Integration of multiple developmental signals in Bacillus subtilis through the Spo0A transcription factor.

K Ireton, D Z Rudner, K J Siranosian and A D Grossman

Genes & Dev. 1993 7: 283-294
Access the most recent version at doi:10.1101/gad.7.2.283

Integration of multiple developmental signals in *Bacillus subtilis* through the Spo0A transcription factor

Keith Ireton, David Z. Rudner,[1] Kathryn Jaacks Siranosian, and Alan D. Grossman[2]

Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139 USA

Multiple physiological and environmental signals are needed to initiate endospore formation in *Bacillus subtilis*. One key event controlling sporulation is activation of the Spo0A transcription factor. Spo0A is a member of a large family of conserved regulatory proteins whose activity is controlled by phosphorylation. We have isolated deletion mutations that remove part of the conserved amino terminus of Spo0A and make the transcription factor constitutively active, indicating that the amino terminus normally functions to keep the protein in an inactive state. Expression of an activated gene product is sufficient to activate expression of several sporulation genes in the absence of signals normally needed for initiation of sporulation. Our results indicate that nutritional, cell density, and cell-cycle signals are integrated through the phosphorylation pathway that controls activation of Spo0A.

[*Key Words*: sporulation; pheromones; development; regulation; phosphorylation; signal transduction]

Received November 5, 1992; revised version accepted December 17, 1992.

Gene expression and developmental processes in both eukaryotes and prokaryotes are often controlled by multiple environmental and physiological signals. Transcription of the *lac* operon, the relatively simple textbook paradigm, is controlled by multiple signals. Inducer is required for expression, and the catabolic state of the cell has significant effects on levels of transcription. Different transcription factors (LacI and CAP–cAMP) independently respond to and transduce each of these signals (Reznikoff 1992). More complex developmental systems are also often regulated by multiple signals, including nutritional, cell-cycle, and intercellular signals, with a defined set of conditions necessary for the developmental process to occur. Sporulation in the yeast *Saccharomyces cerevisiae* is subject to both nutritional (Fantes 1989; Gibbs and Marshall 1989) and cell-cycle control (Weber and Byers 1992). Aggregation into fruiting bodies and subsequent spore formation in the bacterium *Myxococcus xanthus* requires starvation for amino acids as well as at least four different intercellular signals (Kim et al. 1992). Starvation and signaling between cells or organisms are also required for heterocyst formation in the cyanobacterium *Anabaena* (Buikema and Haselkorn 1991) and for dauer formation in the nematode *Caenorhabditis elegans* (Riddle et al. 1981; Golden and Riddle 1985). In all of these organisms, mechanisms must exist to sense and integrate the multiple signals that trigger a given developmental process.

Several physiological and environmental signals are required for the initiation of endospore formation in the Gram positive bacterium *Bacillus subtilis*. Starvation for a carbon, nitrogen, or phosphorus source is the primary signal needed to initiate spore formation (Sonenshein 1989). In addition, cells must be at a relatively high density (Grossman and Losick 1988) and undergoing DNA synthesis (Dunn et al. 1978; Shibano et al. 1978; Ireton and Grossman 1992) for spore formation to begin. All three of these conditions must be met for the successful initiation of sporulation, and *B. subtilis* must have mechanisms for sensing and integrating these signals to ensure the appropriate developmental response.

One of the key regulatory factors required for the initiation of sporulation is the *spo0A* gene product. *spo0A* encodes a transcription factor that can activate or repress transcription depending on the DNA target (Strauch et al. 1990; Satola et al. 1991, 1992; York et al. 1992). Spo0A belongs to a large family of bacterial regulatory proteins called response regulators that have conserved amino termini (Ferrari et al. 1985; Kudoh et al. 1985). The activity of the response regulators is modulated by phosphorylation of an aspartate residue in the conserved amino terminus (Albright et al. 1989; Stock et al. 1989b; Bourret et al. 1991).

Response regulators comprise one member of so-called two-component regulatory systems. The second component is a histidine protein kinase that autophosphorylates at a conserved histidine residue and serves as a

[1]Present address: Department of Molecular and Cellular Biology, University of California, Berkeley, California 94720 USA.
[2]Corresponding author.

substrate for transfer of the phosphate to the amino terminus of its cognate response regulator (Albright et al. 1989; Stock et al. 1989b; Bourret et al. 1991). The *kinA* gene product is one of the histidine protein kinases involved in the activation of Spo0A and the initiation of sporulation (Perego et al. 1989; Antoniewski et al. 1990).

Unlike most kinases of two-component systems, which appear to transfer phosphate directly to their cognate response regulators, transfer of phosphate from KinA to Spo0A is indirect. Two other genes that are required for the initiation of sporulation (*spo0F* and *spo0B*) encode products involved in activation of Spo0A by phosphorylation. These proteins form a phospho-relay in which phosphate is transferred from KinA (and probably other histidine protein kinases) to Spo0F, from Spo0F to Spo0B, and finally from Spo0B to Spo0A (Burbulys et al. 1991). In addition, genetic evidence indicates that several other loci, including *spo0E*, *spo0J*, and *spo0K*, might regulate one or more steps in the phospho-relay (for discussion, see Burbulys et al. 1991; Grossman 1991).

Speculation abounds concerning the role of this phospho-relay in the sensing and integration of developmental signals (Burbulys et al. 1991; Grossman 1991). In principle, all developmental signals could regulate the activity of Spo0A, with each physiological signal affecting one or more steps in the phospho-relay. In this way, the phospho-relay would serve to integrate the multiple signals needed for the initiation of sporulation, ensuring that Spo0A is activated only in the presence of all appropriate signals. Alternatively, some or all developmental signals could affect other transcription factors, for example σ^A and σ^H, the *rpoD* and *spo0H* gene products, both of which are needed for the initiation of sporulation.

Mutations affecting Spo0A, or the phospho-relay, that by-pass developmental signals would provide evidence that those signals normally affect the pathway leading to activation of Spo0A. Using an altered function mutation in *spo0A*, we recently presented evidence that DNA synthesis signals control the activation of Spo0A by regulating a step in the phospho-relay (Ireton and Grossman 1992). Expression of genes that are activated by Spo0A early during sporulation is inhibited when DNA synthesis is inhibited. Furthermore, mutations in *spo0A* that make phosphorylation of the Spo0A transcription factor independent of the phospho-relay (mutations that by-pass the need for *spo0B* and *spo0F*) (Kawamura and Saito 1983; Sharrock et al. 1984; Hoch et al. 1985; Shoji et al. 1988; Spiegelman et al. 1990) allow early gene expression, even when DNA synthesis is inhibited (Ireton and Grossman 1992). Thus, DNA synthesis signals affect some of the earliest changes in gene expression during sporulation by affecting a step in the phospho-relay.

In this report, we present evidence that other developmental signals, including nutrient deprivation and cell density, regulate the activation of Spo0A through the phospho-relay. We have identified mutations in *spo0A* that by-pass the requirement for nutrient deprivation in expression of several sporulation genes. These mutations activate expression of target genes in the absence of a component of the phospho-relay or of the putative phosphorylation site, indicating that they encode forms of Spo0A that are constitutively active. One of these mutations also suppresses the sporulation defect normally observed when cells are starved at low density. Taken together, our results indicate that all three signals that affect the initiation of sporulation, nutritional, cell density, and DNA synthesis are integrated through the phospho-relay to affect the activation of Spo0A.

Results

Rationale

We set out to identify mutations in *spo0A* that would make the transcription factor constitutively active. These mutations should by-pass the activation pathway and no longer require phosphorylation to be active. Such mutations should also by-pass developmental signals that regulate the activation pathway. Thus, by determining which developmental signals become unnecessary for induction of sporulation gene expression in these mutants, we hoped to determine which signals normally control activation of Spo0A.

It seemed likely that expression of a constitutively active form of Spo0A that was able to activate expression of sporulation genes might also inhibit vegetative growth, even in the presence of *spo0A*$^+$. We used this putative toxicity to screen for mutations in *spo0A* that might make the gene product constitutively active. We isolated dominant mutations in *spo0A* that, when expressed, caused a defect in vegetative growth. Expression of mutagenized *spo0A* was controlled from a fusion to the LacI-repressed/IPTG-inducible promoter, P_{spac} (Yansura and Henner 1984; Henner 1990), so that any mutant form of Spo0A would be expressed only in the presence of IPTG (Fig. 1). Replacing the endogenous regulatory region of *spo0A* with the heterologous promoter P_{spac} provided the added benefit of removing sites that might be involved in autogenous regulation. There are three Spo0A binding sites in the *spo0A* promoter region (Strauch et al. 1992), and an activated Spo0A might repress its own synthesis, masking effects on expression of other genes.

