Levels of mRNAs which code for small, acid-soluble spore proteins and their *LacZ* gene fusions in sporulating cells of *Bacillus subtilis*

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ABSTRACT

The levels of mRNAs from genes (*sspA*, *B* and *E*) which code for major small, acid-soluble, spore proteins of *Bacillus subtilis* have been determined, as well as the levels of mRNAs from *ssp-lacZ* gene fusions. Increasing the gene dosage of *ssp-lacZ* fusions resulted in parallel increases in both the *ssp-lacZ* mRNA level and the rate of β -galactosidase accumulation. Similarly, an 11-fold increase in *sspE* gene dosage gave a comparable increase in *sspE* mRNA, but at most a 1.5-fold increase in the amount of *sspE* gene product accumulated. In contrast, an 11-fold increase in the dosage of the *sspA* or *B* genes had no significant effect on the level of total *sspA* plus *sspB* mRNA, but did alter the ratios of these mRNAs as well as the amount of their gene products, to reflect the altered ratio of the two genes. These results suggest that intact *ssp* genes, but not *ssp-lacZ* gene fusions, are subject to feedback regulation of gene expression, with this regulation of the *sspA* and *B* genes effected by modulation of mRNA levels, while the feedback regulation of the *sspE* gene is at the post-transcriptional level.

INTRODUCTION

Approximately 10% of the protein of dormant spores of *Bacillus subtilis* is made up of three small, acid-soluble, proteins (SASP) termed SASP- α , β and γ (1). The genes for these three proteins have been cloned and sequenced and have been termed *ssp* genes, with the *sspA* gene coding for SASP- α , the *sspB* gene for SASP- β , and the *sspE* gene for SASP- γ (2,3). SASP- α and β exhibit approximately 85% amino acid sequence identity, and the *sspA* and *B* genes are a part of a multigene family of at least seven members which code for SASP of almost identical amino acid sequence (2). Other genes in this family (*sspC* and

D) are expressed at much lower levels than the sspA and B genes (4,5). The proteins produced by this multigene family, in particular SASP- α and β , play a key role in the resistance of the spore to ultraviolet light, and are associated with spore DNA in vivo (6,7). In contrast, the amino acid sequence of SASP- γ is quite different from those of SASP- α and β , and there is only a single gene for γ -type SASP in B. subtilis (2,3). In addition, SASP- γ plays no role in spore UV resistance (8) and is not associated with spore DNA in vivo (S. Francesconi and P. Setlow, unpublished results).

Despite differences in the structure and function of the three major SASP of B. subtilis, all three proteins are synthesized in parallel during sporulation (1,9,10). The mRNAs for these proteins are undetectable in log phase and early stationary phase cells, but appear only within the developing forespore midway in sporulation and then disappear (2,3,5,10). This data strongly suggests that ssp genes are transcribed for only a brief time during sporulation, and there is strong evidence that synthesis of a new RNA polymerase σ factor during sporulation is essential for ssp gene transcription (D. Sun and P. Setlow unpublished results). However, it seems likely that additional mechanisms are used to regulate ssp genes, since their expression appears subject to some type of feedback control (5,10). Thus, increasing the sspE gene copy number up to 17-fold has no significant effect on the SASP- γ level in spores nor on levels of SASP- α or β (3,8,10). Similarly, increasing the sspA gene dosage does not result in an increase in total SASP- α plus β , but only decreases SASP- β levels in spores with a comparable increase in the spore SASP- α level; increasing the sspB gene dosage has an analogous effect, but an increased sspA or B gene dosage has no effect on SASP- γ levels (5,10). In contrast to the feedback regulation seen with intact ssp genes, ssp-lacZ gene fusions show no such regulation, as the amount of β -galactosidase accumulated is directly proportional to gene dosage (10). At present, there is no understanding of how feedback regulation of ssp gene expression is effected, including whether it is exerted at the transcriptional or translational level. In this report we present measurements of ssp and ssp-lacZ fusion mRNA levels as a function of gene dosage in an attempt to begin to address this question.

