Role of interactions between SpoIIAA and SpoIIAB in regulating cell-specific transcription factor σ^F of *Bacillus subtilis*

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Genetic experiments have suggested that σ^F , the first compartment-specific transcription factor in sporulating *B. subtilis*, is regulated by an anti- σ factor SpoIIAB and an anti-anti- σ factor SpoIIAA. Previously, we reported biochemical results demonstrating that SpoIIAB is both a phosphokinase whose substrate is SpoIIAA and an inhibitor of σ^F -directed transcription. We now show that in the presence of SpoIIAB and ATP or ADP, SpoIIAA can undergo two alternative reactions. When ATP is present, SpoIIAA is phosphorylated rapidly and completely to SpoIIAA-phosphate, and SpoIIAB is immediately released; but in the presence of ADP, SpoIIAA forms a long-lasting complex with SpoIIAB. ADP is an inhibitor of the phosphorylation by ATP. Furthermore, we have mutated SpoIIAA at residue Ser 58, the target for phosphorylation, to aspartate or alanine. SpoIIAAS58D, which apparently resembles SpoIIAA-phosphate, is unable to make a complex with SpoIIAB and is devoid of anti-anti- σ^F activity, whereas SpoIIAAS58A, which cannot be phosphorylated, makes complexes with SpoIIAB in the presence of ADP or ATP and has constitutive anti-anti- σ^F activity both in vivo and in vitro. It seems likely that the alternative reactions of SpoIIAA and SpoIIAB, involving ADP or ATP, regulate the anti-anti- σ capacity of SpoIIAA and hence the activity of σ^F .

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The establishment of different patterns of transcription in sister cells is a basic problem of development. Spore formation in *Bacillus subtilis* provides a simple system in which such problems are amenable to study at the molecular level (Errington 1993). Soon after the initiation of sporulation, an asymmetrical cell division produces a smaller prespore and a larger mother cell. The two cells immediately initiate different programs of transcription as a result of the cell-specific activation of transcription factors, σ^F in the prespore and σ^E in the mother cell (Errington et al. 1990; Driks and Losick 1991; Margolis et al. 1991; Losick and Stragier 1992; Errington 1993). These factors redirect the catalytic portion of RNA polymerase (E) to new sets of promoters, appropriate to the differentiating cell types. Although σ^F and σ^E are encoded by genes that are active before asymmetrical division (Gholamhosseinian and Piggot 1989; Min et al. 1993; Partridge and Errington 1993), their products

become active only after the division, and then only in a compartment-specific manner (Driks and Losick 1991; Margolis et al. 1991; Errington and Illing 1992). Because σ^F activity is dependent on that of σ^E (Stragier et al. 1988; Jonas and Haldenwang 1989), Losick and colleagues (Margolis et al. 1991; Losick and Stragier 1992) have proposed that σ^F becomes active first, specifically in the prespore. There, it directs the transcription of an as yet unidentified gene, the product of which somehow triggers σ^E activation in the mother cell. If this model is correct, the key event in the establishment of cell-specific transcription is the activation of σ^F in the prespore.

σ^F is encoded by the promoter-distal gene *spoIIAC* in an operon containing three genes (Fort and Piggot 1984; Errington et al. 1985; Sun et al. 1989). Genetic experiments suggest that the product of the second gene in the operon, *spoIIAB*, is an inhibitor of σ^F (i.e., an anti- σ factor) and that the product of the first gene, *spoIIAA*, antagonizes or counteracts the action of SpoIIAB (Schmidt et al. 1990; Margolis et al. 1991; Partridge et al. 1991). These experiments also raised the possibility that this regulatory system could be involved in keeping σ^F inactive in the predivisional cell and the mother cell and in

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releasing σ^F activity in the prespore (Schmidt et al. 1990; Margolis et al. 1991). We and others have shown recently that the purified SpoIIAB protein can inhibit transcription directed by σ^F in vitro (Duncan and Losick 1993; Min et al. 1993); this inhibition apparently depends on binding of SpoIIAB to σ^F (Duncan and Losick 1993). The finding that SpoIIAB catalyzes phosphorylation of SpoIIAA (Min et al. 1993) showed that these proteins can interact directly and suggested that phosphate transfer might be involved in regulating the ability of SpoIIAA to displace SpoIIAB from σ^F and thus liberate the activity of σ^F (Min et al. 1993). Phosphotransferase reactions have been shown to play a crucial role in regulating the initiation of sporulation (Burbulys et al. 1991).

We now report the results of a number of investigations into the role of SpoIIAA. By using site-directed mutagenesis, we have replaced Ser-58 of SpoIIAA with aspartate or alanine. The *spoIIAAS58D* cells are devoid of σ^F activity, whereas *spoIIAAS58A* cells are constitutive for σ^F . Although neither of the purified mutant proteins can be phosphorylated, SpoIIAAS58A forms complexes with SpoIIAB in the presence of ATP or ADP; however, similar complexes are not observed with SpoIIAAS58D. Moreover, SpoIIAAS58A, but not SpoIIAAS58D, reverses the inhibition by SpoIIAB of transcription initiated by σ^F . Wild-type SpoIIAA is rapidly and quantitatively phosphorylated by ATP in a reaction catalyzed by SpoIIAB; but in the presence of ADP, the two proteins form stable complexes resembling those found with SpoIIAB and SpoIIAAS58A. These results, together with those published recently by Alper et al. (1994), allow us to present a model that may account for the activation of σ^F in the prespore.

