Transcription of the Genome of Bacteriophage ϕ 29: Isolation and Mapping of the Major Early mRNA Synthesized In Vivo and In Vitro

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Received for publication 8 February 1977

The ϕ 29 early mRNA's synthesized in infected *Bacillus subtilis* were studied by using sedimentation velocity analysis, polyacrylamide gel electrophoresis, and hybridization of ϕ 29 DNA fragments generated by the restriction endonuclease *Eco*RI. Viral RNAs synthesized in vivo in the presence of chloramphenicol were found to hybridize to *Eco*RI-A, -C, and -D fragments, but not to *Eco*RI-B and -E fragments, of the viral genome. Major early mRNA sedimenting as 16S material in neutral sucrose gradients was examined in detail. Radioactive ϕ 29 RNA, purified by sucrose gradient centrifugation, was hybridized to either the *Eco*RI-A or *Eco*RI-C DNA fragment. The RNA was eluted from the hybrids and then tested for complementary hybrid formation with *Eco*RI-A and -C fragments. RNA eluted from the *Eco*RI-A fragment annealed only to the *Eco*RI-A fragment and not to the *Eco*RI-C fragment. Similarly, RNA eluted from the *Eco*RI-C fragment hybridized to the *Eco*RI-C and -D fragments. Viral RNAs synthesized in vitro using *B. subtilis* RNA polymerase hybridized to both *Eco*RI-A and -C DNA fragments. Furthermore, RNA initiated with [γ -³²P]GTP also hybridized to both *Eco*RI-A and -C fragments. These results indicate that there are at least two efficient promoters for early transcription on the ϕ 29 chromosome. In addition, a low-molecular-weight RNA initiated with [γ -³²P]ATP was found to hybridize exclusively with the *Eco*RI-A fragment. Kinetic studies of ϕ 29 mRNA synthesis during the lytic cycle have shown that viral RNAs hybridizable to the *Eco*RI-A and -C fragments are synthesized immediately after phage infection. On the other hand, mRNA specific for the *Eco*RI-B fragment was not synthesized for several minutes after phage infection. Based on the results of the in vivo and in vitro transcription studies, a transcription map of the ϕ 29 chromosome is proposed.

Gene expression of bacteriophage ϕ 29 in infected vegetative cells of *Bacillus subtilis* during the lytic cycle appears to be regulated in an orderly sequence. Immediately after ϕ 29 infection, the host RNA polymerase transcribes "early" mRNA from the light strand of the ϕ 29 DNA (low density in CsCl-polyuridylic acid-polyguanylic acid gradients) (36, 42, 43), and early mRNA is produced throughout the infectious cycle (29, 30, 42). Later in ϕ 29 infection, "late" mRNA is transcribed from the heavy strand of the viral genome (36, 42, 43). Late transcription is independent of ϕ 29 DNA replication (29); however, at least one of the early gene products appears to be required for late transcription (3, 5). When ϕ 29 infects UV-irradiated *B. subtilis* cells, early and late proteins encoded by early and late mRNA also appear sequentially (7, 17, 39).

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It has been shown (42, 43) that the synthesis of ϕ 29 mRNA requires those components of the host RNA polymerase that are sensitive to rifampin, a specific inhibitor of the initiation of RNA synthesis (8). Furthermore, ϕ 29-specific mRNA synthesis becomes resistant to rifampin in infected *B. subtilis* mutants that are resistant to this drug (42).

Holland and Whiteley (21) have observed that some of the host RNA polymerase is modified after ϕ 29 infection. The modified RNA polymerase differs from the host enzyme in template specificity, sedimentation properties, and subunit composition. Whether the modified RNA polymerase transcribes ϕ 29 late genes is not known, however. In vitro transcription of the ϕ 29 genome by *B. subtilis* RNA polymerase yields only early mRNA (F. Kawamura and J. Ito, unpublished data; M. Salas, personal communication).

Thus, in contrast to coliphage T₇, where a

single early gene product, T7 RNA polymerase, transcribes all the late genes (32, 45), ϕ 29 late genes appear to be transcribed by the modified host RNA polymerase. Modified *B. subtilis* RNA polymerase has recently been extensively studied in two phage systems, SP01 and SP82 (9, 19, 28). In these systems, it has been shown that at least two phage-coded regulatory proteins interact with host RNA polymerase and direct the "late" transcription of the viral genomes (11, 13-15, 19, 48).

