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# Synthesis of $\sigma^{29}$, an RNA Polymerase Specificity Determinant, Is a Developmentally Regulated Event in *Bacillus subtilis*

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**Using an immunological probe, we have determined that the synthesis of the *Bacillus subtilis* RNA polymerase promoter specificity determinant $\sigma^{29}$ is a developmentally regulated event. $\sigma^{29}$ is absent from vegetatively growing cells but is abundant in sporulating cells for a restricted (2-h) period during differentiation (hour 2 to hour 4 into the sporeforming process). The narrowness of this period suggests that $\sigma^{29}$ is a regulatory factor that directs the transcription of a subpopulation of genes at a precise, intermediate stage of spore formation. This view predicts that $\sigma^{29}$ should be dispensable for early sporulation events. We verified this prediction by an analysis of $\sigma^{29}$ accumulation in mutants that are blocked at different stages of sporulation in which we show that cells can advance to at least an intermediate point in development (stage III) in the absence of detectable $\sigma^{29}$. Lastly, our anti-$\sigma^{29}$ antibody probe detected a second, previously unrecognized protein in *Bacillus* cell extracts that may be a precursor to $\sigma^{29}$. This protein, $P^{31}$ (molecular weight, 31,000) is synthesized earlier in sporulation than is $\sigma^{29}$. It has a peptide profile that is similar to $\sigma^{29}$ and is present in all *Bacillus subtilis* Spo$^-$ mutants that were tested and found to still be able to accumulate $\sigma^{29}$.**

Endospore formation in *Bacillus subtilis* is a simple form of cellular differentiation. In response to nutrient deprivation, the bacterium undergoes a sequential alteration in morphology and physiology, ultimately transforming itself into a new cell form, the dormant spore. The mechanisms by which sporulation genes are activated in the correct sequence to drive the program of differentiation properly are unknown. Although there are over 30 genetic loci whose products are required for successful endospore development, no regulatory factor has as yet been identified as the product of any of these genes (18, 22, 23).

Biochemical studies, in which cloned segments of the *B. subtilis* chromosome were used as templates for in vitro transcription, have implicated RNA polymerase as a possible regulatory molecule in *B. subtilis* gene control. Five forms of *B. subtilis* RNA polymerase have been isolated which transcribe unique populations of cloned vegetative and sporulation genes in vitro. Each enzyme has the subunit composition of the core RNA polymerase ($\beta\beta'\alpha_2$) plus an additional protein (sigma factor) of 55,000 ($\sigma^{55}$), 37,000 ($\sigma^{37}$), 32,000 ($\sigma^{32}$), 29,000 ($\sigma^{29}$), or 28,000 ($\sigma^{28}$) daltons that is responsible for the in vitro promoter specificity of the resulting holoenzyme. $\sigma^{55}$ is the predominant sigma factor that is found in association with the RNA polymerase isolated from vegetatively growing cells (26). The $\sigma^{55}$-containing RNA polymerase enzyme complex (E-$\sigma^{55}$) is likely to direct most of the RNA synthesis during vegetative growth. $\sigma^{37}$, $\sigma^{32}$ and $\sigma^{28}$ are also found in association with vegetative cell RNA polymerase; however, holoenzymes carrying these subunits compose only a minor fraction of the extractable polymerase population (9, 14, 31). In vitro, E-$\sigma^{37}$ and E-$\sigma^{32}$ transcribe several cloned *B. subtilis* genes that are active during the early stages of sporulation (9, 10, 14, 32), whereas two promoters that are uniquely recognized by E-$\sigma^{28}$ in vitro seem to be active during vegetative growth (7). E-$\sigma^{29}$ differs from the preceding RNA polymerase holoenzymes in that this enzyme has been isolated only from cultures of sporulating *B. subtilis* (6, 8, 16). In vitro, $\sigma^{29}$ directs the transcription of several cloned *B. subtilis* genes that are active during sporulation (8). Based on the conditions under which each of these polymerases has been isolated and on the types of genes that they transcribe in vitro, it has been proposed that RNA polymerase modification by sigma factor substitution could be a mechanism for sequential gene activation during sporulation (19). In such a model, vegetative cell sigma factors (e.g., $\sigma^{55}$, $\sigma^{37}$, $\sigma^{32}$, and $\sigma^{28}$) would direct the transcription of genes whose products were required during growth and the early stages of spore development, whereas those genes whose transcription may be required at later times in spore development could be activated by the appearance and joining to RNA polymerase of sporulation-specific sigma factors (e.g. $\sigma^{29}$).

$\sigma^{29}$, by virtue of its presence in sporulating bacteria, is the most likely of the known sigma proteins to be a sporulation-specific transcriptional determinant; however, the necessity of isolating it as a component of an RNA polymerase holoenzyme has limited our ability to analyze the genetic and developmental conditions that influence its synthesis. For example, is $\sigma^{29}$ synthesized only during sporulation or is this protein also present in vegetatively growing bacilli and merely more readily isolated in association with RNA polymerase from extracts of sporulating cultures? Such a preferential isolation does, in fact, occur with $\sigma^{55}$ protein, a protein normally associated with vegetative cell RNA polymerase: $\sigma^{55}$ is present in extracts of sporulating *B. subtilis* (25), but it is inconsistently isolated in association with RNA polymerase from these extracts (reviewed in reference 5).

