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A developmental checkpoint couples the initiation of sporulation to DNA replication in *Bacillus subtilis*

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Spore formation in *Bacillus subtilis* requires the generation of two distinct cell types, each with an active chromosome that becomes committed to a defined program of gene expression. Here we show that a developmental checkpoint couples the initiation of sporulation, and the subsequent formation of these two cell types, to DNA replication early during development. Inhibiting the initiation of chromosomal replication prevents the onset of sporulation and inhibits expression of several genes that are normally induced early during development. This defect in gene expression is due to inhibition of the multi-component phosphorylation pathway needed to activate the developmental transcription factor encoded by *spo0A*. The target affected by inhibiting the initiation of replication is neither Spo0A nor the major kinase, KinA, needed for production of Spo0A ~ P. Rather, the target appears to be one of the proteins that transfers phosphate from the kinase to the Spo0A transcription factor. The signal that couples activity of the phosphorelay to the initiation of DNA replication is different from the previously described DNA damage signal that inhibits the phosphorelay during SOS induction in a *recA*-dependent response. Thus, DNA replication as well as DNA damage signals control production of Spo0A ~ P and initiation of sporulation.

Key words: Bacillus subtilis/DNA replication/phosphorylation/*spo0A*/sporulation

Introduction

Normal growth and development in prokaryotes and eukaryotes require the proper replication and segregation of chromosomes, and cells often possess regulatory mechanisms that couple division or development to the fidelity of chromosomal transmission. In *Escherichia coli* and other prokaryotes, cell division is prevented by DNA damage through induction of the 'SOS' response and production of the cell division inhibitor, SulA (Walker, 1987). In eukaryotes, 'checkpoint' mechanisms exist that couple entry into mitosis to successful completion of chromosomal replication and repair of DNA (Murray, 1992).

The formation of dormant endospores by the Gram-positive soil bacterium *Bacillus subtilis* is a developmental process that occurs in response to nutrient deprivation (Sonenshein, 1989). Approximately 1–2 h after starvation, an asymmetric division septum is formed that establishes two distinct cell types: the forespore and mother cell. The smaller cell, the forespore, develops into the spore within the mother cell. Proper development of the spore requires gene expression and an intact chromosome in each cell type (Piggot and Coote, 1976; Losick and Stragier, 1992).

The generation of the two cell types needed for sporulation requires DNA replication early during development. Studies with mutants that are temperature-sensitive for the initiation of replication (*dnaBts*) have shown that cells subjected to sporulation-inducing conditions in the absence of chromosome replication are unable to sporulate (Dawes and Mandelstam, 1970; Dworkin *et al.*, 1972; Mandelstam and Higgs, 1974; Clarke and Mandelstam, 1980) and do not form the asymmetric division septum that generates the two cell types (Mandelstam *et al.*, 1971). Inhibiting the elongation of DNA synthesis early in development also inhibits spore formation (Dunn *et al.*, 1978; Shibano *et al.*, 1978; Ireton and Grossman, 1992a).

In this paper, we demonstrate the existence of a regulatory mechanism that couples the onset of development to DNA replication. We show that inhibiting the initiation of DNA replication causes a decrease in transcription of genes that are usually induced very early during sporulation. The genes affected are controlled by the Spo0A transcription factor, and the activation (phosphorylation) of Spo0A is inhibited when the initiation of replication is inhibited.

Spo0A, a member of a large family of prokaryotic proteins called response regulators, plays a critical and essential role in the initiation of sporulation. Response regulators control gene expression or behavior in response to environmental and physiological signals. Their activity is regulated by phosphorylation of an aspartate residue in a conserved domain, usually in the amino-terminus. Phosphate is transferred to a response regulator from a histidine protein kinase. These kinases, or sensor proteins, autophosphorylate on a histidine residue, usually in their conserved carboxy-terminal domain. Together, a given response regulator and its cognate histidine protein kinase comprise a particular two-component regulatory system (Albright *et al.*, 1989; Stock *et al.*, 1989; Bourret *et al.*, 1991).

kinA encodes a major histidine protein kinase that is involved in sporulation and phosphorylation of Spo0A (Perego *et al.*, 1989; Antoniewski *et al.*, 1990; Burbulys *et al.*, 1991). However, unlike other characterized two-component systems, KinA does not transfer phosphate directly to Spo0A. Instead, the *spo0F* and *spo0B* gene products serve as intermediaries in the transfer of phosphate from KinA, and/or other histidine protein kinases, to Spo0A. Phosphorylated KinA serves as a donor of phosphate to Spo0F, which is also a member of the response regulator family. Phosphate is then transferred from Spo0F to Spo0B protein, and then finally from Spo0B to Spo0A (Burbulys *et al.*, 1991).

Efficient sporulation and expression of Spo0A-controlled target genes requires the simultaneous presence of several

physiological and environmental conditions, including nutrient deprivation (Sonenshein, 1989), high cell density (Grossman and Losick, 1988; Ireton *et al.*, 1993) and uninterrupted DNA synthesis (Ireton and Grossman, 1992a). At least one of the ways in which these multiple signals control the initiation of sporulation is by regulating the phosphorelay and phosphorylation of Spo0A. Mutations in *spo0A* that bypass the requirement for the phosphorelay (called *sad*, *rvtA* and *sof*), or for phosphorylation (*sad*) also bypass the requirement for these developmental signals in expression of Spo0A-controlled target genes (Ireton and Grossman, 1992a; Ireton *et al.*, 1993).

