The *Bacillus subtilis* gene for the developmental transcription factor $\sigma^K$ is generated by excision of a dispensable DNA element containing a sporulation recombinase gene

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The structural gene (*sigK*) for the mother-cell RNA polymerase $\sigma^K$ in *Bacillus subtilis* is a composite of two truncated genes, named *spoIVCB* and *spoIIIC*, which are brought together by site-specific recombination during sporulation. We now show that the recombination event is compartmentalized in that the mother cell, but not the forespore chromosome, undergoes rearrangement. We also show that *spoIIIC* (encoding the carboxy-terminal portion of $\sigma^K$) lies ~42 kb downstream of *spoIVCB* (encoding the amino-terminal portion) and that the joining of the truncated coding sequences is a reciprocal recombination event in which intervening DNA is deleted from the chromosome as a circle. The rearrangement is governed by the product of a gene named *spoIVCA* located in the excised DNA, as demonstrated by the observations (1) that the product of *spoIVCA*, but not the product of any other stage-IV sporulation gene tested, is required for the rearrangement, and (2) that the presence of a cloned copy of the rearranged *sigK* gene in the chromosome bypasses the requirement for the *spoIVCA* gene product in sporulation. Because cells engineered to contain an intact copy of *sigK* sporulate normally, we conclude that the *sigK* rearrangement is not essential for the control of gene expression during sporulation, and we infer the existence of an additional mechanism for restricting $\sigma^K$-directed transcription to the mother-cell chamber of the sporangium. Finally, the construction of a strain deleted for the entire *sigK* intervening sequence shows that the 42-kb element contains no genes essential for viability.

[Key Words: Sporulation, *Bacillus subtilis*, $\sigma$-factor, DNA rearrangement, compartmentalization]

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Endospores of the gram-positive soil bacterium *Bacillus subtilis* are produced in a sporangium that consists of two compartments known as the mother cell and the forespore (Losick and Kroos 1989). Gene expression in the two compartments, which each contain a chromosome generated during the last round of vegetative DNA replication, is regulated differentially. Thus, for example, *cot* genes (Donovan et al. 1987), which encode polypeptide components of the tough protein coat that encases the spore, are expressed selectively in the mother cell [Cutting et al. 1989], whereas *ssp* genes (Conners et al. 1986), which encode a family of acid-soluble proteins located in the core of the spore, are expressed in the forespore (Mason et al. 1988). This differential gene expression is controlled, in part, by compartment-specific RNA polymerase $\sigma$-factors, named $\sigma^K$ [Kroos et al. 1989; Stragier et al. 1989] and $\sigma^C$ [Karmazyn-Campelli et al. 1989; Sun et al. 1989]. The $\sigma^K$-factor is synthesized in the mother cell and directs transcription of certain *cot* and other mother-cell-expressed genes; $\sigma^C$, on the other hand, is produced in the forespore, where it governs transcription of *ssp* and other forespore-expressed genes.

Although the mechanisms that govern the compartmentalized synthesis of $\sigma^C$ and $\sigma^K$ are not well understood, restricted synthesis of $\sigma^K$ in the mother-cell chamber of the sporangium can be partly attributed to a chromosomal rearrangement that generates the structural gene (*sigK*) for the mother-cell $\sigma$-factor [Stragier et al. 1989]. The coding sequence for $\sigma^K$ in the chromosome of vegetative cells is split between two truncated genes, named *spoIVCB* and *spoIIIC*. During sporulation, *spoIVCB*, which encodes the amino-terminal portion of $\sigma^K$, and *spoIIIC*, which encodes the carboxy-terminal portion of the factor, are brought together in-frame by site-specific recombination between repeated 5-bp sequences to create an intact coding sequence. As we show here, the recombination causes the excision of the intervening DNA between *spoIVCB* and *spoIIIC* as a circle and is governed by a gene (*spoIVCA*) that is located within the excised element. Our present results and those of Sato et al. [1990], who have determined recently
that the product of spoIVA is highly similar in its predicted amino acid sequence to that of several prokaryotic DNA recombinases and resolvases, strongly suggest that the spoIVA gene product is a recombinase that catalyzes the excision of the intervening DNA.

In agreement with the view that the rearrangement is partly responsible for the compartmentalized synthesis of $\sigma^K$, we show that the joining of spoIVCB to spoIIIC only occurs in the mother-cell chromosome; hence, only the mother-cell chromosome contains the intact $\sigma^K$ coding sequence. On the other hand, the rearrangement is dispensable for the regulation of gene expression during sporulation in that the introduction of a cloned copy of the intact sigK gene into cells of a mutant blocked in the rearrangement enables the mutant cells to undergo sporulation normally. On this basis, we infer the existence of a mechanism(s) in addition to the compartment-specific chromosomal rearrangement for restricting $\sigma^K$ synthesis to the mother-cell chamber of the sporangium. Finally, we show that the sigK intervening sequence does not contain any essential genes and can be deleted completely from the vegetative chromosome without impairing growth or sporulation.

