Analysis of the Upstream Activating Sequence and Site of Carbon and Nitrogen Source Repression in the Promoter of an Early-Induced Sporulation Gene of *Bacillus subtilis*

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The transcription from the *spoVG* promoter of *Bacillus subtilis* is induced at the start of the stationary phase of growth and is dependent on the expression of the *spo0A*, *spo0B*, and *spo0H* genes. It is repressed in cells grown in the presence of excess glucose and glutamine and is under the negative control of the *abrB* gene. The *spo0A* and *spo0B* gene products function to suppress the negative control exerted by *abrB*. Transcription initiation requires the form of RNA polymerase holoenzyme that contains the *spo0H* gene product, σ^H . Optimal transcription also requires an upstream A-T-rich region termed the upstream activating sequence (UAS). The mechanism of UAS function was examined through mutational analysis of the *spoVG* promoter region. Deletion of the UAS or positioning the UAS one half turn or one full turn of the DNA helix upstream of its location in wild-type *spoVG* resulted in a severe reduction in promoter activity. Deletion of most of the UAS abolished the *abrB*-dependent repression of *spoVG* transcription. Higher activity was observed when the UAS was inserted 10 bp (one turn of the helix) upstream than when the sequence was repositioned either 5 or 13 bp upstream. Sequences upstream of the UAS were found not to be involved with the position-dependent function of the UAS. Positioning the UAS 42 or 116 bp upstream eliminated the stimulatory effect of the sequence on *spoVG* transcription. These data indicate that the UAS functions effectively when it is in close proximity to the -35 region. In vitro transcription analysis indicated that the deletion and insertion mutations affecting the UAS impair RNA polymerase-*spoVG* promoter interaction. Deletion of the UAS showed that the negative effect of exogenous glucose and glutamine is not dependent on the UAS but is exerted at a site within or near the -35 and -10 regions.

Endospore formation in *Bacillus subtilis* is a developmental process triggered by nutritional deprivation. It can be divided into seven well-defined stages, beginning with the initiation of sporulation (stage 0) and ending with mother cell lysis (stage VII) and liberation of the mature spore (24). Initiation of sporulation requires the products of at least seven *spo0* genes (*spo0A*, *spo0B*, *spo0E*, *spo0F*, *spo0H*, *spo0J*, and *spo0K*) to begin the cascade of events leading to the expression of numerous sporulation-associated genes (13, 24). A mutation in any one of the *spo0* genes blocks the sporulation process before any morphological changes within the cell are observed. The *spoVG* gene has served as a system for examining the activation of gene transcription at the onset of sporulation because it is under *spo0* control and is induced as cells enter the stationary phase of growth in sporulation medium (23, 33). Furthermore, transcription of *spoVG* is repressed when cells are grown in a medium containing a high concentration of glucose and glutamine, preferred carbon and nitrogen sources which inhibit sporulation when present in excess (24, 27).

Transcription of *spoVG* is dependent upon the products of *spo0A*, *spo0B*, and *spo0H* (23, 33). The defect in *spoVG* promoter utilization caused by mutations in *spo0A* or *spo0B*, but not *spo0H*, is overcome by a mutation in *abrB* (34). The product of the *abrB* gene is a DNA-binding protein which negatively regulates the transcription of *spoVG* and a number of other late growth-induced genes (26, 28). The *spo0H* gene encodes an RNA polymerase sigma factor, σ^H (5, 7),

which is necessary for the expression of many early-induced sporulation genes and other genes which are activated when cells enter stationary phase (2, 5, 7). In vitro studies had identified two transcriptional start sites utilized by two different RNA polymerase holoenzymes, $E\sigma^B$ and $E\sigma^C$ (14). However, in vivo experiments have shown that *spoVG* transcription initiation is catalyzed by the $E\sigma^H$ form of RNA polymerase bearing the sigma factor σ^H , the product of the *spo0H* gene (7, 33, 34). Since *spoVG* promoter mutations that disrupt $E\sigma^H$ -DNA interaction eliminate *spoVG* transcriptional activity (6, 32), it is likely that $E\sigma^H$ is primarily responsible for the utilization of the *spoVG* promoter. An A+T-rich upstream activation sequence (UAS), positioned 4 bp away from the *spoVG* -35 promoter element, is necessary for optimal transcription from the *spoVG* promoter (3) as well as for the interaction of the AbrB protein with the *spoVG* promoter in vitro (9, 26).

