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The Chromosomal Location of the *Bacillus subtilis* Sporulation Gene *spoIIR* Is Important for Its Function

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Formation of the asymmetrically located septum during sporulation of *Bacillus subtilis* results in enclosure of the origin-proximal 30% of the chromosome in the prespore compartment. The rest of the chromosome is then translocated into the prespore from the mother cell. Transcription of *spoIIR* is initiated in the prespore by RNA polymerase containing σ^F soon after the septum is formed. The SpoIIR protein is required for the activation of the transcription program directed by σ^E in the mother cell. The *spoIIR* locus is located at 324°, near the origin of replication (0/360°). We show here that movement of *spoIIR* to 28° had little effect on sporulation. However, movement to regions not in the origin-proximal part of the chromosome substantially reduced sporulation efficiency. At 283° sporulation was reduced to less than 20% of the level obtained when *spoIIR* was at its natural location, and movement to 190° reduced sporulation to about 6% of that level. These positional effects were also seen in the transcription of a *spoIIR-lacZ* fusion. In contrast, movement of other *spo-lacZ* fusions from 28° to 190° had little effect on their expression. These results suggest that *spoIIR* is the subject of "positional regulation," in the sense that the chromosomal position of *spoIIR* is important for its expression and function.

During sporulation *Bacillus subtilis* undergoes an asymmetrically located cell division. This division is a modified form of the vegetative division (6, 16). However, formation of the sporulation septum results in enclosure of only about 30% of a chromosome in the smaller cell, the prespore (also called the forespore), that results from the division; the rest of the chromosome is then translocated from the larger cell, the mother cell, into the prespore by an active process requiring SpoIIIE (Fig. 1) (23, 25). A second copy of the chromosome remains in the mother cell. The prespores of SpoIIIE mutant cells contain only about 30% of a chromosome, with the other 70% remaining in the mother cell together with the whole of the mother cell chromosome (23). Formation of the asymmetrically located septum is followed by activation of two sporulation-specific transcription factors, σ^F in the prespore and σ^E in the mother cell, which specify different programs of gene expression in the two compartments (reviewed in reference 21). In a *spoIIIE36* mutant the σ^F-directed prespore genes that are located in the 70% of the chromosome distal to the origin of replication (for example, *dacF* and *gpr*) are not transcribed, whereas those located in the origin-proximal 30% are transcribed (for example, *spoIIR* and *spoIIQ*) (9, 12, 20, 22, 23, 25). Thus, it has been known for some time that chromosome position is important for expression of σ^F-directed genes in a *spoIIIE36* mutant (22). It seemed plausible that there could be some prespore-specific gene (or genes) that needed to be expressed as soon as the septum was formed and so needed to be located at the origin-proximal part of the chromosome in the parental, *spo*$^+$ strain. If such a gene were to be relocated distal to the origin, its expression during sporulation might be impaired, resulting in a sporulation-deficient phenotype.

We considered that the *spoIIR* locus might be a possible candidate for this "positional" type of regulation of its activity. The *spoIIR* locus is near the origin and is transcribed only by RNA polymerase containing σ^F (9, 13). It links the activation of σ^E in the mother cell to the activation of σ^F in the prespore, and it is the only σ^F-directed gene needed for the σ^E activation (9, 13). Its activation is thought to ensure that σ^E is not activated until after the septum is formed (9), and rapid activation of σ^E following septation may be important in preventing further septation (1). Thus, a delay in *spoIIR* expression may disrupt the complex network of transcription regulation that is necessary for spore formation. Below we describe experiments indicating that the *spoIIR* gene is the subject of such positional regulation.

MATERIALS AND METHODS

Media. *B. subtilis* was grown in modified Schaeffer's sporulation medium (MSSM) and on Schaeffer's sporulation agar (17, 19). When required, 5-bromo-4-chloro-3-indolyl-β-D-galactoside at 40 μg/ml, chloramphenicol at 5 μg/ml, neomycin at 3 μg/ml, and erythromycin at 1 μg/ml were added.

