form prior to binding DNA (47). No consensus sequence can be drawn for AbrB binding sites; instead, the protein is thought to recognize a specific three-dimensional DNA structure associated with high local A+T content (48). Genes regulated by SpoVT also have no obvious common sequence, but it remains to be shown where within these genes SpoVT might bind.

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