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Differential Regulation of *spo0A* Transcription in *Bacillus subtilis*: Glucose Represses Promoter Switching at the Initiation of Sporulation

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We have shown by S1 nuclease mapping with in vivo transcripts that the differential expression of a sporulation-regulatory gene, *spo0A*, is regulated by switching of two discrete promoters during the initiation of sporulation in *Bacillus subtilis*; vegetative mRNA was transcribed from an upstream promoter (P_v, vegetative promoter), and sporulation-specific mRNA was transcribed from the other promoter (P_s, sporulation-specific promoter) about 150 bp downstream of the P_v promoter. Transcription from the P_v promoter was at a low level and shut off at $T_{0.5}$. On the other hand, transcription from the P_s promoter was strongly induced at $T_{0.5}$ and increased until $T_{2.5}$. In the presence of 2% glucose, P_v-directed transcription was not shut off and was observed even at $T_{1.5}$, whereas the induction of P_s-directed transcription was completely repressed. A mutant in which the *spo0A* gene was transcribed only from the P_s promoter could sporulate normally in the presence of 0.1% glucose but could not sporulate at all in the presence of 2% glucose. In a catabolite-resistant sporulation mutant carrying *crsA47* (*sigA47*), a mutation within the gene encoding σ^A, normal promoter switching from P_v to P_s was observed in the presence of 2% glucose.

Sporulation in *Bacillus subtilis*, which responds to nutritional starvation in the environment, proceeds through an ordered series of morphological changes leading from a vegetative cell to a dormant spore. This differential process is recognized as a result of the sequential expression of a set of *spo* genes, whose mutations arrest the process at characteristic intermediate morphological stages. Recently, a number of *spo* genes have been cloned, and some functions of their products have been characterized, especially for those defined as sporulation-specific sigma factors (for a review, see reference 19). The functions of many of the other *spo* gene products, however, are still obscure.

The transition from the vegetative to the sporulation phase requires at least seven genes known as *spo0*, since *spo0* mutants cannot form an asymmetric septum, which is the first morphological change in the sporulation process. Among these genes, the *spo0A* gene is thought to play a central role in the initiation of sporulation. First, a *spo0A* mutant exerts the most-pleiotropic effects on sporulation-associated phenotypes such as antibiotic formation, extracellular protease synthesis, and competence development for transformation (22). Second, the *sof-1* mutation, a missense mutation in the 12th codon of the *spo0A* gene, suppresses the sporulation-deficient phenotype of *spo0B*, *spo0E*, and *spo0F* mutants (10). Third, the *spo0A* gene product shares homology with a group of proteins called receivers, which are elements of prokaryotic two-component signal-transducing systems (24, 28), and has been shown to be a phosphoacceptor like the other receiver proteins (21). In addition, we have recently found that sporulation in a *spo0A* temperature-sensitive mutant could be normally induced by a shift-down of the culture temperature even in the late stationary phase (2a). These results strongly suggest that the Spo0A protein is a key factor in initiating sporulation.

We have previously reported that a *spo0A-lacZ* translational fusion was expressed at a low level in the vegetative phase and that this expression was significantly stimulated in the early stationary phase (31). This stimulation was shown to be repressed by an excess of glucose (30), which blocks sporulation mostly at the initial stage (27). These results suggested that the *spo0A* gene could be one of the targets for glucose repression of sporulation. We have also shown that the stimulation of *spo0A-lacZ* expression as well as sporulation was restored by mutation *crsA47* (*sigA47*), which has been identified as a mutation in the gene encoding the major sigma factor σ^A (13), in the presence of an excess of glucose (30).

In order to clarify the mechanism by which the *spo0A* expression is differentially regulated and to study how glucose exerts its effect on this regulation, we analyzed the transcription of *spo0A* during the transition phase from vegetative growth to sporulation in both the absence and the presence of an excess of glucose. In vivo transcriptional mapping indicated that the differential expression of the *spo0A* gene is regulated by a switching of two promoters during the initiation of spore development. These two promoters exhibit opposite responses to glucose: the P_v promoter, which functions in the vegetative phase, is resistant to glucose, and the P_s promoter, from which transcription is induced at the early sporulation phase, is sensitive to glucose. A mutant in which the *spo0A* expression depends only on the P_s promoter cannot sporulate in the presence of an excess of glucose. We also suggest that σ^A and the Spo0A protein itself, in addition to σ^H, a minor sigma factor which is necessary to stimulate *spo0A* expression in the early stationary phase (31), are involved in the regulation of promoter switching from P_v to P_s during spore development

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and the repression of transcription from the P_s promoter by glucose.

MATERIALS AND METHODS

Bacteria. The *B. subtilis* strains used in this study were UOT-1285 (*trpC2 lys-1 aprAΔ3 nprE18 nprR2*) and UOT-1296 (*trpC2 lys-1 aprAΔ3 nprE18 nprR2 crsA47* [*sigA47*]) (30). *Escherichia coli* DH1 (9) was used as the host for all the plasmids used in this study.

Plasmids. The construction of plasmid pSPTA1 is shown in Fig. 1A. A 359-bp *Bam*HI fragment containing the promoter and N-terminal coding region of the *spo0A* gene was isolated from a recombinant phage, φCAZ1 (31), and was subcloned into a *Bam*HI site in pSPT18 (Pharmacia Biochemicals). The construction of plasmid pSPTA5, a pSPT18 derivative carrying a 0.5-kb *Cla*I-*Bgl*II fragment of the *spo0A* gene, was described previously (3). In order to construct integrational plasmids pCPA1 and pCPA6 (see Fig. 5), we isolated a 359-bp *Bam*HI fragment from φCAZ1 and a 249-bp *Bam*HI fragment from φCAZ6 (30). The former fragment contains both the P_v and P_s promoters, and the latter fragment contains only the P_s promoter. These fragments were subcloned into a *Bam*HI site in an integrational plasmid, pCP112, which cannot replicate autonomously in *B. subtilis* cells (23). Plasmid pCPA6R was constructed in the same manner as pCPA6, except that the orientation of the inserted *spo0A* fragment was reversed (see Fig. 6A).

