Three sites of contact between the *Bacillus subtilis* transcription factor σ^F and its antisigma factor SpoIIAB

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The developmental regulatory protein σ^F of *Bacillus subtilis*, a member of the σ^{70} -family of RNA polymerase sigma factors, is regulated negatively by the antisigma factor SpoIIAB, which binds to σ^F to form an inactive complex. Complex formation between SpoIIAB, which contains an inferred adenosine nucleotide binding pocket, and σ^F is stimulated strongly by the presence of ATP. Here we report that SpoIIAB contacts σ^F at three widely spaced binding surfaces corresponding to conserved regions 2.1, 3.1, and 4.1 of σ^{70} -like sigma factors. This conclusion is based on binding studies between SpoIIAB and truncated portions of σ^F , the isolation of mutants of σ^F that were partially resistant to inhibition by SpoIIAB in vivo and were defective in binding to the antisigma factor in vitro, and the creation of alanine substitution mutants of regions 2.1, 3.1, or 4.1 of σ^F that were impaired in complex formation. Because the interaction of SpoIIAB with all three binding surfaces was stimulated by ATP, we infer that ATP induces a conformational change in SpoIIAB that is needed for tight binding to σ^F . Finally, we discuss the possibility that another antisigma factor, unrelated to SpoIIAB, may interact with its respective sigma factor in a similar topological pattern of widely spaced binding surfaces located in or near conserved regions 2.1, 3.1, and 4.1.

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Gene transcription in bacteria is governed in part by RNA polymerase sigma factors, which mediate the recognition of promoter sequences (Gross et al. 1992). Bacteria have multiple sigma factors, each capable of recognizing and directing transcription from a cognate set of promoters. Recent evidence indicates that the activity of sigma factors frequently is subject to regulation by a class of proteins called antisigma factors (reviewed in Brown and Hughes 1995). Antisigma factors bind directly to their respective sigma factors, disabling their capacity to direct transcription. Examples of antisigma factors include: FlgM of *Salmonella typhimurium*, which antagonizes the flagellar sigma factor FlhA (Ohnishi et al. 1992); CarR of *Myxococcus xanthus*, which inhibits the carotenoid pigment sigma factor CarQ (Gorham et al. 1996); and AsiA of bacteriophage T4, which suppresses transcription directed by the *Escherichia coli* host sigma factor σ^{70} (Orsini et al. 1993). Broadly speaking, the chaperone DnaK of *E. coli* also can be considered an antisigma factor in the sense that it inactivates the heat shock sigma factor σ^{32} (for example, see Straus et al. 1990; Gamer et al. 1992; Liberek et al. 1992). Rather than forming a stable complex with σ^{32} , DnaK causes σ^{32} to become a substrate for degradation by the protease FtsH (Herman et al. 1995; Tomoyasu et al. 1995).

Two additional examples of antisigma factors, both from *Bacillus subtilis*, are RsbW, which inhibits the stress response sigma factor σ^B (Benson and Haldenwang 1993), and SpoIIAB, which regulates negatively the sporulation sigma factor σ^F (Duncan and Losick 1993; Min et al. 1993). RsbW and SpoIIAB are highly similar to each other (Kalman et al. 1990) and both are regulated negatively by the anti-antisigma factors RsbV and SpoIIAA, respectively. In addition, RsbW and SpoIIAB each contain an adenosine nucleotide-binding pocket and are capable of phosphorylating, and thereby inactivating, RsbV or SpoIIAA (Duncan and Losick 1993; Min et al. 1993; Dufour and Haldenwang 1994). When in their unphosphorylated state, however, RsbV and SpoIIAA are able to induce the release of σ^B or σ^F from the sigma factor-antisigma factor complex, thus allowing σ^B and σ^F to become transcriptionally active (Alper et al. 1994; Diederich et al. 1994; Dufour and Haldenwang 1994; Alper et al. 1996; Duncan et al. 1996). A key difference between the two systems is that RsbW can bind tightly to σ^B in the absence of nucleotides (Alper et al. 1996), whereas SpoIIAB requires ATP for efficient binding to σ^F (Alper et al. 1994).

Here we are concerned with the interaction between σ^F and SpoIIAB. The σ^F and SpoIIAB proteins are of special interest because they are involved in the determination of cell fate during sporulation (Losick and Stragier 1992). At the start of sporulation, each progenitor cell

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divides asymmetrically to produce two unequally sized cellular compartments: the forespore (the smaller compartment) and the mother cell. Although synthesized prior to septation, σ^F becomes active only following septation and exclusively in the forespore (Gholamhosseinian and Piggot 1989; Margolis et al. 1991; Min et al. 1993; Partridge and Errington 1993). SpoIIAA and SpoIIAB are also synthesized prior to septation (Gholamhosseinian and Piggot 1989; Min et al. 1993; Partridge and Errington 1993) and it has been postulated that σ^F is bound by SpoIIAB in both the predivisional cell and the mother cell, and that some feature of the forespore (possibly a reduction in the ratio of ATP/ADP combined with a high local concentration of a SpoIIAA-P-specific phosphatase) activates SpoIIAA to release σ^F from the SpoIIAB- σ^F complex (Alper et al. 1994; Arigoni et al. 1995; Diederich et al. 1994; Duncan et al. 1995).

In this report, we investigate the interaction between σ^F and SpoIIAB. Specifically, we present evidence that σ^F uses three surfaces located in conserved regions 2.1, 3.1, and 4.1 of σ^{70} -like sigma factors to make hydrophobic contacts with SpoIIAB. We discuss our findings in terms of the topology of the SpoIIAB- σ^F complex, the role of ATP in the SpoIIAB- σ^F interaction, and in comparison with the interaction of the flagellar sigma factor, FlhA, with its antisigma factor, FlgM.

