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Transcriptional Control of Synthesis of Acid-Soluble Proteins in Sporulating *Bacillus subtilis*

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The major acid-soluble spore proteins (ASSPs) isolated from mature spores of *Bacillus subtilis* are designated α, β, and γ (about 60, 60, and 100 amino acids in length, respectively). α and β are very similar, and γ is very similar to a less predominant ASSP called δ (about 115 amino acids). A minor and very basic ASSP called ε is the same size as α and β but is unrelated antigenically. These and several minor ASSPs comprise at least three related families of sporulation-specific gene products. Expression of the α and β genes, detectable as functional mRNA in vitro, coincides with the time of synthesis of all of the major ASSPs in vivo. This apparently coordinate expression is dependent on at least the *spo0A*, *spoIIA*, and *spoIIIA* loci, but not on the *spoIVA* or *spoVA* loci, consistent with the late stage of this expression (initiating at 3.5 h after the start of sporulation and peaking at 5 h after start of sporulation). A few minor ASSPs may be asynchronously expressed.

Sporulation of bacilli such as *Bacillus megaterium*, *Bacillus subtilis*, and *Bacillus cereus* is accompanied by the synthesis of acid-soluble spore proteins (ASSPs; 13, 19). Those best characterized are the A, B, and C ASSPs of *B. megaterium*, which have been sequenced and which are 61, 96, and 71 amino acids long, respectively (14–16). A and C are closely related to each other, but not to B. These ASSPs and the related ASSPs of *B. subtilis* spores have been shown to be synthesized during sporulation, stored in the mature spore, rapidly degraded to free amino acids during germination, and reutilized for protein synthesis (7, 12, 13).

Extraction of ASSPs from dry-broken spores of *B. subtilis* strain 168 or SMY with 3% acetic acid produces a mixture of α, β, and γ proteins (approximately 60, 60, and 100 amino acids in length, respectively; 7). We have recently observed (unpublished data) that the N-terminal 38 amino acids of α and β are almost identical and share 27 residues with the *B. megaterium* C protein. α, β, A, and C are clearly components of a highly conserved family of ASSPs. Cloning of the C protein gene (3) and its use as a probe for related sequences (2) has demonstrated that, at least in *B. megaterium*, this family includes a group of related genes resembling, in complexity and function, those for the storage proteins of plant seeds (11).

Rupture of intact *B. subtilis* spores in 2 N HCl or extraction of dry-broken spores with 0.2 to 2 N HCl produces several minor ASSPs in addition to α, β, and γ (7). The most prominent of these is δ, which migrates on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) more slowly than γ and has an estimated size of 115 amino acids. Under all extraction conditions, γ remains the most prominent ASSP. We have recently found (unpublished data) that γ and δ have identical N-terminal sequences (37 residues). Since an oligonucleotide probe based on the common γ and δ sequence detects a single fragment in *B. subtilis* DNA hydrolyzed with several restriction endonucleases (unpublished data), it seems likely that both γ and δ derive from a single gene or two closely linked genes. If a single gene is involved, γ is derived from δ, either in vivo or during isolation.

α, β, and γ have pI values of 6.58, 6.67, and 7.96, respectively (7), and δ is similar in charge to γ. Besides α, β, γ, and δ, extraction of spores with 2 N HCl also produces a number of more basic minor ASSPs. The most prominent and basic of these we have called ε. In this paper, we present preliminary biochemical and immunochemical characterization of δ and ε, demonstrate the presence in sporulating *B. subtilis* cells of mRNAs encoding α, β, and γ, and show that during sporulation the specific activities of α and β mRNAs and the rates of synthesis of the major ASSPs increase in parallel. This is consistent with coordinate transcriptional control of ASSP gene expression. The kinetics of synthesis of some minor, uncharacterized ASSPs appears to overlap but not coincide with that of the major ASSPs. These results confirm and extend the similar results obtained in *B. megaterium* (4).

The absence of production of any detectable ASSP or mRNA coding for α and β (αβ mRNA) in several *B. subtilis spo* mutants demonstrates that synthesis of all ASSPs is part of the sequence of sporulation-specific events under control of early *spo* genes, as previously demonstrated by Mason and Setlow (10) for in vivo-synthesized α and β proteins in similar mutants.

MATERIALS AND METHODS

Bacterial strains and media. *B. subtilis* 168 *trpC2* and *B. subtilis* SMY, a wild-type Marburg strain, were the strains previously used for ASSP production (7). The *spo* mutants were obtained from the Bacillus stock center except for the *spoVA89* mutant, which came from J. Mandelstam. Strains were grown and sporulated (by glucose exhaustion) in MSM medium (7) at 37°C (250 ml per 4.2-liter Fernbach flask). Growth was followed by densitometry using a Klett colorimeter (green filter). The start of sporulation was assumed to be the time at which logarithmic growth (with a doubling time of 23 to 25 min) ceased abruptly, followed by a period of linear increase in culture density. Subsequent hours of sporulation at 37°C are called t_1, t_2, etc. Under these conditions, spore septum formation, separating the progen-

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itors of the mother cell and forespore compartments, occurs at $t_{2.5}$ (7).

Spore preparation and ASSP extraction. Mature spores were harvested from MSM cultures at t_{20}, cleaned, and dry-broken as previously described (7). A modified acid extraction procedure gave more reproducible yields of ASSPs, as follows. The fragmented spores and glass beads were twice extracted for 20 min at 0°C with 250 ml of either 3% (0.52 M) acetic acid, 2 N HCl, or acetic acid followed by HCl. HCl extracts were made 3% in acetic acid and neutralized at 0°C to pH 2 to 3 by the slow addition of 6 N NaOH with rapid stirring. After brief dialysis (three times for 3 h each) in Spectrapor no. 3 dialysis tubing (molecular weight cutoff, 3,500) at 4°C against 3% acetic acid, the material retained was concentrated to about 100 ml by lyophilization and desalted by fractionation on a Sephadex G50 column (500-ml bed volume) in 3% acetic acid.

Alternatively, spores were directly ruptured by suspension in 2 N HCl (5 mg/ml) at 20°C for 25 min, and the extracted ASSPs were desalted as described above.

Recovery of ASSPs from sporulating cells. At suitable intervals, phenylmethylsulfonyl fluoride and sodium EDTA were added to sporulating cultures to final concentrations of 3 and 5 mM, respectively. Cells were immediately poured over crushed ice, centrifuged, washed once in 100 mM sodium phosphate buffer (pH 6.8) containing phenylmethylsulfonyl fluoride and EDTA, and lyophilized. Dry cells (150 mg) were ruptured in a Wig-L-Bug dental amalgamator (13) and extracted twice with 3% acetic acid, 2 N HCl, or acetic acid followed by HCl (7).

