Identification of a control region for expression of the forespore-specific *Bacillus subtilis* locus *spoVA*

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The role of a 20 bp conserved region located 45–64 nucleotides 5' of the *spoVA* transcription start point in *Bacillus subtilis* and *Bacillus licheniformis* was investigated by deletion analysis and by mobility shift assay. Deletions 5' of this conserved sequence had little effect on expression of a *spoVA-lacZ* fusion, whereas deletions extending into the sequence reduced expression of the *spoVA-lacZ* fusion by 85%. The timing of expression of *spoVA* was not affected by deletion of the sequence. The region was shown by mobility shift assays to bind specifically to a protein. Binding activity was detected in protein extracts prepared from bacteria 1 h or more after they had started to sporulate, but not in extracts prepared from vegetative bacteria. Mutations in all known *spoO* loci were screened but none prevented appearance of the binding activity; nor did mutations in any of the stage II and III loci tested. It is concluded that the 20 bp conserved region is the binding site of an activator that is subject to temporal regulation independent of known *spo* loci.

Keywords: *Bacillus subtilis*, spore formation, *spoVA* locus, transcription

INTRODUCTION

Spore formation in *Bacillus subtilis* is a primitive system of cellular differentiation. Under conditions of nutrient depletion, *B. subtilis* stops growing exponentially and enters the developmentally regulated process of spore formation. The resultant spore is dormant and is resistant to heat, organic solvents and UV damage. The process requires the precise interaction of genes and their products in both a temporal and spatial fashion. *spoVA* is one of approximately 50 genetic loci in *B. subtilis* involved in sporulation (Hranueli *et al.*, 1974; Piggot & Coote, 1976; Stragier, 1994). The focus of this work is to study how *spoVA* is regulated.

Mutations in *spoVA* result in defective spores which are sensitive to chloroform and heat (Piggot & Coote, 1976). *spoVA* was the first *spo* locus whose expression could be assigned to the forespore (de Lencastre & Piggot, 1979). The *spoVA* locus is a large polycistronic operon encoding five or six putative proteins of unknown function (Fort & Errington, 1985; Errington, 1993). It is located im-

mediately downstream of *spoIIA* and lies in a separate transcriptional unit (Fort & Piggot, 1984; Piggot *et al.*, 1984; Fort & Errington, 1985). Through primer extension analysis and through the use of *spoVA-lacZ* fusions, transcription has been shown to begin about 3 h after the start of sporulation (T_3) (Errington & Mandelstam, 1986; Moldover *et al.*, 1991). RNA polymerase holoenzyme containing σ^G has been shown to transcribe *spoVA* both *in vitro* and *in vivo*, suggesting that the *spoVA* locus is regulated at least in part by the temporal and spatial activity of σ^G (Nicholson *et al.*, 1989; Moldover *et al.*, 1991). The timing of transcription is similar to that of other genes transcribed by σ^G (Errington & Mandelstam, 1986; Nakatani *et al.*, 1989; Nicholson *et al.*, 1989).

The region upstream of the *spoVA* coding sequence of *Bacillus licheniformis* has also been cloned and sequenced. It contains –10 and –35 promoter sequences identical to those of *B. subtilis*; these conform to the σ^G consensus (Moldover *et al.*, 1991). Control of gene expression is likely to be similar in both species, with E- σ^G regulating at least one level of expression. There is little conservation of sequence in the 17 bp spacer region between the –10 and –35 regions, suggesting that this region is not important for control of *spoVA*. There is a region of 20 bp extending from –45 to –64 which is perfectly conserved between the two species (Moldover *et al.*, 1991).

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and is well conserved (17 out of 20 bp) in *Bacillus megaterium* (Tao *et al.*, 1992). In this work, the role in *spoVA* regulation of this upstream conserved region is explored.

METHODS

Strains. The *Escherichia coli* strain used was DH5 α [F⁻ *endA1* *hsdR17*(r_Km_K) *supE44* *thi-1* *recA1* *gyrA96* *relA1* Δ (*lacZYA-argF*) U169 ϕ 80d/*lacZ*AM15]. The *B. subtilis* 168 strains used are listed in Table 1. Antibiotics were added at the following concentrations, as needed: for *E. coli*, ampicillin (100 μ g ml⁻¹); for *B. subtilis*, chloramphenicol (3 μ g ml⁻¹) and erythromycin (1 μ g ml⁻¹).