In the present study, we analyzed ϕ 29 early mRNA synthesized both in vivo and in vitro by using specific DNA fragments generated with the restriction endonuclease *Eco*RI (R. N. Yoshimori, Ph.D. thesis, University of California, San Francisco, 1971). These studies have allowed us to construct a transcriptional map of the ϕ 29 genome, in which there are at least two distinct promoters for early transcription. A preliminary report of these studies has been presented (F. Kawamura and J. Ito, Fed. Proc. 35:164, 1976).

MATERIALS AND METHODS

Bacterial strains. *B. subtilis* SR22 (*spoA12 trpC2*), an asporogenous mutant of *B. subtilis* 168, was used for the preparation of RNA polymerase from uninfected cells (25). *Bacillus amyloliquefaciens* H was used for the preparation of ϕ 29 lysates.

Extraction of ϕ 29 DNA. Purified phage ϕ 29 was diluted to approximately 5×10^{12} PFU/ml in a solution consisting of 0.05 M Tris-hydrochloride (pH 7.4), 0.05 M NaCl, 20 mM EDTA, and 0.5% sodium dodecyl sulfate (SDS), and was incubated for 10 min at 50°C. After phenol extraction, DNA was precipitated with 3 volumes of cold ethanol in the presence of 0.2 M NaCl and collected by centrifugation (14,000 $\times g$, 20 min, 4°C). The pellet was dissolved in 0.02 M Tris-hydrochloride (pH 7.4) containing 0.1 mM EDTA, dialyzed extensively against this buffer at 4°C, and stored at 4°C. This DNA was used as template for in vitro RNA synthesis.

Labeling and preparation of in vivo RNA. *B. subtilis* SR22 was grown to approximately 2×10^8 cells/ml at 37°C in 10 ml of Spizizen minimal medium, supplemented with 0.25% casein acid hydrolysate and 50 μ g of tryptophan per ml (Min-CH). Cells in 15-cm glass petri dishes were irradiated for 5 min with twin UV lights (General Electric germicidal lamps, 15 W) located 30 cm above the surface of the liquid (approximately 30,000 ergs/mm²) at room temperature. After irradiation, cells were centrifuged (8,000 $\times g$, 5 min at 4°C) and the pellet was resuspended in one-half the original volume of fresh Min-CH medium. After incubation for 5 min at 37°C with shaking, the cells were infected with ϕ 29 at a multiplicity of infection of 50 and incubated further at 37°C with shaking. At selected times after infection, infected bacteria were labeled with [³H]uridine (New England Nuclear Corp.; 46.2 Ci/mmol) as described in the figure legends. Under these conditions, the rate of ϕ 29 DNA synthesis in UV-irradi-

ated, infected cells was found to be approximately 8% that of the unirradiated, infected bacteria. However, the onset of ϕ 29 DNA synthesis was similar in UV-irradiated and unirradiated cells.

RNA was prepared by a modification of the diethylpyrocarbonate method originally adopted for the isolation of bacterial RNA by Summers (46, 47). After labeling, each sample was mixed with an equal volume of ice-cold protoplasting buffer (0.05 M Tris-hydrochloride [pH 7.4], 0.1 M NaCl, 0.01 M EDTA, 0.02 M NaN₃, 30% sucrose, 200 μ g of chloramphenicol per ml, and 1 mg of lysozyme per ml), and the mixture was kept on ice for 4 min.

Spheroplasts were collected by centrifugation (6,000 $\times g$, 5 min, 4°C), and the cells were lysed by addition of one-tenth volume of lysing buffer (0.01 M Tris-hydrochloride [pH 7.4], 0.01 M NaCl, 1.5% SDS, and diethylpyrocarbonate [Eastman Organic Chemicals, Rochester, N.Y.]) to a final concentration of 3%. The resulting clear, viscous lysate was incubated for 5 min at 37°C; then one-half volume of cold, saturated NaCl was added, and the solution was chilled. The resulting precipitate was removed by centrifugation (12,000 $\times g$, 10 min, 4°C). RNA was precipitated from the supernatant with 3 volumes of ethanol, collected by centrifugation, and resuspended in a small volume of 1 $\times$ Loening buffer (27) containing 0.02% SDS. Approximately 90% of the total radioactivity incorporated as acid-insoluble material was recovered under these conditions. More than 99.9% of the labeled material in the final RNA preparation was alkali labile.