The mutagenesis strategy used to generate activated mutations in *spo0A* was inspired by studies on certain prokaryotic and eukaryotic transcription factors. The activity of several regulatory proteins is masked by an inhibitory domain on the same protein (Godowski et al. 1987; Hollenberg et al. 1987; Menon and Lee 1990; Choi and Greenberg 1991). For these proteins, deletion of the inhibitory region results in a constitutively active transcription factor. In addition, for certain bacterial response regulator proteins, the conserved amino terminal region appears to inhibit the activity of the rest of the protein (Simms et al. 1985; Kahn and Ditta 1991). These results suggested that it might be possible to generate constitutively active mutations in *spo0A* by deleting parts of the amino-terminal domain. In addition, we were encouraged by recent work indicating that small deletions in the amino terminus of Spo0A made the pro-

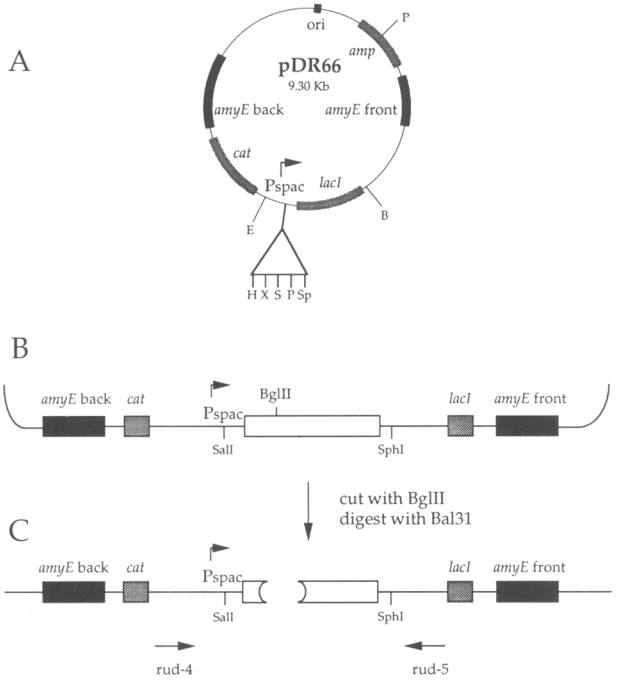

Figure 1. Construction of P_{spac}–*spo0A* and generation of the *sad* deletions. (*A*) Map of pDR66, the cloning vector that was used to construct a fusion of *spo0A* to the LacI-repressed/IPTG-inducible promoter, P_{spac}. pDR66 was made from pDH32 (Shimotsu and Henner 1986) and pAG58 (Jaacks et al. 1989) and contains the selectable marker *amp* and an origin of replication (*ori*) for growth and maintenance in *E. coli*. pDR66 also contains a selectable marker for *B. subtilis* (*cat*) conferring Cm^R, and regions of homology to the nonessential *amyE* locus (*amyE* front and *amyE* back). A similar plasmid, pDR67 contains the multiple cloning site from pDH88 (Henner 1990). In the course of constructing both pDR66 and pDR67, we first deleted the *Dra*III–*Sph*I fragment from pDH32. Next, the *Eco*RI–*Bam*HI fragment from pAG58 or pDH88, which contains the P_{spac} cassette, including *lacI*, was cloned between the *Eco*RI site (found in the cluster of initial cloning sites in pDH32), and the *Sac*I site (located approximately in the middle of *lacZ*). Thus, pDR66 contains unique *Hin*dIII, *Xba*I, *Sal*I, and *Sph*I sites downstream of P_{spac} that are suitable for cloning. In addition, there is no longer an intact *lacZ*. (*B*) The *spo0A* structural gene, without the promoters, was cloned into the *Sal*I–*Sph*I sites of pDR66 to produce pDR69. *Bacillus subtilis* DNA containing *spo0A* extends from the *Hpa*I site, that is between the promoter and the 5′ end of the structural gene, to the *Hin*cII site, that is downstream of the structural gene (Ferrari et al. 1985; Kudoh et al. 1985). (*C*) Deletions in *spo0A* were generated by linearizing pDR69 at the unique *Bgl*II site, located approximately one-fourth of the way into the structural gene, digesting with *Bal*31 exonuclease, and ligating with T4 DNA ligase as described (Materials and methods). Mutant alleles of *spo0A* fused to P_{spac} were then placed into the chromosome of *spo0A*$^+$ cells by recombination into the *amyE* locus. The resulting *sad* (*spo0A* dominant) alleles were tested for the ability to inhibit growth when expressed by addition of IPTG, and mutations that produced the desired phenotype (*sad54*, *sad57*, *sad67*, and *sad76*) were cloned from the chromosome by PCR using primers rud-4 and rud-5 as described (Materials and methods).

tein independent of phosphorylation (Green et al. 1991). It is not clear, however, if these mutations by-pass any of the normal developmental signals.

Identification of mutations in spo0A *that make the gene product constitutively active*

Deletion mutations in *spo0A* were made beginning at the *Bgl*II restriction site located approximately one-fourth of the way into the coding region (Fig. 1). Mutagenized DNA was transformed into wild-type cells and transformants were tested for their ability to form colonies when the mutagenized copy of *spo0A* was expressed, that is in the presence of IPTG. Out of about 800 transformants, ~60 had impaired growth on LB plates containing IPTG, compared to growth on plates lacking IPTG. These mutations were called *sad* (*spo0A* dominant), because they caused a defect in growth even in the presence of the *spo0A*$^+$ allele.

The strength of the different *sad* alleles varied greatly. In the presence of IPTG, the most severe mutants formed very small, pinpoint colonies, whereas the less severe mutants formed colonies only slightly smaller than wild type. We have characterized in detail two mutations (*sad67* and *sad76*) that, when expressed, cause the strongest inhibition of growth. *sad67* is a deletion of 19 codons, removing amino acids 63 through 81, whereas *sad76* is a deletion of 21 codons, removing amino acids 61 through 81 (Fig. 2, Materials and methods). In addition, we have characterized in less detail two other alle-

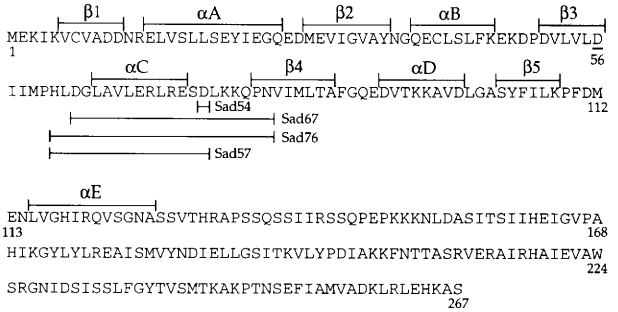

Figure 2. Sequence of Spo0A protein and location of the *sad* mutations. The amino-terminal phospho-acceptor domain, conserved among response regulators, is located within the first 126 amino acids of Spo0A. The α-helical regions and β strands in Spo0A are predicted on the basis of the structure of the response regulator protein CheY (Stock et al. 1989a) and have been presented elsewhere (Stock et al. 1989b; Spiegelman et al. 1990; Green et al. 1991). The conserved aspartate (asp56) that corresponds to the site of phosphorylation in two of the well-characterized response regulators, NtrC and CheY (Sanders et al. 1992a,b) is underlined. The amino acids deleted by the *sad54*, *sad57*, *sad67*, and *sad76* mutations are underlined, and the deletion end points are indicated by vertical lines. In addition to the random *Bal*31 deletions made from the *Bgl*II restriction site, we constructed a deletion, by site-directed mutagenesis, that removed amino acids 4 through 81. This allele did not encode a functional protein as it was unable to complement a *spo0A* null mutation (K. Ireton and A.D. Grossman, unpubl.).

les (*sad54* and *sad57*) that caused a less severe growth defect. *sad54* and *sad57* are deletions of one codon, removing amino acid 75, and 15 codons, removing amino acids 61 through 75, respectively (Fig. 2).

The sad *mutations activate expression of sporulation genes during growth*

We tested the ability of the *sad* mutations to activate transcription of sporulation genes in the absence of starvation signals by monitoring expression of two different *lacZ* fusions, *spoIIA–lacZ* and *spoIIG–lacZ*. The *spoIIA* and *spoIIG* operons are essential for sporulation and encode σ factors that are involved in cell-type-specific control of gene expression (Losick and Stragier 1992). These operons both require Spo0A for expression, but are transcribed by different forms of RNA polymerase holoenzyme. The *spoIIA* promoter is recognized by RNA polymerase containing σ^H, the *spo0H* gene product, whereas the *spoIIG* promoter is recognized by RNA polymerase containing σ^A, the major vegetative σ factor. Transcription from both of these promoters is normally very low or off during growth, is induced early during sporulation, and requires nutrient deprivation (Kenney and Moran 1987; Kenney et al. 1989; Wu et al. 1989) and DNA synthesis (Ireton and Grossman 1992). In vivo, *spo0A* and the phospho-relay are required for transcription of *spoIIA* and *spoIIG* (Kenney et al. 1989; Wu et al. 1989). In vitro, Spo0A binds to these promoter regions and activates transcription (Burbulys et al. 1991; Satola et al. 1991, 1992; York et al. 1992).

Both the *sad67* and *sad76* gene products were able to activate transcription from the *spoIIA* and *spoIIG* operon promoters during vegetative growth (Fig. 3). Cells containing either the *sad67* or *sad76* allele under control of P_{spac} and either a *spoIIA–lacZ* or *spoIIG–lacZ* transcriptional fusion were grown in LB medium. Transcription of the *spoIIA–lacZ* or *spoIIG–lacZ* reporter fusions was induced within 1 hr after induction of expression of the *sad* allele (+ IPTG) (Fig. 3). Accumulation of β-galactosidase continued for at least 2.5 hr after induction of expression of the *sad* gene product, whereas no significant accumulation of β-galactosidase was observed in cells that were not expressing the *sad* gene products (in the absence of IPTG). In contrast, expression of the wild-type Spo0A transcription factor from P_{spac}–*spo0A*$^+$, in parallel experiments, did not induce expression of either sporulation gene (Fig. 3).