Strains. *B. subtilis* 168 strain BR151 *trpC2 metB10 lys-3* and *B. subtilis* ZB307 SPβc2Δ2::Tn*917*::pSK10Δ6 were used as the parent strains. These and the other *B. subtilis* strains used are listed in Table 1. *Escherichia coli* strain DH5α (GIBCO/BRL) was used to maintain plasmids.

The *spoIIR* promoter region was cloned as a *Not*I-*Hin*dIII fragment (9), via pBluescript to provide additional sites, into the following plasmids: pMLK83, a *neo gusA* fusion vector designed for the integration of constructs by a double-recombination event at the *amyE* locus (10); pDG793, an *erm lacZ* fusion vector designed for the integration of constructs by a double-recombination event at the *thrC* locus (a gift from P. Stragier, Institut de Biologie Physico Chimique, Paris, France); pGV34 (4, 26), a *cat lacZ* fusion vector designed for the integration of constructs by a double-recombination event at the SPβ locus, and also used for Campbell-like recombination at *spoIIR*. An intact copy of *spoIIR* was cloned as a 1.2-kb *Not*I-*Xho*I fragment into the same plasmids. The genetic linkage was verified for each chromosomal insertion.

The *spoIIE* promoter region was cloned as an *Eco*RI-*Pvu*II fragment in pMLK83 (10). This construct was then used to introduce the *spoIIE-gusA* fusion into *amyE* by double crossover. The *spoIID-gusA* fusion at *amyE* was derived from pMLK87 (10). The *spoIID-lacZ* fusion at *spoIID* resulted from integration of pMLK23 by a single crossover (10). P. Youngman (Millennium Pharmaceuticals, Cambridge, Mass.) kindly provided strains containing the *spoIIE-lacZ* and *spoIID-lacZ* fusions at SPβ. A strain containing a *spoIIQ-gfp* transcriptional fusion (12) was kindly provided by P. Stragier. The fusion was introduced by

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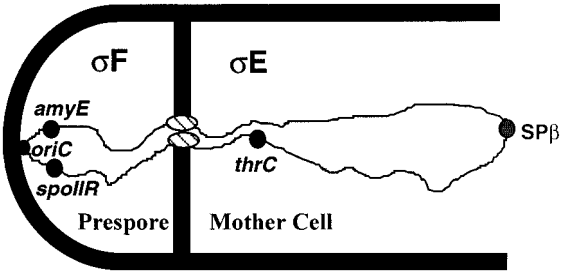

FIG. 1. Model for chromosomal translocation through the sporulation septum showing the approximate location of loci used in this study (adapted from reference 24). The SpoIIIE protein is indicated as ovals at the position where the chromosome transverses the recently formed sporulation septum.

transformation into strains containing *spoIIR* at different chromosomal locations. Details of the construction of strains are available on request.

β-Galactosidase and β-glucuronidase assays. Assays were performed essentially as described previously (10), using lysozyme to permeabilize the cells. Specific activity (in units) is expressed as nanomoles of *o*-nitrophenyl-β-D-galactoside or *p*-nitrophenyl-β-D-glucuronide hydrolyzed per minute per milligram of bacterial dry weight. The endogenous β-galactosidase and β-glucuronidase activities were determined in each experiment for an isogenic parental strain lacking a fusion and subtracted from the corresponding values for the fusion-containing strains.

Other methods. *B. subtilis* transformation, transduction, sporulation by exhaustion in MSSM, and all genetic engineering methods were performed essentially as previously described (7, 15, 17, 28). Sporulation was assayed 18 h after the end of exponential growth by diluting cultures and determining the heat-resistant count (80°C, 20 min) and the viable count in the diluted cultures. The viable count varied somewhat from experiment to experiment: for strains with *spoIIR* at SPβ, SL7256, and SL7344, the range was 1.0×10^8 to 2.5×10^8 per ml; for all other strains the range was 2.5×10^8 to 6.0×10^8 per ml; there was no significant chain formation.