Preparation of RNA polymerase. *B. subtilis* SR22 cells were grown to a cell density of approximately 3×10^8 /ml at 37°C in 20 liters of Min-CH medium. The cells were then harvested, frozen in a dry ice-acetone bath, and stored at -70°C. RNA polymerase was prepared in a manner similar to that described by Duffy and Geiduschek (10). All operations were performed at 4°C. Frozen cells (40 g, wet weight) were suspended in 100 ml of buffer G (0.05 M Tris-hydrochloride [pH 8.0], 10 mM MgCl₂, 0.2 M KCl, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 10% [vol/vol] glycerol containing 3 mM phenylmethylsulfonyl fluoride), sonically treated five times for 1 min, and centrifuged at 10,000 $\times g$ for 30 min. The supernatant was brought to 25% saturation with 33 ml of saturated ammonium sulfate (pH 8.0). After being stirred on ice for 30 min, the suspension was centrifuged at 60,000 $\times g$ for 3 h in a type 30 rotor. The supernatant was collected, made 70% saturated with ammonium sulfate by adding the saturated ammonium sulfate, and stirred on ice for 30 min. The precipitate was collected by centrifugation at 12,000 $\times g$ for 30 min. The pellet was dissolved in 60 ml of buffer A (10 mM Tris-hydrochloride [pH 8.0], 10 mM MgCl₂, 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.2 M KCl, and 10% [vol/vol] glycerol), applied to an agarose gel column (Bio-Gel A-0.5 m, 2 by 130 cm), and eluted at 34 ml/h with buffer A. The peak of RNA polymerase activity was pooled and centrifuged at 130,000 $\times g$ for 3 h in a type 65 rotor. The supernatant was applied to a DEAE-Sephadex A-50 column (2 by 10 cm) equilibrated with buffer A and eluted with a 700-ml linear gradient of 0.2 to 0.6 M KCl. RNA polymerase activity eluted as a single peak at

approximately 0.38 M KCl; the activity was pooled, made 70% saturated with ammonium sulfate, and stirred for 30 min on ice. The precipitate was collected by centrifugation at $35,000 \times g$ for 1 h in a type 30 rotor, dissolved in 8 ml of buffer A, and dialyzed against buffer A. RNA polymerase was further purified by 15 to 30% (vol/vol) linear glycerol gradient centrifugation at $120,000 \times g$ for 16 h in an SW41 rotor in 0.01 M Tris-hydrochloride (pH 8.0)–10 mM MgCl_2 –0.2 M KCl–0.1 mM EDTA–0.5 mM dithiothreitol.

RNA polymerase activity was assayed at 30°C in 0.1 ml of reaction mixture containing 0.1 M Tris-hydrochloride (pH 8.0), 0.1 mM dithiothreitol, 0.1 mM EDTA, 0.8 mM spermidine-hydrochloride, 10 mM MgCl_2 , 100 μg of bovine serum albumin per ml, 10 μg of $\phi 29$ DNA, 0.1 mM each UTP, GTP, CTP, and [^{14}C]ATP (9 cpm/pmol), and 21 to 10 μl of enzyme fraction. After incubation for 10 min, cold trichloroacetic acid-insoluble radioactivity was collected on membrane filters (Millipore Corp.), and its radioactivity was determined in a Packard scintillation spectrometer.

RNA polymerase activity was expressed as nanomoles of AMP incorporated in 10 min at 30°C with 10 μg of $\phi 29$ DNA as template. The peak RNA polymerase fraction in a glycerol gradient had 500 U per mg of protein. Protein was determined by the method of Lowry et al. (31).

Extraction of in vitro $\phi 29$ RNA. In vitro RNA transcription was stopped by mixing with an equal volume of phenol containing 100 μg of *Escherichia coli* tRNA. The aqueous layers separated by centrifugation were passed through a Sephadex G-75 column (1 by 10 cm). The eluted labeled RNA was treated with electrophoretically purified DNase I (Worthington Biochemicals Corp.) and 10 mM MgCl_2 , heated for 3 min at 95°C with 0.2% SDS, precipitated with ethanol, and dissolved in 0.1% SDS.