The *sad54* and *sad57* mutations were also able to activate expression of *spoIIA* in the absence of starvation conditions, although activation by these two alleles was somewhat weaker than the activation caused by expressing *sad67* or *sad76*. Two hours after induction of *sad54* during vegetative growth in LB medium, accumulation of β-galactosidase from the *spoIIA–lacZ* reporter fusion was typically 30–40% the level of β-galactosidase accumulated in cells expressing the *sad67* allele. Induction of the *sad57* allele during growth in LB typically resulted in accumulation of β-galactosidase 2 hr after induction that

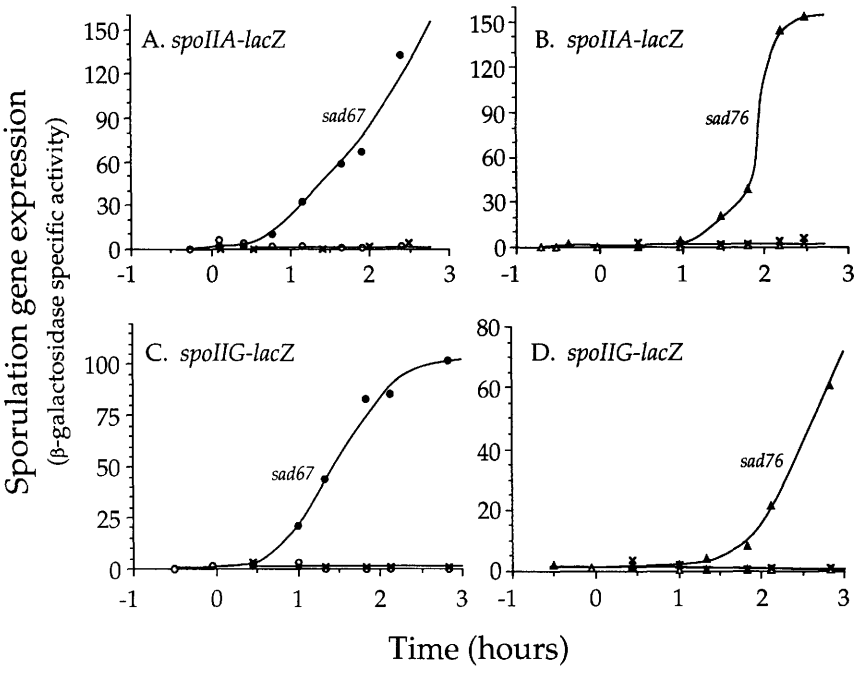

Figure 3. Expression of *sad67* and *sad76* induces expression of Spo0A-controlled target genes in the absence of starvation. The indicated strains were grown in LB medium, and samples were taken for determination of β-galactosidase specific activity as described in Materials and methods. Time zero (T_0) represents the time at which IPTG was added to induce expression of the *sad67*, *sad76*, or *spo0A*$^+$ alleles from P_{spac}. In *A*, each culture was divided into two parts, one with and one without IPTG. In *B*, *C*, and *D*, cells were grown in LB and diluted into prewarmed LB with and without IPTG. T_0 represents the time of addition of IPTG (1 mM). [Normal expression of these *spoIIA–lacZ* and *spoIIG–lacZ* fusions during sporulation in wild-type cells results in peak specific activity values of ~170–240 units and ~80–120 units, respectively. For example, see (Ireton and Grossman 1992).] (*A*) Strain SIK83 [*spoIIA–lacZ amyE*::(P_{spac}–*sad67 cat*)]: (●) plus IPTG; (○) no IPTG. Strain SIK85 [*spoIIA–lacZ amyE*::(P_{spac}–*spo0A*$^+$ *cat*)]: (X) plus IPTG. (*B*) Strain SIK84 [*spoIIA–lacZ amyE*::(P_{spac}–*sad76 cat*)]: (▲) plus IPTG; (△) no IPTG. Strain SIK85 [*spoIIA–lacZ amyE*::(P_{spac}–*spo0A*$^+$ *cat*)]: (X) plus IPTG. (*C*) Strain SIK103 [*spoIIG–lacZ amyE*::(P_{spac}–*sad67 cat*)]: (●) plus IPTG; (○) no IPTG. Strain SIK105 [*spoIIG–lacZ amyE*::(P_{spac}–*spo0A*$^+$ *cat*)]; (X) plus IPTG. (*D*) Strain SIK104 [*spoIIG–lacZ amyE*::P_{spac}–*sad76 cat*)]: (▲) plus IPTG; (△) no IPTG. Strain SIK105 [*spoIIG–lacZ amyE*::P_{spac}–*spo0A*$^+$ *cat*)], (X) plus IPTG. The data for P_{spac}–*spo0A*$^+$ are from the same experiment as *C*.

was 60–90% the amount accumulated in cells expressing *sad67* (data not shown).

sad67 *and* sad76 *repress transcription of* abrB *during vegetative growth*

In addition to activating transcription of several sporulation genes (e.g. *spoIIA* and *spoIIG*), Spo0A represses expression of other genes, most notably *abrB* (Perego et al. 1988; Strauch et al. 1990). Repression of transcription of *abrB* is one of the mechanisms by which Spo0A indirectly activates expression of other genes. The *abrB* gene product is a transcriptional repressor, inhibiting expression of several genes involved in sporulation, including *spoVG* (Zuber and Losick 1987), *spo0E* (Perego and Hoch 1991), *spo0H* (Weir et al. 1991), and *csh163* (Jaacks et al. 1989) (Y.H. Wang, K. Shim, and A.D. Grossman, unpubl.). Spo0A binds to a site just downstream of one of the promoters for *abrB*, inhibiting synthesis of AbrB at the end of exponential growth (Strauch et al. 1990), thereby activating expression of AbrB-repressed genes.

Expression of *sad67* caused decreased transcription of *abrB* in the absence of starvation conditions. When expression of *sad67* was induced with IPTG, β-galactosidase specific activity from an *abrB–lacZ* fusion decreased, compared to no IPTG (Fig. 4). In the course of these experiments, the cells continue to double every 20 to 30 min. The decrease in β-galactosidase specific activity had a half-life of ~20 to 30 min, indicating that the amount of enzyme per cell was depleted as the cells divided. Expression of *sad76* had a similar effect on *abrB–lacZ* expression (data not shown). In contrast, expression of *spo0A*$^+$ from P_{spac} under similar conditions had little or no effect on expression of *abrB–lacZ* (data not shown). As expected, expression of a gene (*csh163*) that is strongly repressed by AbrB was induced when expression of *sad67* (Fig. 4B) or *sad76* (data not shown) was induced. These data demonstrate that the *sad* mutations allow Spo0A to function as both an activator and repressor of transcription in the absence of starvation signals.

sad67 *and* sad76 *encode proteins that are active in the absence of phosphorylation*

Activation of response regulators requires phosphorylation of an aspartate residue in the conserved amino terminus, corresponding to amino acid 56 of Spo0A (Sanders et al. 1992a,b). Although this aspartate is essential for Spo0A function (Burbulys et al. 1991; Green et al. 1991), it was not essential for function of the *sad67* and *sad76* gene products. *sad67* and *sad76* encode mutant proteins that retain the conserved aspartate at amino acid 56 (see Fig. 2). Changing the codon 56 in these alleles from GAT (aspartate) to AAT (asparagine) did not impair the ability of these mutants to activate expression of *spoIIA–lacZ* in conditions of nutrient excess. *sad67* and *sad67D56N* were indistinguishable in their ability to activate expression of *spoIIA–lacZ* during vegetative growth in LB medium (Fig. 5A). Surprisingly, the *sad76D56N* allele of *spo0A* was more effective at activating expression of *spoIIA* than *sad76* (Fig. 5B), suggesting that phosphorylation might inhibit the activity of the *sad76* gene product.