Results

The joining of spoIVCB to spoIIIC is restricted to the mother-cell chromosome

To investigate whether the rearrangement is compartmentalized, we extracted DNA from the mother-cell and forespore compartments of sporangia that had been purified by density gradient centrifugation in Renografin [see Materials and methods]. To obtain mother-cell DNA, the purified sporangia were treated with lysozyme to release selectively the contents of the mother-cell compartments. To obtain forespore DNA, forespores were purified as described in Materials and methods and then disrupted by treatment with dithiothreitol (DTT) and sodium dodecyl sulfate (SDS) to remove the outer protein coat followed by treatment with lysozyme to dissolve the cortex. DNA from the mother-cell and forespore compartments was then cleaved with the restriction enzyme HindIII and subjected to DNA hybridization analysis with radioactive spoIVCB-containing DNA as a probe. The Southern hybridization experiment of Figure 1 shows that the spoIVCB probe reacted almost exclusively with a HindIII fragment of 7.7 kb in forespore DNA (track b), a fragment size indicative of spoIVCB that had not undergone rearrangement (Stragier et al. 1989). In contrast, hybridization of the probe with fragments of purified mother-cell DNA showed that spoIVCB sequences were principally contained within a HindIII fragment of 4.2 kb (track c), a fragment-size diagnostic of spoIVCB that had undergone joining to spoIIIC (Stragier et al. 1989). For comparison, tracks a and d show hybridization to both the 7.7- and 4.2-kb HindIII fragments in total DNA extracted from Renografin-purified sporangia. (Somewhat stronger hybridization is observed to the 4.2-kb fragment than to the 7.7-kb fragment, presumably because DNA is more readily extracted from the mother cell of intact sporangia than from the forespore.) We conclude that the joining of spoIVCB to spoIIIC is compartmentalized and that essentially all mother-cell chromosomes undergo rearrangement.

Figure 1. Compartmentalization of the chromosomal rearrangement. Mother-cell (track c) and forespore (track b) DNAs and total sporangium DNA (tracks a and d) were obtained from purified sporangia, as described in Materials and methods. The DNAs were digested with HindIII and subjected to electrophoresis in a 0.8% agarose slab gel. After electrophoresis, the DNA fragments were denatured, transferred to nitrocellulose, and incubated under hybridization conditions with the radioactive spoIVCB-containing DNA probe described previously (Stragier et al. 1989). The arrowheads identify the 4.2-kb fragment that contains the rearranged sigK gene, the larger band is the 7.7-kb fragment that contains the unrearranged spoIVCB gene (Stragier et al. 1989).

spoIIIC is located downstream of spoIVCB

The spoIVCB gene is one of two convergently transcribed genes [the other being spoIVA] that constitute the spoIVC locus [Farquhar and Yudkin 1988; Kunkel et al. 1988; Sato et al. 1990]. The spoIVCB and spoIVC genes map between the markers aroDI20 and pheA1 in the order aroD-spoIVCB-spoIVC-spoIVCB-pheA. The spoIIIC gene also maps in the aroD pheA region of the chromosome, but its location relative to spoIVCB and spoIVA was unknown. To map spoIIIC more precisely, we carried out three-factor crosses using the generalized transducing phage PBS1. As a marker for the position of spoIIIC, we used a chloramphenicol-resistance determinant [cat] that had been integrated into the chromosome at the site of the spoIIIC gene by single-reciprocal [Campbell-like] recombination between the chromosomal spoIIIC gene and a cloned copy of spoIIIC contained in a plasmid integration vector [see Materials and methods]. In extension of the work of Farquhar and Yudkin [1988], our three-factor crosses [Table 1] indicate the gene order aroD-spoIVCB-spoIVC-spoIVCB-pheA. Therefore, we deduce that spoIIIC, which encodes the carboxy-terminal portion of $\sigma^K$, is located downstream of spoIVCB, which encodes the amino-terminal portion of the transcription factor, and that the second member of the spoIVC locus [spoIVA] is located in the interval between the truncated genes.

The distance between spoIVCB and spoIIIC was estimated by means of a two-factor cross in which a lysate of phage PBS1 that had been propagated on cells containing the spoIIIC-integrated cat gene was used to...
**Table 1. Phage PBS:-mediated three-factor crosses to map a silent cat gene insertion at the spoIIC locus**

| Donor    | Recipient   | Recombinant types | Number | Inferred order |
|----------|-------------|-------------------|--------|----------------|
| BK137    |BK565        |aro spo cat        | 82     |                |
| spoIVCA133|spoIVLB23    |aro spo cat        | 42     |                |
| BK137    |BK565        |aro spo cat        | 101    |                |
| Selection for Aro*|       |D D D           | 16     |aro spo cat   |
| BK136    |BK565        |aro spo cat        | 48     |                |
| Selection for Aro*|       |D D D           | 10     |aro spo cat   |
| BK137    |BK565        |aro spo cat        | 9      |                |
| spoIVCA133|pheA1        |R D D            | 8      |spo cat phe   |
| Selection for Phe+|       |R R D           | 83     |                |

*(D) Donor genotype; (R) recipient genotype.