Enzymes. SP6 RNA polymerase and S1 nuclease were purchased from Takara Shuzo Co., and T7 RNA polymerase was purchased from Pharmacia Biochemicals. Restriction endonucleases were purchased from Takara Shuzo, Boehringer Mannheim-Yamanouchi, Bethesda Research Laboratories, and Nippon Gene.

RNA preparation. *B. subtilis* cells were grown in 2× SG medium (15) containing 0.1 or 2% glucose at 37°C, and the culture was diluted with 9 volumes of the same fresh medium when the optical density of the culture reached 0.5 A_{660} units. During the subsequent growth, 40 ml of the culture was withdrawn as the vegetative-cell fraction in the mid-logarithmic phase (optical density = 0.5 A_{660} units), and 12 ml of the culture was withdrawn at various time points after the end of the logarithmic phase of growth (defined as T_0). The cells were immediately frozen in dry ice-ethanol after centrifugation and stored at −70°C until use. Total cellular RNAs were extracted by the procedure of Kirby et al. (14) with some modifications. The cells were resuspended in 6 ml of modified Kirby mixture (1% sodium triisopropylnaphthalene-sulfonic acid [Kodak], 3% sodium *p*-aminosalicylic acid [Sigma], 50 mM Tris-HCl [pH 8.0], 10% Tris-EDTA-saturated phenol) and sonicated for 2 to 5 min with a Branson Sonifier 200. The subsequent procedures were carried out according to the method described by Hopwood et al. (11).

S1 protection assay. The A1 probe, a ^{32}P-labeled anti-*spo0A* RNA probe complementary to the *Sau*3AI-*Sau*3AI region of the *spo0A* gene, was prepared with *Eco*RI-digested pSPTA1 and SP6 RNA polymerase according to the method described by Melton et al. (18). The A5 probe, which is complementary to the *Cla*I-*Bgl*II region of the *spo0A* gene, was prepared with *Eco*RI-digested pSPTA5 and SP6 RNA polymerase in the same manner. These RNA probes (>10^5 cpm) were used for hybridization with 100 μg of the *B. subtilis* total cellular RNAs or *E. coli* tRNA (Sigma) as described previously (17). The hybridization mixture (20 μl) was incubated at 45°C for 2 h and then diluted into 200 μl of ice-cold S1 buffer (50 mM NaCl, 30 mM sodium acetate [pH 4.8], 1 mM $ZnCl_2$, 5% glycerol) containing 500 U of S1 nuclease. After incubating at 37°C for 60 min, protected hybrids were recovered by phenol-chloroform (1:1) extraction and ethanol precipitation, rinsed with 70% ethanol, and redissolved in 5 μl of loading buffer (80% formamide, 10 mM Tris-HCl [pH 7.9], 1 mM EDTA, 10% glycerol, 0.05% dyes). Samples were denatured at 95°C for 2 min, immediately chilled in ice water, and resolved by electrophoresis in 6% polyacrylamide–7 M urea gels (10 by 10 cm; 1 mm thick) with RNA size markers. ^{32}P-labeled RNA size markers were prepared as described elsewhere (18) with *Nde*I-digested pSPT18 and SP6 RNA polymerase (producing a 335-nucleotide [nt] transcript), *Hpa*I-digested pSPTA1 and T7 RNA polymerase (267-nt transcript), *Hpa*I-digested pSPTA1 and SP6 RNA polymerase (160-nt transcript), *Sau*96I-digested pSPT18 and SP6 RNA polymerase (106-nt transcript), and *Hin*dIII-digested pSPT18 and T7 RNA polymerase (65-nt transcript).

Southern hybridization. Southern blotting was performed as described elsewhere (17). The DNA blot filter was hybridized with the A5 RNA probe (>10^5 cpm) in 5× SSPE (4) containing 0.1% each Ficoll, polyvinylpyrrolidone, and bovine serum albumin; 50% formamide; and 50 mg of *E. coli* tRNA per ml. After incubation at 45°C for 16 h, the filter was treated with 10 μg of RNase A (Sigma) per ml in 2× SSPE at 37°C for 10 min and washed twice in 2× SSPE at room temperature.

Assay for sporulation. *B. subtilis* cells were grown in 2× SG medium containing 0.1 or 2% glucose at 37°C for 20 h, and heat-resistant spores were counted by heating the cells at 80°C for 10 min and then plating them.