Plasmids. Plasmids were maintained in *E. coli* DH5 α unless otherwise stated. Preparation of plasmids from *E. coli* was according to the method of Birnboim & Doly (1979), and for small-scale preparations, by the method of Ish-Horowitz & Burke (1981). Plasmid pPP170 was constructed by inserting a 2250 bp *Pst*I fragment from pPP33 (Piggot *et al.*, 1984) into the *Pst*I site of pBluescript II SK⁺ (Stratagene). The *Pst*I fragment extends from -92 to +2158 relative to the start site for transcription of *spoVA* (Moldover *et al.*, 1991). This plasmid contained a unique *Sma*I site at -95 relative to the *spoVA* transcription start site, and a unique *Hind*III site at +2172.

Deletion analysis. For creating nested deletions of DNA the Erase-a-Base System (Promega) based on the method of Henikoff (1984) was used. Plasmid pPP170 containing the region of interest was linearized with *Sma*I and subjected to digestion with exonuclease III for varying times. The reactions were terminated and ends made flush by S1 nuclease digestion followed by treatment with Klenow fragment of DNA polymerase. This material was digested with *Hind*III and ligated into pGV34 (Youngman *et al.*, 1989) previously digested with *Sma*I and *Hind*III. The extent of the *spoVA* promoter region was determined by dideoxy sequencing (Sanger *et al.*, 1977).

Manipulation of phage SP β . The phage SP β derivative used was SP β _{C2} Δ 2::Tn917::pSK10A6 (Zuber & Losick, 1987). The temperature-inducible prophage was contained in strain ZB307. Plasmid pGV34 (Youngman *et al.*, 1989) was used to construct transcriptional fusions to *lacZ*, and then to transfer the fusions into the SP β derivative for further study. Manipulations of the SP β -containing strains were performed according to the methods of Zuber & Losick (1987).

Isolation of protein. Cultures of *B. subtilis* were grown in 100 ml MSSM (modified Schaeffer's sporulation medium; Piggot & Curtis, 1987) and the cells harvested at the time of interest. Protein was extracted using the method described by

Fouet *et al.* (1990). The pellets were washed with 10 ml of a buffer containing 20 mM Tris/HCl (pH 8.0), 50 mM NaCl, 0.5 mM EDTA (pH 8.0), 1 mM DTT, 5% (v/v) glycerol, and resuspended in 10 ml of the same buffer freshly supplemented with PMSF (360 μ g ml⁻¹). The mixture was then sonicated for 3 min (15 s on, 15 s off; total 3 min actual sonication) while being cooled with ice water. The debris was removed by centrifugation at 12000 *g* for 15 min at 4 °C. The protein concentration in the supernatant was determined using a Bio-Rad Protein Assay Kit and the supernatants were stored at -70 °C.

Mobility shift assay (Fried & Crothers, 1981). This assay was performed as described by Ausubel *et al.* (1990), with modification. DNA fragments and synthetic oligonucleotides were labelled at the 5' ends by incubation with T4 polynucleotide kinase in the presence of [γ -³²P]ATP at 37 °C for 60 min. Protein (15 μ g) and radiolabelled probe DNA (10 ng) were mixed in a final volume of 40 μ l buffer (20 mM Tris/HCl, pH 7.6, 50 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 5% glycerol). The reactions were allowed to incubate at 30 °C for 15 min. DNA loading buffer (2 μ l) was added (50% glycerol, 0.001% bromophenol blue), and the samples were loaded onto a 4% polyacrylamide (401 acrylamide/bisacrylamide) gel. The buffer was Tris/HCl/glycine, and the gel was electrophoresed at 25 mA constant current for 2 h at 4 °C. The gel was transferred to Whatman paper, dried, and autoradiographed.

The oligonucleotides used were 5' GTGGTAATTTATGGTCTTTT 3' and 5' AAAAGACCATAAATTACCAC 3'.