Results

Nonoverlapping fusion proteins containing either the amino-terminal or the carboxy-terminal portion of σ^F bind to SpoIIAB

The σ^F factor has been divided into nine subregions on

the basis of its amino acid sequence similarity to other members of the σ^{70} family of sigma factors (Lonetto et al. 1992). Distinct functions have been attributed to several of these subregions (Fig. 1A). For example, region 2.1 is involved in binding of the sigma factor to core RNA polymerase (Lonetto et al. 1992 and references therein); region 2.3 may be involved in formation of the open complex during transcription initiation (Jones and Moran 1992; Juang and Helmann 1994; Rong and Helmann 1994); region 2.4 contacts the -10 promoter DNA (Dombroski et al. 1992; Lonetto et al. 1992 and references therein); and region 4.2 contacts the -35 promoter DNA (Dombroski et al. 1992; Lonetto et al. 1992 and references therein). To determine which portions of the σ^F protein interact with SpoIIAB, we constructed a series of fusion proteins in which either full-length or various truncated portions of σ^F were joined in-frame to the carboxyl-terminus of maltose binding protein (MBP). Then, we tested the ability of each fusion protein to bind to SpoIIAB by means of chemical cross-linking (Alper et al. 1994; Duncan and Losick 1993). [35 S]Methionine labeled SpoIIAB and unlabeled fusion protein were incubated in the presence of disuccinimidyl suberate (DSS), a homobifunctional cross-linker that covalently links lysine residues located ~11 Å apart. The mixtures then were subjected to electrophoresis through sodium dodecyl sulfate (SDS)-polyacrylamide gels and complexes were visualized by autoradiography. As observed previously (Duncan and Losick 1993; Alper et al. 1994), SpoIIAB appeared as a ~14-kD monomer in the absence of DSS (Fig. 2A, lane 1) and as both the monomer and a 29-kD dimer in the presence of the cross-linker (Fig. 2A, lane 2). When [35 S]SpoIIAB was incubated with MBP- σ^F fusion protein containing full-length σ^F (255 residues), the subsequent

Figure 1. Anatomy of σ^F . (A) The nine subregions identified by amino acid sequence similarity to other σ^{70} -like sigma factors are shown as boxes and labeled above the diagram, as are subregions that are associated with distinct functions of the sigma factor. Amino acid positions representing the MBP- σ^F fusion protein endpoints are numbered below the diagram, as are positions of the amino acid substitutions. (B) Summary of the ability of the MBP- σ^F fusion proteins to bind to SpoIIAB. Residues from σ^F contained within each fusion protein are listed at left and shown schematically by lines in the middle. At right are the results of binding experiments between each fusion protein and SpoIIAB. (+++) Strong binding, (++) intermediate binding, (+) weak binding, and (-) no detectable binding. In all cases the degree of binding was assessed by side-by-side comparisons of the strength of the signal corresponding to complex formation.

Figure 2. Binding of MBP- σ^F fusion proteins to SpoIIAB. The figure is an autoradiograph of cross-linking reactions between radiolabeled SpoIIAB and unlabeled MBP- σ^F fusion proteins that had been subjected to SDS-polyacrylamide gel electrophoresis. Cross-linking was achieved by incubating the proteins with 1 mg/ml DSS for 2–3 hr on ice. Numbers to the left of the autoradiographs indicate the position of protein size markers in kD. Reactions that contained 1 mM ATP are indicated above each autoradiograph. (A) The binding between SpoIIAB and full-length MBP- σ^F fusion protein is as follows: [³⁵S]SpoIIAB in the absence (lane 1) and presence (lane 2) of DSS; [³⁵S]SpoIIAB, DSS, and 100 ng of purified MBP- σ^F 1-255 (lanes 3,4); and [³⁵S]SpoIIAB, DSS, and 500 ng of purified MBP (lanes 5,6). (B,C) The binding between SpoIIAB and various truncated MBP- σ^F fusion proteins. (B) [³⁵S]SpoIIAB in the absence (lane 1) and presence (lane 2) of DSS; [³⁵S]SpoIIAB, DSS, and 20 μ g of total soluble proteins from an *E. coli* strain over-producing either MBP- σ^F 115-255 (lanes 3,4), MBP- σ^F 136-255 (lanes 5,6), or MBP- σ^F 1-114 (lanes 7,8). (C) [³⁵S]SpoIIAB, DSS, and 20 μ g of total soluble proteins from an *E. coli* strain over-producing either MBP (lanes 2,7,8), MBP- σ^F 55-201 (lane 1), MBP- σ^F 184-255 (lanes 3,4), or MBP- σ^F 115-201 (lanes 5,6). Lanes 1–6 of B and lanes 2–4 of C represent a single gel. Reactions corresponding to all lanes of B and lanes 2–4 of C were performed and analyzed on the same day.

addition of DSS revealed the presence of a slowly migrating radiolabeled species that appeared at the expense of the SpoIIAB dimer (Fig. 2A, lane 4). We interpret this band to represent a complex between SpoIIAB and the MBP- σ^F fusion protein. Formation of the SpoIIAB * MBP- σ^F complex was stimulated by the pres-

ence of ATP in the binding reaction (Fig. 2A, lanes 3,4) in agreement with previous results that adenosine nucleotides stimulate the SpoIIAB- σ^F interaction (Alper et al. 1994). Complex formation was also dependent on the presence of σ^F amino acid sequences in the fusion protein as no slowly migrating species were observed when [³⁵S]SpoIIAB was incubated with MBP alone (Fig. 2A, lanes 5,6).

We began our deletion series by systematically removing amino acids from the amino-terminus of σ^F . Fusion proteins lacking either the first 54 or first 114 amino acids of σ^F were capable of binding SpoIIAB effectively; and at least in the case of the MBP- σ^F 115-255 fusion, this binding occurred in an ATP-dependent manner (Fig. 2B, lanes 3,4). However, removal of an additional 21 amino acids to residue 136 diminished binding greatly, and, further, this residual level of binding was not enhanced by the addition of ATP (Fig. 2B, lanes 5,6). Together, these results suggest the existence of an ATP-dependent binding site for SpoIIAB in the end of region 2.4 or in the beginning of region 3.1 of σ^F (see Fig. 1B). Next, we constructed fusion proteins that were lacking 54 or 142 amino acids from the carboxyl terminus of σ^F . As expected, the fusion protein lacking the final 54 amino acids of σ^F was able to bind to SpoIIAB. Surprisingly, however, the fusion protein lacking the final 142 amino acids of σ^F (MBP- σ^F 1-114) was also capable of binding to SpoIIAB, and this binding was also stimulated by ATP (Fig. 2B, lanes 7,8). Because the fusion proteins MBP- σ^F 1-114 and MBP- σ^F 115-255 do not overlap, there must be at least two binding regions for SpoIIAB: one located in the amino-terminal portion of σ^F and the other located in the carboxy-terminal portion of σ^F . The ATP dependence of the binding of MBP- σ^F 1-114 and MBP- σ^F 115-255 to SpoIIAB does not demonstrate unambiguously the functional significance of these two binding domains but is consistent with the known strong dependence of the SpoIIAB- σ^F interaction on ATP (Alper et al. 1994).