RESULTS

Promoter switching of the *spo0A* gene during the initial stage of sporulation. We recently reported that the expression of the *spo0A-lacZ* fusion was subject to at least two different regulatory signals; one functions in the vegetative phase, and the other is induced at the early sporulation phase (30). In order to examine whether this differential expression of the *spo0A* gene was derived from two different units of transcription, we carried out an S1 nuclease protection assay using a ^{32}P-labeled anti-*spo0A* RNA probe complementary to the *Sau*3AI-*Sau*3AI region of the *spo0A* gene (named the A1 probe [Fig. 1A]). *B. subtilis* UOT-1285 cells were grown in 2× SG medium containing 0.1% glucose, and total cellular RNA was extracted at various time points in the vegetative and stationary phases. As shown in Fig. 1B, the A1 probe was protected by two discrete transcripts. The lengths of the S1-protected bands were 296 and 148 nucleotides, as determined by high-resolution S1 mapping experiments (data not shown). This indicates that one transcript initiated 198 bp upstream from the N-terminal GTG codon of the *spo0A* gene and the other initiated 50 bp upstream from the GTG codon. The former was weakly transcribed within the phase from vegetative growth to $T_{0.5}$, while the latter was induced at T_1 and then markedly increased until $T_{2.5}$. These results indicate that the differential expression of the *spo0A* gene is regulated by the switching of two discrete promoters during the initial stage of sporulation. Therefore, the upstream promoter, which functions in the vegetative phase, was named P_v (vegetative promoter), and the downstream promoter, which is induced at the initial stage of sporulation, was named P_s (sporulation-specific promoter). Although the *spo0A* transcript derived from P_s seems to be the same as

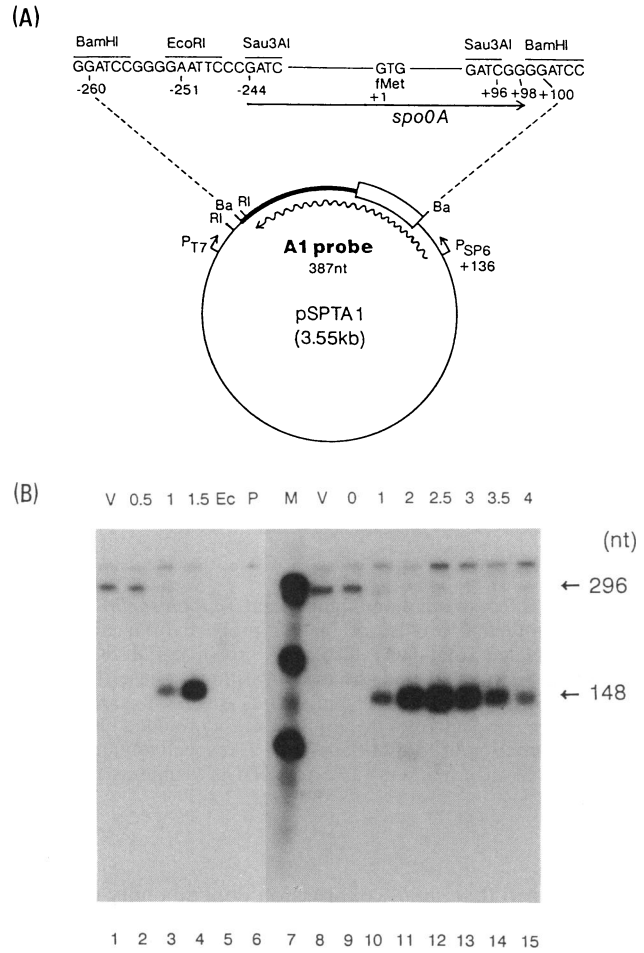

FIG. 1. Time-course profiles of *spo0A* transcription. (A) Construction of pSPTA1. Thick and thin lines represent the DNA fragments derived from ϕCAZ1 (31) and pSPT18, respectively. The N-terminal coding region of *spo0A* is shown as an open box. The A1 probe transcribed from a SP6 promoter is shown as a wavy arrow. Abbreviations for the restriction sites: RI, *Eco*RI; Ba, *Bam*HI. (B) S1 protection assays of the *spo0A* transcripts at various time points. The A1 probe was hybridized with the RNAs extracted from UOT-1285 cells in the vegetative phase (lanes 1 and 8) and at T_0 (lane 9), $T_{0.5}$ (lane 2), T_1 (lanes 3 and 10), $T_{1.5}$ (lane 4), T_2 (lane 11), $T_{2.5}$ (lane 12), T_3 (lane 13), $T_{3.5}$ (lane 14), and T_4 (lane 15) and with *E. coli* tRNA as a negative control (lane 5). Lane 6, Diluted sample of A1 probe (387 nt); lane 7, RNA size markers (from top to bottom: 335, 267, 160, and 106 nt).

FIG. 2. Comparison of the nucleotide sequences of the two *spo0A* promoters and the other *B. subtilis* promoters. (A) P_v promoter and consensus sequence for σ^A promoters (20). The transcription initiation site of the P_v promoter is indicated as an arrow. Solid and dashed lines above and below the sequence indicate the two overlapping sets of putative promoter sequences. (B) P_s promoter and σ^H promoters (25). The transcription initiation sites of the promoters are indicated as arrows. Underlining indicates homology between sequences. Arrows above the P_s promoter sequence indicate an inverted repeat structure observed upstream of the promoter.

that previously reported by Ferrari et al. (7), the P_v-derived transcript was newly identified in this study.

The P_v region contains two overlapping sets of putative promoter sequences which closely resemble the consensus sequence recognized by the major sigma factor σ^A (20; Fig. 2A). Each of the sets shares four of six bases in the −35 region and four or five of six bases in the −10 region with the consensus sequence and contains a 17-bp spacing which is typical for the σ^A promoters. We have previously shown that the region between positions −203 and −244, which contains both of the putative promoter sequences, is essential for *spo0A* expression during vegetative growth (30). These results strongly suggest that the P_v promoter is recognized by σ^A. At present, however, we do not know which set of the putative promoter sequences actually functions in vivo. On the other hand, the P_s region contains the sequence which in part resembles the consensus sequence observed for σ^H promoters (7, 25; Fig. 2B). These promoters had formerly been thought to be recognized by σ^B, but *spoVG* P1 and *rpoD* P3 promoters have been shown to be transcribed by $E\sigma^H$ RNA polymerase in vitro (1, 2, 6). It has been reported that all of these promoters are induced during the early stage of sporulation (16, 29, 32). We have also shown that the stimulation of *spo0A* expression in the initial stage of sporulation depends on the *spo0H* gene product (31), which has been identified as σ^H (6). It is thus likely that the P_s promoter is recognized by σ^H.