Copy number determination. The number of tandem repeats of pPP153 in the chromosome was determined essentially as described by Piggot & Curtis (1987). Bacteria from part of the culture used for determination of β -galactosidase activity during sporulation were harvested and embedded in agarose beads. DNA was prepared from the embedded bacteria and digested with a restriction endonuclease that cut near, but not in, the reiterated region. The size of the restriction fragment flanking the insert was determined by Southern blotting of restricted DNA that had been fractionated by pulsed-field gel electrophoresis. DNA was also digested with endonucleases that cut within the reiterated region, and examined to ensure that rearrangements had not occurred in the reiterated region. Only cultures that yielded a single predominant copy number were used for β -galactosidase assays.

Other methods were as described by Wu *et al.* (1989).

RESULTS

Deletion analysis of the region 5' to the *spoVA* operon

Analysis of the region 5' to *spoVA* employing integrative plasmids had indicated that the region extending to the *Pst*I site located 92 bp upstream of the *spoVA* transcriptional start site (i.e. at -92) was sufficient for efficient expression of *spoVA* (Moldover *et al.*, 1991). To test the hypothesis that the conserved region 45-64 nucleotides upstream of the *spoVA* transcription start site is important for transcription, a series of nested deletions was constructed. Deletions were generated from the *Pst*I site at -92. Truncated fragments of the *spoVA* promoter region were fused in the correct orientation to *lacZ* in pGV34. The promoter-*lacZ* fusions were then introduced into the SP β prophage of *B. subtilis* strain ZB307 using the

Table 1. *B. subtilis* strains used

Strain	Genotype
MB24	<i>trpC2 metC3 rif-2</i>
SL493	<i>spoOJ93 metC3 rif-2 trpC2</i>
SL513	<i>spoOH17 trpC2 rif-2</i>
SL566	<i>spoO.A34 phe-12 rif-2 tal-1</i>
SL741	<i>spoOK141 trpC2</i>
SL964	<i>spoOB136 metC3 tal-1</i>
SL965	<i>spoOF221 metC3 tal-1</i>
ZB307	SP β _{C2} Δ 2::Tn917::pSK10A6

Fig. 1. Effect of deletions into the region 5' to *spoVA* on expression of *spoVA*. Transcriptional fusions of different extents of the 5' region to *lacZ* were integrated into the chromosome of *B. subtilis* strain MB24 at *SPβ*. The extent of the 5' region is indicated as the distance in bp to the deletion from the transcription start point of *spoVA*. The start of sporulation is defined as the end of exponential growth. ■, -92; ●, -76; ▲, -69; ◆, -64; □, -57; ○, -42; △, -23; ◇, strain MB24 containing no *lacZ* fusion.

method of Zuber & Losick (1987). The strains were assayed for β -galactosidase production during sporulation.

A deletion that removed the region further than 76 bp upstream of the *spoVA* transcription start (i.e. a deletion to -76) gave essentially the same β -galactosidase activity as a deletion to -92 (Fig. 1). A deletion to -69 resulted in a slight decrease in β -galactosidase expression. A deletion to -64 bp, which changes one nucleotide at the 5' end of the upstream homologous region [hereafter referred to as the '*spoVA* box' (Fig. 2)], resulted in 41% of the β -galactosidase expression compared to the peak expression obtained with the -92 deletion. Deletions extending to -57 bp and -42 bp each reduced activity almost to the background level of endogenous activity

displayed by the parent strain MB24, which contained no *lacZ* fusion. Further deletion to -23 bp, which lies within the promoter for *spoVA*, reduced activity to that background level. It is concluded that the *spoVA* box is required for efficient expression of *spoVA*.

Southern analysis of the *B. subtilis* chromosome was performed using an oligomer complementary to the *spoVA* box to determine whether similar regions existed elsewhere on the *B. subtilis* chromosome. Southern analysis was performed at very low stringency, yet failed to detect homology with sequences other than that contained immediately upstream of *spoVA* (data not shown). This does not rule out the possibility of other homologous sequences, but if they exist they were not detected.

Gel retardation analysis

It was of great interest to determine the nature of the regulatory moiety acting on the *spoVA* box. The possibility that this moiety was a DNA-binding protein was explored through the use of mobility shift assays (Fried & Crothers, 1981). A 310 bp DNA fragment extending from the *Ava*I site at -181 to the *Pvu*II site at +129 with respect to the transcription start point for *spoVA* was used as a probe to see if a specific protein was able to bind to the region. Protein was prepared from cultures of *B. subtilis* during growth and sporulation, and used in this assay.