Isolation of σ^F mutants resistant to inhibition by SpoIIAB

To further characterize the binding sites on σ^F for SpoIIAB, we sought mutants of σ^F that were active in directing transcription, yet were resistant to inhibition by SpoIIAB. We performed PCR mutagenesis (see, for example, Zhou et al. 1991) on the gene encoding σ^F , *spoIIAC*, and then screened for mutants exhibiting elevated levels of σ^F -directed gene expression as measured by the use of *lacZ* fused to a gene under the control of σ^F . Two classes of mutants were recovered. The first class of mutants exhibited high levels of σ^F -directed gene expression (8- to 25-fold higher than wild type; see Fig. 3B), was blocked during sporulation prior to septation (as visualized by DAPI staining) (Setlow et al. 1991), and lysed within 24 hr after being plated on sporulation agar. Consistent with the idea that these mutants are defective in the SpoIIAB- σ^F interaction, this phenotype is similar to that of mutants that lack SpoIIAB (Schmidt et al. 1990; Coppolecchia et al. 1991). The second class of mutants

exhibited higher levels of σ^F activity than did wild-type cells (2- to 8-fold higher; see Fig. 3, A,C); but unlike the class I mutants, they were not blocked at an early stage of sporulation (see Materials and Methods).

Sequence analysis revealed that the class I mutants contained single amino acid substitutions (V137E, E149K, E156K) in region 3.1 of the σ^F protein, whereas the class II mutants contained amino acid substitutions in either region 2 or region 4.1 (Fig. 1A). Specifically, three class II mutants contained single amino acid substitutions in region 4.1 (L209Q, Y212H, L213H), and one class II mutant contained two closely spaced amino acid substitutions in region 2 (V48A and G56R, whose individual contributions to the mutant phenotype are considered below).

To investigate whether the elevated levels of σ^F activity observed in the mutants were attributable to increased amounts of the σ^F protein, we determined the level of σ^F in the mutants by Western blot analysis using anti- σ^F antibodies. In none of the cases tested did the mutants accumulate σ^F to higher levels than cells containing the wild-type σ^F gene (Fig. 3D). Because the in-

Figure 3. Western blot analysis of σ^F in mutant cells exhibiting high levels of σ^F activity. Mutants of *spoIIAC* were isolated using a strain with the following three features: the *E. coli lacZ* gene fused to a σ^F -dependent promoter, two copies of the *spoIIAA* and *spoIIAB* genes, and a fusion of the *spoOH* gene encoding σ^H to the IPTG-inducible *P_{spac}* promoter [see Materials and Methods]. Because the *spoIIA* operon is under the control of σ^H , transcription of the *spoIIAA*, *spoIIAB*, and *spoIIAC* genes in this strain was induced by the addition of IPTG. In this specially constructed strain, σ^F -directed β -galactosidase synthesis commences several hours later during sporulation than that observed in wild-type cells. A, B, and C show the accumulation of β -galactosidase at the indicated hour after the exponential phase of growth in sporulating cells containing σ^F mutant in region 2 (A), region 3 (B), or region 4 (C). The time course of accumulation of β -galactosidase for strain AB414, which contains a wild-type copy of the σ^F gene, is repeated in each panel for comparison. For each time point from A, B, and C, duplicate samples were subjected to Western blot analysis using antibodies against σ^F as displayed in D.

creased σ^F activity observed in the mutants cannot be explained by an increase in the synthesis or stability of σ^F , it seemed possible that the mutants were defective in their interaction with SpoIIAB.

σ^F proteins mutant in region 2, region 3.1, or region 4.1 are defective in binding to SpoIIAB

To investigate whether σ^F mutant in region 2 was defective in binding to SpoIIAB, we introduced the V48A and G56R substitutions into an MBP- σ^F fusion protein containing the amino-terminal portion of σ^F (amino acid residues 1–114). Unlike the wild-type version of this fusion, MBP- σ^F_{1-114} V48A, G56R was unable to bind to SpoIIAB (Fig. 4A, lanes 1–4). Next, we separated the two substitutions to determine the effect of each one individually. MBP- σ^F_{1-114} fusion proteins separately containing the V48A or the G56R substitution were unable to bind to SpoIIAB (Fig. 4A, lanes 5–8). Thus, each of these two amino acid substitutions was capable of disrupting the interaction between SpoIIAB and the amino-terminal portion of σ^F .

To investigate whether σ^F proteins mutant in region 3.1 or region 4.1 were defective in binding to SpoIIAB, we constructed MBP- σ^F fusion proteins that contained the carboxy-terminal portion of σ^F (amino acid residues 115–255) and either the V137E, the E149K, the E156K, or the L213H amino acid substitution. Carboxy-terminal σ^F fusion proteins separately containing each of the above amino acid substitutions were impaired in binding to SpoIIAB, with those containing substitutions in region 3.1 being the most severely impaired (Fig. 4B). Thus, amino acid substitutions that caused elevated σ^F activity in vivo were found to cause defective binding to SpoIIAB in vitro.

Alanine substitution mutations suggest that σ^F residues V48, V137, and L213 are contact sites for SpoIIAB

Amino acid substitutions at six different positions in the σ^F protein (V48, G56, V137, E149, E156, and L213) adversely affected the binding of σ^F to SpoIIAB. To investigate whether some of these positions were sites of contact with SpoIIAB, we constructed MBP- σ^F fusion proteins containing either the amino-terminal portion (residues 1–114) or the carboxy-terminal portion (residues 115–255) of σ^F and an alanine residue at position 48, 137, 149, 156, or 213. Substitution of an alanine residue (which lacks a side chain beyond the β carbon) would be expected to remove any positive energetic contribution of the wild-type amino acid side chain to the binding interaction, yet still maintain protein structure (Cunningham and Wells 1989). As noted above, the fusion protein MBP- σ^F_{1-114} V48A was unable to bind to SpoIIAB (Fig. 4A, lanes 5–6). Similarly, MBP- $\sigma^F_{115-255}$ fusion proteins containing the V137A or the L213A substitution were impaired in binding to SpoIIAB either severely (V137A) or partially (L213A) (Fig. 4C, lanes 3,4,9,10). In contrast, MBP- $\sigma^F_{115-255}$ fusion proteins containing ei-