We expected that the *sad* mutations would by-pass the need for the phosphorylation pathway, because the *sad* gene products were active during vegetative growth and were able to function with the asp to asn change at amino acid 56. As expected, the *sad* mutations were able to induce expression of *spoIIA* in the absence of the phospho-relay, that is, in a *spo0B* mutant (data not shown). Mutations in *spo0B* normally cause a severe defect in sporulation and block expression of many *spo* genes, including *spoIIA*.

sad67 *and* sad76 *have different abilities to support sporulation*

Expression of *sad67* or *sad76*, in the absence of nutrient deprivation, was not sufficient to induce sporulation, even though it was sufficient to induce expression of several sporulation genes. Cells expressing either allele were unable to form an asymmetric division septum,

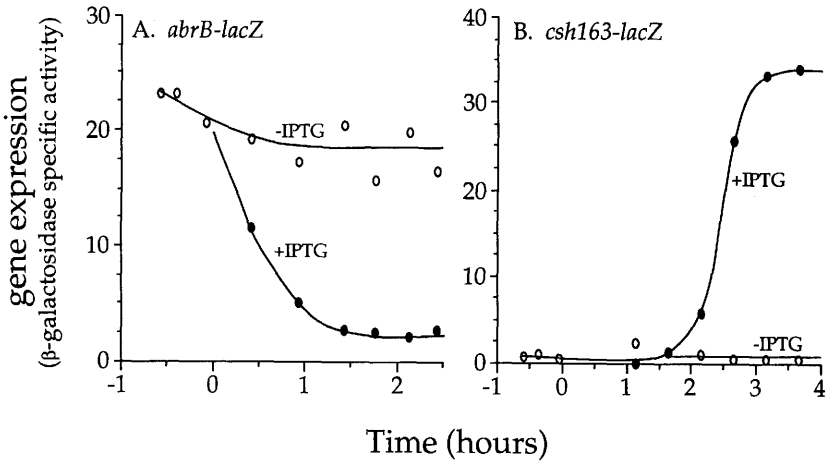

Figure 4. Expression of *sad67* inhibits expression of *abrB* and induces expression of *csh163* in the absence of starvation. Strains (*A*) SIK27 [SPβ::(*abrB–lacZ*) *amyE*::(P_{spac}–*sad67 cat*)] and (*B*) SIK13 [*csh163–lacZ amyE*::(P_{spac}–*sad67 cat*)] were grown in LB medium and samples were taken for determination of β-galactosidase specific activity as described in the Materials and methods. The time at which cells were diluted into LB medium lacking IPTG or containing 1 mM IPTG to induce expression of *sad67* or *spo0A*$^+$ from P_{spac} is defined as time zero (T_0). (●) Plus IPTG; (○) no IPTG.

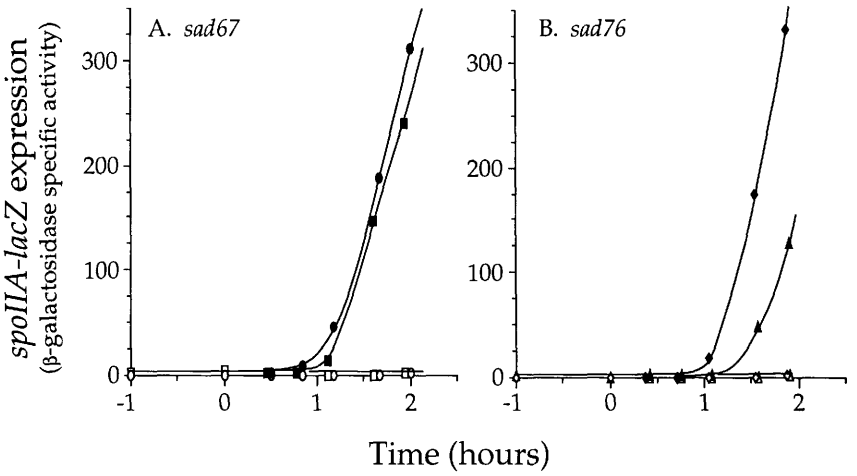

Figure 5. Sad67 and Sad76 activate expression of *spoIIA–lacZ* in the absence of phosphorylation. Cultures were grown in LB medium, and samples were taken for determination of β-galactosidase specific activity as described above. Time zero (T_0) is defined as time at which the culture was split and IPTG was added to one part of newly divided culture to induce expression of *sad67* or *sad76*. (Closed symbols) Plus IPTG; (open symbols) no IPTG. (*A*) Strain SIK83 [SPβ::(*spoIIA–lacZ*) *amyE*::(P_{spac}–*sad67 cat*)]; (circles) strain SIK183 [SPβ::(*spoIIA-lacZ*) *amyE*::(P_{spac}–*sad67D56N cat*)], (squares). (*B*) Strain (▲) SIK84 [SPβ::(*spoIIA–lacZ*) *amyE*::(P_{spac}–*sad76 cat*)]; (◆ SIK184) [SPβ::(*spoIIA–lacZ*) *amyE*::(P_{spac}–*sad76D56Ncat*)]. Similar results to those shown in *A* and *B* were obtained when experiments were performed with strains containing a deletion in *spo0A* (*spo0A::erm*) or with strains containing a null mutation in *spo0B*, which encodes a component of the phospho-relay that normally activates Spo0A in vivo.

which is one of the first morphological indicators of spore development, and genes normally expressed just after formation of the sporulation septum were not expressed (data not shown). It is not surprising that simply making activated Spo0A is not sufficient to cause efficient sporulation. Spo0A does not control expression of all genes that are induced early during sporulation. For example, both *citB* (Freese et al. 1979; Dingman et al. 1987; Fouet and Sonenshein 1990) and *ald* (K. Jaacks Siranosian, K. Ireton, A.D. Grossman, in prep.) are required for sporulation and are expressed early during sporulation, independently of *spo0A*. As expected, these genes were not induced by expression of the *sad* gene products in nutrient excess (data not shown). In addition, starvation might also affect metabolic processes and protein turnover, which are thought to be important for sporulation and might not depend totally on *spo0A*.

In combination with nutrient deprivation, expression of *sad67* was sufficient to cause sporulation. Sporulation of wild-type (*spo0A*$^+$) cells is very efficient, typically >50% of the cells give rise to heat-resistant spores, corresponding to ~2×10^8 spores per milliter of culture. When *spo0A*$^+$, or *sad67*, is expressed from P_{spac}, with a *spo0A* null mutation at the normal chromosomal locus, a similar sporulation frequency was observed (Table 1). Cells expressing the *sad67D56N* allele from P_{spac} typically produced ~10–50% as many spores as cells expressing *sad67* (Table 1), indicating that the ability of the Sad67 mutant protein to support sporulation is largely independent of phosphorylation at asp56, in striking contrast to the D56N mutation in the otherwise wild-type *spo0A* (Table 1). Consistent with the ability of *sad67D56N* to support sporulation, was the finding that it was also able to induce expression of *spoIIA–lacZ* (Fig. 5A).

In contrast to *sad67*, *sad76* was considerably less effective in supporting sporulation under these conditions. A culture of cells expressing *sad76* from P_{spac} produced 3–4 orders of magnitude fewer spores than a culture expressing a wild-type copy of *spo0A*. Interestingly, the inability of the Sad76 mutant protein to support sporulation seems to be attributable, in part, to inhibition of this protein by phosphorylation. Cells expressing *sad76D56N* were able to sporulate at a reasonably high frequency (typically ~10–50% of cells expressing *spo0A*$^+$) and produced about 3 orders of magnitude more spores than cells expressing *sad76*. Genetic inactivation of the phospho-relay, with a *spo0B* mutation, also increased the ability of cells expressing *sad76* to sporulate. Typically, the *sad76 spo0B* strain was able to sporulate at a frequency of ~10–100% of the *sad76D56N* strain. These results indicate that the increased ability of the protein encoded by *sad76D56N* to support sporulation

Table 1. sad67 *and* sad76 *have different abilities to support sporulation*

Strain	Relevant genotype[a]	Sporulation frequency[b]
SIK31	P_{spac}–*sad67*	5.9×10^{-1}
SIK190	P_{spac}–*sad67D56N*	1.6×10^{-1}
SIK30	P_{spac}–*sad76*	4.1×10^{-4}
SIK191	P_{spac}–*sad76D56N*	3.2×10^{-1}
DZR160	P_{spac}–*spo0A*$^+$	7.0×10^{-1}
SIK167	P_{spac}–*spo0AD56N*	$<10^{-7}$

Cells were grown in 2 × SG medium, and IPTG was added in late exponential growth, approximately one to two doublings before stationary phase. Similar results were obtained in several independent experiments.

[a]All strains contain a *spo0A* allele fused to P_{spac}, as indicated, and integrated into the chromosome at the *amyE* locus. In addition, all strains have a null mutation in the endogenous *spo0A* gene (*spo0A::erm*).

[b]Sporulation frequency is the number of heat-resistant spores per viable cell. In these experiments, the sporulation frequency is primarily an indication of the number of spores in the culture and not due to a drastic decrease in cell viability. The viable cell counts ranged from a low of 6.8×10^7 (SIK190) to a high of 2.8×10^8 (DZR160).

was probably attributable to an inability of this protein to be phosphorylated and probably not attributable to a structural alteration that fortuitously increased the activity of the protein. These effects of *sad76* and *sad76D56N* on sporulation are consistent with the effects of these alleles on expression of *spoIIA–lacZ* in conditions of nutrient excess (Fig. 5B).

sad67 *by-passes the requirement for high cell density for efficient sporulation*

In addition to nutrient deprivation, efficient sporulation requires that *B. subtilis* be at high cell density. Cells at low density, even when starved, sporulate poorly (Vasantha and Freese 1979; Grossman and Losick 1988). Efficient sporulation requires the presence of at least one extracellular factor that accumulates in culture medium as cells reach high density. Conditioned medium containing this factor stimulates sporulation of cells at low density (Grossman and Losick 1988).