*b*cat gene integrated in the chromosome at the spoIIC locus by homologous recombination between the chromosomal spoIIC gene and a cloned copy of spoIIC contained as a 0.92-kb BssHII–HindIII fragment (Stragier et al. 1989) in the integrative plasmid pSK2.

transduce a spoIVCB mutant to chloramphenicol resistance. The observed frequency of cotransduction ranged from 60–80% in several independent experiments. Using the relationship between physical and genetic distance described by Henner and Hoch (1980), we estimate that spoIIC is located 17–45 kb downstream of spoIVCB, a value that is in agreement with the distance between the genes, as estimated by physical methods [see below].

spoIVCB is joined to spoIIC by reciprocal recombination

In earlier work (Stragier et al. 1989), we showed that spoIVCB is joined to spoIIC by means of Southern hybridization experiments, in which we used as hybridization probes radioactive DNA from upstream of the site of recombination in spoIVCB and downstream of the recombination site in spoIIC. To ask whether the recombination is reciprocal, we carried out an additional Southern hybridization experiment in which we used as a probe (the thick bar in Fig. 2A) radioactive DNA from downstream of the recombination site in spoIVCB. The Southern hybridization experiment of Figure 2B shows that the probe reacted exclusively with EcoRI and HindIII fragments of 3.6 and 7.7 kb, respectively, in DNA purified from cells at an early stage of sporulation. These fragments are diagnostic of spoIVCB that has not been joined to spoIIC. Figure 2B also shows that in DNA purified from cells at a late stage of sporulation, the probe additionally reacted with EcoRI and HindIII fragments of 5.5 and 4.8 kb, respectively. These fragment sizes correspond to those expected if DNA downstream of the recombination site in spoIVCB had been joined to DNA upstream of the recombination site in spoIIC. Together with our previously published Southern hybridization analyses, these results are consistent with the idea that the joining of spoIVCB to spoIIC is a reciprocal recombination event.

To investigate the reciprocity of the recombination at the nucleotide sequence level, we cloned DNA overlapping the junction of sequences downstream of the recombination site in spoIVCB and upstream of the recombination site in spoIIC in rearranged chromosomal DNA (see Materials and methods). A determination (data not shown) of the nucleotide sequence across the junction [1] confirmed that DNA downstream of the recombination site in spoIVCB had indeed been joined to DNA upstream of the recombination site in spoIIC and [2] demonstrated that the site of joining of upstream and downstream sequences was the same 5-bp sequence [AATGA; Stragier et al. 1989] at which spoIVCB and spoIIC are joined in sigK. Hence, we conclude that the chromosomal rearrangement is perfectly reciprocal.

Intervening DNA between spoIVCB and spoIIC is excised from the chromosome as a circle

Without knowing the relative orientation of spoIVCB and spoIIC, we could not distinguish whether reciprocal recombination caused excision or inversion of the intervening DNA (see Discussion in Stragier et al. 1989). If the sigK gene was generated by the excision of the intervening DNA, then DNA between spoIVCB and spoIIC should be released from the chromosome as a circle, because our demonstration of reciprocal recombination showed that the borders of the intervening DNA become joined to each other as a consequence of the re-arrangement. If sigK is generated by a DNA inversion, on the other hand, then the intervening DNA should not be released from the chromosome.

To investigate the possibility that intervening DNA is excised as a circle, we studied the migration of intervening sequences by subjecting total uncut [i.e., uncut
A nation. (A) An endonuclease restriction map of the
Figure 2. spoIVCB is joined to spoIIC by reciprocal recombi-
nation. [A] An endonuclease restriction map of the spoIVCB
and spoIIC regions of the chromosome. The hatched boxes
show the location, and the arrows indicate the orientation
of the two genes. The thick horizontal bar identifies the DNA
segment (a 1.1-kb StuI-EcoRI fragment containing the 5' region
of the spoIVA gene) that was used as the probe in the
Southern hybridization of B. The vertical dotted line is the site
of recombinational joining of the two genes. (B) An autoradio-
graph of the hybridization of the radioactive probe to electrophoretically separated EcoRI (tracks 1 and 2) and HindIII (tracks
3 and 4) fragments of total DNA from cells harvested at hours 1
(T1; lanes 1 and 3) and 5 (T5; lanes 2 and 4) of sporulation. The
numbers indicate the sizes [in kb] of the fragments that reacted
with the radioactive probe.

spoIVCB is the only stage IV gene required for the
chromosomal rearrangement

As a first step in identifying spo genes whose products
are involved in the chromosomal rearrangement, we
surveyed mutants blocked at stage IV of sporulation for
those in which the joining of spoIVCB to spoIIC was
prevented. We reasoned that recombination-defective
mutants would be blocked at stage IV, because this is
the stage at which spoIVCB and spoIIC mutants are
blocked. [Note that spoIIC is a misnomer in that
spoIIC mutants are actually blocked at stage IV (Piggot
and Coote 1976).] Among the stage IV mutants exam-
ined, strains containing the mutations spoIIIC94,
spoIVC133, spoIVD92, spoIVD::Tn917HU10 [not
shown], and spoIE11 were found to be prevented from
creating the composite gene [Fig. 4]. The spoIIIC94 mu-
tation is known to be a complete deletion of the spoIIIC
gene [Errington et al. 1988]; hence, the failure of this
mutant to undergo the chromosomal rearrangement is
explained by the absence of the site within spoIIIC at
which the recombination occurs. In other experiments
data not shown] in which radioactive spoIIIC DNA was
used as a hybridization probe, both spoIVD mutants and
the spoIVE mutant were also found to contain large de-
letions encompassing spoIIIC. Thus, the spoIVD92,
spoIVD::Tn917HU10, and spoIE11 mutations in
these strains are evidently misidentified alleles of
spoIIIC, and the failure of mutants harboring these mu-
tations to undergo rearrangement is once again ex-
plained by the absence of one of the two sites at which
recombination occurs. Thus, among stage IV genes
spoIVC, which is itself located in the intervening se-
quence between spoIVCB and spoIIC, is the only can-
date for a gene whose product is involved in the recom-
bination. Several stage III mutants were also examined
for their capacity to undergo the chromosomal rearr-
angement. In confirmation and extension of earlier
work (Stragier et al., 1989), spoIIID is the only stage III
gene so far identified whose product is required for the

