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Genetics

Coupling between gene expression and DNA synthesis early during development in *Bacillus subtilis*

(differentiation/transcription/protein phosphorylation/*spo0A*)

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ABSTRACT **Endospore formation in the bacterium *Bacillus subtilis* involves generation of two cell types, each with different developmental fates. Each cell type contains an active chromosome, and treatments that inhibit DNA synthesis at the beginning of development inhibit spore formation. We describe experiments demonstrating that gene expression early during sporulation is coupled to DNA synthesis. Expression of several genes that are induced early during sporulation, before the formation of two cell types, is inhibited when DNA synthesis is inhibited. Genes that are affected require the transcription factor encoded by *spo0A* for normal induction. Spo0A protein is normally activated early in development by a multicomponent phosphorylation pathway, or phospho-relay. Altered function mutations in *spo0A* that bypass the need for the phospho-relay allow early sporulation gene expression, even when DNA synthesis is inhibited. These results indicate that inhibition of DNA synthesis prevents activation of the Spo0A transcription factor by inhibiting a step in the phospho-relay. It seems likely that coupling early developmental gene expression to DNA synthesis is a general mechanism to prevent inappropriate or unnecessary gene expression.**

During development, the generation of different cell types with characteristic patterns of gene expression often requires DNA replication and cell division. In many organisms, intricate regulatory mechanisms couple DNA synthesis to cell division and gene expression. Identifying the regulatory circuits and factors involved in the coordinate control of DNA synthesis, cell division, and developmental gene expression is essential to understand mechanisms that regulate differentiation.

Spore formation in the bacterium *Bacillus subtilis* is an accessible system for studying the mechanisms and regulatory pathways that control cell division and development. Rapidly growing cells of *B. subtilis* can be induced to differentiate into dormant heat-resistant endospores upon nutrient deprivation (1). One characteristic feature of endospore formation is generation of an asymmetric cell division septum ≈1 hr after nutrient deprivation. Asymmetric septation creates two cell types—the mother cell and forespore—with different developmental fates (reviewed in refs. 2 and 3). Generation of the two cell types, with intact chromosomes, requires DNA synthesis. Inhibition of DNA synthesis early in development inhibits spore formation (4, 5), while blocking DNA synthesis ≈1 hr into sporulation has little or no effect (4).

One of the central regulatory factors required for entry into the developmental pathway and the formation of the two cell types is the *spo0A* gene product. Spo0A is a DNA binding protein that controls transcription of genes involved in the transition from growth to stationary phase and the initiation of sporulation (6–9). Early during sporulation, the Spo0A transcription factor is activated by phosphorylation (10–12), presumably in response to several developmental signals (12, 13). Spo0A is a member of a large family of regulatory proteins (called response regulators) that are part of the two-component regulatory systems (14–18). These proteins are homologous in their N termini and are involved in signal transduction and regulation of gene expression in response to changing environmental conditions. The activity of these regulatory proteins is modulated by phosphorylation of an aspartate residue in the conserved N terminus. Most response regulators are thought to interact directly with their cognate histidine protein kinase (17, 18), the other component of the two-component system. The histidine protein kinases are homologous in their C termini, autophosphorylate on a histidine residue, and transfer the phosphate to the cognate regulator (17, 18).

In contrast to most response regulators, activation of Spo0A seems to occur by a multicomponent phospho-relay that includes products of the sporulation genes *spo0B* and *spo0F* (12) and may allow for integration of multiple developmental signals (12, 13). Most of the other *spo0* genes necessary for initiation of sporulation seem to affect the activation of *spo0A*. There are mutations in *spo0A* (*sof, rvtA*) that bypass the need for the other *spo0* genes except *spo0H* (19–23). They could act by making Spo0A activation independent of phosphorylation or, more likely, by making Spo0A a direct substrate for one or more of the histidine protein kinases (23).

