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RNA Polymerase from *Bacillus amyloliquefaciens* Infected with ϕ29 Bacteriophage

(properties/subunit composition/time of modification)

MICHAEL HOLLAND* AND H. R. WHITELEY

Department of Microbiology, University of Washington, Seattle, Wash. 98195

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ABSTRACT **DNA-dependent RNA polymerase was purified from uninfected and ϕ29-infected *Bacillus amyloliquefaciens*. Differences were observed in the specific activities, template specificities, stability, and sedimentation properties of the two enzymes. A polypeptide of 30,000 molecular weight was found in association with the polymerase of high specific activity from phage-infected cells and was absent from polymerase isolated from uninfected cells. The change in polymerase properties and the appearance of the polypeptide occurred early in phage infection.**

Studies with the coliphages T4, T7, and T3 suggest that DNA-dependent RNA polymerase is involved in control of differential transcription (1–3). Several changes in polymerase have been reported to occur during T4 development: a rapid loss of the σ subunit (4, 5), an increase in the molecular weight of the α subunit (6), attachment of 5′-AMP to the α subunit (7), and a change in the net charge of the β' subunit (1). It has also been proposed (8) that a new factor might be produced that would direct transcription of specific regions of the T4 genome. Polypeptides of low molecular weight have been detected in association with RNA polymerase after T4 infection (9), but their function has yet to be established. In contrast, phage-specific polymerases appear to be synthesized *de novo* early in T7 and T3 infections (2, 3).

In the present study, we have examined the properties of polymerases isolated from uninfected and ϕ29-infected *Bacillus amyloliquefaciens*. ϕ29 is a small virulent *Bacillus* phage that contains double-stranded DNA having a molecular weight of 11×10^6 (10). Genetic studies have identified 18 (11, 12) or 13 (13) complementation groups, as compared to 19 for T7 (14). The phage has an elaborate structure (10) and commits at least 60% of its genetic information to synthesis of seven structural proteins (15, 16). Another interesting property of this phage is that both strands of the DNA are transcribed during the infection cycle (17): only the L strand is transcribed early in infection, but RNA is produced from both strands late in infection (18). Protein synthesis is required for transcription from the H strand. These properties indicate that this phage infection may provide a simple, yet sophisticated, system for studying the role of RNA polymerase in control of transcription.

The present communication reports the purification and properties of polymerases from uninfected and ϕ29-infected *B. amyloliquefaciens*. The template activities of host and phage polymerases are described, as well as the time-course of polymerase modification during the latent period of ϕ29.

* Present address: Department of Biochemistry and Biophysics, University of California at San Francisco, San Francisco, Calif. 94122.

MATERIALS AND METHODS

Bacterial Strains and Phages. Uninfected and ϕ29-infected *B. amyloliquefaciens* were grown in Penassay broth, and ϕ29 was titered on *B. subtilis* A12 according to Schachtele *et al.* (19). *B. subtilis* 3610 (ATCC 6051) was used for preparation of ϕe and SP82 lysates as described by Roscoe (20). *B. amyloliquefaciens* strain H, *B. subtilis* strain A12, and ϕ29 were obtained from J. Spizizen. *B. subtilis* strain SB11, a derivative of W23, was obtained from E. W. Nester. Phage ϕe was donated by R. H. Doi; phage SP82 was provided by C. Stewart.

Preparation of DNA. Phages were sedimented by high-speed centrifugation (21) or were precipitated with 10% polyethyleneglycol, (22) then suspended in buffer, sedimented at 78,000 $\times$ *g* for 1 hr, and used without further purification for preparation of DNA. DNA from SP82 and ϕe was extracted with phosphate-buffered phenol at 0° (21). ϕ29 DNA was extracted according to Schachtele *et al.* (19); *B. subtilis* DNA was prepared from log-phase cells of strain SB11 by the Marmur method (23); sea-urchin DNA was extracted from sperm (24).