DNA-RNA hybridization. DNA-RNA hybridization in formamide was carried out according to the procedures described by Hayashi and Hayashi (18). Denatured $\phi 29$ DNA and DNA fragments by restriction endonuclease *EcoRI* were immobilized on nitrocellulose filters (Sartorius Membrane Filter, GmbH; 25 mm) as described by Gillespie and Spiegelman (16). Hybridization was performed in 0.1 ml of hybridization fluid (5 $\times$ SSC, 0.1% SDS [SSC = 0.15 M NaCl + 0.015 M sodium citrate]) for 20 h at 64°C. The filters were washed with 2 $\times$ SSC, incubated in the presence of 30 μg of pancreatic RNase per ml (Worthington) and 10 U of RNase T1 per ml (Sankyo, Tokyo) for 60 min at 25°C, washed again with 2 $\times$ SSC, dried, and counted. When RNA was eluted, RNase treatment was omitted.

Isolation of $\phi 29$ DNA fragments generated by *EcoRI*. $\phi 29$ DNA cleaved by endonuclease *EcoRI* was separated by electrophoresis on preparative agarose slab gels. Fragments were eluted as described previously (24) and dialyzed against 0.1 $\times$ SSC.

Sucrose gradient centrifugation. Sucrose gradients were made at room temperature by layering 0.9 ml each of 20, 15, 10, and 5% sucrose solutions in

0.05 M Tris-hydrochloride (pH 7.4)–0.1 M NaCl–0.01 M EDTA–0.1% SDS and stored overnight before use. RNA samples (20 to 200 μl) were layered on the gradients and centrifuged in a Beckman SW56 rotor at 20°C as described in the figure legends. rRNA used as a reference marker was prepared as described previously (24).

Polyacrylamide gel electrophoresis. RNA was analyzed by polyacrylamide gel electrophoresis. Polyacrylamide gels (2.5%) were prepared by mixing 2.5 ml of a stock solution of 20% acrylamide and 1% bisacrylamide, 15.3 ml of water, 2 ml of 10 $\times$ Loening buffer (27), 0.014 ml of *N,N,N',N'*-tetramethylethylenediamine, and 0.15 ml of fresh 10% ammonium persulfate solution. After polymerizing overnight at room temperature, the gels were run at a constant current of 4 mA per gel at room temperature. After electrophoresis, the gels were removed from the glass tubes, frozen in an acetone-dry ice bath, and cut into 1-mm slices. The gel slices were placed in vials containing 0.1 ml of 30% hydrogen peroxide and incubated overnight at 55°C. After the gels were completely dissolved, the radioactivity was counted in 10 ml of scintillation fluid containing 12.5% Biosolv BBS3 (Beckman).

RESULTS

Isolation of $\phi 29$ early RNA. A typical zonal sedimentation profile of the early RNA, extracted from UV-irradiated cells infected with $\phi 29$ in the presence of chloramphenicol, is shown in Fig. 1. Three major RNA peaks, designated peaks A, B, and C, sedimented at 16, 11

FIG. 1. Sedimentation profile of early $\phi 29$ RNA extracted from UV-irradiated, infected cells. *B. subtilis* SR22 cells, UV irradiated, were treated with chloramphenicol (300 $\mu\text{g}/\text{ml}$) for 5 min before infection at a multiplicity of infection of 50. Cells were labeled with [^3H]uridine (100 $\mu\text{Ci}/\text{ml}$) from 10 to 14 min after infection, and RNA was extracted as previously described (23) for analysis by sedimentation through a 5 to 20% linear sucrose gradient at 48,000 rpm in a Beckman SW56 rotor at 20°C for 150 min. Ten-drop fractions were collected directly on filter papers (Discs GFC, Whatman), dried, and counted. Symbols: (●) ^3H -labeled $\phi 29$ RNA; (○) ^{14}C -labeled rRNA.

to 13S, and 6 to 8S, respectively, relative to the *B. subtilis* rRNA markers. Figure 2 shows the electrophoretic profile of early $\phi 29$ mRNA. At least eight RNA peaks (indicated by Roman numerals) were seen reproducibly. To analyze further the early RNA by polyacrylamide gel electrophoresis, each RNA peak (Fig. 1) was pooled and repurified by zonal sedimentation through a 5 to 20% sucrose gradient (Fig. 3B-D). Peaks A and B were each divided into three sections (Fig. 3B and C). The subdivided fractions were electrophoresed in polyacrylamide gels (Fig. 4 and 5). The electrophoretic profile of unfractionated peak C (Fig. 3D) in polyacrylamide gel is shown in Fig. 6. All RNA fractions were treated with 90% formamide before elec-