A retarded band corresponding to a DNA-protein complex was detected at 1 h and increased in intensity up to 5 h after the start of sporulation (Fig. 3). The band was not detected in vegetatively growing cells, or at 0 h. Repetition of the above experiments using the synthetic double-stranded oligomer as labelled probe showed the same temporal pattern of appearance of retarding activity (data not shown); a 50-fold molar excess of unlabelled oligomer largely prevented appearance of the retarded band, whereas 50-fold molar excess of an unrelated oligomer did not. This indicates that the binding activity was specific for the *spoVA* box. The binding moiety was found to be in the cell supernatant fraction (100 000 g, 30 min); it was sensitive to heat, to protease treatment and to EDTA, and is presumed to be a protein.

The dependence pattern of the DNA-protein complex was explored using protein prepared from various mutant strains of *B. subtilis*. As the appearance of the DNA-

Fig. 2. Location of deletion end-points on the DNA sequence of the non-transcribed strand of the *spoVA* promoter region. The transcription start-point is shown as +1. The positions of deletions used are indicated with an asterisk, and β -galactosidase activities associated with the deletions (Fig. 1) are given as a percentage of the maximum obtained with the -92 deletion. The *spoVA* box is underlined.

Fig. 3. Mobility shift assay of proteins extracted from cultures at different stages of growth and sporulation with DNA from the *spoVA* promoter region. A 317 bp DNA fragment extending from -182 to +120 was used as labelled probe. Protein was extracted from vegetative cultures (lane V) and at various times during sporulation (0, 1, 2, 3, 4 and 5 h after the start of sporulation) and incubated with 10 ng labelled probe. Fifty nanograms protein was used for each sample, as described in Methods. Lane P contained probe without any protein extract.

protein complex was occurring early in sporulation, it was possible that a mutation at stage 0 would have an effect. Mutations in *spoOA*, *spoOB*, *spoOF*, *spoOH*, *spoOJ* and *spoOK* surprisingly had no effect on formation of the DNA-protein complex (Fig. 4). In the example shown, the retarded band was fainter for the *spoOF* and *spoOH* mutants. In other experiments the band obtained with these mutants was of comparable intensity to the band obtained with strain MB24. The retarding activity was also present in extracts of *spoOE*, *spoIIAA*, *spoIIAC*, *spoIIE*, *spoIIG*, *spoIIIA*, *spoIIIC*, *spoIIIE*, *spoIIIG* and *spoIIIJ* mutants (data not shown).

Effect of copy number on expression of *spoVA*

The manipulation of gene copy number has been used to analyse the expression of the *spoIIA* (Piggot & Curtis, 1987) and *spoOF* loci (Chapman & Piggot, 1987). To examine further the role of the 5' region in controlling transcription of *spoVA*, the region was fused to *lacZ* in plasmid pPPP153. This plasmid replicates autonomously in

Fig. 4. Mobility shift assay of extracts from strains containing *spoO* mutations. Protein was extracted from cultures at 1 h and incubated with 10 ng radiolabelled oligomer. The particular mutations were *spoOA34*, *spoOB136*, *spoOF221*, *spoOH17*, *spoOJ93* and *spoOK141* (lanes 1, 2, 3, 4, 5 and 6, respectively). Lane P contained labelled probe without any protein extract. The extract from the parental *spo*⁺ strain MB24 is also shown (lane 7).

E. coli, but is unable to replicate in *B. subtilis*. Transformants of *B. subtilis* strain MB24 with pPPP153 were selected that expressed the plasmid-encoded *cat* gene. A particular transformant clone was chosen for subsequent study in which the plasmid was shown by Southern blotting to have integrated into the homologous region of the chromosome by a single crossover (data not shown).

Derivatives of MB24 with tandem repeats of the integrated plasmid pPPP153 were selected by growth on media with elevated levels of chloramphenicol. β -Galactosidase activity during sporulation was determined for strains containing different numbers of tandem repeats (Piggot & Curtis, 1987). In none of the strains tested was sporulation impaired. Strains having a range of copy numbers began synthesizing β -galactosidase at about 3 h. The rate of induction was approximately proportional to copy number up to a copy number of 18 (Fig. 5). This indicates that *spoVA* is not under negative control. When the copy number increased above 18, enzyme activity did not increase greatly, suggesting that an activator had been titrated out; the data are not precise enough to permit a firm conclusion. We were unable to obtain any strain with a stable copy number above 26 and so were not able to exclude unregulated expression unequivocally.