In vivo labeling of ASSPs. (i) Pulse-harvest. Samples (10 ml) of a culture (150 ml) of *B. subtilis* 168 growing in MSM at 37°C were distributed in midexponential-growth phase into prewarmed conical flasks (125 ml) which continued to incubate at 37°C. At appropriate intervals, a single flask was selected, samples (0.1 ml) were removed for assay of heat and UV resistance, and L-[^{3}H]valine (50 μCi) was added. After 10 min, samples (0.5 ml) were removed for analysis of the rate of labeling of total protein, and ASSPs were immediately isolated from the cells as described above.

(ii) Pulse-chase-harvest. Alternatively, nonradioactive L-valine was added at the end of the 10-min labeling period to a final concentration of 5 mM, a 500-fold excess over the [^{3}H]valine. Kinetic data (not shown) demonstrated that this addition effectively prevented further incorporation of radioactivity. Spores were isolated at t_{20}, and ASSPs were isolated by rupture in 2 N HCl. Valine (5 mM), when added early in sporulation, delayed the appearance of phase-white spores by about 15 min without affecting efficiency or synchrony of sporulation. No effect was seen if addition was made after t_4.

(iii) Continuous label, harvest. Alternatively, labeled cultures were incubated to t_{20} without the addition of cold valine, and ASSPs were extracted as described above.

Analyses of labeled cells and ASSPs. Incorporation of total radioactivity was measured by precipitating samples of labeled cultures (0.1 ml) with trichloroacetic acid. We have previously demonstrated (6) that the pulsed rate (10 min) of L-[^{35}S]methionine incorporation in *B. subtilis* 168 cells, sporulating in MSM, is a close approximation of the rate of total protein synthesis, since equilibration with internal pools is apparently rapid at all stages. Using cultures (10 ml) labeled for 10 min, it was found that the efficiency of valine incorporation, relative to methionine incorporation, increased 2.5-fold over the period from t_2 to $t_{4.5}$ and then remained constant. Rates of ASSP synthesis, based on rates of [^{3}H]valine incorporation, have been corrected for this discrepancy.

The specific activity of total ASSPs is defined as the ratio of corrected [^{3}H]valine incorporation to total protein in acetic acid extracts of dry-broken sporulating cells. Accumulated total and individual ASSP contents were determined by fractionation of samples of ASSP extracts, corresponding to constant culture volumes, by SDS-PAGE, staining with Coomassie brilliant blue, and densitometry.

^{3}H and ^{35}S label incorporation into individual ASSPs was determined by SDS-PAGE of ASSP extracts of labeled cells, fluorography, and densitometry. Samples with equal total radioactivity were fractionated. Fluorgraphic exposures were adjusted to give responses in the linear range for the film (preflashed Kodak X-AR).

Resistance to heat and UV. To measure heat-resistant colony-forming units, culture samples (0.5 ml) were heated in covered tubes for 20 min at 80°C, and appropriate dilutions were plated on Difco tryptose blood agar base medium and counted after overnight incubation at 37°C.

To measure resistance to UV irradiation (UV^r), 0.1-ml culture samples diluted with 0.9 ml of 0.15 M saline were irradiated for 1 min at 30 cm with a General Electric model μ bactericidal light. Residual colony-forming units were determined by diluting and plating as described above.

Column chromatography. ASSPs were fractionated on carboxymethyl cellulose in Tris-maleate buffer (pH 5.6) (5 mM) with a linear gradient of increasing NaCl concentration to 0.35 M, as previously described (7). Phosphocellulose columns were eluted in the same buffer in a gradient of 0 to 1.8 M NaCl.

Gel electrophoresis. ASSPs were fractionated by SDS-PAGE using a 7.5 to 15% linear acrylamide gradient or by electrophoresis in 6 M urea at pH 4.7 (7) or in aluminum lactate buffer at pH 3.6, and detected in each case by staining with Coomassie brilliant blue.

Antisera and immunoprecipitations. γ and an approximately equimolar mixture of α and β were purified as previously described (7). Samples of 5 mg of α+β and 5 mg of γ (0.8 and 0.4 nmol, respectively) were separately cross-linked in 1 ml of 100 mM sodium phosphate buffer (pH 7.0) for 90 min at 23°C with a 50-fold molar excess of glutaraldehyde. After addition of 500 mM lysine (1 ml), the mixture was dialyzed at 4°C for 16 h against 100 mM sodium phosphate (pH 7). Fractionation of samples by SDS-PAGE and staining with Coomassie blue revealed a series of oligomers for each protein.

New Zealand white rabbits were inoculated with 1.25 mg of polymerized protein in complete Freund adjuvant. After 30 days, they were boosted with 0.7 mg of polymerized protein in Freund incomplete adjuvant. Blood samples (30 to 50 ml) were collected by cardiac puncture 7 and 14 days after the boost. One month later, rabbits were again boosted and bled as above. Immunoglobulin G (IgG) fractions were obtained from total serum by precipitation with 33% saturated ammonium sulfate and fractionation on DEAE and carboxymethyl cellulose (5). Quantitative radioimmunoassay demonstrated that 7 mol of anti-α+β IgG was required to bind 1 mol of α+β labeled with *p*-[^{125}I]hydroxymethylbenzimidate hydrochloride (18), while 45 mol of anti-γ IgG was required to bind 1 mol of similarly labeled γ.

For immunoprecipitations, translation products (50 μl) or samples of labeled cell extracts (100 μl, containing maximally 10 pmol of α+β or γ) were incubated with anti-α+β IgG or anti-γ IgG (0.5 and 2 mg/ml, respectively; 85 μl) in 1 ml of buffer I (50 mM KPO_4, 150 mM NaCl, 2 mM EDTA,

pH 7.4, containing 2 mg of bovine serum albumin per ml) for 30 min at 20°C. The immune complexes were then bound to fixed, washed *Staphylococcus aureus* Cowan I cells (100 μl of a 10% suspension) for 15 min at 0°C. The *S. aureus* cells plus bound immune complexes were washed at 0°C in buffer I plus 0.5% Triton X-100 containing 500 mM NaCl and then twice with the same buffer containing 150 mM NaCl. For samples labeled with [^{35}S]methionine, the wash buffers contained 5 mM L-methionine. For samples labeled with [^{3}H]valine, wash buffers contained 5 mM L-valine. Bound proteins were eluted by boiling in 2% SDS for 3 min, fractionated by PAGE either in the presence of SDS or at pH 4.7 after precipitation with 9 volumes of acetone to remove SDS, and finally detected by fluorography.