Figure 4. σ^F mutant in region 2, 3.1, or 4.1 is defective in binding to SpoIIAB. The figure is an autoradiograph of the products of cross-linking reactions between radiolabeled SpoIIAB and unlabeled MBP- σ^F fusion proteins that had been subjected to SDS-polyacrylamide gel electrophoresis. The MBP- σ^F fusion proteins contained amino acid substitutions in regions 2, 3.1, or 4.1. Numbers to the left of the autoradiographs indicate the position of protein size markers in kD. Reactions which contained 1 mM ATP are indicated above each autoradiograph. (A) The binding between SpoIIAB and MBP- σ^F fusion proteins containing the amino-terminal portion of σ^F . All lanes contain [35 S]SpoIIAB, DSS, and 20 μ g of total soluble proteins from an *E. coli* strain over-producing either MBP- σ^F_{1-114} (lanes 1,2), or MBP- σ^F_{1-114} containing the indicated amino acid substitutions (lanes 3–8). (B, C) The binding between SpoIIAB and MBP- σ^F fusion proteins containing the carboxy-terminal portion of σ^F . All lanes contain [35 S]SpoIIAB, DSS, and 20 μ g of total soluble proteins from an *E. coli* strain over-producing either MBP- $\sigma^F_{115-255}$ (lanes 1,2, in each panel), or MBP- $\sigma^F_{115-255}$ containing the indicated amino acid substitutions (lanes 3–10, in each panel). All of the reactions shown in B and C were performed and analyzed on the same day.

ther the E149A or the E156A substitution were each able to bind to SpoIIAB, although the E156A substitution resulted in a slightly lower level of binding than that observed with the wild-type fusion protein (Fig. 4C, lanes 5–8). We take these results as evidence that the side

chains of residues V48, V137, and L213 make energetically favorable interactions required for SpoIIAB- σ^F complex formation. In contrast, the side chains of E149 and E156 evidently are not involved importantly in the SpoIIAB- σ^F interaction. Instead, the lysine substitutions at these positions likely caused interference with the binding of σ^F to SpoIIAB, presumably by virtue of being near the inferred contact site at position 137.

Our original fusion protein binding studies distinguished between two binding regions for SpoIIAB: one in the amino-terminal portion and one in the carboxy-terminal portion of σ^F . The alanine substitution analysis described above supports this inference through the identification of inferred contact sites at positions 48, 137, and 213. Because the contact sites at positions 137 and 213 are spaced widely, this analysis additionally suggests the existence of two separate binding regions (one in region 3.1 and one in region 4.1) within the carboxy-terminal portion of σ^F . To investigate this possibility further, we built two additional MBP- σ^F fusion proteins: one containing region 3 of σ^F (amino acids 115–201) and another containing region 4 of σ^F (amino acids 184–255). Although these two fusion proteins partially overlap, each contained only one of the identified contact sites (see Fig. 1). MBP- $\sigma^F_{115-201}$ and MBP- $\sigma^F_{184-255}$ were each capable of binding to SpoIIAB in an ATP-dependent manner (Fig. 2C, lanes 3–6), although the level of binding was weaker than that observed between SpoIIAB and the entire carboxy-terminal region of σ^F (Fig. 2B, lanes 3,4). Perhaps correct folding and stabilization of the presumed binding domains require larger portions of the σ^F polypeptide than were included in these two fusion proteins. Consistent with this interpretation, a MBP- σ^F fusion protein (MBP- σ^F_{55-201}) that did not contain the V48 or L213 contact sites, but did contain the presumed region 3.1 binding domain plus additional upstream amino acids, was able to bind efficiently to SpoIIAB (see Fig. 1B and Fig. 2C, lane 1). Taken together, the genetic and binding results are consistent with the existence of three ATP-dependent binding regions for SpoIIAB on σ^F : one in region 2.1, one in region 3.1, and one in region 4.1.

Transcription directed by the E149K mutant of σ^F is partially resistant to inhibition by SpoIIAB

The mutants described in this study were isolated on the basis of their enhanced level of σ^F -directed gene expression. The results of our binding studies suggest that the enhanced transcription was attributable to impaired interaction with SpoIIAB. As a direct test of this interpretation, we carried out transcription reactions with core RNA polymerase supplemented with wild-type σ^F or with the mutant sigma factor (E149K) that exhibited the highest level of σ^F -directed gene expression in vivo. In the absence of σ^F , core RNA polymerase was unable to generate a run-off transcript (Fig. 5, lane C). However, when supplemented with equal amounts of either wild-type σ^F or σ^F E149K, core RNA polymerase generated similar levels of a run-off transcript of the expected size of 77 nucleotides (Fig. 5, lanes 1,6). To assess the effect of

Figure 5. Transcription directed by the E149K mutant of σ^F is partially resistant to inhibition by SpoIIAB. The figure displays an autoradiograph of the products of transcription reactions that had been subjected to electrophoresis on an 8% polyacrylamide sequencing gel. The transcription reactions contained 2 μ g of linearized template DNA, 200 ng of core RNA polymerase, and either no σ^F (lane C), 150 ng of wild-type σ^F (lanes 1–5), or 150 ng of σ^F E149K (lanes 6–11). In addition, each transcription reaction contained the following amounts of SpoIIAB: none (lanes C, 1, 6), 127 ng (lanes 2, 7), 169 ng (lanes 3, 8), 254 ng (lanes 4, 9), 338 ng (lanes 5, 10), or 423 ng (lane 11), which correspond to the indicated molar ratios of SpoIIAB to σ^F .

the antisigma factor, we incubated σ^F or σ^F E149K, SpoIIAB, and template DNA at 37°C for 5 min prior to the addition of core RNA polymerase. Synthesis of the transcript directed by wild-type σ^F was inhibited partially at a molar ratio of SpoIIAB to σ^F of 1.5 (Fig. 5, lane 2), and was undetectable at a molar ratio of 3 (Fig. 5, lane 4). In contrast, the transcript generated by σ^F E149K was abundant when synthesis was carried out at a molar ratio of 2 (Fig. 5, lane 8). Moreover, σ^F E149K-directed synthesis was still detected at a molar ratio as high as 5 (Fig. 5, lane 11).

Discussion

We have investigated the topology of the interaction of the sporulation transcription factor σ^F with its antisigma factor SpoIIAB. Our evidence indicates the existence of three binding regions for SpoIIAB at widely spaced intervals on σ^F . This conclusion is based on four lines of evidence. First, binding studies between SpoIIAB and portions of σ^F showed that the amino-terminal portion (regions 1,2) and the carboxy-terminal portion (regions 3,4) were each capable of binding to SpoIIAB. Second, amino acid substitutions in σ^F that conferred partial resistance to SpoIIAB in vivo were obtained in three regions of the σ^F protein (region 2, region 3.1, and region 4.1), and representative substitutions from each region were shown to impair the binding of σ^F to SpoIIAB in vitro. Third, MBP- σ^F fusion proteins separately containing substitutions with an amino acid (alanine) lacking a side chain beyond the β carbon at positions 48, 137, or 213 of σ^F were found to be defective in binding to SpoIIAB. We interpret this as evidence that the side chains of the wild-type residues at these positions, which are located in regions 2.1, 3.1, and 4.1, respectively, are contact sites for SpoIIAB. Interestingly, side chain truncation substitutions at two other positions (149, 156), at which lysine substitutions had been found to impair binding, did not cause a strong inhibitory effect on complex formation.