Preparation of Cells. Cultures of *B. amyloliquefaciens* were grown at 37° in 100-liter batches in Penassay broth to an optical density of 0.28 measured at 600 nm. Growth was stopped by addition of sodium azide (0.01 M final concentration) and ice to lower the temperature below 10°. Cells were harvested by centrifugation and stored frozen. For preparation of ϕ29-infected cells, cultures at an optical density of 0.28 were infected at a multiplicity of 20, the infection was stopped at the designated times by addition of sodium azide and ice, and the cells were harvested by centrifugation.

Enzyme Purification. In brief, the following steps were used: extracts were prepared by treatment of 40–100 g of cells with lysozyme in the presence of 0.05 M Tris·HCl (pH 7.9), 1 mM EDTA, and 0.1 mM phenylmethylsulfonylfluoride. After sonication to reduce viscosity, endogenous DNA was removed by the polyethyleneglycol/dextran phase separation method (25); the enzyme was eluted from the polyethyleneglycol precipitate with 4 M NaCl and fractionated with ammonium sulfate. The 30–70% ammonium sulfate fraction, containing most of the polymerase activity, was resuspended and chromatographed on DEAE-Sephadex. Active fractions were eluted from this column by a 0.05–1 M gradient of KCl; they were concentrated by ultrafiltration and applied to a column of Sepharose 6B. The active fractions eluted from the latter column by buffer containing 1 M

NaCl were again concentrated by ultrafiltration, chromatographed on a sea-urchin DNA–cellulose column (26), and sedimented through a 10–30% glycerol gradient in the presence of 1 M NaCl. Protein was determined by the method of Bramhall *et al.* (27).

Sodium dodecyl sulfate–polyacrylamide gels were run according to Laemmli (28), stained with Coomassie blue, and scanned with a Gilford scanning accessory. Bovine-serum albumin, ovalbumin, and immunoglobulin G were used as standards; the accuracy of the molecular weight estimates was ±10% (29).

Assay. RNA polymerase activity was assayed in reaction mixtures containing 0.08 μCi of [8-^{14}C] ATP (Calbiochem., 27.7 Ci/mol), 0.8 mM GTP, UTP, and CTP, 0.05 M Tris buffer (pH 7.9), 1 mM EDTA, 10 mM $MgCl_2$, 1 mM 2-mercaptoethanol, and 12.5 μg of DNA (usually SP82 or φe DNA) in a total volume of 0.5 ml and incubated at 37° for 10 min. The reaction was stopped by addition of 0.5 ml of 10% trichloroacetic acid in the presence of 100 μg of bovine-serum albumin. The precipitated material was collected on Gelman GF/C glass fiber filters, washed with 5% trichloroacetic acid, dried, and counted in a liquid scintillation counter. Specific activity is defined in terms of nmol of ATP incorporated per μg of protein per 10 min.

RESULTS AND DISCUSSION

Template Activities. As a first step in investigating the possibility that RNA polymerase may be modified during infection of *B. amyloliquefaciens* with φ29, studies were done with different DNA templates with partially purified fractions obtained from extracts of uninfected and infected cells. DNAs from three *Bacillus* phages and from *B. subtilis* were selected as templates. A single high-molecular-weight preparation of each template was used in parallel assays, since activity depended on the intactness of the DNA. Saturating amounts of template were used in each experiment, as determined from saturation curves with each polymerase preparation.

As shown in Table 1, the activity of polymerase from φ29-infected cells was substantially higher than that of polymerase from uninfected cells when φ29 DNA was used in the assay. No significant differences in apparent K_m values for DNA were observed for each of the DNA templates with the two polymerase preparations. The last two columns of Table 1 show the ratios of activity of each of the three fractions with each DNA relative to the activity observed with φ29 DNA. This ratio is a measure of the "template specificity" of each polymerase and should be identical for the two preparations if each transcribed a given template with the same efficiency. The results show that the ratios for the two enzyme preparations were different through three steps of purification, suggesting that the polymerases from infected and uninfected cells were different.