To analyze the regulation of $\sigma^{29}$ protein synthesis divorced from needing to isolate it as an RNA polymerase component, we have prepared an immunological probe against the $\sigma^{29}$ protein. We have examined extracts prepared from *B. subtilis* at various stages of growth and differentiation with this probe and have determined that $\sigma^{29}$ is present in *B. subtilis* for only a limited period during an intermediate stage in spore development. It is undetectable in vegetatively growing cells. An analysis of the appearance of $\sigma^{29}$ in Spo$^-$ mutants that are blocked at different stages of spore formation revealed both that cells can develop at least to an

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intermediate stage of sporulation (forespore protoplast formation within the spore mother cell) (stage III), in the absence of detectable $\sigma^{29}$ and that the pathway controlling $\sigma^{29}$ accumulation branches from that which controls the simultaneous morphological development.

Lastly, our $\sigma^{29}$-specific probe detected a previously undescribed sporulation-specific protein in crude cell extracts that is a potential precursor to $\sigma^{29}$. This protein (apparent molecular weight [mw], 31,000) $P^{31}$, appears to be structurally related to $\sigma^{29}$ and is regulated in a similar but not identical fashion to $\sigma^{29}$.

## MATERIALS AND METHODS

**Bacterial strains.** All of the *B. subtilis* strains were obtained from the Bacillus Stock Center (Ohio State University), with the exception of strain SMY ($Spo^+$) which was from R. Losick. The strains and their relevant genotypes are listed in the figure legends.

**Growth of bacteria and purification of RNA polymerase.** *B. subtilis* vegetative cells were obtained by growing cells in either DS or LB medium (28) to a cell density that was one-half of the maximum density obtainable in that medium. Sporulating cells were obtained by harvesting cells at the indicated times after they completed exponential growth in DS medium.

RNA polymerase was purified as previously described (8, 26).

**Immunization and production of hybridomas.** BALB/c mice received two intraperitoneal injections 10 days apart of 50 μg of purified RNA polymerase containing $\sigma^{29}$ protein (the first injection was emulsified in Freund complete adjuvant; the second injection was emulsified in Freund incomplete adjuvant). A third intraperitoneal injection of 10 μg of purified RNA polymerase containing $\sigma^{29}$ protein was given 3 days before the mice were killed. Hybridomas were produced by a modified procedure of Oi and Herzenberg (21), with the non-immunoglobulin-secreting SP2/0-Ag14 BALB/c myeloma cell line (27). Supernatants from wells containing proliferating hybridomas were screened for antibody production in two separate enzyme-linked immunosorbent assays (ELISAs) as described by Voller (30). The two ELISA screens differed only in the antigen used: RNA polymerase containing $\sigma^{29}$ protein or RNA polymerase core enzyme. In these assays, 111 of the screened clones produced antibodies that reacted with either core RNA polymerase or RNA polymerase containing $\sigma^{29}$ protein. Hybridomas demonstrating antibody activity exclusive for $\sigma^{29}$ protein were single cell cloned by limiting dilution, and the isotype of the monoclonal antibodies was determined by ELISA.

**Preparation of samples for SDS polyacrylamide gels.** RNA polymerase with and without $\sigma^{29}$ protein was purified as described previously (8, 26). Crude *B. subtilis* extracts were prepared as follows. Cells were harvested at specified time points and washed in 50 mM Tris (pH 8.4)–1 M KCl. The pellet was suspended in buffer I (10 mM Tris [pH 8], 100 mM EDTA, 10 mM MgCl, 100 mM dithiothreitol, 5% [vol/vol] of a phenylmethylsulfonylfluoride solution [6 mg per ml ethanol]). The cells were lysed by passing them twice through a French pressure cell at 15,000 $lb/in^2$. This treatment, as judged by light microscopy, routinely disrupts 98% of the cells and 70% of the forespores that are present at 6 h into sporulation. After clarification by centrifugation at 10,000 × *g* for 30 min, the supernatant was adjusted to 63% saturation with ammonium sulfate. The precipitate was collected by centrifugation at 10,000 × *g* for 30 min, dissolved in buffer I, and dialyzed against two changes of buffer I over a 2-h period. All steps were carried out at 0 to 4°C.

The protein concentration of each extract was determined by the Coomassie method (BioRad Laboratories). Total protein (100 μg) was precipitated with 20% [vol/vol] cold trichloroacetic acid, washed with 0.1 M HCl, and lyophilized. The final pellets were suspended and boiled in 50 μl of sample buffer then electrophoresed on one-dimensional sodium dodecyl sulfate (SDS)-polyacrylamide gels (15). The gels were either stained with Coomassie brilliant blue R or used in protein blotting.