MATERIALS AND METHODS

Plasmids and bacterial strains

The parental *B. subtilis* strain used for all work was *B. subtilis* 168. The construction of the *sspA*, *B* and *E-lacZ* gene fusion derivatives in plasmid pJF751, their integration into the *B. subtilis* chromosome and the amplification of these fusions and determination of their copy number have been described previously (10). The cloning of the complete *sspA*, *B* and *E* genes in plasmid pUB110 (11) and the determination of the copy number of these plasmids in *B. subtilis* spores was also described previously (10). Fragments of the *sspA*, *B* and *E* genes were cloned in plasmid pT7-1 (United States Biochemicals) which has a T7 RNA polymerase promoter just prior to the cloning sites. The cloned fragments all contained the amino-terminal part of the *ssp* gene's coding sequence and 150 to 650 nucleotides of upstream sequence. In all cases, the genes were cloned with the most carboxyl terminal portion of the coding sequence adjacent to the T7 promoter in order to generate anti-sense mRNA using T7 RNA polymerase. Specific fragments cloned were: *sspA* gene - a 0.49 kb *EcoR*I-*Hinc*II fragment (2) cloned into *EcoR*I and *Sma*I cut pT7-1; *sspB* gene - a 0.28 kb *EcoR*I-*Hind*III fragment (2) cloned into *EcoR*I and *Hind*III cut pT7-1; and *sspE* gene - a 0.8 kb *EcoR*I-*Hind*III fragment (3) cloned into *EcoR*I and *Hind*III cut pT7-1. These plasmids as well as pJF751 were grown in *Escherichia coli* RR101 and plasmid DNA isolated and purified as previously described (2).

Enzymes and reagents

Restriction enzymes, DNA ligase and the large fragment of *E. coli* DNA polymerase were obtained from New England Biolabs. Deoxynucleoside triphosphates, *E. coli* tRNA and S1 nuclease were obtained from the Sigma Chemical Company, and T7 RNA polymerase from the United States Biochemical Company. [α - 32 P]dATP (2000 Ci/mmol) and [α - 32 P] ATP (800 Ci/mmol) were obtained from New England Nuclear.

Sporulation, cell harvest, and RNA extraction and purification

B. subtilis strains were grown and sporulated at 37°C in 2 x SG medium (12) with chloramphenicol (3 μ g/ml for single copy *ssp-lacZ* fusions, and 30-50 μ g/ml for amplified *ssp-lacZ* fusions) and

kanamycin (10 µg/ml for strains carrying pUB110 derivatives) as needed (10). The use of ssp-lacZ fusion strains for all experiments allowed determination not only of the onset of ssp gene expression, but also allowed comparison of results with different strains. As sporulation proceeded, samples (1-ml) of strains carrying various ssp-lacZ fusions were harvested by centrifugation and permeabilized and assayed as described previously (10), except that lysozyme was at 250 µg/ml, and these preliminary assays were only for five min. Immediately after the time at which β-galactosidase activity was first detected, 1- and 25-ml samples of culture were taken every 45 min for the next 3 hr, the time during which the rate of β-galactosidase accumulation was found to be maximal. The 1-ml samples were centrifuged, the pellets frozen and stored at -20°C prior to assays for β-galactosidase (10) to determine the amount of enzyme accumulated at the times when samples were collected for RNA extraction. The 25-ml samples were added to crushed ice in 40-ml centrifuge tubes containing sufficient MgCl₂ and NaN₃ to give final concentrations of 1 and 10 mM respectively (13). Samples were centrifuged in a pre-cooled SS-34 rotor for 4 min at 8,000 rpm, the pellets frozen at -70°C and lyophilized.