In the study reported herein, the *spoVG* promoter-regulatory region was subjected to in vitro mutagenesis in an effort to elucidate the mechanism by which the UAS functions. Our results indicate that UAS function is dependent upon its linear distance from the *spoVG* -35 promoter element. Also presented are data from experiments designed to examine the carbon and nitrogen source-dependent control of *spoVG* transcription. The repression exerted on *spoVG* requires the presence both of excess glucose and of a preferred nitrogen source for *B. subtilis*, glutamine. *spoVG* promoter mutants were utilized to identify the region necessary for glucose- and glutamine-dependent repression (Ggr). We show that sequences downstream of -46 with respect to the start site

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype	Source or reference
<i>B. subtilis</i>		
JH642	<i>trpC2 pheA1</i>	J. Hoch
ZB307A	SP β c2 Δ 2::Tn917::pSK10 Δ 6	34
LAB569	<i>trpC2 pheA1</i> SP β c2 Δ 2::Tn917::spoVG-lacZ	This study
LAB570	<i>trpC2 pheA1 spo0A12</i> SP β c2 Δ 2::Tn917::spoVG-lacZ	This study
LAB571	<i>trpC2 pheA1</i> SP β c2 Δ 2::Tn917::spoVGD-lacZ	This study
LAB572	<i>trpC2 pheA1 spo0A12</i> SP β c2 Δ 2::Tn917::spoVGD-lacZ	This study
LAB573	<i>trpC2 pheA1</i> SP β c2 Δ 2::Tn917::spoVG1B-lacZ	This study
LAB574	<i>trpC2 pheA1 spo0A12</i> SP β c2 Δ 2::Tn917::spoVG1B-lacZ	This study
LAB575	<i>trpC2 pheA1</i> SP β c2 Δ 2::Tn917::spoVG210-lacZ	This study
LAB576	<i>trpC2 pheA1 spo0A12</i> SP β c2 Δ 2::Tn917::spoVG210-lacZ	This study
LAB577	<i>trpC2 pheA1</i> SP β c2 Δ 2::Tn917::spoVG Δ 1B-lacZ	This study
LAB578	<i>trpC2 pheA1</i> SP β c2 Δ 2::Tn917::spoVG Δ 210-lacZ	This study
LAB579	<i>trpC2 pheA1</i> SP β c2 Δ 2::Tn917::spoVG213-lacZ	This study
LAB580	<i>trpC2 pheA1</i> SP β c2 Δ 2::Tn917::spoVG2D-lacZ	This study
LAB581	<i>trpC2 pheA1</i> SP β c2 Δ 2::Tn917::spoVG105-lacZ	This study
LAB582	<i>trpC2 pheA1</i> SP β c2 Δ 2::Tn917::spoVGL1-lacZ	This study
LAB583	<i>trpC2 pheA1</i> SP β c2 Δ 2::Tn917::spoVGL4-lacZ	This study
LAB585	<i>trpC2 pheA1 spo0A12 abrB703</i> SP β c2 Δ 2::Tn917::spoVG-lacZ	This study
LAB691	<i>trpC2 pheA1</i> SP β c2 Δ 2::Tn917::rpsD-lacZ (pTMH115)	This study (11)
<i>E. coli</i>		
AG1574	<i>araD139</i> Δ (<i>ara leu</i>)7697 Δ <i>lacX74 galU,K hsr m⁺ strA recA56 srl</i>	A. Grossman
NK7085	Δ (<i>lac pro</i>) NalA ^r /F' <i>lacZYA536 proA⁺ proB⁺ mutS104::Tn5</i>	18
XL1-blue	<i>recA1 endA1 gyrA96 thi hsdR17</i> (rk ⁻ mk ⁻) <i>supE44 relA1 lac</i> [F' <i>proAB lacI^q ZΔM15 Tn10</i> (Tet ^r)]	Stratagene
Plasmids		
pDF100	Ap ^r Cm ^r <i>spoVGD-lacZ</i>	This study
pDF101	Ap ^r Cm ^r <i>spoVG1B-lacZ</i>	This study
pDF102	Ap ^r Cm ^r <i>spoVG210-lacZ</i>	This study
pDF103	Ap ^r Cm ^r <i>spoVGΔ1B-lacZ</i>	This study
pDF104	Ap ^r Cm ^r <i>spoVGΔ210-lacZ</i>	This study
pDF105	Ap ^r Cm ^r <i>spoVG213-lacZ</i>	This study
pDF106	Ap ^r Cm ^r <i>spoVG2D-lacZ</i>	This study
pDF107	Ap ^r Cm ^r <i>spoVG105-lacZ</i>	This study
pDF108	Ap ^r Cm ^r <i>spoVGL1-lacZ</i>	This study
pDF109	Ap ^r Cm ^r <i>spoVGL4-lacZ</i>	This study
pZ Δ 327	Ap ^r Cm ^r <i>spoVG327-lacZ</i>	31
pZL207	Ap ^r Cm ^r <i>spoVG-lacZ</i>	33

of transcription are required and that Ggr regulation is not exerted at the posttranscriptional level.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study are listed in Table 1. All of the *B. subtilis* strains are derived from JH642 (obtained from J. Hoch).