Results

Mutations affecting Ser-58 of SpoIIAA abolish phosphorylation in vitro and have contrasting effects on σ^F activity in vivo

Previously we showed that as well as inhibiting the activity of transcription factor σ^F , the SpoIIAB protein could also phosphorylate the SpoIIAA protein (Min et al. 1993). We suggested that this phosphorylation of SpoIIAA might be involved in regulating the activity of SpoIIAB. If this view is correct, we would expect the regulation of σ^F to be severely disturbed by *spoIIAA* mutations that prevent phosphorylation. Therefore, we used site-directed mutagenesis to make changes in amino acid residues that were likely candidates for the residue or residues that are phosphorylated, in the hope that the phenotypic properties of such mutants might shed light on the role of phosphorylation in vivo.

Previously, we showed that SpoIIAA is phosphorylated on one or more serine residues (Min et al. 1993). Although SpoIIAA contains 10 serines (Fort and Piggot 1984), comparison of its sequence with that of the closely related RsbV protein, which is thought to play an analogous role in regulation of the SigB protein (Benson

and Haldenwang 1992, 1993; Boylan et al. 1992; Dufour and Haldenwang 1994), revealed that only three of these are conserved, Ser-54, Ser-58, and Ser-84 (Kalman et al. 1990). Site-directed mutagenesis was used to make mutations that would change each of the three serines to alanine. After verification of the sequences of the mutated plasmids, each mutation was introduced separately into *B. subtilis* strain SG38, by a plasmid integration procedure that should ensure that a significant proportion of the transformants would express the mutant phenotype (see Materials and methods). Only one of the substitutions, S58A, produced a visible phenotype (the colonies were Spo⁻ and lytic), suggesting that Ser-58 was the most likely site of phosphorylation. This result was in accordance with our previous finding that a classical *spoIIAA* mutation, *spoIIAA562*, causes a substitution (S → N) at this residue (Errington and Mandelstam 1983; Challoner-Courtney and Yudkin 1993).

If phosphorylation of Ser-58 is involved in regulating the activity of SpoIIAA, we would expect to obtain contrasting phenotypes by replacing the serine with alanine, which is similar in size to serine but cannot be phosphorylated, and with aspartic acid, which would partially resemble the larger, charged, phosphoserine residue. The phenotypic effects of such substitutions might indicate the properties of the phosphorylated and nonphosphorylated forms of SpoIIAA. Therefore, we constructed a S58D substitution, which, on introduction into *B. subtilis*, was also found to produce an asporogenous phenotype. To test whether the failure to make spores was attributable to effects on σ^F activity, the new mutations were introduced into a strain containing a *spoIIIG-lacZ* fusion. The promoter of the *spoIIIG* gene is recognized both by σ^F and by the product of the *spoIIIG* gene itself, σ^G (Karmazyn-Campelli et al. 1989; Sun et al. 1991). To avoid the problem of the contribution made by σ^G to transcription from the promoter, the fusion used also inactivated the *spoIIIG* gene.

The results of measurements of β -galactosidase activity during vegetative growth and sporulation are shown in Figure 1A. The wild-type pattern of gene expression was as described previously (Sussman and Setlow 1991; Partridge and Errington 1993): There was little or no β -galactosidase activity in vegetative cells, but strong induction of expression began about 2 hr after the initiation of sporulation. The S58D mutant behaved like a classical *spoIIAA* mutant, in that β -galactosidase activity was virtually abolished; thus, a SpoIIAA protein that mimics a constitutively phosphorylated form of SpoIIAA was apparently unable to antagonize the SpoIIAB anti- σ factor. In contrast, the S58A substitution not only caused expression of *spoIIIG* during the later stages of sporulation but also in vegetative cells and early in sporulation. This phenotype was in sharp contrast with that of all *spoIIAA* mutations studied previously, which abolish or severely reduce σ^F activity (Challoner-Courtney and Yudkin 1993; Partridge and Errington 1993). The behavior of the mutant resembled that caused by mutations in the *spoIIAB* gene, in which σ^F activity is released prematurely (Schmidt et al. 1990; Coppolecchia et

Figure 1. Effects of specific mutations in the *spoIIAA* gene of *B. subtilis* on the transcription of a σ^F -dependent gene in vivo and on phosphorylation in vitro. (A) Effects of *spoIIAAS58A* and *spoIIAAS58D* on expression of the σ^F -dependent *spoIIIG'-'lacZ* fusion. Derivatives of strain 687 [*trpC2* Ω (*spoIIIG'-'lacZ ermC*) 687] containing plasmids pSG912 (*spoIIAAS58A*) (○) and pSG913 (*spoIIAAS58D*) (●) were induced to sporulate in parallel with a control strain consisting of a nonmutant segregant of 687 transformed with plasmid pSG913 (▲). The cultures were assayed at intervals for β -galactosidase. (B) Phosphorylation of SpoIIAA and mutant derivatives by SpoIIAB. Purified SpoIIAA, or its mutant derivatives with substitutions at Ser-58, were incubated with SpoIIAB and [γ - 32 P]ATP for 30 min at 37°C in phosphorylation buffer as described by Min et al. (1993). After nondenaturing PAGE, the gel was soaked in 7% acetic acid for several hours, dried, and exposed to X-ray film to visualize the labeled proteins. The reaction mixtures all contained SpoIIAB, together with wild-type and/or mutant SpoIIAA, as shown at the top. The horizontal arrows show the positions in the gel of wild-type SpoIIAA (AA), phosphorylated SpoIIAA (AA-P), and SpoIIAB (AB).

al. 1991). This result suggested that the nonphosphorylatable form of SpoIIAA can antagonize SpoIIAB constitutively, that is, in the absence of the signals that are normally recognized only after the initiation of sporulation and specifically in the prespore compartment.

