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Two Polypeptides Associated with the Ribonucleic Acid Polymerase Core of *Bacillus subtilis* During Sporulation

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The ribonucleic acid (RNA) polymerase from log-phase and sporulating cells of *Bacillus subtilis* was analyzed to determine whether any structural changes occurred during sporulation. The elution pattern of RNA polymerase from a deoxyribonucleic acid (DNA)-cellulose column revealed that sporulating cells at stages III and IV contained a new RNA polymerase fraction in addition to the vegetative holoenzyme ($\alpha_2\beta\beta'\sigma$). Stage III cells contained the vegetative holoenzyme and a new enzyme with the composition $\alpha_2\beta\beta'\delta^1$; the molecular weight of δ^1 was 28,000. Stage IV cells contained the vegetative holoenzyme, the δ^1-containing enzyme, and another enzyme with the composition $\alpha_2\beta\beta'\delta^2$. The δ^2 factor had a molecular weight of around 20,000. The δ-containing enzymes have a higher affinity for the DNA-cellulose column and a higher specific activity on various templates than vegetative holoenzyme. The simultaneous appearance of these enzymes with vegetative holoenzymes in sporulating cells is consistent with the data found previously with DNA-RNA hybridization studies, which showed that sporulating cells contained both vegetative and sporulation messenger RNAs.

Several types of evidence indicate that specificity of transcription is altered during sporulation of *Bacillus subtilis*. Analyses of messenger ribonucleic acid (RNA) present in sporulating cells have indicated the presence of transcripts from genes expressed during both vegetative growth and sporulation and from genes expressed only during sporulation (5, 6, 24). The reduced ability of sporulating cells to replicate certain phage also suggests that the RNA polymerase (RPase) in sporulating cells may have altered specificity in recognizing or transcribing phage genes (10, 19, 25). In addition, the isolation of conditional RPase mutants that are temperature sensitive only during sporulation (12; C. Sumida, L. Santo, and R. H. Doi, Abstr. Annu. Meet. Am. Soc. Microbiol. 1973, G214, p. 61) suggests that the RPase in sporulating cells has functions that are different from those found in vegetative cells.

One possible mechanism for changing the specificity of RPase would be to modify the structure of RPase by addition of a new sigma-like factor (4, 18). Recent studies have indicated that sporulating cells of *B. subtilis* contain sporulation-specific polypeptides associated with RPase core (8, 9, 15, 21). The data in this paper indicate that sporulating cells contain two new polypeptides independently associated with RPase core in addition to a sigma-containing holoenzyme, which was very similar or identical to the vegetative cell holoenzyme. When the RPase core contained the new polypeptide, sigma factor was no longer attached to that core.

MATERIALS AND METHODS

Bacterial strains and media. *B. subtilis* 168 wild type was grown at 37°C in modified Schaeffer medium (2× SG) (13) for all preparations of enzyme. To obtain maximum synchronous sporulation, cells were transferred three times at log stage to fresh 2× SG medium as described previously (24). The final (fourth) 10-liter culture in the fermentor was stopped at the proper time by adding NaN_3 (final concentration, 1 mM) and $MgCl_2$ (final concentration, 1 mM) and by chilling rapidly by the addition of −70°C ice. Cells were washed once in buffer B (see below) and stored at −70°C.

B. subtilis 168 Ts-1 Rif^r (C. Sumida, L. Santo, and R. H. Doi, Abstr. Annu. Meet. Am. Soc. Microbiol. 1973, G214, p. 61) was grown at either the permissive temperature (32°C) or at the nonpermissive temperature (47°C) in the same medium; rifampin (1 μg/ml) was added to the first and second cultures to prevent the growth of revertants. The final culture was discarded if revertants were found.

Buffers. Buffer A contained 0.01 M tris(hydroxymethyl)aminomethane (Tris)hydrochloride (pH 7.8), 0.01 M $MgCl_2$, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.3 mM dithiothreitol (DTT), and 0.1 M KCl.

Buffer B contained 0.1 M Tris-hydrochloride (pH 7.8), 0.01 M $MgCl_2$, 1 mM EDTA, 0.3 mM DTT, 10% (vol/vol) glycerol, and 2 mM phenylmethylsulfonyl fluoride (PMSF).

Buffer D contained 0.02 M Tris-hydrochloride (pH 7.8), 1 mM EDTA, 0.3 mM DTT, 10% (vol/vol) glycerol, and 2 mM PMSF.

PMSF was dissolved in the buffers just before use.

RPase purification. The RPase was purified by a modification of the phase-separation method of Babinet (2). Others (1, 11, 23) have reported favorably on his method.

(i) Crude extract. Cells (50 g wet weight) were suspended in an equal volume (50 ml) of buffer B and combined with approximately fivefold (wt/vol) cold (−20°C), washed, 0.1-mm glass beads. The mixture was exposed to maximum vibration in a Vibrogen Mill for 10 to 15 min and extracted with a threefold excess (vol/wt, 150 ml) of buffer B. The extract was centrifuged at 30,000 × *g* for 30 min to obtain the crude supernatant extract.

(ii) Phase separation. Phase separations 1 and 2 were performed as described by Babinet (2). Polyethylene glycol phase 2 (PEG2) thus obtained was dialyzed against two changes of seven- to eightfold excess buffer B containing 8% (wt/wt) PEG 6000 for 8 h. Dextran T500 was added to a final concentration of 1% (wt/wt) to the dialyzed PEG2. The mixture was stirred for 60 min at 0°C and centrifuged at 30,000 × *g* for 10 min. The dextran (DEX) phase and precipitates thus obtained contained more than 95% of total RPase activity and were dissolved in a fivefold excess (vol/vol) of buffer D plus 0.02 M KCl. This fraction was called DEX3.