Plasmids. The plasmids used in this study were derivatives of pZL207 (33) or pZ Δ 327 (31). To construct plasmid pDF100, containing the deletion mutation *spoVGD*, pZL207, a plasmid containing the *spoVG* promoter, was digested with the restriction enzymes *Dra*I and *Sal*I (Fig. 1) and the *Dra*I-*Sal*I restriction fragment, containing the -35 and -10 promoter elements, was isolated. The plasmid pZ Δ 328, a derivative of pZ Δ 327, was first cleaved with *Hind*III and treated with T7 DNA polymerase (U.S. Biochemical Corp.) and deoxynucleotides to create a blunt end and then cleaved with *Sal*I. The linearized plasmid was combined with the *Dra*I-*Sal*I restriction fragment in a ligation reaction. Plasmids pDF101, pDF102, pDF106, and pDF107, bearing the mutant promoters *spoVG1B*, *spoVG210*, *spoVG2D*, and *spoVG105*, respectively, were constructed by subcloning *Hind*III-*Sal*I restriction fragments, containing each mutant

promoter, into pZ Δ 329 (described below). *spoVG Δ 1B* and *spoVG Δ 210* were constructed by removing DNA in plasmids pDF101 and pDF102 between the *Hind*III and *Bam*HI sites, giving rise to plasmids pDF103 and pDF104, respectively. To construct pDF105, bearing the *spoVG213* mutant promoter, pDF102 was digested with *Bam*HI, filled in by T7 DNA polymerase (U.S. Biochemical Corp.) treatment, and

FIG. 1. Nucleotide sequence of the *spoVG* promoter region. The 159-bp *Hind*III-*Sal*I fragment containing the *spoVG* promoter is fused with the *lacZ* open reading frame of the *lacZ* gene at the *Sal*I site. Positions -46 and -11 are indicated to show the sequences necessary for Ggr regulation of *spoVG* transcription. The letters within the box indicate the nucleotide positions which are essential for σ^H -dependent transcription. The ribosome binding site (rbs), the fMet initiation codon, and the UAS (bold underlining) are indicated. The start site of σ^H -dependent transcription (+1) is indicated by the arrow.

ligated together. The final insertion should have been 4 bp, but nucleotide sequence analysis revealed that the actual insertion was only 3 bp. Restriction fragments of phage lambda DNA in the size range of approximately 30 bp was inserted into the *Bam*HI site of pDF102, creating pDF108 and pDF109.

Oligonucleotide-directed mutagenesis. Single-stranded template DNA from a recombinant M13mp9 phage (propagated in *Escherichia coli* XL1-Blue; Stratagene) bearing a 159-bp *Hind*III-*Sal*I restriction fragment containing the *spoVG* promoter region was subjected to oligonucleotide-directed mutagenesis. The protocol described previously (32) was followed in this study, with the exception that T7 DNA polymerase instead of the Klenow fragment was used to fill in the gapped heteroduplex. The gapped heteroduplex was created by annealing denatured *Hind*III-*Sal*I-digested M13mp9* (32) replicative-form DNA and the mismatch oligonucleotide with the single-stranded M13mp9 template containing the *spoVG* promoter. *E. coli* NK7085 (18) was transfected with the sealed heteroduplex. The presence of the desired mutations was confirmed by nucleotide sequence analysis.

β -Galactosidase assays. *spoVG-lacZ*-directed β -galactosidase activity was monitored in cells grown in Difco sporulation (DS) medium. Samples (1 ml) were taken at 0.5-h intervals at the indicated times and were assayed for β -galactosidase specific activity as previously described (21, 33).

In vitro transcription assays. Transcription reactions were assembled as described previously (5). Plasmids containing the wild-type and mutant *spoVG* promoters were used as DNA templates in the reactions. The plasmid pZSP6-189 (34), containing the wild-type promoter, was digested with *Eco*RI to give a runoff RNA 120 bases long and was included in each reaction as an internal control. pZL207 (*P_{spoVG}*), pDF100 (*P_{spoVGD}*), pDF101 (*P_{spoVG1B}*), and pDF102 (*P_{spoVG210}*) were digested with *Sal*I to give runoff transcripts of 62 bases. Each of the test plasmids was present in the reactions in a twofold molar excess over the internal control plasmid. $E\sigma^H$ RNA polymerase (obtained from C. Moran) and 1 μ g of template DNA were combined in a reaction mixture. The mixture was incubated at 37°C for 10 min to allow for the prebinding of RNA polymerase to the promoters. ATP, CTP, and UTP were added to 0.5 mM and [α -³²P]GTP was added to 0.5 μ M (10 μ Ci), and incubation continued at 37°C. After 1 min, 6 μ g of heparin was added to prevent reinitiation. Following another 10 min at 37°C, unlabeled GTP was added to 0.5 mM and incubation was allowed to continue for an additional 5 min. The reactions were terminated by the addition of 2 volumes of ethanol and 5 mM ammonium acetate, after which they were placed at -80°C for 20 min. Precipitated RNA was collected by centrifugation, and the pellets were resuspended in sample buffer (10 M urea, 0.02% bromophenol blue, 0.02% xylene cyanol) and electrophoresed in 8% (wt/vol) polyacrylamide gels containing 7 M urea.