Little is known about the mechanisms that couple development and developmental gene expression to DNA synthesis (discussed in ref. 24). At the least, blocking DNA synthesis early during sporulation in *B. subtilis* should prevent the normal patterns of gene expression after formation of the two cell types, as each cell needs a chromosome to direct gene expression. Furthermore, if cell division is coupled to or dependent on DNA replication, then blocking DNA synthesis should prevent asymmetric septation during sporulation, perhaps in a manner similar to the normal coupling of cell division and DNA synthesis during growth. The primary mechanism by which growing cells sense and respond to DNA damage and disruptions in DNA synthesis is to activate RecA protein, which induces an SOS response and causes a block in cell division (25, 26). Alternatively, there may be mechanisms that couple early predivision sporulation gene expression to DNA synthesis such that disruptions in DNA synthesis could affect the developmental program well before septation.

We describe experiments that demonstrate that early sporulation gene expression in *B. subtilis* is coupled to DNA synthesis. Expression of several genes that are normally induced early during sporulation, before asymmetric septation, is inhibited when DNA synthesis is inhibited. The genes that are affected all require the transcription factor encoded

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Abbreviation: HPUra, 6-(*p*-hydroxyphenylazo)uracil.

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by *spo0A* for normal induction. In addition, at least one of the altered function mutations in *spo0A, rvtA11*, relieves the requirement for DNA replication in the induction of early sporulation gene expression. These results indicate that inhibition of DNA synthesis prevents activation of the Spo0A transcription factor by inhibiting a step in the phospho-relay.

MATERIALS AND METHODS

Strains and *lacZ* Fusions. The *B. subtilis* strains used are listed in Table 1. All were derived from JH642 (27) and contain the *trpC2* and *pheA1* mutations. The *ald–lacZ* fusion is a transcriptional fusion recombined into the nonessential *amyE* locus. *ald* encodes alanine dehydrogenase and is required for normal sporulation (K. Jaacks Siranosian, K.I., and A.D.G., unpublished data). The *amyE*::(*spo0A–lacZ*) transcriptional fusion was made with the *Hin*dIII/*Bgl* II restriction fragment from pJF1361 (14) that contains both the vegetative and sporulation promoters of *spo0A* (28), cloned into the *lacZ* fusion vector pDG268 (29), and recombined into the *B. subtilis* chromosome. The *spoVG–lacZ* translational fusion (30) and the *spoIIA–lacZ* (31) and *spoIIG–lacZ* (32) transcriptional fusions were all carried in the specialized transducing phage SPβ and have been described. The *spoIID–lacZ* transcriptional fusion recombined into *amyE* was provided by P. Stragier (33). The *rvtA11* mutation is a missense mutation in *spo0A* that bypasses the need for *spo0F* and *spo0B* (20). Strains containing *rvtA11* were constructed by transformation of *rvtA11* ≈90% linked to a silent *cat* insertion downstream of *spo0A* (34). The *recA260* mutation is disruption of *recA* and was kindly provided by D. Cheo, K. Bayles, and R. Yasbin (35).

Media. LB medium (36) was used for routine strain growth and maintenance. The minimal medium used was based on the S7 minimal salts medium described by Vasantha and Freese (37), except that Mops buffer was used at 50 rather than 100 mM. The minimal medium was supplemented with 1% glucose, 0.1% glutamate, and 40 μg of all other amino acids per ml. Antibiotic selections used for strain constructions were by standard procedures essentially as described (38, 39).

6-(*p*-Hydroxyphenylazo)uracil (HPUra) is a specific inhibitor of DNA polymerase III of *B. subtilis*, blocks elongation of DNA synthesis (40, 41), and has been used in many of the studies on DNA synthesis and spore formation (4, 5). HPUra was a generous gift from G. Wright (University of Massachusetts Medical Center, Worcester) and was used at a final concentration of 5 μg/ml unless otherwise indicated.

β-Galactosidase Assays. Cells were grown in S7 minimal medium supplemented with glucose, glutamate, and all other amino acids essentially as described (42). Sporulation was initiated by the addition of decoyinine (U-7984; Upjohn) to a final concentration of 1 mg/ml to cultures at an OD_{600} between 0.4 and 0.8. HPUra was added (as indicated) at the same time as, or 20 min before, the addition of decoyinine.