**Binding of protein to nitrocellulose paper and probing with monoclonal antibody.** Purified protein or *B. subtilis* crude extracts fractionated by SDS-polyacrylamide gel electrophoresis were transferred electrophoretically to nitrocellulose paper and probed with monoclonal antibody by using a modification of the method described by Towbin et al. (29). The nitrocellulose blots were either blocked with TSGA-bovine serum albumin-Tween 20 (1) or Blotto (13) at room temperature for 6 h. Blots were then incubated overnight at room temperature with $\sigma^{29}$ monoclonal antibody in blocking buffer. After a brief washing, the blots were incubated with either a 1:3000 dilution of horseradish peroxidase-conjugated goat immunoglobulin G against mouse immunoglobulin (HyClone) or $10^5$ cpm or $^{125}I$-labeled rabbit immunoglobulin G ($1.30 \times 10^6$ cpm per μg of immunoglobulin G, a gift of K. Krolick, University of Texas Health Science Center at San Antonio) against mouse immunoglobulin. Blots reacted with enzyme-conjugated second antibody were developed by the method of Hawkes et al. (12). Blots reacted with $^{125}I$-labeled second antibody were dried and exposed to Kodak X-Omat XAR film at −70°C with an intensifying screen. Quantitation of the amount of $^{125}I$ antibody that bound at the site of a particular protein on the nitrocellulose was accomplished by densitometry. Autoradiographs of different extract concentrations (100 to 400 μg) that were analyzed in parallel were found to give a linear response with increasing extract concentration.

**Peptide analysis of $P^{31}$ and $\sigma^{29}$.** Crude protein extracts (500 μg) of SMY cells that contained either $P^{31}$ (extracts from 1 h into the sporeforming process [$T_1$ extracts]) or 10-fold more $\sigma^{29}$ than $P^{31}$ (extracts from 3 h into the sporeforming process [$T_3$ extracts]) were each incubated with 170 U of *Staphylococcus aureus* $V_8$ protease as described previously (11). The resulting peptide mixtures were separated on SDS-polyacrylamide gels (15 to 20% acrylamide), electrophoretically transferred to nitrocellulose, and probed with monoclonal antibody; the antigen-antibody complexes were visualized with $^{125}I$-labeled rabbit anti-mouse immunoglobulin and autoradiography.

## RESULTS

**Specificity of anti-$\sigma^{29}$ monoclonal antibody.** As described above, monoclonal antibody was prepared by immunizing mice to the $\sigma^{29}$ holoenzyme. After spleen cell-myeloma fusion and selection, ca. 700 hybridoma clones were screened in two separate ELISAs. The ELISA screens differed only in the antigen used: E-$\sigma^{29}$ or RNA polymerase core enzyme (E). A total of 30 clones appeared to react only with E-$\sigma^{29}$ and were further characterized by Western blot analysis (29). Purified RNA polymerase core and E-$\sigma^{29}$ were resolved into their subunit components by SDS-PAGE transferred to nitrocellulose, and probed with the putative anti-$\sigma^{29}$ antibody. Figure 1 illustrates the result obtained with the antibody used in the present study. It is specific for a protein of mw 29,000 that appears only in the E-$\sigma^{29}$ preparation.

To insure that our probe recognized an epitope that was not common to many *B. subtilis* proteins, we reacted the monoclonal antibody to crude cell extracts after the proteins had been electrophoretically separated and transferred onto nitrocellulose. The extracts were prepared either from *B. subtilis* cells that were vegetatively growing in a rich medium which would inhibit sporulation (24) or from *B. subtilis* cells that had been permitted to begin sporulation 2 h earlier. The former extract contains vegetative cell sigma factors, whereas the later extract contains $\sigma^{29}$ (8). We observed that although the monoclonal antibody detected no proteins in the vegetative extract (Fig. 2A), it recognized two proteins of similar size (30,000 mw) in the extract from sporulating cells (Fig. 2B).

To identify which of these proteins was $\sigma^{29}$, we added purified E-$\sigma^{29}$ to a sample of vegetative extract and electrophoresed it in parallel with a portion of the sporulation extract (Fig. 2C). The more rapidly migrating of the two proteins had the mobility of the authentic $\sigma^{29}$ antigen. The more slowly migrating protein has an apparent mw of 31,000 ($P^{31}$). Thus, this monoclonal antibody recognizes a determinant that is absent from vegetative cell proteins and present on only two proteins ($P^{31}$ and $\sigma^{29}$) in sporulating cells. This result not only demonstrates that this monoclonal antibody has a high specificity for $\sigma^{29}$ but also indicates that $\sigma^{29}$ protein is not present in vegetatively growing bacteria.

**Temporal appearance of $\sigma^{29}$ during sporulation.** To examine when $\sigma^{29}$ protein is synthesized and maintained in *B. subtilis*, the organism was grown in medium (DSM) in which the cells begin sporulating at the end of logarithmic growth. We prepared protein extracts from vegetatively growing cells and at hourly intervals as the cells proceeded into sporulation. The formation of heat-resistant spores took ca. 8 h under these culture conditions. Figure 3 depicts a Western blot analysis of these extracts. $T_0$ represents the time point at which logarithmic growth ceases and the sporeforming process commences ($T_1$ represents 1 h into the process, etc.). Neither $\sigma^{29}$ nor $P^{31}$ is present at detectable levels in vegetatively growing *B. subtilis*. Both proteins are however present in sporulating cells, with the appearance of $P^{31}$ preceding that of $\sigma^{29}$.