Fig. 5. β -Galactosidase synthesis during sporulation for *B. subtilis* MB24(pPP153) clones with different numbers of tandem repeats of the integrated plasmid. $\diamond$, 1 copy; $\blacksquare$, 2 copies; $\triangle$, 5 copies; $+$, 11 copies; $\circ$, 18 copies; $\ast$, 21 copies; $\square$, 26 copies.

DISCUSSION

The recognition of sequence similarities between *B. subtilis* and *B. licheniformis* made it possible to suggest regions necessary for regulation of the sporulation locus, *spoVA* (Moldover *et al.*, 1991). Thus, identification of -10 and -35 sequences in *B. licheniformis* that are identical to those shown to be recognized by σ^G in *B. subtilis* suggested that transcription of the *B. licheniformis* locus required the *B. licheniformis* homologue of σ^G (Moldover *et al.*, 1991). The analysis reported here of the homologous region between -45 and -64 , the *spoVA* box, has revealed another regulatory region for the *spoVA* locus.

Expression of *spoVA* was dependent on the presence of an intact *spoVA* box. Deletions to -57 (8 bp into this box) or -42 (box removed completely) reduced *spoVA* expression to less than 15% of the expression obtained when the upstream region extended to -92 , and deletion to -64 (which removed just 1 bp of the box) reduced expression to 41% of that of constructs where the box was intact (Fig. 1). Deletion to -69 , which is only 4 bp upstream of this box, had very little effect on expression compared to deletion to -92 . The box region is therefore presumed to be the target of some unknown protein, which regulates *spoVA* expression. It is important to note that while the level of expression was substantially affected by deletion into the box, the temporal regulation of *spoVA* appeared not to be affected (Fig. 1).

Deletion of the *spoVA* box greatly impaired transcription, indicating that the box was the target of an activator. This conclusion was consistent with experiments in which the

number of copies of the *spoVA* 5' region was selectively amplified. The position of the *spoVA* box relative to the -35 region of the promoter is also consistent with an activator binding to the box (Collado-Vides *et al.*, 1991). Too few σ^G promoters have been characterized to say if the *spoVA* promoter conforms poorly to the optimal σ^G promoter (as might be expected of a promoter under positive control), although *spoVA* is weakly expressed compared to other genes in the σ^G regulon such as *sspA*, *sspB* and *sspE*. None of the known sequences upstream of loci in the σ^G regulon (other than *spoVA*) have a region resembling the *spoVA* box.

Specific binding of a protein to the *spoVA* box during sporulation was indicated by mobility shift assays. Binding was not seen during vegetative growth, nor prior to 1 h of sporulation. The binding was specific to the 20 bp box, but appeared to be stabilized by surrounding sequences, as unlabelled oligonucleotide competed more efficiently with labelled oligonucleotide than with the labelled 310 bp fragment (unpublished observations). We infer that the protein detected by the mobility shift assay is an activator of *spoVA* transcription.

The binding activity appears about 2 h prior to transcription of *spoVA*. Transcription is presumably delayed until the requisite sigma factor, σ^G (Nicholson *et al.*, 1989), is active. It is possible that the early appearance of the binding activity relative to *spoVA* expression indicates a need for the protein elsewhere, and earlier, in sporulation, although we were unable to detect hybridization of the *spoVA* box to anything other than itself. In any case, the timing suggests that the protein may be present before the sporulation septum is formed, even though it acts on a locus that is expressed only in the forespore.

Of major interest is the pattern of the DNA-protein complex formation in *spo* mutants. No sporulation mutation tested, including mutations in the *spoOA*, *spoOB*, *spoOE*, *spoOF*, *spoOH*, *spoOJ* and *spoOK* loci which are associated with the start of sporulation, had any effect on formation of the DNA-protein complex. Nor had mutations in two vegetatively expressed loci, *spoIIIE* and *spoIIJ*, that are required for expression of late sporulation genes (Foulger & Errington, 1989; Errington *et al.*, 1992). These results were unexpected. The presumed activator of *spoVA* displays temporal regulation, as the binding activity is only detected during sporulation. Thus, the activator, or its binding activity, must be subject to temporal regulation independent of all the known *spo* loci.

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