Identification of the promoter and the transcriptional start site of the *spoVA* operon of *Bacillus subtilis* and *Bacillus licheniformis*

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The region upstream of the coding sequence of the *spoVA* operon was studied by several techniques to identify the promoter and to determine the start point for transcription of *spoVA*. The results of plasmid integration analysis in *Bacillus subtilis* showed that no more than 119 bp upstream of the coding sequence is needed for expression. A comparison of the sequence of this upstream region with the corresponding sequence from *Bacillus licheniformis* showed four stretches that were perfectly conserved, interspersed with poorly conserved stretches; the second and third of these conserved stretches appeared to represent the '–35' and '–10' regions of a promoter recognized by RNA polymerase containing σ^G . Primer extension analysis in *B. subtilis* revealed a *spoVA* transcript which had apparently initiated 6 bp downstream of the putative '–10' heptanucleotide CATACTA, that is, 27 bp upstream of the coding sequence. This transcript was observed 4 h and 5 h after the initiation of sporulation, but not at earlier times.

Introduction

The formation of endospores in bacilli requires the temporally regulated expression of several dozen genes. One of the mechanisms that ensures the correct regulation of expression is the sequential synthesis of a number of sigma factors, which interact successively with the core RNA polymerase. Each sigma factor, in combination with core RNA polymerase, recognizes its cognate promoters and hence leads to transcription of a specific set of genes, and each set of promoters is presumed to differ in nucleotide sequence from the other sets.

One genetic locus that regulates sporulation is *spoIIA*, which both of our laboratories have studied extensively. The *spoIIA* locus comprises three genes (Fort & Piggot, 1984; Piggot *et al.*, 1984), and is expressed in *Bacillus subtilis* within an hour of the onset of sporulation, from a promoter that resembles promoters recognized by RNA polymerase containing σ^H (Wu *et al.*, 1989). In *B. licheniformis*, the three genes of *spoIIA* are identical in length, and very similar in sequence, to those of *B.*

subtilis, and the putative promoter is precisely conserved between the two species (Yudkin *et al.*, 1989). About 120 bp downstream of *spoIIA* in *B. subtilis* lies another sporulation locus, *spoVA*. There are several reasons for believing that in *B. subtilis* *spoVA* is transcribed separately from *spoIIA*: first, experiments with integrational plasmid vectors suggest that the two loci belong to different transcriptional units (Piggot *et al.*, 1984); secondly, the sequence immediately downstream of *spoIIA* includes what appears to be a terminator of transcription (Wu *et al.*, 1989; Yudkin *et al.*, 1989); thirdly, results of experiments with fusions of *spoVA* to *lacZ* show that *spoVA* is expressed much later in sporulation than *spoIIA* (Errington & Mandelstam, 1986); fourthly, expression of *spoVA*, unlike that of *spoIIA*, must take place in, and is confined to, the prespore compartment (Errington & Mandelstam, 1986; Gholamhosseini & Piggot, 1989); fifthly, the size of *spoIIA* mRNA estimated from Northern blots agrees with that predicted for a tricistronic *spoIIA* message (Savva & Mandelstam, 1986). We now describe experiments whose results have identified the startpoint of transcription of *spoVA*, and show that the *spoVA* promoter, which is identical in sequence in *B. subtilis* and *B. licheniformis*, resembles promoters that are known to be recognized by RNA polymerase containing σ^G .

The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession number X53991.

Methods

Bacterial strains. The *Escherichia coli* strain used was DH5 α [F⁻endA1 hsdR17 (r_K⁻m_K⁺) supE44 thi-1 λ -recA1 gyrA96 relA1 Δ (lacZYA-argF)U169 Φ 80dlacZ Δ M15]. The *B. subtilis* 168 strain used was MB24 [trpC2 metC3 rif-2]. *B. subtilis* strains containing a single copy of an integrative plasmid were constructed by transformation with a plasmid containing the chloramphenicol drug resistance marker, and maintained on media containing 3–5 μ g chloramphenicol per ml⁻¹.