Recently we showed that inhibiting elongation of DNA replication causes a *recA*-dependent SOS response that prevents expression of genes normally induced very early during sporulation by blocking the activation of the Spo0A transcription factor (Ireton and Grossman, 1992a). In this paper we show that the initiation of DNA replication also regulates the activation of Spo0A through the phosphorelay. Inhibiting the initiation of chromosomal replication inhibits expression of genes that are directly activated by Spo0A ~ P. Initiation of DNA synthesis was inhibited by shifting a *dnaBts* mutant to a non-permissive temperature. *dnaB* is required for the initiation of replication and for attachment of the *oriC* region of the chromosome to the cell membrane (Winston and Sueoka, 1980; Hoshino *et al.*, 1987). The decrease in early developmental gene expression caused by inhibiting the initiation of replication was not dependent on *recA*, indicating that the regulatory response is distinct from the previously described DNA damage response (Ireton and Grossman, 1992a).

Experiments with altered function mutations in *spo0A* that bypass the requirement for the phospho-transfer pathway indicate that this *recA*-independent control of sporulation gene expression acts by regulating a step in the phosphorelay. Additional experiments indicate that the target of this regulation is Spo0F and/or Spo0B, the two proteins that are needed to transfer phosphate from histidine protein kinases to Spo0A.

Results

Inhibition of DNA replication causes a defect in sporulation

dnaB encodes a protein that is required for the initiation of DNA synthesis and the attachment of the replication origin to the cell membrane (Winston and Sueoka, 1980; Hoshino *et al.*, 1987). Temperature-sensitive mutations in *dnaB* have been shown to cause a defect in sporulation at non-permissive temperature (Mandelstam *et al.*, 1971; Dworkin *et al.*, 1972; Mandelstam and Higgs, 1974).

We repeated some of these experiments and found a substantial decrease in the production of spores when cells containing the *dnaB19ts* mutation were induced to sporulate in the absence of DNA synthesis at the restrictive temperature of 42°C. Measurements of [^{3}H]thymidine incorporation in *dnaB19ts* cells indicated that within 1 h after shift to the restrictive temperature, most ongoing rounds of DNA synthesis were completed (data not shown). When starvation conditions were imposed (addition of decoyinine) 1 h after shift to 42°C, the sporulation frequency of the *dnaB19ts* mutant was ~0.1%. In contrast, the sporulation frequency at 32°C was typically ~10%. In parallel experiments, the sporulation frequency of wild type (*dnaB*$^+$) cells at 32°C or 42°C was typically between 20 and 30%.

Somewhat surprisingly, there was also a decrease in sporulation in the *dnaBts* mutant when cells were starved at the same time as (rather than 60 min after) the shift to 42°C. The sporulation frequency at the restrictive temperature was somewhat variable, but was always between 0.2 and 1.0%. Under these conditions, we expected that most cells would be continuing ongoing rounds of replication, as the replication cycle is ~45–55 min and the *dnaB* mutations specifically prevent initiation of DNA replication while having little or no effect on elongation (Winston and Sueoka, 1980; Hoshino *et al.*, 1987). These results indicate that inhibiting the initiation of replication is sufficient to cause a defect in spore formation, and that sporulation might require initiation of a round of DNA replication after the cells have starved.

It is clear that under some starvation conditions *B.subtilis* can initiate a round of DNA replication. The stringent response (amino acid starvation) inhibits DNA synthesis in both *E.coli* and *B.subtilis*. In *E.coli*, initiation of DNA replication at the origin seems to be blocked. However, during a stringent response in *B.subtilis*, DNA replication is inhibited at 'stringent control sites' ~190 kb from either side of the origin, but initiation at the origin can occur (Levine *et al.*, 1991). It is therefore not surprising that cells might be able to initiate a round of DNA replication after sensing starvation conditions for sporulation.

Inhibition of DNA replication prevents expression of genes normally transcribed early during sporulation

Some of the earliest regulatory events in development were affected when DNA replication was inhibited with the *dnaB19ts* mutation. The *spoIIA* and *spoIIE* operons are required for spore formation and are normally transcribed before the formation of the two cell types (Beall and Lutkenhaus, 1991). Expression of *spoIIA* and *spoIIE* (as judged by *lacZ* transcriptional fusions) was significantly reduced in the *dnaB19ts* mutant when sporulation was induced 60 min after shift to non-permissive temperature, when most ongoing rounds of DNA synthesis should be finished (Figure 1A and B). In wild type cells under similar conditions, significant accumulation of β-galactosidase was evident 30–60 min after the initiation of sporulation (Figure 1). Two hours after sporulation was induced, β-galactosidase accumulation in *dnaB19ts* cells at the restrictive temperature was typically 5–10% of that in isogenic *dnaB*$^+$ cells (Figure 1A and B). Similar effects on expression of *spoIIG*, which is regulated similarly to *spoIIE*, were also observed (data not shown).

