Synthesis of *spoIIA* and *spoVA* mRNA in *Bacillus subtilis*

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The expression of the *spoIIA* and *spoVA* sporulation loci of *Bacillus subtilis* was examined by using DNA–RNA hybridization to detect the time of appearance of their corresponding mRNA molecules in wild-type and asporogenous mutants of *B. subtilis*. From the size of the mRNA molecules it is clear that both the *spoIIA* and *spoVA* loci are polycistronic operons. Neither of the mRNA molecules is polyadenylated. The results also indicate the *spoIIA* operon is regulated by two promoters which become functional at different times.

INTRODUCTION

Sporulation in *Bacillus subtilis* is determined by over 50 identified operons (Piggot & Hoch, 1985), and the means by which they are regulated remains an unsolved problem, though several models have been proposed. Among these is the suggestion that they are expressed in a linear dependent sequence in which one of the translational products of each operon induces or derepresses the next (Mandelstam, 1969, 1976). In variants of this model the possibilities have been considered that there are parallel or branched sequences (Coote & Mandelstam, 1973; Piggot & Coote, 1976). In a rather different model it has been proposed that the sequence is regulated by successive replacements of the sigma factor of RNA polymerase (see Losick & Pero, 1981; Westpheling *et al.*, 1985). Some involvement of sigma factors in sporulation is also supported by the report (Trempey *et al.*, 1985) that the *spoIIG* locus in *B. subtilis* has a region of close homology with the *E. coli rpoD* gene, which encodes a sigma factor. An equally intriguing observation followed from the sequence determination of *spoIIA* (Fort & Piggot, 1984), which showed that the locus contains three genes. It is now apparent that the third of these genes, 'ORF 3', has a striking similarity both to *spoIIG* in *B. subtilis* and to the *rpoD* gene of *E. coli* already referred to (Errington *et al.*, 1985).

Clearly, further facts are needed before we can make any choice between these models and others that can easily be suggested. The work described here was undertaken to examine the expression of the *spoIIA* and *spoVA* loci, which have been cloned and sequenced recently (Savva & Mandelstam, 1984; Fort & Piggot, 1984; Fort & Errington, 1985). We used portions of both operons to determine transcription in a number of sporulation mutants. These results were published in a preliminary form (Savva & Mandelstam, 1985). Here we report the results for 14 additional mutants and show that the two transcripts have markedly different half-lives.

METHODS

Bacterial strains. The strains used in this investigation are shown in Table 1 of an accompanying paper (Errington & Mandelstam, 1986a), except for strains 69 (*trpC2 spoIIA69*) [strain NG18.6 of Piggot (1973)], 487 (*trpC2 pheA12 spo0C9*) [strain 9V of Ionesco *et al.* (1970)] and SL980(*trpC2 metB10 spoIIA4*) (Liu *et al.*, 1982). Bacteria were grown and resuspended in sporulation medium as described by Sterlini & Mandelstam (1969); at different times after resuspension, samples (100 ml) of culture were removed and used for the isolation of RNA. All experiments were done at 37 °C, and the time (h) after resuspension is denoted t_0 , t_1 , etc.

Buffer and other solutions. The following solutions were used: (i) lysozyme [1% (w/v) in 50 mM-Tris/HCl pH 8.0]; (ii) guanidinium isothiocyanate [6 M-guanidinium isothiocyanate, 0.1 M- β -mercaptoethanol, 5 mM-sodium citrate pH 7.0, 0.5% (w/v) sarcosyl]; (iii) RNA loading buffer [20% (w/v) glycerol, 50 mM-boric acid, 5 mM-sodium tetraborate, 10 mM-sodium sulphate, 25 mM-methyl mercuric hydroxide, 0.02% (w/v) bromophenol blue]; (iv) gel-running buffer (50 mM-boric acid, 5 mM-sodium tetraborate, 10 mM-sodium sulphate); (v) SSC (0.15 M-NaCl, 0.015 M-sodium citrate, pH 7.0); (vi) prehybridization solution [50% (v/v) formamide, 0.9 M-NaCl, 5 mM-EDTA (disodium salt), 50 mM-sodium phosphate buffer pH 7.7, 0.1% (w/v) sodium dodecyl sulphate, 0.02% (w/v) bovine serum albumin, 0.02% (w/v) Ficoll, 0.02% (w/v) polyvinylpyrrolidone, 0.01% (w/v) denatured salmon sperm DNA]; (vii) wash solution 1 [0.36 M-NaCl, 2 mM-EDTA (disodium salt), 20 mM-sodium phosphate buffer pH 7.7, 0.1% (w/v) sodium dodecyl sulphate]; (viii) wash solution 2 [0.018 M-NaCl, 0.1 mM-EDTA (disodium salt), 1 mM-sodium phosphate buffer pH 7.7, 0.1% (w/v) sodium dodecyl sulphate].