RNA isolation and translation. Vegetative cells, or cells from synchronously sporulating cultures, were chilled rapidly by being poured over an equal volume of crushed ice plus sufficient $MgSO_4$ and NaN_3 to give final concentrations of 10 mM and 20 mM, respectively. Cells were harvested rapidly in a Sorvall GSA rotor (7 min at 7,000 rpm) or, for larger cultures, by passage at 0.5 to 1 liter/min through a Sharples centrifuge. Cell pellets were immediately suspended in 5 ml of *m*-cresol per liter of original culture and frozen at −80°C. Partially thawed suspensions were ruptured in a French pressure cell, and RNA was isolated (8). Translations using L-[^{35}S]methionine label or a mixture of ^{3}H-amino acids were performed using an *Escherichia coli* S30 system (1). The tritiated amino acids used were L-leucine, L-tyrosine, L-alanine, and L-phenylalanine at specific activities of 120, 78, 83, and 102 Ci/mmol, respectively. Concentrations of Mg^{2+} and K^{+} were optimized for each S30 preparation to give maximum incorporation into identifiable proteins. Concentration of *B. subtilis* vegetative- and sporulating-cell RNA preparations were adjusted to give maximal incorporation into protein species of interest. RNA recoveries were reproducible, and amounts of RNA corresponding to equal culture volumes and giving maximal incorporation into α+β in the t_5 sample were used to compare αβ mRNA specific activity as a function of time in sporulation. Products were fractionated by SDS-PAGE, with or without immunoprecipitation, and detected by fluorography (17).

RESULTS

Fractionation and characterization of δ and ε. Dry-broken spores of strains 168 and SMY, extracted with 3% acetic acid, produce ASSPs consisting almost entirely of α+β and γ (7). Extraction with 2 N HCl alone, or a combination of extracts made sequentially with 3% acetic acid and 2 N HCl, gives an ASSP pattern in which γ and α+β still predominate, but which also contains a substantial quantity of δ, a slower-moving species (estimated M_r, 12,700; 115 amino acids), and a reproducible pattern of minor bands migrating between γ and α+β (7) (Fig. 1A, lane 1). When such a mixture was fractionated on carboxymethyl cellulose, α+β eluted in the wash-through fraction (Fig. 1A, lanes 4 and 5), while δ eluted near the start of the salt gradient (Fig. 1B, lanes 2 through 5). γ eluted later, overlapping δ (Fig. 1B, lane 6), and was followed by the minor species. Thus this simple procedure gave electrophoretically pure δ. Pure γ is obtained by carboxymethyl cellulose chromatography of ASSPs extracted with acetic acid alone (7).

The most predominant of the minor ASSPs was also the last ASSP eluted from carboxymethyl cellulose. It had the same mobility on SDS-PAGE as α+β and was called ε (Fig. 1B, lanes 8 through 11). On phosphocellulose, all ASSPs

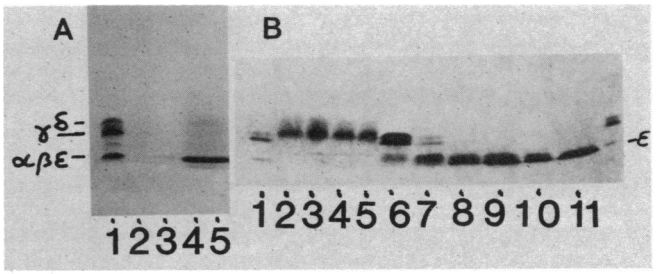

FIG. 1. Fractionation of *B. subtilis* ASSPs on carboxymethyl cellulose. Samples were fractionated by SDS-PAGE on 7.5 to 15% gradient gels and detected by staining with Coomassie brilliant blue. (A) Flow-through fractions. Lane 1, Mixture of ASSPs from acid rupture of *B. subtilis* 168 spores in 2 N HCl, as applied to the column; lanes 2, 3, 4, and 5, sequential fractions eluting in the starting buffer (5 mM Tris-maleate, pH 5.6). (B) Fractions eluted with a linearly increasing gradient of NaCl in the starting buffer. Lane 1, Standard mixture of α, β, γ, and δ ASSPs. A second sample is shown to the right of lane 11. Lanes 2 to 5 correspond to alternating fractions from 10 to 20, covering a broad protein peak (δ). Lane 6 is from fraction 26, containing a sharper peak (mostly δ+γ). Lanes 7 through 11 are from fractions 28, 30, 32, 34, and 36, respectively. The component in these fractions comigrating with α and β is ε.

bound in the 5 mM, pH 5.6 loading buffer, and the order of elution was, again, α+β (free of all other ASSPs but unfractionated from each other) followed by δ, a mixture of δ plus γ, and finally the minor ASSPs (data not shown).

Electrophoresis at pH 3.6 fractionated α from β, while γ migrated a little faster than β and δ overlapped both β and γ (Fig. 2A, lanes 3, 4, and 5). The ε fraction from Fig. 1B, lane 11, migrated at pH 3.6 with lysozyme (not shown) and corresponds to the most predominant minor ASSP seen in Fig. 2A, lane 3.

α, β, and ε were also distinguished from one another by electrophoresis at pH 4.7 (7). ASSPs were extracted with 2 N HCl from spores of strains 168 and SMY which had been labeled during sporulation with L-[^{35}S]methionine and fractionated on carboxymethyl cellulose. A mixture of the wash-through and low salt-eluted fractions, consisting mostly of γ+δ (approximately 80% of the total) plus α and β (20% of the total), was fractionated at pH 4.7, and the labeled species were detected by fluorography (Fig. 3, lanes 1 and 2). Neither γ nor δ was labeled with methionine. Their positions, detected by staining, were as indicated. α and β are barely resolved by this gel procedure, which gives rather broad bands. Strain SMY extracts consistently contained a lower ratio of β to α than did extracts of strain 168. Methionine-labeled α and β were also separated at pH 3.6 (Fig. 2B, lane 2).

The fraction of strain SMY ASSPs eluted with high salt from carboxymethyl cellulose is shown in Fig. 3, lane 3. It consisted mainly of ε, which was shown by staining to coincide with the predominant labeled spot. Thus ε contains methionine, like α and β, but is very basic. It migrated slightly faster than lysozyme at pH 4.7. pI values estimated by isoelectric focusing (7) for δ and ε were 7.4 and >9.8, respectively.