Some comments on the phase partitioning method are necessary at this point, since Babinet's phase partitioning method (2) was altered after phasing step 2. To analyze the change in RPase during sporulation, it is important to recover as much enzyme from the cells as possible. In this respect, Babinet's method was favorable, starting from the cell extract after removal of cell debris by low centrifugation. Because the ammonium sulfate fractionation step did not give a consistent recovery of the enzyme, the method was modified. This modification was based on the observation that purified RPase, which was free from nucleic acid, was partitioned between the phases in the same way as the enzyme in the crude extract (Table 1). The purified enzyme moved into the dextran phase (DEX1) in the presence of 2 M NaCl at the first phasing but was recovered in the PEG phase (PEG2) from the DEX1 phase in the presence of 4.5 M NaCl at the second phasing. The recovery was always very good.

To concentrate the RPase in the PEG2 phase, the salt was removed by dialysis, and a small amount of dextran was added. Upon removal of the salt, the RPase was found in the dextran phase. By this method, we were always able to recover more than 90% of the RPase from a crude extract in a small volume (Table 2).

(iii) DEAE-cellulose column chromatography. The DEX3 fraction was applied to a diethylaminoethyl (DEAE)-cellulose column and eluted with a linear gradient of KCl (from 0.15 to 0.6 M) in buffer D. RPase activity was eluted as a single peak with some tailing of activity (Fig. 1). Essentially the same elution pattern was found for all enzyme preparations reported in this paper.

TABLE 1. *Phase partitioning of purified RPase*[a]

Step of phase separation	NaCl (M)	Recovery of activity (%)
Enzyme solution	0	100
PEG1	2	3.3
PEG2	4.5	81.8
DEX2	4.5	5.2
PEG3	0	6.7
DEX3	0	78.2

[a] RPase was purified from vegetative *B. subtilis* through phase separation, DEAE-cellulose, DNA-cellulose, and Bio-Gel A-1.5m in high-salt buffer (see Materials and Methods). The purity of the enzyme was more than 95% without any detectable contamination of nucleic acids. A 2-ml portion of the enzyme solution (30 μg/ml) was treated as above.

The active fractions were combined and dialyzed against a 20-fold excess (vol/vol) of buffer D for 4 h.

(iv) DNA-cellulose column chromatography. Calf thymus deoxyribonucleic acid (DNA)-cellulose was prepared by the method of Litman (17) and equilibrated with buffer D containing 0.02 M KCl.

The dialyzed DEAE-cellulose fraction was applied to the DNA-cellulose column. After washing, the column was eluted with a linear gradient of KCl (from 0.02 to 1.22 M) in buffer D.

(v) Glycerol gradient centrifugation. Each peak of activity of DNA-cellulose column was pooled and concentrated in dialyzing tubing in vacuo. Then the samples were brought to an ammonium sulfate concentration of 65% by dialysis against an ammonium sulfate solution in the presence of 0.1 M Tris-hydrochloride (pH 7.8), 1 mM EDTA, 0.3 mM DTT, 10% glycerol, and 1 mM PMSF for 12 h. The precipitates thus formed were collected by centrifugation and dissolved in a small volume of buffer A containing 1 mM PMSF. After dialysis against the same buffer containing 10% glycerol, they were centrifuged through a 13-ml linear glycerol gradient (15 to 35%) in buffer A containing 0.5 mM PMSF using a Beckman RPS40 rotor at 38,000 rpm for 21 h at 4°C.

(vi) Use of diisopropylfluorophosphate as a protease inhibitor. Diisopropylfluorophosphate (final concentration, 1 mM) was added at the following steps of the enzyme preparation: PEG2 before dialysis, DEX3 before DEAE-cellulose column chromatography, and the pooled enzyme fractions from the DEAE-cellulose column. The recovery of the enzyme was improved considerably without any detrimental effects on the properties of the enzyme.

(vii) Recovery of RPase. Table 2 shows the purification steps of RPase from the various growth stages. The yield of enzyme from sporulating cells varied from preparation to preparation.

(viii) RPase assay. The volume of the assay mixture was 0.125 ml or 0.25 ml. It contained Tris-

TABLE 2. *RPase purification*

Phase	Purification steps	Protein (mg)	Total act (U[a])	Sp act[b]	Purification	Yield (%)
Log	Crude extract	3,960	54,000	14	1	100
	PEG2	540	65,675	122	9	122
	DEX3	244	69,275	284	21	128
	DEAE column[c]	115	56,215	488	36	104
	DNA column[d]	11	30,355	480 (A)[e]	35	
				1,055 (B)	78	56
$t_{1.5}$	Crude extract	3,120	51,698	17	1	100
	PEG2	648	63,010	97	6	122
	DEX3	307	32,018	104	6	62
	DEAE column	140	20,000	112	7	39
	DNA column	9	9,605	70 (A)	4	
				350 (B)	21	19
$t_{3.5}$	Crude extract	2,463	15,334	6	1	100
	PEG2	434	34,784	80	13	227
	DEX3	162	22,950	142	23	150
	DEAE column	90	16,026	214	35	105
	DNA column	12	7,310	860 (A)	139	
				1,091 (B)	176	47
				1,270 (C)	205	
$t_{5.5}$	Crude extract	3,599	20,248	9	1	100
	PEG2	369	29,579	80	9	146
	DEX3	174	15,756	91	10	78
	DEAE column	80	7,500	228	25	37
	DNA column	3	2,480	800 (A)	90	
				1,600 (B)	180	12
				3,400 (C)	384	

[a] Nanomoles of UMP incorporated per 10 min.
[b] Units/milligram of protein.
[c] DEAE-cellulose column.
[d] DNA-cellulose column.
[e] (A), (B), and (C) indicate peaks A, B, and C, respectively (see text).