The effects of mutations affecting Ser-58 of SpoIIAA strongly suggested that Ser-58 was the site of phosphorylation of the protein. If so, the purified mutant proteins should not be phosphorylated in vitro in the presence of

ATP and the SpoIIAB protein. To test this, the S58A and S58D forms of the protein were cloned into the T7 expression vector, overproduced in *Escherichia coli* and purified by the methods used for wild-type SpoIIAA. Parallel phosphorylation reactions were set up containing in each case SpoIIAB and [γ - 32 P]ATP, and the wild-type or mutant SpoIIAA proteins; the reaction products were separated by nondenaturing PAGE, on which wild-type SpoIIAA and SpoIIAAS58D, and phosphorylated SpoIIAA and SpoIIAB all proved to have different mobilities (see below). As expected, the wild-type SpoIIAA protein was phosphorylated, but neither of the mutant proteins with substitutions of Ser-58 showed detectable phosphorylation (Fig. 1B). To confirm that the failure to be phosphorylated was not attributable to nonspecific inhibitors or phosphatases in the mutant protein preparations, reactions containing a mixture of wild-type and mutant proteins were prepared. Phosphorylation (presumably of the wild-type protein) was found to occur normally in the presence of either of the Ser-58 mutant proteins. In similar experiments the S54A mutant protein, which had no apparent phenotypic effects in vivo, was purified and found to be phosphorylated normally (results not shown).

These results strongly supported the view that Ser-58 was the site of phosphorylation of SpoIIAA and also suggested that this is the only serine residue that is phosphorylated. Proof of this conclusion has been obtained by M. Najafi, A. Willis, and M. Yudkin (unpubl.), who analyzed SpoIIAA that had been phosphorylated with [γ - 32 P]ATP. After protease digestion, separation, and Edman degradation of the only peptide that was radioactive, all of the 32 P was found in phosphoserine derived from Ser-58 of SpoIIAA.

SpoIIAA is phosphorylated rapidly and completely in the presence of ATP and SpoIIAB

If our proposal above—that SpoIIAAS58D resembles a permanently phosphorylated form of SpoIIAA and SpoIIAAS58A, a form that cannot be phosphorylated—is correct, the phenotypes of the corresponding mutant cells suggest that the role of phosphorylation of SpoIIAA in wild-type cells is to eliminate its anti-SpoIIAB activity. Therefore, we wanted to see whether the mutant SpoIIAA proteins could interact in any way with SpoIIAB, even though they were incapable of being phosphorylated. Figure 2A shows that in the presence of ATP, the constitutive mutant protein SpoIIAAS58A formed what appeared to be complexes with SpoIIAB, with the result that none of the SpoIIAB could be detected at its normal position on the gel; on the other hand, the loss-of-function mutant protein SpoIIAAS58D apparently failed to interact with SpoIIAB. These results support the earlier suggestion (Duncan and Losick 1993; Min et al. 1993; Alper et al. 1994) that the formation of complexes between SpoIIAB and SpoIIAA might be a means of counteracting the anti- σ activity of SpoIIAB; we shall return to this point below.

Next, we incubated wild-type SpoIIAA and SpoIIAB

Figure 2. Reactions between wild-type or mutant SpoIIAA and SpoIIAB in the presence of ADP or ATP. (A–C) Coomassie-stained nondenaturing gels. The horizontal arrows show the positions in the gels of wild-type SpoIIAA (AA), SpoIIAAS58A (AA S58A), SpoIIAAS58D (AA S58D), phosphorylated SpoIIAA (AA-P), SpoIIAB (AB), and the putative SpoIIAA–SpoIIAB complex ([AA.AB]). (A) Effect of incubation with SpoIIAB and ADP or ATP on mutant SpoIIAA proteins. (Lanes 1,3,4,5) SpoIIAAS58A; (lanes 6–9) SpoIIAAS58D; (lanes 2–5, 7–9) SpoIIAB; (lanes 4,8) ADP; (lanes 5,9) ATP. (B) Stoichiometry of the reaction between SpoIIAA and ATP. Lane 1 contained SpoIIAB only and lane 2 SpoIIAA only. For lanes 3–8, SpoIIAA and SpoIIAB (each 7 μM) were incubated without nucleotide (lane 3) or with the concentration of ATP (μM) shown at the top (lanes 4–8). (C) Time course of phosphorylation of SpoIIAA. SpoIIAA and SpoIIAB were incubated at 20°C in phosphorylation buffer. The reaction was started by adding ATP, and samples were taken before ATP was added (lane 1) or after the times indicated (in sec) at the top (lanes 2–7). (D) Inhibition by ADP of phosphorylation of SpoIIAA. SpoIIAA and SpoIIAB were incubated at 0°C in phosphorylation buffer containing [γ -³²P]ATP (20 μM final concentration) with or without 20 μM ADP. Samples were taken and treated as described in Materials and methods. (●) Without ADP; (□) with ADP.

together in the presence of ATP and separated the products by nondenaturing PAGE. We showed previously that when the two proteins are incubated together with ATP, SpoIIAA is phosphorylated but SpoIIAB is not (Min et al. 1993); we have now found that phosphorylation changes SpoIIAA to a form with increased electrophoretic mobility in nondenaturing polyacrylamide gels, as expected for a more negatively charged species (Fig. 2B; see also Fig. 1B). Incubation of SpoIIAA with ATP in the absence of SpoIIAB did not lead to a shift in the mobility of the protein. Therefore, we refer to this species, which migrates on nondenaturing gels between SpoIIAA and SpoIIAB, as SpoIIAA-P. Figure 2B also shows that as long as SpoIIAB was present, the fraction of SpoIIAA that became phosphorylated depended only on the molar ratio of ATP to SpoIIAA. When this ratio was unity or greater, all the SpoIIAA present was converted to the phosphorylated form.