Cross-reactivity of antisera to α+β and γ. Samples of α+β (the approximately equimolar mixture from strain 168 spores) and γ (also from strain 168 spores), each free of any contamination by the other that would be detectable by SDS-PAGE, were polymerized with glutaraldehyde and used to raise rabbit antisera. Neither whole sera nor purified IgG fractions gave precipitation bands against native ASSPs

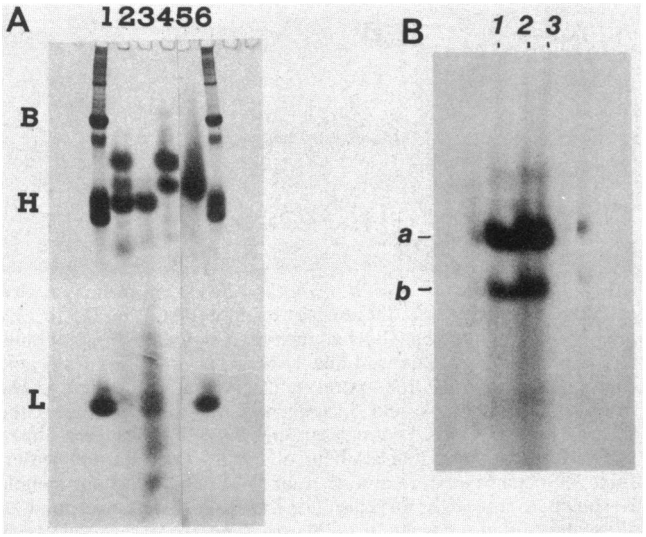

FIG. 2. Fractionation of *B. subtilis* ASSPs by electrophoresis at pH 3.6. (A) Lanes 1 and 6 contain marker proteins which, in order of increasing mobility, are bovine serum albumin (B), hemoglobin (H), and lysozyme (L). Lane 2, Total acetic acid extract of dry-broken *B. subtilis* spores comprising α, β, and γ plus traces of more basic ASSPs. Lane 3, Preparation of γ purified from an HCl extract of dry-broken spores by carboxymethyl cellulose chromatography. It contains a higher proportion of basic ASSPs including ε, the component migrating with lysozyme. Lane 4, Flow-through from carboxymethyl cellulose chromatography, consisting of almost pure α (upper band) + β (lower band). Lane 5, Purified δ, fractionated from an HCl extract of *B. subtilis* spores (see Fig. 1). It overlaps β in this electrophoretic system. (B) ASSPs were isolated by acetic acid extraction from dry-broken *B. subtilis* SMY spores, labeled during sporulation with L-[^{35}S]methionine. A fluorogram of samples fractionated by electrophoresis at pH 3.6 is shown: lane 2, total extracted ASSPs; lane 3, the fraction precipitated by anti-α+β IgG; lane 1, translation products of t_5 RNA precipitated by anti-α+β IgG. The positions of α (a) and β (b), as detected by staining with Coomassie blue, are as indicated.

in an Ouchterlony double-diffusion assay. However, the antisera did form specific complexes which bound to the protein A-rich cells of *S. aureus* Cowan I.

Immunocompetition (Fig. 4), using constant amounts (0.24 μg) of ^{125}I-α+β or ^{125}I-γ and sufficient homologous antiserum to bind half of the antigen, showed that a 50% reduction in binding of ^{125}I-α+β by anti-α+β required 0.8 μg of α+β or 10 to 15 μg of γ. A 50% reduction in binding of ^{125}I-γ by anti-γ required 0.5 μg of γ or 5 μg of α+β. Thus cross-reactive binding with each antiserum was 5 to 10% of the efficiency of binding of the homologous antigen. Gel electrophoresis of immunoprecipitates demonstrated that this cross-reactivity was not due to cross-contamination of the iodinated antigen preparations; however, cross-contamination of the eliciting antigen preparations, below the level of detectability by SDS-PAGE, cannot be ruled out. α and β, labeled in vivo with [^{35}S]methionine, were both efficiently bound by anti-α+β IgG (Fig. 2B, lane 3).

Neither anti-α+β nor anti-γ bound ε, although both bound both α and β and [^{3}H]valine-labeled γ and δ (data not shown).

Detection of mRNA for α and β. Total RNA was prepared (8) from *B. subtilis* cells in late exponential growth phase and at t_5 of sporulation. Translation in an *E. coli* S30 system, using L-[^{35}S]methionine, fractionation by SDS-PAGE, and fluorography, gave the results shown in Fig. 5 (lanes 2 and 3). A distinct band comigrating with α and β was seen, but only in the t_5 products, where it accounted for approximately 7% of the total ^{35}S incorporated, if the prominent band of endogenous product migrating just above it is ignored. The characteristic products of translation of phage MS2 RNA are shown in lane 4. The α+β band was bound by anti-α+β serum (lanes 10 and 12), but not by preimmune serum (lane 7). Addition of 1 μg of competitor α+β (lanes 11 and 13) prevented binding of the in vitro product, demonstrating the specificity of this binding.

Fractionation of the immunoprecipitate at pH 3.6 demonstrated that both α and β were synthesized and precipitated (Fig. 2B, lane 1). Fractionation of total translation products at pH 4.7 failed to detect synthesis of ε (data not shown).

Detection of mRNA for γ. Since neither γ nor δ is labeled in vivo with methionine (Fig. 3), it was anticipated that this would also be true in vitro. No band comigrating with γ was seen in the total or immunoprecipitated [^{35}S]methionine-labeled translation products of t_5 RNA (Fig. 5, lanes 3, 10, and 12). However, if the in vitro products were labeled with a ^{3}H-amino acid mix, the products of precipitation with anti-α+β IgG (Fig. 6, lane 6) or anti-γ IgG (Fig. 6, lane 9) contained a relatively faint species comigrating with γ, in addition to a strong band of α+β. No δ production was

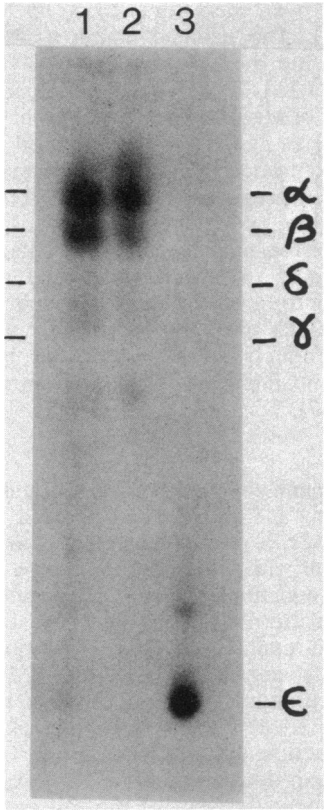

FIG. 3. Fractionation of [^{35}S]methionine-labeled ASSPs at pH 4.7. ASSP preparations from cells of strains SMY and 168, continuously labeled with [^{35}S]methionine from $t_{2.5}$ to t_{24}, were fractionated on carboxymethyl cellulose. Fractions were analyzed by electrophoresis at pH 4.7 and detected by fluorography. Lanes 1 and 2 show the combined flow-through and low salt-eluted fractions (α, β, γ, and δ) from strain 168 and SMY spores, respectively. Lane 3 shows the high-salt eluate from strain SMY spores. The predominant methionine-labeled spot in lane 3 comigrated with ε, the only ASSP detected in this lane by staining with Coomassie blue.

visible, and no γ band was visible in the products of translation of vegetative RNA (Fig. 6, lanes 5 and 8). Preimmune serum or IgG derived from antiserum against a *B. subtilis* coat protein failed to bind γ (Fig. 6, lanes 3 and 18). The specificity of precipitation of both α+β and γ by both anti-α+β and anti-γ was shown by immunocompetition with 1 μg of α+β and γ (Fig. 6, lanes 12 and 15).