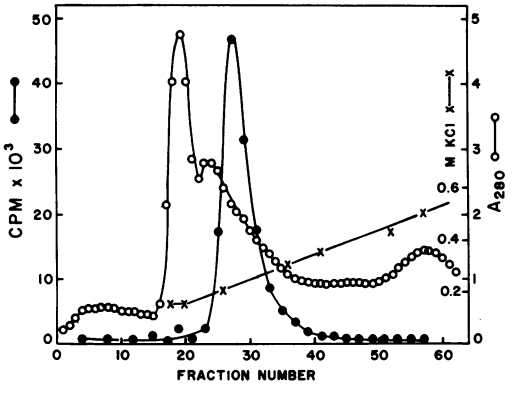

FIG. 1. *Elution pattern of RPase from a DEAE-cellulose column. The DEX3 fraction from log-phase cells (see Table 2) was loaded onto a column (1.9 by 20 cm) in the presence of 0.02 M KCl and eluted with a linear KCl gradient from 0.15 M (150 ml) to 0.60 M (150 ml). The column was equilibrated with buffer D containing 0.15 M KCl before the DEX3 fraction was added. Buffer D was also used for the gradient buffer. Fractions of 5 ml were taken, and 25-μl samples were assayed for RPase activity.* ●, *Counts per minute of the* ^{3}*H-labeled uridine monophosphate incorporated into RNA. The KCl concentration of the fractions was determined from the refractive index of the solution.* A_{280}, *Absorbance at 280 nm.*

hydrochloride (pH 7.8), 120 mM; $MgCl_2$, 10 mM; $MnSO_4$, 2 mM; DTT, 0.4 mM; KCl, 150 mM; adenosine 5'-triphosphate, guanosine 5'-triphosphate, and cytidine 5'-triphosphate, each at 0.4 mM; uridine 5'-triphosphate (UTP), 0.2 mM; ^{3}H-labeled UTP, 2 μCi; and poly[d(A-T)], 2 to 3 μg. Other details are as described elsewhere (14).

(ix) SDS-polyacrylamide gel electrophoresis. The sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis method was performed according to the procedure of Neville (20). The β and β' subunits were clearly separated by this method. When the protein concentration was very low, the samples were dialyzed against an SDS solution containing 2 mM Tris-hydrochloride, 0.3 mM DTT, 1 mM PMSF, and 0.1% SDS, pH 7.9, and then lyophilized to dryness. The lyophilized sample was dissolved in a small volume of a solution containing 1% SDS, 1 mM DTT, and 1 mM PMSF and then dialyzed against

upper gel buffer containing 1 mM DTT, 1 mM PMSF, 1% SDS, and 10% glycerol.

Materials. Tris base, ^{3}H-labeled UTP, enzyme-grade ammonium sulfate, and calf thymus DNA were obtained from Schwarz/Mann; PEG 6000–7500 was from MCB Co.; dextran T500 was purchased from Pharmacia; DTT, diisopropylfluorophosphate, and PMSF were obtained from Calbiochem; glass beads (Glasperlen, 0.1 to 0.15 mm) were from B. Braun Melsungen Apparateban; DEAE-cellulose (DE52) was from Whatman; cellulose (Cellex410) was purchased from Bio-Rad; ribonucleotide triphosphates were from P-L Biochemicals; polyacrylamide, *N,N'*-methylenebisacrylamide, *N,N,N',N'*-tetramethylethylenediamine (optical grade), glycerol, and ammonium persulfate were from Eastman Kodak Co.; sodium dodecyl sulfate was obtained from Sigma; and nonenzymic protein molecular weight markers were from Mann Laboratories. Rifampin was generously provided by E. R. Newman and H. Heymann, and phospholipase A was a gift from T. Nishihara.

RESULTS

DNA-cellulose pattern of sporulating cells. Two peaks of RPase activity were eluted from a DNA-cellulose column when the enzyme was prepared from vegetative cells. The first peak (peak A) eluted at 0.55 to 0.60 M KCl, and the second one (peak B) eluted at 0.65 to 0.70 M KCl (Fig. 2a). The subunit composition of peak A