RNA was extracted from lyophilized sporulating cells by dry rupture for 2 min in a dental amalgamator (Wig-L-Bug) with 100 mg glass beads as abrasive (13). The dry powder was dissolved in a 6 M guanidinium thiocyanate solution and RNA was purified by density gradient centrifugation with CsCl (14). The final RNA pellets were dissolved in diethylpyrocarbonate treated sterile H₂O and stored at -70°C.

Quantitation of mRNA levels

mRNA levels were quantitated by three different procedures - dot blots, Northern blots, and quantitative S1 nuclease protection assays. Dot blots were carried out using a 96 well manifold from Bethesda Research Laboratories. For each strain tested, RNA extracted from three different time points in sporulation was analyzed; for each time point, duplicate samples of three different amounts of RNA over a 9-fold concentration range were tested. RNA samples were treated with glyoxal, transferred to Nytran paper (Schleicher and Schuell) and the paper baked and hybridized for 18 hr to the appropriate probe as described by Thomas (15). The paper was washed (15), dried and

autoradiographed and the hybridizing dots cut out and counted in a scintillation counter.

For Northern blots two different amounts of RNA (usually 0.7 and 3 µg) from at least two different time points of cell harvest were treated with glyoxal, run in duplicate on 1 or 2% agarose gels, blotted to Nytran paper (Schleicher and Schuell), treated, hybridized, and washed as described previously (4,15). Again hybridizing bands were cut from the paper and counted in a scintillation counter. In experiments where the effect of gene dosage on ssp gene mRNA levels was to be measured, two different amounts of RNA from the single copy ssp gene strain were included in all gels to provide an internal standard.

Measurement of mRNA levels by quantitative S1 nuclease protection assays were performed essentially as described by Quarless and Heinrich (16). In these experiments five different quantities of RNA encompassing a ten-fold concentration range were hybridized to a constant amount of gel-purified ³²P-labelled antisense RNA probe (~2000 cpm). Quantities of RNA were chosen in order to obtain a linear plot of protected cpm vs RNA added. For all strains analyzed, at least two RNA samples collected at different times in sporulation were measured in parallel with two RNA samples collected similarly from the wild type strain.

Using the wild type strain carrying the sspA-lacZ fusion, we also measured the relative amounts of mRNA from different ssp genes using both Northern blot analysis and quantitative S1 nuclease protection. For these assays all RNA probes were synthesized with the same ³²P-ATP, and all incubations, both for hybridization and nuclease protection, contained the same number of probe molecules. Following measurement of the hybridized or protected cpm (15,16), values obtained for different ssp genes were corrected relative to each other for differences in the number of AMP residues in either the whole probe (Northern blot analysis) or the protected region of the probe (nuclease protection).

The probes used for lacZ fusion mRNAs were: a) 5'-region of the lacZ gene - an 0.83 kb ClaI-EcoR1 fragment from plasmid pJF751 (17); and b) 3'-region of the lacZ gene- a 0.54 kb AvaI fragment from plasmid pJF751. These fragments were isolated by electrophoresis on agarose gels and labeled as described by Feinberg and Vogelstein (18). The ssp gene probes used were all antisense RNA probes synthesized by

T7 RNA polymerase using as a template HindIII (sspA and B genes (2)) or HincII (sspE gene (3)) linearized plasmid pT7-1 DNA carrying the appropriate ssp gene fragment cloned as described above. The length of the runoff antisense RNAs for each of the genes is: a) sspA-301 nucleotides of which 111 hybridize to sspA mRNA; b) sspB - 284 nucleotides of which 182 hybridize to sspB mRNA; and c) sspE - 283 nucleotides of which 195 hybridize to sspE mRNA (B. Setlow and P. Setlow, unpublished results). These probes were purified by electrophoresis on 2% agarose gels prior to use.

Isolation of spores and extraction and analysis of SASP

Spores of various strains carrying pUB110 and its derivatives were produced as described previously in 2 x SG medium (12). After 48 hr of sporulation, aliquots (25-ml) were harvested by centrifugation, washed twice with distilled water and the pellet lyophilized. The spores were then dry-ruptured, SASP extracted, dialyzed, separated by polyacrylamide gel electrophoresis and gels stained and immunoblotted and SASP from equivalent amounts of spores quantitated as described previously (5,6,10).

