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Spore Coat Protein Synthesis in Cell-Free Systems from Sporulating Cells of *Bacillus subtilis*

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Cell-free systems for protein synthesis were prepared from *Bacillus subtilis* 168 cells at several stages of sporulation. Immunological methods were used to determine whether spore coat protein could be synthesized in the cell-free systems prepared from sporulating cells. Spore coat protein synthesis first occurred in extracts from stage t_2 cells. The proportion of spore coat protein to total proteins synthesized in the cell-free systems was 2.4 and 3.9% at stages t_2 and t_4, respectively. The sodium dodecyl sulfate-urea-polyacrylamide gel electrophoresis patterns of immunoprecipitates from the cell-free systems showed the complete synthesis of an apparent spore coat protein precursor (molecular weight, 25,000). A polypeptide of this weight was previously identified in studies in vivo (L. E. Munoz, Y. Sadaie, and R. H. Doi, J. Biol. Chem., in press). The synthesis in vitro of polysome-associated nascent spore coat polypeptides with varying molecular weights up to 23,000 was also detected. These results indicate that the spore coat protein may be synthesized as a precursor protein. The removal of proteases in the crude extracts by treatment with hemoglobin-Sepharose affinity techniques may be preventing the conversion of the large 25,000-dalton precursor to the 12,500-dalton mature spore coat protein.

A highly specific probe to analyze transcriptional and translational regulation during bacterial sporulation is very desirable. One component of spores which has been shown to be spore specific and present in high amounts is the spore coat protein (1). In fact, it is one of the few sporulation-specific gene products that have been well characterized. The characterization of its properties and time of synthesis during sporulation would aid in its use to monitor the expression of a sporulation-specific gene.

The presence of precursor spore coat proteins and mature coat proteins has been reported in cellular extracts of sporulating cells of *Bacillus subtilis* (17; L. E. Munoz, Y. Sadaie, and R. H. Doi, J. Biol. Chem., in press) and in *Bacillus cereus* (4). The spore coat proteins were identified by antibodies made against mature spore coat protein (Munoz et al., in press). The spore coat protein precursor of *B. subtilis* had a molecular weight of 25,000, compared to the mature coat protein with a molecular weight of around 13,000 (17; Munoz et al., in press). The precursor protein was synthesized as early as 1 h after the cells had reached stationary phase (t_1), and the mature coat protein appeared about 1 h later (t_2).

To investigate the synthesis of the precursor spore coat protein and to develop a Zubay type system (23) in *B. subtilis* to study the regulation of gene expression during sporulation, an in vitro system of protein synthesis has been developed with *B. subtilis* cells which can synthesize the precursor of the spore coat protein in reasonably good yields. The use of a protease affinity system (18) has resulted in an in vitro system relatively free of protease activity, which may explain the good yield and the accumulation of the precursor spore coat protein.

MATERIALS AND METHODS

Bacterial strain and growth media. *B. subtilis* 168 wild type was grown at 37°C in a modified Schaeffer (2× SG) medium (13) for preparation of polysomes and cell sap. To obtain maximum synchronous sporulation, cells were transferred three times at log stage to fresh 2× SG medium as described previously (13). The final (fourth) 10-liter culture was stopped at the proper time of sporulation (Fig. 1) by adding NaN_3 and $MgCl_2$ to final concentrations of 1 mM and by the addition of −10°C ice. The stages of sporulation are indicated by t_0, t_1, t_2, t_3, and t_4, with the subscripts indicating hours after the end of the log stage of growth (21).

Cells were collected with a Sharples continuous-flow centrifuge and washed immediately four times after harvesting with 400 ml of ice-cold buffer A (see below) to remove extracellular proteases as described previously (18). Washed cells were frozen in a dry ice-acetone bath and stored at −70°C until used.

