Bacillus subtilis SpoIIIE Protein Required for DNA Segregation During Asymmetric Cell Division

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Sporulation in *Bacillus subtilis* begins with an asymmetric cell division, producing a smaller prespore and a larger mother cell, both of which contain intact copies of the chromosome. The *spoIIIE* gene is required for chromosome segregation into the prespore compartment. The effects of the *spoIIIE36* mutation on σ^F-dependent transcription are an indirect consequence of the failure of certain genes to enter the cellular compartment in which their transcription factor has become active. SpoIIIE may also be required to prevent σ^F from becoming active in the mother cell.

Cell differentiation often begins with an asymmetric cell division (*1*). In response to starvation, the Gram-positive bacterium *Bacillus subtilis* undergoes an asymmetric cell division to produce a small prespore cell and a much larger sporangium or mother cell (*2–5*). The formation of the spore septum probably contributes to the activation of the compartment-specific transcription factors σ^F and σ^E (*3, 6*), which initiate differentiation in the two cells. Little is known about how the spore septum is positioned or how a newly replicated chromosome is accurately segregated into the smaller polar compartment. Chromosome partitioning mechanisms probably operate during vegetative growth (*7*). Here we describe a chromosome segregation mechanism that operates during the early stages of sporulation.

The prespore chromosome, compressed into a relatively small compartment, can normally be distinguished from the more expansive chromosome of the sporangium by fluorescence microscopy in the presence of DNA-specific fluorophores (Fig. 1A) (*8, 9*). Strains carrying the *spoIIIE36* mutation appeared to be defective in chromosome segregation at the onset of sporulation. Although discrete polar fluorescence was visible in the cells of such mutants, the intensity was less than that of the remainder of the cell (Fig. 1B). Quantitative analysis of the images obtained from 20 cells indicated that the prespore cells of the *spoIIIE36* mutant contain only about 17% of the total DNA in each sporulating organism (Fig. 1 and Table 1). Thus, only about 30% of the "prespore" chromosome reaches a polar position in this mutant.

Mutations in the *spoIIG* gene result in an "abortively disporic" phenotype (*5, 10*) in which both chromosomes of the early sporulating organism are packaged into polar prespore-like bodies, leaving a central anucleate compartment (*8, 9*) (Fig. 1C). In *spoIIG spoIIIE* double mutants, the majority of the DNA remained in the central compartment (Fig. 1D), confirming that the *spoIIIE36* mutation prevents the complete migration of the prespore chromosome to the pole of the cell. A small proportion of the stained material (again about the equivalent of DNA in 30% of one chromosome) appeared in the expected polar positions (now at both poles of the cell because of the effects of the *spoIIG* mutation) (Fig. 1 and Table 1).