RESULTS

The importance of the UAS in the activation of *spoVG* transcription has been clearly demonstrated (3, 31), but the mechanism by which it functions has yet to be determined. In an effort to understand this mechanism, the *spoVG* promoter-regulatory region was subjected to mutational analysis. Derivatives of the *spoVG* promoter bearing deletions and insertions were constructed in vitro. The mutant promoters were propagated in *E. coli* on plasmids in which

FIG. 2. Nucleotide sequence of DNA upstream of the -35 region of the mutant derivatives of the *spoVG* promoter. The UAS sequence is indicated by bold underlining. The -35 region is underlined with a dotted line. Pertinent restriction sites are indicated. Bracketed restriction enzyme designations indicate the positions which lost restriction sites during construction. The boxed sequence indicates which nucleotides were deleted in the construction of *spoVG2D*.

the 5' end of the *spoVG* coding sequence and the *lacZ* coding sequence are joined in a translational fusion (33). The plasmids were then introduced by transformation into ZB307A, a strain of *B. subtilis* in which all of the plasmids recombined into the chromosome by marker replacement recombination with the SP β prophage derivative SP β c2del2::Tn917::pSK10 Δ 6 (34). Lysates of SP β phage containing each *spoVG-lacZ* fusion were generated to facilitate the transfer of the translational fusions by specialized transduction into various mutant strains of *B. subtilis*.

Deletion of 20 nucleotides of the UAS reduces promoter activity and affects regulation of *spoVG*. One promoter mutant, designated *spoVGD*, was constructed by removing all of the DNA between the *Dra*I site (-46) within the UAS and a unique *Hind*III site located at -95 (Fig. 2). The deletion resulted in the removal of all but 6 bp of the UAS. Figure 3 shows the pattern of β -galactosidase expression in strains bearing a *spoVGD-lacZ* fusion. In a Spo⁺ background, β -galactosidase activity was induced at the end of exponential growth (a point called T₀) in a fashion similar to that observed with a wild-type *spoVG-lacZ* fusion. However, the maximal level of activity from the mutant promoter (at T₂) was approximately 18-fold less than that of the wild-type *spoVG* promoter (Table 2). Expression of *spoVGD-lacZ* in a *spo0A* background was identical to expression in a Spo⁺ background (Fig. 3), in direct contrast with *spoVG-lacZ*, which is repressed in a *spo0A* strain (33). Since a *spo0A* mutation does not affect *spoVGD-lacZ* expression, we postulate that AbrB can no longer efficiently bind to this promoter and repress transcription. This is in keeping with previous studies which showed that the UAS was required for the efficient binding of the AbrB protein (26).

***spoVG-lacZ* expression is reduced when the position of the UAS relative to the -35 promoter element is altered.** A 159-bp *Hind*III-*Sal*I restriction fragment containing the entire *spoVG* promoter region and the 5' coding sequence was subjected to oligonucleotide-directed site-specific mutagenesis in order to alter the position of upstream DNA sequences relative to the -35 region of the promoter. Insertion mutations were made between the -35 region and the UAS

FIG. 3. Effects of *spoVGΔ1B*, *spoVGΔ210*, and *spoVG210* promoter mutations on *spoVG-lacZ* expression in *Spo*⁺ and *spo0A* strains. β-Galactosidase specific activity was measured in samples collected at 0.5-h intervals from cultures of cells grown in liquid DS medium. (A) ●, *Spo*⁺ *spoVGΔ1B-lacZ*; ○, *spo0A12 spoVGΔ1B-lacZ*. (B) ■, *Spo*⁺ *spoVG1B-lacZ*; □, *spo0A12 spoVG1B-lacZ*. (C) ▲, *Spo*⁺ *spoVG210-lacZ*; △, *spo0A12 spoVG210-lacZ*.

within a region in which base substitutions have little, if any, effect on the transcription of *spoVG* (32, 35). Insertions which moved the UAS 5 bp (approximately one half turn of the DNA helix) or 10 bp (approximately one full turn of the DNA helix) further upstream resulted in two mutant derivatives of the *spoVG* promoter that were designated *spoVG1B* and *spoVG210*, respectively (Fig. 2). In a *Spo*⁺ background, *spoVG1B-lacZ* expression is induced at *T*₀ (Fig. 3), but the level of β-galactosidase activity is approximately 10-fold less than that of the wild type at maximum induction at *T*₂ (Table 2). *spoVG210-lacZ* expression in a *Spo*⁺ background is also induced at *T*₀ and peaks at around *T*₂ (Fig. 3), but the amount of activity at peak induction is consistently higher than that of *spoVG1B-lacZ* (Fig. 3 and Table 2). In a *spo0A* background, the expression of both mutant promoters is reduced. However, the effect of a *spo0A* mutation on *spoVG1B* and

TABLE 2. Expression of *spoVG-lacZ* and mutant derivatives

Promoter	% Wild-type β-galactosidase sp act ^a	No. of times assay performed
<i>spoVG</i>	100	
<i>spoVGΔ1B</i>	5.6 ± 0.05	2
<i>spoVGΔ210</i>	9.2 ± 0.3	2
<i>spoVG1B</i>	3.8 ± 0.1	2
<i>spoVG210</i>	14.9 ± 1.5	4
<i>spoVGΔ105</i>	1.0 ± 0.6	2
<i>spoVGΔ13</i>	8.8 ± 0.5	3
<i>spoVG105</i>	88.7 ± 1.3	2
<i>spoVG2D</i>	103 ± 31	2
<i>spoVGL1</i>	2.1 ± 0.45	3
<i>spoVGL4</i>	0.7 ± 0.17	2