Isolation, electrophoresis and blotting of RNA. The methods used were those described by Savva & Mandelstam (1985), as were the procedures for DNA-RNA hybridizations. The specific activity of the probe DNA was 1.5×10^7 c.p.m. per μ g DNA.

Isolation of polyadenylated RNA. RNA (0.5 mg) isolated from *B. subtilis* 168 at t_5 after resuspension was separated into the polyadenylated (poly-A⁺) and non-polyadenylated (poly-A⁻) fractions using oligo-dT-cellulose chromatography (Aviv & Leder, 1972). The procedure used was that described by Maniatis *et al.* (1982) for batch absorption and elution of RNA.

Electrophoresis of DNA and isolation of restriction fragments from agarose gels. Restriction enzyme reaction, electrophoresis of DNA and isolation of DNA restriction fragments from agarose gels were done as described by Savva & Mandelstam (1984).

RESULTS

Hybridization probes

The isolation of the recombinant phage ϕ 105DS1 (derived from phage ϕ 105), which contains the *B. subtilis spoIIA* and *spoVA* loci, has been reported earlier (Savva & Mandelstam, 1984).

By using the DNA sequences of the *spoIIA* and *spoVA* loci (Fort & Piggot, 1984; Fort & Errington, 1985), a number of DNA restriction fragments which lie totally within each locus were chosen, isolated from low melting point agarose gels, labelled by nick translation (Rigby *et al.*, 1977) and used as probes for the detection of the transcripts of these loci. Fig. 1 shows the restriction fragments used as probes for the detection of the *spoIIA* and the *spoVA* mRNA molecules; these were the 0.67 kb *EcoRI*-*BglII* and the 1.89 kb *EcoRV*-*EcoRV* fragments respectively. Identical results were obtained with a number of other probes in each locus (data not shown).

Times of transcription of *spoIIA* and *spoVA*

Samples of RNA (4 μ g) isolated from wild-type *B. subtilis* at different times after resuspension in sporulation medium were spotted on each of two nitrocellulose filters and hybridized to either the *spoIIA* probe or the *spoVA* probe (Fig. 2). Both probes failed to hybridize to the RNA samples isolated immediately after resuspension in the sporulation medium (i.e. at t_0); this indicates that transcription of both the *spoIIA* and the *spoVA* loci does not occur during vegetative growth.

The *spoIIA* probe was found to hybridize to the RNA samples isolated 1 h or more after resuspension (i.e. t_1 - t_5 samples). However, at t_1 the hybridization was weak, indicating that the amount of *spoIIA* mRNA was low. In the case of the *spoVA* probe, weak hybridization was obtained with the RNA sample isolated at t_3 , with signals becoming more intense at t_4 and t_5 . (Results, not shown indicated that at $t_{3.5}$ the hybridization signal was as strong as at t_4 .) Therefore, it appeared that the transcripts for *spoIIA* and *spoVA* began to be synthesized at about t_1 and t_3 respectively, and that both transcripts were present until at least t_5 .

The more informative and reliable method of 'northern' hybridizations on gels confirmed these results (Fig. 3) and provided further information about the *spoIIA* and *spoVA* mRNA molecules. Initially, the *spoIIA* probe hybridized to only one mRNA species, the size of which, about 1.7 kb, was in agreement with the results of Fort & Piggot (1984) which showed that the *spoIIA* locus is a polycistronic operon. However, from t_3 onwards the *spoIIA* probe hybridized to a second, larger species of mRNA of about 2.6 kb (Fig. 3). As the *spoVA* probe did not hybridize

Fig. 1. Genetic map of the cloned *B. subtilis* DNA based on the DNA sequence of the *spoIIA* and *spoVA* loci (Fort & Piggot, 1984; Fort & Errington, 1985). The DNA restriction fragments used as probes in the hybridization experiments and some restriction enzyme cleavage sites are shown.

Fig. 2. Timing of transcription of the (a) *spoIIA* and (b) *spoVA* loci as determined by dot hybridizations. Samples labelled t_0 – t_5 were isolated at the indicated time (h) after resuspension of *B. subtilis* 168.

to this mRNA species, this larger transcript was not the result of anti-termination of *spoIIA* transcription.

In the case of the *spoVA* mRNA, the exact size could not be determined because of the smearing of the RNA samples due, presumably, to degradation of this much larger mRNA species during preparation.