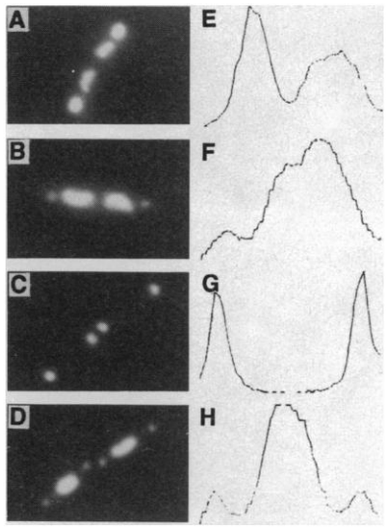

Fig. 1. Nucleoid segregation during sporulation in *B. subtilis* viewed by staining with 4,6-diamidino-2-phenylindole (DAPI). Each strain was induced to sporulate (*35*), and 4 hours later a sample was stained with DAPI (*8*) and viewed by epifluorescence microscopy. Microscopic images of individual cells were acquired with a cooled 1024 by 1024 pixel charge-coupled device (CCD) camera (Hamamatsu) through a Perceptrics frame grabber (Knoxville, Tennessee), and processed and analyzed with NIH Image Software (Image 1.49, Research Services Branch, National Institute of Mental Health, Image Processing and Analysis, National Institutes of Health) (Macintosh Quadra). (**A** to **D**) Images of pairs of sporulating cells of various genotypes. (**E** to **H**) Fluorescence intensity profiles through the leftmost cell image in (A) to (D), respectively. A straight line was drawn through the middle of the image of the chosen cell, and the intensity of fluorescence was measured along its length. The strains used were SG38 (Spo$^+$, *trpC2*) (*43*) (A and E) and isogenic derivatives carrying mutations *spoIIIE36* (B and F), *spoIIGA::aphA-3* (C and G), or *spoIIGA::aphA-3* and *spoIIIE36* (D and H).

A defect in DNA segregation should affect the formation of the asymmetric septum and subsequent gene expression. In vegetatively growing bacteria, a block in DNA replication or chromosome segregation can lead to the inhibition of septation (*11*), or the septum can close around the DNA (*12*), sometimes severing it (*13*). Complete septation is believed to be essential for the activation of the cell-specific transcription factors σ^E and σ^F (*3, 6*). The effects of *spoIIIE* mutations on gene expression could thus be indirect consequences of the failure in chromosome segregation. However, electron microscopy images showed that, although the membranes surrounding the prespore appeared unusually irregular, most *spoIIIE36* mutant cells proceeded beyond the completion of septation to engulfment (stage III) (*5, 14, 15*). Thus, septation and engulfment are not tightly linked to chromosome segregation.

Complete closure of the septum in the *spoIIIE36* mutant would ultimately require chromosome breakage. In *spoIIG* mutants, polar septa are made at both ends of the cell (*8*) and engulfment is blocked (*5*), so cells trapped at the stage of septum completion accumulate. By electron microscopy, sporulating cells of a *spoIIG spoIIIE* double mutant appeared quite similar to those of the *spoIIG* single mutant. Few cells had partially completed asymmetric septa (<5%, about the same proportion as in *spoIIG* single mutants), whereas most or all of the septa should have been incomplete if the

Table 1. DNA content of small and large cellular compartments of wild-type and mutant cells. Cells (all *trpC2* auxotrophes) were induced to sporulate, samples were stained with DAPI after 4 hours and viewed by epifluorescence microscopy, and the images were processed as described (Fig. 1). To measure the amount of fluorescence within a region, a line encircling the area was drawn and the area and mean density within that region were computed. The mean fluorescence from the smaller fluorescent body in each cell (*P*, usually the compressed prespore chromosome) and the whole cell (*T*) were measured from 20 cells of each genotype.

Strain	Genotype	Ratio (*P/T*)	SD
SG38	Wild type	0.45	0.034
36.3	*spoIIIE36*	0.17	0.04
647	Δ*(spoIIIE::aphA-3)647*	0.14	0.04
901	Δ*(spoIIGA::aphA-3)901*	0.46	0.029
902	Δ*(spoIIGA::aphA-3)901 spoIIIE36*	0.11	0.021

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presence of the incompletely segregated chromosome had prevented septum closure (*15*). Thus, the failure in DNA segregation caused by the *spoIIIE* mutation does not cause large discontinuities in the septum.

The SpoIIIE protein may not be the only component of the polar chromosome segregation system because, even in the *spoIIIE36* mutant, about 30% of the prespore chromosome is correctly localized near the pole (Table 1). The establishment of new sites of chromosomal attachment near the cell poles before or during the initiation of sporulation could help to explain both our results and previous reports of the elongated appearance of the nuclear material early in sporulation (*5, 16*).