Thus RNA recovered from t_5 cells contains a high level of mRNA for α+β and a much weaker level of detectable mRNA for γ. mRNAs for δ or ε could not be detected. Although a heterologous (*E. coli*) system was used for translation, this seems unlikely to be a cause for discrimination. Similar results, at least for α+β, have been obtained using a homologous system (9). Precipitation of labeled γ by anti-γ appears to be more efficient than precipitation by anti-α+β (compare Fig. 6, lanes 6 and 9). These data confirm both the identities of the in vitro products and the cross-reactivity of anti-α+β and anti-γ sera against α+β and γ.

Kinetics of accumulation of ASSPs and other spore characteristics. Samples were withdrawn at intervals from a culture of *B. subtilis* 168 sporulating in MSM at 37°C and were assayed for cell density, resistance to heat and UV, and percentage of phase-white spores (Fig. 7). A portion of each sample was labeled with L-[^{3}H]valine for 10 min at 37°C, and ASSPs were extracted with acetic acid after dry-breakage. The specific activities of the total ASSP extracts (corrected for labeling efficiency; see above) and their contents of α+β and γ, relative to those of the t_{20} sample, are also shown in Fig. 7. α, β, and γ accumulated in parallel, so that a single set of data points (ASSPs accumulated) is shown.

The corrected specific activity of [^{3}H]valine in ASSPs was barely above background at t_3, but peaked at t_4, declined about 30%, and then remained roughly constant between $t_{4.5}$ and t_7 before declining again. Accumulation of α, β, and γ was not detectable until $t_{4.5}$ and occurred almost exactly parallel to appearance of phase-white spores, being half maximal at t_6. Acquisition of UV and heat resistance started at t_6 and t_7, respectively, being half maximal at about $t_{6.5}$ and $t_{7.5}$, distinctly after 50% accumulation of ASSPs (t_6) and well after ASSP synthesis commenced.

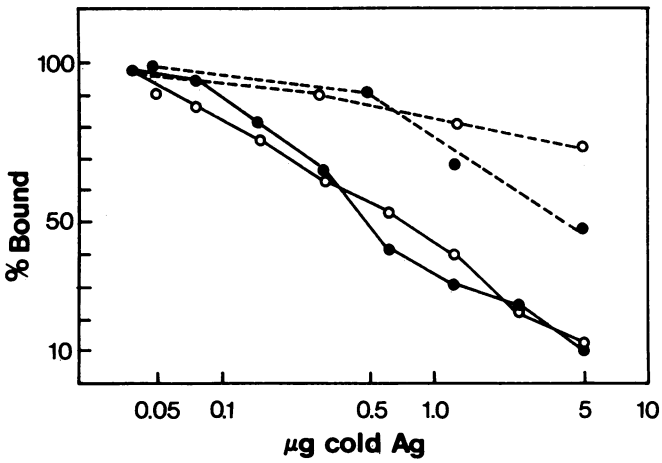

FIG. 4. Specificity of anti-α+β and anti-γ IgG preparations. ^{125}I-labeled α+β (0.24 μg) was incubated with sufficient anti-α+β IgG (30 μg) to bind half of the label to *S. aureus* Cowan I cells. O——O, Competition for binding by unlabeled α+β; O- - -O, competition for binding by unlabeled γ. ^{125}I-labeled γ (0.24 μg) was similarly incubated with sufficient anti-γ IgG (200 μg) to bind half of the label. ●——●, Competition for binding by unlabeled γ; ●- - -●, competition for binding by unlabeled α+β.

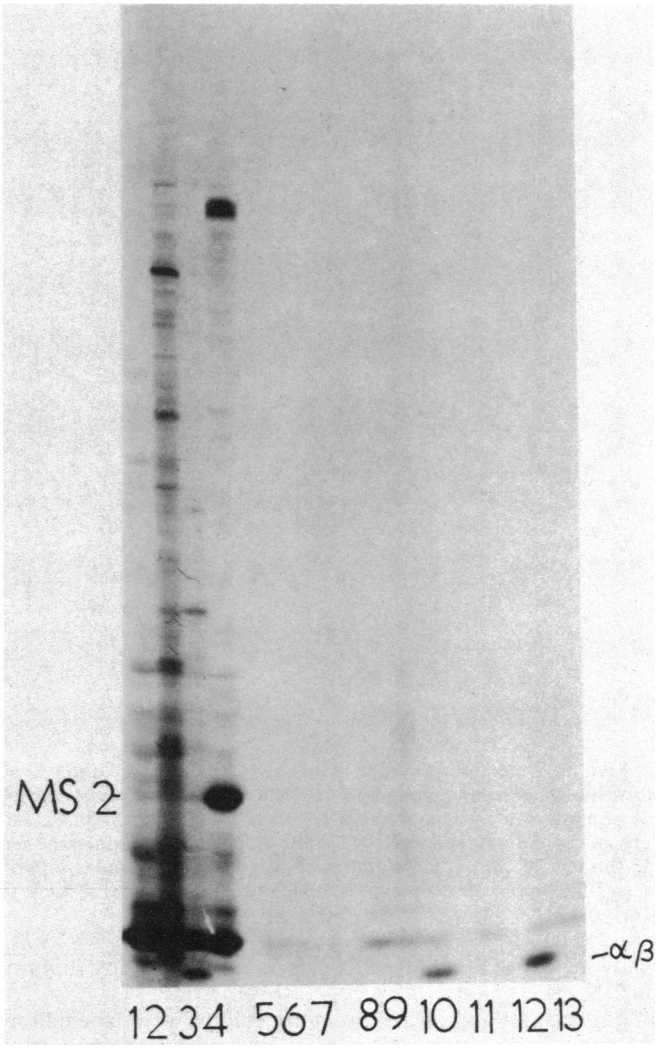

FIG. 5. Identification of α and β mRNA by specific immunoprecipitation of its translation product. The L-[^{35}S]methionine-labeled translation products of various RNA preparations were fractionated by SDS-PAGE on 7.5 to 15% gradient gels and detected by fluorography. Lane 1, Endogenous products of the *E. coli* S30 system (no added RNA); lane 2, *B. subtilis* 168 vegetative RNA; lane 3, *B. subtilis* 168 t_5 RNA; lane 4, MS2 phage RNA. The positions of Coomassie blue-stained standards of MS2 coat protein and α+β ASSPs were as shown. Immune complexes bound to *S. aureus* Cowan I cells are shown in lanes 5 through 13. The samples from lanes 1, 2, and 3, bound by preimmune serum, are shown in lanes 5, 6, and 7, respectively. The fractions of the same samples bound by anti-α+β IgG (25 μg) are shown in lanes 8, 9, and 10, respectively. The labeled translation product, bound by anti-α+β IgG and comigrating with α+β (lane 10), was not bound if an excess (1 μg) of unlabeled α+β was present (lane 11). Lanes 12 and 13 are identical to lanes 10 and 11, respectively, except that twice as much anti-α+β IgG was employed.