Cultures used for visualization of green fluorescence protein (GFP) were grown in MSSM at 33.5°C and harvested 6 h after the end of exponential growth, by which time the bulk of the population had reached the sporulation division stage. Culture samples of 10 μl of unfixed cells were transferred to 0.1% poly-lysine-coated slides and examined by fluorescence microscopy essentially as described previously (28).

RESULTS

Complementation of the Spo$^-$ phenotype associated with *spoIIR*::*neo* by placing the intact *spoIIR* gene at different chromosomal locations. A knockout of the *spoIIR* gene with an insertion of a *neo* cassette in codon 98 of the *spoIIR* open reading frame (9) resulted in an asporogenous phenotype (less than 1 spore in 10^8 cells). To test the possibility that the chromosomal location (at 324°) of the *spoIIR* gene near the origin of replication (at 0/360°) might be important for its proper functioning, we chose three different chromosomal locations (11) for the integration of *spoIIR*: near the origin but on the other side of the origin to *spoIIR* (at *amyE*, 28°), approximately halfway from the origin to the terminus (at *thrC*, 283°), and near the terminus (at SPβ, 190°). The intact copy of *spoIIR* as a *Not*I-*Xho*I fragment was cloned in different plasmids, pDH32, pDG793, and pGV34, designed to facilitate integration of *spoIIR* by double crossover at *amyE*, *thrC*, and SPβ, respectively. The results of sporulation efficiency assays of strains carrying the knockout of *spoIIR* at its original location (324°) and integration of an intact copy of *spoIIR* at the different locations are summarized in Table 2. Movement of the intact copy of *spoIIR* to *amyE* had little effect on sporulation. However, movement to *thrC* reduced sporulation to less than 20% of the efficiency of the isogenic parent strain. Movement to SPβ reduced sporulation to about 6% of that of the parent (Table 2); the method of determining sporulation may overestimate this figure because the viable counts of strains with *spoIIR* at SPβ were about twofold lower than those of the other strains. The same effect of movement to SPβ was also observed with a *B. subtilis* strain of a different lineage, ZB307 (29). The results suggest that the efficiency of sporulation depends on the distance between the location of the *spoIIR* gene and the origin of replication.

Expression of a *spoIIR-lacZ* fusion in different chromosomal locations. To test *spoIIR* transcription at the different locations, we employed a *spoIIR-lacZ* transcriptional fusion in strains that also contained a *spoIIR-gusA* fusion inserted at *amyE* as an internal control. No significant differences were observed in the timing or the level of expression of *spoIIR-lacZ* integrated at *spoIIR* compared to the *spoIIR-gusA* fusion at *amyE* (Fig. 2A). Expression of *spoIIR-lacZ* was reduced at *thrC* (Fig. 2B) and was barely detectable at SPβ (Fig. 2C). Introduction of an inducible copy of the gene for σ^F, *spoIIAC*, under the control of the P*spac* promoter (5) into the latter strain established that the *spoIIR-lacZ* at SPβ fusion was still functional (data not shown).