Stability of the mRNA transcribed from spoIIA and spoVA

B. subtilis 168 was resuspended in sporulation medium and incubated at 37 °C for 4 h before actinomycin D was added to a final concentration of 1 µg ml⁻¹. Samples of culture were removed immediately and after 3, 6 and 9 min, and were used for isolation of RNA as described in Methods. These RNA samples were used in dot hybridizations with the two probes. The results (Fig. 4) show that a 3 min treatment with actinomycin D causes a considerable reduction in the amount of the *spoIIA* mRNA; this suggests that the *spoIIA* mRNA has a short half-life (possibly 2–3 min). However, the *spoVA* mRNA appeared to be more stable since it was still detectable even after 9 min of treatment with actinomycin D. Identical results were obtained using 'northern' hybridization analysis (results not shown).

Polyadenylated RNA

Total RNA from *B. subtilis* 168 was separated into a poly-A⁺ and a poly-A⁻ fraction by oligo-dT-cellulose chromatography; the amount of poly-A⁺ RNA (as estimated on agarose gels after staining with ethidium bromide) was about 5% of the total (results not shown).

Fig. 3. 'Northern' hybridizations for the detection of (a) *spoIIA* mRNA and (b) *spoVA* mRNA. Samples labelled t_0 – t_5 were isolated at the indicated time (h) after resuspension of *B. subtilis* 168. The origin is at the top of the figure. The positions of the size markers used (phage λ cleaved with *Hind*III) are indicated.

Fig. 4. Dot hybridizations showing the stability of the transcripts of the (a) *spoIIA* and (b) *spoVA* loci. Actinomycin D ($1 \mu\text{g ml}^{-1}$) was added to *B. subtilis* 168 at t_4 and RNA was isolated after 0, 3, 6 and 9 min.

Table 1. Transcription of the *spoIIA* and *spoVA* loci in asporogenous mutants of *B. subtilis*

The *spoIIA* and *spoVA* probes were hybridized to the $t_{2.5}$ and t_5 RNA samples respectively; both probes were also hybridized to a t_0 sample of RNA from each strain and in every case no hybridization occurred. +, Hybridization; -, no hybridization; w, weak hybridization (<10% of normal strength). Where a signal of intermediate strength was obtained this is expressed as a percentage of the normal strength.

Mutation	Hybridization to:	
	<i>spoIIA</i> probe	<i>spoVA</i> probe
<i>spo0A43</i>	10-15%	w
<i>spo0B136</i> , <i>spo0C9</i> , <i>spo0D486</i>	w	-
<i>spo0E11</i>	10-15%	w
<i>spo0F221</i> , <i>spo0G485</i> , <i>spo0H17</i>	w	-
<i>spo0J93</i>	10-15%	w
<i>spo0K141</i>	w	-
<i>spoIIA4</i>	-	w
<i>spoIIA69</i> , <i>spoIIB131</i> , <i>spoIID298</i> , <i>spoIIE48</i> , <i>spoIIG55</i>	+	w
<i>spoIIIA65</i> , <i>spoIIIB2</i> , <i>spoIIIC496</i> , <i>spoIIID497</i> , <i>spoIIIE36</i> , <i>spoIIIF590</i>	+	w
<i>spoIVA67</i> , <i>spoIVB165</i> , <i>spoIVC23</i> , <i>spoIVD92</i> , <i>spoIVE498</i> , <i>spoIVF88</i> , <i>spoIVG25</i>	+	+
<i>spoVA89</i> , <i>spoVB91</i> , <i>spoVC134</i> , <i>spoVD156</i> , <i>spoVE85</i> , <i>spoVF224</i>	+	+
<i>spoVIA513</i> , <i>spoVIB520</i> , <i>spoVIC610</i>	+	+
<i>gerE36</i>	+	+

Samples of poly-A⁺ and poly-A⁻ RNA were used in dot hybridizations with the *spoIIA* and *spoVA* probes. Neither probe hybridized to the poly-A⁺ RNA fraction (results not shown), so apparently the mRNA molecules for *spoIIA* and *spoVA* were not polyadenylated.

Transcription of the spoIIA and spoVA operons in asporogenous mutants blocked at various stages of sporulation

The transcription of the *spoIIA* operon in a number of asporogenous mutants of *B. subtilis* was studied by hybridizing the *spoIIA* probe to RNA isolated from these mutants 2.5 h after resuspension; both dot and 'northern' hybridizations were used. Similar experiments were done using the *spoVA* probe but the RNA samples were isolated 5 h after resuspensions. For both probes RNA samples isolated from each mutant at t_0 were also used.

The results of these hybridizations are shown in Table 1. It is apparent that neither the *spoIIA* probe nor the *spoVA* probe hybridized to the RNA isolated from the *spoIIA4* mutant (strain SL980). This was expected because DNA from this strain shows no homology to plasmids pHM2 (Liu *et al.*, 1982) and pPP33 (Piggot *et al.*, 1984) and therefore the whole of the *spoIIA* operon and most of the *spoVA* must be deleted (P. Piggot, personal communication).