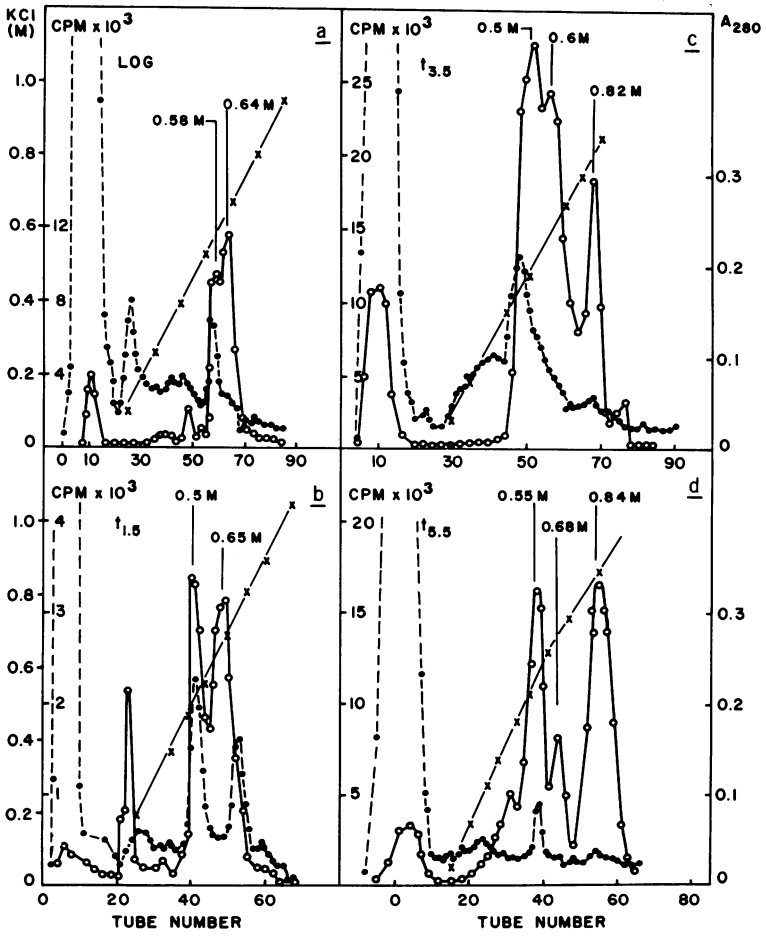

FIG. 2. *Elution pattern of RPase from cells harvested at various stages of growth and sporulation from DNA-cellulose columns. The fractions from the DEAE-cellulose column containing RPase activity (Fig. 1) were dialyzed against buffer D containing 0.02 M KCl. The sample was then applied to a DNA-cellulose column (1.5 by 15 cm), which had been equilibrated with buffer D containing 0.02 M KCl, at a flow rate of 5.7 ml/h. The enzyme was eluted from the column with a linear gradient of 0.02 M KCl-buffer D (80 ml) to 1.22 M KCl-buffer D (80 ml) at a flow rate of 10 ml/h. Fractions of about 3.3 ml were taken, and 25-μl samples were assayed for RPase activity* (○). *The absorbance at 280 nm (A_{280})* (●) *and KCl concentration* (×) *were also monitored. (a) Log phase; (b) $t_{1.5}$ cells; (c) $t_{3.5}$ cells; and (d) $t_{5.5}$ cells. The KCl concentration of the fractions was determined from the refractive index of the solution.*

was $\alpha_2\beta\beta'$ and that of peak B was $\alpha_2\beta\beta'\sigma$ (see Fig. 5A, lanes a and b, respectively). Therefore, peak A is RPase core and peak B is sigma-containing holoenzyme.

The elution patterns from the DNA-cellulose column for those enzymes that were prepared from sporulating cells at $t_{1.5}$ (stage I), $t_{3.5}$ (stage III), and $t_{5.5}$ (stage IV) are shown in Fig. 2b–d. At $t_{1.5}$ (Fig. 2b), two peaks were observed: one eluted at 0.5 M KCl, and the other eluted at 0.65 M KCl. These were found to correspond to peaks A and B of the vegetative enzyme (Fig. 2a), respectively. In addition to peaks A and B, another sharp peak of activity was observed to elute at 0.82 M KCl for $t_{3.5}$ cells (Fig. 2c), which was designated as peak C. At $t_{5.5}$ (Fig. 2d) compared to peaks A and B, the activity eluted at peak C increased further as a slightly broader peak. A similar elution pattern was observed for $t_{7.0}$ cells (data not shown). Thus, the new peak C at $t_{3.5}$ and $t_{5.5}$ was found only in sporulating cells and is thus specific for sporulation.

Some characteristics of RPase from peaks A, B, and C. (i) Specific activity of the sporulating enzymes. The specific activities of peaks A, B, and C have been summarized in Table 3 for the various sporulation stages. The ratio of specific activities for peaks A and B was similar to that for the vegetative enzymes. The specific activity of peak C was the highest, usually being three to five times higher than the other two peaks, but only a small amount of protein was obtained in peak C.

(ii) DNA templates. Figure 3 illustrates the activity of vegetative enzyme (peaks A and B combined) and sporulation enzymes with poly-[d(A-T)], phage ϕe DNA, and with *B. subtilis* DNA as template. There is a significant difference in activity between vegetative and sporulation RPase for phase ϕe DNA, which has already been reported (3, 16). For all three enzyme peaks the activity with phage ϕe DNA was reduced compared to the activity with poly[d(A-T)] as sporulation proceeded.

(iii) Activity with Mg^{2+} or Mn^{2+}. A test for the optimum concentration of Mg^{2+} ion showed that there was no difference among those enzyme peaks from vegetative or sporulating cells. An optimum concentration of about 4 mM

TABLE 3. *Specific activity of RPase in peak tubes from a DNA-cellulose column*[a]

Peak	KCl (M)	Log	$t_{1.5}$	$t_{3.5}$	$t_{5.5}$
A	0.5–0.58	480	70	860	800
B	0.6–0.68	1,055	350	1,091	1,600
C	0.82–0.84			1,270	3,400

[a] Nanomoles of UMP incorporated/10 min per milligram of protein. The template used was poly[d(A-T)].

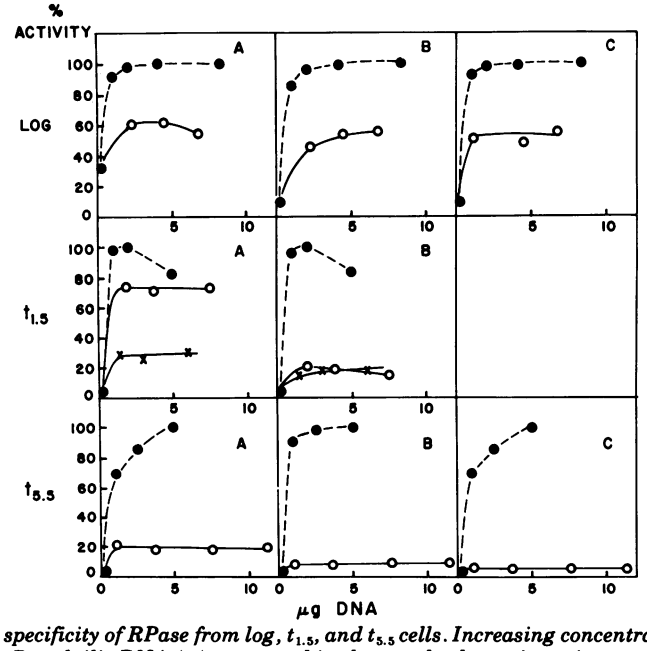

FIG. 3. *Template specificity of RPase from log, $t_{1.5}$, and $t_{5.5}$ cells. Increasing concentrations of poly[d(A-T)]* (●), *Φe DNA* (○), *or B. subtilis DNA* (×) *were used in the standard reaction mixtures with enzyme from log cells (A, crude enzyme; B, DEX3 fraction; C, DNA-cellulose fraction containing a mixture of peaks A + B), $t_{1.5}$ cells (A, DC 41; B, DC 48), or $t_{5.5}$ cells (A, DC 38; B, DC 44; C, DC 55). DC, DNA-cellulose column fractions from Fig. 2.*

Mg^{2+} was noted. Sporulating enzymes ($t_{5.5}$, peaks A and C) required a slightly higher Mn^{2+} ion concentration (3 to 4 mM) compared to the vegetative enzymes (2 mM) (data not shown).