RESULTS

Choice of RNA samples to be analyzed

A major problem we faced in trying to compare levels of ssp- and ssp-lacZ fusion mRNAs in different strains was that the levels of these RNAs change drastically during growth and sporulation (2,3,4). Consequently, we needed some internal measurement to allow us to compare mRNA levels from cells at the same point in sporulation. The measurement we chose to use was the rate of β -galactosidase synthesis in an ssp-lacZ fusion, since previous work (10) has shown that: a) during sporulation the timing of synthesis of β -galactosidase from all ssp-lacZ fusions is identical and parallels SASP synthesis; and b) variation in the dosage of the sspA, B or E genes has no effect on the synthesis of β -galactosidase from any ssp-lacZ fusion. Furthermore, examination of the rate of accumulation of β -galactosidase from a number of single copy and multicopy ssp-lacZ fusions indicated that there was a period in sporulation of at least 1-1/2 hrs when the rate of accumulation of enzyme was both high and constant (Fig. 1 and data not shown). This suggests that during this period there was a constant rate of enzyme synthesis, and thus a constant level of ssp-lacZ mRNA.

Fig. 1. Rates of β -galactosidase accumulation in strains carrying various ssp-lacZ fusions. Strains were grown and samples harvested during sporulation and assayed for β -galactosidase. The three arrows denote times during which the rates of β -galactosidase accumulation are constant, and thus the times when samples were isolated for extraction and analysis of mRNA levels. The symbols used are: $\blacktriangle$ - sspE-lacZ fusion strain carrying 17 copies of the fusion; $\square$ - single copy sspB-lacZ fusion strain; $\bullet$ - single copy sspE-lacZ fusion strain; and Δ - single copy sspA-lacZ fusion strain.

Indeed, with any one ssp-lacZ fusion strain, when levels of lacZ mRNA were quantitated at three times when the rate of β -galactosidase accumulation was constant, these levels differed by less than twenty percent (Table 1). Since β -galactosidase and SASP accumulation take place in parallel during sporulation (10), this finding further suggested that we could use the rate of β -galactosidase synthesis at the time of

Table 1
Rate of accumulation of β -galactosidase and level of ssp-lacZ fusion mRNAs in various strains of *B. subtilis*^a

<u>ssp-lacZ</u> fusion ^b	Rate of β -galactosidase accumulation Miller units/ml culture/min ^c	<u>ssp-lacZ</u> mRNA units/ml culture ^d
<u>sspA</u> (1)	15	1.0 ^e
<u>sspA</u> (10)	175	9.9
<u>sspB</u> (1)	135	0.8
<u>sspE</u> (1)	20	0.8
<u>sspE</u> (17)	200	8.7

^aCells were grown and samples harvested and RNA extracted, purified and analyzed for lacZ mRNA level by dot blots using the 5'-lacZ probe.

^bValues in parentheses are the copy numbers of the ssp-lacZ fusion genes.

^cThese values are the constant rate of β -galactosidase accumulation as shown in Fig. 1, and are the result of duplicate assays of at least five time points in sporulation.

^dThese are the averages of the relative lacZ mRNA levels in cells harvested at three different times during the period of constant β -galactosidase accumulation (arrows, Fig. 1). Three different aliquots of each RNA sample were assayed in duplicate. Average values for different time points differed by less than 20%.

^eThe amount of lacZ mRNA in this strain was arbitrarily given a value of 1 unit/ml of culture.

cell isolation to normalize the mRNA levels from different strains carrying the same ssp-lacZ fusion. However, the correction required for normalization between different strains was small, because for the fusion we chose for use (single copy sspA-lacZ fusion), the high constant rate of β -galactosidase synthesis was very similar ($\pm 15\%$) during sporulation of all strains tested.