field gels after extraction of the DNA from cells em-
bedded in agarose (Canard and Cole 1989). In such an
electrophoresis system, large circular DNAs either do
not enter the gel or exhibit a pulse time-dependent mo-
tility [Beverley 1988]. In these experiments [data not
shown], the radioactive probe for the intervening se-
quence reacted with a DNA species that substantially
entered the gel and was present in chromosomal DNA
from late sporulating cells but not in DNA from early
sporulating cells. The apparent size of the extrachromo-
somal DNA increased dramatically [from 170 to 1250
kb] as the pulse time was increased from 10 to 100 sec.
Combining the demonstration [above] that the recombi-
nation is perfectly reciprocal with the observation (Fig.
3) that the intervening sequence migrates as an ex-
trachromosomal element of aberrant electrophoretic be-
havior, we conclude that the intervening sequence is re-
leased from the chromosome as a circle.
Figure 3. The intervening sequence is excised from the chromosome as an extrachromosomal element during sporulation. Sporulating cells of strain PY79 were harvested at the indicated hourly intervals after the end of exponential growth in DS medium. DNA was purified from the cells and subjected to electrophoresis in a 0.7% agarose gel. After electrophoresis, the DNA was denatured, transferred to nitrocellulose, and incubated under hybridization conditions with the radioactive DNA probe described in Fig. 2. The position of bulk chromosomal DNA (chr) was identified by ethidium bromide staining of the agarose gel. The unlabeled arrowhead identifies the position of the extrachromosomal element.

rearrangement. spoIIIID encodes a small DNA-binding protein, and the basis for its requirement for the chromosomal rearrangement is considered in the Discussion.

If spoIVCA encodes a protein (e.g., a recombinase) that is involved in the recombination, then its product should be diffusible and should act in trans to promote recombination. To investigate this, we inserted a DNA fragment containing spoIVCA and spoIVCB into the amylase E [amyE] locus of the chromosome. The complementation experiment of Table 2 shows that when introduced into the chromosome of a spoIVCA mutant, the amyE::spoIVCA–spoIVCB insertion restored the capacity of the mutant cells to sporulate. In contrast, the amyE:: spoIVCA–spoIVCB insertion was inefficient in restoring sporulation to spoIVCB mutant cells. (spoIVCB mutant cells bearing the amyE::spoIVCA–spoIVCB insertion sporulated ~100-fold less efficiently than did spoIVCA cells containing the insertion.) We interpret these findings to indicate that spoIVCB must be close to spoIIIIC for the rearrangement to occur efficiently [as expected, as spoIVCB contains one of the two sites of recombinational joining] but that spoIVCA encodes a diffusible product that can direct the joining of spoIVCB to spoIIIIC even when the putative recombinase gene is located far from the site of the chromosomal rearrangement. Our results confirm and extend work by Fujita and Kobayashi (1985), who demonstrated complementation of a spoIVCA mutation by a prophage-borne copy of the spoIVCA gene.

The introduction of a cloned copy of the rearranged gene into the chromosome bypasses the requirement for the spoIVCA product in sporulation

As a more direct test of the involvement of the product of spoIVCA in the recombination, we asked whether the requirement for the spoIVCA gene product could be bypassed by introducing a cloned copy of the rearranged gene [i.e., the composite spoIVCA–spoIIIIC or sigK gene] into the amyE locus of the chromosome. We reasoned that if the spoIVCA product is a recombinase or an auxiliary protein involved in the recombination and if it has no essential function in sporulation other than to catalyze the rearrangement, then the presence of the intact σK-coding sequence should enable spoIVCA mutant cells to produce spores. A possible complication in this experiment, however, was the possibility that cells engineered to contain a cloned copy of the composite gene would be blocked in sporulation, because such cells would be expected to pass sigK on to both the mother-cell and the forespore chromosomes during sporulation and, hence, might produce σK in the inappropriate compartment of the sporangium.

Therefore, we first investigated whether introducing a copy of sigK into the chromosome would impair the capacity of wild-type [spo+] cells to sporulate. To do this, we cloned sigK from the DNA of late sporulating cells (as described in Materials and methods) and inserted it into the amyE locus of the chromosome. Cells bearing this amyE::sigK insertion were found to produce normal-looking, heat-resistant spores at normal efficiency (Table 2). Evidently, the presence of sigK in both compartments of the sporangium does not cause impairment of sporulation. Our interpretation of this observation, as presented below, is that the mechanism for restricting sigK to the mother cell by a compartment-specific chromosomal rearrangement is redundant to a separate mechanism that restricts transcription of sigK to the mother cell, whether or not the composite gene is present in the forespore.