In further studies of the phosphorylation of SpoIIAA we found that the reaction is extremely rapid (Fig. 2C); after 10 sec at 20°C the conversion of SpoIIAA to SpoIIAA-P, catalyzed by SpoIIAB in the presence of ATP, was complete (apart from a very small fraction of the protein that could never be converted to SpoIIAA-P and that we believe to have been inactivated during isolation). No less important, SpoIIAB appeared to be liberated at the end of the reaction, as it can be seen running at its normal position in nondenaturing polyacrylamide gels (Fig. 2C). Finally, we wondered whether the phosphorylation of SpoIIAA might be inhibited by ADP. Figure 2D shows that it was.

SpoIIAA makes long-lasting complexes with SpoIIAB in the presence of ADP

Alper et al. (1994) have shown that while ATP stimu-

lates the binding of SpoIIAB to σ^F , ADP stimulates the binding of SpoIIAB to wild-type SpoIIAA. We found that the same was not true of the mutant SpoIIAA proteins. Each of them, when incubated with SpoIIAB and ADP, behaved just as it had when incubated with SpoIIAB and ATP (Fig. 2A; cf. lanes 4 and 5, and 8 and 9). SpoIIAAS58A appeared to make complexes with SpoIIAB, whereas SpoIIAAS58D showed no interaction with SpoIIAB, at least as judged by its behavior in nondenaturing polyacrylamide gels.

Wild-type SpoIIAA was quite different in this respect. After SpoIIAA and SpoIIAB had been incubated in the presence of ADP, nondenaturing PAGE showed the presence of protein complexes that ran with mobilities intermediate between those of SpoIIAA and SpoIIAB (Fig. 3A). The fact that no distinct band is seen in lane 3 of Figure 3A, which contains the products of the interaction between SpoIIAA, SpoIIAB and ADP, suggested that the complexes might be dissociating in the course of the lengthy electrophoresis run, and we wondered whether the presence of ADP during the run might help to stabilize them. The gel displayed in Figure 3B shows (lane 3) a fairly well-defined band running with a mobility somewhat greater than that of SpoIIAA. This result suggests that the formation of complexes between the two proteins was promoted by the continued presence of ADP, a result consistent with the report of Alper et al. (1994) that ADP stimulates binding of SpoIIAA to SpoIIAB. To confirm that these complexes contained SpoIIAB, we subjected a parallel gel to immunoblotting with anti-SpoIIAB IgG. The results (Fig. 3C) show that most of the SpoIIAB that had been incubated with SpoIIAA and ADP migrated much slower than normal, in the position where Coomassie staining (Fig. 3B) had suggested the bulk of the protein to lie. In contrast, the SpoIIAB that had been involved in the phosphorylation of SpoIIAA could not be seen in a complex with SpoIIAA but instead ran at the normal SpoIIAB position, confirming our previous conclusion that SpoIIAB is rapidly released from the reaction between SpoIIAA and ATP.

SpoIIAAS58A can reverse the inhibition of transcription attributable to SpoIIAB

We and others reported earlier (Duncan and Losick 1993; Min et al. 1993) that transcription initiated by RNA polymerase containing σ^F was inhibited by SpoIIAB; but at that time we failed to reverse the inhibition by adding SpoIIAA to the system (Min et al. 1993). In light of the results described above, we suspected that we could, in retrospect, interpret this failure in terms of the conversion of SpoIIAA to SpoIIAA-P: In the reaction mixture prepared for testing reversal of inhibition, both ATP (as a substrate for RNA polymerase) and SpoIIAB (as inhibitor of the σ^F -containing holoenzyme) were necessarily present. However, we were then in a position to test the effect of SpoIIAA in a more educated manner, basing our experiments on the results described in this paper and on the tentative conclusions that we had derived from them.

Figure 3. Complex formation between SpoIIAA and SpoIIAB in the presence of ADP. (A) Dissociation of SpoIIAA–SpoIIAB complex during electrophoresis. Samples of SpoIIAA (lane 1), SpoIIAB (lane 2), SpoIIAA and SpoIIAB incubated with ADP (lane 3), and SpoIIAA and SpoIIAB incubated with ATP (lane 4) were subjected to nondenaturing PAGE. At the end of the electrophoresis run, the gel was stained with Coomassie blue. (B,C) Effect of the addition of ADP to the running buffer. Samples prepared as in A were subjected to nondenaturing PAGE in two parallel gels with ADP (100 μ M) added to the running buffer. (B) The gel was stained with Coomassie blue; (C) the gel was subjected to immunoblotting with anti-SpoIIAB IgG. The horizontal arrows show the positions in the gel of SpoIIAA (AA), phosphorylated SpoIIAA (AA-P), SpoIIAB (AB), and the putative SpoIIAA–SpoIIAB complex ([AA.AB]).

We first determined the minimum amount of SpoIIAB protein necessary to inhibit transcription directed by σ^F in a runoff transcription assay. As shown in Figure 4A, transcription was eliminated by addition of SpoIIAB approximately equal in mass to that of σ^F . We added to the system, in separate reactions, the mutant or wild-type forms of SpoIIAA in the presence of ADP or ATP and studied their effect on the inhibition attributable to SpoIIAB. SpoIIAAS58D failed to reverse inhibition by SpoIIAB, whether ADP was present or not. Conversely, SpoIIAAS58A counteracted inhibition effectively, whether added with ADP or with ATP (Fig. 4B). We also confirmed the result obtained by Alper et al. (1994)—that wild-type SpoIIAA was able to reverse inhibition when