Table 1. *B. subtilis* strains used

Strain	Genotype
JH642	*trpC2 pheA1*
AG1157	JH642 *amyE*::(*ald–lacZ*)
KI1179	JH642 SPβ::(*spoVG–lacZ*)
SIK86	JH642 SPβ::(*spoIIA–lacZ*)
SIK134	JH642 SPβ::(*spoIIA–lacZ*) *rvtA11*
SIK122	JH642 SPβ::(*spoIIG–lacZ*)
KI1267	JH642 SPβ::(*spoIIG–lacZ*) *rvtA11*
KI1276	JH642 *amyE*::(*spoIID–lacZ*)
KI1281	JH642 *amyE*::(*spoIID–lacZ*) *rvtA11*
KI1261	JH642 *amyE*::(*spo0A–lacZ*)
KI1257	JH642 SPβ::(*spoIIA–lacZ*) *recA260*

Samples were taken at the indicated times for determination of β-galactosidase specific activity. Due to the orange color of the medium containing HPUra, cells were removed from the medium by centrifugation and resuspended in Spizizen salts (43) containing 10 mM dithiothreitol. β-Galactosidase specific activity is presented as the (ΔA_{420} per min per ml of culture per OD_{600} unit) × 1000 (44).

RESULTS

DNA Replication Is Required for Spore Formation. To generate the two cell types needed for endospore formation, two intact genomes are needed and conditions that disrupt DNA replication before the generation of the two cell types inhibits spore formation (4, 5). Spore formation can be initiated in several ways, one of which is by the inhibition of GTP biosynthesis by addition of the drug decoyinine (45, 46). As expected, DNA replication is required for spore formation when sporulation is induced with decoyinine. Typically, the sporulation efficiency was between 10% and 30% (usually corresponding to $\approx 2 \times 10^7$ spores per ml) in the absence of HPUra. The sporulation efficiency was typically reduced by a factor of $\approx 10^{-4}$ ($\approx 10^3$ spores per ml) with little or no effect on cell viability when HPUra (5 μg/ml) was added to cells at the same time as (or 20 min before) the addition of decoyinine. [HPUra binds to DNA polymerase III and inhibits elongation of DNA synthesis (40, 41).]

Inhibition of DNA Synthesis Inhibits Gene Expression Early in Sporulation. We measured expression of several genes that are induced early during sporulation, before asymmetric septation, in the presence and absence of HPUra. In all cases, transcription of the gene of interest was monitored by a *lacZ* fusion. The addition of HPUra greatly reduced expression of *spoIIA–*, *spoIIG–*, and *spoVG–lacZ* fusions (Fig. 1). *spoVG* encodes a product that is needed at late times during sporulation (47). The *spoIIA* and *spoIIG* operons encode σ factors that are essential for sporulation and are involved in cell type-specific control of transcription (reviewed in refs. 3 and 48). σ^F, encoded by *spoIIAC*, is active only in the developing forespore (49), while σ^E, encoded by *spoIIGB*, appears to be active only in the mother cell (50).

Transcription of *spoVG, spoIIA*, and *spoIIG* occurs before the asymmetric cell division event that generates the two cell types (51–53) and depends on the transcription factors encoded by *spo0A* and *spo0H* (reviewed in ref. 54). Spo0A directly regulates expression of *spoIIA* and *spoIIG* by binding to the promoter regions and activating transcription (7–9). Spo0A indirectly activates transcription of *spoVG* by inhibiting expression of a protein (AbrB) that directly represses transcription of *spoVG* (6, 55–57). The *spo0H* gene product is a σ factor, σ^H (58), that recognizes promoters upstream of *spoVG* (59) and *spoIIA* (31, 60). Its role in expression of *spoIIG* (and *spoIIE*) is indirect and is through its role in expression of *spo0A* (K. Jaacks Siranosian and A.D.G., unpublished data).