^a Percent wild-type activity was determined for samples collected 2 h after the end of exponential growth, when peak levels of activity are observed.

spoVG210 expression is less severe than the previously reported effect on expression caused by the wild-type *spoVG* promoter (33), suggesting that the repression caused by *ArbB* is partially relieved when the UAS is positioned further upstream. However, *AbrB* apparently can still bind to both mutant promoters and interfere with transcription, since the reduction of β-galactosidase activity caused by a *spo0A* mutation is relieved in cells which also bear a mutation in *abrB* (data not shown). No β-galactosidase activity in *spo0H* mutant cells bearing any of the *spoVG-lacZ* fusion derivatives is observed (data not shown).

It is possible that the repositioned UAS of both *spoVG1B* and *spoVG210* is no longer functional in transcriptional enhancement and that the insertion of additional nucleotides between the UAS and the -35 promoter element created new sequences that influence the level of transcription from both mutant promoters. To determine whether the intact UAS elements of *spoVG1B* and *spoVG210* are functioning, the entire UAS was deleted from both promoters. This was accomplished by deleting all of the DNA between the upstream *Hind*III site and the *Bam*HI site introduced into the sequence between the UAS and the -35 element of each promoter. *lacZ* fusions containing the deletion promoters (*spoVGΔ1B* and *spoVGΔ210*; Fig. 2) directed lower levels of β-galactosidase activity than *spoVG1B-lacZ* and *spoVG210-lacZ*. However, the ratio of *spoVG1B-lacZ* activity to *spoVGΔ1B-lacZ* activity (less than 3:1) is significantly lower than the ratio of *spoVG210-lacZ* activity to *spoVGΔ210-lacZ* activity (15:1) (Table 2). A possible explanation for this variance is that the specific sequences inserted in the region between -35 and the UAS affect the level of *spoVG* transcription in the *spoVGΔ1B* and *spoVGΔ210* derivatives. Part of the explanation could be that the UAS in *spoVG210* functions more efficiently than in *spoVG1B* because of its rotational orientation (one turn of the helix) with respect to the *spoVG* promoter region. In light of the observed loss in activity when the UAS is deleted, we conclude that the UAS is functioning in the transcriptional activation of *spoVG1B* and *spoVG210*, albeit less efficiently than in the wild-type promoter.

These data indicate that the UAS functions in transcriptional activation and *AbrB* control in a position-dependent manner. The difference in expression between *spoVG210-lacZ* and *spoVG1B-lacZ* suggests that UAS function may also depend upon which face of the DNA helix the element is oriented toward in relation to the -35 region. Another insertion mutation was constructed in *spoVG210* in order to

further investigate this possibility. The *Bam*HI site, located between the -35 promoter element and the UAS of the mutant promoter, was cleaved and filled in using T7 DNA polymerase and deoxynucleotides. The blunt ends were then ligated together. This should have created a 4-bp insertion, but nucleotide sequence analysis revealed that the actual insertion was only 3 bp. This mutant promoter (*spoVG213*; Fig. 2) contained an insertion of 13 bp between the -35 region and the UAS. *spoVG213*-directed β -galactosidase activity was induced after T_0 , but at T_2 the level of activity was only about one-half that observed from a *spoVG210-lacZ* fusion (Fig. 3 and Table 2). The level of *spoVG213* promoter activity is similar to the level observed with *spoVG1B* (Fig. 3 and Table 2). Since sequences within the UAS of both mutants are rotationally displaced, it is tempting to speculate that UAS function relies, at least to some extent, upon the rotational orientation of the UAS relative to the -35 promoter sequence. The differences in the observed expressions of *spoVG210*, *spoVG1B*, and *spoVG213* may, however, be the result of the specific sequences that were inserted into the region between -35 and the UAS in creating the mutant *spoVG* promoter derivatives.

UAS does not activate transcription of *spoVG* from a distance. To test whether the UAS could affect *spoVG* transcription from a long range, DNA from *E. coli* lambda bacteriophage was inserted into the *Bam*HI site between the UAS and the -35 promoter element of *spoVG210*. Lambda DNA was subjected to partial *Sau*3A1 digestion, and fragments in the range of 30 bp in length were isolated. These fragments were combined with *Bam*HI-digested pDF102 in a ligation reaction. Plasmids containing inserts were isolated and subjected to nucleotide sequence analysis. Two plasmids were selected for further study, one of which contained an insert of 32 bp. The other plasmid contained three fragments of the *Sau*3A1-digested lambda DNA that were joined together end to end, resulting in the insertion of 106 bp of foreign DNA. The lambda DNA inserted into both plasmids came from the nonregulatory coding sequences of the lambda J gene. The 32-bp insertion repositioned the UAS 42 bp upstream of its original location, which represents about four full turns of the DNA helix. This mutant, called *spoVGL1*, was introduced into cells of strain ZB307A, as described above. β -Galactosidase assays were performed with a Spo^+ strain bearing a *spoVGL1-lacZ* fusion. The notably low expression of β -galactosidase from this promoter (Table 2) indicates that the UAS is unable to function in transcriptional activation of *spoVG* from this distance. Similar results were obtained with the 106-bp insertion (*spoVGL4-lacZ*; Table 2), consistent with the previous experiments which indicated that the UAS of *spoVG* must be in close proximity to the -35 promoter element in order to function efficiently in transcriptional activation.

