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 sporulation. 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 immediately 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 (T3) (Errington & Mandelstam, 1986; Moldover et al., 1991). RNA polymerase holoenzyme containing $\sigma^B$ 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 $\sigma^B$ (Nicholson et al., 1989; Moldover et al., 1991). The timing of transcription is similar to that of other genes transcribed by $\sigma^B$ (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 $\sigma^B$ consensus (Moldover et al., 1991). Control of gene expression is likely to be similar in both species, with E-$\sigma^B$ 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)
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 DH5α [F- endA1 hsdR17(c m6 recA1 thr-1 leu-169 proA16 galK16 dcm] and is well conserved (17 out of 20 bp) in B. MOLDOVER, I,.

**Plasmids.** Plasmids were maintained in E. coli DH5α 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-Horowicz & Burke (1981). Plasmid pPP170 was constructed by inserting a 2250 bp PstI fragment from pPP33 (Piggot et al., 1984) into the PstI site of pBluescript II SK+ (Stratagene). The PstI fragment extends from -92 to +2158 relative to the start site for transcription of spoVA (Moldover et al., 1991). This plasmid contained a unique Smal site at -95 relative to the spoVA transcription start site, and a unique HindIII site at +2173.

**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 Smal 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 HindIII and ligated into pGV34 (Youngman et al., 1989) previously digested with Smal and HindIII. The extent of the spoVA promoter region was determined byideoxy sequencing (Sanger et al., 1977).

**Manipulation of phase SPβ.** The phase SPβ derivative used was SPβΔ2::Tn917::pSK10A6 (Zuber & Losick, 1987). The temperature-inducible prophage was prepared 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 SPβ derivative for further study. Manipulations of the SPβ-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 μg 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 BioRad 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 μg) and radiolabelled probe DNA (10 ng) were mixed in a final volume of 40 μl 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 μl) 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′ GGTGTAATTATGCTGTCTTTT 3′ and 5′ AAAAGACATATAATCACCAC 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 within 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 PstI 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 PstI 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 SPβ prophage of B. subtilis strain ZB307 using the
Regulation of the spoVA locus in Bacillus subtilis

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; Δ, -23; ◤, strain MB24 containing no lacZ fusion.

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 complimentary 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 AraI site at -181 to the PvuII 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 (100000 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–
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, spoIAC, spoIIE, spoIIIG, spoIII A, spoIII C, spoIII E, spoIII G and spoIII J 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 spoV’A, the region was fused to lacZ in plasmid pPP153. This plasmid replicates autonomously in *E. coli*, but is unable to replicate in *B. subtilis*. Transformants of *B. subtilis* strain MB24 with pPP153 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 pPP153 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 spoV’A 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.
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 *spoA*, *sspB* and *spoE*. None of the known sequences upstream of *spoVA* (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 mutations tested, including mutations in the *spoOA*, *spoOB*, *spoOE*, *spoOF*, *spoOH*, *spoOF* 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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