Effect of glucose on promoter switching of the *spo0A* gene. We have recently shown that the stimulation of *spo0A* expression in the initial stage of sporulation was repressed in the presence of an excess of glucose (30). We therefore examined the effect of glucose on the promoter switching of the *spo0A* gene by the S1 protection assay. Figure 3 shows the S1 protection profiles of the A1 RNA probe hybridized with the RNA extracted from UOT-1285 cells grown in 2× SG medium containing 0.1 or 2% glucose. In the presence of 2% glucose, the P_s-derived transcription was completely repressed, while the P_v-derived transcription continued until at least $T_{1.5}$. These results clearly indicate that the responses of the two promoters to an excess of glucose are opposite; the P_s promoter is sensitive to glucose, and the P_v promoter is resistant to glucose.

To study further these opposite responses of the two promoters to glucose, we next examined the effects of adding an excess of glucose immediately after the induction of the P_s promoter. UOT-1285 cells were grown in 2× SG

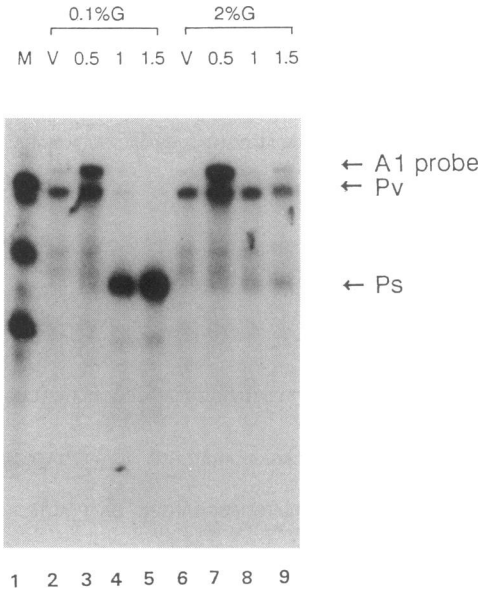

FIG. 3. Effect of glucose on *spo0A* transcription. S1 protection profiles of the A1 probe hybridized with the RNAs extracted from UOT-1285 cells grown in 2× SG medium containing 0.1% glucose (lanes 2 to 5) or 2% glucose (lanes 6 to 9) are shown. Lane 1, RNA size markers.

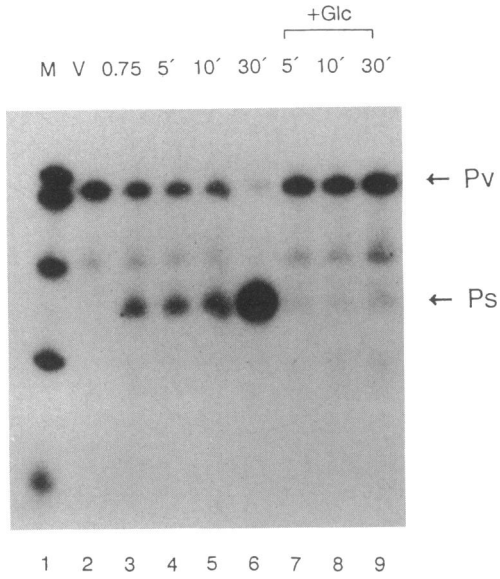

FIG. 4. Effect of addition of glucose at $T_{0.75}$ on *spo0A* transcription. S1 protection profiles of the A1 probe hybridized with the RNAs extracted from UOT-1285 cells grown in 2× SG medium containing 0.1% glucose in the mid-logarithmic phase (lane 2), at $T_{0.75}$ (lane 3), and 5, 10, and 30 min after $T_{0.75}$ without added glucose (lanes 4 to 6, respectively) or with an excess of added glucose at a final concentration of 55 mM (lanes 7 to 9, respectively) are shown. Lane 1, RNA size markers (a 65-nt RNA is visible as the lowest band).

medium containing 0.1% glucose, and an excess of glucose was added to half of the culture at $T_{0.75}$. The final concentration of glucose was 55 mM (ca. 1%), which has been shown to be sufficient to reduce sporulation frequency to less than 1% (26). RNAs were extracted 5, 10, and 30 min after $T_{0.75}$ from both the culture with added glucose and the culture without added glucose, and the S1 protection assay was carried out with the A1 probe. As shown in Fig. 4, P_s-derived transcription was fully repressed within 5 min after the addition of glucose (lane 7). On the other hand, P_v-derived transcription was enhanced after the addition of glucose (lanes 7 through 9), while it was repressed after $T_{0.75}$ in the cells from the culture without added glucose (lanes 4 through 6). These results indicate that the promoter switching from P_v to P_s is reversible in the early stationary phase.