Early developmental gene expression was also inhibited in *dnaB19ts* cells when sporulation was induced at the same time that cells were shifted to the restrictive temperature (Figure 1C). Under these conditions, ongoing rounds of replication were occurring in most cells when sporulation (starvation) was induced. These results indicate that inhibiting the initiation of replication might be sufficient to affect early sporulation gene expression. In addition, at the permissive growth temperature of 32°C, the *dnaB19ts* mutation caused a delay of ~30 min in the induction of expression of *spoIIA* and *spoIIE*. These results indicate that initiation of replication might be slightly defective even at the permissive temperature and are consistent with the possibility that induction of gene

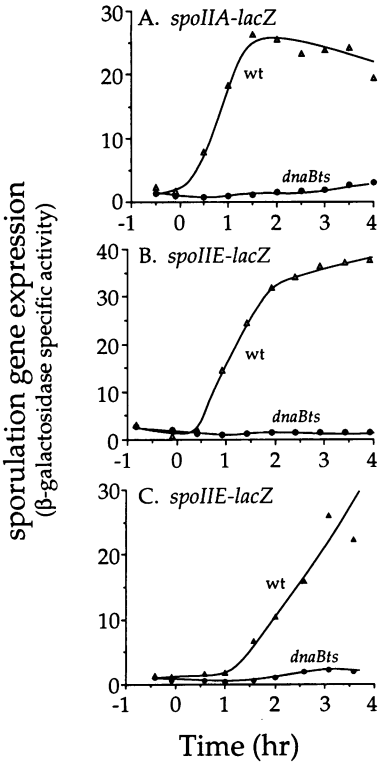

Fig. 1. Expression of genes normally transcribed early during sporulation is inhibited when DNA replication is inhibited with the *dnaB19ts* mutation. The indicated strains were grown in defined minimal medium and samples were taken for determination of β-galactosidase specific activity. Time 0 indicates the time at which sporulation was induced by addition of decoyinine. Cultures were grown at the permissive temperature of 32°C to an OD_{600} of between 0.25 and 0.40. At this time, each culture was split into two parts that were then incubated at either 32°C or the restrictive temperature of 42°C. Sporulation was initiated by addition of decoyinine either 60 min after the shift to 42°C (**A** and **B**) or at the same time as the temperature shift (**C**). For the sake of simplicity, we only show data from the cells that were incubated at 42°C after division of a given culture. (A) Closed circles, strain KI1339 (*spoIIA−lacZ*; *dnaB19ts*); open triangles, strain KI1340 (*spoIIA−lacZ*; *dnaB*$^+$). (B and C) Closed circles, KI1463 (*spoIIE−lacZ*; *dnaB19ts*); open triangles, KI1469 (*spoIIE−lacZ*; *dnaB*$^+$). Similar inhibition of *spoIIA−lacZ* expression in *dnaB19ts* cells was observed when cells were induced to sporulate at the same time initiation of replication was inhibited by upshift to 42°C (data not shown).

expression early in sporulation requires the initiation of a round of DNA replication after the cells have starved.

Inhibition of sporulation gene expression by dnaB19ts is recA-independent

Previously, we had found that inhibition of elongation of DNA synthesis with HPUra [6-(*p*-hydroxy-phenylazo)uracil] induces the SOS response and prevents expression of several developmental genes, including *spoIIA*, *spoIIE* and *spoIIG*. This inhibition of sporulation gene expression is largely relieved by a null mutation in *recA* (Ireton and Grossman, 1992a). In contrast, inhibition of DNA replication with the *dnaB19ts* mutation did not induce the SOS response (Figure 2A). SOS induction was monitored by use of a *lacZ* reporter fusion to the damage-inducible gene, *dinC* (Cheo *et al.*, 1991). When cells containing the *dnaB19ts* mutation were shifted to the restrictive temperature of 42°C no significant accumulation of β-galactosidase from the damage-inducible reporter was observed. In contrast, addition of the

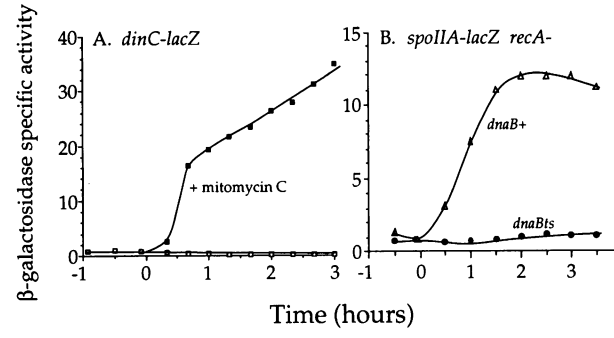

Fig. 2. (**A**) Inhibition of initiation of chromosomal replication with the *dnaB19ts* mutation does not induce expression of the damage-inducible fusion, *dinC18*::Tn*917lac*. Strain KI1359 (*dinC18*::Tn*917lac*; *dnaB19ts*) was grown in defined minimal medium at the permissive temperature of 32°C until an OD_{600} of ~0.40 was reached. At this time (designated as time 0) the culture was shifted to 42°C to inhibit the initiation of replication. At time 0 the culture was also divided into two parts, with one part receiving the SOS-inducing agent mitomycin C at a final concentration of 500 ng/ml (Cheo *et al.*, 1991). Open squares, absence of mitomycin C; closed squares, presence of mitomycin C. (**B**) Inhibition of DNA replication with *dnaB19ts* affects *spoIIA* expression by a *recA*-independent mechanism. Cells were grown and treated as described in the legend to Figure 1, except that the permissive and restrictive temperatures used were 34°C and 43°C, respectively. Again, only data from cells incubated at the restrictive temperature are shown. Closed circles, KI1365 (*spoIIA−lacZ*; *dnaB19ts*; *recA260*); open triangles, KI1366 (*spoIIA−lacZ*; *dnaB*$^+$; *recA260*).