Although mutations in a large number of *spo* genes affect gene expression during sporulation, the *spoIIIE36* mutation abolishes virtually all transcription of prespore-specific genes but has little effect on the mother cell (*2, 17*). However, the effects on gene expression are dependent on chromosome position (*18*). In certain chromosomal locations [originally *amyE* (*19*) but also at *ctc* (*20*)], reporter gene expression was independent of *spoIIIE*. Activity of the σ^F factor, which initiates prespore-specific gene expression, is probably regulated by a mechanism that recognizes some feature of the small prespore compartment (*8, 20*). Although DNA segregation partially fails in *spoIIIE* mutants, σ^F becomes active (as indicated by the expression of reporter genes at the *amyE* or *ctc* loci). To test whether this activity was localized in the prespore region, we used digital microscopy and the fluorogenic β-galactosidase substrate fluorescein di-(β-D-galactopyranoside) (FDG) (*8, 21, 22*) (Fig. 2). The activity of β-galactosidase was detected predominantly in small, discrete, roughly spherical regions of some of the sporulating cells. The pattern of staining was stable for more than an hour, although some fading and loss of cell specificity followed prolonged incubation (>2 hours) (Fig. 2, C to E). Staining patterns were similar to that of a wild-type strain carrying the σ^F-dependent *gpr'-'lacZ* fusion at its natural location (Table 2). Some individuals of both the wild type and mutant showed weak staining throughout the cell (*23*). To determine which compartment was being stained by FDG in the *spoIIIE36* mutant, some of the preparations were also stained with propidium iodide (PI) to label nucleic acids (*24*). The β-galactosidase activity (Fig. 2A) colocalized with the incompletely segregated "prespore" nucleoids (Fig. 2B).

To confirm that the localization of β-galactosidase to the prespore in the *spoIIIE36* mutant was not a side effect of the morphological impairment of the cells, we tested the ability of FDG to detect the expression of a typical σ^E-dependent gene, *spoIVA* (*25, 26*), in the *spoIIIE36* background (Fig. 2, F to I). The activity of β-galactosidase in this strain appeared to be associated with the mother cell rather than the prespore. The pattern of staining was again stable for prolonged periods, and secondary staining with PI confirmed that the β-galactosidase activity colocalized with the mother cell nucleoid. We conclude that both σ^F and σ^E transcriptional activities are correctly compartmentalized in the *spoIIIE36* mutant, despite the failure in DNA segregation.

To determine whether the phenotypic effects of the *spoIIIE36* mutation indicated the complete absence of SpoIIIE function, we constructed a null mutation, *spoIIIE647*, in which most of the coding sequence of *spoIIIE* was replaced with a selectable kanamycin resistance determinant (*27*). The *spoIIIE647* mutation blocked DNA segregation and perturbed the ultrastructure of the sporulating cell in a manner indistinguishable from that of *spoIIIE36* (Table 1). However, the null mutation had a strikingly different effect on gene expression (Fig. 3A): The expression of *gpr'-'lacZ* (placed at its normal position) was increased, rather than blocked, although normal temporal control of expression was retained. Analysis of the cellular location of σ^F activity in the *spoIIIE647* mutant, with the use of the two *gpr'-'lacZ* fusions, indicated a strong chromosome position effect and substantial σ^F activity in both the prespore (most obvious with the fusion located at *amyE*) and mother cell (detected with the fusion at its natural location). Thus, a null mutation in *spoIIIE* affects not only chromosome partitioning but also the localization of σ^F activity.

Our results are not specific to these two alleles of *spoIIIE*. Several other *spoIIIE* mutants examined have phenotypic effects similar to those of either *spoIIIE36* (designated class I mutations) or *spoIIIE647* (class II mutations), both in the blockage of DNA segregation and in the effects on gene expression (*28*). Class I mutants also differ from class II mutants immunologically. An