FIG. 2. Electropherograms of ^3H -labeled $\phi 29$ CAM-RNA in UV-irradiated cells. UV-irradiated *B. subtilis* SR22 cells were treated with chloramphenicol (300 $\mu\text{g}/\text{ml}$) for 5 min before phage infection. Cells infected with $\phi 29$ at a multiplicity of infection of 400 were labeled with [^3H]uridine (50 $\mu\text{Ci}/\text{ml}$) from 10 to 14 min after infection. The RNA was extracted and layered on a 10-cm 2.5% acrylamide gel as described in Materials and Methods. Electrophoresis was carried out for 2 (A) and 3.5 (B) h at a constant current of 4 mA per gel. The gels were cut into 1-mm slices and dissolved, and the radioactivity was determined. Symbols: (O) ^{14}C -labeled rRNA; (●) ^3H -labeled $\phi 29$ CAM-RNA.

FIG. 3. Separation of early $\phi 29$ RNA by sucrose gradient centrifugation. Early $\phi 29$ RNA, prepared as described in the legend to Fig. 1, was centrifuged at 45,000 rpm in a Beckman SW56 rotor at 20°C for 150 min, and 10-drop fractions were collected. Samples (5 μl) from each fraction were placed on filter paper, dried, and counted. The three peaks (A, B, and C) migrating at about 16S, 11 to 13S, and 6 to 8S, respectively (indicated by three brackets), were pooled and again centrifuged at 48,000 rpm in a Beckman SW56 rotor at 20°C for 150 min, and 10-drop fractions were collected. Samples (5 μl) from each fraction were counted as above. (A) Early $\phi 29$ RNA; (B) 16S RNA peak (peak A); (C) 11–13S RNA peak (peak B); (D) 6–8S RNA peak (peak C).

trophoresis to denature the RNA molecules.

The higher-molecular-weight RNA species (peak A in Fig. 1 and 3B) migrated much more slowly in acrylamide gels than did 16S rRNA, but slightly faster than did the 23S rRNA in acrylamide gel electrophoresis. This indicates that $\phi 29$ early mRNA may have an extensive secondary structure. The size of the higher-molecular-weight RNA, e-I RNA, was estimated to be 0.9×10^6 daltons. From Fig. 5, it appears that the peak B RNA fraction (Fig. 1 and 3C) contained e-II, e-III, e-IV, and e-V

FIG. 4. Analysis of peak A $\phi 29$ early RNA in a sucrose gradient by polyacrylamide gel electrophoresis. Each of the fractions of Fig. 3B was pooled and applied to a 2.5% polyacrylamide gel and then subjected to electrophoresis for 3.5 h at a constant current of 4 mA/gel at room temperature. Gels were cut into 1-mm slices and counted, after incubation with 0.2 ml of 30% hydrogen peroxide at 60°C. (A) Faster-sedimenting portion of peak A (fraction 7-9 in Fig. 3B); (B) center portion of RNA peak A (fraction 10-13 in Fig. 3B); (C) slower-sedimenting portion of RNA peak A (fraction 14-15 in Fig. 3B). Symbols: (●) ^3H -labeled $\phi 29$ DNA; (○) ^{14}C -labeled rRNA.

RNA species (Fig. 2). On the other hand, peak C RNA appeared to consist of e-VI, e-VII, and e-VIII RNA components (Fig. 2, 3D, and 6).

Characterization of the e-I RNA. The purified e-I RNA, which sedimented at 16S in neutral sucrose gradients (Fig. 7A), was further analyzed by hybridization experiments, using $\phi 29$ DNA fragments generated by restriction endonuclease *EcoRI* (24). Previously, it had been shown that the *EcoRI* endonuclease cleaves $\phi 29$ DNA into five fragments (22, 24, 44). The order of these five DNA fragments has been established as A-B-E-D-C (22-24).

e-I RNA hybridized preferentially to the *EcoRI*-A and -C fragments and to a lesser extent to the *EcoRI*-B and -D fragments (Table 1). Since the preparation of the *EcoRI*-B fragment