Kinetics of synthesis of individual ASSPs. Because of their extreme protease sensitivity, the time of appearance of ASSPs in sporulating cells may merely represent the time at which they become recoverable. Thus the accumulation data presented above indicate that accumulation commences at or before t_5. Alternative procedures for extracting ASSPs from sporulating cells (e.g., cryoimpacting in liquid N_2; 7) proved insufficiently reproducible, so pulse-chase procedures were employed to assay ASSP synthesis kinetics,

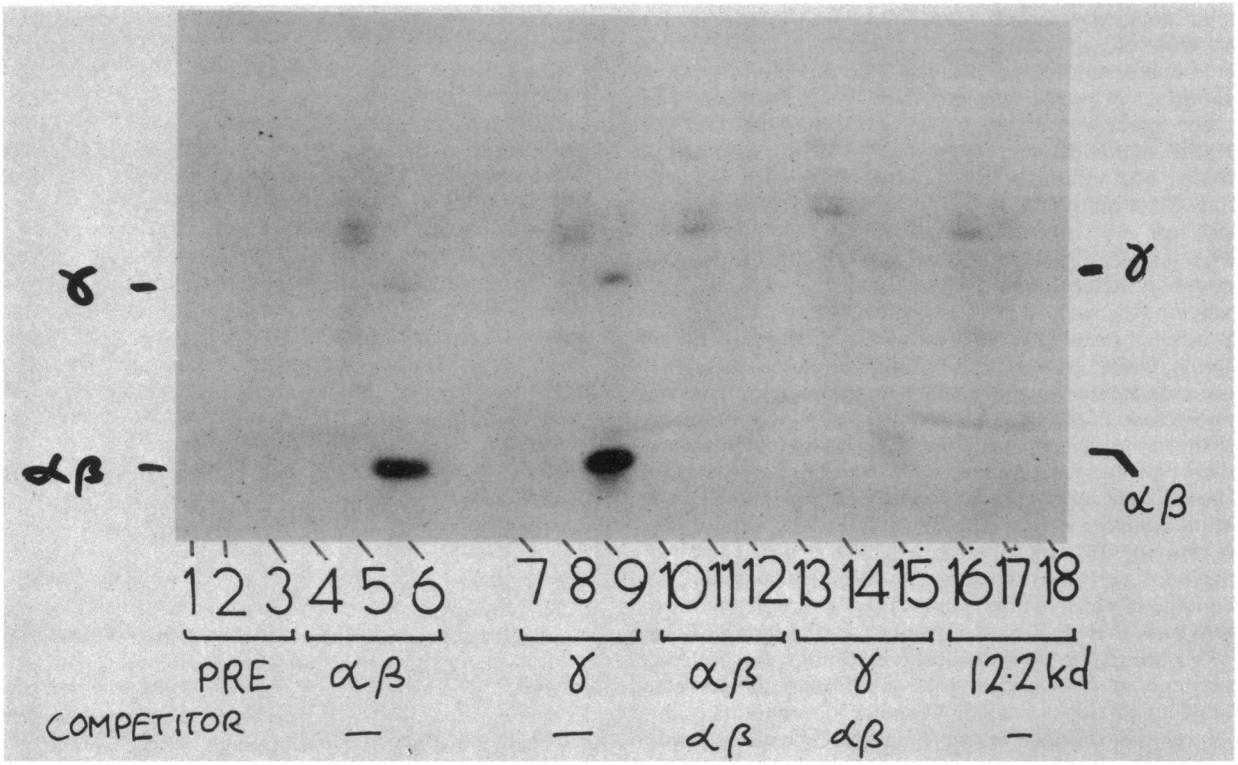

FIG. 6. Detection of γ mRNA in t_5 RNA. RNA preparations were translated, as shown in Fig. 5, but using a mixture of L-[^{3}H]leucine, tyrosine, alanine, and phenylalanine. The products were analyzed as described for Fig. 5 after immunoprecipitation. Lanes 1, 4, 7, 10, 13, and 16, Endogenous products (no RNA added). Lanes 2, 5, 8, 11, 14, and 17, Vegetative RNA. Lanes 3, 6, 9, 12, 15, and 18, t_5 RNA. IgG preparations employed were: lanes 1 through 3, from preimmune serum; lanes 4 through 6 and 10 through 12, anti-α+β; lanes 7 through 9 and 13 through 15, anti-γ; lanes 16 through 18, from antiserum to a purified 12.2-kilodalton *B. subtilis* spore coat protein (6). Competitor ASSPs, 1 μg of an approximately equimolar mixture of α, β, and γ, were present during incubation with IgG in lanes 10 through 12 and 13 through 15.

allowing extraction of ASSPs from mature spores by rupture in 2 N HCl.

Samples (10 ml) of a sporulating culture were labeled for 10 min at 37°C with [^{3}H]valine, followed by addition of a 500-fold molar excess of nonradioactive valine. Spores were isolated at t_{24}. Acid rupture of these spores gave completely uniform yields of ASSPs, as determined by SDS-PAGE and staining. A fluorogram of equal quantities of these ASSPs, fractionated by SDS-PAGE, is shown in Fig. 8A. The content of individual bands (Fig. 8B) was determined by densitometry of appropriately exposed fluorograms of a different gel, containing equal amounts of counts in each sample applied.

Labeling of α, β, and ε was considerably more efficient than labeling of γ and δ, although staining (not shown) demonstrated that γ was the predominant ASSP as usual. Valine incorporation also emphasized some of the minor ASSPs migrating between γ and α+β (Fig. 8A). Nevertheless, band positions were characteristic, as shown by staining with Coomassie blue (not shown). Fractionation at pH 4.7 of the t_5 ASSPs demonstrated that both α and β were labeled and that about 15% of the label in the α+β position represented incorporation into ε (data not shown).

Synthesis of all ASSPs was initiated between $t_{3.5}$ and t_4 and was declining by t_7. The major ASSPs, including α, β, γ, and δ (and presumably ε), all became readily detectable by t_4 (Fig. 8A), and their rates of synthesis peaked between $t_{4.5}$ and t_5, declining noticeably by t_6. The ASSPs labeled at $t_{3.5}$ consisted mostly of minor species of mobility intermediate between those of α+β and γ. Synthesis of these minor species appeared to precede that of the major species. Synthesis of one minor species, migrating just below γ, seemed to be delayed until t_5 to t_6 (Fig. 8A). Analysis of fractions labeled continuously with [^{3}H]valine, from various times in sporulation until t_{20}, gave the same result for the kinetics of synthesis of the major ASSPs (data not shown).