DNA damaging agent mitomycin C caused a rapid induction in expression of the *dinC−lacZ* fusion (Figure 2A). In addition, *recA* was not required for the inhibition of sporulation gene expression in the *dnaB* mutant (Figure 2B). At 42°C in the *dnaB19ts* mutant, expression of *spoIIA* was typically ~10% of that in wild type (*dnaB*$^+$), regardless of whether cells were *recA*$^+$ (Figure 1A) or *recA*$^-$ (Figure 2B).

DNA replication affects activation of the Spo0A transcription factor

Expression of the *spoIIA* and *spoIIE* operons during sporulation requires the phosphorelay and the Spo0A transcription factor. Spo0A plays a direct role in controlling transcription of these operons, as it has been shown to bind to their respective promoters and/or to activate transcription of these promoters *in vitro* (Satola *et al.*, 1991, 1992; Trach *et al.*, 1991; York *et al.*, 1992).

Inhibition of DNA replication with the *dnaB19ts* mutation appears to inhibit expression of the *spoIIA* and *spoIIE* operons by preventing the activation of Spo0A. An altered function mutation in *spo0A* (*rvtA11*) that bypasses the requirement for the normal phosphorylation pathway (Sharrock *et al.*, 1984) bypasses the requirement for DNA replication in *spoIIA* and *spoIIE* expression (Figure 3A and B). *dnaB19ts* cells containing the *rvtA11* mutation in *spo0A* expressed *spoIIA−lacZ* and *spoIIE−lacZ* fusions at levels comparable to those seen in wild type (*dnaB*$^+$) cells, even when DNA replication was inhibited by incubation at the restrictive temperature 1 h before sporulation induction. Similar results were obtained using the *sof-1* mutation in *spo0A* (Hoch *et al.*, 1985; Spiegelman *et al.*, 1990) which also bypasses the requirement for *spo0F* and *spo0B* in sporulation (data not shown).

Inappropriate entry into the sporulation pathway in the

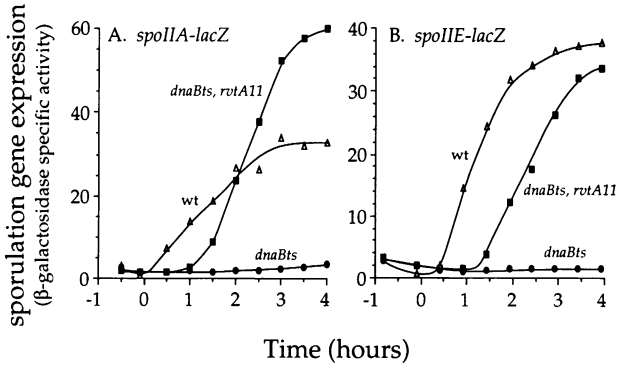

Fig. 3. The *rvtA11* mutation in *spo0A* restores expression of developmental genes when DNA replication is inhibited in the *dnaB19ts* mutant. Cells were grown and treated as described in the legend to Figure 1. Only data from cells that were induced to sporulate at the restrictive temperature (42°C) are presented. (**A**) Closed circles, strain KI1453 (*spoIIA–lacZ; dnaB19ts*); open triangles, KI1457 (*spoIIA–lacZ*; *dnaB*$^+$); closed squares, KI1361 (*spoIIA–lacZ*; *dnaB19ts*; *rvtA11*). (**B**) Closed circles, strain KI1463 (*spoIIE–lacZ*; *dnaB19ts*); open triangles, strain KI1469 (*spoIIE–lacZ*; *dnaB*$^+$); closed squares, strain KI1462 (*spoIIE–lacZ*; *dnaB19ts*; *rvtA11*). The data from strains KI1463 and KI1469 in part B are the same as in Figure 1B. Expression of the *spoII–lacZ* fusions is delayed in the *rvtA11* and *sof-1* mutants when initiation of DNA replication is inhibited. This is consistent with the finding that sporulation of a *spo0F sof-1* mutant is delayed (T.Lewis and A.D.Grossman, unpublished data). In the absence of a functioning phosphorelay, activation of the *sof-1* and *rvtA11* forms of Spo0A depends on MskA (KinC), and may be less efficient than activation through the phosphorelay (see text).