(iv) Rifampin sensitivity. Figure 4 shows typical results of rifampin inhibition of RPase from crude extracts and PEG2 and DEX3 fractions from $t_{3.5}$ and $t_{5.5}$ cells. At no time during sporulation was any rifampin-resistant RPase activity found. The same pattern of inhibition was observed for enzymes purified with a DNA-cellulose column.

(v) SDS-polyacrylamide gel electrophoresis pattern. The sporulation RPase subunits of peaks A and B were identical with those for vegetative cells, showing $\alpha_2\beta\beta'$ for A and $\alpha_2\beta\beta'\sigma$ for B (Fig. 5A, lanes a and b, and Fig. 5B, lanes a and b).

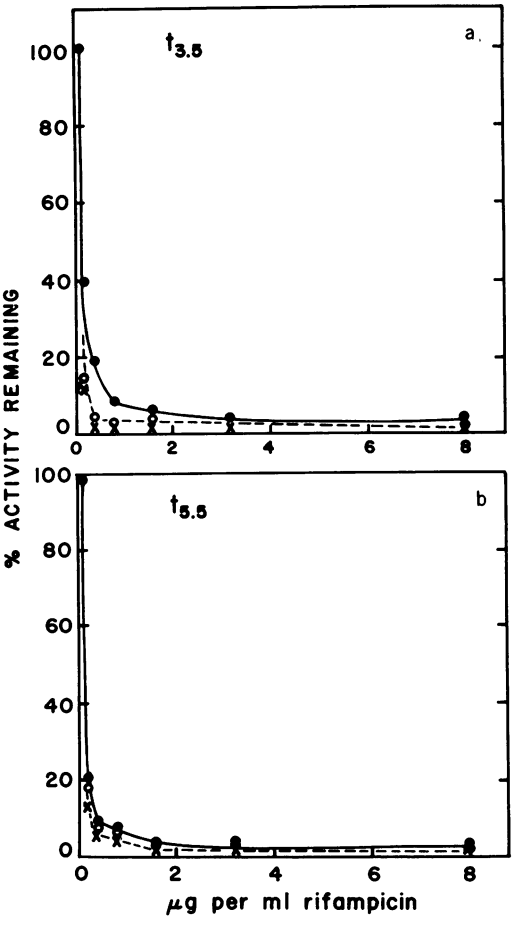

FIG. 4. *Rifampin sensitivity at various stages of purification of RPase. Increasing amounts of rifampin were added to reaction mixtures containing crude extract (●), PEG2 (○), and DEX3 (×) fractions (see Table 2) from (a) $t_{3.5}$ and (b) $t_{5.5}$ cells.*

Peak C RPase from $t_{3.5}$ cells (Fig. 5B, lane c) contained a new subunit (δ^1) with a molecular weight of 28,000 (Fig. 6). The new subunit was present in a molar ratio to α_2 of about 1.0 (Table 4), thus making the subunit composition $\alpha_2\beta\beta'\delta^1$.

The peak C for $t_{5.5}$ cells contained an RPase that eluted in a rather broad peak at 0.84 M KCl. This broad peak was analyzed for subunits, and another small subunit (δ^2) of molecular weight 20,000 (Fig. 6) was associated with the early-eluting fractions of the peak (Fig. 5C, lanes b–d) and δ^1 was found with the late-eluting fractions of the peak (Fig. 5C, lanes c–e).

Therefore, peak C is thought to be composed of two overlapping peaks of enzymes, one with a subunit composition of $\alpha_2\beta\beta'\delta^2$, eluting at the lower KCl concentration, and the other with $\alpha_2\beta\beta'\delta^1$, eluting at the higher KCl concentration. The combined molarity of δ^1 and δ^2 was about equal to that of α_2 (Table 4).

(vi) Glycerol gradient centrifugation pattern. To see whether the small δ^1 peptide was truely associated with the core subunits, the enzyme was prepared from sporulating $t_{4.5}$ cells, and the three enzyme peaks were eluted from the DNA-cellulose column. Each peak fraction was pooled separately and concentrated and subjected to glycerol gradient centrifugation in the presence of 0.1 M KCl (see above). Each enzyme was recovered as a single peak of about 15S (Fig. 7), which was analyzed by SDS-polyacrylamide gel electrophoresis. These gels (Fig. 8) showed that δ^1 (Fig. 8c) as well as σ (Fig. 8b) were associated with core subunits. The poor separation of the DNA-cellulose peaks resulted in overlapping of peaks B and C. However, it is clear that the σ and δ^1 factors sediment with the RPase core. Subsequent studies have shown that δ^1 factor is in fact more tightly associated with the core than σ factor (T. Nakayama and R. H. Doi, unpublished data).