Construction of a mutant in which the *spo0A* gene is transcribed only from the P_s promoter. To study further the functions of the P_v and P_s promoters, we constructed a mutant in which *spo0A* expression depends only on the P_s promoter. We used integrational plasmids pCPA1 and pCPA6 (see Materials and Methods; Fig. 5) for transforming UOT-1285 cells to chloramphenicol resistance (Cm^r). We expected that pCPA1 or pCPA6 would be integrated into the *spo0A* locus of the host chromosome by a Campbell-type recombination (8) in these Cm^r transformants, resulting in the genomic structures around the locus shown in Fig. 6A. Figure 6B shows the Southern hybridization profiles of *Bcl*I- and *Hin*dIII-digested chromosomal DNAs of those Cm^r transformants, obtained by using an anti-*spo0A* RNA probe complementary to the *Cla*I-*Bgl*II region of the *spo0A* gene (named the A5 probe). The results were consistent with our expectations, so we gave the designation UOT-1371 to the transformant in which both the P_v and P_s promoters exist adjacent to the intact *spo0A* coding region and the designation UOT-1372 to the transformant in which only the P_s promoter is present (Fig. 6A, rows 1 and 2, respectively).

To confirm that *spo0A* expression depends only on the P_s promoter in strain UOT-1372, the S1 protection assay was carried out. UOT-1371, UOT-1372, and UOT-1285 cells were grown in 2× SG medium containing 0.1% glucose, and RNAs were extracted from the cells at various time points in the vegetative and stationary phases. We used the A5 probe for the assay with the UOT-1371 RNA and the A1 probe for the assay with the UOT-1372 RNA. As shown in Fig. 7A (lanes 3 through 6), five bands (a through e) were detected in the assay with the UOT-1371 RNA. Bands a and c resulted from the protection by the P_v-derived transcripts, and bands d and e resulted from that by the P_s-derived transcripts, as illustrated in Fig. 7C. Bands a and c decreased in amount after T_1, and then bands d and e increased in amount, indicating that promoter switching from P_v to P_s occurred normally in this strain, as it did in the wild type. We found another band, b, which was approximately 400 nt long and appeared at every time point. This seemed to correspond to the region from the *Cla*I site to position +98 in Fig. 7C, suggesting that there was another unidentified transcript initiating upstream from the *Cla*I site. The probe-length bands often detected in the other S1 protection assays (Fig. 1 and 3) may be partly ascribed to this transcript.

Figure 7B shows the S1 protection profiles of the A1 probe with the UOT-1372 RNA (lanes 3 through 6). Three bands (f through h) were detected from this assay. Band f corresponded to the P_v-derived transcripts, and bands g and h corresponded to the P_s-derived transcript, as illustrated in Fig. 7D. If a message is transcribed from the upstream P_v or P_s promoter to the downstream *spo0A* gene via the integrated vector region, it should protect 302 nt of the probe (Fig. 7D). Since we could not detect a 302-nt band in the autoradiograph, we concluded that the *spo0A* gene was

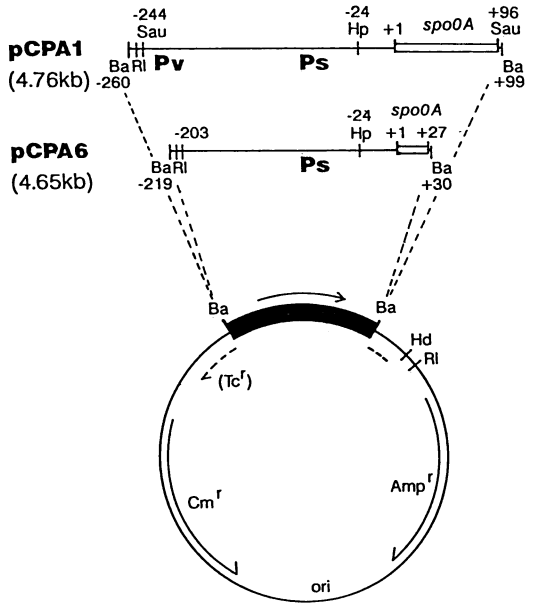

FIG. 5. Construction of integrational plasmids pCPA1 and pCPA6. Thick and thin lines represent the DNA fragments derived from ϕCAZ1 (or ϕCAZ6) and pCP112, respectively. Open boxes in the maps at the top represent the N-terminal coding region of *spo0A*. Abbreviations: Sau, *Sau*3AI; Hp, *Hpa*I; Hd, *Hin*dIII; Amp^r and Tc^r, ampicillin and tetracycline resistance genes derived from pBR322, respectively; Cm^r, chloramphenicol resistance gene derived from pC194 (12); ori, pBR322 *ori* region.

transcribed only from the P_s promoter adjacent to the intact *spo0A* coding region in strain UOT-1372. Although promoter switching from P_v to P_s occurred in this strain, the time of switching was between T_1 and T_2 (Fig. 7B, lanes 4 and 5), which was later than that observed in strain UOT-1371.

Effects of glucose on the P_s promoter and on sporulation in strain UOT-1372. We examined the effects of glucose on *spo0A* transcription in the strains UOT-1371 and UOT-1372 described above. The cells were grown in 2× SG medium containing 2% glucose, and RNAs were extracted from the cells in the vegetative and stationary phases (at $T_{1.5}$ for UOT-1371 and at T_3 for UOT-1372). The results of an S1 protection assay with these RNAs are shown in Fig. 7A and B (lanes 7 and 8). In both strains, the induction of the transcription from the P_s promoter (bands d, e, g, and h) was fully repressed, and the transcription from the P_v promoter (bands a, c, and f) was continued in the stationary phase in the presence of an excess of glucose. These results indicate that the responses of the P_v and P_s promoters to glucose in these strains are the same as those in the wild type (Fig. 3 and 4).