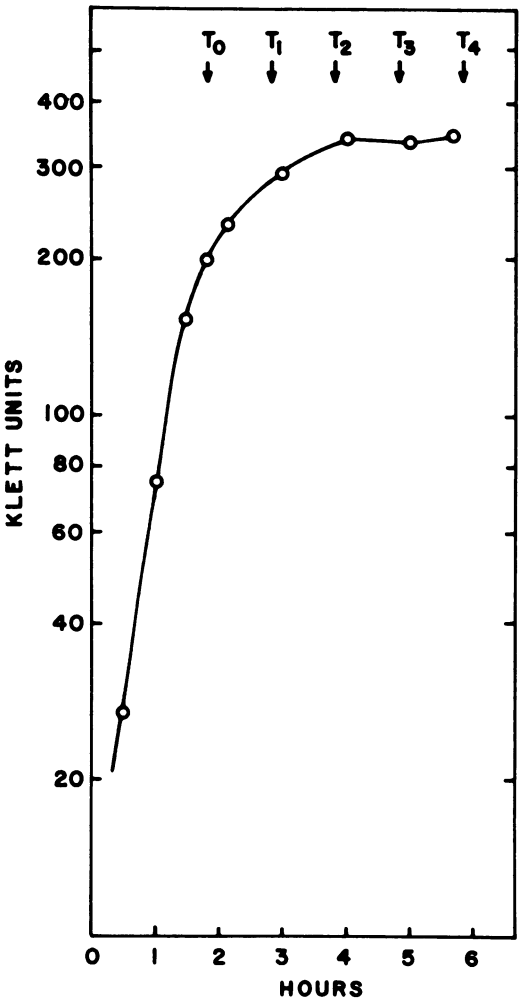

FIG. 1. *Growth curve of B. subtilis 168. Cells were grown in 10 liters of 2× SG medium at 37°C. The symbol t_0 represents time at the end of the log phase of growth. The symbols t_1 through t_4 represent 1-h intervals after t_0. The 10-liter cultures were stopped by chilling at the proper time of growth.*

Buffers. Buffer A: 0.25 M sucrose, 10 mM $MgCl_2$, 1 M KCl, 10 mM tris(hydroxymethyl)aminomethane-hydrochloride (Tris) (pH 7.6), and 2 mM phenylmethylsulfonylfluoride.

Buffer B: 0.25 M sucrose, 10 mM $MgCl_2$, 10 mM KCl, 10 mM Tris (pH 7.6), and 2 mM phenylmethylsulfonylfluoride.

Buffer C: 0.5 M sucrose, 10 mM $MgCl_2$, 10 mM KCl, 10 mM Tris (pH 7.6), 2 mM phenylmethylsulfonylfluoride, and sodium heparin (100 μg/ml) as ribonuclease inhibitor (19).

Preparation of polysomes and cell sap from the cells. Cells (15 g wet weight) were suspended in 20 ml of buffer B and were broken by use of a French press twice (17,000 to 18,000 lb/in^2) at 4°C. After addition of 10 ml of buffer B to the mixture, deoxyribonuclease (ribonuclease free, from which ribonuclease was again removed by a Sephadex G-50 column) was added to a concentration of 30 μg/ml, and hemoglobin-Sepharose (10 g) was also added to remove proteases by a batch method which was modified from that described previously (18). After stirring at 4°C for 30 min, the mixture was centrifuged at 30,000 × *g* for 30 min to obtain the S-30 fraction. The S-30 fractions obtained were centrifuged at 175,700 × *g* for 3 h. The upper one-third phase of the supernatant obtained was used as cell sap (enzyme fraction). The amount of proteins was estimated according to the method of Lowry et al. (15) with bovine serum albumin as a standard.

Polysome fractions were obtained from the pellets by a method described previously (19, 20). The pellets were washed with a small volume of buffer B several times and suspended in 9 ml of buffer B. The suspension was used as crude polysome fraction. The crude polysome fraction was centrifuged at 175,700 × *g* for 3 h. The pellet was washed with a small volume of buffer B and again suspended in 6 ml of the same buffer. The suspension was centrifuged at 25,500 × *g* for 10 min to remove insoluble materials. The supernatant was layered over 5 ml of buffer C, which contained 0.5 M sucrose. After centrifugation at 175,700 × *g* for 3 h, the pellets obtained were washed with a small volume of buffer B and suspended in 5 ml of buffer B. The opalescent supernatant obtained was used as the pure polysome fraction. The amounts of the polysomes were measured based on absorbance at 260 nm. The ratio of absorbance at 260 nm to that at 280 nm of the polysomes was about 1.9 (Table 2).

Hemoglobin-Sepharose column chromatography. This affinity system was made according to the method of Chua and Bushuk (5) and used for removal of protease activity from crude extracts as described previously (18).

Amino acid incorporation systems. The small-scale incubation mixture (total volume, 0.25 ml) contained ATP, 2 mM; GTP, 0.25 mM; creatine phosphate, 10 mM; creatine kinase, 0.005%; dithiothreitol, 3 mM; $MgCl_2$, 5 mM; KCl, 10 mM; Tris (pH 7.6), 10 mM; [^{3}H]leucine, 1 μCi; polysomes, 20 μl; and the cell sap, 0.25 μl. The optimum concentrations of the components for [^{3}H]leucine incorporation into acid-insoluble proteins were used in the present study unless otherwise stated. The time course of [^{3}H]leucine incorporation was followed at 37°C for proper time periods. The amino acid incorporating reaction was usually performed at 37°C for 30 min except for the time course experiments. To terminate the reaction, cold 10% trichloroacetic acid was added to the reaction mixture to a final concentration of 5%. After keeping at 4°C overnight, the precipitates were washed successively with 5% trichloroacetic acid (six times) and ethanol (twice) on filter papers. The filter papers were put into vials, 5 ml of scintillation fluid (12 g of 2,5-diphenyloxazole [PPO], 0.3 g of 1,4-bis-(5-phenyloxazolyl)benzene [POPOP] in 3 liters of toluene) was added, and then the radioactivity was counted with a Packard scintillation counter. Large scale: to isolate the radioactively labeled complete and nascent spore

coat proteins, the above system was enlarged 20-fold (5 ml). The concentrations of the components were kept the same. DL-[^{3}H]phenylalanine was used instead of [^{3}H]leucine. The amounts of polysomes and cell sap are given below for each experiment.