We interpret this as evidence that the side chains of the wild-type residues at these positions (glutamate in both cases) do not make important energetic contributions to the SpoIIAB- σ^F interaction. Rather, the lysine substitutions at these positions could impair the SpoIIAB- σ^F interaction by interfering with the nearby contact site at position 137. Fourth, subsegments from the carboxy-terminal portion of σ^F that separately contained the putative contact sites at region 3.1 or 4.1 were each capable of binding to SpoIIAB. Taken together, these results suggest that σ^F contacts SpoIIAB by means of at least three surfaces located in regions 2.1, 3.1, and 4.1.

Affinity chromatography experiments have shown that the SpoIIAB- σ^F complex is highly stable, having a dissociation constant of less than 10^{-7} M and being resistant to 1 M salt (Duncan et al. 1996). The high salt resistance is consistent with the SpoIIAB- σ^F interaction being at least partly hydrophobic in character. Strikingly, all three positions that we identify as potential contact sites on the basis of the alanine substitution analysis are amino acids bearing hydrophobic side chains (V48, V137, and L213). Thus, we infer that σ^F binds to SpoIIAB by making at least three widely separated hydrophobic contacts.

The inference that σ^F residues V48, V137, and L213 contact SpoIIAB rests on the assumption that these residues are displayed on the surface of the σ^F protein. Although no structural information is available for regions 3 or 4 of σ^{70} -like factors, A. Malhotra, E. Severinova, and S. Darst (pers. comm.) recently solved the crystal structure of region 2 of σ^{70} of *E. coli*. In the σ^{70} structure, residue I390, which occupies the homologous position in σ^{70} to V48 in σ^F , may be shielded from solvent by an abutting residue (R436). However, taking into account that the σ^F residue (I94) corresponding to R436 has a smaller side chain, the side chain of V48 could be exposed on the surface of σ^F . If so, this inference would support our contention that V48 contacts SpoIIAB. Interestingly, I390 of σ^{70} is adjacent to an exposed hydrophobic patch that Malhotra et al. (A. Malhotra, E. Severinova, and S. Darst, pers. comm.) suggest may be a surface with which the sigma factor contacts core RNA polymerase. The close proximity of the inferred contact site at V48 to this putative core-binding surface is relevant to our current investigation in two respects. First, it suggests that the binding of SpoIIAB to σ^F could prevent σ^F from associating with core RNA polymerase. Although it is not known whether binding of SpoIIAB sequesters σ^F from core RNA polymerase, Benson and Haldenwang (1993) have presented evidence that a close homolog of SpoIIAB, RsbW, blocks the binding of σ^B (a close homolog of σ^F) to core RNA polymerase. Second, the close proximity of V48 to a core-binding surface suggests that σ^F residues important for the SpoIIAB- σ^F interaction may also be important for the core polymerase- σ^F interaction. If so, this may explain why region 2 mutants were relatively difficult to isolate given that our genetic screen demanded that σ^F be transcriptionally active.

Experiments with another member of the σ^{70} family of sigma factors suggest that region 3 may also be involved

in the binding of sigma to core RNA polymerase. In this work, a mutant of the heat shock sigma (σ^{32}) of *E. coli* that contained a 24-amino-acid deletion in region 3 was found to have impaired affinity for core RNA polymerase (Zhou et al. 1992). This 24-amino-acid deletion lies immediately downstream from the region that corresponds to the sites of residues V137, E149, and E156 in σ^F . If region 3 of σ^F serves a similar function, then binding of SpoIIAB to region 3.1, like binding of SpoIIAB to region 2.1, could interfere with the association of σ^F with core RNA polymerase. Finally, we note that the inferred involvement of regions 2 and 3 in binding to core polymerase raises the possibility that substitutions V137E, E149K, and E156K in region 3.1, and V48A in region 2.1, increase the σ^F -core polymerase interaction, thereby causing (or contributing to) the increased σ^F activity that we observed in the mutants. However, this is evidently not the case for the E149K mutant whose activity in directing transcription in vitro was no higher (or lower) than the wild-type sigma factor in the absence of SpoIIAB.

B. subtilis contains an additional sporulation sigma factor (σ^G) that is highly similar to σ^F , and like σ^F , is also bound by SpoIIAB (Kellner et al. 1996). Interestingly, of the residues in σ^F that we infer to be contact sites for SpoIIAB, two are not conserved in σ^G : σ^G contains an alanine at the position corresponding to V137 and a lysine at the position corresponding to L213. Thus, if we are correct that the side chains of σ^F residues V137 and L213 contact SpoIIAB, then σ^G must contact SpoIIAB differently in detail. Nevertheless, the glutamate residue at position 149 in σ^F , at which a lysine substitution was found to interfere with binding to SpoIIAB, is conserved in σ^G , and a lysine substitution at this position in σ^G also impairs binding of σ^G to SpoIIAB (Kellner et al. 1996). This finding is consistent with the existence of a contact site for SpoIIAB in or near region 3.1 of σ^G , even if the specific amino acid contacts are different from that in σ^F .

SpoIIAB is believed to contain an adenosine nucleotide-binding pocket (Duncan and Losick 1993; Min et al. 1993), and previous work has shown that formation of the SpoIIAB $\cdot$ σ^F complex is stimulated by the presence of ATP and its nonhydrolyzable analogs (Alper et al. 1994). Two models (which are not mutually exclusive) for how ATP stimulates complex formation are as follows: ATP could be directly involved in the binding between SpoIIAB and σ^F by electrostatic interaction between the γ -phosphate of the nucleotide and residues on σ^F . Alternatively, ATP could induce a conformational change in SpoIIAB that is needed for efficient binding to σ^F . Our data supports the second model for the following reason. The interaction of SpoIIAB with each of the three proposed binding sites on σ^F is ATP dependent. However, each SpoIIAB molecule is inferred to contain only a single binding site for ATP (Duncan and Losick 1993; Magnin et al. 1996). Because the SpoIIAB $\cdot$ σ^F complex contains an equal number of SpoIIAB and σ^F molecules (SpoIIAB₂ $\cdot$ σ^F ₂) (Duncan and Losick 1993), ATP could at most contact one binding site on σ^F . Thus, we infer that ATP induces a conformational change in SpoIIAB that is

needed for tight association with at least two of the three contact sites on σ^F (See Fig. 6). Consistent with the idea that ATP is not involved directly in the contact between SpoIIAB and σ^F , work by Duncan et al. (1996) indicates that the ATP-binding pocket of SpoIIAB in the SpoIIAB $\cdot$ σ^F complex is exposed and is capable of interacting directly with the anti-antisigma factor SpoIIAA, which induces the release of σ^F from the SpoIIAB $\cdot$ σ^F complex.