FIG. 5. Analysis of peak $\phi 29$ early RNA in a sucrose gradient by polyacrylamide gel electrophoresis. Each of the fractions was pooled separately, as shown in Fig. 3C by the bracket, and was analyzed by polyacrylamide gel electrophoresis as described in Fig. 4. (A) Faster-sedimenting portion of RNA peak B (fraction 16-17 in Fig. 3C); (B) center portion of RNA peak B (fraction 18-22 in Fig. 3C); (C) slower-sedimenting portion of RNA peak B (fraction 23-24 in Fig. 3C). Symbols (●) ^3H -labeled $\phi 29$ RNA; (○) ^{14}C -labeled rRNA.

used in these experiments was contaminated slightly with the *EcoRI*-A fragment (~5%), we consider the hybridization value of the *EcoRI*-B fragment not to be significant. On the other hand, the hybridization value for the *EcoRI*-D fragment is significant since the *EcoRI*-D fragment represents only 4.0% of the $\phi 29$ genome (24). Since the *EcoRI*-A and -C fragments are both terminal ends of the $\phi 29$ genome, the hybridization data suggest that $\phi 29$ DNA may be functionally circular in vivo, at least early in infection; alternatively, the e-I RNA may consist of at least two RNA components. To examine whether the e-I RNA is a single species, we hybridized e-I RNA in formamide to either the *EcoRI*-A or -C fragment. Hybridized RNA was eluted, and portions of the eluates were rehy-

FIG. 6. Analysis of peak C $\phi 29$ early RNA in a sucrose gradient by polyacrylamide gel electrophoresis. The fractions were pooled from fraction 28 to fraction 35, as indicated by the bracket in Fig. 3D, and were analyzed by polyacrylamide gel electrophoresis as described in Fig. 4, except that the electrophoresis was for 2 h. Symbols: (●) ^3H -labeled $\phi 29$ RNA; (○) ^{14}C -labeled rRNA.

bridized to the *EcoRI*-A and -C fragments. The e-I RNA eluted from fragment A hybridized only to the *EcoRI*-A fragment, and RNA eluted from the *EcoRI*-C fragment hybridized to the *EcoRI*-C and -D fragments (Table 1). To exclude the possibility that the eluted RNA may have been degraded during hybridization and elution, we examined the size distribution of the e-I RNA eluted from the *EcoRI*-C hybrid by sedimentation through a 5 to 20% sucrose gradient. The eluted e-I RNA from the *EcoRI*-C hybrid sedimented at about 16S (Fig. 8), indicating that the e-I RNA was not degraded during the procedures used here. This observation clearly indicates that there are at least two species of early RNA that sediment at 16S in neutral sucrose gradient: one is the transcript from the *EcoRI*-A region, and the other is from the *EcoRI*-C region of the $\phi 29$ genome.

Analysis of $\phi 29$ early RNA specific for *EcoRI*-A and -C fragments. $\phi 29$ early mRNA consists of at least eight components ranging in molecular weight from about 0.9×10^6 to 0.05×10^6 (Fig. 2; Kawamura and Ito, manuscript in preparation). However, an analysis of the e-I RNA described above suggested strongly that even the sharp single band of RNA in the case of e-I RNA in a polyacrylamide gel may contain more than one species of RNA. Therefore, we attempted to analyze $\phi 29$ early RNAs by hybridization to specific DNA fragments and elution of RNA from the hybrids, followed by electrophoresis in polyacrylamide gels. [^3H]RNA, prepared from UV-irradiated, infected cells pretreated with chloramphenicol (CAM-RNA),

FIG. 7. Re-sedimentation profiles of $\phi 29$ early RNA peaks isolated by sucrose gradient centrifugation. Each of the three RNA peaks shown in Fig. 3B, C, and D was analyzed by sedimentation through 5 to 20% linear sucrose gradients at 48,000 rpm in a Beckman SW56 rotor at 20°C for 150 min. Ten-drop fractions were collected directly on filter papers, dried, and counted. (A) Peak A RNA (fraction 10-13 in Fig. 3B); (B) peak B RNA (fraction 18-22 in Fig. 3C); (C) peak C RNA (fraction 28-30 in Fig. 3D). Symbols: (●) ^3H -labeled $\phi 29$ RNA; (○) ^{14}C -labeled rRNA.