In an attempt to quantitate the levels of $P^{31}$ and $\sigma^{29}$ antigens that were present in these extracts, we repeated the experiment depicted in Fig. 3 at several concentrations of crude cell extract (100 to 400 μg of extract per lane) and reacted the nitrocellulose-immobilized antigen-antibody

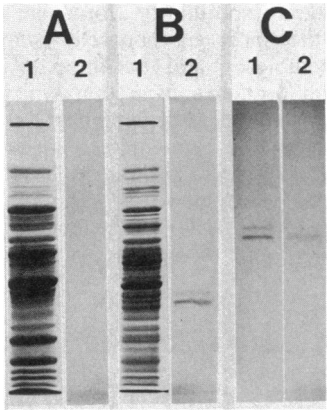


FIG. 2. Specificity of anti-$\sigma^{29}$ monoclonal antibody in probing whole-cell extracts of *B. subtilis*. Protein (100 μg) from crude cell extracts (SMY) (see text) of vegetative (A) or sporulating $T_2$ (B) *B. subtilis* SMY was fractionated in duplicate under denaturing conditions on 12% polyacrylamide gels, either stained with Coomassie blue (lanes 1) or transferred electrophoretically to nitrocellulose (lanes 2) and visualized as in Fig. 1. (C) Lane 1 depicts the $T_2$ extract displayed in B; lane 2 depicts the vegetative extract visualized in A to which 2 μg of E-$\sigma^{29}$ had been added. Both extracts were fractionated, transferred to nitrocellulose, and probed with antibody as in Fig. 1.

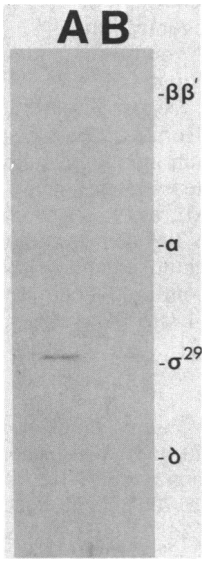


FIG. 1. Western blot analysis of monoclonal antibody that is specific for $\sigma^{29}$ protein. A 1-μg amount of either E-$\sigma^{29}$ (lane A) or core RNA polymerase (lane B) was fractionated under denaturing conditions on an SDS-polyacrylamide gel (12% acrylamide), electrophoretically transferred to nitrocellulose, and reacted with supernatant from a hybridoma demonstrating antibody activity in an ELISA to RNA polymerase containing $\sigma^{29}$ protein. Bound antibody was visualized by a horseradish peroxidase-conjugated goat anti-mouse antibody that catalyzes a colorimetric reaction.

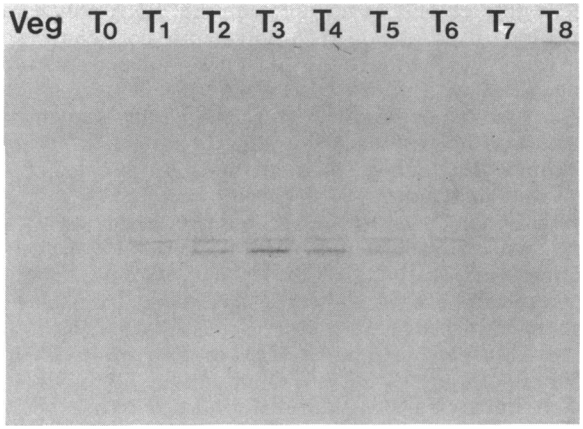


FIG. 3. Time course of accumulation of $P^{31}$ and $\sigma^{29}$. Crude protein extracts (100 μg) from JH642 (Spo$^+$) cells that were gathered during vegetative growth or harvested at hourly intervals during sporulation in DS medium were size fractionated by SDS-PAGE (15% acrylamide), transferred to nitrocellulose, and probed with anti-$\sigma^{29}$ monoclonal antibody. Fixed antibody was visualized as in Fig. 1. The slower and faster migrating bands correspond to $P^{31}$ and $\sigma^{29}$, respectively.

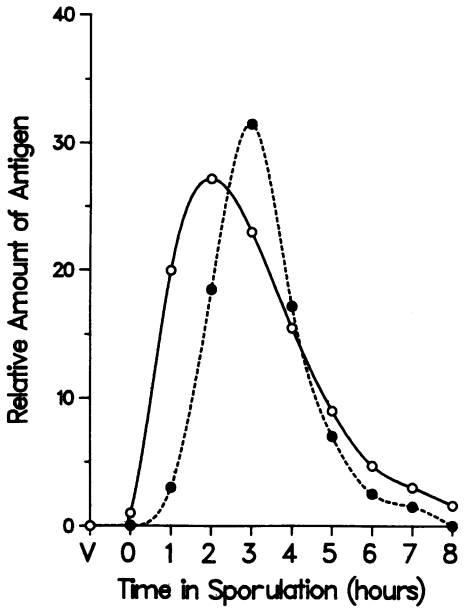


FIG. 4. Quantitation of $P^{31}$ and $\sigma^{29}$ accumulation. Samples of extracts (100 to 400 μg) from JH642 ($Spo^+$) cells that were gathered during vegetative growth or harvested at hourly intervals during sporulation in DS medium, were size fractionated by SDS-PAGE (15% acrylamide), transferred to nitrocellulose, and probed with anti-$\sigma^{29}$ monoclonal antibody. This was followed by incubation with $^{125}$I-rabbit anti-mouse immunoglobulin antibody. The relative amount of $^{125}$I at the positions of the $P^{31}$ (○) and $\sigma^{29}$ (●) bands was determined by autoradiography and densitometry. For the antigen, 1 U represents the amount of $^{125}$I bound at the $P^{31}$ position of a $T_0$ extract. All other extract values are normalized to this unit value.