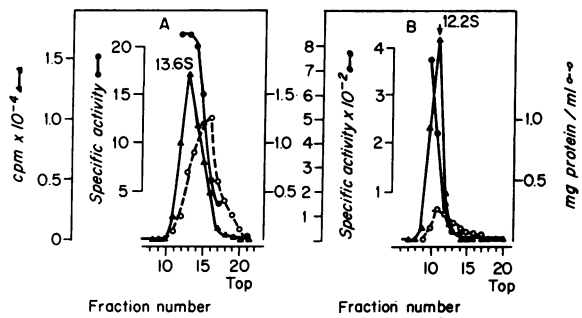

FIG. 1. Sedimentation of RNA polymerase from uninfected and φ29-infected *B. amyloliquefaciens* in a glycerol gradient. (*A*) Uninfected *B. amyloliquefaciens;* polymerase activity, protein profile, and specific activities. (*B*) φ29-infected *B. amyloliquefaciens;* polymerase activity, protein profile, and specific activities. RNA polymerase fractions from DNA–cellulose columns were sedimented in a 10–30% glycerol gradient (11 ml, 38,000 rpm for 24 hr in a B-60 International centrifuge with a 283 rotor). Catalase and hemoglobin were used as markers.

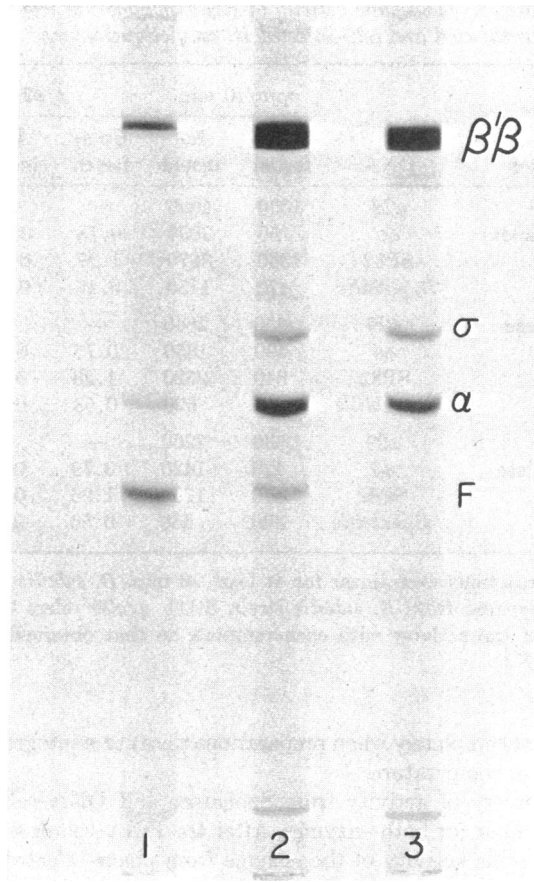

FIG. 2. Sodium dodecyl sulfate–polyacrylamide gels of glycerol gradient fractions of RNA polymerase preparations from φ29-infected and uninfected *B. amyloliquefaciens*. Gel *1*, fraction 10 from Fig. 1*B*, φ29-infected *B. amyloliquefaciens*. Gel *2*, fraction 11 from Fig. 1*B*, φ29-infected *B. amyloliquefaciens*. Gel *3*, fraction 13, from Fig. 1*A*, uninfected *B. amyloliquefaciens*. 10-μl Samples were applied on the gels.

Polymerase Purification and Properties. The purification scheme for polymerase from infected and noninfected cells is summarized in Table 2. The recovery of polymerase activity from phage-infected cells after chromatography of DEAE–Sephadex was routinely 5- to 8-times higher than that of polymerase from uninfected cells. A single peak of polymerase activity, eluting from DEAE–Sephadex at about 0.5 M KCl, was observed with either enzyme preparation. The recovery of host polymerase was low whether chromatography was performed at room temperature or at 3°. In contrast, polymerase activity from phage-infected cells

TABLE 1. *Template activity of RNA polymerase from uninfected and φ29-infected B. amyloliquefaciens*

Fraction	DNA	cpm/10 min		x/φ29	
		Uninfected	Infected	Uninfected	Infected
DEAE-Sephadex	φ29	1030	4690	—	—
	φe	750	3030	0.73	0.65
	SP82	1300	3970	1.27	0.85
	B. subtilis	470	1150	0.46	0.25
Sepharose	φ29	650	2840	—	—
	φe	490	1850	0.75	0.66
	SP82	840	2520	1.28	0.89
	B. subtilis	310	620	0.53	0.22
DNA-cellulose	φ29	530	2200	—	—
	φe	420	1420	0.79	0.65
	SP82	670	1790	1.26	0.82
	B. subtilis	300	550	0.56	0.25

All reactions were linear for at least 20 min. *B. subtilis* DNA was prepared from *B. subtilis* strain SB11. x/φ29 refers to the ratio of the activity with each template to that obtained with φ29 DNA.

was lost completely when preparations were chromatographed at room temperature.