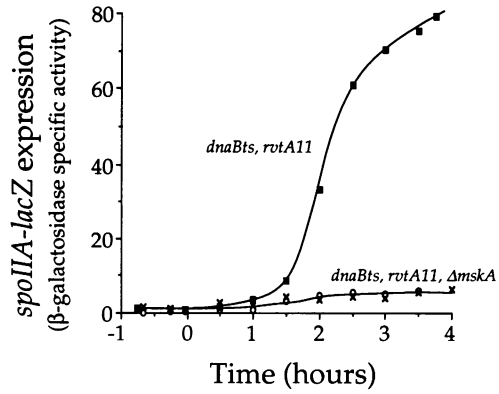

Fig. 4. The kinase encoded by *mskA* is needed for *rvtA11* to bypass the requirement for DNA replication in the expression of genes activated by Spo0A ~ P. Cells were grown and treated as described in the legend to Figure 1. Only data from cells that were induced to sporulate at the restrictive temperature of 42°C are presented. open circles, strain KI1339 (*spoIIA–lacZ*; *dnaB19ts*); closed squares, strain KI1450 (*spoIIA–lacZ*; *dnaB19ts*; *rvtA11*); crosses, strain KI1639 (*spoIIA–lacZ*; *dnaB19ts*; *rvtA11*; Δ*mskA*::*spc*).

absence of DNA replication can be lethal. Fifteen to twenty hours after the initiation of sporulation at the restrictive temperature, the number of viable cells in a culture of the *rvtA11 dnaB19ts* double mutant was typically 0.01–0.10% of the number in a culture of the isogenic *spo0A*$^+$ *dnaB19ts* strain. These results indicate that coupling the initiation of sporulation to proper replication is important for viability as well as for sporulation.

Inhibition of DNA replication regulates Spo0F and/or Spo0B

The possible targets of the replication control include the major kinase that activates the phosphorelay (KinA), a component of the phosphorelay (Spo0F or Spo0B), or Spo0A itself. The *rvtA11* and *sof-1* mutations could bypass the replication control because they bypass the phosphorelay or because they make Spo0A directly resistant to the mechanism of replication control. Experiments below show that the target of the DNA replication control is Spo0F or Spo0B or both.

The *rvtA11* (and *sof-1*) mutations bypass the requirement for components of the phosphorelay, probably by making Spo0A a direct substrate for a histidine protein kinase. We recently identified the gene for this kinase, *mskA* (multicopy suppressor of *spo0K*; also called *kinC*) in a screen for genes that, when present on a multicopy plasmid, suppress the sporulation defect caused by a null mutation in *spo0K* (see below). MskA is needed for cells containing the *rvtA11* (or *sof-1*) mutation to sporulate in the absence of the phosphorelay proteins encoded by *spo0F* and *spo0B* (J.R.LeDeaux and A.D.Grossman, in preparation). The *rvtA11* and *sof-1* forms of Spo0A can be phosphorylated either by MskA or by the phosphorelay, whichever is present.

mskA is needed for the *rvtA11* mutant to activate expression of Spo0A-controlled genes in the absence of DNA replication. A null mutation in *mskA* restores DNA replication control of the early sporulation genes. Cells containing both *rvtA11* and a null mutation in *mskA* are unable to induce significant expression of the *spoIIA* operon when DNA replication is inhibited with *dnaB19ts* (Figure 4). This is in marked contrast to *spoIIA* expression in *mskA*$^+$ *rvtA11* cells in the absence of DNA replication (Figures 3 and 4). These results indicate that DNA replication is not affecting the level or activity of Spo0A directly. For example, if *rvtA11* uncoupled the initiation of sporulation from DNA replication by producing a form of Spo0A that was insensitive to a direct inhibitor, then this form of Spo0A should remain insensitive in the absence of MskA kinase. Rather, DNA replication appears to affect the phosphorylation of Spo0A by somehow regulating the activity of the phosphorelay.

KinA does not seem to be the target of the DNA replication control. Inhibiting DNA replication with the *dnaBts* mutation in a *kinA* null mutant still caused a strong reduction in expression of *spoIIA* (data not shown), indicating that expression of Spo0A-controlled genes in the *kinA* null mutant was still coupled to DNA replication. Together, the results with *rvtA11*, *mskA* and *kinA* indicate that Spo0F and/or Spo0B are the likely targets.

Additional experiments also provided evidence that Spo0F and Spo0B are the targets of the DNA replication control. We utilized two different multicopy clones that bypass the requirement for different parts of the phosphorelay. pLK2 and pLK11 were isolated as multicopy clones that suppress the sporulation defect caused by a null mutation in *spo0K* (J.R.LeDeaux and A.D.Grossman, manuscript in preparation). The *spo0K* operon encodes an oligopeptide permease that is required for the initiation of sporulation (Perego *et al.*, 1991; Rudner *et al.*, 1991) and is required for the activation of Spo0A (Sharrock *et al.*, 1984; Shoji *et al.*, 1988; Rudner *et al.*, 1991). This oligopeptide

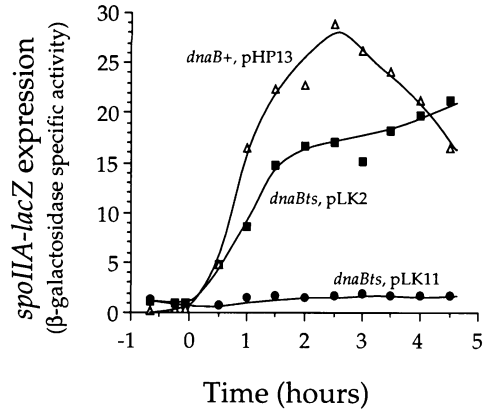

Fig. 5. Multicopy plasmid pLK2 bypasses the requirement for DNA replication in expression of a Spo0A-controlled target gene. Cells were grown and treated as described in the legend to Figure 1, except that chloramphenicol was added to the growth medium to a final concentration of 5 μg/ml to select for the presence of the plasmids pHP13, pLK2 or pLK11. Plasmid pHP13 is the parent vector for pLK2 and pLK11 (see Materials and methods), and in similar experiments this plasmid had no effect on the defect in *spoIIA−lacZ* expression caused by inhibition of DNA synthesis in the *dnaB19ts* mutant. The data shown are from cells induced to sporulate at the restrictive temperature (42°C). Closed circles, strain KI1430 (*spoIIA−lacZ*; *dnaB19ts*; pLK11); open triangles, strain KI1431 (*spoIIA−lacZ*; *dnaB*$^+$; pHP13); closed squares, strain KI1429 (*spoIIA−lacZ*; *dnaB19ts*; pLK2).