Separation of in vitro labeled proteins into several fractions. After the reaction was stopped at 0°C, the incubation mixture, made 0.25 M in sucrose, was layered over buffer C (0.5 M sucrose) and centrifuged at 175,700 × *g* for 3 h. The supernatant was carefully separated into two fractions, upper layer (0.25 M sucrose fraction) and lower layer (0.5 M sucrose fraction), by use of a pipette. The resulting pellets, after removal of the supernatants described above, were washed several times with a small volume of buffer B and suspended in 2 ml of buffer B. This fraction was used as the total ribosome fraction. To determine the counts incorporated into these fractions, 0.15 ml of 10% trichloroacetic acid was added to 0.15 ml from each sample solution. Radioactivity was measured as described in the preceding section.

Purification of complete spore coat protein labeled with [^{3}H]phenylalanine by the immunological method. To 1.5 ml of the 0.25 M sucrose and 0.5 M sucrose fractions (see above) from log, t_0, t_1, t_2, t_3, and t_4 stages of the cells, 0.1 ml of rabbit antibody for pure spore coat proteins was added and kept at 37°C for 15 min and then kept at 4°C overnight. To the above solution, 0.25 ml of goat anti-rabbit immunoglobulin G (IgG) was added at the equivalence ratio and kept at 37°C for 15 min, then kept at 4°C overnight. The precipitates of the [^{3}H]phenylalanine-labeled spore coat protein-rabbit IgG-goat IgG complex were obtained by centrifugation at 10,000 × *g* for 30 min. To remove nonspecific protein labeled with the radioactive amino acid and free [^{3}H]phenylalanine, the immunoprecipitate was washed with cold 1% NaCl-50 mM Tris (pH 7.6) for 15 times, placed on filter paper, and washed 10 times with 5% trichloroacetic acid and twice with ethanol. The radioactivity was measured. In control experiments it was shown that greater than 90% of the immunoprecipitation of radioactive counts could be prevented by the addition of unlabeled mature spore coat protein, thus indicating the specificity of the precipitation reaction.

Purification of nascent spore coat protein labeled in vitro with [^{3}H]phenylalanine by the immunological method. To remove ribosomal proteins and ribonucleic acids from the total ribosome fraction, the method described previously was used (20). The solution of total ribosome fraction was adjusted to 5 mM ethylenediaminetetraacetic acid and treated with ribonuclease (20 μg/ml) at 37°C for 1 h, then dialyzed overnight against 50 mM Tris (pH 7.6)-5 mM ethylenediaminetetraacetic acid to remove nucleotides. Centrifugation was carried out at 30,000 × *g* for 30 min to remove the insoluble ribosomal proteins. The supernatant obtained was adjusted to pH 12 with dilute NaOH and kept at 37°C for 1 h to cleave ester bonds between the C-terminal amino acids of nascent peptides and the 5′-terminal adenosine of the tRNA's, and again dialyzed against 50 mM Tris-5 mM ethylenediaminetetraacetic acid. The solution obtained was referred to as the total nascent peptide fraction. To determine the counts incorporated into these fractions, 0.15 ml of 10% trichloroacetic acid was added to 0.15 ml from each sample solution. Radioactivity of the precipitate was measured.

Samples of 1 ml from each of the total nascent peptide fractions of t_2, t_3, and t_4 cells were combined. To the mixture, 0.1 ml of rabbit antibody for spore coat protein was added and kept at 37°C for 15 min, then at 4°C overnight. To the above solution, 0.25 ml of goat anti-rabbit IgG was added at the equivalence ratio, and the solution was kept at 37°C for 15 min and then at 4°C overnight. The precipitate of [^{3}H]-phenylalanine-labeled nascent spore coat protein-rabbit IgG-goat IgG complex was obtained by centrifugation at 10,000 × *g* for 30 min. To remove free radioactive amino acid and nonspecific labeled proteins from the precipitate, the precipitate was washed six times with cold 1% NaCl-50 mM Tris buffer (pH 7.6).

Disc gel electrophoresis of ^{3}H-labeled spore coat protein and nascent spore coat protein. For this purpose, 1.5 ml each from the 0.25 M sucrose fractions of t_2, t_3, and t_4 cells were combined. From this mixture, spore coat protein labeled in vitro with [^{3}H]phenylalanine was purified by the method described in the preceding section. The method of Wu and Bruening (22) was used for disc gel electrophoresis of the spore coat protein. The gels of standard spore coat protein were stained and destained as described previously (17). The gels of the radioactive samples were frozen at −70°C and then cut into 2-mm pieces with the aid of a gel slicer. The slices were put into a vial, and 0.5 ml of NCS tissue solubilizer was added. After keeping at 45°C overnight, 5 ml of scintillation fluid was added, and then the radioactivity was measured.