Recovery of activity from Sepharose and DNA-cellulose was similar for both enzymes. After the DNA-cellulose step, the specific activity of the enzyme from phage-infected cells was about 3-times greater than that of the host enzyme. At this stage of purification, both enzymes were about 90% pure, as judged by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The overall yield of enzyme activity from phage-infected cells was 8- to 9-times greater than that of the host enzyme, suggesting basic differences in the stability of the two enzymes. It should be noted that saturating concentrations of ATP were not used in determining the specific activities shown in Table 2; when saturating concentrations were used, the values were equal to or higher than those reported by Hermoso *et al.* (30).

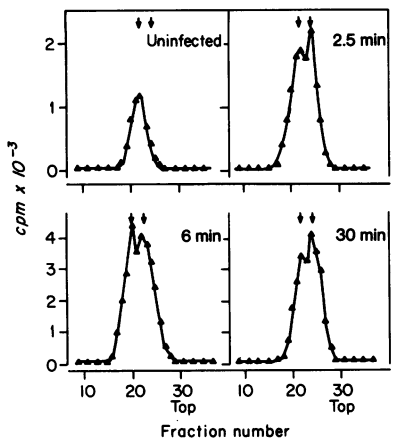

FIG. 3. Sedimentation patterns of RNA polymerase from uninfected and φ29-infected cells harvested at various times after infection. Extracts were prepared from 4 to 6 g of cells harvested at the indicated min after infection. They were fractionated through the 30–70% ammonium sulfate step (step 2 of Table 2), applied to a 5-ml gradient of 10–30% glycerol, and sedimented for 18 hr at 30,000 rpm in a Beckman model L centrifuge with a SW-50 rotor. Catalase and hemoglobin were used as markers. *Arrows* are drawn at 13.6 S and 12.2 S.

Sedimentation of Polymerase and Subunit Composition. The glycerol gradient sedimentation pattern of host polymerase (Fig. 1*A*) purified through the DNA-cellulose step gave a sedimentation constant of 13.6 ± 0.4. The Na dodecyl sulfate-polyacrylamide gel pattern of the glycerol gradient fraction having the highest specific activity (fraction 13 of Fig. 1*A*) is shown in gel 3 of Fig. 2. In agreement with Hermoso *et al.* (30), three major bands were found after staining with Coomassie blue: $\beta'\beta$ (which could be resolved into two closely spaced bands when longer gels were used), σ, and α. The molecular weights of these subunits were estimated to be 170,000–160,000, 48,000, and 40,000, respectively. The subunit stoichiometry, based on densitometer tracings of this gel was $(\beta)_2$, $(\sigma)_{0.5}$, and $(\alpha)_2$,indicating that some σ was lost during purification.

A similar glycerol gradient sedimentation pattern for polymerase from φ29-infected cells after DNA cellulose chromatography is shown in Fig. 1*B*. The sedimentation constant for the "φ29 polymerase" was 12.2 ± 0.4. The comparable gel pattern for the fraction having the highest activity is shown in gel 1 of Fig. 2, whereas gel 2 of this figure shows the fraction with the next highest activity (fractions 10 and 11 of Fig. 1*B*). The β subunits, σ, and α appear to be identical to those found in the host polymerase (gel 3 of Fig. 3). Further evidence (unpublished observations) substantiating the similarities among these subunits comes from electrophoretic patterns seen in urea gels and from studies with inhibitors (rifampicin and streptolydigin) that presumably interact with the β subunit.