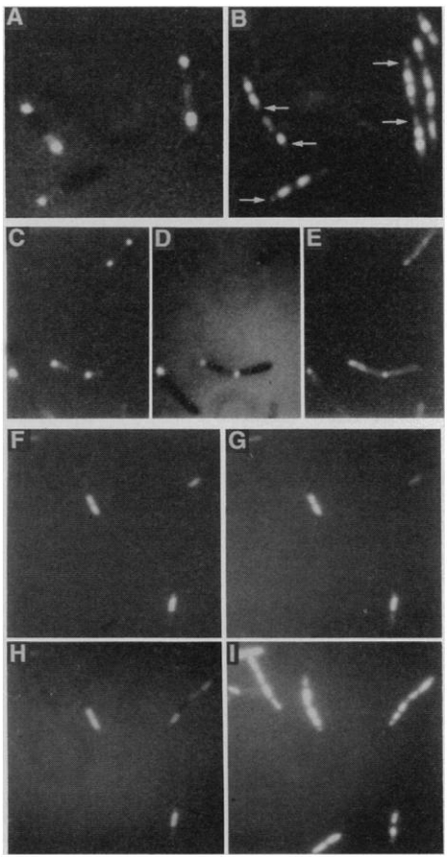

Fig. 2. Localization of reporter gene expression in the *spoIIIE36* mutant. (**A** to **E**) Strain 930 (*trpC2 spoIIIE36 amyE::gpr'-'lacZ cat*) was induced to sporulate (*35*), and 3.5 hours later a sample (1.5 μl) was treated with FDG and viewed by epifluorescence microscopy (*8*). (A) A digital image of a typical field, obtained as in Fig. 1. After the image in (A) was acquired, cells were secondarily stained with PI (*8*) and viewed with a filter set optimized for PI fluorescence (B). Arrows in (B) indicate areas of strong fluorescence in (A). (C to E) The results of prolonged incubation of a similar sample of cells taken 2 hours after the onset of sporulation. The cells were stained with FDG, and images were taken (C) 32, (D) 87, and (E) 140 min later. The insertion of *gpr'-'lacZ* at *amyE* was achieved by transformation with plasmid pPS1326 (*18*). (**F** to **I**) Strain 940 [*trpC2 spoIIIE36 Ω(spoIVA'-lacZ cat)713*] was treated similarly, except that the samples were examined after 2 hours of sporulation. FDG-stained cells are shown (F) 13, (G) 55, and (H) 135 min after staining. (I) PI image of the same field. The *spoIVA'-lacZ* fusion was described previously (*26*).

Table 2. Localization of σ^F activity in sporulating cells. Isogenic strains with or without a *spoIIIE* mutation and carrying a *gpr'-'lacZ* fusion either at its natural location (*gpr*) or at the *amyE* locus were induced to sporulate. After 3 hours, samples of cells were treated with FDG and examined by phase-contrast and epifluorescence microscopies.

Mutation	Location of *gpr'-'lacZ* fusion	Cells of fluorescence type (%)		Cells counted
		Prespore-specific*	Whole cell†	
Spo+	*gpr*	38	5	461
spoIIIE36	*amyE*	22	2	1628
spoIIIE647	*amyE*	13	4	1012
spoIIIE647	*gpr*	1.3	14	461

*Some cells also showed weak fluorescence in the mother cell (*8, 23*). †Could include cells with fluorescence only in the mother cell or in both compartments.

antiserum raised against a 533 amino acid segment of SpoIIIE (*29*) reacts with the wild-type strain and strains carrying class I mutations (*spoIIIE36*, *spoIIIE82*, and *spoIIIE905*) but not with strains carrying class II mutations (*spoIIIE644* and *spoIIIE647*) (Fig. 4).

These results suggest that wild-type SpoIIIE protein has two functions: The first, required for DNA segregation, is absent in *spoIIIE* mutants of both classes; the second, required to prevent σ^F activity from appearing inappropriately in the mother cell, is absent in class II mutants but retained by class I mutants. The formal possibility that the class I phenotype is an artifact produced by the mutant protein that gains some function that the wild-type protein does not possess seems unlikely because the phenotype of the class I mutants resembles that of the wild type more closely than that of the class II mutants and because a class I phenotype was also produced by the *spoIIIE82* nonsense mutation (*30*). Nevertheless, to determine whether this was a dominant gain-of-function mutation, we infected strains carrying *spoIIIE36* or *spoIIIE647* with a transducing phage, ϕ105J115 (*31*), which would stably introduce into the cells a single copy of the wild-type *spoIIIE* gene. The resulting strains showed a pattern of gene expression similar to that of the wild type, indicating that both mutations are recessive (Fig. 3B).