Purification of mature spore coat protein and its antibody. Mature spore coat protein and antibody (rabbit IgG) for pure spore coat protein were purified according to the methods of Munoz et al. (17) and Fahey and Horbett (8), respectively. The mature spore coat protein had a molecular weight of about 12,500, a histidine residue at the amino terminus, and a high content of hydrophobic amino acids (17). The rabbit antibody prepared against the mature spore coat protein showed a single precipitin line in Ouchterlony immunodiffusion plates (17). Furthermore, the antibody prepared against whole spores and against crude spore coat protein also formed a single precipitin line against the purified mature spore coat protein. These results indicated that the spore coat protein was pure and the antibody-antigen reaction was specific.

Measurement of radioactivity. The radioactivity incorporated into proteins was measured as described previously (20).

Materials. L-[4,5-^{3}H]leucine (60 Ci/mmol), ribonuclease-free sucrose, and DL-[*G*-^{3}H]phenylalanine (6.8 Ci/mmol) were obtained from Schwarz/Mann; phenylmethylsulfonylfluoride, diisopropylfluorophosphate, and dithiothreitol came from Calbiochem; heparin, creatine kinase, hemoglobin, and sodium dodecyl sulfate were from Sigma Chemical Co.; creatine phosphate, ATP, and GTP were from Boehringer Mannheim Biochemicals; deoxyribonuclease was from Worthington Biochemical Co.; Sepharose 6B was from Pharmacia Fine Chemicals; polyacrylamide, *N,N′*-methylene bisacrylamide, *N,N,N′,N′*-tetramethylethylenediamine, and ammonium persulfate were ob-

tained from Eastman Organic Chemicals; marker proteins for molecular weights came from Mann Laboratory; NCS tissue solubilizer was obtained from Amersham/Searle; and anti-rabbit immunoglobulin (goat IgG) was from Antibodies, Inc.

RESULTS

Cell-free systems for protein synthesis were prepared from log-phase and sporulating cells. Conditions for optimum incorporation of amino acids were determined initially, followed by an analysis of the products synthesized in vitro.

Time course of [^{3}H]leucine incorporation into acid-insoluble proteins. The time course of [^{3}H]leucine incorporation into acid-insoluble materials in the cell-free system from log-phase cells is shown in Fig. 2. The incorporation of [^{3}H]leucine increased appreciably up to 30 min of incubation time. In cell-free systems from sporulating cells, patterns essentially similar to that in Fig. 2 were obtained. These results indicate that our cell-free systems have high activity of protein synthesis and probably little or no protease activity, because of the hemoglobin-Sepharose treatment (see Materials and Methods and ref. 18).

Optimum conditions for incorporation of [^{3}H]leucine into acid-insoluble proteins. The optimum concentration was determined for the various components of the reaction mixture for incorporation of [^{3}H]leucine into acid-insoluble protein by a log-phase cell-free system. The optimum concentrations are summarized in Table 1.

TABLE 1. *Optimum concentrations of components for the in vitro protein synthesis system*[a]

Component	Concn (mM)
ATP	2.0
GTP	0.3
Dithiothreitol	3.0
$MgCl_2$	5.0
KCl	10.0
Sucrose	250.0
Creatine phosphate	10.0
Tris (pH 7.6)	10.0
Creatine kinase	0.001%

[a] The cell-free system from log-phase cells was used to determine the optimum concentrations.

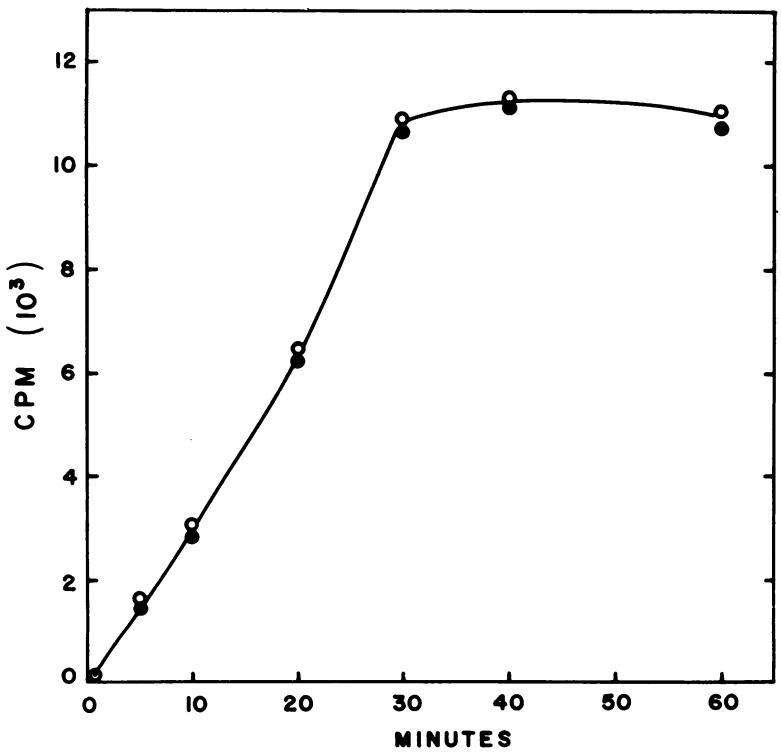

FIG. 2. *Time course of [^{3}H]leucine incorporation into acid-insoluble materials. A cell-free system from log-phase cells was used, and components were used at the optimum concentrations shown in Table 1. The reactions were stopped at the proper time by adding 10% trichloroacetic acid to a final concentration of 5%. The closed and open circles represent results from two reaction mixtures.*