complexes with $^{125}$I-labeled rabbit anti-mouse antibody. After autoradiography, densitometry was used to estimate the amount of $^{125}$I present at the positions of $P^{31}$ and $\sigma^{29}$. The response was linear with the concentrations of extract and antibody that we employed. We still could not detect $P^{31}$ or $\sigma^{29}$ in vegetatively growing cells. $\sigma^{29}$ was also undetectable in $T_0$ extracts. The smallest amount of antigen that we could measure was in the $P^{31}$ portion of the autoradiograph at $T_0$. We normalized all of our data to this value (i.e., 1 U = the amount of $P^{31}$ at $T_0$). The results of this analysis are given in Fig. 4. $P^{31}$ increased 27-fold from its level at $T_0$ to a peak at $T_2$. $\sigma^{29}$ similarly increased to a peak ($T_3$) that was 31 times the minimum level of antigen detected in our assay. Clearly these proteins must be present in sporulating cells at levels at least 30 times their amount in vegetatively growing cells. In addition, there is a strikingly narrow period during which each of these proteins is maximally present (i.e., $P^{31}$ at $T_1$ to $T_3$ and $\sigma^{29}$ at $T_2$ to $T_4$).

We infer from these results that $P^{31}$ and $\sigma^{29}$ are stringently regulated so that they appear at a precise time after the end of vegetative growth in *B. subtilis*.

**$P^{31}$ and $\sigma^{29}$ synthesis requires *spo0* gene products.** There are at least seven *spo0* loci in *B. subtilis*. These loci are defined by mutations that have little or no overt effect on vegetative growth but arrest spore development at a point before the earliest sporulation-specific morphological event (the formation of an asymmetic septum that divides the precursor spore mother cell from the precursor forespore) (22). This asymmetric cell division is normally concluded by $T_2$ (4). Therefore, the products of the *spo0* genes are likely to be exerting their effects before $T_2$ (the time at which $\sigma^{29}$ appears in the cell). We reasoned that if $\sigma^{29}$ protein synthesis is indeed a developmentally regulated phenomenon and not merely a consequence of cell cultures entering a stationary phase of growth, some of the *spo0* mutants should not be able to synthesize $\sigma^{29}$. Extracts prepared from five stage 0 mutants (*spo0A*, *-0B*, *-0E*, *-0F*, *-0H*) at a time ($T_2$) when $\sigma^{29}$ and $P^{31}$ are present in sporulating cells failed to react with the monoclonal antibody probe (Fig. 5, lanes 1 to 5). The probe did, however, detect these proteins in one of the stage 0 extracts (*spo0J*) (Fig. 5, lane 7). Thus, five of six *B. subtilis* mutations that specifically arrest sporulation inhibit the appearance of $P^{31}$ and $\sigma^{29}$ in cell extracts. We conclude that the synthesis of $P^{31}$ and $\sigma^{29}$ is controlled by sporulation-specific regulatory pathways.

**$\sigma^{29}$ expression in *B. subtilis* mutants blocked at different stages of spore development.** There are a large number of *B. subtilis* mutations that arrest spore formation at particular stages of morphological development (22). Although the functions of the mutant genes and the time in development when their products need to be present for normal differentiation to proceed are unknown, these mutant clones provide an experimental system in which we can ask whether cells proceed past the initiation of spore formation in the absence of $\sigma^{29}$. We assumed that some of these mutant cells have lesions that not only stop differentiation but also either directly or indirectly inhibit the synthesis of $\sigma^{29}$. If such a mutant exists, then the stage to which the mutant becteria differentiate would represent a landmark as to how far into sporulation a cell could develop without $\sigma^{29}$-mediated transcription.

We examined several different stage II, III, and IV mutants for the presence of $\sigma^{29}$ protein. Stage II mutants (*spoII* loci) are able to form the spore septum (normally completed by $T_2$) but are unable to form a detached prespore within the mother cell. Stage III mutants (*spoIII* loci) do form the prespore protoplast (normally completed by $T_{3.5}$) but fail to

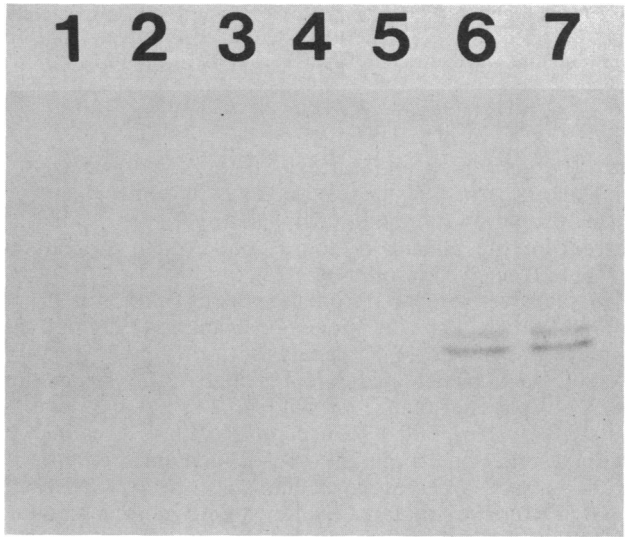


FIG. 5. Analysis of $P^{31}$ and $\sigma^{29}$ accumulation in *B subtilis* stage 0 mutants. Cultures of *B. subtilis* were grown in DS medium and harvested at 2 h after the end of growth phase ($T_2$). Extracts were prepared, and 100-μg samples were analyzed as in Fig. 1. Lane 1, JH646 (*spo0A12*); lane 2, JH648 (*spo0B136*); lane 3, JH647 (*spo0E11*); lane 4, JH649 (*spo0F221*); lane 5, JH651 (*spo0H81*); lane 6, JH696 (*spo0J87*); lane 7, JH642 ($Spo^+$). All strains were isogenic except for the indicated $Spo^-$ mutation.