We suppose that, soon after the onset of sporulation, nascent chromosomes become attached to the polar regions of the cell. After the completion of DNA replication, septum formation begins near one pole of the cell. The chromosome attached to the pole is then transported, by a SpoIIIE-dependent mechanism, into the small compartment (Fig. 5). At some stage in this process, σ^F becomes active. In the wild-type and class I *spoIIIE* mutants, σ^F activity appears in the small compartment, triggering the prespore program of gene expression. In class II *spoIIIE* mutants, an additional defect allows σ^F to become active in both compartments.

In *spoIIIE* mutants of class I and probably class II, a specific segment of the *B. subtilis* chromosome is (likely) trapped in the small compartment. The segment includes the *amyE* locus and excludes the known σ^F-dependent genes (such as *gpr*) (*20*, *32*) at their normal locations (Fig. 5B). The two chromosomal locations where expression is independent of *spoIIIE* both lie near the origin of DNA replication (*oriC*), indicating that this locus may be involved in polar chromosomal attachment (*33*). The partitioning of a specific region of the chromosome into the small compartment would explain why σ^F-dependent gene expression is either fully on or off, depending on chromosome location, in the class I *spoIIIE* mutants. We do not yet know why the localization of σ^F activity is impaired in the complete absence of SpoIIIE, but this protein may yet have a role in the localization of σ^F activity. Alternatively, in cells containing a nearly completed septum and an incompletely segregated prespore chromosome, SpoIIIE protein, by interacting with both DNA and septum (*34*), may seal off the two compartments from each other, allowing normal localization of σ^F and σ^E activities. This sealing function could be retained in the

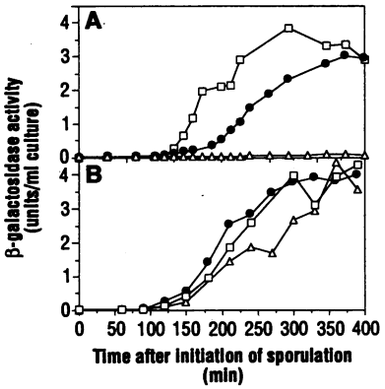

Fig. 3. (**A**) Effects of the *spoIIIE36* and *spoIIIE647* mutations on the expression of *gpr'-'lacZ* at its natural chromosomal location. Each strain was induced to sporulate (*35*) and assayed at intervals for β-galactosidase activity (*43*). The strains used were SG38, containing plasmid pPS1395 (*gpr'-'lacZ*) (*44*) integrated at the *gpr* locus (filled circles), and isogenic strains carrying mutations *spoIIIE36* (open triangles) and *spoIIIE647* (open squares). (**B**) The dominance of wild-type *spoIIIE* over mutations *spoIIIE36* and *spoIIIE647*. Strain SG38 with a *gpr'-'lacZ* fusion at the natural location (filled circles) and isogenic strains carrying the *spoIIIE36* (open triangles) or *spoIIIE647* (open squares) mutations were infected with transducing phage ϕ105J115, which carries a wild-type copy of the *spoIIIE* gene (*31*). Lysogenic derivatives were purified, induced to sporulate, and assayed for β-galactosidase activity.