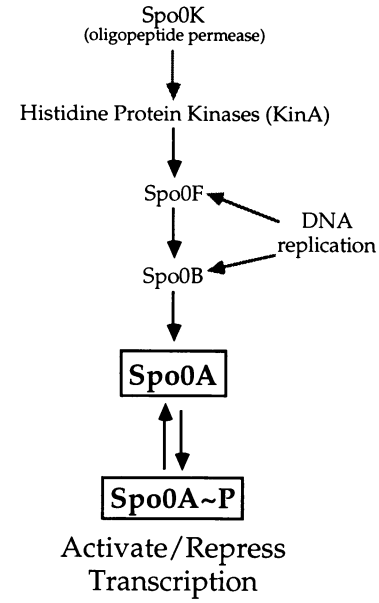

Fig. 6. DNA replication regulates Spo0F and/or Spo0B in the phosphorelay. Spo0K is thought to regulate one or more histidine protein kinases, possibly including KinA (Rudner *et al.*, 1991). Phosphate is transferred through the phosphorelay to activate Spo0A (Burbulys *et al.*, 1991).

permease seems to act upstream of KinA or other histidine protein kinases (Rudner *et al.*, 1991).

Both pLK2 and pLK11 bypass the need for *kinA* in sporulation. In addition, pLK2 partially bypasses the requirement for the phosphorelay components encoded by *spo0F* and *spo0B* in sporulation, while pLK11 does not bypass the need for these components (J.R.LeDeaux and A.D.Grossman, manuscript in preparation). The suppressing activity of pLK2 is due to overexpression of the histidine protein kinase encoded by *mskA* (discussed above). When overexpressed, this kinase causes phosphorylation of Spo0A in the absence of the phosphorelay. We do not yet know the mechanism by which pLK11 bypasses the need for *kinA* in sporulation. However, these two plasmids can be used to test the genetic requirements for control of the phosphorelay by DNA replication.

If DNA replication signals normally affect Spo0A activation by regulating KinA or a component acting upstream of KinA, then both multicopy plasmids, pLK11 and pLK2, should bypass the requirement for DNA replication in the expression of Spo0A-controlled target genes. If, on the other hand, DNA replication signals normally regulate a component that acts downstream of KinA, such as Spo0F and Spo0B, then only pLK2, which is able to bypass the requirement for the entire activation pathway, should restore sporulation gene expression in the absence of DNA replication.

Like the *rvtA11* mutation in *spo0A*, pLK2 was able to bypass the requirement for DNA replication in the expression of a Spo0A-controlled target gene. In contrast, pLK11 was not. Expression of *spoIIA−lacZ* in the *dnaBts* mutant was significantly restored by the presence of pLK2, but not by the presence of pLK11 (Figure 5). These results indicate that the ability to bypass the requirement for *kinA* in sporulation is not sufficient to uncouple sporulation gene expression from DNA replication signals. The simplest interpretation of these results is that DNA replication signals normally control the activation of Spo0A by acting downstream of KinA, to regulate the activity of Spo0F or Spo0B (Figure 6).

Inhibiting elongation of DNA replication and inducing the SOS response seems to control the same step in the phosphorelay as the *recA*-independent pathway. Expression of *spoIIA−lacZ* was inhibited in the presence of HPUra (which blocks elongation and induces SOS) in cells containing pLK11, but expression was nearly normal in cells containing pLK2 (data not shown). These results indicate that DNA damage signals (*recA*-dependent) also control Spo0A activation by affecting Spo0F or Spo0B, and suggest that DNA replication and DNA damage signals may regulate the same target in the phosphorelay.

Discussion

DNA replication and DNA damage controls converge to regulate phosphorylation of the Spo0A transcription factor

Both DNA replication and DNA damage signals control activation of the Spo0A transcription factor. In this way, the activation of Spo0A serves as a developmental checkpoint that couples the initiation of sporulation to the proper replication and repair of chromosomes that are required for the two different cell types generated later in development. Both DNA replication and DNA damage signals appear to control the activation of Spo0A by regulating the activity of Spo0F and/or Spo0B (Figure 6). In principle, the activity of a given phosphorelay component could be modulated by regulation of the ability to transfer or accept phosphate, or by control of a phosphatase activity that acts on that component. It is not presently known whether Spo0F alone or Spo0B alone is the target of responses to DNA replication and DNA damage signals, or whether the activity of both of these phosphorelay components is affected by these signals. In addition, it is possible that the signaling pathways

for DNA damage and DNA replication converge before the phosphorelay. In this case, there would be genes involved in both regulatory responses. Characterization of mutations that allow the phosphorelay to function in the absence of DNA replication, or in the presence of DNA damage, should identify components of the signaling pathways.