Plasmids. Plasmids were maintained in *E. coli* DH5 α unless otherwise noted. Plasmid pPP151 contains a 952 bp fragment covering the 3' end of *spoIIAC* and the 5' end of *spoVAA*. It was constructed by cloning the 952 bp PvuII fragment of pPP33 (Piggot *et al.*, 1984) into the *Sma*I site of pUC19. Plasmid pPP170 was constructed by cloning a 2.2 kb PstI fragment of pPP33, extending from the putative terminator for *spoIIA* to the middle of *spoVAD*, into the PstI site of pJH101 (Ferrari *et al.*, 1983). A pUC13 clone carrying the *spoIIA* region, and part of *spoVA*, from *B. licheniformis* was described by Yudkin *et al.* (1989).

DNA preparation. Plasmid DNA was prepared from *E. coli* by the method of Birnboim & Doly (1979), and, for small-scale preparation, by the method of Ish-Horowicz & Burke (1981).

RNA preparation. RNA from *B. subtilis* was prepared as previously described (Wu *et al.*, 1989).

Primer extension. Primer extension analysis was by the method of Inoue & Cech (1985). Synthetic primers 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. 10 pg labelled primer was mixed with 100 μ g RNA in a final volume of 20 μ l hybridization buffer (0.2 M-KCl, 0.1 M-Tris/HCl [pH 8.3]). The mixture was first heated to 90 °C, and then incubated at 55 °C for 10 min, and on ice for 5 min, to allow for DNA-RNA hybridization. The mixture was then precipitated with 2.5 vol. ethanol, dried, and resuspended in 9 μ l reverse transcription buffer (50 mM-Tris/HCl, pH 8.3, 50 mM-KCl, 6 mM-MgCl₂, 1 mM-dithiothreitol, 1 mM-dNTP's, 2 units RNasin). To this was added 3.5 units avian myeloblastosis virus reverse transcriptase, and the mixture was incubated at 42 °C for 1 h. The reaction was stopped by the addition of 2 μ l loading buffer (80% w/v formamide, 0.1% bromophenol blue, 0.1% xylene cyanol, 1 mM-EDTA). Samples were heated at 75 °C for 2 min, and 3 μ l was loaded on an 8% (w/v) urea-8% (w/v) polyacrylamide sequencing gel for analysis. The same primer was used for DNA sequencing reactions with pPP153 as template, and the products were loaded on the same gels.

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

Results

Previous experiments with integrative plasmids had suggested that *spoVA* is in a different transcriptional unit from *spoIIA* (Piggot *et al.*, 1984). We therefore delimited the 5' end of the *spoVA* transcriptional unit of *B. subtilis* by the use of a new integrational plasmid. Plasmid pPP170 contains the 2250 bp PstI fragment that extends from 119 bp upstream of the *spoVA* coding sequence to the middle of the fourth ORF in *spoVA*. Chloramphenicol-resistant transformants of *B. subtilis* MB24 were obtained in which pPP170 had integrated by a Campbell-like mechanism (as confirmed by Southern analysis of appropriately restricted chromosomal DNA). These transformants sporulated normally. This indicated that

the cloned region in pPP170 extends outside the *spoVA* transcription unit, indicating that no more than 119 bp upstream of the coding sequence is needed for expression of *spoVA*. We then examined this upstream region.

Yudkin *et al.* (1989) isolated a clone from *B. licheniformis* that contained the *spoIIA* region. Sequencing showed that the third gene of the region was succeeded by a sequence that resembled a strong transcription terminator. We have now obtained the sequence of a further 150 bp downstream of *spoIIA*: this sequence is compared with the corresponding region of *B. subtilis* in Fig. 1. We note the following features.