DNA-cellulose column elution pattern of RPase from a temperature-sensitive asporogenic mutant Ts-1. Asporogenic mutant Ts-1 was selected from rifampin-resistant mutants (C. Sumida-Yasumoto and R. H. Doi, unpublished data). This mutant can grow normally at both 32 and 47°C during the vegetative-growth phase; however, whereas sporulation can occur at 32°C, it does not occur at 47°C. The sporulation process is stopped at stage I (C. Sumida-Yasumoto, L. Santo, and R. H. Doi, unpublished data), and the species of RNAs synthesized in vivo were only those of stage I, as shown by hybridization-competition experiments. This mutation is a single-site (point) mutation on the rifampin locus as concluded by

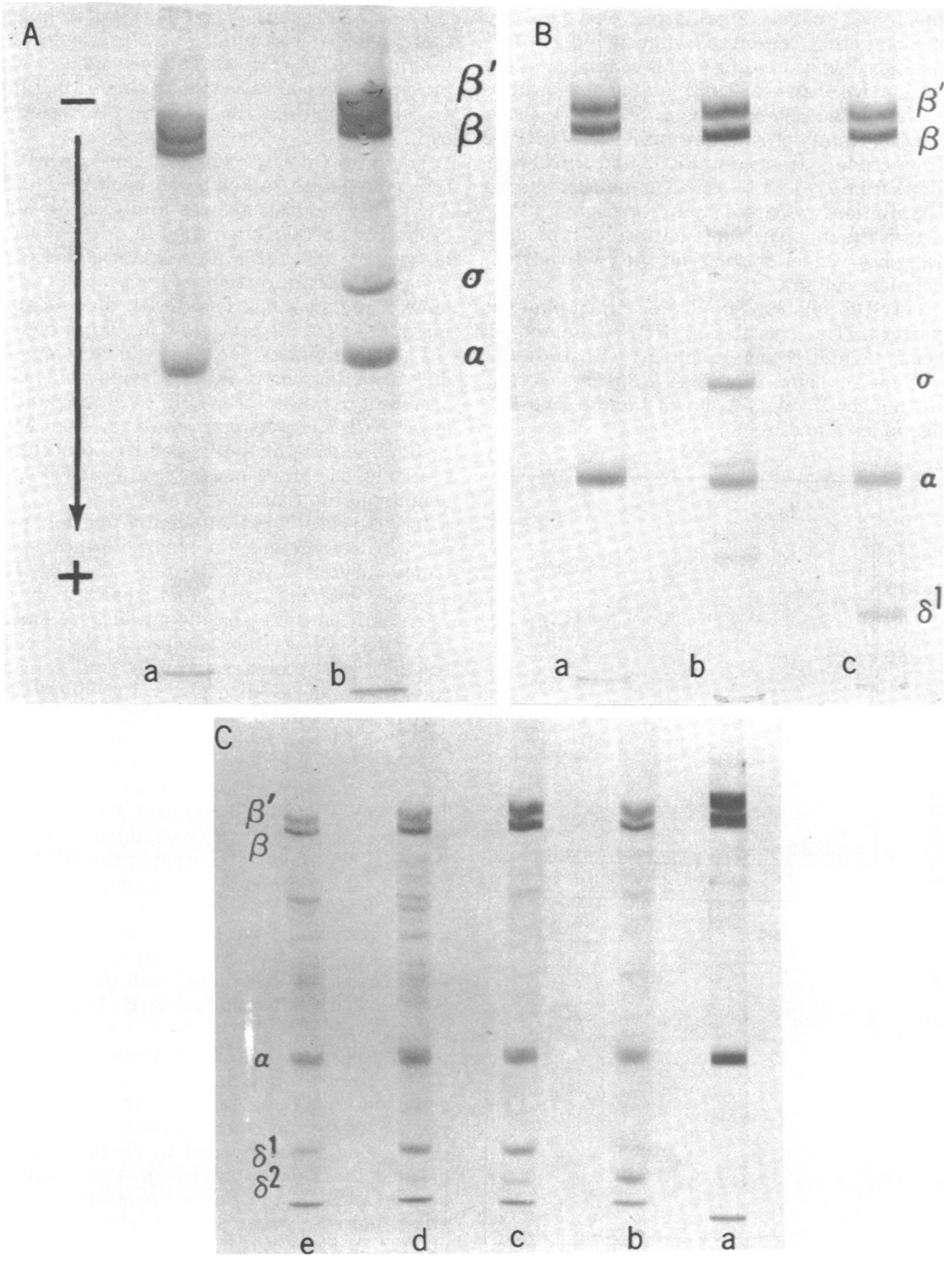

FIG. 5. *SDS-polyacrylamide gel electrophoresis of subunits of various RPase species. (A) Vegetative cell: (a) peak A or core enzyme; (b) peak B or holoenzyme. (B) $t_{3.5}$ cells: (a) peak A or core enzyme; (b) peak B or holoenzyme; (c) peak C or δ^1 enzyme. (C) $t_{5.5}$ cells: (a) peak A or core enzyme; (b–e) peak C or δ enzyme [(b) DC fractions (Fig. 2) 51 to 52, (c) DC fractions (Fig. 2) 53 to 54, (d) DC fractions (Fig. 2) 58 to 59, (e) DC fractions (Fig. 2) 61 to 62]. DC, DNA-cellulose column. The percentage of gel used in these experiments was 11%; however, these gels are low cross-linked gels (19).*

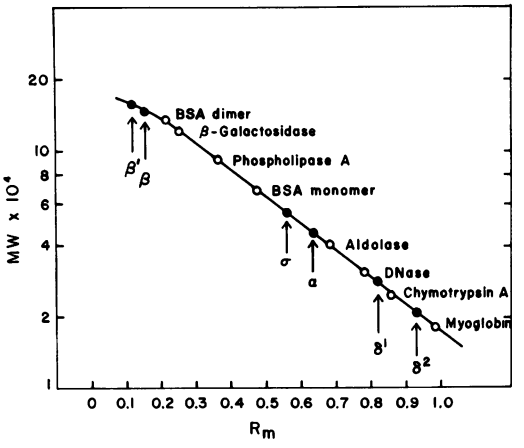

FIG. 6. *Molecular weights of δ^1 and δ^2 factors. The data were obtained from the results shown in Fig. 5B and 5C. The R_m has been plotted against the log of the molecular weight.*

TABLE 4. *Molar ratio of δ^1 and δ^2 to α_2 (core enzyme)*[a]

Stage	DC no.[b]	δ^1/α_2	δ^2/α_2	$\delta^1 + \delta^2/\alpha_2$
$t_{3.5}$	68	1.08		1.08
$t_{5.5}$	51		0.92	0.92
	53	0.48	0.50	0.98
	57	0.62	0.38	1.00
Ts-1 grown at 32°C	60	0.62		0.62

[a] The quantitative distribution of the protein components was determined by weighing the area of paper corresponding to the optical density peaks of the densitometer tracings.