Regulation of *spoVG* is unaffected by the repositioning of sequences upstream of the UAS. Oligonucleotide-directed mutagenesis was again used to test the possibility that the displacement of sequences upstream of the UAS by the introduction of insertion mutations actually caused the reduction in *spoVG* transcriptional activity. A 5-bp insertion at position -68 , with respect to the start site of transcription, repositions sequences upstream of the UAS one half turn of the DNA helix further upstream, creating a *spoVG105* promoter derivative called *spoVG105* (Fig. 2). A 6-bp deletion, removing sequences -76 through -82 , relocates upstream sequences approximately one half turn of the DNA helix closer to the UAS. This mutant, called *spoVG2D*, was originally constructed to delete a 6-bp sequence that resembled an

FIG. 4. Effects of various promoter mutations on *spoVG-lacZ* expression in a Spo^+ background. β -Galactosidase specific activity of samples collected at 0.5-h intervals from cultures of cells grown in liquid DS medium. (A) ●, *spoVG210-lacZ*; △, *spoVG213-lacZ*; ■, *spoVG1B-lacZ*; □, *spoVGΔ1B-lacZ*; ○, *spoVGΔ210-lacZ*. (B) ●, *spoVG-lacZ*; □, *spoVG105-lacZ*; ▲, *spoVG2D-lacZ*.

operator sequence for catabolite repression of the *Bacillus licheniformis* α -amylase structural gene (16). β -Galactosidase assays using *spoVG105*- and *spoVG210-lacZ* fusions revealed that the displacement of sequences upstream of the UAS has no effect on *spoVG* transcription, since both promoters had wild-type levels of activity, were induced normally at T_0 (Fig. 4 and Table 2), and were repressed when cells were propagated in medium containing excess glucose and glutamine. This further supports the conclusion that displacement of the UAS results in a severe reduction in *spoVG* promoter activity.

Promoter mutations affect the transcription of *spoVG* in vitro. The effect of the *spoVGD*, *spoVG1B*, and *spoVG210* promoter mutations on RNA polymerase-promoter interaction was examined in vitro by using purified σ^H RNA polymerase holoenzyme (obtained from C. Moran) and plasmids bearing the mutant *spoVG* promoters for runoff

FIG. 5. In vitro transcription from mutant *spoVG* promoters by σ^H RNA polymerase holoenzyme. Transcriptions were conducted in vitro by using purified σ^H and plasmid templates containing wild-type and mutant derivatives of the *spoVG* promoter. The 120-base runoff transcript was synthesized from *Eco*RI-digested pZSP6-189 (34), a plasmid that contains the wild-type *spoVG* promoter. pZSP6-189 template DNA was included as an internal control in all of the transcription reactions. The test plasmids, containing wild-type or mutant forms of the *spoVG* promoter, were cleaved at the *Sal*I site downstream of the promoter and were present in the transcription reactions in a twofold molar excess over the control template. Transcription of the *Sal*I-cleaved templates resulted in the synthesis of 62-base runoff transcripts. Equal amounts of each reaction were loaded onto the gel. The transcripts were resolved by electrophoresis in 8% polyacrylamide gels containing 7 M urea and were visualized by autoradiography. Lanes: a, molecular weight markers; b, pZL207 (P_{spoVG}); c, pDF102 ($P_{spoVG210}$); d, pDF101 ($P_{spoVG1B}$); e, pDF100 ($P_{spoVG1D}$); f, pZSP6-189 only (control); g, no DNA template (control). Molecular sizes (in bases) are indicated.

transcription assays. The preparation of RNA polymerase that was used has been shown to catalyze transcription from the *spoVG* promoter and the *citG* P2 promoter (25, 29). If the UAS affects *spoVG* transcription by enhancing the affinity of RNA polymerase for the promoter without the aid of any additional factors, such as a positive regulatory protein, then the reduction in promoter activity caused by the *spoVGD*, *spoVG1B*, and *spoVG210* mutations should be observed in vitro by using purified σ^H . The reactions were performed in the presence of a control template, the wild-type *spoVG* promoter, transcription from which gives rise to a RNA of 120 bases.