The decreased expression of *spoVG, spoIIA*, and *spoIIG* could not be explained by decreased transcription of *spo0A*, as transcription of *spo0A* did not require DNA synthesis. Addition of HPUra at the beginning of sporulation had little or no effect on expression of a *spo0A–lacZ* fusion (Fig. 2).

In contrast to *spoVG, spoIIA*, and *spoIIG*, transcription of *ald*, encoding alanine dehydrogenase, is not dependent on *spo0A* or *spo0H* (K. Jaacks Siranosian, K.I., and A.D.G., unpublished data). Transcription of *ald* is induced early during sporulation, and this induction is not coupled to DNA synthesis. Addition of HPUra at the time of (or 20 min before) decoyinine addition had little or no effect on expression of an *ald–lacZ* fusion (Fig. 1*D*).

An Altered Function Mutation in *spo0A* Bypasses the DNA Synthesis Requirement for Early Sporulation Gene Expres-

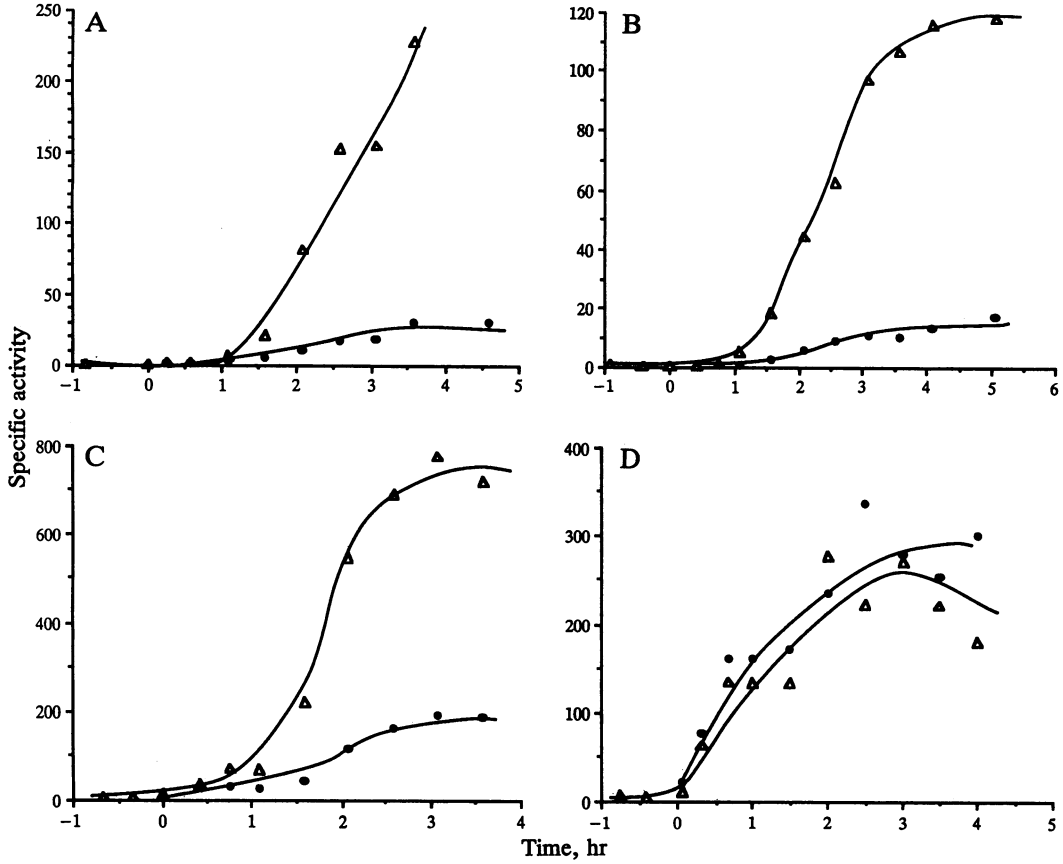

FIG. 1. Expression of genes early in sporulation is inhibited when DNA synthesis is inhibited. The indicated strains were grown in defined minimal medium and samples were taken for determination of β-galactosidase specific activity. Time 0 indicates the time of addition of decoyinine to initiate sporulation. HPUra (5 μg/ml) was added at the same time as (*A* and *B*) or 20 min before (*C* and *D*) addition of decoyinine. △, No HPUra; ●, presence of HPUra. (*A*) Strain SIK86 (*spoIIA–lacZ* in the specialized transducing phage SPβ). Similar results were obtained with a *spoIIA–lacZ* fusion integrated into the *spoIIA* locus in the chromosome, indicating that the effects of HPUra on *spoIIA* expression were not due to possible effects on prophage induction. (*B*) Strain SIK122 (*spoIIG–lacZ*). Similar results were obtained with a *spoIIE–lacZ* fusion, which is normally regulated similarly to *spoIIG* (data not shown). (*C*) Strain KI1179 (*spoVG–lacZ*). (*D*) Strain AG1157 (*ald–lacZ*). In the case of *spoVG* and *ald*, similar results were obtained when HPUra was added at time 0.