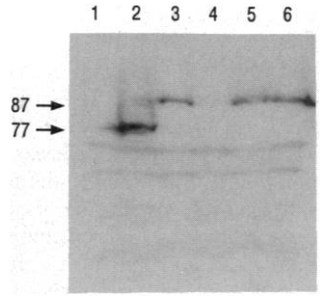

Fig. 4. Western blot analysis (*41*) of the SpoIIIE proteins produced by wild-type and mutant strains of *B. subtilis*. Total protein from vegetative growing cells was separated on 10% polyacrylamide, 0.1% SDS gels. After electrophoresis, the proteins were transferred to nitrocellulose filters and probed with polyclonal antiserum raised against a GST-SpoIIIE fusion protein (*29*). The bound antibodies were detected with x-ray film, after the application of a secondary antibody conjugated to horseradish peroxidase and the use of an enhanced chemiluminescence detection kit (Amersham International). Lane 6 contains protein from vegetative cells of Spo$^+$ strain SG38. Lanes 1 to 5 contain similar amounts of protein prepared from isogenic strains containing the following *spoIIIE* mutations: 1, *spoIIIE647*; 2, *spoIIIE82*; 3, *spoIIIE905*; 4, *spoIIIE644*; 5, *spoIIIE36*. Arrows indicate the full-length 87-kD (*38*) form of SpoIIIE and the truncated form (77 kD) (*30*) produced by the *spoIIIE82* mutant. The two faster migrating bands present in all tracks represent proteins of unknown identity that are also detected by the primary antibody preparation.

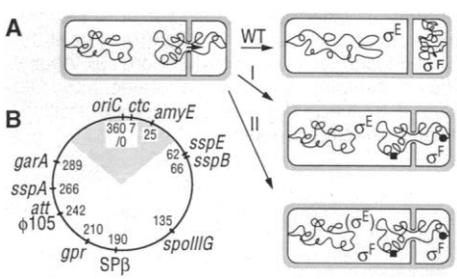

Fig. 5. Chromosome segregation during sporulation and the effects of *spoIIIE* mutations. (**A**) Depiction of the early stages of sporulation. During the transition to sporulation, points of DNA attachment arise at the poles. After DNA replication is completed, a polar septum is formed. The wild-type (WT) SpoIIIE protein, perhaps located in the nascent septum, then drives the chromosome attached at the pole into the prespore compartment, and σ^F and σ^E activities appear in their respective compartments. In class I *spoIIIE* mutants (I), chromosome segregation fails but σ^F and σ^E activities are localized normally. The σ^F activity can drive the transcription of genes that are trapped in the small compartment [for example, *amyE* (filled circle)] but not of genes remaining in the large compartment [such as *gpr* (filled square)]. In class II *spoIIIE* mutants (II), DNA segregation also fails but σ^F becomes active in both compartments. (**B**) The correlation of chromosomal location with σ^F-dependent transcription in a *spoIIIE36* mutant. Map positions on the chromosome (*45*) are marked in degrees. The abbreviation *oriC* represents the origin of bidirectional DNA replication. The white sector includes σ^F-dependent genes known to be blocked by the *spoIIIE36* mutation (*2*). The shaded sector represents the portion (approximately 30%) of the chromosome found in the prespore compartment of *spoIIIE* mutants (Table 1). This sector has been centered on the origin of replication for reasons discussed in the text and includes the two locations where σ^F-dependent promoters are independent of *spoIIIE36*.

class I *spoIIIE* mutants but lost in class II mutants, in which no SpoIIIE-like protein accumulates.