TABLE 1. *B. subtilis* strains

Strain	Relevant characteristics	Origin or reference
BR151	*trpC2 metB10 lys-3*	Laboratory stock
ZB307	SPβc2Δ2::Tn*917*pSK10Δ6	P. Youngman (26, 30)
SL7205	SPβc2Δ2::Tn*917*pSK10Δ6 *amyE*::*spoIIR-gusA spoIIR*::*spoIIR-lacZ*	This study
SL7213	*amyE*::*spoIIE-gusA* SPβc2Δ2::Tn*917*pSK10Δ6::*spoIIE-lacZ*	This study
SL7256	SPβc2Δ2::Tn*917*pSK10Δ6::*spoIIR spoIIR*::*neo*	This study
SL7258	SPβc2Δ2::Tn*917*pSK10Δ6 *spoIIR*::*neo amyE*::*spoIIR*	This study
SL7303	*amyE*::*spoIIR-gusA* SPβc2Δ2::Tn*917*pSK10Δ6::*spoIIR-lacZ*	This study
SL7304	*amyE*::*spoIIR-gusA* SPβc2Δ2::Tn*917*pSK10Δ6::*spoIIR-lacZ spoIIAC561*	This study
SL7310	*trpC2 metB10 lys-3 amyE*::*IIR-gusA thrC*::*spoIIR-lacZ*	This study
SL7313	*trpC2 metB10 lys-3 spoIIR*::*neo thrC*::*spoIIR*	This study
SL7321	SPβc2Δ2::Tn*917*pSK10Δ6 *spoIID-lacZ@spoIID*[a]	This study
SL7322	SPβc2Δ2::Tn*917*pSK10Δ6::*spoIIR spoIIR*::*neo spoIID-lacZ@spoIID*	This study
SL7340	*trpC2 metB10 lys-3 spoIIR*::*neo amyE*::*spoIIR*	This study
SL7344	*trpC2 metB10 spoIIR*::*neo* SPβc2Δ2::Tn*917*pSK10Δ6::*spoIIR*	This study
SL8344	*trpC2 metB10 lys-3 spoIIQ-gfp*	This study
SL8345	*trpC2 metB10 lys-3 spoIIR*::*neo spoIIQ-gfp*	This study
SL8378	*trpC2 metB10 lys-3 spoIIR*::*neo amyE*::*spoIIR spoIIQ-gfp*	This study
SL8380	*trpC2 metB10 lys-3 spoIIR*::*neo* SPβc2Δ2::Tn*917*pSK10Δ6::*spoIIR spoIIQ-gfp*	This study
SN178	SPβc2Δ2::Tn*917*pSK10Δ6 *amyE*::*spoIIR-gusA spoIIR-lacZ@spoIIR spoIIAC561*	This study

[a] @ indicates the fusion is located at the locus by a Campbell-like recombination.

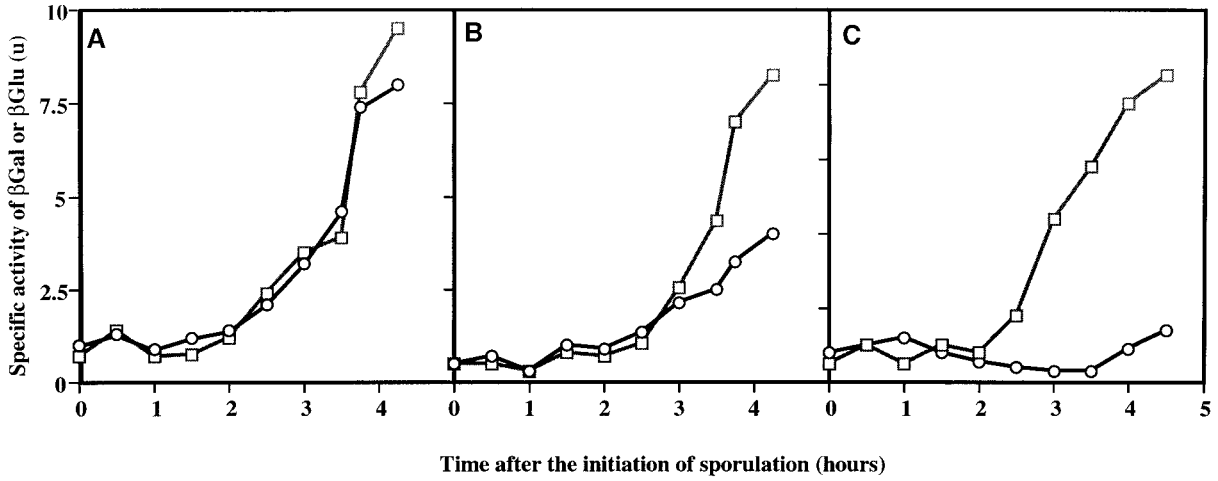

FIG. 2. Expression of *spoIIR* at various chromosomal locations in a *spoIIA*$^+$ background. □, expression of *amyE*::*spoIIR-gusA*. ○, expression of *spoIIR-lacZ* at *spoIIR* (SL7205) (A), *thrC* (SL7310) (B), or SPβ (SL7303) (C). SL7310 is a derivative of BR151; SL7205 and SL7303 are derivatives of ZB307. βGal, β-galactosidase; βGlu, β-glucuronidase.