sion. SpoOA transcription factor is activated by phosphorylation (10, 11), at least in part by a multicomponent phospho-relay (12). The finding that DNA synthesis is needed for expression of genes controlled by *spo0A* indicated that DNA synthesis might be needed to activate SpoOA. Altered function mutations in *spo0A* (*sof, rvtA*) have been described that bypass the normal phosphorylation pathways and allow sporulation in strains that are missing components of the phospho-relay (19–23). We measured early sporulation gene expression in such a mutant (*rvtA11*) under conditions that block DNA replication (+HPUra).

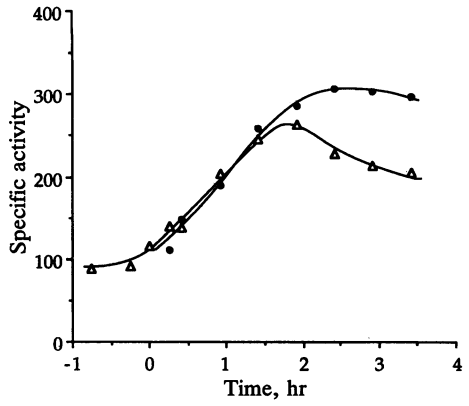

FIG. 2. Transcription of *spo0A* is not inhibited by HPUra. Strain KI1261 (*spo0A–lacZ*) was grown as described in Fig. 1. △, No HPUra; ●, HPUra was added at time 0.

The DNA synthesis requirement for expression of *spoVG*, *spoIIA*, and *spoIIG* was bypassed by the *rvtA11* mutation in *spo0A*. Expression of the *spoIIA–* and *spoIIG–lacZ* fusions was not blocked by HPUra in the *rvtA11* mutant (Fig. 3), even though sporulation was still inhibited by at least 3 orders of magnitude. Similar results were obtained with *spoVG–* and *spoIIE–lacZ* fusions in the *rvtA11* mutant (data not shown).

Expression of the sporulation gene *spoIID* normally depends on the products of *spoIIA*, *spoIIE*, and *spoIIG* (61); is restricted to the mother cell (50); and is thought to depend on and to occur after septation (53). In the presence of HPUra, expression of *spoIID* was blocked in wild-type cells and restored in the *rvtA11* mutant (Fig. 3), consistent with the finding that expression of *spoIIA*, *spoIIE*, and *spoIIG* was restored. This result also suggests that septation might be occurring in the *rvtA11* mutant, even in the absence of DNA synthesis.