Recently, amino acid substitutions in the flagellar sigma factor FliA of *S. typhimurium* that resulted in increased FliA-directed transcription in the presence of its antisigma factor, FlgM, have been described by two groups (Kutsukake et al. 1994; K. Hughes, pers. comm.). In striking similarity to the results obtained here, both groups recovered amino acid substitutions in regions 2.1, 3.1, and 4 of the FliA protein (See Fig. 7). Indeed, two of the FliA region 4 substitutions (at L199) and one of the σ^F region 4 substitutions (at L213) occur at exactly homologous positions. At present, only the substitutions in region 4 of FliA are inferred (by in vivo titration experiments) to impair binding of FliA to FlgM (Kutsukake et al. 1994). If, however, all of the amino acid substitutions described by Kutsukake et al. and Hughes do impair the FliA-FlgM interaction, then SpoIIAB and FlgM, which show no significant amino-acid similarity (Duncan and Losick 1993), may interact in topologically similar ways with their respective sigma factors. If so, this could indicate that binding in or near regions 2, 3.1, and 4 of σ^{70} -like sigma factors is an especially effective strategy for suppression of sigma factor activity.

Materials and methods

General methods

Routine manipulations of *B. subtilis* and *E. coli* strains were carried out as described (Harwood and Cutting 1990; Sambrook et al. 1989) with two exceptions. First, in preparation of competent *B. subtilis* cells, 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) was added to both competence media whenever cells contained the *P_{spac}-spoOH* fusion (Jaacks et al. 1989). Second, to reduce uninduced expression of the MBP- σ^F fusion proteins, *E. coli* host cells were propagated on NZYM medium (Sambrook et al. 1989) containing 0.2% glucose. Synthetic oli-

Figure 6. Model for the SpoIIAB- σ^F interaction. In the absence of ATP, SpoIIAB is unable to form a tight interaction with σ^F . Upon the addition of ATP, SpoIIAB undergoes a conformational change that allows it to interact efficiently at three widely spaced points on the σ^F molecule.

Figure 7. Comparison of amino acid substitutions in σ^F and FliA that confer SpoIIAB- or FlgM-resistance. The figure is a schematic representation of the sigma factors σ^F and FliA in which the positions of amino acid substitutions that conferred resistance of the sigma factor to its respective antisigma factor (this study and Kutsukake et al. 1994) are shown in relation to each other and to the conserved regions of σ^{70} -like sigma factors.

gonucleotides used in this work are: OL36, 5'-ACAGTCGTGT-CAGAGGC-3'; OL209, 5'-ATGGATGTGGAGGTTAAGAA-AAACG-3'; OL210, 5'-CGAAAAGACCATAAATTACCA-CGC-3'; OL218, 5'-GCTGCTGAATTCAGAGGATATGAG-CCTGACG-3'; OL219, 5'-GCTGCTGGATCCCTAGCTGAT-CGCTTCTTTCAGCGC-3'; OL227, 5'-GGAGAACTACTCGCT-GAAAGTCCTG-3'; OL231, 5'-AGCGTCTTCCATCAGCTGC-CGTTCC-3'; OL261, 5'-GCTGCTGAATTCGACCTTGGAAA-CAAAATCCGC-3'; OL262, 5'-GCTCGCTGGATCCCTACGGC-ACTCTGCCAGTGT-3'; OL276, 5'-GCTGCTGAATTCGA-CAACTCAGAAGAAAATGG-3'; OL299, 5'-GCTGCTGGATC-CCTATTTTAATGACCGTGATACCTTTAC-3'; OL300, 5'-AAA-AAAGAATTCACGGTGCAGGAGATCGCTGAC-3'; OL389, 5'-CCGACGGCGCAGGAGCTCGC-3'; OL390, 5'-TTGAAGCTGC-GGATGTTGTCACTGG-3'; OL391, 5'-CTGGCCCCAAGCGCGC-GTAAGG-3'; and OL392, 5'-CTAATCGTCTATGCCAGATAT-TATAAAG-3'.

B. subtilis strain construction

Strain AB407 (*spoVA* Δ ::*spec*) was constructed by insertion of the spectinomycin resistance gene into the chromosome in place of part of the *spoVA* operon. First, a *SacII*-*HincII* fragment of 850 bp from pPP33 (Pigot et al. 1984) containing sequences internal to *spoVA* was cloned into pJL74 (Ledeaux and Grossman 1995) that had been digested with *Ecl136II* and *SacII* to create pAB55. Second, a *PstI*-*PvuII* fragment of 200 bp from pPP33, which contained the 5' end of *spoVA*, was cloned into pUC18 that had been digested with *PstI* and *SmaI*. Third, this fragment was re-isolated as a *HindIII*-*EcoRI* fragment and cloned into pAB55 that had been digested with *HindIII* and *EcoRI* to create pAB56. Finally, PY79 (Youngman et al. 1984) was transformed to spectinomycin resistance with pAB56 that had been linearized with *ScaI* to create the presence of the *spoVA* Δ ::*spec* insertion in AB407. AB407 was verified by Southern blot hybridization.

Strain AB409 (*amyE*::*spoIIAA*,*spoIIAB* Ω *kan*) was constructed to contain a truncated copy of the *spoIIA* operon lacking *spoIIAC* at the *amyE* locus. First, a *ScaI*-*Sall* fragment of 1.3 kb from pHM2 (Liu et al. 1982), containing the *spoIIA* promoter, *spoIIAA*, and *spoIIAB*, was cloned into pBluescript II SK+ (Stratagene) that had been digested with *SmaI* and *Sall*. Next, the truncated operon was reisolated as a *BamHI*-*HincII* fragment and cloned into pER82 (an *amyE* integration vector) (Ricca et al. 1992) that had been digested with *EcoRI*, filled in with DNA polymerase I Klenow fragment, and then digested with

BamHI, to create pAB54. Finally, PY79 was transformed with pAB54 that had been linearized with *ScaI*, selecting for kanamycin resistance and screening for loss of amylase activity, to create AB409. The presence of this construct was confirmed by its ability to complement a *spoIIAA69* (Yudkin et al. 1985), a *spoIIAB1* (Rather et al. 1990), and a *spoIIAB1* (Rather et al. 1990) mutation.