was labeled from 4 to 8 min after infection and subjected to hybridization with *EcoRI* fragments of $\phi 29$ DNA (Table 2). CAM-RNA hybridized exclusively to the *EcoRI*-A, -C, and -D fragments. Labeled CAM-RNA was hybridized in formamide to either the *EcoRI*-A or the *EcoRI*-C fragment, and RNA from the hybrids was eluted. Samples of the eluates were then analyzed by polyacrylamide gel electrophoresis. Figure 9 shows the electropherogram of $\phi 29$ early RNA specifically hybridized to the *EcoRI*-A and -C fragments. About ten *EcoRI*-A-spe-

TABLE 1. Hybridization of *e*-I RNA with *Eco*RI fragments of ϕ 29 DNA^a

DNA fragment	Per-cent of ϕ 29 DNA ^b	Hybridized cpm		
		<i>e</i> -I RNA	RNA eluted from <i>Eco</i> RI-A	RNA eluted from <i>Eco</i> RI-C
<i>Eco</i> RI-A	51.0	1,473	238	28
<i>Eco</i> RI-B	31.3	485	41	67
<i>Eco</i> RI-C	9.6	4,537	39	732
<i>Eco</i> RI-D	4.7	342		95
<i>Eco</i> RI-E	3.4	119		39

^a The ϕ 29 *e*-I RNA was isolated from CAM-RNA, which was extracted from UV-irradiated cells pretreated with chloramphenicol by sedimentation through 5 to 20% sucrose gradients, as described in the text. ϕ 29 *e*-I RNA was hybridized to *Eco*RI-A or *Eco*RI-C in 1 ml of 65% formamide-2 $\times$ SSC-0.2% SDS and eluted as described by Hayashi and Hayashi (18). Each RNA was hybridized to ϕ 29 DNA fragments cleaved by *Eco*RI in 0.2 ml of 65% formamide-2 $\times$ SSC-0.2% SDS, washed with 2 $\times$ SSC, treated with pancreatic RNase (30 μ g/ml) and RNase T1 (10 U/ml) for 1 h at room temperature, dried, and counted. Input radioactivities of *e*-I RNA, *e*-I RNA eluted from *Eco*RI-A, and *e*-I RNA eluted from *Eco*RI-C were 15,500, 490, and 1,550 cpm, respectively. Radioactivity bound on blank filters was 17 to 25 cpm.

^b Percentage estimates are based on the distribution of radioactivity in a complete *Eco*RI digest of ³²P-labeled ϕ 29 DNA (24).

FIG. 8. Sedimentation profile of ϕ 29 *e*-I RNA eluted from the *Eco*RI-C DNA fragment. ϕ 29 *e*-I RNA was hybridized with *Eco*RI-C fragments, washed, and eluted as described in Table 1. Eluted *e*-I RNA was centrifuged at 48,000 rpm in a Beckman SW56 rotor at 20°C for 150 min. Twelve-drop fractions were collected, and radioactivity was determined.

cific RNA bands can be seen in Fig. 9A, and three *Eco*RI-C-specific RNA bands can be seen in Fig. 9B. These results clearly indicate that each of the RNA bands observed in Fig. 2A and B represented more than a single class of RNA. The estimated molecular weights of the 10 RNA components specific for the *Eco*RI-A fragment,

TABLE 2. Hybridization of ³H-labeled *in vivo* RNA to *Eco*RI DNA fragments^a

DNA fragment (0.4 μ g)	Hybridized cpm
<i>Eco</i> RI-A	850
<i>Eco</i> RI-B	60
<i>Eco</i> RI-C	578
<i>Eco</i> RI-D	97
<i>Eco</i> RI-E	20

^a [³H]CAM-RNA was prepared from UV-irradiated, infected cells that were labeled from 4 to 8 min after infection. Hybridization experiments were performed in 0.2 ml of 65% formamide-0.2% SDS-2 $\times$ SSC for 24 h at 25°C, as described by Hayashi and Hayashi (18). The filters were counted after being washed with 2 $\times$ SSC and treated with pancreatic RNase (30 μ g/ml) and RNase T1 (10 U/ml) for 1 h at room temperature. Input radioactivity was 6,500 cpm.