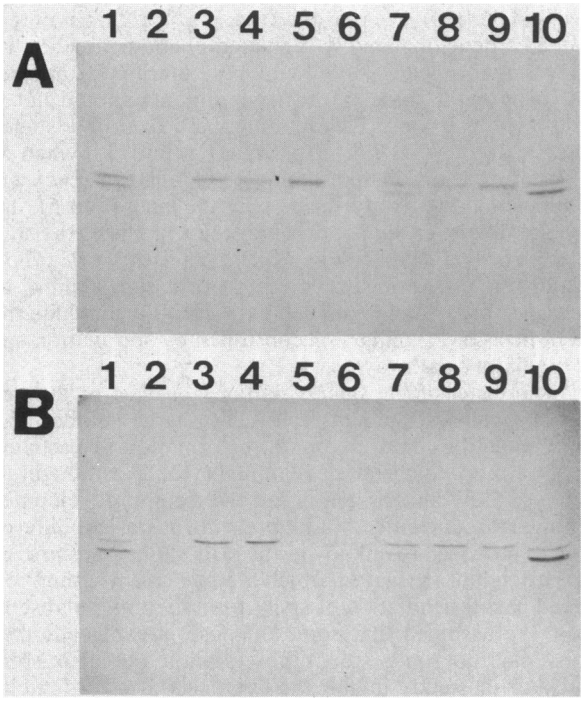


FIG. 6. Analysis of $P^{31}$ and $\sigma^{29}$ accumulation in *B. subtilis* stage II, III, and IV Spo$^-$ mutants. (A) Cultures of *B. subtilis* were grown in DS medium and harvested at 2 h after the end of growth phase ($T_2$). Extracts were prepared and analyzed as in Fig. 3. Lane 1, JH642 (Spo$^+$ control); lane 2, 26u (*spoIIA26*); lane 3, $Z_3$ (*spoIIB131*); lane 4, $P_9$ (*spoIIc298*); lane 5, NG17.15 (*spoIIE64*); lane 6, 7Z (*spoIIIA7*); lane 7, 94u (*spoIIIc94*); lane 8, NG1.67 (*spoIIIE36*); lane 9, $Z_7$ (*spoIVc133*); lane 10, 11T (*spoIVE11*). (B) *B. subtilis* strains described in A that failed to accumulate typical amounts of $\sigma^{29}$ at $T_2$ were cultured as in (A) and harvested at 3.5 h after the end of growth phase ($T_{3.5}$). The extracts were analyzed as in (A). Lane 1, JH642 (Spo$^+$, control) $T_2$; lane 2, 26u (*spoIIA26*) $T_{3.5}$; lanes 3 and 4, NG17.15 (*spoIIE64*) $T_2$ and $T_{3.5}$, respectively; lane 5, 7Z (*spoIIIA7*) $T_{3.5}$; lanes 6 and 7, NG1.67 (*spoIIIE36*) $T_2$ and $T_{3.5}$, respectively, lanes 8 and 9, Z7 (*spoIVC133*) $T_2$ and $T_{3.5}$, respectively; lane 10, *spoIV* mutant with normal $\sigma^{29}$ synthesis at $T_{3.5}$ (*spoIVE11*).

form the germ wall and cortex. Stage IV mutants (*spoIV* loci) allow germ wall deposition and, in some instances, cortex formation (normally completed by $T_{4.5}$ to $T_5$) but do not permit the placing of spore coat protein around the prespore (reviewed in reference 22).

Extracts were prepared from the mutant cultures at $T_2$ and probed with $\sigma^{29}$-specific monoclonal antibody. Figure 6A displays the results of this analysis. Although $P^{31}$ and $\sigma^{29}$ proteins could be detected in representatives of each mutant class, at least one stage II mutant (*spoIIA*) and one stage III mutant (*spoIIIA*) failed to accumulate these proteins. In addition, one stage II mutant (*spoIIE*) contained only $P^{31}$ in its $T_2$ extract, whereas particular stage III (*spoIIIE*) and stage IV (*spoIVC*) mutants had apparently normal amounts of $P^{31}$ but relatively low levels of $\sigma^{29}$ protein.

To determine whether $P^{31}$ or $\sigma^{29}$ synthesis or both were delayed in those mutant cells that displayed anomolous protein patterns in the preceding experiment, we repeated the protocol with extracts prepared from cells at $T_{3.5}$ (Fig. 6B). We observed that those cultures that contained unusually small amounts of $\sigma^{29}$ at $T_2$ (*spoIIIE* and *spoIVC*) only marginally increased their $\sigma^{29}$ content by $T_{3.5}$. Those mutants that failed to accumulate detectable amounts of either $\sigma^{29}$ (*spoIIE*) or both $P^{31}$ and $\sigma^{29}$ (*spoIIA*, *spoIIIA*) at $T_2$ remained negative for the missing proteins at $T_{3.5}$. We conclude that at least one stage II and one stage III mutant lack $\sigma^{29}$ and infer from this that *B. subtilis* can differentiate, at least morphologically, through the formation of the spore protoplast (stage III) in the absence of $\sigma^{29}$-directed transcription.