Knowing that the amyE::sigK insertion does not impair sporulation, we next asked whether the rearranged gene would complement mutations in either spoIVCA or spoIIIIC, the truncated genes from which the composite gene is generated. Table 2 shows that the presence of the amyE::sigK insertion restored efficient sporulation to both the spoIVCA and the spoIIIIC mutants. This is in striking contrast to the observation above that spoIVCB present at amyE was inefficient in complementing a spoIVCA mutation.

Finally, as a direct test of the idea that spoIVCA governs the rearrangement, the amyE::sigK insertion was introduced into a spoIVCA mutant. Table 2 shows that the presence of the rearranged gene restored the capacity of the mutant cells to sporulate efficiently. (As a control, the amyE::sigK insertion failed to restore the capacity of spoIIIID mutant cells to sporulate.) The rearranged gene must be bypassing the requirement for the spoIVCA gene product rather than complementing the spoIVCA mutation, because the amyE::sigK insertion is entirely lacking in spoIVCA sequences.
Expression of a spore coat protein gene is still compartmentalized in cells containing a cloned copy of the rearranged gene

As noted above, cells that had been engineered to contain a cloned copy of sigK as an insertion in the amyE locus were unimpaired within the limits of measurement in their capacity to undergo sporulation, despite the presence of the composite coding sequence in both the mother-cell and forespore chambers of the sporangia. To investigate whether σ^K-controlled gene expression was compartmentalized in such sporangia, cells of the amyE::sigK insertion strain were lysogenized with a specialized transducing phage bearing a lacZ fusion to the spore coat protein gene cotA [Sandman et al. 1988]. Cells containing the cotA–lacZ fusion were then harvested at 8–9 hr after the onset of sporulation and the contents of their mother-cell and forespore compartments were fractionated, as described previously [Cutting et al. 1989]. Table 3 lists the average specific activities of β-galactosidase observed in the two fractions in several independent experiments. Although the levels of enzyme activity varied somewhat from experiment to experiment [see Table 3 footnote], the specific activity of β-galactosidase was consistently much higher in the mother-cell fractions than in the forespore fractions, whether or not the cells contained the amyE::sigK insertion. As verification of the functionality of the amyE::sigK insertion, similar results were obtained (Table 3) with spoIVCB mutant cells bearing the sigK insertion.) For comparison, the β-galactosidase specific activity was much higher in the forespore fraction than in the mother-cell fraction in extracts of sporulating cells containing a lacZ fusion to the forespore-expressed gene sspB [see Table 3 footnote; Mason et al. 1988]. We conclude that the expression of a gene under σ^K control was substantially restricted to the mother-cell chamber of the sporangium whether or not intact sigK was present in the forespore chromosome.

Deletion of the entire intervening sequence does not impair growth or sporulation

Because the presence of the composite gene bypassed the requirement for the chromosomal rearrangement, we wondered whether the intervening sequence was dispensable for sporulation and whether it contained any genes essential for viability. To investigate this possi-
Wild type 26 2 13
and spolVCB23 amyE :: sigK activity varied 4- to 10-fold from experiment to experiment because of the variability in the efficiency of lysing the mother-cell compartment and permeabilizing the forespores in each experiment. Sporulating cells of the indicated strains containing amyE :: sigK were fractionated into mother-cell and forespore fractions as described previously (Cutting et al. 1989). The specific activity of §-galactosidase in each fraction was determined as described by Miller (1972) and is expressed as nanomoles of ONPG hydrolyzed per min per microgram of protein. Background specific activity of ONPG hydrolyzing activity in the mother-cell [1-2 nmole/min • mg protein] and in the forespore [0.5-1 nmole/min • mg protein] fractions from cells lacking a gene fusion were subtracted.

The following evidence indicates that the Spo ÷ Er s strain was -87 kb, on which basis we infer that the intervening sequence had been replaced by the intact sigK gene in the Spo ÷ Er s transformant strain.

The intervening sequence is ~42 kb in length
Having created a strain lacking the intervening sequence, we could estimate the size of the intervening sequence from the decrease in the size of the NotI fragment that contains the spoIVCB-spoIIC locus (Ventra and Weiss 1989). DNA was prepared from vegetative cells of wild-type and deletion-mutated bacteria that had been embedded in agarose. The DNAs were cut with NotI and were subjected to field inversion electrophoresis (Canard and Cole 1989). The size of the NotI fragment containing the spoIVCB-spoIIC locus was estimated to be 129 kb [data not shown], in agreement with the measurement of Ventra and Weiss [1989]. The corresponding NotI fragment in DNA from the deletion-mutated strain was ~87 kb, on which basis we infer that the intervening sequence is ~42 kb in length. This value is not inconsistent with our estimate of the distance between spoIVCB and spoIIC based on phage-mediated transduction (above).