Kinetics of accumulation of mRNA for α and β. Larger volumes of sporulating cultures (200 ml) were used as sources of total RNA samples which were assayed for αβ mRNA activity by translation and immunoprecipitation with anti-α+β IgG (Fig. 5). αβ mRNA activity was first detectable above background at $t_{3.5}$ and was maximal between t_4 and t_5, declining about 30% by t_6 (Fig. 7).

Production of ASSPs and of αβ mRNAs in Spo$^-$ mutants. Cells of *B. subtilis spo0A*, *spoIIA-26*, *spoIIIA-7*, *spoIVA-67*, and *spoVA-89* mutants were grown into stationary phase in MSM and harvested at the time when a parallel culture of the parent 168 strain had reached t_6. Total RNA preparations were assayed for αβ mRNA as shown in Fig. 5. The *spoIVA* and *spoVA* strains gave essentially normal amounts of αβ mRNA activity, whereas the others, including the *spoIIIA* strain, gave none. Dry-breakage and extraction with 2 N HCl at t_{10} gave similar results for accumulation of ASSPs; identifiable ASSPs were detectable only in the *spoIVA* and *spoVA* cell extracts (data not shown).

DISCUSSION

The similarity of δ and γ, indicated by amino acid composition and N-terminal sequence analysis (unpublished data), is confirmed by the data presented here: they are similar in

size and charge, although γ is somewhat smaller and more basic, and both lack methionine. These data, their immunological cross-reactivity, and the results of oligonucleotide probing of *B. subtilis* DNA (unpublished data) suggest that γ may be derived from δ. No precedent or rationale for in vivo maturation of an ASSP from a precursor exists, and fragmentation of δ under conditions of ASSP isolation seems unlikely. Moreover, the synthesis, by in vitro translation, of a protein cross-reacting immunologically with γ and comigrating with γ on SDS-PAGE (Fig. 4B) is not consistent with this interpretation. This suggests that γ is a primary gene product. Its gene is presumably closely linked to that for δ, and γ and δ form a distinct subgroup of *B. subtilis* ASSPs.

The preliminary analyses of ε suggest that, although it is the same size as α and β and is labeled with methionine, it is probably not a member of the αβ ASSP subgroup since it differs markedly in charge and does not cross-react with anti-α+β IgG. Thus *B. subtilis* seems to contain at least three groups of ASSP's: α+β, γ+δ, and ε. The status of the many minor species seen by SDS and pH 4.7 PAGE is unknown at present. Because of the extreme protease sensitivity of ASSPs, fragmentation during isolation is always a possible source of artifacts. However, yields and patterns of ASSPs, particularly in extracts isolated by 2 N HCl rupture, are highly reproducible (7). Analyses of C-like protein genes in *B. megaterium* (2) suggest that the apparent complexity is probably real.

The accumulation of α+β and γ in recoverable form in sporulating cells is first significant at $t_{4.5}$, and the subsequent kinetics of ASSP accumulation closely follows the acquisition of phase whiteness in the forespore (Fig. 7). However, the specific activity of total ASSPs rises rapidly to a maximum about 0.5 h previously, at t_4 (Fig. 7), and pulse-chase experiments also show that the rate of synthesis of major ASSPs is half maximal by t_4 and peaks between $t_{4.5}$ and t_6 (Fig. 8). It is thus probable that recovery of ASSPs from sporulating cells is inefficient until forespores commence phase whitening (dehydration?) at $t_{4.5}$ and that their synthesis precedes this event by about 30 min.

The levels of α and β mRNA activities recovered (Fig. 7) are proportional to the in vivo rates of α and β synthesis (Fig. 8), indicating that synthesis of these ASSP's is transcriptionally controlled, as found by Dignam and Setlow (4) for the A and C proteins of *B. megaterium* and by Leventhal and Chambliss (9) for an uncharacterized mixture of immunoprecipitated ASSPs in *B. subtilis*.

The levels of αβ mRNA activities recovered from cells after $t_{4.5}$ are much higher than those for other ASSPs and represent a disproportionate fraction of the total mRNA activity present in these preparations (Fig. 5). We demonstrated that αβ mRNAs are unusually stable in vivo in t_5 cells treated with rifampin (17), and it was subsequently shown that they have a half-life of 11 min under these conditions (9). Perhaps this relative resistance to in vivo degradation and their small size (unpublished data) results in much less degradation during RNA isolation. Alternatively, if these mRNA levels are unusually high in vivo, they may have inefficient ribosome binding sites not reflected by translation efficiency in the *E. coli* S30 system. Preferential recovery seems a more likely explanation.

Synthesis of γ and δ, as well as synthesis of some of the minor ASSPs, appears to be synchronous with that of α and β (Fig. 8), implying coordinate expression of this group of sporulation-specific genes, as anticipated from the similarities in structure and function of their products (13, 19). The

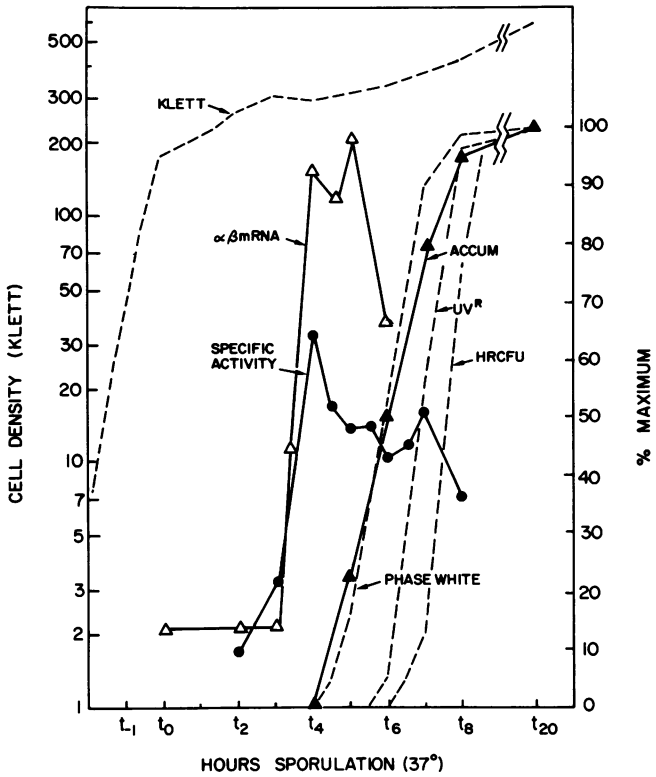

FIG. 7. Kinetics of accumulation of recoverable ASSPs and αβ mRNA. A sporulating culture of *B. subtilis* 168 was analyzed for cell density (Klett colorimeter, green filter) (KLETT), phase-white forespores (phase-contrast microscopy) (PHASE WHITE), resistance to UV irradiation (UV^R), and heat-resistant colony-forming units (HRCFU). At intervals during sporulation, culture samples were pulse-labeled with L-[^{3}H]valine. ASSPs were immediately isolated, fractionated by SDS-PAGE, and quantitated by densitometry. The data for α+β, γ, and δ were identical. The percentage of maximum accumulated radioactivity is shown (ACCUM). The specific activity of the total recovered ASSPs is also shown. A separate culture, sporulating with the same kinetics, was the source of RNA preparations. Equal quantities of RNA were translated to determine the relative αβ mRNA activity (αβ mRNA).