Expression of *sad67* suppressed the sporulation defect normally observed when cells are starved at low density (Table 2). Cells expressing the wild-type copy of *spo0A* from P_{spac} (in addition to *spo0A*$^+$ from the normal chromosomal locus) sporulated poorly at low density (OD_{600} ~0.06) compared to sporulation at high density (OD_{600} ~0.6). In contrast, cells expressing the *sad67* allele (in addition to *spo0A*$^+$ from the normal chromosomal locus) did not exhibit a sporulation defect at low density. In fact, the sporulation efficiency of these cells at low density was similar to that at high density and somewhat greater than that of *spo0A*$^+$ cells at low density in the presence of conditioned medium (Table 2). These results demonstrate that cell density signals are bypassed by production of activated Spo0A. Normally, it seems that cell density signals, in addition to nutritional signals, regulate activation of Spo0A through the phospho-relay.

Discussion

We have isolated and characterized mutations in *spo0A* of *B. subtilis* that make the gene product constitutively active. These mutations are in-frame deletions and provide information about the function of the Spo0A transcription factor. In addition, these mutations uncouple expression of sporulation-specific genes from the normal developmental signals and provide evidence that these developmental signals are normally integrated through the pathway that activates Spo0A.

Spo0A transcription factor contains an inhibitory amino-terminal domain

The fact that a variety of deletion mutations in the amino terminus of Spo0A make the transcription factor constitutively active indicates that the amino-terminal domain inhibits the ability of the carboxyl terminus to function as a transcriptional activator. The *sad* deletions could produce constitutively active forms of Spo0A either by physically deleting the region responsible for inhibition or by disrupting the structural integrity of this region. The latter possibility is more likely because a deletion of a single codon (*sad54*) creates an activated Spo0A. In the wild-type protein, phosphorylation of an aspartate in the amino terminus probably relieves the inhibition and unmasks the ability of the protein to activate transcription.

The amino-terminal domain of many response regulators might normally function to inhibit the activity of the carboxy-terminal domain. Amino-terminal deletions of at least two other response regulators seem to create activated forms of these proteins. A form of the CheB methylesterase lacking the amino-terminal two-fifths of the protein exhibits elevated methylesterase activity in vitro (Simms et al. 1985), and an amino-terminal deletion of the FixJ transcriptional activator creates a protein

Table 2. sad67 *suppresses the sporulation defect of cells at a low density*

Strain		IPTG	Sporulation frequency[a] high density	low density	low + CM
SIK221	P_{spac}–*spo0A*$^+$	–	1.4×10^{-1}	8.1×10^{-5}	1.3×10^{-2}
		+	2.3×10^{-1}	9.6×10^{-5}	1.6×10^{-2}
SIK219	P_{spac}–*sad67*	–	1.4×10^{-1}	9.6×10^{-5}	1.4×10^{-1}
		+	2.6×10^{-1}	1.7×10^{-1}	6.4×10^{-1}

Cultures of SIK219 (*spo0A*$^+$, P_{spac}–*sad67*) and SIK221 (*spo0A*$^+$, P_{spac}–*spo0A*$^+$) were grown in defined minimal medium in the absence (–) or presence (+) of IPTG to induce expression of the *sad67* or *spo0A*$^+$ alleles from P_{spac}. (Note that even in the absence of IPTG these strains express the *spo0A*$^+$ gene product from the normal location in the chromosome. (At OD_{600} of ~0.60, cells were induced to sporulate by addition of decoyinine (high density) or were diluted 1 : 10 into fresh medium containing decoyinine (low density), or into conditioned medium (CM) containing decoyinine (low + CM). Conditioned medium was made from strain AG132 (*spo0A abrB*), as described (Grossman and Losick 1988).

[a]Sporulation frequency is the number of heat-resistant spores per viable cell. In these experiments, the sporulation frequency is primarily an indication of the number of spores in the culture and not due to a drastic decrease in cell viability. At high density, the viable cell count ranged from 6.9×10^7 to 1.5×10^8. At low density, the final viable cell counts ranged from 1.0×10^7 to 1.6×10^7, except for SIK219 in the presence of IPTG, which were 1.2×10^6 and 1.5×10^6 (low density + CM).

that is active in the absence of its cognate kinase (Kahn and Ditta 1991).

The type of regulation in which one domain masks the activity of a DNA-binding or transcriptional-activating domain appears to be relatively common among prokaryotic and eukaryotic transcription factors. The steroid hormone binding domain of the glucocorticoid receptor inhibits this protein's activity as a transcriptional enhancer in the absence of hormone (Godowski et al. 1987; Hollenberg et al. 1987). The AraC and LuxR transcriptional activators contain amino-terminal regions that inhibit the ability of these proteins to activate transcription in the absence of their respective inducer molecules (Menon and Lee 1990; Choi and Greenberg 1991). In addition, the major σ factor of *Escherichia coli* contains a region that masks the ability of free σ^{70} to bind DNA (Dombroski et al. 1992), and inhibition of DNA binding by this region is presumably relieved by interaction of σ with core RNA polymerase. In *B. subtilis*, two of the sporulation-specific σ factors are synthesized as inactive precursors. In both cases, several amino acids are removed from the amino terminus, causing activation of the σ factor (for review, see Losick and Stragier 1992).

Mutations in spo0A *that bypass developmental signals*

Spore formation in *B. subtilis* involves the sensing and integration of several environmental and physiological signals, including nutritional, cell-density, and cell-cycle signals. For cells to initiate the process of sporulation, they must be starved for a carbon, nitrogen, or phosphorus source (Sonenshein 1989), and be at a relatively high density (Grossman and Losick 1988). Spore formation also requires DNA replication (Dunn et al. 1978; Shibano et al. 1978; Ireton and Grossman 1992), and blocking DNA synthesis affects the ability of cells to enter into the earliest stages of sporulation (Ireton and Grossman 1992).

Our results indicate that the multiple environmental and physiological signals that control the initiation of sporulation affect the activity of the transcription factor encoded by *spo0A*. The *sad* mutations make the *spo0A* gene product constitutively active and by-pass the need for the activation (phospho-relay) pathway and by-pass the need for the aspartate at position 56, the putative site of phosphorylation. These mutations also uncouple expression of sporulation-specific genes from the environmental and physiological conditions normally required for their induction.

Many mutations (called *sof*, *rvtA*, *sur0B*, *coi*) that alter the conserved amino-terminal phospho-acceptor domain of Spo0A by-pass the normal phosphorylation pathway (Kawamura and Saito 1983; Sharrock et al. 1984; Hoch et al. 1985; Shoji et al. 1988; Olmedo et al. 1990; Spiegelman et al. 1990). Unlike the *sad* mutations, however, these mutations do not appear to encode constitutively active proteins because they still require starvation signals to activate expression of sporulation genes (Olmedo et al. 1990; Ireton and Grossman 1992). In addition, most (if not all) of the proteins encoded by these mutations still appear to require phosphorylation to be active (Spiegelman et al. 1990). We have also shown that the *rvtA11* mutation (Sharrock et al. 1984) does not allow sporulation when combined with an aspartate to asparagine change at amino acid 56 (K. Ireton and A.D. Grossman, unpubl.), again in contrast to the *sad* mutations. Two mutations in Spo0A (small deletions in the amino-terminus) have been described that suppress the sporulation defect of an aspartate to glutamate change at amino acid 56 (*spo0AD56Q*). These *pin* (phosphorylation independent) mutations also bypass the need for the phospho-relay (Green et al. 1991), but it is not known if these alleles encode proteins that are active in the absence of developmental signals.

Integration of multiple developmental signals by the Spo0A transcription factor during the initiation of sporulation

Nutritional, cell density, and cell-cycle signals could all regulate activation of the Spo0A transcription factor by affecting one or more steps of the phospho-relay (Fig. 6). We speculate that cell density signals regulate the activation of Spo0A by affecting the activity of one or more histidine protein kinases. Cells sense high density by sensing the presence of an extracellular factor that accumulates in the culture fluid. This factor is at least in part a peptide (Grossman and Losick 1988) and may stimulate sporulation by acting through the oligopeptide permease encoded by the *spo0K* operon. *spo0K* is required for the initiation of sporulation (and the development of genetic competence), and the five genes encode an oligopeptide uptake system with extensive similarity to oligopeptide permease from *Salmonella typhimurium* (Perego et al. 1991; Rudner et al. 1991). The requirement for *spo0K* in sporulation can be by-passed by overproduction of the histidine protein kinase encoded by *kinA*, indicating that the normal role of *spo0K* might be to activate one

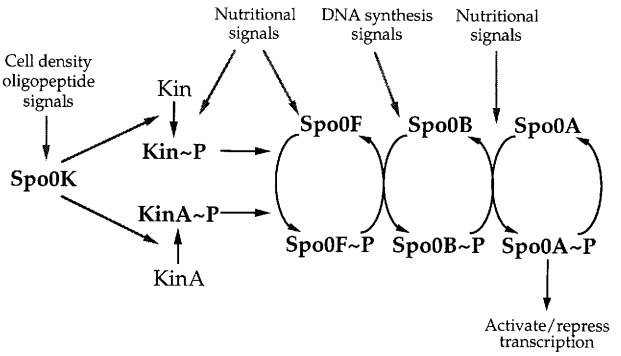

Figure 6. Integration of developmental signals through the phospho-relay affects the activity of the Spo0A transcription factor. Lighter arrows indicate speculative points of regulation. We speculate that nutritional signals might regulate more than one step.

of the kinases needed for sporulation (Rudner et al. 1991).