Next, we examined the effects of glucose on the sporulation of these strains. UOT-1371, UOT-1372, and UOT-1285 cells were grown in 2× SG medium containing 0.1 or 2% glucose for 20 h to determine the number of heat-resistant spores (Table 1). In strains UOT-1285 and UOT-1371, the numbers of spores were reduced to 2.0×10^6/ml and 5.5×10^6/ml, respectively, by the presence of 2% glucose. In strain UOT-1372, however, the number of spores was reduced to less than 20/ml by 2% glucose, indicating that sporulation in

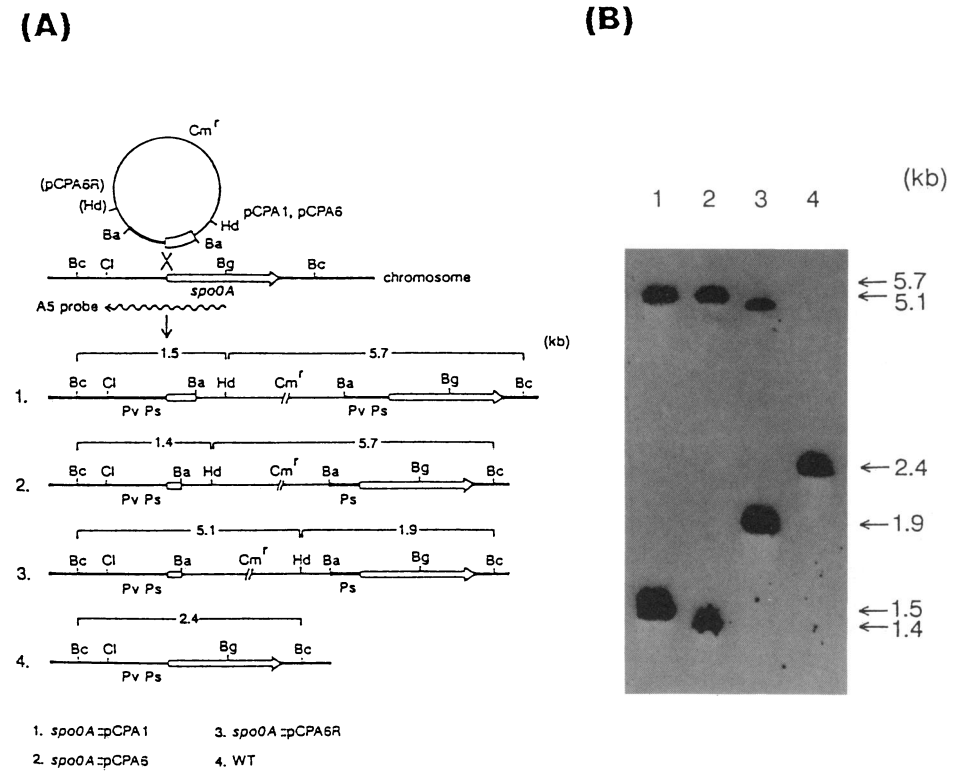

FIG. 6. Integration of pCPA1 and pCPA6 into the *spo0A* locus. (A) Genomic structures around the *spo0A* loci in the chloramphenicol-resistant (Cm^r) transformants. Thick and thin lines represent the *B. subtilis* chromosomal DNA and pCP112, respectively. Open boxes and arrows represent the *spo0A* coding region. Abbreviations: Cl, *Cla*I; Bc, *Bcl*I; Bg, *Bgl*II; Ba, *Bam*HI; Hd, *Hin*dIII. (B) Southern hybridization profiles of the *Bcl*I- and *Hin*dIII-digested chromosomal DNAs of the Cm^r transformants. Lane 1, UOT-1371 (*spo0A*::pCPA1); lane 2, UOT-1372 (*spo0A*::pCPA6); lane 3, UOT-1372R (*spo0A*::pCPA6R) as a size control; lane 4, UOT-1285 (*spo0A*$^+$).