Figure 5 shows the results of in vitro transcriptions in which equal amounts of each reaction were loaded onto a denaturing polyacrylamide gel. The *spoVG210* promoter has a much lower capacity than the wild-type *spoVG* promoter to compete with the control template for RNA polymerase. This is demonstrated by the decreased intensity of the bottom band and increased intensity of the upper band in lane c compared with lane b. Even further reductions in activity are observed with templates bearing the *spoVG1B* (lane d) and *spoVGD* (lane e) promoters.

Data from the in vitro transcription experiments are con-

FIG. 6. Glucose-glutamine effect on *spoVG-lacZ* expression in wild-type and mutant strains, and *spoVGD-lacZ* and *rpsD-lacZ* expression in wild-type cells. β -Galactosidase specific activity was measured at 0.5-h intervals in cells grown in liquid DS medium either supplemented with 1% glucose and 0.1% glutamine or unsupplemented. (A) $\bullet$, Spo^+ *spoVG-lacZ* in DS medium; $\circ$, Spo^+ *spoVG-lacZ* in DS medium with 1% glucose and 0.1% glutamine. (B) $\blacktriangle$, *spo0A abrB spoVG-lacZ* in DS medium; $\triangle$, *spo0A abrB spoVG-lacZ* in DS medium with 1% glucose and 0.1% glutamine. (C) $\bullet$, Spo^+ *spoVGD-lacZ* in DS medium; $\circ$, Spo^+ *spoVGD-lacZ* in DS medium with 1% glucose and 0.1% glutamine. (D) $\blacksquare$, Spo^+ *rpsD-lacZ* in DS medium; $\square$, Spo^+ *rpsD-lacZ* in DS medium with 1% glucose and 0.1% glutamine.

sistent with the in vivo β -galactosidase assay data. The level of activity observed in vitro for each mutant promoter, relative to that observed for the wild-type *spoVG* promoter, correlates well with the level of β -galactosidase activity produced in vivo from *lacZ* fusions containing the mutant promoters. We therefore conclude that the UAS mutations are acting at the level of transcription by affecting the interaction between RNA polymerase and the promoter.

Glucose- and glutamine-dependent repression of *spoVG* is exerted at a region downstream of the UAS. *spoVG* expression is inhibited in cells grown in rich media, as is the expression of a number of late-growth genes in *B. subtilis*. When either wild-type Spo^+ cells or *spo0A abrB* mutant cells that contain a *spoVG-lacZ* fusion are grown in DS medium supplemented with both 1% glucose and 0.1% glutamine, β -galactosidase synthesis is repressed (Fig. 6), implying that *spoVG* transcription is under a form of carbon and nitrogen source-dependent control which operates independently of *spo0A* and *abrB*. This form of repression has been designated Ggr.

Each of the promoter mutants *spoVGD*, *spoVG1B*, and *spoVG210* exhibited the Ggr phenotype (Fig. 6; data not shown for *spoVG1B* and *spoVG210*). Results from assays of *spoVGD-lacZ*-directed β -galactosidase synthesis (Fig. 6) show that Ggr regulation of the *spoVG* promoter requires sequences downstream of position -46.

To determine whether Ggr regulation of *spoVG-lacZ* could be exerted at the posttranscriptional level, β -galactosidase specific activity was directed from a *rpsD-lacZ* transcriptional fusion (obtained from F. Grundy [11]), which contains

sequences from -134 to +350 of the *rpsD* gene (including the *rpsD* promoter and part of the coding sequence for ribosomal protein S4 of *B. subtilis*) joined to the *spoVG-lacZ* translational fusion at position -11 of the *spoVG* gene. Expression of this fusion, which lacks the intact *spoVG* promoter but retains the *spoVG* DNA corresponding to the untranslated leader region and ribosome binding site, in DS medium containing glucose and glutamine was examined (Fig. 6). Repression was not observed, which strongly suggests that Ggr regulation is exerted at a site downstream of -46 and at the level of transcription initiation.

DISCUSSION

Our results suggest that the UAS sequence of *spoVG* functions in a position-dependent manner and without the involvement of a positive regulatory protein. We have shown by mutational analysis, in which the UAS was rotationally and linearly displaced, that the position of the activation sequence relative to the -35 promoter element of *spoVG* affects transcriptional enhancement. Additionally, we present evidence from in vitro transcription analysis that the UAS is sufficient to stimulate transcription of *spoVG* in the absence of any additional positive control factors (1). A similar conclusion was reported by Bracco et al., who fused the UAS of *spoVG* to the promoter of the *E. coli gal* operon and observed that transcription was activated in a cyclic AMP-independent manner (4). Their work and the work reported here also demonstrates that the UAS is not specific for a particular form of RNA polymerase, such as $E\sigma^H$, since the sequence can stimulate transcription from a promoter that is recognized by the major form of RNA polymerase of *E. coli*, $E\sigma^{70}$.