We considered it unlikely that the local context of SPβ would explain the reduced expression of *spoIIR-lacZ*. The SPβ system (29) has been used extensively, and we had noted no reduction in expression of the strong σ^H promoter, *ftsA*p2, when it was located at SPβ (3). However, we considered it necessary to retest the possibility that expression of *lacZ* fusions at SPβ might somehow be inhibited by the local gene context. We compared the expression at *amyE* and SPβ of the σ^A-dependent sporulation-specific gene *spoIIE*, which is also very weakly expressed. There was no difference in the level and pattern of expression, regardless of chromosomal position (Fig. 3). Fusions to *gusA* were used at *amyE*, and *lacZ* fusions were used at SPβ; previous studies had shown that the *gusA* and *lacZ* fusions gave activities similar to each other (10).

The transcription of *spoIIR* is normally weak and is substantially higher in *spoIIAC(P)* mutants (9). Consequently we checked *spoIIR-lacZ* expression at the different locations in a *spoIIAC561* background in order to visualize more clearly possible differences in expression patterns. The *spoIIAC561* mutation is a V233M change in the 4.2 promoter recognition region of σ^F and does not affect regulation of σ^F activity (14, 27). It curtails transcription of some σ^F-directed genes (14, 27) but enhances transcription of *spoIIR* (9). Again, no significant differences were observed in the timing or level of expression of *spoIIR-lacZ* at *spoIIR* compared to *spoIIR-gusA* at *amyE* (Fig. 4). When the fusion was integrated at SPβ, there was a delay in expression of *spoIIR-lacZ*, and expression was also reduced compared to that of *spoIIR-gusA* at *amyE*. The delay was difficult to measure accurately; in different experiments it was about 15 to 20 min. These data indicate that chromosome position is important for the expression of *spoIIR*; they suggest that the SpoIIIE-mediated active transport of the distal 70% of the chromosome through the sporulation septum into the prespore (25) may take 15 to 20 min.

Activity of σ^E is significantly decreased when the *spoIIR* chromosomal location is altered. Having the level of *spoIIR* expression at SPβ significantly decreased and delayed, it was reasonable to expect that expression of σ^E-dependent genes would be impaired. To test this expectation, we introduced a *spoIID-lacZ* fusion into strain ZB307 and into a derivative of ZB307 carrying a single functional copy of the *spoIIR* gene at SPβ. The pattern of *spoIID-lacZ* expression in cultures of these two strains is shown in Fig. 5. Relocation of the *spoIIR* gene to the terminus region resulted in reduction in expression by about 80%, and this was accompanied by a delay compared to *spoIID-lacZ* expression in the parent strain. It seems plausible that the sporulation-deficient phenotype of strains containing *spoIIR* located only at SPβ is the result of a decrease in σ^E-dependent gene expression.

TABLE 2. Efficiency of sporulation of strains carrying a single intact copy of *spoIIR* at different chromosomal locations

Location of intact copy of *spoIIR*	% Sporulation[a]	
	A	B
spoIIR	47, 55 (BR151)	55, 71 (ZB307)
amyE	42, 46 (SL7340)	59, 81 (SL7258)
thrC	7, 10 (SL7313)	ND, ND
SPβ	3.3, 2.7 (SL7344)	0.9, 2.9 (SL7256)

[a] Results are for two different experiments with BR151 (A) and ZB307 (B) as parent strains. ND, not determined.

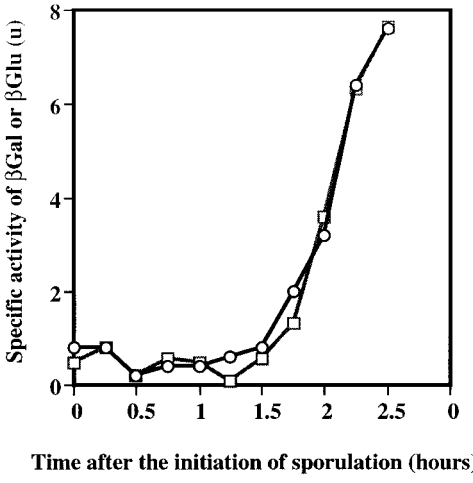

FIG. 3. Expression of *spoIIE* at different chromosomal locations. □, *amyE*::*spoIIE-gusA*; ○, SPβ::*spoIIE-lacZ* (SL7213). βGal, β-galactosidase; βGlu, β-glucuronidase.