Figure 4. Stoichiometry of the SpoIIAB anti- σ factor activity and its reversal by mutant SpoIIAA proteins. (A) Inhibition of $E\sigma^F$ -directed transcription by SpoIIAB. σ^F was incubated with purified SpoIIAB, and the transcription assay was carried out as described in Materials and methods. The reaction mixtures all contained 24 pmoles of σ^F , without SpoIIAB (lane 1), or with the quantity of SpoIIAB (in pmoles) shown at the top (lanes 2–8). (B) Reversal by mutant SpoIIAA proteins of anti- σ factor activity of SpoIIAB. Mutant SpoIIAA was incubated with SpoIIAB and either ADP or ATP, σ^F was then added, and the transcription assay was carried out as described in Materials and methods. All reaction mixtures contained σ^F , and all except lane 1 contained SpoIIAB. (Lane 2) No SpoIIAA; (lane 3) SpoIIAAS58A and ADP; (lane 4) SpoIIAAS58A and ATP; (lane 5) SpoIIAAS58D and ADP; (lane 6) SpoIIAAS58D and ATP. The arrow shows the expected size of transcript from this template.

accompanied by ADP but not by ATP (results not shown).

Discussion

The results of previous genetic experiments suggested that SpoIIAA is required for σ^F activity and that it works by reversing the inhibitory action of SpoIIAB (Schmidt et al. 1990). In attempts to investigate the system biochemically, we and others succeeded in showing that purified SpoIIAB can inhibit σ^F in vitro (Duncan and Losick 1993; Min et al. 1993), but we were unable to reverse the inhibitory effect of SpoIIAB by adding purified SpoIIAA (Min et al. 1993). We thought it probable that this failure was attributable to our inability to reproduce in vitro the particular conditions needed for activation of SpoIIAA in vivo. To examine this question further, we have studied the interactions between SpoIIAA and SpoIIAB, and the effects of making mutations in a crucial SpoIIAA residue, Ser-58. The results of these experiments, complemented by those of Alper et al. (1994), allow us to propose a model for the anti-anti- σ activity of SpoIIAA (see below).

The effect of mutations in SpoIIAA residue Ser-58

We showed in a previous paper that a serine residue of SpoIIAA is phosphorylated by SpoIIAB (Min et al. 1993). A sequence comparison of SpoIIAA with the analogous protein RsbV suggested that the site of phosphorylation was likely to be Ser-54, Ser-58 or Ser-84. Changing Ser-54 or Ser-84 proved to be without effect, but mutations affecting Ser-58 had striking phenotypic effects both in vivo and in vitro, suggesting that this residue is important in the function of the protein. A strong indication that Ser-58 is the only residue phosphorylated in SpoIIAA comes from the fact that mutant proteins with an alanine or aspartate residue at position 58 were not detectably phosphorylated in vitro (see Fig. 1B), and direct analysis of phosphorylated SpoIIAA confirmed the position of the phosphoserine (M. Najafi, A. Willis, and M. Yudkin, unpubl.). Although the S58A substitution resembled the S58D substitution in abolishing phosphorylation, the two mutant proteins were strikingly different in other respects. We shall consider their effects under three headings: σ^F activity in the whole cell; the ability of the mutant proteins to make complexes with SpoIIAB; and the reversal of anti- σ activity.

Mutant cells with *spoIIAAS58D* showed no σ^F activity when resuspended in sporulation medium (as judged by expression of a *lacZ* fusion driven by a σ^F -dependent promoter); and purified SpoIIAAS58D protein was unable to make complexes with SpoIIAB in the presence of ADP or ATP, or to reverse the anti- σ activity of SpoIIAB in a transcription assay. In contrast, the *spoIIAAS58A* mutant showed constitutive anti-anti- σ activity, suggesting that its mutant SpoIIAA protein can reverse inhibition of σ^F by SpoIIAB in the absence of the normal signals; moreover, purified SpoIIAAS58A protein formed complexes with SpoIIAB in the presence of either ADP or ATP and exhibited anti-anti- σ activity in vitro. Our interpretation is that SpoIIAAS58D, with its negatively charged Asp-58 that mimics phosphoserine, resembles SpoIIAA-P and can never function as an anti-anti- σ factor. An alternative possibility, that SpoIIAAS58D is highly unstable in the cell, would not account for the failure of the purified mutant protein to reverse the activity of SpoIIAB in vitro, as shown in Figure 4B. Conversely, SpoIIAAS58A cannot be phosphorylated and is permanently active as an anti-anti- σ factor. In this view, it is the ability of SpoIIAAS58A to sequester SpoIIAB into a complex, even in the presence of ATP, that prevents SpoIIAB from interacting with σ^F .

The role of wild-type SpoIIAA and SpoIIAB

How may these conclusions be applied to wild-type SpoIIAA? Our experiments show that in the presence of ADP, SpoIIAA and SpoIIAB interact to make complexes, which can be detected on nondenaturing polyacrylamide gels. Alper et al. (1994) have detected SpoIIAA–SpoIIAB complexes by another method, chemical cross-linking, and in their experiments the formation of these complexes was also stimulated by ADP. In the presence of ATP, on the other hand, SpoIIAA reacts quite differently with SpoIIAB, being rapidly phosphorylated (see Fig. 2C). After catalyzing this phosphorylation SpoIIAB dissociates, and the free species can function as an anti- σ factor. The rapidity of this reaction probably explains why SpoIIAA–SpoIIAB complexes in the presence of ATP were not detected by Alper et al. (1994). Thus, we propose that the role of SpoIIAB is to take part in either of two reactions, depending on the relative concentrations of ATP and ADP: When the concentration of ATP is relatively high, SpoIIAB phosphorylates SpoIIAA and is then released to act as an anti- σ factor, but when the concentration of ADP is relatively high SpoIIAB forms complexes with SpoIIAA. The effect of ADP is reinforced by its ability to inhibit the phosphorylation reaction (see Fig. 2D). Equally, the role of SpoIIAA is twofold: It acts as a substrate for phosphorylation by SpoIIAB when the ATP/ADP ratio is high and as a ligand when the ratio is low; and the fact that the SpoIIAA–SpoIIAB complex has these alternative possibilities allows the system to function as a sensor of the ATP/ADP ratio. Because SpoIIAAS58A can act as a ligand but not as a substrate, the *spoIIAAS58A* strain is constitutive for σ^F function; and because SpoIIAAS58D can act neither as a ligand nor a substrate, the *spoIIAAS58D* strain is devoid of σ^F activity.