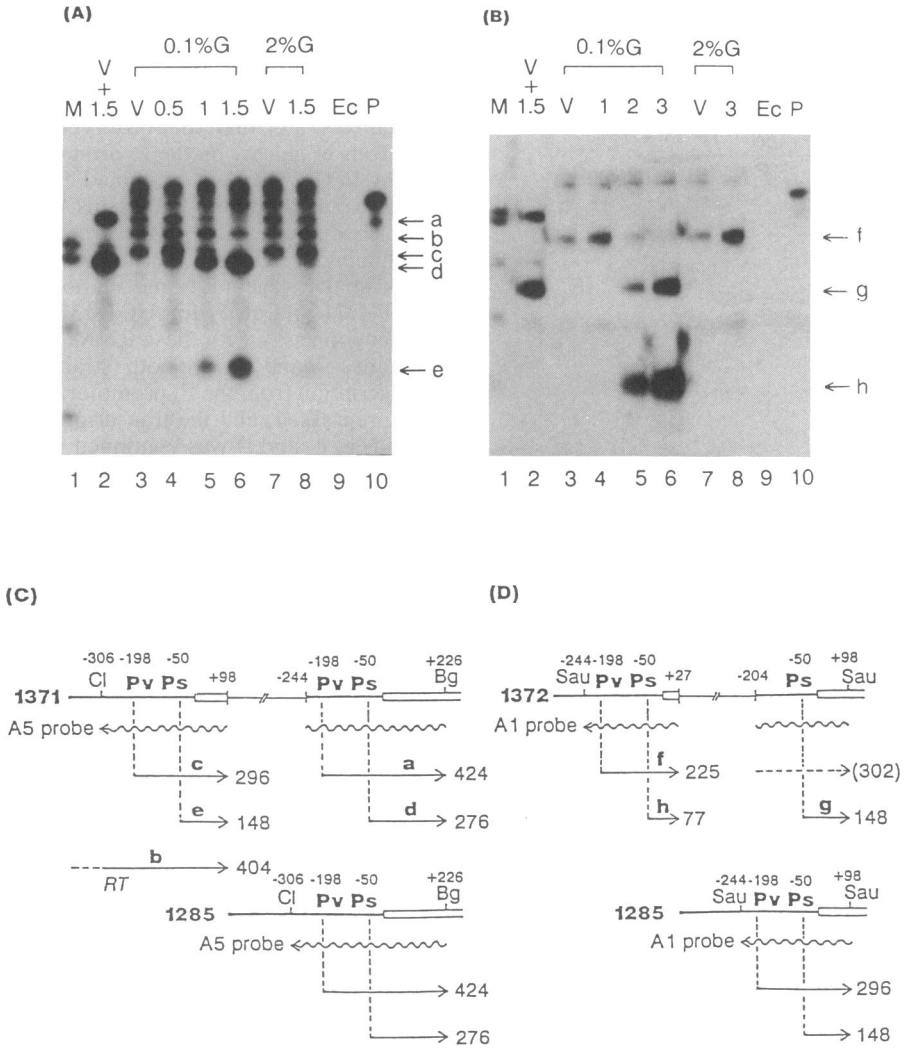

FIG. 7. Mapping of *spo0A* transcripts in strains UOT-1371 and UOT-1372. S1 protection profiles of the A5 probe hybridized with the RNAs extracted from UOT-1371 cells (A) and the A1 probe hybridized with the RNAs extracted from UOT-1372 cells (B) are shown. The RNAs were extracted from cells grown in 2× SG medium containing 0.1% glucose (lanes 3 to 6) or 2% glucose (lanes 7 and 8) at the time points shown above the lanes. Lane 2 in each panel shows the S1 protection profile of each probe hybridized with the mixture of the vegetative-phase (V) and $T_{1.5}$ RNAs extracted from UOT-1285 cells grown in 2× SG medium containing 0.1% glucose. Lanes 1, RNA size markers; lanes 9, negative control with *E. coli* tRNA; lanes 10, diluted sample of each probe. Maps of the S1-protected bands shown in panels A and B are illustrated in panels C and D, respectively. Arrows and numbers to their right indicate the transcripts protected by the probes and their lengths in nucleotides, respectively. For restriction endonuclease abbreviations, see the legend to Fig. 6.

this strain is hypersensitive to glucose. In this strain, *spo0A* expression depends only on P_s-derived transcription, which is fully repressed by 2% glucose as described above. Thus, it was concluded that the hypersensitivity of sporulation to glucose in strain UOT-1372 can be ascribed to the lack of expression of *spo0A* in the presence of an excess of glucose.

Effect of the *crsA47* (*sigA47*) mutation on *spo0A* transcription. We have recently shown that the *crsA47* (*sigA47*) mutation, which is known to be a mutation in the gene coding for σ^A (13), restores the stimulation of *spo0A* expression as well as sporulation in the presence of an excess of glucose (30). We examined the effect of this mutation on *spo0A* transcription by using an S1 protection assay both in the presence and in the absence of an excess of glucose. As shown in Fig. 8, promoter switching from P_v to P_s was restored in this mutant in the presence of 2% glucose (lanes 5 through 8). On the other hand, promoter switching occurred in the presence of 0.1% glucose, but in a different way (lanes 1 through 4). P_v-derived transcription was continued even at T_1, and P_s-derived transcription was already induced at $T_{0.5}$, which is slightly earlier than in the case of the wild type. We also detected another band, which was located between the P_v- and P_s-derived bands, in the autoradiograph. Since this band was not reproducible the other times the same experiment was performed, we regard it as derived from the degradation of the P_v-derived transcription product (data not shown).

DISCUSSION

In this study, we found that the *spo0A* gene was weakly transcribed from a newly identified upstream promoter, P_v, in the vegetative phase. This transcription was repressed after $T_{0.5}$, at which point the downstream promoter, P_s, was

TABLE 1. Effect of glucose on sporulation in strains UOT-1371 and UOT-1372

Strain (relevant genotype)	No. of spores/ml in 2× SG medium containing[a]:	
	0.1% glucose	2% glucose
UOT-1285 (*spo0A*$^+$)	5.6×10^8 (1.00)	2.0×10^6 (0.004)
UOT-1371 (*spo0A*::pCPA1)	3.3×10^8 (0.59)	5.5×10^6 (0.010)
UOT-1372 (*spo0A*::pCPA6)	1.4×10^8 (0.26)	<20 (<3.6×10^{-7})

[a] Numbers in parentheses are ratios of the numbers of spores per milliliter to that of UOT-1285 cells grown in 2× SG medium containing 0.1% glucose.

strongly induced. Thus, it is now clear that the differential expression of the *spo0A* gene can be ascribed to promoter switching from P_v to P_s during the initial stage of sporulation. Some similar examples of transcriptional regulation during the transition from the vegetative phase to the sporulation phase in *B. subtilis* are known. The *spo0F* gene has two promoters, P1 and P2, which share homologies with σ^H- and σ^A-type promoters, respectively (16, 25). The upstream P2 promoter functions only in the vegetative phase. In contrast, the downstream P1 promoter functions in both the vegetative and early sporulation phases, and transcription from P1 is strongly enhanced after T_0 (16). In this case, therefore, switching of the promoters is not as obvious as in the case of *spo0A*. A clear example of promoter switching has been reported for the *rpoD* gene, which encodes σ^A. The *rpoD* gene has three promoters, two of which (P1 and P2, both of the σ^A type) are transcribed only in the vegetative phase and one of which (P3, of the σ^H type) is transcribed only in the sporulation phase (29). The promoter activity of P3, however, is not stronger than those of P1 and P2, which apparently differs from the strong induction of transcription from the *spo0A* P_s promoter.