In addition to nutritional and cell density signals, cell-cycle or DNA replication signals also regulate activation of Spo0A. Altered function mutations in *spo0A* (*rvtA11*, *sad67*) that do not require the phospho-relay for activity, suppress the defect in early sporulation gene expression normally observed when DNA synthesis is inhibited in starving cells (Ireton and Grossman 1992; K. Ireton and A.D. Grossman, unpubl.). Thus, all three of the main signals required for spore formation, nutrient deprivation, high cell density, and DNA synthesis are involved in the activation of Spo0A.

In principle, integration of multiple signals can be achieved by a variety of different mechanisms. The phospho-relay allows inputs for multiple regulatory signals and provides a way of integrating these signals to affect the activation of a single transcription factor, the *spo0A* gene product (Fig. 6). In contrast, integration could be achieved by a mechanism in which each signal controls the activity of a different transcription factor. The combinatorial interaction of multiple transcription factors on a DNA template then ensures that gene expression occurs only when all relevant conditions are met. For example, expression of *HO* endonuclease in the yeast *Saccharomyces cerevisiae* occurs in response to three signals, cell type, cell cycle, and cell lineage. Transcription occurs only in **a** or α haploid cells, only in late G_1 phase of the cell cycle, and only in mother cells (Herskowitz 1989). Each of these conditions or signals controls the activity of different transcription factors, which interact in a combinatorial fashion to control expression of *HO*. These two contrasting mechanisms of signal integration—integration to affect a single transcription factor, and integration by combinatorial interaction of multiple transcription factors–illustrate different solutions to a common biological problem.

Materials and methods

Strains

The following *E. coli* strains were used for cloning and maintaining plasmids: JM101 [*supE thi Δ(proAB–lac)* (F′ *traD proAB*$^+$ *lacI*^{q} *lacZM15*)]; AG115, which is MC1061 [*araD139 Δ(ara-leu)7697 ΔlacX74 galU galK rpsL hsdR*] containing F′ *lacI*^{q} *lacZ*::Tn*5*; and AG1111, which is MC1061 with F′ *proAB*$^+$ *lacI*^{q} *lacZM15* Tn*10*. Strain CJ236 [*dut-1 ung-1 thi-1 rel-1*, pCJ105] was used for preparing uracil-containing single-stranded DNA for oligonucleotide-directed mutagenesis reactions (Kunkel et al. 1987).

The *B. subtilis* strains used are listed in Table 3. All strains are isogenic with JH642 (*trpC2*, *pheA1*) (Perego et al. 1988). The *spo0A*::*erm* is a deletion–insertion similar to the *spo0A*::*cat* mutation described previously (Grossman et al. 1992). The *spoIIA–lacZ* (Wu et al. 1991) and *spoIIG–lacZ* (Kenney et al. 1989) transcriptional fusions were all carried in the specialized transducing phage SPβ and have been described. The *abrB–lacZ* transcriptional fusion in SPβ was a gift from P. Zuber. The *csh163*::Tn*917lac* is a transcriptional fusion identified in a search for genes controlled by σ^H (Jaacks et al. 1989).

Media

LB medium (Davis et al. 1980) was used for the routine growth and maintenance of *E. coli* and *B. subtilis*, as well as for studies on gene expression with the *sad* mutants. The nutrient sporulation medium used for *B. subtilis* was 2× SG medium (Leighton and Doi 1971). The minimal medium was the S7 minimal salts medium of Vasantha and Freese (1980), except that MOPS (morpholine-propanesulfonic acid) buffer was used at a concentration of 50 rather than 100 mM. The minimal medium was supplemented with 1% glucose, 0.1% glutamate, and 40 μg/ml of each required amino acid as described (Jaacks et al. 1989). Media for plates was solidified with 15 g of agar (Difco Laboratories) per liter. Antibiotics were used at standard concentrations as described (Harwood and Cutting 1990). IPTG (isopro-

Table 3. Bacillus subtilis *strains used*

Strain	Genotype or description
DZR160	*amyE*::(P_{spac}–*spo0A*$^+$ *cat*) *spo0A*::*erm*
KI1317	AG130 (Grossman and Losick 1988) converted to Spec^r, Cm^s (*trp*$^+$, *phe*$^+$)
SIK13	*csh163*::Tn*917lacZ amyE*::(P_{spac}–*sad67 cat*)
SIK27	*amyE*::(P_{spac}–*sad67 cat*) SPβ::*abrB–lacZ*
SIK30	*amyE*::(P_{spac}–*sad76 cat*) *spo0A*::*erm*
SIK31	*amyE*::(P_{spac}–*sad67 cat*) *spo0A*::*erm*
SIK83	*amyE*::(P_{spac}–*sad67 cat*) SPβ::*spoIIA–lacZ*
SIK84	*amyE*::(P_{spac}–*sad76 cat*) SPβ::*spoIIA–lacZ*
SIK85	*amyE*::(P_{spac}–*spo0A*$^+$ *cat*) SPβ::*spoIIA–lacZ*
SIK103	*amyE*::(P_{spac}–*sad67 cat*) SPβ::*spoIIG–lacZ*
SIK104	*amyE*::(P_{spac}–*sad76 cat*) SPβ::*spoIIG–lacZ*
SIK105	*amyE*::(P_{spac}–*spo0A*$^+$ *cat*) SPβ::*spoIIG–lacZ*
SIK167	*amyE*::(P_{spac}–*spo0A*$^+$ *cat*) *spo0A*::*erm*
SIK183	*amyE*::(P_{spac}–*sad67D56N cat*) SPβ::*spoIIA–lacZ*
SIK184	*amyE*::(P_{spac}–*sad76D56N cat*) SPβ::*spoIIA–lacZ*
SIK190	*amyE*::(P_{spac}–*sad67D56N cat*) *spo0A*::*erm*
SIK191	*amyE*::(P_{spac}–*sad76D56N cat*) *spo0A*::*erm*
SIK219	KI1317 *amyE*::(P_{spac}–*spo0A*$^+$ *cat*)
SIK221	KI1317 *amyE*::(P_{spac}–*sad67 cat*)

pyl-thio-β-D-galactoside) was used at a final concentration of 1 mM. Decoyinine, an inhibitor of GMP synthetase, was used at 0.5 mg/ml or 1 mg/ml to initiate sporulation in minimal media (Mitani et al. 1977).

Transformations and transductions

Cells of *E. coli* and *B. subtilis* were made competent and transformed according to standard procedures (Dubnau and Davidoff-Abelson 1971; Sambrook et al. 1989; Rudner et al. 1991). SPβ phage lysates were made and transductions done essentially as described (Harwood and Cutting 1990).

Materials

Molecular biological reagents (enzymes, PCR and DNA sequencing reagents) were purchased from commercial suppliers and used according to standard procedures (Sambrook et al. 1989; Ausubel et al. 1990) or the suppliers instructions. Synthetic oligonucleotides were purchased from Oligos Etc., Inc. (Guilford, Conn.) or the Biopolymers lab of the MIT Center for Cancer Research.

Isolation of sad *mutations*

sad deletion mutations were made by *Bal*31 nuclease digestion of a linearized plasmid containing the wild-type *spo0A* gene. pDR69 contains the *spo0A*$^+$ structural gene cloned downstream of the LacI-repressed/IPTG-inducible promoter P_{spac}, in vector pDR66 (Fig. 1). pDR69 was linearized with *Bgl*II, which cuts approximately one-fourth of the way into *spo0A*, and treated with *Bal*31 exonuclease. Samples were removed at various times between 10 sec and 13 min after the start of *Bal*31 digestion, and reactions were stopped by addition of EDTA to 40 mM. After ethanol precipitation, the DNA ends were repaired with T4 DNA polymerase, ligated, and transformed into wild-type *B. subtilis*, selecting for resistance to chloramphenicol. Individual transformants were tested for growth on LB and/or 2× SG plates with and without IPTG. Transformants that had impaired growth when the *sad* allele was expressed (+IPTG) were chosen for further study.

Cloning and sequence analysis of sad *mutations*

The *sad* alleles were amplified from the chromosome using the polymerase chain reaction (PCR) and oligonucleotide primers rud-4 (5′-ACATCCAGAACAACCTCTG) and rud-5 (5′-GATCTTTCAGCCGACTCAA), which are complimentary to sequences in pDR69 that flank the P_{spac}–*spo0A* fusion (Fig. 1C). Amplified products should contain the *Sal*I and *Sph*I restriction sites upstream and downstream of *spo0A*, respectively. The products of the PCR reactions were digested with *Sal*I and *Sph*I and the ~1.5-kb fragment containing the *spo0A* allele was subcloned between the *Sal*I and *Sph*I sites of pGEM*cat* to generate plasmids pDR80 (*sad76*), pDR81 (*sad54*), pDR83 (*sad57*), pDR88 (*spo0A*$^+$), and pDR89 (*sad67*). The DNA sequence of the *sad* mutations was determined from these plasmids by use of the primer rud-7 (5′-TTTGGTTCAGGCTGGCT), which is complimentary to a sequence ~200 bp downstream of the *Bgl*II site that was used as the starting point for the *Bal*31 digestion.