Excision of sigK intervening element
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Figure 5. Southern hybridization analysis of chromosomal DNA from a strain lacking the intervening sequence. Chromosomal DNA was prepared from wild-type cells harvested at hours 1 (lanes a) and 5 (lanes b) of sporulation and from vegetative cells of a deletion-mutated strain (lanes c) lacking the sigK intervening sequence that was constructed as described in Results and Materials and methods. The DNAs were cut with the indicated restriction enzymes and were subjected to electrophoresis, the DNAs were denatured, transferred to a nitrocellulose filter, and incubated under hybridization conditions with the same radioactive DNA probe used in the experiment of Fig. 1 and as described previously [Stragier et al. 1989]. The arrowheads in b lanes identify spolVCB-containing fragments that occur as a consequence of the rearrangement. The absence of the intervening sequence in the deletion-mutated strain is demonstrated by the perfect match in the pattern of hybridized fragments in vegetative DNA from the mutant strain [lanes c] with the pattern of spolVCB-containing fragments generated by the rearrangement [lanes b].

Discussion

We have shown that the joining of spolVCB (encoding the amino-terminal portion of σ^K) to spolIID [encoding the carboxy-terminal portion of the factor] to create the composite σ^K-coding sequence [sigK] occurs in the mother-cell chamber of the sporangium but not in the forespore. Thus, the existence of a mechanism for creating sigK by a developmentally regulated chromosomal rearrangement provides an explanation [but not the only explanation, as discussed below] for the compartmentalization of the synthesis of the mother-cell σ-factor: The intact σ^K-coding sequence is present in only one of the two compartments of the developing sporangium. It also follows that the rearrangement need not be reversible in that the mother cell and its chromosome are discarded by lysis when maturation of the endospore is complete; only the forespore gives rise to subsequent progeny.

What is the topology of the rearrangement, and how is the rearrangement catalyzed? Our results show that sigK arises as a consequence of the excision of the intervening sequence between spolVCB and spolIIC. We estimate that the intervening sequence is ~42 kb in length, and we infer that it is excised as a circular DNA molecule. We propose that the excised sequence be referred to as skin, for sigK intervening element. The excision event is the result of reciprocal recombination between repeated 5-bp sequences in spolVCB and spolIIC. The spolIIC gene, which—as judged from three-factor mapping experiments—is located downstream of spolVCB, must be in the same orientation as the coding sequence for the amino-terminal coding portion of σ^K; if it were in the opposite orientation [i.e., convergent to spolVCB], then site-specific recombination would be expected to invert rather than excise the intervening DNA.

The DNA recombinase that catalyzes the joining of spolVCB to spolIIC is the product of sporulation gene spolVCA, which is itself located in the skin element. This conclusion is based on the following observations: First, among mutants blocked at the same morphological stage [IV, the stage of cortex formation] as spolVCB and spolIIC mutants [the latter being a misnomer in that spolIIC mutants are blocked at stage IV], only a spolVCA mutant was impaired in the rearrangement. [spolVD92, spolVD :: Tn9170HU10, and spolVE11 mutants were also prevented in the rearrangement, but these mutants were found to contain deletions of the spolIIC gene.] Second, the capacity of a spolVCA mutant to sporulate was restored by the introduction of a cloned copy of the intact sigK gene into the chromosome of the mutant cells. Thus, the spolVCA gene product has no essential function in sporulation other than to cause the joining of spolVCB to spolIIC. Finally and importantly, the predicted product of spolVCA, as determined recently by Sato et al. [1990], is highly similar in its amino acid sequence to members of the Gin Pin Hin TnpR family of site-specific recombinases and resolvases.

In addition to the product of the stage IV gene spolVCA, the joining of spolVCB to spolIIC requires the product of the stage III gene spolIID. The spolIID gene product is a small DNA-binding protein that governs the transcription of sigK [Kroos et al. 1989; Kunkel et al. 1988, 1989]. What is the basis for its requirement in the creation of sigK by rearrangement? We imagine two possibilities, which are not mutually exclusive. One possibility is that the spolIID product is indirectly required for recombination by controlling the transcription of
spolVCA. Another possibility is that spolIID protein participates directly in the recombination event as part of a nucleosome-like, synaptic complex, in analogy with the involvement of small host proteins in Salmonella phase variation and in excisive and integrative recombination by coliphage λ [Pollack and Nash 1983; Johnson et al. 1986; Thompson et al. 1987; Friedman 1988].

Whether the spolIID product participates directly or indirectly in the DNA excision event, its requirement for the joining of spolVCA to spolIIC provides an explanation as to why the rearrangement occurs only in the mother-cell chromosome. Other work has shown that spolIID is not required in the forespore [De Lencastre and Piggot 1979] and that its expression is substantially restricted to the mother-cell chamber of the sporangium (Kunkel et al. 1989). Thus, the compartmentalization of the recombination process is, at least in part, a consequence of the compartmentalization of the expression of a gene required for the rearrangement to occur.