By using cross-linking, Alper et al. (1994) have shown that just as an interaction between SpoIIAB and SpoIIAA is favored by ADP, so an interaction between SpoIIAB and σ^F is favored by ATP. This finding provides independent support for the suggestion above. In experiments involving mixtures of the three proteins, Alper et al. (1994) have found that SpoIIAB binds more strongly to σ^F as the ATP/ADP ratio increases and more strongly to SpoIIAA as the ATP/ADP ratio decreases; these investigators have coined the term “partner-switching” to describe this idea.

If our suggestion is correct, the release of σ^F activity will require a supply of nonphosphorylated SpoIIAA. This SpoIIAA is apparently not made (or not necessarily made) in the prespore, as genetic mosaic experiments (Gholamhosseinian and Piggot 1989) indicate that SpoIIA products synthesized in the predivisional cell are sufficient to ensure that σ^F becomes active in the prespore. Of several possible interpretations, we offer two: Some of the SpoIIAA in the predivisional cell could escape phosphorylation, or SpoIIAA could be regenerated by a phosphatase acting on SpoIIAA-P.

Recent work has suggested that the activity of σ^B is regulated in a similar way to that of σ^F (Dufour and Hal-

denwang 1994). RsbW, which is the analog of SpoIIAB, can bind either to RsbV, which is the analog of SpoIIAA, or to σ^B itself; in the latter case, the binding inhibits activity of the σ -factor. Dufour and Haldenwang (1994) have now shown that RsbV can be phosphorylated by ATP in the presence of RsbW and that this phosphorylation inhibits the interaction of RsbV with RsbW.

Reconstruction of the system in vitro

The previous failure to reverse the inhibitory effect of SpoIIAB on σ^F activity by adding SpoIIAA (Min et al. 1993) has now been overcome. We have found that purified SpoIIAAS58A is an effective anti-anti- σ factor in vitro (see Fig. 4B), and Alper et al. (1994) have shown that the same is true of wild-type SpoIIAA, provided that ADP is added together with the protein. Thus, it is possible to reproduce in vitro both steps in the regulatory hierarchy controlling σ^F in vivo: the inhibition by SpoIIAB, and the release of this inhibition by the action of SpoIIAA. In both cases, it seems that the reactions involve direct protein–protein contacts. The results strongly support the model for the control of σ^F described by Schmidt et al. (1990).

Release of SpoIIAA anti-anti- σ -factor activity during sporulation

The model presented in Figure 5 explains both the present results and those of Alper et al. (1994). The fact that ADP and ATP have different effects on the interaction between SpoIIAA and SpoIIAB suggests that a change in the ratio of these two nucleotides is responsible for the release of σ^F activity in vivo. Alper et al. (1994) have demonstrated that uncouplers of oxidative phosphorylation, which would be expected to change the ATP/ADP ratio of the cell, can induce σ^F activity inappropriately in nonsporulating cells.

Although it is not clear why a change in the ATP/ADP ratio should occur specifically in the prespore, we presume that it is a physiological consequence of some distinctive feature of the prespore morphology. This notion finds some support from recent experiments with *spoIIIG* mutants. These mutants have a distinct morphological phenotype, called abortively disporic, in which prespore-like cells form sequentially at both poles of the parent cell. We have shown that in such mutants σ^F activity can appear at both poles of the cell (Lewis et al. 1994), a result that speaks in favor of the idea that σ^F activity is triggered by some feature of this special cell morphology.

Our results and those of Alper et al. (1994) have defined the main components of the mechanism regulating the establishment of cell-specific transcription during sporulation. The low-molecular-weight effectors to which this mechanism responds appear to be ADP and ATP. The main challenges now will be to determine whether a change in the ATP/ADP ratio does occur in the prespore soon after septation and, if so, how this change is confined to a specific compartment.

Figure 5. A model for the regulation of σ^F activity. In the predivisional cell, and in the mother cell after septation, the ATP/ADP ratio is high, and the phosphorylation of SpoIIAA (A) is catalyzed by SpoIIAB (B) in a rapid reaction. The SpoIIAB that is released binds with the help of ATP to σ^F and inhibits it. In the prespore the ATP/ADP ratio is lower, and SpoIIAB makes a long-lived complex with SpoIIAA; this interaction preempts the binding of SpoIIAB to σ^F , so that the latter is free to initiate prespore-specific transcription.

Materials and methods

Bacterial strains

B. subtilis strains used were SG38 (*trpC2*) (Errington and Mandelstam 1986) and 687 [*trpC2* Ω (*spoIIIG'* - '*lacZ* *ermC*) 687] (Partridge et al. 1991). *E. coli* strain BL21 lysogenic for bacteriophage DE3 (a derivative of λ carrying an IPTG-inducible T7 RNA polymerase) was used for the expression of mutant SpoIIAA proteins. Plasmid pET-3a (Studier et al. 1990) was obtained from G.L. Smith (The Sir William Dunn School of Pathology, Oxford, England). Plasmid constructions were performed by transformation of *E. coli* DH5 α (GIBCO BRL).