Oligonucleotide site-directed mutagenesis

Codon 56 of *spo0A* was changed from GAT (asp) to AAT (asn) by site-directed mutagenesis with the oligonucleotide KI-14 (5′-CTCGTATTAAATATTATTAT) and single-stranded DNA from pDR80 (*sad76*), pDR88 (*spo0A*$^+$) and pDR89 (*sad67*). Mutagenesis was performed by use of standard procedures with uracil-containing DNA (Kunkel et al. 1987). Plasmids pIK152 (*sad76D56N*), pIK153 (*sad67D56N*), and pIK155 (*spo0AD56N*) contained the indicated mutations as determined by DNA sequencing with primer rud-7.

The *sad76D56N*, *sad67D56N*, and *spo0AD56N* alleles were placed under transcriptional control of the LacI-repressed/IPTG-inducible promoter P_{spac} and recombined into the chromosome of *B. subtilis*. The *Sal*I–*Sph*I fragments from pIK152 (*sad76D56N*), pIK153 (*sad67D56N*), and pIK155 (*spo0AD56N*) were subcloned into the *Sal*I–*Sph*I sites of pDR66 generating pIK173 (P_{spac}–*sad76D56N*), pIK165 (P_{spac}–*sad67D56N*), and pIK168 (P_{spac}–*spo0AD56N*). These plasmids were linearized with *Sca*I and transformed into the nonessential *amyE* locus, selecting for resistance to chloramphenicol.

β-Galactosidase assays

Cells were grown and treated as described in figure legends. β-Galactosidase activity was determined as described previously (Miller 1972; Jaacks et al. 1989) and specific activity is presented as the (ΔA420 per min per ml of culture per OD_{600} unit) × 1000.

Spore assays

Cells were grown in either 2× SG or minimal medium as indicated. The number of spores per milliliter of culture was determined as the number of heat-resistant (80°C for 20 min) colony forming units (CFU) on LB plates. The number of viable cells in the same culture was determined as the total number of CFU (before heat treatment) on LB plates. Sporulation frequencies are expressed as the number of spores per milliliter as a fraction of the number of viable cells per milliliter.

Acknowledgments

Decoyinine was a generous gift from the Upjohn Company. We thank P. Zuber for providing the *abrB–lacZ* fusion, S. Vidwans for help with some of the β-galactosidase assays, members of our lab for useful discussions, comments and suggestions on the manuscript, and C. Gross, C. Price, L. Rothman-Denes, and T. Baker for comments on the manuscript. K.I. was supported in part by an National Institutes of Health predoctoral training grant. A.D.G. is a Lucille P. Markey Scholar in Biomedical Sciences and this work was supported in part by a grant from the Lucille P. Markey Charitable Trust, and Public Health Services grant GM41934 to A.D.G. from the NIH.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

References

Albright, L.M., E. Huala, and F.M. Ausubel. 1989. Prokaryotic signal transduction mediated by sensor and regulator protein pairs. *Annu. Rev. Genet.* **23:** 311–336.

Antoniewski, C., B. Savelli, and P. Stragier. 1990. The *spoIIJ* gene, which regulates early developmental steps in *Bacillus subtilis*, belongs to a class of environmentally responsive genes. *J. Bacteriol.* **172:** 86–93.

Ausubel, F., R. Brent, R. Kingston, D. Moore, J. Seidman, J. Smith, and K. Struhl. 1990. *Current protocols in molecular*

biology. John Wiley and Sons, New York.
Bourret, R.B., K.A. Borkovich, and M.I. Simon. 1991. Signal transduction pathways involving protein phosphorylation in prokaryotes. *Annu. Rev. Biochem.* **60:** 401–441.
Buikema, W.J. and R. Haselkorn. 1991. Characterization of a gene controlling heterocyst differentiation in the cyanobacterium *Anabaena*. *Genes & Dev.* **5:** 321–330.
Burbulys, D., K.A. Trach, and J.A. Hoch. 1991. Initiation of sporulation in *B. subtilis* is controlled by a multicomponent phospho-relay. *Cell* **64:** 545–552.
Choi, S.H. and E.P. Greenberg. 1991. The C-terminal region of the *Vibrio fisheri* LuxR protein contains an inducer-Independent *lux* gene activation domain. *Proc. Natl. Acad. Sci.* **88:** 11115–11119.
Davis, R.W., D. Botstein, and J.R. Roth. 1980. *Advanced bacterial genetics*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
Dingman, D.W., M.S. Rosenkrantz, and A.L. Sonenshein. 1987. Relationship between aconitase gene expression and sporulation in *Bacillus subtilis*. *J. Bacteriol.* **169:** 3068–3075.
Dombroski, A.J., W.A. Walter, M.T. Record Jr., D.A. Siegele, and C.A. Gross. 1992. Polypeptides containing highly conserved regions of transcription initiation factor sigma-70 exhibit specificity of binding to promoter DNA. *Cell* **70:** 501–512.
Dubnau, D. and R. Davidoff-Abelson. 1971. Fate of transforming DNA following uptake by competent *B. subtilis*. *J. Mol. Biol.* **56:** 209–221.
Dunn, G., P. Jeffs, N.H. Mann, D.M. Torgersed, and M. Young. 1978. The relationship between DNA replication and the induction of sporulation in *Bacillus subtilis*. *J. Gen. Microbiol.* **108:** 189–195.
Fantes, P. 1989. Yeast cell cycle. *Curr. Opin. Cell Biol.* **1:** 250–255.
Ferrari, F.A., K. Trach, D. LeCoq, J. Spence, E. Ferrari, and J.A. Hoch. 1985. Characterization of the *spo0A* locus and its deduced product. *Proc. Natl. Acad. Sci.* **82:** 2647–2651.
Fouet, A. and A.L. Sonenshein. 1990. Target for carbon source-dependent negative regulation of the *citB* promoter of *Bacillus subtilis*. *J. Bacteriol.* **172:** 835–844.
Freese, E.B., N. Vasantha, and E. Freese. 1979. Induction of sporulation in developmental mutants of Bacillus subtilis. *Mol. Gen. Genet.* **170:** 67–74.
Gibbs, J.B. and M.S. Marshall. 1989. The *ras* oncogene—An important regulatory element in lower eucaryotic organisms. *Microbiol. Rev.* **53:** 171–185.
Godowski, P.J., S. Rusconi, R. Miesfeld, and K.R. Yamamoto. 1987. Glucocorticoid receptor mutants that are constitutive activators of transcriptional enhancement. *Nature* **325:** 365–368.
Golden, J.W. and D.L. Riddle. 1985. A gene affecting production of the *Caenorhabitis elegans* dauer-inducing pheremone. *Mol. Gen. Genet.* **198:** 534–536.
Green, B.D., M.G. Bramucci, and P. Youngman. 1991. Mutant forms of Spo0A that affect sporulation initiation: A general model for phosphorylation-mediated activation of bacterial signal transduction proteins. *Semin. Dev. Biol.* **2:** 21–29.
Grossman, A.D. 1991. Integration of developmental signals and the initiation of sporulation in *B. subtilis*. *Cell* **65:** 5–8.
Grossman, A.D. and R. Losick. 1988. Extracellular control of spore formation in *Bacillus subtilis*. *Proc. Natl. Acad. Sci.* **85:** 4369–4373.
Grossman, A.D., T. Lewis, N. Levin, and R. DeVivo. 1992. Suppressors of a *spo0A* missense mutation and their effects on sporulation in *Bacillus subtilis*. *Biochimie* **74:** 679–688.
Harwood, C.R. and S.M. Cutting. 1990. *Molecular biological methods for* Bacillus. John Wiley and Sons, Chichester, England.
Henner, D.J. 1990. Inducible expression of regulatory genes in *Bacillus subtilis*. *Methods Enzymol.* **185:** 223–228.
Herskowitz, I. 1989. A regulatory hierarchy for cell specialization in yeast. *Nature* **342:** 749–757.
Hoch, J.A., K. Trach, F. Kawamura, and H. Saito. 1985. Identification of the transcriptional suppressor *sof-1* as an alteration in the Spo0A protein. *J. Bacteriol.* **161:** 552–555.
Hollenberg, S.M., V. Giguere, P. Sequi, and R.M. Evans. 1987. Colocalization of DNA-binding and transcriptional activation functions in the human glucocorticoid receptor. *Cell* **49:** 39–46.
Ireton, K. and A.D. Grossman. 1992. Coupling between gene expression and DNA synthesis early during development in *Bacillus subtilis*. *Proc. Natl. Acad. Sci.* **89:** 8808–8812.
Jaacks, K.J., J. Healy, R. Losick, and A.D. Grossman. 1989. Identification and characterization of genes controlled by the sporulation regulatory gene *spo0H* in *Bacillus subtilis*. *J. Bacteriol.* **171:** 4121–4129.
Kahn, D. and G. Ditta. 1991. Modular structure of FixJ: Homology of the transcriptional activator domain with the −35 binding domain of sigma factors. *Mol. Microbiol.* **5:** 987–997.
Kawamura, F. and H. Saito. 1983. Isolation and mapping of a new suppressor mutation of an early sporulation gene *spo0F* mutation in *Bacillus subtilis*. *Mol. Gen. Genet.* **192:** 330–334.
Kenney, T.J. and C.P. Moran Jr. 1987. Organization and regulation of an operon that encodes a sporulation-essential sigma factor in *Bacillus subtilis*. *J. Bacteriol.* **169:** 3329–3339.
Kenney, T.J., K. York, P. Youngman, and C.P. Moran Jr. 1989. Genetic evidence that RNA polymerase associated with sigma-A factor uses a sporulation-specific promoter in *Bacillus subtilis*. *Proc. Natl. Acad. Sci.* **86:** 9109–9113.
Kim, S.K., D. Kaiser, and A. Kuspa. 1992. Control of cell density and pattern by intercellular signaling in *Myxococcus* development. *Annu. Rev. Microbiol.* **46:** 117–139.
Kudoh, J., R. Ikeuchi, and K. Kurahashi. 1985. Nucleotide sequences of the sporulation gene *spo0A* and its mutant genes of *Bacillus subtilis*. *Proc. Natl. Acad. Sci.* **82:** 2665–2668.
Kunkel, T.A., J.D. Roberts, and R.A. Zakour. 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.* **154:** 367–403.
Leighton, T.J. and R.H. Doi. 1971. The stability of messenger ribonucleic acid during sporulation in *Bacillus subtilis*. *J. Biol. Chem.* **252:** 268–272.
Losick, R. and P. Stragier. 1992. Crisscross regulation of cell-type-specific gene expression during development in *B. subtilis*. *Nature* **355:** 601–604.
Menon, K.P. and N.L. Lee. 1990. Activation of *ara* operons by a truncated AraC protein does not require inducer. *Proc. Natl. Acad. Sci.* **87:** 3708–3712.
Miller, J. 1972. *Experiments in molecular genetics*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
Mitani, T., J.F. Heinze, and E. Freese. 1977. Induction of sporulation in *Bacillus subtilis* by decoyinine or hadacidin. *Biochem. Biophys. Res. Commun.* **77:** 1118–1125.
Olmedo, G., E.G. Ninfa, J. Stock, and P. Youngman. 1990. Novel mutations that alter the regulation of sporulation in *Bacillus subtilis*: Evidence that phosphorylation of regulatory protein Spo0A controls the initiation of sporulation. *J. Mol. Biol.* **215:** 359–372.
Perego, M. and J. Hoch. 1991. Negative regulation of *Bacillus subtilis* sporulation by the *spo0E* gene product. *J. Bacteriol.* **173:** 2514–2520.
Perego, M., G.B. Spiegelman, and J.A. Hoch. 1988. Structure of