The last 51 nucleotides shown begin with ATG and represent an open reading frame whose putative translation product is extremely similar to that at the start of *spoVA* in *B. subtilis*. Given this similarity, and the fact that, as in *B. subtilis*, the open reading frame begins about 100 bp downstream of *spoIIA*, we assume that we have sequenced the beginning of the *spoVA* region of *B. licheniformis*. Five to 14 bp upstream of the *B. licheniformis* open reading frame lies a sequence whose transcript could function as a ribosome binding site: base-pairing with the 3' end of 16S RNA would give a structure with free energy of about -14.5 kcal mol⁻¹ (60.1 kJ mol⁻¹) according to the rules of Tinoco *et al.* (1973).

Between the apparent terminator of *spoIIA* and this putative ribosome binding site lie four stretches of sequence that are perfectly conserved within the two species, interspersed with stretches in which the two sequences differ. Since we presume that the *spoVA* promoter will lie in this region, we wondered whether a '-35' and a '-10' sequence could be found among the four conserved stretches.

We therefore determined the initiation site of transcription of *spoVA* in *B. subtilis* by primer extension analysis. We extracted RNA from cultures during exponential growth, at the end of exponential growth (which we define as T₀, the point at which sporulation is induced), and hourly thereafter. Samples of this RNA were annealed to two primers identical in sequence to 17-base sections of the transcribed strand of the DNA, one representing bases 90 to 74, and the other bases 32 to 16 of the published sequence (Fort & Errington, 1985; their numbering is used); these were then used as primer templates for the AMV reverse transcriptase. The same primers were used to initiate dideoxy sequencing reactions (Sanger *et al.*, 1977) with pPP171 as template, and the products of the different enzyme reactions from each primer were fractionated on urea-polyacrylamide gels.

The results (Fig. 2) show that a transcript corresponding to *spoVA* was present at T₄ and T₅, but not in vegetative growth; we also found no transcript at T₀, T₁, T₂, or T₃ (results not shown). [In *spoVAA::lacZ* fusions

Fig. 1. The sequence downstream of the terminator of *spoIIA*. The nucleotide sequence in heavy type is that of *Bacillus licheniformis*; the first nucleotide shown is immediately 3' of the sequence given in Fig. 5 of Yudkin *et al.* (1989). The amino-acid sequence shown (in heavy type) immediately above the nucleotide sequence is that of the predicted N-terminal region of the product of the *spoVA* gene. The nucleotide and amino-acid sequences in normal type are those reported for the corresponding region of *Bacillus subtilis* (Fort & Piggot, 1984; Fort & Errington, 1985). Dots mark the position of gaps that have been introduced to maximize the homology. The dashed underlining and Roman numerals indicate the regions of nucleotide conservation between the two species of *Bacillus*.

β -galactosidase activity is detectable at T₃ and much increased at T₄ and T₅ (Errington & Mandelstam, 1986).] Experiments with each primer clearly showed only a single transcript, which appeared from its length to have its 5' end at the second nucleotide in region IV shown in Fig. 1, which corresponds to nucleotide 2051 of the published sequence.

In the course of the sequencing reactions, we detected a G (open arrow in Fig. 2) which is not present in the published sequence (Fort & Piggot, 1984).

Discussion

The results show unequivocally that in *B. subtilis* *spoVA* – or, to be more precise, the first gene of *spoVA* – lies in a different transcriptional unit from *spoIIA*. Not only are the two loci expressed at different times during sporulation, but the starting point of the transcript that includes *spoVAA* is well downstream of the terminator that

Fig. 2. Determination of the 5' end of the *spoVA* transcript of *B. subtilis*. Primer extension analysis was carried out on mRNA prepared as described in Methods. RNA was extracted from M24 during vegetative growth (sample V) and during sporulation at T₄ (sample 4) and T₅ (sample 5). The primer used was identical in sequence to bases 90 to 74 of the published sequence (Fort & Errington, 1985). A sequencing ladder using the same primer is also shown. The letters above the lanes indicate which dideoxy nucleotide was used to terminate the sequencing reaction. The putative transcription start site is indicated with a solid arrow. The open arrow indicates the extra G found during sequencing.

follows the final open reading frame (*spoIIAC*) of the *spoIIA* locus. The sequence of *spoVA* gives no indication that the five genes of the locus comprise more than one transcription unit, and experiments with integrative plasmids also suggest that the five are expressed from a single promoter (Fort & Errington, 1985), although the results of Northern blots were not definitive on this point (Savva & Mandelstam, 1986).