RNA isolation

Prior to quantitation of ssp and ssp-lacZ mRNA levels, we compared a number of procedures for isolation of RNA from sporulating cells, including dry rupture followed by phenol extraction (13), lysozyme disruption followed by phenol extraction, and dry rupture followed by dissolving in 4 M or 6 M guanidinium thiocyanate and subsequent density gradient purification (14). The quality of the RNA was then assessed by Northern blot analysis using both the 5'-lacZ probe and the sspE probe. In terms of both total RNA yield as well as

Fig. 2. Northern blots of RNA samples from various strains. RNA was isolated from cells exhibiting both high and essentially identical rates of β -galactosidase synthesis during sporulation (Fig. 1, arrows). Three μ g of purified RNA was run on agarose gel electrophoresis and transferred to Nytran paper and treated and hybridized as described in Materials and Methods. **Lane a** - the RNA is from the *sspA-lacZ* fusion strain and was run on a 1% agarose gel and the blot hybridized with the 5'-*lacZ* probe. The arrows marked 23S and 16S beside lane **a** mark the positions of these rRNAs which can be seen as light bands in the hybridizing *lacZ*-mRNA; intact *sspA-lacZ* mRNA should be 4700 nucleotides long. **Lane b** - the RNA is identical to that in lane **a**, but was run on a 2% agarose gel and the blot probed with the purified *sspE* antisense RNA probe. **Lane c** - the RNA is from the *sspA-lacZ* fusion strain carrying pUB110 with the *sspE* gene thus increasing the *sspE* gene's copy number 11-fold; again this RNA was run on a 2% agarose gel and the blot was probed with the *sspE* antisense RNA probe. The abbreviation used is nt - nucleotides.

the highest yield of full length *sspA-lacZ* fusion and *sspE* mRNA, the procedure using dry rupture followed by dissolving in 6 M guanidinium thiocyanate was by far the best (data not shown). With this procedure *sspE* mRNA was 340 nucleotides long with little smaller hybridizing material as found previously (3) (Fig. 2, lanes b and c); similar results were found for *sspA* and *B* mRNA (data not shown), although these mRNAs had different sizes than *sspE* mRNA (2,3). However, even with this RNA isolation procedure, much of the *sspA-lacZ* fusion mRNA was

not full length (Fig. 2, lane a). Since the 3'-lacZ probe gave the same distribution of hybridizing material as seen with the 5'-lacZ probe (data not shown), the shorter lacZ mRNA does not seem to arise from incomplete synthesis, and presumably represents partially degraded mRNA. While we do not know when this degradation took place, we expected the large sspA-lacZ fusion mRNA (~ 4700 nucleotides) to be more sensitive to degradation than the smaller sspE mRNA, in particular to possible nicking during RNA isolation. While the presence of so much degraded lacZ fusion mRNA was not ideal, we did find that the proportion of this smaller hybridizing material was quite similar with the different ssp-lacZ fusions as well as in samples taken at different times in sporulation (data not shown). These findings give us confidence that quantitation of total lacZ mRNA by dot blots is an accurate measure of total ssp-lacZ mRNA *in vivo*.

Quantitation of ssp-lacZ fusion mRNAs

Measurement of the relative levels of lacZ mRNA from the sspA, B and E fusions indicated that there were similar amounts of all three mRNAs in the single copy strains, but that much more enzyme was produced from the sspB-lacZ fusion mRNA (Table 1) (see Discussion). Strikingly, when strains carrying higher copy numbers of the sspA- and sspE-lacZ fusions were analyzed, there was good agreement between the increase in the rate of β -galactosidase synthesis in the high copy number strains and the increase in the level of lacZ mRNA (Table 1). A high copy number sspB-lacZ fusion strain could not be analyzed because this strain does not sporulate (10). It is known that the amount of β -galactosidase accumulated in spores of high copy number ssp-lacZ-fusion strains relative to the amount in the single copy strains is directly proportional to the increase in gene dosage (10). Consequently, the parallel increases in the rate of β -galactosidase synthesis and the level of lacZ mRNA in the high copy number strains is consistent with this finding. The reason that the high copy number sspE-lacZ fusion strain does not exhibit a 17-fold increase in both the level of lacZ mRNA and the rate of β -galactosidase synthesis is that the efficiency of sporulation is significantly decreased in this strain (data not shown).