Yield and specific activity of polysomes from each stage of growth of *B. subtilis*. Polysomes were prepared from 15 g of cells (wet weight) at each stage of growth as described above, and their activity was assayed by the small-scale cell-free system. The ratios of absorbance at 260 nm to that at 280 nm of the polysomes from each stage of growth were about 1.9 to 2.0, similar to those in a previous report (20). The amount of the polysomes was based on their absorbance at 260 nm. The amount of polysome activity was based on the [^{3}H]leucine incorporated into acid-insoluble materials. The polysomes from cells at log phase were used as standard. As shown in Table 2, the amounts of polysomes decreased during the sporulation process, but almost 50% of the log-phase polysome level remained even at stage t_4. The activity of protein synthesis decreased more rapidly than the quantity of polysomes during sporulation, and almost 30% of the activity remained at stage t_4. From these data, the specific activity of the polysomes from each stage of the growth was calculated and is summarized in Table 2. The polysomes from sporulating cells retained more than 60% of the specific activity of polysomes from log-phase cells.

Distribution of in vitro synthesized proteins into various fractions. To analyze the completed proteins, which would be released from the ribosome, and the nascent proteins, which would still be attached to the ribosomes, the reaction mixture (0.25 M sucrose) was layered over 0.5 M sucrose and centrifuged. The rationale for this procedure is that the completed proteins would be in the sucrose fractions and the nascent proteins would be in the ribosome pellet.

After the reaction in the large-scale cell-free system was stopped, the incubation mixture (0.25 M sucrose) was layered over 4 ml of buffer C (0.50 M sucrose) and centrifuged at 175,700 × *g* for 3 h. The 0.25 M and 0.5 M sucrose fractions and nascent peptide fraction (ribosome pellet) were fractionated as described above. Complete spore coat protein (0.25 M sucrose) and nascent spore coat protein (ribosome pellet) were purified from the above-mentioned fractions as described above. The results obtained are summarized in Table 3.

The data indicated that complete spore coat protein appeared as early as t_2, when it comprised 3.74% of the total protein synthesized. This percentage increased slightly at t_3 (3.84%) and at t_4 (5.89%). From earlier experiments it was noted that nascent spore coat proteins associated with the polysomes comprised only a very small part of the total nascent proteins; therefore, the nascent spore coat proteins present on t_2, t_3, and t_4 polysomes were pooled, and their percentage of the total nascent proteins was determined to be 3.83%, which was similar

TABLE 2. *Properties of polysomes from various stages of growth and sporulation*[a]

Stage	A_{260}/A_{280}	Total polysomes (%)	Total activity (%)	Sp act (%)
Log	1.89	100	100	100
t_0	1.94	116	68	59
t_1	1.91	74	50	67
t_2	1.91	62	36	68
t_3	1.99	54	34	64
t_4	1.91	49	30	61

[a] A_{260}/A_{280}, Ratio of absorbance at 260 nm to that at 280 nm. Total polysomes was based on absorption at 260 nm of polysome solutions. Total activity was based on the incorporation of [^{3}H]leucine into acid-precipitable counts by the in vitro system. Specific activity was based on the [^{3}H]leucine counts incorporated per unit of absorbance at 260 nm of polysomes.

TABLE 3. *Distribution of proteins made in vitro in the completed protein and nascent protein fractions*[a]

Stage	0.25 M sucrose fraction		0.5 M sucrose fraction		Nascent protein	
	Total protein (cpm)	Spore coat protein, cpm (%)	Total protein (cpm)	Spore coat protein, cpm (%)	Total protein (cpm)	Spore coat protein, cpm (%)
Log	76,226	50 (0.06)	231,351	14 (0)	75,810	0 (0)
t_0	87,309	0 (0)	147,116	2 (0)	45,473	0 (0)
t_1	82,428	36 (0.04)	99,876	4 (0)	33,463	0 (0)
t_2	31,672	1,184 (3.7)	18,052	19 (0.1)	20,256	
t_3	25,433	977 (3.8)	13,317	0 (0)	11,039	1,575 (3.8)
t_4	20,552	1,210 (5.9)	10,734	0 (0)	9,829	

[a] The large-scale cell-free system was used with a total of 5 ml in the reaction mixture. One-week-old polysomes were used, and DL-[^{3}H]phenylalanine (6.8 Ci/mmol, 40 μCi) was used instead of [^{3}H]leucine for these experiments. After the reaction was stopped, the 0.25 M (small completed proteins) and 0.5 M (large completed proteins) sucrose fractions and nascent peptide (ribosome bound) fraction were obtained as described in the text. The spore coat proteins from these fractions were obtained by the immunological techniques described in the text. Since very small amounts of nascent spore coat protein were made by the t_2, t_3, and t_4 polysomes, these polysome fractions were pooled prior to immunological precipitation of the spore coat protein.

to the figures obtained above for complete spore coat proteins.