Gels 1 and 2 of Fig. 2 show that polymerase from φ29-infected cells contained an additional polypeptide (designated F) having a molecular weight of about 30,000. This polypeptide was not observed in any of the fractions of polymerase obtained from uninfected cells. These observations

TABLE 2. *Preparation of RNA polymerase from uninfected and φ29-infected B. amyloliquefaciens*

Purification step	Uninfected			φ29-Infected		
	Total units	Specific activity	Recovery (%)	Total units	Specific activity	Recovery (%)
PEG eluate*	5051	—	100	1396	—	100
30–70% $(NH_4)_2SO_4$	1913	1.5	38	1104	4.1	79
DEAE-Sephadex	244	2.8	4.8	517	6.5	37
Sepharose 6B	221	6.5	4.4	326	14.2	23
DNA-cellulose	112	39.3	2.2	266	116	19

* Fraction obtained by polyethyleneglycol/dextran phase separation (25) from 100 g of uninfected cells and from 40 g of cells harvested 20 min after infection with φ29. 1 Unit of activity is defined as 1 nmol of ATP incorporated per 10 min; specific activity is defined as units/μg of protein.

suggest that this polypeptide is either coded for by the phage or is produced in the host after phage infection. Furthermore, the σ subunit was either absent or present in greatly reduced amounts in the fraction having the highest specific activity (gel 1 of Fig. 2), whereas the next fraction, which had a lower specific activity, contained a smaller amount of F and some σ. If it is assumed that F does not have any unusual staining properties, the following subunit stoichiometry can be calculated for gel 1 of Fig. 2: $(\beta)_2$, $(\alpha)_2$, and $(F)_2$. The stoichiometry for gel 2 of this figure was $(\beta)_2$, $(\sigma)_{0.4}$, $(\alpha)_2$, and $(F)_{0.6}$. These ratios suggest that F is not a degradation product of the β or α subunits; the possibility that F arises from degradation of σ has not been eliminated. There was no evidence in these gels of $\beta'\beta$ degradation (31).

Since host macromolecular syntheses are not shut down during φ29 infection (18), synthesis of host subunits may continue throughout the latent period, thus accounting for continued production of "early" RNA from the L strand throughout infection (18). Under such conditions, it would be expected that only a fraction of the host polymerase would be associated with the polypeptide of 30,000 molecular weight. By summing up the intensities of the bands from gels of fractions having polymerase activity across the entire glycerol gradient, it could be calculated that only 10–15% of the enzyme was associated with the subunit of 30,000 molecular weight. Since the specific activity of these fractions was, however, at least 20-times higher than that of comparable fractions obtained with host polymerase (Fig. 1*A* and *B*), an alteration of 10–15% of the enzyme could easily account for the 2- to 4-fold increased specific activity observed in crude extracts of φ29-infected cells. No direct evidence has been obtained that F is involved directly in catalysis, and an indirect effect (e.g., stabilization of the enzyme) is compatible with the present evidence.

Time Course of Polymerase Modification During the Latent Period of φ29 Infection. Since the enzyme from uninfected and infected cells differ in sedimentation constants, it was possible to analyze for the presence of "φ29 polymerase" during the infection cycle. Fig. 3 shows the sedimentation patterns of the polymerase activity of 30–70% ammonium sulfate fractions (Table 2) prepared from cells at various times after infection. Activity sedimenting at 12.2 S was detected 2.5 min after infection, and the relative amounts of 13.6S and 12.2S materials were similar at all intervals sampled after 6 min of infection (similar results were obtained with fractions from cells at 9, 12, 15, and 20 min after infection; data not shown). These data suggest that the polymerase is modified early in the latent period and that modification is completed by at least 6 min after infection. Transcription of "late RNA" from the H strands requires protein synthesis and is detected 9–12 min after infection (18). The fact that two peaks of activity were observed in the experiment presented in Fig. 3, whereas only one peak was found after total purification (Fig. 1*B*), can be attributed to large differences in stability of the enzymes during the purification procedure.

Genetic evidence reported by Schachtele *et al.* (32) shows that only a single gene, designated gene 4, is expressed during the first 6 min of the latent period. Recombination studies with conditional lethal mutants (33) suggest that gene 4 is of sufficient size to code for a polypeptide of 30,000 molecular weight. These data are consistent with the hypothesis that the gene-4 product is responsible for modification of polymerase described above.

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