Our results also indicated the opposite responses of the two *spo0A* promoters to glucose. The P_v promoter is resistant and the P_s promoter is sensitive to an excess of glucose

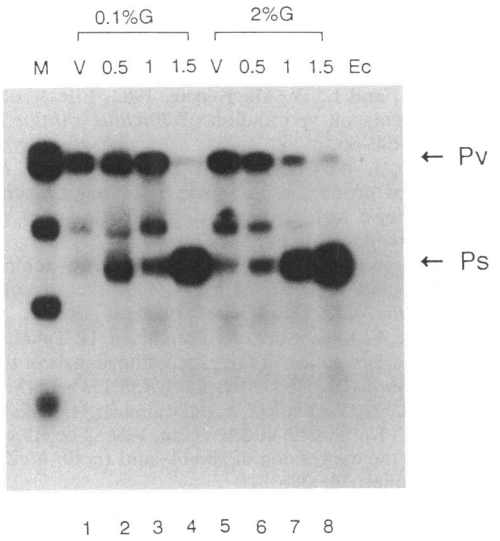

FIG. 8. Effect of glucose on *spo0A* transcription in the *crsA47* (*sigA47*) mutant. S1 protection profiles of the A1 probe hybridized with the RNAs extracted from UOT-1296 cells grown in 2× SG medium containing 0.1% glucose (lanes 1 to 4) or 2% glucose (lanes 5 to 8) are shown. Lane M, RNA size markers.

in the culture medium. This is consistent with our previous data, which showed that the stimulation of *spo0A-lacZ* expression at the initiation of sporulation was repressed, but that a basal-level expression of the fusion in the vegetative phase was not repressed, by an excess of glucose (30). It should be noted that the repression of the P_v promoter after $T_{0.5}$ was reduced by an excess of glucose, which suggests that the P_v promoter may be dependent on glucose. Furthermore, sporulation in wild-type strains was partially repressed to the order of 10^6 spores per ml by an excess of glucose, but when *spo0A* expression depended only on the glucose-sensitive P_s promoter, sporulation was completely repressed (Table 1). Therefore, we suggest that one of the possible biological functions of the glucose-resistant P_v promoter is to ensure the survival of the cells in the event of a drastic change in the environment by supplying an amount of Spo0A protein which is adequate to initiate sporulation at low efficiency. However, we cannot exclude the possibility that the readthrough transcript initiating upstream from the P_v promoter also contributes to the glucose-resistant expression of *spo0A*, since this transcript was detected even in the presence of 2% glucose (Fig. 7A). Like P_v-derived transcription, this readthrough transcription is not necessary for sporulation under normal conditions (e.g., in the presence of 0.1% glucose) because a *spo0A* clone that ends at the *Cla*I site can complement the Spo$^-$ phenotype of a *spo0A* mutant (31).

The P_s promoter shares homology with the consensus sequence for the σ^H promoters. The stimulation of *spo0A-lacZ* expression at the initiation of sporulation depends on the *spo0H* gene product (31), suggesting that σ^H is necessary for the induction of transcription from the P_s promoter. On the other hand, our results shown here indicate that the *crsA47* (*sigA47*) mutation overcomes the glucose repression of the P_s-derived transcription, suggesting that σ^A is also involved in the regulation of P_s induction. The mutant σ^A, however, does not seem to be able to utilize the P_s promoter directly, since P_s-derived transcription was not induced in a *crsA spo0H* double mutant (unpublished data). Therefore, we suggest that the P_s promoter is transcribed by $E\sigma^H$ RNA polymerase. How, then, does the mutant σ^A cause the effect on P_s induction in the presence of an excess of glucose? Since glucose has no effect on *spo0H* expression (25), it is unlikely that the mutant σ^A enhances *spo0H* transcription in the presence of an excess of glucose so that σ^H can induce P_s-derived transcription. In fact, it has been found that σ^H is present and appears to direct transcription from some promoters in vegetative cells (19). However, transcription from the other promoters used by σ^H is induced (or enhanced) after T_0, suggesting that another factor(s) is required for the temporal regulation of these σ^H promoters (19). We propose that such a factor(s), in addition to σ^H, would also be necessary for P_s induction and that the factor(s) might be subject to glucose repression, which is overcome by the *crsA47* (*sigA47*) mutation.

We also suggest that the Spo0A protein itself may play an important role in inducing active transcription from P_s because the delay of the induction in UOT-1372 cells is thought to be due to the lack of *spo0A* transcription in the vegetative phase. This positive effect of the Spo0A protein on P_s induction may be explained by the regulation of the *spo0H* expression by Spo0A. Since the level of *spo0H* expression is extremely low in a *spo0A* deletion mutant (5), the lack of *spo0A* expression in the vegetative phase in UOT-1372 cells would reduce the amount of σ^H to a level which is inadequate to induce P_s-derived transcription effi-

ciently. Alternatively, it is also possible that the Spo0A protein positively regulates the other factor(s) required for efficient P_s induction.

The promoter switching of the *spo0A* gene described here is significant for the purpose of revealing the molecular mechanism of the initiation of sporulation. Our results suggest the possibility that at least two different sigma factors, σ^A and σ^H, and the Spo0A protein itself are involved in the promoter switching of *spo0A* during the early stage of sporulation. However, direct biochemical evidence obtained by using an in vitro transcription system would be necessary to discover their exact functions on the promoter switching of *spo0A*.

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