Strain AB414 (*spoIIIG* Δ 1::*spoIIIG-lacZ**cat*, *P*_{spac}-*spoOH* Ω *cat*, *amyE*::*spoIIAA*,*spoIIAB* Ω *kan*) was constructed by transforming a PY79 derivative containing *P*_{spac}-*spoOH* (Jaacks et al. 1989) to kanamycin resistance with AB409 chromosomal DNA and congressing simultaneously in the *spoIIIG* locus from a strain carrying *spoIIIG* Δ 1::*spoIIIG-lacZ* (Margolis 1993).

PCR mutagenesis and mapping of mutants

Localized mutagenesis of the *spoIIA* operon was performed by PCR using Taq DNA polymerase (see, for example, Zhou et al. 1991). The template for the PCR reactions was *HindIII*-*StuI* digested chromosomal DNA from strain AB407 that contains a spectinomycin resistance gene inserted 200 bp downstream of *spoIIAC*. (Restriction digestion of the template was necessary to prevent the chromosomal DNA from transforming more efficiently than the amplified DNA.) The primers for PCR were OL231, which is located ~2.8 kb downstream of the spectinomycin resistance gene, and OL227, OL36, and OL209, which are located ~2 kb, 1.2 kb, and 0.9 kb upstream, respectively, of the resistance gene. Each PCR reaction contained: 1 μ g of *HindIII*-*StuI* digested AB407 chromosomal DNA, 1 $\times$ thermo buffer (Promega), 2.5 mM MgCl₂, 0.5 mM dNTPs, 40 pmoles of each primer, and 10 units of Taq DNA polymerase (Promega). PCR was carried out for 30 cycles of denaturing at 95°C for 1 min, annealing at 60°C (or 48°C when OL36 was used) for 2 min, and extending at 72°C for 6 min. The resulting 6-kb PCR products were recovered by ethanol precipitation and used to transform AB414 to spectinomycin resistance in the absence of IPTG. Because of the expected toxicity of σ^F mutants resistant to SpoIIAB (Schmidt et al. 1990), expression of the mutated *spoIIA* operon was rendered conditional by means of the presence of a *P*_{spac}-*spoOH* fusion, which produces the transcription factor (σ^{H}) necessary for *spoIIA* expression (Wu et al. 1989) only in response to IPTG (Jaacks et al. 1989). In addition, strain AB414 contained a second copy of the *spoIIAA* and *spoIIAB* genes at the *amyE* locus in order to avoid isolating loss-of-function mutations in either of these two genes. Approximately 4500 transformants from 30 separate PCR reactions were screened for mutants exhibiting elevated levels of σ^F activity by patching the colonies on DS agar (Harwood and Cutting 1990) plates containing 80 μ g/ml 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside and 1 mM IPTG. On average, 1–2% of transformants exhibited the desired phenotype (dark blue patches) whereas 15–20% of transformants showed loss of σ^F activity (white patches).

To distinguish between mutations in *spoIIAA*, *spoIIAB*, and *spoIIAC*, we developed a mapping strategy based on PCR amplification of the mutant chromosomal templates (digested with *HindIII* and *StuI*) with a fixed 3' primer (OL231) and, in separate PCR reactions, different 5' primers (OL36, OL209, OL218, OL300, and OL276) that spanned the *spoIIA* operon. Resulting PCR products were transformed into strain AB414 and spectinomycin-resistant transformants were scored for mutant (dark blue) versus the AB414 parental (light blue) phenotype. By measuring the percentage of transformants that exhibited the mutant phenotype as the 5' primer was moved further downstream into the *spoIIA* operon, we could localize each mutation partially.

We isolated 12 independent *spoIIAC* mutants with elevated

σ^F -directed gene expression. E149K (GAG to AAG) and L213H (CTC to CAC) were each isolated from three independent PCR reactions; V137E (GTG to GAG) was isolated from two independent PCR reactions; and E156K (GAG to AAG), L209Q (CTA to CAA), Y212H (TAT to CAT), and the double mutant V48A,G56R (GTC to GCC at codon 48 and GGA to AGA at codon 56) were each isolated once. Class II mutants were blocked at a late stage of sporulation because of the presence of the *spoVAΔ::spec* insertion/deletion in AB407 [see above].

Nucleotide sequence analysis

A fragment containing all of *spoIIAC* and the 3' end of *spoIIAB* was amplified from mutant chromosomal DNAs by PCR, purified using the QIAquick Spin PCR purification kit (Qiagen), and sequenced using the dideoxy method (Sanger et al. 1977) and the Sequenase kit (U.S. Biochemical).

β -Galactosidase assays and Western blot analysis

Strain AB414 and derivative strains containing the *spoIIAC* mutations were induced to sporulate by nutrient exhaustion in DS media (Harwood and Cutting 1990) supplemented with 1 mM IPTG. Samples (1 ml) were collected in duplicate at hourly intervals during sporulation. The first sample of each duplicate pair was used to determine β -galactosidase activity (Harwood and Cutting 1990), using lysozyme to permeabilize the cells and o-nitrophenol- β -D-galactopyranoside as the substrate, whereas the second sample was used to prepare whole cell extracts for Western blot analysis. Whole cell extracts were prepared as described (Sambrook et al. 1989) except that cells were first incubated with 0.5 mg/ml lysozyme for 15 min at 37°C. Approximately equal number of cells (as determined by optical density) were electrophoresed on SDS-PAGE gels and electroblotted to Immobilon-P membranes (Millipore). Immunodetection was achieved using polyclonal anti- σ^F antibodies (L. Duncan, unpubl.) followed by secondary antibodies conjugated to alkaline phosphatase (Promega).