Formally, we have identified the 5' end of the *spoVA* mRNA. We consider this to be the transcriptional start point used *in vivo*, as it agrees exactly with the transcription start point obtained *in vitro* with purified E- σ^G (Sun *et al.*, 1989), and there is no evidence of mRNA processing. The identification of the starting point for transcription of *spoVA* allows us to conclude that the sequences TGAATG and CATACTA (regions II and III in Fig. 1) are the '-35' and '-10' recognition sequences of the *spoVA* promoter and that they are separated by a 17 bp 'spacer'. The 5' nucleotide of the mRNA then corresponds to the A located six nucleotides downstream of the CATACTA sequence. These recognition sequences correspond exactly to the consensus sequences of σ^G promoters; and the conclusion that *spoVA* is transcribed by RNA polymerase containing σ^G is consistent with the fact that this form of the enzyme functions specifically to transcribe forespore-specific genes, of which *spoVA* is one, and with the time at which transcription begins (Sun *et al.*, 1989; Nicholson *et al.*, 1989).

The heptanucleotides TGAATGA and CATACTA are perfectly conserved between *B. subtilis* and *B. licheniformis*, but the 17 bp spacer of the two species differs greatly, with only the -15, -16, -18, -26 and -27 nucleotides being identical. We note that in promoters of *Escherichia coli* that are recognized by σ^{70} (the equivalent of σ^A of *B. subtilis*), 17 bp spacers are less well conserved than those with 16 bp or 18 bp (O'Neill, 1989), but it is not clear whether this finding is pertinent to *B. subtilis* promoters, still less to the subset that are recognized by σ^G . It is known from the work of Nakatani *et al.* (1989) that mutations in the spacer region of the *B. subtilis* *gdh* promoter affect utilization by the RNA polymerase; again, a systematic study of other σ^G -specific promoters has not yet been published.

The apparent ribosome binding site, which is located 14–22 bases from the 5' end of the *spoVA* mRNA in *B. licheniformis*, and 12–20 bases from the 5' end in *B. subtilis*, is much less well conserved than the '-35' and '-10' regions. The ribosome binding is, however, strong in both species: our discovery of an additional G in the *B. subtilis* sequence increases the calculated binding energy of the site to $-17.4 \text{ kcal mol}^{-1}$ (72.8 kJ mol^{-1}). Our evidence suggests that the sequence of ribosome binding sites may be able to drift in evolution without much

affecting the expression of their genes. By contrast, '-35' and '-10' regions may be stabilized by the need to interact specifically with one of the several available sigma factors.

Two other regions (I and IV, Fig. 1) are strongly conserved in addition to regions II and III which are the '-35' and '-10' regions. Region IV includes the transcription start point of *spoVA* and the first 7 nucleotides of the transcribed region. The striking conservation of the 20 bp region I, extending from 45 to 64 bp upstream of the deduced *spoVA* transcription start point, leads us to think that the region may be important for the regulation of *spoVA* expression. No homology was detected between region I and the corresponding upstream regions of genes *gerA* (Feavers *et al.*, 1985), *sspA*, *sspB* (Connors *et al.*, 1986), *sspC* (Connors & Setlow, 1985), *sspD* (Connors *et al.*, 1986), and *sspE* (Hackett & Setlow, 1987). In contrast, the '-35' and '-10' regions of these genes share extensive homology with the '-35' and '-10' regions of *spoVA*, and indeed are transcribed by E- σ^G . Thus region I may be a control region that is specific to *spoVA*.

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