Compartmentalized expression of spolIID also explains the observation that the presence in vegetative cells of a copy of the rearranged σK-coding sequence did not measurably impair the capacity of such cells to sporulate. When vegetative cells that have been engineered to contain intact sigK undergo sporulation, both the mother-cell and the forespore chambers of the sporangium inherit a copy of the composite σK-coding sequence. Nevertheless, as our results show, the presence of sigK in both compartments neither interferes with the formation of normal-looking spores nor allows for the expression in the forespore of a gene (spore coat protein gene cotA) that is under σK control. Because the transcription of sigK depends on the spolIID gene product and because spolIID expression is compartmentalized, the synthesis of σK and, in turn, the transcription of genes that are under σK control, are expected to be restricted to the mother cell, whether or not the forespore contains an intact copy of the σK-coding sequence. That is, the rearrangement process for compartmentalizing the synthesis of σK is both redundant to and dependent upon compartmentalized expression of spolIID.

As a further and more direct demonstration of the redundancy of the rearrangement process, it was possible to construct a strain in which the entire sigK intervening element skin was deleted from the σK coding sequence such that the chromosome simply contained an intact sigK gene. This strain was unimpaired in growth and sporulation, a finding that not only demonstrates that the rearrangement is not required for sporulation but that the skin element does not contain any genes that are essential for viability. In light of this result and in light of the similarity of the spolVCA gene product to the TnpR resolvase of transposon Tn3, it is attractive to imagine that skin is the remnant of a transposable element that inserted into the sigK gene and whose capacity to undergo site-specific recombination came under sporulation control. On the other hand, excision as a circle is characteristic of the genome of lysogenic bacteriophages. Thus, skin might be a cryptic derivative of an ancestral temperate phage whose lytic cycle is induced under sporulation control specifically within the cell compartment programmed to lyse.

Finally, our results extend the analogy between the joining of spolVCA to spolIIC in the mother-cell chromosome of B. subtilis sporangia and the deletion of an 11-kb “excision” from the chromosome of heterocysts of the cyanobacterium Anabaena sp. strain PCC 7120 (Golden et al. 1985; Lammers et al. 1986). In both organisms, the chromosomal DNA rearrangement is the result of the excision as a circle of a DNA element that interrupts the coding sequence for a gene [sigK in B. subtilis and the nitrogen fixation gene nifD in Anabaena] whose expression is confined to the cell type in which the DNA excision occurs. In both organisms, the DNA excision occurs by site-specific recombination between short (5 bp in B. subtilis and 11 bp in Anabaena) repeated sequences, and in both organisms the putative recombinase gene is located within the element that is excised [although the B. subtilis recombinase is a member of the Gin Pin Hin TnpR family, whereas the corresponding Anabaena enzyme is not]. Finally, in both B. subtilis and Anabaena, the DNA excision need not be [and evidently is not] reversible in that the mother cell and the heterocyst are terminally differentiated cell types. It will be interesting to learn the extent to which irreversible chromosomal rearrangements of this sort are a feature of other developmental systems involving terminally differentiating cell types.

Materials and methods

Bacterial strains

B. subtilis strains SC620 [spolIAD59] and SC622 [spolIIE36] (Cutting et al. 1989), BK395 [spolIIIC83], BK558 [spolVCA133], BK556 [spolVCB23], BK410 [spolIC94] [Kunkel et al. 1988, 1989], and KS10 [spolVD : Tn971H10179] [Sandman et al. 1987] were isogenic with the Spor + strain PY79. Strains 67 [spolVA67 trpC2], 292 [spolVD92 trpC2], 498.2 [spolVE11 trpC2], and 88.7 [spolVF88 trpC2] were isogenic with strain SG38 [trpC2] and were provided by J. Errington [Errington and Mandelstam 1986]. Strain SL765 [spolVB165 trpC2] was kindly provided by J. Hoch. The spolVF insertional mutation was previously referred to as a spolVL insertion [Sandman et al. 1987], but recent work [S. Roels, S. Cutting, and R. Losick, unpubl.] indicates that it is allelic to spolVF.

Purification of sporangia and isolation of chromosomal DNA from mother-cell and forespore compartments

Mother-cell and forespore DNA were obtained from sporangia that had been purified by centrifugation through a solution of Renografin. Cells [strain PY79] [250 ml] that had been harvested 9–10 hr after the onset of sporulation in Difco Sporulation [DS] medium were washed in 50 ml of lysis buffer [100 mM NaCl, 50 mM EDTA [pH 7.5]] and suspended in 25 ml of 10% Renografin [Squibb Diagnostics]. Three milliliters of the cell suspension were gently layered onto 9 ml of 50% Renografin in a 15-ml disposable plastic centrifuge tube and centrifuged at 12,000g for 30 min at room temperature. The lower band of cells, consisting mostly of sporangia, was removed from the gradient by piercing the wall of the tube with an 18-gauge needle and by
gently pulling the cells into a syringe. The sporangia were washed in lysis buffer and subjected to a second round of centrifugation through Renografin. Total DNA was obtained by lysing the sporangia in lysis buffer containing 1.5 mg/ml lysozyme for 15 min at 37°C, followed by treatment with Sarkosyl at a final concentration of 1.5%. The lysed sporangia were extracted with phenol/chloroform, and the DNA was ethanol-precipitated. To obtain mother-cell DNA, the sporangia were gently lysed in lysis buffer containing 0.8 mg/ml lysozyme for 15 min at 37°C. The lysed sporangia were centrifuged at 14,000g for 20 min at 4°C to remove spores and unlysed sporangia. The supernatant was removed and extracted twice with phenol/chloroform, and the DNA was ethanol-precipitated. To obtain forespore DNA, forespores were prepared from purified sporangia by lysing the sporangia in lysis buffer containing 0.8 mg/ml lysozyme for 15 min at 37°C, followed by treatment with Sarkosyl at a final concentration of 1.5%. The forespores were collected by centrifugation and subjected in lysis buffer and were subjected to two rounds of sonication for 10 sec at 50 W to disrupt any remaining sporangia. The purified forespores were then suspended in coat extraction buffer [10 mM Tris-HCl (pH 6.8), 50 mM DTT, 2% SDS, 0.025% phenylmethylsulfonyl fluoride (PMSF)] and incubated at 70°C for 30 min, followed by a 10-min incubation at 100°C. The forespores were washed twice in lysis buffer, subjected in lysis buffer containing 2.5 mg/ml lysozyme, and incubated at 37°C for 15 min. Sarkosyl was added to a final concentration of 1.5% to lyse the forespores. The lysed forespores were extracted with phenol/chloroform, and the DNA was ethanol-precipitated.