Cells sense DNA replication and DNA damage by different mechanisms

The signals generated or modulated by inhibition of DNA replication and DNA damage are transmitted to the phosphorelay, at least in part, by different mechanisms. Inhibition of elongation of DNA synthesis or DNA damage prevents the activation of Spo0A by a mechanism that is mostly dependent on *recA* (Ireton and Grossman, 1992a). Apparently the SOS response induces production of a product, perhaps a phosphatase, that inhibits a step in the phosphorelay.

In contrast, inhibiting the initiation of DNA replication with the *dnaB19ts* mutation affects the activation of Spo0A independently of *recA* (Figure 2). It is not known how the signals generated or modulated by inhibition of DNA replication with *dnaB19ts* are sensed and transmitted to the phosphorelay. One possibility is that the DnaB protein itself is specifically involved in regulation of the phospho-transfer pathway. For example, the activity of a phosphorelay component could be coupled to the assembly of an active replication initiation complex that requires the presence of functional DnaB. In this regard it is interesting to note that *dnaB* appears to be in an operon with two other genes, *orfY* and *orfZ*, which may also be required for the initiation of replication. The products of these genes and *dnaB* have been hypothesized to form a membrane-bound complex that is involved in attachment of the chromosome to the membrane and in the initiation of replication (Hoshino *et al.*, 1987; Sueoka *et al.*, 1988). One or more of the proteins present in such a complex could directly or indirectly regulate the phosphorelay, with this regulation depending on the ability of the complex to initiate replication successfully.

It is also possible that the activity of the phosphorelay is coupled to the passage of a replication fork through a region in the chromosome, as previously suggested (Mandelstam and Higgs, 1974). Replication through a particular chromosomal region could alter the concentration of a protein or other diffusible factor, or stimulate transcription of a particular gene that regulates the phosphorelay. Several examples of coupling between transcription of certain genes and replication through a particular region of DNA exist. The replication fork of the bacteriophage T4 appears to function as a mobile enhancer of expression of late phage promoters, and transcription of these promoters is activated by replication through an upstream region of DNA (Herendeen *et al.*, 1989; Geiduschek, 1991). In addition, transcription of genes encoding transposase from Tn*10* and Tn*5* is activated by replication through their respective promoter regions (Roberts *et al.*, 1985; Yin *et al.*, 1988).

Regulatory responses controlled by DNA replication or damage in other organisms

Like the initiation of sporulation in *B.subtilis*, progression through the cell cycle in several eukaryotic organisms is prevented by inhibition of DNA replication or DNA damage. Mutations have been identified, in organisms as diverse as yeast and mammalian cells, that abolish the cell cycle arrest normally caused by inhibition of DNA replication or DNA damage, suggesting that these organisms all possess regulatory mechanisms that couple cell cycle progression to the replication and repair of chromosomes (Nishimoto *et al.*, 1978; Osmani *et al.*, 1988; Weinert and Hartwell, 1988; Matsumoto and Beach, 1991; Al-Khodairy and Carr, 1992; Enoch *et al.*, 1992; Kuerbitz *et al.*, 1992; Murray, 1992; Rowley *et al.*, 1992). The mechanisms by which DNA damage or inhibition of DNA replication control cell cycle progression are generally unclear at this point, although substantial progress in this area has been made for a few of these organisms. Genetic evidence indicates that in the yeast *Schizosaccharomyces pombe*, inhibition of DNA replication prevents the onset of mitosis by regulating the activity of the protein kinase encoded by *cdc2* (Gould and Nurse, 1989; Enoch and Nurse, 1990). In addition, like the initiation of sporulation, the initiation of S-phase in mammalian cells is coupled to DNA damage by post-translational regulation of a protein, p53, that can function as a transcriptional regulator (Kastan *et al.*, 1991; Kuerbitz *et al.*, 1992; Prives and Manfredi, 1993). Coupling of cell cycle progression to DNA damage by p53 appears to be important for preventing the generation of cells with chromosomal aberrations, since mice null for p53 exhibit an increased frequency of tumor formation and mutational events in tumors (Donehower *et al.*, 1992). Coupling of development or cell cycle progression to DNA replication and repair appears to be a general mechanism for preventing the wasteful, and sometimes harmful, generation of cell types with defective chromosomes.