A+T-rich sequences, such as the UAS of *spoVG*, are found upstream of several strong bacterial promoters such as *rrnA* (20), *rrnB* (10), *tyrT* (15), and *tRNA-tufB* (30) in *E. coli*. Primarily on the basis of electrophoretic mobility studies, it has been proposed by Gourse et al. that these A+T-rich sequences create a "bent" conformation in the DNA (10). The bent conformation may help to open the DNA helix, which could enhance the binding of RNA polymerase to the promoter by allowing additional polymerase-DNA contacts to occur. This is consistent with evidence presented in two recent publications addressing "factor-independent activation" of *rrnB* P1 in *E. coli*. Leirimo and Gourse (17) have shown that the upstream curved DNA increases the rate of stable complex formation between RNA polymerase and the promoter at the initial binding step. In addition, Newlands et al. (22) observed increased protection of sequences upstream of the *rrnB* P1 promoter from hydroxyl radical attack in footprinting experiments with $E\sigma^{70}$ RNA polymerase when the "upstream activation region" was intact, suggesting that the presence of the upstream curved DNA allows for additional RNA polymerase-DNA contacts to occur.

An insertion of 5 bp (*spoVG1B*) between the -35 promoter element and the UAS, displacing the UAS both rotationally and linearly, reduces promoter utilization about 10-fold. However, an insertion which linearly displaces the UAS by 10 bp (*spoVG210*) restores the activation sequence to its original rotational orientation and results in a six- or seven-fold reduction in promoter activity from wild-type levels. Deletion of the UAS from *spoVG1B* and *spoVG210* mutant promoters resulted in a reduction in expression from both mutant promoters. The higher activity observed with *spoVGΔ1B* than with *spoVGΔ210* may be due to a more favorable overall conformation of the DNA surrounding the

spoVGΔ1B promoter, allowing for more efficient promoter recognition in the absence of the UAS. The observation that *spoVGΔ1B* is only threefold less active than *spoVG1B* may indicate that the UAS of the *spoVG1B* promoter is not effective in enhancing transcriptional activity. This is unlike the *spoVG210* promoter, in which the removal of the UAS, giving rise to *spoVGΔ210*, causes a 15-fold decrease in transcriptional activity. This observation suggests that the UAS functions more efficiently when it is rotationally oriented on its original face of the DNA helix. However, the small differences in activity between the *spoVG1B*, *spoVG210*, and *spoVG213* promoters could also be due to an effect caused by the insertion of the specific sequences between -35 and the UAS in creating the mutant promoters. The requirements for the positioning of the UAS in *spoVG* contrasts with the *Alu156* promoter of the *B. subtilis* bacteriophage SP82 (19). In this case, the linear displacement of the curved DNA upstream of the *Alu156* promoter was much less of a factor than rotational displacement. These data support a model for *Alu156* in which the upstream curved sequence creates a loop which brings more remote upstream elements into close proximity to the promoter, where they may facilitate interactions involving RNA polymerase and other effectors. For *spoVG*, this seems less likely since insertion (*spoVG105*) or deletion (*spoVG2D*) of nucleotides altering the spacing and orientation of sequences upstream of the UAS had no effect on *spoVG-lacZ* expression. Therefore, the UAS of *spoVG* most likely functions more directly in transcriptional activation by creating a DNA conformation that is favorable for the formation of a stable complex between RNA polymerase and the promoter or by enhancing RNA polymerase-DNA contacts.

Repression of *spoVG* by AbrB was also affected by displacement of the UAS. As stated previously, the UAS is required for the binding of the AbrB protein to the *spoVG* promoter. Footprint analysis has shown that AbrB binds to a region of *spoVG* from -6 to -75, which includes the UAS and promoter sequences (9). That AbrB interacts with a long DNA sequence suggests that AbrB monomers bind cooperatively to promoter DNA. Moving the UAS upstream, as with *spoVG1B* and *spoVG210*, may reduce the cooperativity which enhances AbrB-*spoVG* DNA interaction.

Analysis of the *spoVGΔ-lacZ* fusion showed that Ggr of *spoVG* requires sequences downstream of -46. Additionally, an *rpsD-lacZ* transcriptional fusion, which was constructed from a derivative of the *spoVG-lacZ* translational fusion in which sequences upstream of -11 had been eliminated but the *spoVG* untranslated leader and translation initiation region were retained (11), was not repressed in DS medium supplemented with glucose and glutamine. This strongly suggests that the observed repression is exerted at the level of transcription.

The transcriptional regulation of *spoVG* is different from that of the *citB* promoter, which is also repressed in cells grown in DS medium supplemented with glucose and glutamine. A sequence centered at -66 has been shown to be necessary for glucose-glutamine regulation of *citB* and is thought to be the binding site for a negative regulatory protein (8). At present, it is unclear whether there exists a Ggr-associated negative regulatory protein that binds to the *spoVG* promoter. It is possible that Ggr control of *spoVG* is through a mechanism involving the control of σ^H protein levels. Healy et al. (12) have reported that the level of σ^H is apparently regulated by a posttranscriptional mechanism. Since σ^H is not involved in the regulation of *citB* and there is no similarity in the sequences of *citB* and *spoVG* that are

responsible for the glucose-glutamine effect, it seems unlikely that the regulatory factor responsible for *citB* repression is also involved in Ggr regulation of *spoVG*.

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