Transcription of the *spoIIA* operon was found to occur in all the stage 0 mutants investigated but the amount of the *spoIIA* mRNA was different in different mutants. In the case of *spo0B*, *spo0C*, *spo0D*, *spo0F*, *spo0G*, *spo0H* and *spo0K* only trace amounts of *spoIIA* mRNA were observed, whilst *spo0A*, *spo0E* and *spo0J* produced markedly higher amounts, which were roughly judged to be 10-15% of normal. All the mutants tested which were blocked at stage II or later, apart, of course, from the *spoIIA4* strain, were found to produce normal amounts of *spoIIA* mRNA.

The *spoVA* probe did not hybridize to the RNA samples isolated from most stage 0 mutants; however, trace amounts of *spoVA* mRNA were observed in *spo0A*, *spo0E* and *spo0J* mutants. Traces of *spoVA* mRNA were also found in all the stage II and stage III mutants examined.

Transcription of the *spoVA* operon was found to be normal in the stage IV, stage V, stage VI and *gerE* mutants investigated.

Neither probe hybridized to the RNA isolated from the mutants tested at t_0 . For a number of mutants the time of transcription was also determined; in these cases, transcription of both operons was found to occur at the same time as in the wild-type strain (results not shown).

DISCUSSION

Although the *spoIIA* and *spoVA* operons are closely linked and have been cloned as a single insert in phage $\phi 105$ (Savva & Mandelstam, 1984), they are transcribed at quite different times. The mRNA corresponding to *spoIIA* becomes detectable at t_1 and the signal in 'northern' blot experiments is at full strength by $t_{1.5}$. The transcript is roughly 1.7 kb in size, which is what would be expected if the three genes of *spoIIA*, coding for a total of 507 amino acid residues (divided between three polypeptides) (Fort & Piggot, 1984), gave rise to a single polycistronic message. An unexpected finding was the appearance at t_3 of a second and larger species of mRNA molecule (about 2.6 kb) which also hybridized with the *spoIIA* probe (see Fig. 3). This observation remained puzzling until S1 nuclease studies indicated the presence of a second promoter about 1 kb upstream from the first (D. Savva, unpublished); it therefore seems likely that at t_3 transcription of the operon starts from this second promoter. This may be relevant to the fact that in some strains containing the β -galactosidase gene, *lacZ*, fused into the *spoIIA* operon (Errington & Mandelstam, 1986a), there is a preliminary output of β -galactosidase beginning at t_1 that reaches a plateau at about t_2 and is followed by renewed enzyme synthesis at t_3 , about the time that the larger mRNA species becomes detectable.

Transcription of the *spoVA* operon began at t_3 and was undiminished at t_5 , when the last samples were taken. Experiments with strains containing a *spoVA/lacZ* fusion showed that the messenger for this operon is also translated without delay (Errington & Mandelstam, 1986b). It is worth noting that the defect in spore development resulting from mutations in this locus only becomes manifest about 2 h after the appearance of the translation products. This is in keeping with the earlier findings of Dion & Mandelstam (1980) and Jenkinson *et al.* (1981), which showed that the spore properties associated with stages V and VI result from the self-assembly of proteins made at earlier stages.

In view of the report (Kerjan *et al.*, 1982) that sporulation involves the production of polyadenylated mRNA, we looked for polyadenylation in the messenger molecules for both *spoIIA* and *spoVA* and did not find it in either. It thus appears that polyadenylation is not an invariable property of sporulation mRNA molecules.

The very rough estimates of half-lives show that these are different for the two species of mRNA, being very approximately 2–3 min for *spoIIA* mRNA and rather longer for *spoVA* mRNA. It is of interest in this context that Segall & Losick (1977) have shown that the mRNA for *spoVG* has an extended half-life and is detectable in quantity even after 30 min.

The fact that some species of mRNA have fairly long half-lives, as implied by earlier experiments with actinomycin D (Sterlini & Mandelstam, 1969), raises the possibility that translational control may be a factor in regulation of the sporulation sequence. The evidence, already cited and based on *lacZ* fusions, that translation occurs without delay once the mRNA molecules for these two sporulation operons have been synthesised does not formally rule out the intervention of a translational control step, though it does make it less likely.

We studied the transcription of the two operons in mutants blocked at various stages; *spoIIA* was transcribed poorly or not at all in all the types of *spoO* mutants that were tested (Table 1). By contrast, transcription at the normal rate was found in all the stage II mutants tested except for the mutant carrying *spoIIA4*, which is a deletion of the whole operon. Transcription also took place normally, as might have been expected, in mutants blocked at later stages. Similarly, *spoVA* was transcribed poorly or not at all in mutants blocked at stages up to and including stage III, but the transcript was found in normal amounts in all the stage IV, stage V and stage VI mutants tested in the mutant carrying *gerE36*.

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