[b] DNA-cellulose column fraction number (Fig. 2 and 5 for $t_{3.5}$ and $t_{5.5}$; Fig. 9 for Ts-1).

transformation experiments and reversion frequency.

To determine the relationship between peak C and sporulation, we analyzed the DNA-cellulose column pattern of this mutant. The cells were grown to stage III of sporulation at the permissive temperature (32°C). This was 11 h after inoculation into the final culture. As shown in Fig. 9b, two peaks were observed to elute from the DNA-cellulose column, one at 0.69 M KCl, with a small shoulder of activity at 0.79 M KCl, and the other at 1.0 M KCl.

Subunit analysis of these two peaks revealed their identities to be those of the wild-type enzymes. The first peak was $\alpha_2\beta\beta'$, and the second one was $\alpha_2\beta\beta'\delta^1$. Very little holoenzyme ($\alpha_2\beta\beta'\sigma$) was detected in these preparations.

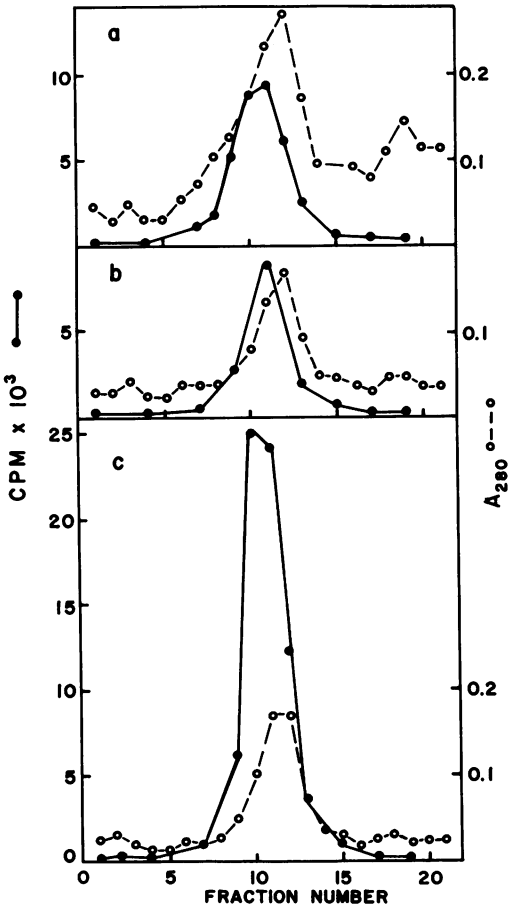

FIG. 7. *Glycerol gradient centrifugation pattern of $t_{4.5}$ cell RPase peaks A, B, and C. The method is described in Materials and Methods. RPase from $t_{4.5}$ cells was fractionated into peaks A, B, and C by DNA-cellulose column chromatography as described in the legend to Fig. 2. Each peak of activity was pooled, concentrated, and analyzed by glycerol gradient centrifugation. As a reference, β-galactosidase (15S) and catalase (11.5S) were centrifuged in the fourth tube. RPase activity (●) and absorbance at 280 nm (A_{280}; ○) are shown. (a) Peak A, (b) peak B, and (c) peak C.*

The molar ratio of δ^1 to α_2 was 0.6 in this case (Table 4); the reason for this lower ratio is not known, although it is possible that the core with the mutated β subunit may bind the δ^1 subunit less tightly, resulting in some loss of δ^1 during the purification procedure. It also appears that the amino acid substitution of the mutation resulted in stronger binding of the Ts-1 core enzyme to the DNA-cellulose column, thus making every peak elute at a higher salt concentration than that of the wild-type enzymes.

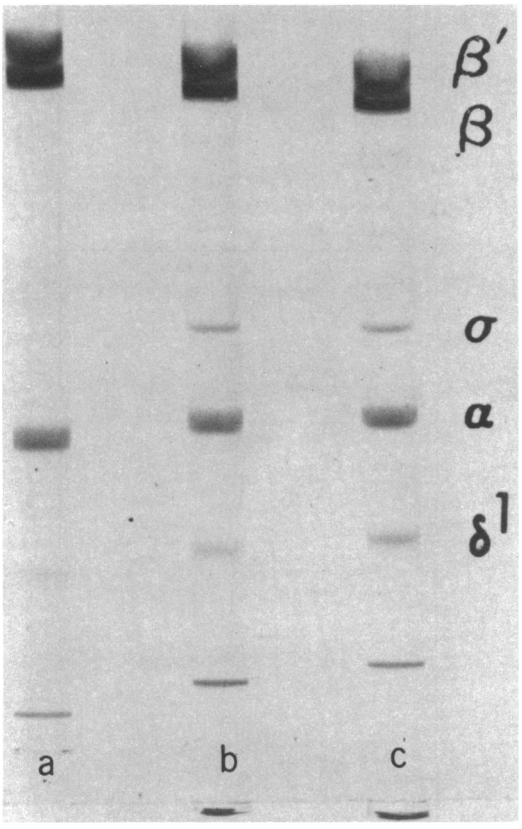

FIG. 8. *SDS-polyacrylamide gel electrophoresis pattern of peak fractions of glycerol gradient centrifugation (Fig. 7). (a) Peak A; (b) peak B; (c) peak C.*

Next, the mutant was grown at 47°C for 7 h after inoculation into the final culture. At this nonpermissive temperature, the sporulation development was stopped at stage I. The DNA-cellulose column elution pattern of extracts obtained from cells grown at 47°C showed only one peak, which eluted at 0.69 M KCl with a shoulder of activity at 0.79 M KCl (Fig. 9a). The former corresponded to peak A, and the shoulder corresponded to peak B (data not shown). Neither activity nor enzyme protein was eluted at the higher KCl concentration of 1.0 M.