Quantitation of ssp mRNA levels

As was found with the various ssp-lacZ gene fusions, an increase in the sspE gene dosage from one to eleven copies resulted in an almost parallel increase in the level of sspE mRNA as determined by Northern

Table 2
Northern blot measurements of ssp mRNA levels in strains with varying ssp gene dosage^a

<u>B. subtilis</u> <u>sspA-lacZ</u> strain also carrying	<u>sspA</u>	<u>sspB</u>	<u>sspE</u>
	mRNA level-units/ml culture		
pUB110	1.0	1.0	1.0
pUB 110 with <u>sspA</u> gene (10) ^b	1.6	0.4	-
pUB110 with <u>sspB</u> gene (10) ^b	0.4	1.8	0.9
pUB110 with <u>sspE</u> gene (10) ^b	1.1	-	7.8

^a RNAs were extracted from sporulating cells in which the rate of accumulation of β -galactosidase was constant (arrows, Fig. 1) and RNA extracted, purified and quantitated by Northern blot analysis. All mRNA levels are expressed relative to the level of that mRNA in the strain carrying pUB110; these levels were given a value of 1 unit/ml culture. Values have also been corrected for slight differences in the constant rates of β -galactosidase accumulation in the different cultures. This difference was small (< 15%) between different cultures.

^b Values in parentheses are the copy numbers of these plasmids in spores relative to the chromosomal gene's copy number (10).

blot analysis (Fig. 2, lanes b and c, and Table 2). This increase in sspE mRNA level was also evident upon analysis of these samples by quantitative S1 nuclease protection (Table 3). These latter results are particularly compelling because of the precision of this technique (Fig. 3). In contrast, an 11-fold increase in the sspA gene dosage gave only a 1.4- to 1.6-fold increase in the sspA mRNA level, but was accompanied by a 2.5- to 4-fold decrease in the level of sspB mRNA depending on the method used for analysis (Tables 2 and 3). Analogous results were obtained when the sspB gene copy number was increased 11-fold (Tables 2 and 3). For all ssp genes examined, the amounts of mRNA found in a single strain at the two to three time points analyzed (arrows, Fig. 1) were within 20% as was described above for the lacZ mRNA (data not shown). Similarly, the size of the various ssp mRNAs as

Table 3
S1 nuclease protection measurements of ssp mRNA levels in strains
with varying ssp gene dosage^a

<u>B. subtilis</u> <u>sspA-lacZ</u> strain also carrying	<u>sspA</u>	<u>sspB</u>	<u>sspE</u>
	mRNA level-units/ml culture		
pUB110	1.0	1.0	1.0
pUB110 with <u>sspA</u> gene (10)	1.4	0.2	-
pUB110 with <u>sspB</u> gene	0.3	2.3	-
pUB110 with <u>sspE</u> gene (10)	-	-	14.6

^aThe RNA samples analyzed in Table 2 were analyzed by quantitative S1 nuclease protection for determination of various ssp mRNA levels. All mRNA levels are expressed relative to the level of that mRNA in the strain carrying pUB110; these levels were given a value of 1 unit/ml culture. Values were also corrected for slight differences in the constant rate of b-galactosidase accumulation in the different cultures. This difference was small (< 15%).

^bValues in parentheses are the copy numbers of these plasmids in spores relative to the chromosomal gene's copy number (10).

determined by Northern blot analysis was identical in both high and low copy number strains (Fig. 2, lanes b and c, and data not shown).