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15. Strain SG38 (Spo$^+$) and isogenic strains carrying the *spoIIIE36* mutation with or without the *spoIIG55* mutation were induced to sporulate (*35*). Samples taken 3.5 hours later were fixed, embedded, sectioned, and prepared for transmission electron microscopy as described (*10, 28*).
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17. Mutations of the *spoIIIE* gene have little effect on the expression of σ^E-dependent genes in the early stages of mother cell development. However, during the later stages of mother cell development, transcription is directed by the σ^K form of RNA polymerase, the activity of which is coupled to gene expression in the prespore (*36*). Because prespore gene expression is blocked in *spoIIIE* mutants, the later stages of mother cell development are also impaired (*2, 3, 36*).
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23. The weak fluorescence sometimes associated with the mother cell compartment of both wild-type and *spoIIIE* mutant cells containing the *gpr'-'lacZ* fusion may indicate that σ^F activity is not completely localized to the prespore immediately after septation. Although this ambiguity complicates the interpretation of the results, it nevertheless does not affect the conclusion that the distribution of σ^F activity in sporulating cells of the *spoIIIE36* mutant is similar to that of wild-type cells.
24. PI was used to visualize DNA in preference to 4,6-diamidino-2-phenylindole (DAPI) because its absorbance and emission spectra are distinct from FDG and because it stains unfixed cells.
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27. To construct a deletion of the *spoIIIE* gene, plasmids containing cloned segments from the flanking regions of the *spoIIIE* gene (*37*) were linearized by digestion at restriction sites just inside the beginning and end of the coding region of the gene. Plasmid pSG204 was cleaved with *Bam HI*, which cleaves in a polylinker adjacent to the *Pvu II* site after base 1184 in the published sequence [(*37*), corrected in (*38*)]. Plasmid pSG209 was cleaved with *Hind III*, at base 3039 in the published sequence. The two digested plasmids were ligated to plasmid pSG122 (*39*), which had been digested with Bam HI and Hind III to release the selectable *aphA-3* gene encoding kanamycin resistance. The ligation products were transformed into Spo$^+$ *B. subtilis* strain 168 (*trpC2*), with selection for kanamycin resistance (5 μg/ml). Southern blot analysis confirmed that the *spoIIIE* gene was replaced by the *aphA-3* insertion.
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29. A 58-kD segment of SpoIIIE (residues 158 to 691) was overproduced in *Escherichia coli* as a COOH-terminal fusion to glutathione-*S*-transferase (GST), with the use of the pGEX system (*40*). The gel-purified fusion protein was used to raise polyclonal antiserum (*41*) (R. Allmansberger and J. Errington, unpublished results).
30. DNA sequence analysis revealed that *spoIIIE82* is a nonsense mutation close to the COOH-terminal coding end of the gene (codon 697 of 787) (*28, 38*).
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33. Most reports of DNA-membrane association relate to associations between the replication origin and the membrane [for example, (*42*)].
34. The SpoIIIE protein has extensive stretches of hydrophobic residues in an NH_2-terminal domain (*38*), which could anchor it to the nascent septum. The protein's apparent role in DNA transfer suggests a direct interaction with DNA, and preliminary attempts to purify the protein (*22*) suggest that it has affinity for phosphocellulose and heparin agarose, as is commonly found with DNA binding proteins.
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A Neurochemically Distinct Third Channel in the Macaque Dorsal Lateral Geniculate Nucleus

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The primate visual system is often divided into two channels, designated M and P, whose signals are relayed to the cerebral cortex by neurons in the magnocellular and parvicellular layers of the dorsal lateral geniculate nucleus. We have identified a third population of geniculocortical neurons in the dorsal lateral geniculate nucleus of macaques, which is immunoreactive for the α subunit of type II calmodulin–dependent protein kinase. This large third population occupies interlaminar regions (intercalated layers) ventral to each principal layer. Retrograde labeling of kinase-immunoreactive cells from the primary visual cortex shows that they provide the geniculocortical input to cytochrome oxidase–rich puffs in layers II and III.

The primate visual system is commonly viewed as an amalgam of two functional channels, designated M and P (*1*), that include the magnocellular and parvicellular layers in the dorsal lateral geniculate nucleus (LGN) of the thalamus (*2, 3*). Projections from M and P reach the primary visual cortex (V1), where the P channel is said to split into two streams, one that is selective for stimulus color and is represented by the cytochrome oxidase–rich puffs (or blobs) in the superficial layers, and another that is selective for stimulus form and is located in the surrounding cytochrome oxidase–poor interpuffs (*4*). From those points, cortical connections that are segregated to greater or lesser degrees reach the many extrastriate visual areas (*5*). A central issue in this division of labor in the macaque visual system is the origin of visual input to the puffs. Although in other primate species

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