The sum of the counts incorporated into acid-insoluble proteins of the 0.25 M sucrose, 0.5 M sucrose, and nascent peptide fractions of t_0, t_1, t_2, t_3, and t_4 stages of the growth were 73.0, 50.3, 18.3, 19.1, and 10.7%, respectively, compared to that of log-phase cells. The percentages of the t_2, t_3, and t_4 stages were lower than those (36.5, 34.5, and 30.2%) shown in Table 2, since in this experiment the polysomes were used after storage at −70°C for a week. These results indicated that the polysomes from sporulating cells were more unstable at −70°C than those from vegetative cells.

The distribution of the counts incorporated into acid-insoluble materials of the 0.25 M sucrose, 0.50 M sucrose, and ribosomal pellet fractions by polysomes from various stages of the growth is shown in Fig. 3. Two classes of free proteins were present, a slowly sedimenting class in the 0.25 M sucrose fraction and a faster-sedimenting class in the 0.50 M sucrose fraction. Although the percentage of the nascent peptide fractions was constant (about 20%), those of the 0.25 M and 0.5 M sucrose fractions changed dramatically during the growth stages of *B. subtilis* 168. The percentages of the 0.5 M sucrose fractions decreased from 60% (log stage) to 26% (t_4 stage), but the percentages of the 0.25 M sucrose fractions increased from 20% (log stage) to 50% (t_4 stage). Between t_1 and t_2, the relative amounts of these two types of proteins were reversed. Although the exact characteristics of these two protein fractions are still unknown, it is unlikely that this reversal in relative amounts is due to the protease activities of sporulating cells for the following reasons: (i) the batch method of hemoglobin-Sepharose was used to remove protease activity from crude extracts (18); (ii) polysomes used in these studies were almost pure, and a very small amount of cell sap was used as enzyme fraction; (iii) the incorporation of [^{3}H]leucine into acid-insoluble proteins increased linearly up to 30 min. The data in Fig. 3 suggest that the rate of expression of specific genes changed during the sporulation stages, yielding relatively more slowly sedimenting (smaller) proteins at late sporulation.

Sodium dodecyl sulfate-urea-polyacrylamide gel electrophoretic patterns of complete and nascent spore coat proteins syn-

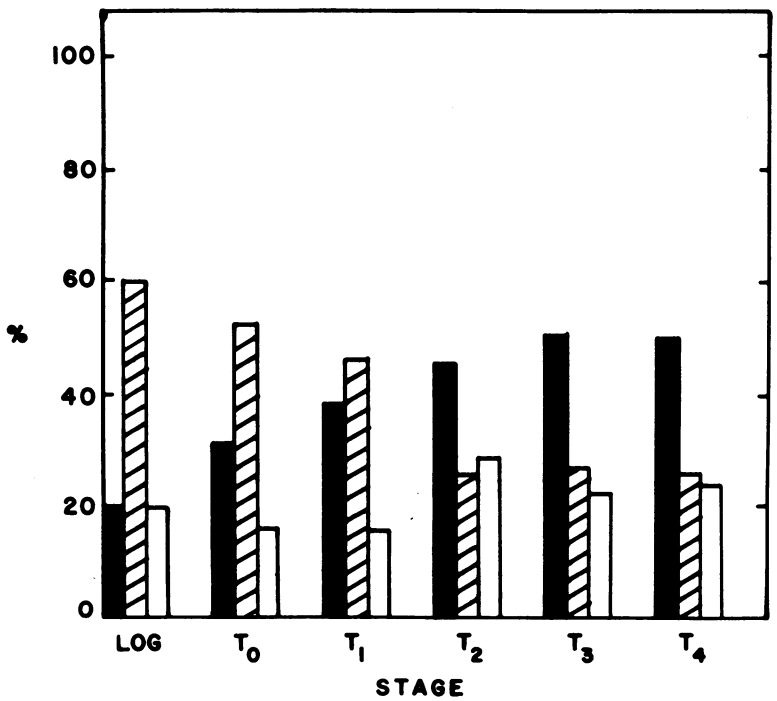

FIG. 3. *Distribution of in vitro synthesized proteins by the polysomes from each stage of growth into three fractions. The data were obtained from the results shown in Table 3. The sum of counts incorporated into the acid-insoluble materials of the 0.25 M sucrose, 0.5 M sucrose, and nascent peptide fractions was considered as 100% for each stage of growth. Symbols: counts incorporated into acid-insoluble materials of (■) the 0.25 M sucrose fraction; (▨) the 0.5 M sucrose fraction; (□) the nascent peptide fraction.*

thesized in vitro. To determine the nature of the in vitro synthesized protein, complete and nascent spore coat proteins synthesized in vitro and labeled with [^{3}H]phenylalanine were purified immunologically by combining the 0.25 M sucrose fraction from t_2, t_3, and t_4 stages, and combining the polysome-associated nascent peptide fractions from t_2, t_3, and t_4 stages.