Effect of *recA* on Inhibition of Early Gene Expression Caused by HPUra. The primary mechanism by which bacteria are known to sense and respond to inhibition of DNA synthesis is via RecA and the SOS response (reviewed in ref. 26). Addition of HPUra to cells induced *recA*-dependent expression of the SOS-inducible gene *dinC* (data not shown). We tested the effects of HPUra on expression of *spoIIA* in a *recA* null mutant. Expression of *spoIIA* in the *recA* mutant was

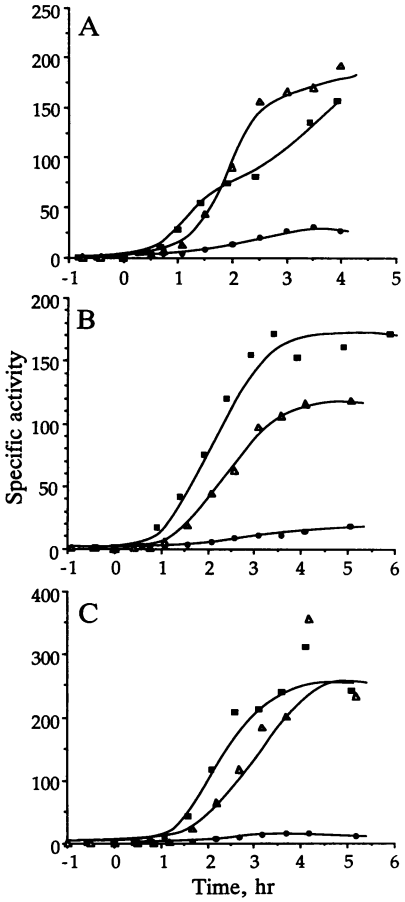

FIG. 3. An altered function mutation in *spo0A* (*rvtA11*) restores expression of *spo* genes in the presence of HPUra. △, *spo0A*$^+$ strains in the absence of HPUra; ●, *spo0A*$^+$ strains in the presence of HPUra (5 μg/ml added at time 0); ■, *rvtA11* mutants in the presence of HPUra (5 μg/ml added at time 0). (*A*) Strains SIK86 (*spoIIA–lacZ; spo0A*$^+$) and SIK134 (*spoIIA–lacZ; rvtA11*). Similar results were obtained with a *spoIIA–lacZ* fusion integrated into the *spoIIA* locus in the chromosome. (*B*) Strains SIK122 (*spoIIG–lacZ; spo0A*$^+$) and KI1267 (*spoIIG–lacZ; rvtA11*). Data for SIK122 are the same as shown in Fig. 1. (*C*) Strains KI1276 (*spoIID–lacZ; spo0A*$^+$) and KI1281 (*spoIID–lacZ; rvtA11*).

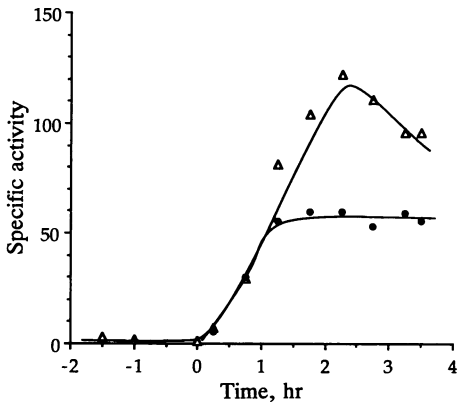

FIG. 4. Null mutation in *recA* partly relieves inhibition of *spoIIA* expression in the presence of HPUra. △, KI1257 (*spoIIA–lacZ; recA260*) in the absence of HPUra; ●, KI1257 in the presence of HPUra (5 μg/ml added at time 0).

reduced only 2- to 3-fold in the presence of HPUra (Fig. 4), while that in *recA*$^+$ cells was typically reduced 5- to 10-fold (Fig. 1), suggesting a possible role for *recA* in inhibiting expression of *spoIIA* in response to HPUra. However, *spoIIA* expression in the *recA* mutant in the presence of HPUra is only 2- to 3-fold greater than that in wild-type cells, indicating that there may be a *recA*-independent mechanism. If *recA* is involved in inhibiting expression of *spoIIA* in response to disruptions in DNA synthesis, presumably this is due to induction of the SOS response and production of a factor that inhibits activation of Spo0A. Identification of such an SOS-induced inhibitory factor would provide better evidence for a role for *recA* in coupling early sporulation gene expression to DNA synthesis.