**Relatedness of $P^{31}$ to $\sigma^{29}$.** The monoclonal antibody used in this study had been prepared with purified E-$\sigma^{29}$ antigen and was found to react with an epitope on the $\sigma^{29}$ protein. It reacts with no other known *B. subtilis* sigma protein, but does react with one additional protein ($P^{31}$) whose synthesis is under sporulation-specific regulation in *B. subtilis*. The specificity of the anti-$\sigma^{29}$ antibody prompted us to perform a preliminary peptide analysis to determine whether $P^{31}$ is related to $\sigma^{29}$ by more than the presence of a single antigenic site. *S. aureus V8* protease digests $\sigma^{29}$ protein into a number of fragments that can be resolved by SDS-PAGE (11). We performed a protease digestion on crude cell extracts that contained either $P^{31}$ protein ($T_1$ extracts) or predominantly $\sigma^{29}$ protein ($T_3$ extracts) and probed the resulting fragments, after electrophoresis and transfer to nitrocellulose, with the anti-$\sigma^{29}$ monoclonal antibody. The protease treatment that we used yielded a mixture of overlapping, partially digested small proteins. A number of these proteins could still carry the antigenic site that was recognized by the monoclonal antibody. The extracts used in this study were isolated from the *B. subtilis* SMY. $P^{31}$ levels in this bacterium fall precipitously after $T_2$ so that extracts prepared at $T_3$ have 10-fold more $\sigma^{29}$ than $P^{31}$. Bound antibody was detected with $^{125}$I-labeled rabbit antimouse antibody and autoradiography. The partial digestions yielded four major and two minor protein bands that had identical mobilities in both extracts (Fig. 7). We conclude that $P^{31}$ and $\sigma^{29}$ have similar peptide substructures.

## DISCUSSION

The occurrence of multiple RNA polymerase specificity determinants (sigma factors) within a single organism poten-

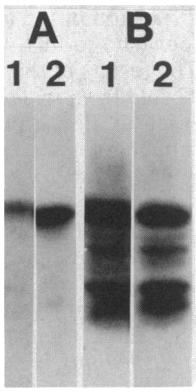


FIG. 7. Peptide analysis of $P^{31}$ and $\sigma^{29}$. Crude cell extracts (500 μg) containing either $P^{31}$ (lanes 1) or greater than 90% $\sigma^{29}$ (lanes 2) were size fractionated by SDS-PAGE (15% acrylamide), transferred to nitrocellulose, and probed with anti-$\sigma^{29}$ monoclonal antibody. Antigen-antibody complexes were detected by reaction with an $^{125}$I-labeled rabbit anti-mouse immunoglobulin antibody followed by visualization by autoradiography. (A) undigested $P^{31}$ and $\sigma^{29}$ extracts (lanes 1 and 2, respectively). (B) $P^{31}$ and $\sigma^{29}$ extracts digested with *S. aureus* $V_8$ protease (170 U per reaction) before size fractionation.

tially represents a powerful control mechanism for gene regulation. The association of one or another of these polymerase subunits with the core transcriptase could activate or suppress a large number of genes by a single regulatory event. This type of gene control would seem to be well adapted to orchestrating the program of gene expression that occurs during differentiation, in which groups of genes appropriate for a particular stage in development are required to be activated coordinately.

We have developed an immunological probe with which we have demonstrated that the *B. subtilis* promoter specificity determinant, $\sigma^{29}$, is apparently synthesized under the control of sporulation-specific regulatory factors: not only does it appear at a precise time during differentiation but its appearance is contingent on the presence of several *spo0* gene products. The strikingly narrow period during which $\sigma^{29}$ is abundant ($T_2$ to $T_4$) argues that it would be a potential vehicle for activating a subpopulation of genes that are required to be expressed at a particular stage of development. As a consequence of $\sigma^{29}$ being present only at an intermediate stage of development, we would predict that, if sigma factor substitution is orchestrating spore gene expression, other sigma-like proteins are likely to be needed to direct transcription of sporulation-specific genes that are activated before or after this period of $\sigma^{29}$ abundance. $\sigma^{37}$ and $\sigma^{32}$ are likely candidates for the transcription of early-activated spore genes; however, no sigma-like protein has as yet been isolated from cells that have progressed to an advanced stage of spore formation.

$\sigma^{29}$ is absent from at least one cell line that is blocked after completion of stage III in spore development. A cell can therefore advance morphologically to that stage (i.e., formation of a forespore protoplast within the mother cell) without $\sigma^{29}$. Protoplast engulfment normally occurs between $T_2$ and $T_{3.5}$ (24), the times during which $\sigma^{29}$ levels are maximal. $\sigma^{29}$-directed transcription is therefore not needed for the overt morphological changes that are occurring at the time at which it is most abundant in the cell. We infer that $\sigma^{29}$ is probably involved in the activation of genes whose products are required for differentiation beyond the stage at which the protein is maximally present (e.g., E-$\sigma^{29}$ could transcribe genes involved in stage IV development). There is precedent for the synthesis of spore-related gene products before their products have a direct effect on differentiation. The spore coat protein, for example, which is deposited around the forespore as a late event in sporulation, has been detected as a large precursor molecule early in development (3). In addition, a number of the *spo0* genes alter the pattern of protein synthesis in vegetative cells although their overt effect is not on vegetative growth but on the initiation of sporulation (2, 17). Alternatively, this particular *spoIIIA* mutant (*spoIIIA7*) may not be blocked as late in development as believed. We have attempted to repeat this experiment with three additional stage III mutants which map at the *spoIIIA* locus (*spoIIIA26*, *spoIIIA59*, and *spoIIIA65* [all from P. Piggot]). In each case, normal amounts of $P^{31}$ and $\sigma^{29}$ were detected (data not shown).