Construction of plasmids encoding the MBP- σ^F fusion proteins

The *malE-spoIIAC* fusions were constructed in pMAL-c2 [New England Biolabs, (NEB)], in which expression of the *malE* gene is under the control of an IPTG-inducible promoter. Specific fragments of *spoIIAC* were generated by PCR using Vent DNA polymerase (NEB). OL209 and OL210 were used to amplify the entire *spoIIAC* coding sequence and the resulting fragment was cloned into *XmnI-PstI* digested pMAL-c2 using the blunt 5' end and a natural *PstI* site located immediately after the *spoIIAC* stop codon. Because full-length σ^F is toxic to *E. coli* (Yudkin 1986), we chose to clone the 561 allele of *spoIIAC* that is less toxic to *E. coli* (Yudkin and Harrison 1987), yet is still sensitive to SpoIIAB inhibition (Margolis et al. 1991), to create MBP- σ^F_{1-255} . To facilitate cloning of partial *spoIIAC* fragments, 5' PCR primers complementary to sequences internal to *spoIIAC* (OL218, OL261, OL300, and OL276) were engineered to contain an *EcoRI* site, and 3' PCR primers complementary to sequences internal to *spoIIAC* (OL219 and OL299) were engineered to contain a *BamHI* site. (OL219 and OL299 also were engineered to contain an amber stop codon.) Thus, partial *spoIIAC* fragments were cloned as either blunt-*BamHI* fragments, *EcoRI-BamHI* fragments, or *EcoRI-PstI* fragments into pMAL-c2 that had been digested with either *XmnI* and *BamHI*, *EcoRI* and *BamHI*, or *EcoRI* and *PstI*, respectively. Fusion proteins containing the V48A and G56R, V137E, E149K, E156K, or L213H substitutions were constructed as described above except that the template

for PCR amplification was chromosomal DNA of the appropriate mutant. All plasmids encoding fusion proteins that contained amino acid substitutions were sequenced, as well as plasmids encoding wild-type fusion proteins MBP- $\sigma^F_{115-255}$, MBP- $\sigma^F_{136-255}$, and MBP- σ^F_{55-135} .

To create MBP- σ^F_{1-114} V48A and MBP- σ^F_{1-114} G56R, we took advantage of a *DraI* site that exists in between codons 48 and 56 of *spoIIAC* and a second *DraI* site that exists in the pMAL-c2 vector to exchange *DraI* fragments between pAB70, which encodes the wild-type MBP- σ^F_{1-114} fusion protein, and pAB79, which encodes the doubly mutant MBP- σ^F_{1-114} V48A,G56R fusion protein. However, we first needed to remove a third *DraI* site in the pMAL-c2 vector. To this end, we made small deletions in the vector backbone of both pAB70 and pAB79 as follows: pAB70 and pAB79 were digested separately with *DraIII*, rendered flush with T4 DNA polymerase, digested with *SwaI*, and religated. The resulting plasmids, pAB87 and pAB88, respectively, then were digested with *DraI* to generate two fragments each of sizes 5.7 kb and 1.1 kb. Next the 5.7-kb fragment from pAB87 was ligated to the 1.1-kb fragment from pAB88 and vice versa to create pAB89, which encodes MBP- σ^F_{1-114} V48A, and pAB90, which encodes MBP- σ^F_{1-114} G56R. The presence of each single mutation was confirmed by sequencing.

MBP- $\sigma^F_{115-255}$ fusion proteins containing the V137A, E149A, E156A, or L213A substitutions were created by site-directed mutagenesis using the Sculptor in vitro mutagenesis system (Amersham) and oligos OL389, OL390, OL391, and OL392, respectively. To create a single-stranded template for the mutagenesis, a fragment of *spoIIAC* containing codons 115–255 was generated by PCR amplification using oligonucleotides OL261 and OL210, digested with *EcoRI* and *PstI*, and cloned into M13mp19 that had also been digested with *EcoRI* and *PstI*. The site-directed mutations were verified by single-strand sequencing and then replicative form M13 DNA was isolated from each candidate and digested with *EcoRI* and *PstI* to liberate a fragment of 430 bp that was then ligated to pMAL-c2 that had been digested also with *EcoRI* and *PstI*. All resulting plasmids were sequenced to confirm the presence of the desired mutation.

Production and purification of fusion proteins

Fusion proteins were produced in *E. coli* strain TB1 (NEB). Cells were grown at 37°C to mid-log at which time expression of each MBP- σ^F fusion protein was induced with 1 mM IPTG for 1–2 hr. Cell pellets from induced cultures were resuspended in one tenth volume 20 mM HEPES, 150 mM NaCl, 1 mM EDTA buffer and frozen overnight at –20°C. Cells were thawed, sonicated in the presence of 1 mM PMSF, and spun at 9000 g at 4°C for 20 min. Supernatants were used as crude extracts. For purification of MBP and MBP- σ^F_{1-255} , affinity chromatography using amylose beads (NEB) was performed according to manufacturer's guidelines.

[³⁵S]Methionine labeling of SpoIIAB

Radiolabeling of SpoIIAB was performed as described previously using strain LDE15 (Duncan and Losick 1993) except that the radiolabeling of induced cells was carried out for 30 min.

Chemical cross-linking reactions

Chemical cross-linking reactions were performed as described (Alper et al. 1994) except that the buffer contained 2 mg/ml BSA, the cross-linker DSS (Pierce) was used exclusively, cross-

linking was carried out for 2–3 hr on ice, and samples were electrophoresed on 12.5% SDS–polyacrylamide gels. To confirm that approximately equal amounts of the different fusion proteins were used in each cross-linking reaction, equivalent amounts of each crude extract were electrophoresed on SDS–polyacrylamide gels and stained with Coomassie.

Purification of σ^F and E149K σ^F

Production and purification of σ^F using strain LDE7 was performed as described (Duncan et al. 1996). To create an expression strain for E149K σ^F , a 1.1-kb fragment containing the E149K mutant allele of *spoIIAC* was generated by a PCR reaction containing OL36 and OL210 as primers, E149K mutant chromosomal DNA as template, and Vent DNA polymerase. This fragment was digested with *Bgl*II and *Pst*I and cloned into pT713 (Bethesda Research Laboratories) that had been digested with *Bam*HI and *Pst*I to create pAB75. pAB75 was sequenced to confirm that it contained the E149K allele of *spoIIAC*, and then transformed into the T7 expression host, BL21(DE3)pLysS (Novagen). Production and purification of E149K σ^F was carried out as described for wild-type σ^F (Duncan et al. 1996).

In vitro transcription

Transcription reactions were carried out as described (Alper et al. 1994) using a linearized template (*Hinc*II digested pLD14) (Duncan et al. 1996) containing the σ^F -dependent promoter *sspE-2G* (Sun et al. 1991). A sequencing ladder was used as an approximate size marker. Purified SpoIIAB (Duncan et al. 1996) and core RNA polymerase (Duncan and Losick 1993) were gifts of L. Duncan and S. Alper (Harvard University, Cambridge, MA).

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Three sites of contact between the *Bacillus subtilis* transcription factor sigmaF and its antisigma factor SpoIIAB.

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