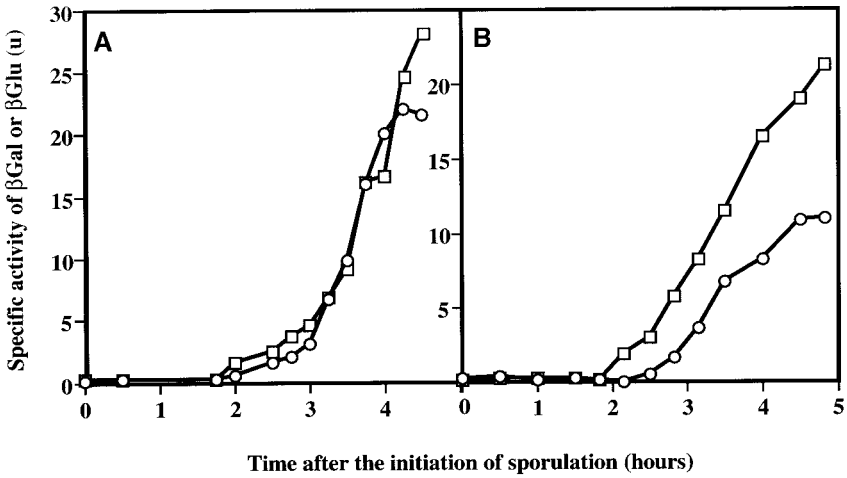

FIG. 4. Expression of *spoIIR* at different chromosomal locations in a *spoIIA561* background. □, expression of *amyE*::*spoIIR-gusA*; ○, expression of *spoIIR-lacZ* at *spoIIR* (SN178) (A) or SPβ (SL7304) (B). βGal, β-galactosidase; βGlu, β-glucuronidase.

The major role of *spoIIR* is thought to be to ensure that activation of σ^E requires prior activation of σ^F, and so σ^E activation follows formation of the sporulation septum (9). Mutations in the structural gene for σ^E, *spoIIGB*, result in the abortively disporic phenotype in which a sporulation division has occurred near both cell poles, and it is inferred that a role of σ^E during sporulation is to prevent the formation of the second, asymmetrically located division septum (1). Mutation of *spoIIR* also results in this abortively disporic phenotype (in which each of the prespores contains a nucleoid, whereas the mother cell is nucleoid free), although at a slightly reduced frequency (9) (our unpublished results). Appearance (or lack thereof) of the abortively disporic phenotype is used here as a separate test of the effect on σ^E activation of moving *spoIIR*. The prespore-specific expression of a *spoIIQ-gfp* transcriptional fusion (12) was used as an indicator of prespore formation. In this system, a *spoIIGB* mutant gave 45% disporic and 55% monosporic organisms displaying GFP fluorescence 6 h after the start of sporulation at 33.5°C. When an intact copy of *spoIIR* was located at SPβ, the strain exhibited an abortively disporic phenotype, similar to that of a *spoIIR* null mutant, with about 30% of fluorescing organisms displaying the disporic pattern (Table 3); a similar pattern was obtained for strains constructed in the ZB307 background (data not shown). Disporic forms were very rare when *spoIIR* was located at *spoIIR* or at *amyE* (Table 3).