The response of ssp mRNA levels to gene dosage was further compared to the level of gene product accumulated - i.e. the amount of SASP accumulated in the mature spore (Table 4). Strikingly, the increased sspE gene dosage and mRNA level resulted in at most a 50% increase in the spore level of SASP- γ (Table 4). However, the alteration in sspA/sspB mRNA ratio seen upon variation of the sspA/sspB gene dosage ratio was reflected in comparable variations in the ratio of SASP- α /SASP- β in mature spores (Table 4) (5,10).

By using probes of identical specific radioactivity it was also possible to determine the relative amounts of different ssp mRNAs in the same strain (Table 5). The level of sspA mRNA was highest with sspE and B mRNA levels ~ 2- and 5-fold lower, respectively.

Fig. 3. Quantitative S1 nuclease protection assay of the *sspE* mRNA level in strains with one or eleven copies of the *sspE* gene. The *sspA-lacZ* fusion strain and the *sspA-lacZ* fusion strain carrying pUB110 with the *sspE* gene were grown and sporulated. RNA was extracted from cells exhibiting high and essentially identical rates of β -galactosidase synthesis (see Fig. 1) and purified. Various amounts of these RNAs were mixed with a constant amount of *sspE* antisense RNA probe, hybridized, treated with S1 nuclease, and the protected counts quantitated. The slopes of lines obtained in these and similar experiments were used to calculate the relative levels of *ssp* mRNAs. The amount of probe which remained undigested with addition of no RNA was ~ 2% of the total and this value has been subtracted from the data prior to plotting.

DISCUSSION

For the three intact *ssp* genes we examined, the *sspE* mRNA appeared to be translated most efficiently, with the most SASP- γ synthesized per unit of mRNA (Table 5). The reason for the different translation efficiencies of the *ssp* mRNAs are not clear, but may be related to differences in the numbers of nucleotides preceding the ribosome binding sites as well as the nucleotide sequences in and around the ribosome binding sites of the different *ssp* mRNAs (2,3; and B. Setlow and P. Setlow, unpublished results). Strikingly, the ratio of the amount of *sspA-* to *sspE-lacZ* fusion mRNA was similar to the ratio of the intact mRNAs (compare Tables 1 and 5), and the relative translation efficiencies of the intact and *lacZ* fusion mRNAs from these two genes were within a factor of two of each other. However, there was more *sspB-lacZ* fusion mRNA present than was expected based on

Table 4

Levels of SASP in spores of strains with varying *ssp* gene dosage^a

<i>B. subtilis</i> <i>sspA-lacZ</i> strain		Relative SASP level in spores			
also carrying		SASP- α	SASP- β	SASP- $\alpha+\beta$	SASP- γ
pUB110		100	40	140	170
pUB+10 with <i>sspA</i> gene (10) ^b		160	5	165	180
pUB110 with <i>sspB</i> gene (10) ^b		30	100	130	160
pUB110 with <i>sspE</i> gene (10) ^b		95	40	135	230

^aStrains were grown and sporulated, SASP extracted from equivalent amounts of spores and quantitated as described in Materials and Methods. The value for SASP- α in spores with pUB110 was set at 100 and all other values are expressed relative to this number (10).

^bValues in parentheses are the copy numbers of these plasmids in spores relative to the chromosomal gene's copy number (10).

Table 5

Relative level of *ssp* mRNAs in the *sspA-lacZ* strain^a

Gene tested	mRNA level ^b units/ml culture	SASP level in spores ^c units/ml culture	Ratio SASP/mRNA
<i>sspA</i>	4.7(3.8,5.6)	100(a)	21
<i>sspB</i>	1.0	40(b)	40
<i>sspE</i>	2.5(2.8,2.2)	170(g)	68

^aThe *sspA-lacZ* fusion strain was sporulated and RNA extracted at three times in sporulation when the rate of β -galactosidase synthesis was constant (arrows, Fig. 1). The relative levels of various mRNAs were determined in these samples by Northern blot analysis and S1 nuclease protection and values corrected as described in Materials and Methods. A separate culture was allowed to sporulate completely, and SASP extracted, separated and quantitated.