Our analysis of $\sigma^{29}$ accumulation in various mutant backgrounds emphasizes not only the dependence of this protein on the ability of the bacterial cell to proceed into sporulation but also that the sporulation process is not a single pathway of dependent steps leading from vegetative cell to endospore. This follows from the lack of correlation between the morphological stage in development to which a particular mutant advances and the presence or absence of $\sigma^{29}$ protein. At least one mutant cell line with a lesion (*spo0J*) that prevents the earliest sporulation-specific morphological events accumulates $\sigma^{29}$ normally, whereas another mutant (*spoIIIA*) which could proceed to an intermediate stage of spore development fails to display detectable amounts of $\sigma^{29}$. Hence $\sigma^{29}$, which normally appears as the cell is completing stage II of its development and has even been reported to be compartmentalized in the forespore protoplast (20), can accumulate in the absence of the morphological changes that occur before or shortly after its time of synthesis. We infer from this result that the pathway controlling $\sigma^{29}$ accumulation branches from that which controls the morphological progression to stage III, even though these events are occurring simultaneously.

The monoclonal antibody that was used in this study is specific for the sigma factor which elicited its synthesis. It fails to detect any known *B. subtilis* sigma protein except $\sigma^{29}$ in crude cell extracts. Nevertheless, it recognizes a second *B. subtilis* protein, 31,000 molecular weight ($P^{31}$) which, based upon a similar peptide profile with $\sigma^{29}$ after limited proteolysis, is structurally related to $\sigma^{29}$. Both $P^{31}$ and $\sigma^{29}$ appear to be common sporulation-dependent proteins among members of the genus *Bacillus*. Anti-$\sigma^{29}$ antibody detects 31,000- and 29,000-dalton proteins not only in extracts of sporulating *B. subtilis* but also in extracts prepared from *Bacillus amyloliquifaciens*, *Bacillus cereus*, *Bacillus licheniformis*, and *Bacillus natto* (J. Trempy, unpublished data). Although $P^{31}$ and $\sigma^{29}$ both depend upon sporulation-specific regulatory factors for their appearance, $P^{31}$ accumulates earlier in development than $\sigma^{29}$. In addition, there is at least one Spo$^-$ *B. subtilis* mutant (*spoIIE*) which accumulates $P^{31}$ but not $\sigma^{29}$. Presumably the *spoIIE* mutant lacks a critical element in the pathway of $\sigma^{29}$ synthesis.

What is the relationship of $P^{31}$ to $\sigma^{29}$? An obvious possible relationship is that $P^{31}$ and $\sigma^{29}$ are the products of a single gene and that $\sigma^{29}$ is processed from a 31,000-dalton precursor. It could even be argued that $\sigma^{29}$ protein is an artifact produced during extract preparation by a proteolytic activity that accumulates during sporulation and that the physiologically significant sigma factor has a molecular weight of 31,000. We feel that $\sigma^{29}$ is unlikely to be an artifact for several reasons. First, we don't detect a 31,000-dalton sporulation-specific protein in our purified preparations of E-$\sigma^{29}$. If E-$P^{31}$ exists, it must have different properties from E-$\sigma^{29}$. Second, Spo$^-$ mutants of *B. subtilis* that are blocked at the same stage of development can have $P^{31}$ (*spoIIE*) or $P^{31}$ and $\sigma^{29}$ (*spoIIC*). It would have to be argued that the artifact-generating proteolytic activity is present under very restrictive conditions and is not merely a common sporulation-associated protease. Finally, an analysis in our laboratory of the in vivo transcription of six *B. subtilis* DNA segments that are transcribed by E-$\sigma^{29}$ in vitro reveal that RNAs that hybridize to the E-$\sigma^{29}$-transcribed regions are synthesized in a stage II Spo$^-$ mutant that accumulates $\sigma^{29}$ (*spoIIC*), but not in mutants (*spoIIA*, *spoIIE*) that fail to accumulate this protein, regardless of whether $P^{31}$ is present (G. L. Ray, unpublished data). This result implies that $P^{31}$ cannot substitute for $\sigma^{29}$ in vivo. If $\sigma^{29}$ is processed from $P^{31}$, we believe that this processing has biological relevance and is likely to be a novel form of gene regulation in *Bacillus* spp. There are, however, alternative possibilities. $P^{31}$ and $\sigma^{29}$ may, for example, be distinct sporulation-specific proteins which based on their structural relatedness have similar functions (i.e., $P^{31}$ is a previously undescribed sigma factor with a unique resemblance to $\sigma^{29}$). The ultimate explanation of the relationship of $P^{31}$ to $\sigma^{29}$ will come only after the structural genes that code for these proteins have been

cloned and analyzed. We are currently attempting to use our $\sigma^{29}$-specific immunological probes toward this end.

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