Table I. *Bacillus subtilis* strains used

Strain	Genotype or description[a]
JH642	*trpC2 pheA1*
KI1339	JH642 *spoIIA–lacZ dnaB19ts-zhb83*::Tn*917*
KI1340	JH642 *spoIIA–lacZ zhb83*::Tn*917*
KI1359	JH642 *dinC18*::Tn*917lac dnaB19ts-zhb83*::Tn*917*::*cat*
KI1361	JH642 *spoIIA–lacZ dnaB19ts-zhb83*::Tn*917 rvtA11-spc*
KI1365	JH642 *spoIIA–lacZ dnaB19ts-zhb83*::Tn*917 recA260*
KI1366	JH642 *spoIIA–lacZ zhb83*::Tn*917 recA260*
KI1429	JH642 *spoIIA–lacZ dnaB19ts-zhb83*::Tn*917* pLK2
KI1430	JH642 *spoIIA–lacZ dnaB19ts-zhb83*::Tn*917* pLK11
KI1431	JH642 *spoIIA–lacZ zhb83*::Tn*917* pHP13
KI1450	JH642 *spoIIA–lacZ dnaB19ts-zhb83*::Tn*917 rvtA11-cat*
KI1453	JH642 *spoIIA–lacZ dnaB19ts-zhb83*::Tn*917* (*spo0A*$^+$)-*spc*
KI1457	JH642 *spoIIA–lacZ zhb83*::Tn*917* (*spo0A*$^+$)-*spc*
KI1462	JH642 *spoIIE–lacZ dnaB19ts-zhb83*::Tn*917 rvtA11-spc*
KI1463	JH642 *spoIIE–lacZ dnaB19ts-zhb83*::Tn*917* (*spo0A*$^+$)-*spc*
KI1469	JH642 *spoIIE–lacZ zhb83*::Tn*917* (*spo0A*$^+$)-*spc*
KI1683	JH642 *spoIIA–lacZ dnaB19ts-zhb83*::Tn*917 rvtA11-cat* Δ*mskA*::*spc*

[a]The *spo–lacZ* fusion in all strains is associated with neomycin resistance. Unless otherwise indicated, Tn*917* confers resistance to erythromycin (and lincomycin). pLK2, pLK11 and pHP13 confer resistance to both chloramphenicol and erythromycin. The *recA260* mutation is associated with both chloramphenicol and erythromycin resistance. *cat* confers resistance to chloramphenicol and *spc* confers resistance to spectinomycin.

Materials and methods

Strains, lacZ fusions and plasmids

The *B.subtilis* strains used are derived from JH642 (*trpC2 pheA1*) (Perego *et al.*, 1988) and are listed in Table I. *dinC18*::Tn*917lac* is a transcriptional

fusion/insertion located in the damage-inducible gene, *dinC* (Cheo *et al.*, 1991), and was kindly provided by D.Cheo, K.Bayles and R.Yasbin. The *spoIIA–lacZ* (Wu *et al.*, 1989) and *spoIIE–lacZ* (Guzman *et al.*, 1988) transcriptional fusions were integrated by a single crossover into the *spoIIA* and *spoIIE* loci, respectively, and have been described. The selectable marker associated with the *spoIIA* and *spoIIE* fusions was converted from *cat* to *neo* (*cat*::*neo*), that is, from chloramphenicol resistance to chloramphenicol sensitivity and neomycin resistance, using pIK105, as described by Ireton and Grossman (1992b). The *dnaB19ts* mutation is a temperature-sensitive mutation in the *dnaB* gene that specifically prevents the initiation of chromosomal replication at the restrictive temperature (Karamata and Gross, 1970; Winston and Sueoka, 1980; Hoshino *et al.*, 1987). Strains containing *dnaB19ts* were constructed by transformation and selection for a silent Tn*917* insertion, *zhb83*::Tn*917* (Vandeyar and Zahler, 1986), that we found to be ~25% linked to *dnaB19ts* by transformation. The *recA260* (*cat* and *erm*) mutation was provided by D.Cheo, K.Bayles and R.Yasbin (Cheo *et al.*, 1992). *rvtA11* is a missense mutation in *spo0A* that suppresses that sporulation defects normally caused by *spo0F* and *spo0B* mutations (Sharrock *et al.*, 1984). Strains containing the *rvtA11* mutation were constructed by transformation with DNA from a strain with *rvtA11* ~90% linked to a spectinomycin resistance (*spc*) or chloramphenicol resistance (*cat*) cassette inserted just downstream of *spo0A* (Grossman *et al.*, 1992; Ireton and Grossman, 1992a). The plasmids pLK2 and pLK11 are derivatives of the *B.subtilis*/*E.coli* shuttle vector pHP13 (*cat* and *erm*) (Bron, 1990) and contain inserts of chromosomal DNA from *B.subtilis* (J.R.LeDeaux and A.D.Grossman, manuscript in preparation).

Media and transformations

Routine growth and maintenance of *B.subtilis* was carried out in LB medium (Davis *et al.*, 1980). Minimal medium used for β-galactosidase and sporulation assays was S7 medium (Vasantha and Freese, 1980), except that MOPS buffer was used at 50 mM instead of 100 mM. The minimal medium was supplemented with 1% glucose, 0.1% glutamate and 50 mg/ml of each required amino acid. Media for plates contained 15 g of agar (Difco Laboratories) per liter. Antibiotics were used at standard concentrations (Harwood and Cutting, 1990). *Bacillus subtilis* cells were made competent and transformed by either of two methods previously described (Dubnau and Davidoff-Abelson, 1971; Rudner *et al.*, 1991). The GMP synthetase inhibitor decoyinine was added to a final concentration of 0.5–1.0 mg/ml to initiate sporulation in minimal media (Mitani *et al.*, 1977).

β-Galactosidase assays

Cells were grown and treated as described in the figure legends. All experiments were done at least three times, except those in Figure 4 (Δ*mskA*) which were done twice. β-galactosidase assays were done as described (Miller, 1972; Jaacks *et al.*, 1989), and specific activity is expressed as the (ΔA_{420} per min per ml of culture per OD_{600} unit) × 1000 (Miller, 1972). β-galactosidase specific activity in cells grown at 42°C is typically lower than that in cells grown at lower temperatures.

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