gene duplication events presumably responsible for the proliferation of functional ASSP genes would necessarily include duplication of the control regions. The only suggestion of variation is seen in Fig. 6A, where it is apparent that unidentified minor ASSPs of mobility intermediate between those of α+β and γ, and possibility one large species of about 20 kilodaltons, get an early start at $t_{3.5}$, whereas one minor species just below γ appears late, at about t_6. These apparent variations from coordinate expression will be clarified only when genes for these minor ASSPs become available for analysis.

Accumulation of ASSPs in *B. subtilis* precedes the acquisition of UV resistance by a full 1 to 1.5 h (Fig. 5). A role for ASSPs in spore UV resistance has been suggested (13, 17). It appears that, if ASSPs play a part in this resistance, their synthesis is not sufficient to cause it. A primary role for ASSPs in spore heat resistance, which follows a further 1.5 h late, seems even less likely. Only the creation of null mutations in genes for major ASSP components such as γ will allow a direct test of the potential roles of these proteins in the resistance properties of dormant spores.

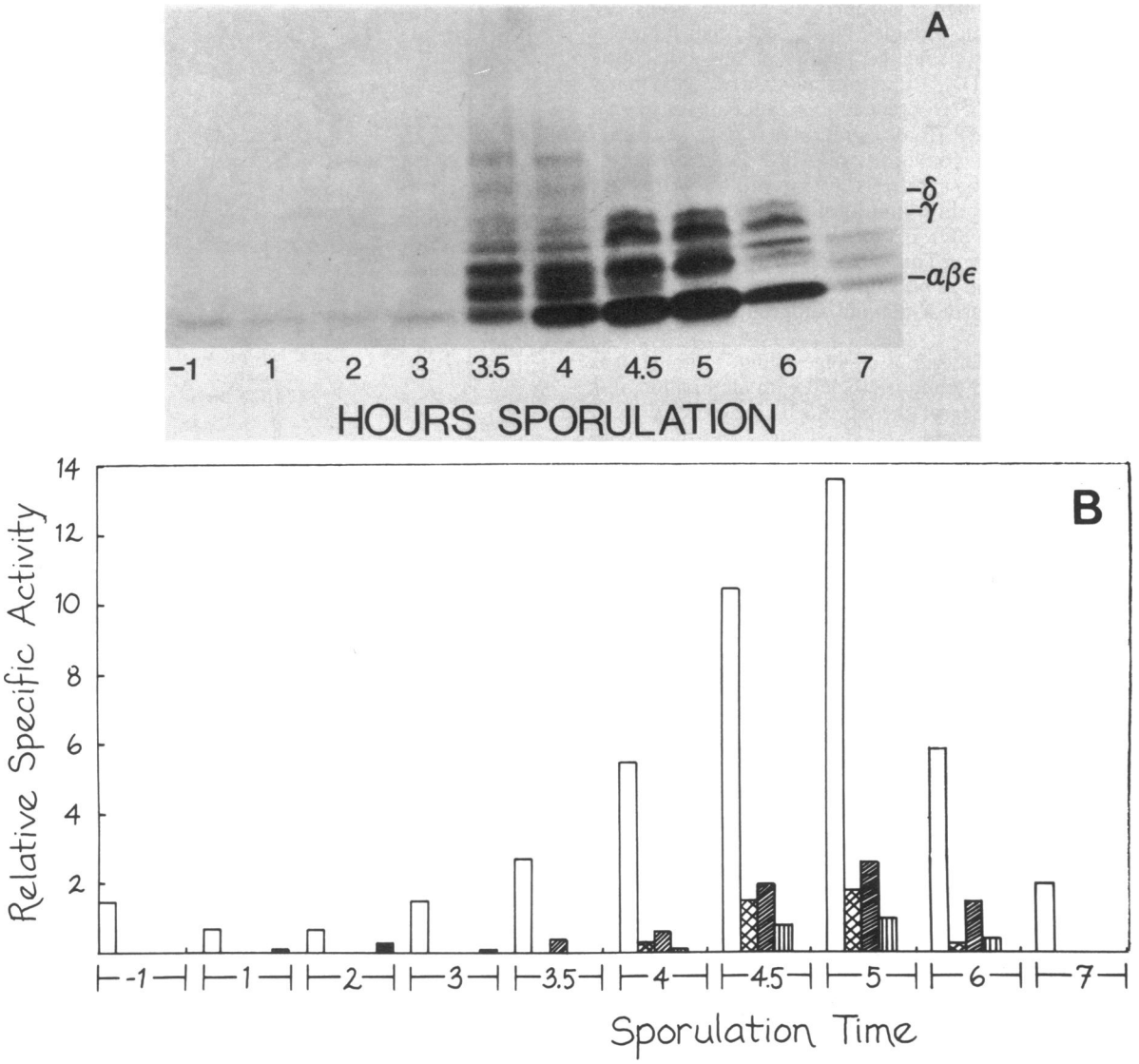

FIG. 8. Pulse-chase determination of the in vivo kinetics of synthesis of *B. subtilis* ASSPs. Samples (9.5 ml) of a sporulating culture of *B. subtilis* 168 were pulse-labeled for 10 min with L-[^{3}H]valine at the indicated times and then chased to t_{24} in the presence of excess cold valine. (A) Equal quantities of ASSPs, isolated by rupture of spores in 2 N HCl, were fractionated by SDS-PAGE in a 7.5 to 15% gradient and detected by fluorography. (B) Relative specific activities of various ASSP components were determined by densitometry of a fluorogram, essentially identical to that shown in part A, but containing equal amounts of radioactivity in each lane. Columns: □, α+β+ε; ■, γ; ▥, δ; ▩, prominently labeled component running halfway between α+β and γ.

Investigation of the effects of Spo$^-$ mutations on ASSP accumulation and αβ mRNA synthesis indicates that lack of accumulation, for α and β at least, is due to lack of transcription rather than to rapid turnover of synthesized ASSPs. Expression of these ASSP genes appears to be dependent on *spo0A*, *spoIIA*, and *spoIIIA* genes. Thus clones of these genes will be valuable probes for analysis of factors controlling late transcription events, dependent on expression of an array of earlier *spo* genes.

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