Plasmids

Plasmid pSG901, used for site-directed mutagenesis, was constructed by cloning a 0.91-kbp *ScaI*-*ClaI* fragment of DNA containing the *spoIIAA* gene, originally from pHM2 (Liu et al. 1982), into pSG1301 (Stevens et al. 1992) that had been digested with *ClaI* and *EcoRV*. Integration of this plasmid into the *B. subtilis* chromosome, which occurs by a Campbell-type mechanism (Duncan et al. 1978) would lead to duplication of the *spoIIAA* gene. To avoid this complication, the mutant *spoIIAA*

alleles were subcloned into plasmid pSG902. This plasmid was constructed by cloning the 1.6-kbp *EcoRI*-*PstI* fragment of DNA containing the *spoIIAA*, *spoIIAB*, and *spoIIAC* genes, but not the promoter of the operon, from plasmid pSG634 (Partridge et al. 1991), into pSG1301 that had been cut with the same enzymes.

Plasmids for expression of the wild-type *spoIIAA*, *spoIIAB*, and *spoIIAC* genes, pEAA, pEAB and pEAC, were described previously (Min et al. 1993). They consist essentially of one of the pET-3 plasmids (pET-3a or pET-3d; Studier et al. 1990) containing polymerase chain reaction (PCR)-amplified copies of the wild-type *spo* genes. Each plasmid carries the promoter and ribosome-binding site of the phage T7 gene 10 protein. Synthesis of T7 RNA polymerase in a host containing the plasmid results in strong transcription and translation of the cloned gene. Mutant versions of the *spoIIAA* gene (see below) were cloned into plasmid pET-3a as follows. The plasmids derived by site-directed mutagenesis of *spoIIAA* were used as templates for PCR amplifications with the following oligonucleotides: 5'-GAG-GAAACCATATGAGCCTTGAATTG-3', creating an *NdeI* site overlapping the ATG translation initiation codon of the *spoIIAA* gene, and 5'-GGGCAGAAGGATCCAGGTGCATTTCAT-3', creating a *BamHI* site just after the termination codon of *spoIIAA*. The amplified fragments were purified from low-melting-point agarose gels, digested with *NdeI* and *BamHI*, and ligated with pET-3a that had been digested with the same enzymes. The ligation products were used to transform *E. coli* DH5 α . The plasmids derived were used to transform *E. coli* BL21 (DE3) for protein overproduction and purification.

pSG148, used as template for the in vitro transcription reactions, was derived by digestion of pSG139 (Foulger and Errington 1989) with *BamHI*, followed by religation. The resultant plasmid contains a 1.38-kb *PvuII* fragment covering the *spoIIIG* promoter.

Site-directed mutagenesis

Single-stranded DNA of plasmid pSG901 was mutagenized by the uracil template method of Kunkel (1985), by means of the following mutagenic oligonucleotides (the amino acid substitutions generated in the SpoIIAA protein are given in parentheses): 5'-GCTGTCCATAAAGGCAAGGTCCTCC-3' (S54A, pSG911); 5'-GCCAAGCCCCGAGGCGTCCATAAAGG-3' (S58A, pSG912); 5'-GCCAAGCCCCGAGTCTGTCATATAAGG-3' (S58D, pSG913); 5'-CTTACCCGACGAGCGATAGCGCAAAC-3' (S84A, pSG914). The double-stranded molecules were used to transform *E. coli* DH5 α with selection for ampicillin resistance. The complete DNA sequences of the inserts of mutant plasmids were checked. To facilitate analysis of the phenotypic effects of the plasmids, their inserts were subcloned into plasmid pSG902, giving rise to plasmids pSG915, pSG916, pSG917, and pSG918, respectively (see above).

Analysis of the phenotypic effects of *spoIIAA* mutations

Mutant plasmids derived from pSG901 were used to transform (Anagnostopoulos and Spizizen 1961) *B. subtilis* SG38 or 687 with selection for chloramphenicol resistance. Transformants of SG38 with a defect in spore formation were apparent by the translucent appearance of their colonies. Transformants of 687 with an effect on spore formation were apparent by an altered blue coloration on plates containing X-gal. The plasmid carrying the *spoIIAAS58A* mutation gave ~40% of intensely blue colonies as well as wild-type pale blue colonies. The plasmid carrying *spoIIAAS58D* gave a similar proportion of white colo-

nies in addition to pale blue ones. Because integration of pSG901 derivatives results in duplication of the *spoIIAA* gene, the mutant colonies could have arisen either by gene conversion, which occurs at a significant rate in such experiments, or by partial dominance of the mutant alleles. To confirm that the S54A and S84A mutations did not give a detectable phenotype, the inserts in these plasmids were subcloned into pSG902. Insertion of pSG902 derivatives leaves only a single functional copy of the *spoIIAA* gene. None of the transformants with plasmids carrying the *spoIIAAS54A* or *spoIIAAS84A* mutations showed a mutant phenotype.

Overproduction of SpoIIA proteins

BL21 (DE3) cells (Studier et al. 1990) harboring the pET-3 derivatives carrying wild-type or mutant copies of *spoIIAA*, *spoIIAB*, or *spoIIAC* were grown in 2×YT medium supplemented with 50 µg/ml of ampicillin, at 37°C with shaking until an OD₆₀₀ of 0.8 was reached. Protein overproduction was induced by the addition of 1 mM IPTG. One hr later, 200 µg/ml of rifampicin was added and incubation was continued for 3–4 hr.