^bThese values are expressed relative to the level of *sspE* mRNA which was set at 1 unit/ml culture, and are an average of the values determined by the two procedures. The numbers in parentheses are the values determined by Northern blot analysis and S1 nuclease protection respectively.

^cThese values are expressed relative to the level of SASP- α in spores, which was set at 100 units/ml of culture.

the levels of the intact ssp mRNAs. Similarly the sspB-lacZ mRNA generated more β -galactosidase than would be predicted based on the amount of SASP- β produced from sspB mRNA. Again, the reasons for these anomolous results are not clear, but may be related to the fact that the sspB gene generates an mRNA with an 80 nucleotide leader sequence prior to the ribosome binding site (2, and B. Setlow and P. Setlow, unpublished results). In contrast, sspA and E mRNAs have only 2 nucleotides before the ribosome binding site (2,3; and B. Setlow and P. Setlow, unpublished results).

While different ssp-lacZ fusion mRNAs gave different rates of b-galactosidase accumulation, for at least two of the ssp-lacZ fusions (sspA and E) gene dosage experiments indicated that the rates of b-galactosidase accumulation from these fusions were directly proportional to the level of lacZ mRNA over at least a 10-fold range. Since forespore proteins such as SASP do not turn over significantly (19), it seems reasonable to conclude that the rates of b-galactosidase synthesis are also directly proportional to the levels of lacZ mRNA, and that these levels respond directly to the gene dosage of ssp-lacZ fusions. Consequently, these ssp-lacZ fusion genes show no feedback control at either the transcriptional or post-transcriptional level. This further suggests that if ssp gene transcription is turned on by a positive control mechanism (10), then there must be a significant excess of any required positive control factors.

As was found with the ssp-lacZ fusions, the levels of sspE mRNA also increased in direct proportion to gene dosage. However, for this gene there was only a slight increase in the amount of gene product accumulated for an 11-fold increase in level of sspE mRNA. Consequently, SASP-g levels are subject to feedback regulation at the post-transcriptional level. One can imagine a number of mechanisms to accomplish this, including an increased degradation of SASP-g in the high copy number strain as well as some type of translational control of SASP- γ synthesis. However, any type of translational control, if it exists, would probably involve that portion of the sspE mRNA not present in the sspE-lacZ fusion, since the latter exhibits no feedback regulation.

As was found with the intact sspE gene, the sspA and B genes also exhibit feedback regulation. However, these latter genes are reciprocally regulated while sspE gene dosage has no effect on

synthesis of SASP- α or β and vice-versa (10). This reciprocal regulation of the sspA and B genes is accomplished by modulation of the level of mRNA such that the total amount of sspA plus B mRNA, in terms of its ability to generate SASP, remains relatively constant. Again one can imagine various mechanisms to effect this regulation, including control of both mRNA synthesis and degradation. While control at the level of mRNA synthesis is certainly possible, the findings that an increased gene dosage of intact sspA or B genes does not affect levels of β -galactosidase synthesized from sspA or B-lacZ fusions, and vice versa (10) are difficult to reconcile with such a possibility. This suggests that regulation of sspA or B mRNA degradation rates might be the mechanism whereby the feedback regulation of these genes is effected. Interestingly, the one study of the stability of sspA and B mRNAs in wild type B. subtilis found them to be significantly more stable than other B. subtilis mRNAs (20). Recent work on the regulation of histone mRNA levels in eukaryotic cells has indicated that the levels of these mRNAs are subject to feedback regulation by histones themselves, which appear to act by increasing the rates of degradation of their own mRNAs (21). Since like histones, both SASP- α and β , (but not SASP- γ !) are associated in vivo with DNA (7,22), it is tempting to speculate that a similar mechanism may regulate sspA and B mRNA levels. Studies on the rates of degradation of sspA, B and E mRNAs in various strains are currently in progress.

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