As a control, Fig. 4 shows the electrophoretic pattern of the total acid-insoluble proteins synthesized in cell-free systems from t_2, t_3, and t_4 stages of *B. subtilis*. As shown in this figure, various-sized proteins were synthesized in the cell-free systems from the sporulating cells.

The electrophoresis of complete (1,000 cpm) and nascent (1,400 cpm) spore coat proteins obtained by immunoprecipitation was then carried out. As shown in Fig. 5a, the putative complete spore coat protein (17) had only one sharp peak and its molecular weight was 25,000; the polysome-bound nascent spore coat polypeptides (Fig. 5b) were present in a broader peak, with most of the proteins with a molecular weight around 23,000. The large peaks near the origin in Fig. 5 were probably due to large aggregates of the protein. It is unlikely that the peak at 25,000 daltons in Fig. 5a is a dimer of the mature spore coat protein, since no dimers were observed with mature spore coat protein under the conditions used for gel electrophoresis. The nascent spore proteins had a broad peak whose molecular weight was less than that of the complete spore coat protein (25,000) and larger than that of mature spore coat protein (12,500).

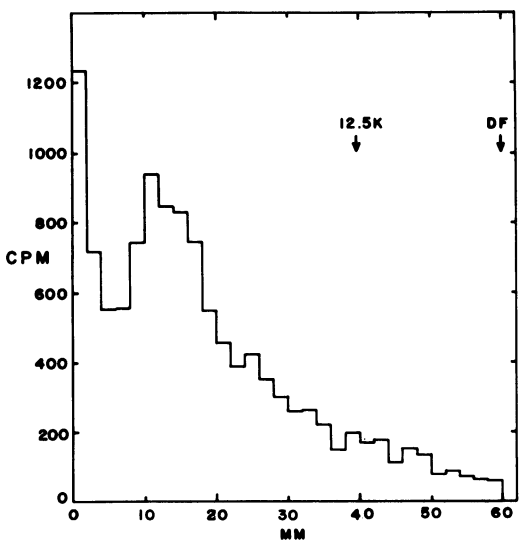

FIG. 4. *Sodium dodecyl sulfate-urea-polyacrylamide gel electrophoresis patterns of total proteins synthesized in cell-free systems from sporulating cells of B. subtilis. The proteins of the 0.25 M sucrose fraction from t_2, t_3, and t_4 cells were combined. To 0.3 ml of the mixture was added 10% trichloroacetic acid to a final concentration of 5%. After washing with 5% trichloroacetic acid (five times) and ethanol (twice), the precipitate was dissolved in 0.5 ml of sample application buffer, and then electrophoresis (12% gel) was carried out as described in the text. The arrows indicate the mobility of mature spore coat protein (12.5 K) and the dye front (DF).*

DISCUSSION

These results indicate that cell-free systems for protein synthesis can be developed from cells at all stages of growth and sporulation in *B. subtilis*. Although the total and specific activities are decreased in extracts of sporulating cells, a significant amount remains which can yield completed proteins. These studies did not indicate whether protein synthesis could be initiated in vitro; however, earlier studies with log-phase cells of *B. subtilis* indicated that proteins could be initiated with formyl methionine and completely synthesized in vitro (16).

Sporulating cells from stages t_2 and t_4 are making large amounts of spore coat protein, since from 2.4 to 3.9% of the total protein synthesized in vitro was comprised of spore coat protein. These numbers compare well with in vivo studies which indicate that 1.8 to 2.4% of the total protein was comprised of spore coat protein (17). Since the isolated polysomes were active as templates for protein synthesis, they may be excellent sources for isolating spore coat protein mRNA which can be used as probes for DNA containing the spore coat protein gene.

The isolation of complete in vitro synthesized precursor and nascent spore coat proteins similar in molecular weight to that isolated from whole cells (17) indicates that the system is operating with a high degree of fidelity. Furthermore, the use of the hemoglobin-Sepharose affinity method (18) eliminates virtually all protease and peptidase activities from *B. subtilis* extracts. This method has allowed the purification of undegraded RNA polymerase, which has been used in the isolation of RNA polymerase subunits (12) and in subunit reconsititution studies (11). This protease removal method was also necessary for the isolation of the precursor spore coat protein and nascent protein. It is highly likely that a protease activity is necessary for the conversion of precursor spore coat protein to the mature coat protein (4).