DISCUSSION

Normal cell growth and division require DNA synthesis and the proper segregation of chromosomes to daughter cells. Mechanisms exist in both prokaryotes and eukaryotes that couple cell division and mitosis to DNA synthesis. In many bacteria, disruptions in DNA synthesis and DNA damage stimulate the activity of the *recA* gene product and cause induction of the SOS response. One of the functions of the response is to produce products (*sulA* or *sfiA* in *Escherichia coli*) that block septation and cell division (reviewed in ref. 26). In eukaryotes, disruptions in DNA synthesis and DNA damage block entry into mitosis (reviewed in ref. 62). In the budding yeast *Saccharomyces cerevisiae* coupling between DNA synthesis and mitosis is controlled by *RAD9*, a nonessential gene that negatively regulates progression into mitosis and ensures proper transmission of chromosomes (63, 64). Genes involved in coupling mitosis and DNA synthesis have been identified in other organisms as well, including the fission yeast *Schizosaccharomyces pombe* (62, 65) and *Aspergillus nidulans* (66).

Spore formation in *B. subtilis* requires DNA synthesis and proper partitioning of an intact chromosome to each of two cells with different developmental fates. Our results indicate that coupling between spore formation and DNA synthesis occurs well before septation. One of the earliest regulatory events required for initiation of sporulation is the activation (phosphorylation) of the transcription factor encoded by *spo0A*. We suggest that activation of Spo0A is coupled to DNA synthesis and have shown that genes that require activated Spo0A for expression are inhibited when DNA synthesis is inhibited. Remarkably, an altered function mutation in *spo0A* that bypasses the normal activation pathway (the phospho-relay) also uncouples early gene expression and DNA synthesis. These results suggest that one of the steps in the phospho-relay that leads to the activation of Spo0A is blocked when DNA synthesis is inhibited.

The phospho-relay that is required for activation of Spo0A involves several steps. The first step is autophosphorylation of one or more histidine protein kinases (10, 12, 29). The phosphate is transferred to an aspartate residue in Spo0F, then to Spo0B, and finally from Spo0B to Spo0A (12). Conceivably, any of the steps in the phosphotransfer pathway, as well as the stability of one of the phosphorylated proteins could be regulated. It has been proposed that the phospho-relay serves to integrate several different developmental signals that are required for initiation of sporulation (12, 13). Our results indicate that cell cycle/DNA synthesis signals feed into the phospho-relay to regulate the activation of Spo0A. It is not yet known how any of the developmental signals needed to initiate sporulation (including DNA synthesis, nutrient deprivation, and cell density) regulates the phosphotransfer pathway.

Coupling of gene expression to DNA synthesis is a general mechanism to prevent inappropriate or unnecessary gene expression and to ensure proper cell cycle control of gene expression. Flagellum biosynthesis in *Caulobacter crescentus* is coupled to DNA replication (67, 68). It is not known

whether this coupling involves *recA* and the SOS response or the regulated activity of a transcription factor (69). Late gene expression in bacteriophage T4 requires DNA replication, and the replication fork seems to function directly as a mobile enhancer of transcription initiation (70, 71). Transcription of the genes encoding transposase from Tn*10* and Tn*5* is stimulated immediately after DNA replication of the promoter region. The transposase promoters contain DNA adenine methylation sites, and, when fully methylated, transcription initiation is inhibited. After replication, these promoters are hemimethylated and are much more active than when fully methylated (72, 73).

It is almost certainly advantageous for *B. subtilis* to have a mechanism to delay or inhibit the initiation of development in response to disruptions in DNA synthesis or DNA damage. Inhibiting the activation of the Spo0A transcription factor under conditions that disrupt DNA synthesis ensures that early developmental gene expression will be inhibited. *B. subtilis* is a soil bacterium and is often exposed to UV irradiation. We speculate that inhibition of early sporulation gene expression in response to inhibition of DNA synthesis, perhaps by the SOS response, is an important mechanism regulating development.

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