Thus, it was shown that peak C appeared only after stage II of the normal sporulation process, whereas peaks A and B were common to vegetative cells and to sporulating cells.

DISCUSSION

The results indicate strongly that the RPase of *B. subtilis* cells is modified during sporulation by the addition of new polypeptides to the RPase core. We have based our conclusion on the following observations. (i) There is a stoichiometric amount of δ factor bound to the core. Several other larger polypeptides are occasionally associated with the core, but never in stoichiometric quantities. (ii) The polypeptide is tightly associated with the core and is not separated by passage through a DNA-cellulose column nor through a glycerol gradient. Furthermore, Nakayama and Doi (Fed. Proc. 35:1638, 1976) have shown that δ factor is not removed from the core by passage through a phosphocellulose column under conditions that readily remove sigma factor. (iii) The δ factor does not appear when the normal sporulation process is blocked in a temperature-sensitive mutant (Fig. 9a); also, little or no δ enzyme appears when sporulation is specifically blocked by the antibiotic netropsin at stage II (Nakayama and Doi, unpublished observation). (iv) The specific activity of the δ enzyme is higher than that of the vegetative holoenzyme, and the only apparent difference is the presence of the δ^1 factor. The results of Linn et al. (15) support the presence of the δ^1 at t_3. Although they also reported the presence of an 85,000-dalton polypeptide with their sporulation RPase, we have not been able to find this polypeptide consistently associated with our enzyme in stoichiometric amounts.

The relationship of the sporulation δ factors to the RPase core-associated polypeptides reported in log-phase cells of *B. subtilis* by Pero et al. (22), Duffy and Geiduschek (7), and Holland and Whiteley (9) is still uncertain. Polypeptides with molecular weights of 9,500 (7), 11,000 (7, 22), 21,500 (22), and 60,000 (9) were observed associated with the core in log-phase cells. The different results obtained by these various investigators probably reflect differences in purification procedures, which could effect differential removal of RPase core-associated polypeptides. It seems likely that the fine regulation of RPase activity must involve polypeptides in addition to the sigma factor. Since the δ factors reported in this article were observed only with sporulation RPase and not with vegetative cell RPase purified in an identical manner, it is likely that they are different from the polypeptides associated with vegetative cell core. We have also noted small polypeptides associated with vegetative cell core and are currently determining their molecular weights and stoichiometric relationship with the core enzyme.

Another significant observation was the simultaneous presence of sigma-containing holoenzyme in sporulating cells through stage IV (Fig. 2). We have consistently observed the presence of large quantities of sigma enzyme in sporulating cells. This result actually is con-

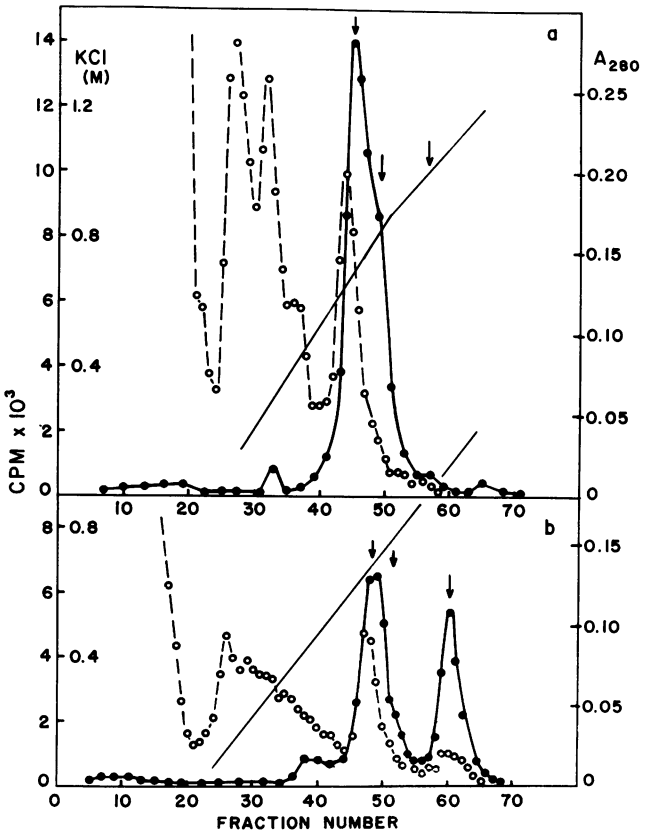

FIG. 9. *Elution pattern of RPase of Ts-1 cells from the DNA-cellulose column. The column chromatography was performed as described in the legend of Fig. 2 except that the enzyme was eluted from the column with a linear gradient of 0.02 M KCl-buffer D (80 ml) to 1.5 M KCl-buffer D (80 ml). (a) The cells were grown at the permissive temperature (32°C) to stage III of sporulation. (b) The cells were grown at the nonpermissive temperature (47°C) to a time equivalent to stage III for wild-type cells. RPase activity (●), absorbance at 280 nm (A_{280}; ○), and KCl concentration (solid straight line) are shown.*

sistent with earlier observations that sporulating cells contain mRNA's found in vegetative cell (5, 6, 24). Since vegetative holoenzyme would recognize vegetative gene promoters, it is likely that the sigma enzyme is continuing to transcribe these "vegetative genes" during sporulation.

Finally, the sequential appearance of the δ-containing enzymes suggests that the sequential appearance of sporulation mRNA's (24) could be related to some sequential change in the property of the RPase during spore formation. One possibility is that these new forms of the enzyme recognize a new set of genes necessary for the morphological development of the spore.

Currently studies are being pursued to demonstrate that the δ factors definitely differ in primary amino acid sequence from the sigma factor and from each other and that they have different specificities in transcription. These results should clarify the role of the δ factors in sporulating cells.

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