The soluble nature of the precursor spore coat protein versus the relatively insoluble property of the mature spore coat protein indicates that the precursor form may be synthesized and then transported to the surface of the forespore prior to conversion to the mature form. Since a large

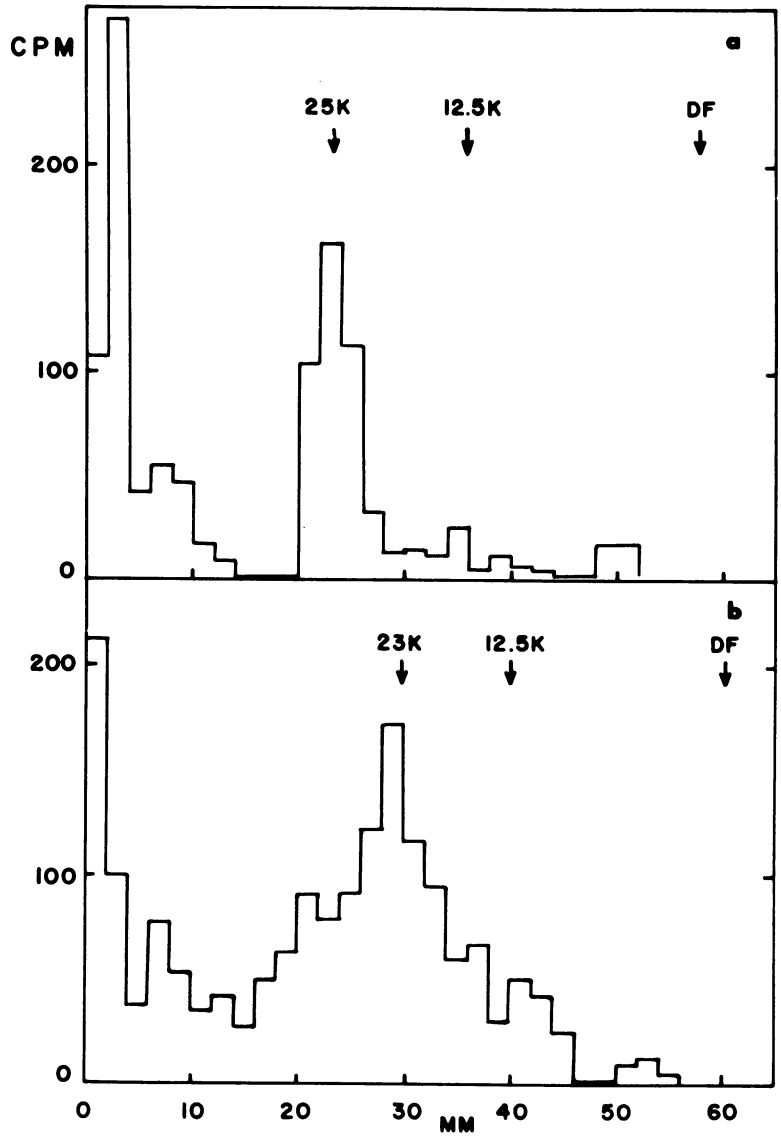

FIG. 5. *Sodium dodecyl sulfate-urea-polyacrylamide gel electrophoresis patterns of in vitro synthesized complete and nascent (1,400 cpm) spore coat proteins. (a) Complete (1,000 cpm) and (b) nascent (1,400 cpm) spore coat proteins synthesized in vitro with [^{3}H]phenylalanine were immunologically purified from the reaction mixtures of cell-free systems from t_2, t_3, and t_4 stages as described in the text. After the sample was applied to the top of the stacking gel, electrophoresis was carried out, and the counts incorporated into the gels (12%) were measured as described in the text. Standard markers were run simultaneously, and molecular weights were determined from their mobilities. The arrows indicate the mobility of complete precursor coat protein (25 K), nascent precursor coat protein (23 K), mature spore coat protein (12.5 K), and the dye front (DF).*

amount of spore coat protein is necessary (1), the precursor form may serve as a soluble pool in the early sporulation stages (t_1 to t_3) and then be converted at around t_4 to the mature form as it is laid down on the forespore.

A functional modification of the translational system in *B. subtilis* during sporulation has been reported (2, 3). These reports indicate the necessity for a homologous translational system, since it is possible that the spore coat mRNA may only be translated with high fidelity by the modified translation system. The use of an in vitro system from sporulating cells freed of proteases is therefore particularly important, since

the high level of proteases present in sporulating cells might yield anomalous results.

The early initiation of precursor spore coat protein synthesis at t_{1-2} makes it a product of an "early" sporulation gene. It is interesting that most of the RNA polymerase modifications that have been noted in sporulating *B. subtilis* cells occur at t_3 or later (9, 10, 14). The analysis of $t_{1.5}$ RNA polymerase (9, 10) indicated that the cells apparently contained only vegetative holoenzyme. However, a finer analysis of the property of RNA polymerase from t_0 to t_3 cells may indicate some modifications in its properties. It is also possible that the expression of the sporulation coat protein gene is not dependent on modification of RNA polymerase structure, but on other types of control, such as release from catabolite repression or antitermination (6, 7).

The use of suitable DNA fragments, sporulation RNA polymerase, and an in vitro protein-synthesizing system should be valuable in future studies on the regulation of gene expression during sporulation.

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