the gene for the transition state regulator, *abrB*: Regulator synthesis is controlled by the *spo0A* sporulation gene in *Bacillus subtilis*. *Mol. Microbiol.* **2:** 689–699.

Perego, M., S.P. Cole, D. Burbulys, K. Trach, and J.A. Hoch. 1989. Characterization of the gene for a protein kinase which phosphorylates the sporulation-regulatory proteins Spo0A and Spo0F of *Bacillus subtilis*. *J. Bacteriol.* **171:** 6187–6196.

Perego, M., C.F. Higgins, S.R. Pearce, M.P. Gallagher, and J.A. Hoch. 1991. The oligopeptide transport system of *Bacillus subtilis* plays a role in the initiation of sporulation. *Mol. Microbiol.* **5:** 173–185.

Reznikoff, W.S. 1992. The lactose operon controlling elements: a complex paradigm. *Mol. Microbiol.* **6:** 2419–2422.

Riddle, D.L., M.M. Swanson, and P.S. Albert. 1981. Interacting genes in nematode dauer larva formation. *Nature* **290:** 668–671.

Rudner, D.Z., J.R. LeDeaux, K. Ireton, and A.D. Grossman. 1991. The *spo0K* locus of *Bacillus subtilis* is homologous to the oligopeptide permease locus and is required for sporulation and competence. *J. Bacteriol.* **173:** 1388–1398.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: A laboratory manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sanders, D.A., B.L. Gillece-Castro, A.L. Burlingame, and D.E. Koshland. 1992a. Phosphorylation site of NtrC, a protein phosphatase whose covalent intermediate activates transcription. *J. Bacteriol.* **174:** 5117–5122.

Sanders, D.A., B.L. Gillece-Castro, A.M. Stock, A.L. Burlingame, and D.E. Koshland. 1992b. Identification of the site of phosphorylation of the chemotaxis response regulator protein, CheY. *J. Biol. Chem.* **264:** 21770–21778.

Satola, S., P.A. Kirchman, and C.P. Moran Jr. 1991. Spo0A binds to a promoter used by sigma-A RNA polymerase during sporulation in *Bacillus subtilis*. *Proc. Natl. Acad. Sci.* **88:** 4533–4537.

Satola, S.W., J.M. Baldus, and C.P. Moran Jr. 1992. Binding of Spo0A stimulates *spoIIG* promoter activity in *Bacillus subtilis*. *J. Bacteriol.* **174:** 1448–1453.

Sharrock, R.A., S. Rubenstein, M. Chan, and T. Leighton. 1984. Intergenic suppression of *spo0* phenotypes by the *Bacillus subtilis* mutation *rvtA*. *Mol. Gen. Genet.* **194:** 260–264.

Shibano, Y., K. Tamura, M. Honjo, and T. Komano. 1978. Effect of 6-(para-hydroxyphenylazo)-uracil on sporulation in *Bacillus subtilis*. *Agric. Biol. Chem.* **42:** 187–189.

Shimotsu, H. and D.J. Henner. 1986. Construction of a single-copy integration vector and its use in analysis of regulation of the *trp* operon of *Bacillus subtilis*. *Gene* **43:** 85–94.

Shoji, K., S. Hiratsuka, F. Kawamura, and Y. Kobayashi. 1988. New suppressor mutation *sur0B* of *spo0B* and *spo0F* mutations in *Bacillus subtilis*. *J. Gen. Microbiol.* **134:** 3249–3257.

Simms, S.A., M.G. Keane, and J. Stock. 1985. Multiple forms of the CheB methylesterase in bacterial chemosensing. *J. Biol. Chem.* **260:** 10161–10168.

Sonenshein, A.L. 1989. Metabolic regulation of sporulation and other stationary phase phenomena. In *Regulation of procaryotic development* (ed. I. Smith, R. Slepecky and P. Setlow), pp. 109–130. American Society for Microbiology, Washington, D.C.

Spiegelman, G., B. Van Hoy, M. Perego, J. Day, K. Trach, and J.A. Hoch. 1990. Structural alterations in the *Bacillus subtilis* Spo0A regulatory protein which suppress mutations at several *spo0* loci. *J. Bacteriol.* **172:** 5011–5019.

Stock, A.M., J.M. Mottonen, J.B. Stock and C.E. Schutt. 1989a. Three-dimensional structure of CheY, the response regulator of bacterial chemotaxis. *Nature* **337:** 745–749.

Stock, J.B., A.J. Ninfa, and A.M. Stock. 1989b. Protein phosphorylation and regulation of adaptive responses in bacteria. *Microbiol. Rev.* **53:** 450–490.

Strauch, M., V. Webb, G. Spiegelman, and J.A. Hoch. 1990. The Spo0A protein of *Bacillus subtilis* is a repressor of the *abrB* gene. *Proc. Natl. Acad. Sci.* **87:** 1801–1805.

Strauch, M.A., K.A. Trach, and J.A. Hoch. 1992. Spo0A activates and represses its own synthesis by binding at its dual promoters. *Biochimie* **74:** 619–626.

Vasantha, N. and E. Freese. 1979. The role of manganese in growth and sporulation of *Bacillus subtilis*. *J. Gen. Microbiol.* **112:** 329-336.

———. 1980. Enzyme changes during *Bacillus subtilis* sporulation caused by deprivation of guanine nucleotides. *J. Bacteriol.* **144:** 1119–1125.

Weber, L. and B. Byers. 1992. A RAD9-dependent checkpoint blocks meiosis of *cdc13* yeast cells. *Genetics* **131:** 55–63.

Weir, J., M. Predich, E. Dubnau, G. Nair, and I. Smith. 1991. Regulation of *spo0H*, a gene coding for the *Bacillus subtilis* σ^H factor. *J. Bacteriol.* **173:** 521–529.

Wu, J.-J., M.G. Howard, and P.J. Piggot. 1989. Regulation of transcription of the *Bacillus subtilis spoIIA* locus. *J. Bacteriol.* **171:** 692–698.

Wu, J.J., P.J. Piggot, K.M. Tatti, and C.P. Moran Jr. 1991. Transcription of the *Bacillus subtilis spoIIA* locus. *Gene* **101:** 113–116.

Yansura, D.G. and D.J. Henner. 1984. Use of the *Escherichia coli lac* repressor and operator to control gene expression in *Bacillus subtilis*. *Proc. Natl. Acad. Sci.* **81:** 439–443.

York, K., T.J. Kenney, S. Satola, C.P. Moran Jr., H. Poth, and P. Youngman. 1992. Spo0A controls the sigma-A dependent activation of *Bacillus subtilis* sporulation-specific transcription unit *spoIIE*. *J. Bacteriol.* **174:** 2648–2658.

Zuber, P. and R. Losick. 1987. Role of AbrB in Spo0A- and Spo0B-dependent utilization of a sporulation promoter in *Bacillus subtilis*. *J. Bacteriol.* **169:** 2223–2230.