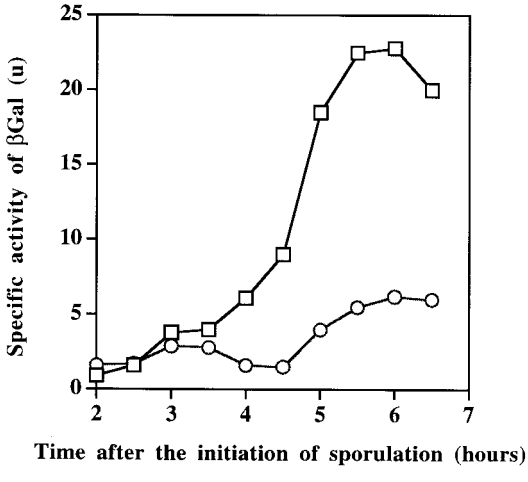

FIG. 5. Effect of *spoIIR* location on expression of *spoIID-lacZ*. □, *spoIIR* at *spoIIR* (SL7321); ○, *spoIIR* at SPβ (SL7322). βGal, β-galactosidase.

DISCUSSION

An early stage in sporulation of *B. subtilis* is an asymmetric cell division that forms the prespore and the mother cell (1, 16, 21). Shortly after the division, σ^F becomes active and governs gene expression in the prespore (21). However, the asymmetrically located division septum, when first formed, traps the origin-distal 70% of the prespore-destined chromosome in the mother cell (25) (Fig. 1); movement of the rest of the chromosome into the prespore is an active process requiring the membrane-associated DNA translocase SpoIIIE (23). Thus, a σ^F-dependent gene whose activity is required early in the prespore may need to be located near the chromosome origin. Our results demonstrate impaired sporulation when the σ^F-dependent *spoIIR* locus is moved away from the origin to either the *thrC* locus (283°; less than 20% of the sporulation of the isogenic parent with *spoIIR* at its natural position) or SPβ (190°; about 6%) (Table 2). This phenotype can be explained by the observed impairment in *spoIIR* expression at the origin-distal locations. With a *spoIIAC561* background, which en-

TABLE 3. Frequency of monosporic and disporic phenotypes in strains having *spoIIR* at different chromosomal locations[a]

Strain	Location of intact copy of *spoIIR*	No. of organisms with expression pattern of *spoIIQ-gfp*	
		Disporic	Monosporic
SL8344	*spoIIR*	0	200
SL8378	*amyE*	1	199
SL8380	SPβ	54	146
SL8345	None	61	139

[a] The phenotypes were assayed for each strain by scoring 200 organisms that were displaying fluorescence from GFP resulting from prespore-specific expression of a *spoIIQ-gfp* transcriptional fusion (12). Organisms were sampled 6 h after the end of exponential growth at 33.5°C in MSSM; similar results were obtained with samples taken at 5 and 8 h (data not shown). The strains are in the BR151 genetic background.

hances *spoIIR* transcription (9), we were able to observe that *spoIIR-lacZ* expression was delayed when the fusion was located at SPβ (Fig. 4). This delay is thought to represent the time required for SpoIIIE-dependent chromosome translocation through the sporulation septum. The 15- to 20-min estimate agrees with that of Pogliano et al. (18) obtained from microscopy studies. These observations are in agreement with the hypothesis that chromosome partitioning during sporulation is an active, unidirectional, and time-requiring process (23, 25). Frandsen et al. (2) have utilized the transient gene asymmetry resulting from this slow chromosome partitioning to engineer activation of σ^F independent of its normal regulators, SpoIIAA, SpoIIAB, and SpoIIE.

Why may a delay in the prespore localization of the *spoIIR* gene result in significantly lower expression? The native *spoIIR* promoter is weak (9, 13). Thus, when *spoIIR* is located near the terminus, one possibility is that the observed decrease in its expression results from competition with other σ^F-directed genes that now precede *spoIIR* into the prespore. In this regard, Ju et al. (8) have shown that expression of another σ^F-directed gene is higher when the gene is located nearer to the origin. The role of SpoIIR in spore formation is to activate σ^E in the mother cell (9, 13), and a consequence of σ^E activation is to block further septation (1). It is thought that *spoIIR* needs to be expressed very soon after the septum is formed (9). Reducing and/or delaying *spoIIR* expression is presumably sufficient to disrupt the delicate balance of controls that coordinate transcription between mother cell and prespore (21). The positional type of transcription regulation for *spoIIR* may thus be critical to the complex sporulation process.

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