Cloning DNA overlapping the reciprocal recombination site of joining of sequences downstream of spoIVC and upstream of spoIIC

To obtain DNA overlapping the junction of sequence downstream of spoIVC and upstream of spoIIC, a 2.5-kb EcoRI–HindIII fragment upstream of spoIIC [from previously cloned DNA of vegetative cells (Errington et al. 1988)] was cloned in a derivative of pBR322 containing a erythromycin resistance gene (the erm gene from pE194) capable of expression in B. subtilis. The hybrid plasmid was introduced into the B. subtilis chromosome by single-reciprocal (Campbell) recombination by transformation into the mutant strains listed in Table 2.

Integration of the spoIVCA and spoIVCB genes and of the sigK gene at the amyE locus

The 3.6-kb EcoRI fragment containing the spoIVCA and spoIVCB genes [see Fig. 2] was cloned into pDG268 (Antoniewski et al. 1990), a derivative of the amyE integration vector of Shimotsu and Henner (1986) that had been linearized with EcoRI. The resulting plasmid was linearized with XhoI and used to transform competent wild-type cells of strain PY79 to chloramphenicol resistance. The resulting transformants were screened for those exhibiting an Amy− phenotype (Shimostu and Henner 1986). Chromosomal DNA from one chloramphenicol-resistant Amy− transformant was purified and used to introduce the spoIVCA and spoIVCB genes at the amyE locus by transformation into the mutant strains listed in Table 2.

To introduce sigK at amyE, it was first necessary to clone the composite gene. To do this, the 0.74-kb PvuII–SaiI fragment carrying the 5′ region of the spoIVC gene (Stragier et al. 1989) was subcloned in the integrative vector pDC271 (Antoniewski et al. 1990). The resulting plasmid was introduced by transformation into B. subtilis cells. Next, DNA flanking the integrated plasmid was recovered by digesting chromosomal DNA extracted from cells harvested 5 hr after the onset of sporulation with HindIII, by ligating the cut DNA at low DNA concentration, and by using the ligated DNA to transform E. coli cells. A 1.52-kb Psfl–HindIII fragment containing the sigK gene was then cloned in pUC19, from which it was recovered as an EcoRI–HindIII fragment. The sigK-bearing EcoRI–HindIII fragment was then cloned in the amyE integration vector pDC268. To introduce sigK into the amyE locus, the resulting plasmid was linearized with XhoI and used to transform competent wild-type cells to chloramphenicol resistance. The resulting transformants were screened for those exhibiting an Amy− phenotype (Shimostu and Henner 1986). Chromosomal DNA from one chloramphenicol-resistant Amy− transformant was purified and used to introduce the sigK gene at the amyE locus by transformation into the mutant strains listed in Table 2.

Construction of a deletion-mutated strain lacking the entire intervening sequence

A spoIVCA::erm insertion mutant was constructed by replacement of the wild-type gene in the chromosome by an in vitro-constructed mutant gene in which an erythromycin-resistance-bearing fragment was inserted between the two StuI sites in spoIVC. The pUC19 derivative plasmid bearing intact sigK (see above) was linearized with EcoRI and used to transform competent cells of the spoIVCA::erm mutant. After exposure to the sigK DNA, the competent cells were suspended in DS medium and incubated for 20 hr in the absence of erythromycin. Cells that had sporulated were selected by incubation at 80°C for 10 min. Transformants that survived the heat treatment were plated on DS agar plates without erythromycin and screened for those that were spo+ and Er5. The significance of the Er5 phenotype is that it allowed us to distinguish between transformants expected to be Er5 in which the sigK-bearing plasmid had integrated into the chromosome by Campbell recombination at spoIVC or at spoIIC and transformants in which the intervening sequence had been replaced by the intact sigK by double recombination expected to be Er5.

Transformation of B. subtilis

Competent cells were prepared and transformed as described by Dubnau and Davidoff-Abelson (1971).

Growth and sporulation

Growth and sporulation in DS medium were carried out as described by Sandman et al. (1988).

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