Purification of proteins

As described previously (Min et al. 1993), σ^F was gel purified. The purified protein was renatured from guanidine-HCl according to the method of Hager and Burgess (1980). Core RNA polymerase was purified from *B. subtilis* CU267 by the method of Burgess and Jendrisak (1975) as described previously (Min et al. 1993). The first stage in the purification of wild-type and mutant SpoIIAA was by FPLC anion exchange chromatography as described previously (Min et al. 1993). The absorbed proteins were eluted with a linear gradient of 50–600 mM NaCl in buffer A over 20 min. Wild-type SpoIIAA and SpoIIAAS58A eluted at ~200 mM NaCl, and SpoIIAAS58D at ~250 mM NaCl. The column fractions enriched in SpoIIAA were subjected to gel filtration over Sephadex G-50 equilibrated in 20 mM Tris-Cl (pH 8.5), which removed a contaminant of high molecular weight. To purify SpoIIAB, cell extracts were subjected to ammonium sulfate fractionation, and the proteins that precipitated between 10% and 40% saturation were applied to an FPLC anion exchange column (Min et al. 1993). The column fractions containing SpoIIAB, eluting at ~450 mM NaCl, were subjected to non-denaturing 12% PAGE [in 200 mM Tris-Cl (pH 8.5)], containing 20% glycerol. SpoIIAB migrates more rapidly in this system than any visible contaminants; at the end of the run, it was recovered by diffusion into 100 mM Tris-Cl (pH 7.5), 20% glycerol, 100 mM NaCl, 0.1% sodium azide, and 2 mM dithiothreitol. The proteins were stored in 50% glycerol at –20°C.

In vitro phosphorylation assays

Phosphorylation assays with wild-type and mutant proteins were performed in the buffer described previously (Min et al. 1993). Unless specified otherwise, the reactions contained 7 µM SpoIIAA, 7 µM SpoIIAB, and 20 µM ATP and were incubated for 10 min at 37°C. To test the ability of mutant SpoIIAA proteins to be phosphorylated, 2 µCi of [γ -³²P]ATP was added to a total reaction volume of 30 µl. At the end of the reaction, 30-µl samples were added to 10 µl of a solution (sample buffer) at 0°C containing 100 mM EDTA, 10% glycerol, and 0.02% bromophenol blue (final concentrations) and kept on ice until they were subjected to nondenaturing 12% PAGE at 0–4°C in 200 mM Tris-HCl (pH 8.5), and developed with a running buffer of 25 mM Tris and 192 mM glycine. The proteins were detected by stain-

ing with Coomassie blue or by autoradiography as appropriate, or treated by immunoblotting as described by Min et al. (1993). To study the effect of ADP on the phosphorylation of SpoIIAA, 2 µCi of [γ -³²P]ATP was added to a reaction mixture, already containing ADP, with a final volume of 200 µl. The reaction was run at 0°C, and 30-µl samples were precipitated with 10% TCA in the presence of BSA (1 mg). After 30 min on ice, the samples were passed through a Whatman GF/B filter, which was washed four times with 5% TCA (2 ml) and twice with ethanol (2 ml), dried, and counted by Cerenkov radiation.

Interaction of SpoIIAA and SpoIIAB in the presence of ADP

SpoIIAA (wild type or mutant) and SpoIIAB, both at 7 µM, were incubated together for 10 min at 37°C in the presence of 20 µM ADP in phosphorylation buffer (Min et al. 1993). Samples were added to sample buffer and subjected to nondenaturing polyacrylamide gels as described above. In some experiments ADP (100 µM) was added to the running buffer. Proteins were detected by staining with Coomassie blue or by immunoblotting (Min et al. 1993).

In vitro transcription assays

BamHI-digested pSG148, which contains the *spoIIIG* promoter, was used as the template. RNA polymerase holoenzyme containing σ^F ($E\sigma^F$) was reconstituted by incubation of 0.7 µg of core enzyme and 0.7 µg of gel-purified, renatured σ^F on ice for 10 min. This $E\sigma^F$ was mixed with template (1 µg) in 40 µl (final volume) of transcription buffer, which contained 33 mM Tris-acetate (final pH 7.5), 100 mM potassium acetate, 10 mM magnesium acetate, 10 mM dithiothreitol, and 160 µg/ml of BSA. After incubation for 10 min at 37°C, 1 µl each of ATP, GTP, and UTP (all 1.5 mg/ml) and 4–5 µCi of [α -³²P]CTP were added, followed 2 min later by heparin (1 µl of a 6 mg/ml solution). After incubation for a further 10 min at 37°C, unlabeled CTP (1 µl of a 1.5 mg/ml solution) was added. Five minutes later, the RNA was precipitated by addition of 200 µl of ethanol and cooled to –70°C. The dried RNA pellet was dissolved in 10 µl of urea stop mixture (Moran 1990). The sample was heated for 3 min at 85°C, and 2 µl was loaded on to a 6% polyacrylamide denaturing gel. After electrophoresis the gel was washed and fixed in 15% methanol and 5% acetic acid for 5–10 min. The reaction products were detected by autoradiography.

To test for the inhibition of σ^F activity by SpoIIAB, native SpoIIAB was mixed with σ^F in transcription buffer and incubated at ambient temperature for 20 min before addition of core RNA polymerase and DNA template.

To test the ability of mutant SpoIIAA to counteract the inhibition of σ^F activity by SpoIIAB, SpoIIAB was mixed with mutant SpoIIAA in transcription buffer. The reaction mixtures contained (where shown) 700 ng of SpoIIAB, with or without SpoIIAAS58A (4.5 µg) or SpoIIAAS58D (6 µg). ATP or ADP (where added) was at a final concentration of 250 µM. After 20 min at ambient temperature, σ^F (700 ng) was added. After another 20 min period at ambient temperature, core RNA polymerase and pSG148 template were added, and the transcription reaction was carried out as described above.

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