relative to rRNA, are: 1.0×10^6 , 0.9×10^6 , 0.64×10^6 , 0.5×10^6 , 0.38×10^6 , 0.32×10^6 , 0.25×10^6 , 0.11×10^6 , 0.09×10^6 , and 0.5×10^6 . Three major RNA components specific for the *Eco*RI-C fragment had molecular weights of 0.9×10^6 , 0.43×10^6 , and 0.28×10^6 . Although RNA components with molecular weights of 0.9×10^6 and 0.28×10^6 appeared to be a single RNA species, the RNA band with a molecular weight of 0.43×10^6 had a shoulder (Fig. 9B), suggesting that this RNA band may contain more than one RNA species. These results indicate that ϕ 29 early RNA consists of at least 13 RNA species that can be resolved by polyacrylamide gel electrophoresis; 10 of them are transcripts of the *Eco*RI-A region, and the others are from the *Eco*RI-C region of the ϕ 29 genome.

Analysis of ϕ 29 RNA synthesized *in vitro* with *B. subtilis* RNA polymerase. These studies on ϕ 29 early RNA synthesis *in vivo* suggest that initiators for early RNA transcription may be localized on the *Eco*RI-A as well as the *Eco*RI-C DNA fragments. To confirm these observations and to extend our study of gene expression in ϕ 29, we performed *in vitro* ϕ 29 transcription experiments, using purified *B. subtilis* RNA polymerase.

B. subtilis RNA polymerase and template ϕ 29 DNA were preincubated with all the components necessary for *in vitro* transcription except for Mg^{2+} , which is an absolute requirement for RNA chain elongation. RNA synthesis was begun by the addition of Mg^{2+} , and further initiations were prevented by simultaneous addition of rifampin.

The time course of RNA synthesis *in vitro* is shown in Fig. 10. The incorporation of radioactivity into acid-insoluble material increased linearly for 6 min and reached a plateau at 10 min under the conditions used here. The size

FIG. 9. Analysis of $\phi 29$ early RNA specific for *EcoRI*-A and -C fragments by polyacrylamide gel electrophoresis. Early $\phi 29$ RNA, extracted as described in Fig. 1, was hybridized with DNA fragments of *EcoRI*-A and -C and eluted as described previously (18), followed by polyacrylamide gel electrophoresis at a constant current of 4 mA/gel for 140 min at room temperature. (A) *EcoRI*-A-specific $\phi 29$ early RNA; (B) *EcoRI*-C-specific $\phi 29$ early RNA. Symbols: (●) ^3H -labeled $\phi 29$ RNA; (○) ^{14}C -labeled rRNA.

FIG. 10. Kinetics of $\phi 29$ RNA synthesis directed by *B. subtilis* RNA polymerase. RNA was synthesized at 30°C in 0.2 ml of reaction mixture containing 17 μg of $\phi 29$ DNA, 10 μg of RNA polymerase, 0.1 mM dithiothreitol, 0.1 M Tris-hydrochloride (pH 8.0), 1 mM EDTA, 0.8 mM spermidine, 100 μg of bovine serum albumin per ml, and 0.1 mM nucleotide triphosphates (GTP, CTP, ^3H UTP, and ^3H ATP; 0.1 $\mu\text{Ci/mol}$ each), which were preincubated for 3 min at 30°C . RNA synthesis was started by addition of 10 mM MgCl_2 and 10 μg of rifampin per ml (zero time). At the indicated times, 20- μl portions were removed, and radioactivity in trichloroacetic acid-insoluble material was determined.

distribution of $\phi 29$ RNA synthesized during the limited time of incubation at 30°C was analyzed by polyacrylamide gel electrophoresis. The

chain length of the major RNA products increased as a function of incubation time (Fig. 11). This suggests that RNA chain elongation is synchronized to a considerable extent. In addition, several discrete RNA products were seen (Fig. 11).

To determine the location of the *in vitro* promoters of $\phi 29$ RNA synthesis, the following experiments were performed. RNA was synthesized *in vitro* for 30 s at 30°C in the presence of either ^3H -labeled nucleotide triphosphates or $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ and hybridized to the *EcoRI* fragments of $\phi 29$ DNA. Under the conditions used here, RNA synthesized had an average molecular weight of about 0.2×10^6 (data not shown). ^3H -labeled RNA synthesized *in vitro* hybridized with the *EcoRI*-A and -C fragments but not with the other *EcoRI* fragments of $\phi 29$ DNA.

It has been previously shown that the initiation of $\phi 29$ RNA synthesis *in vitro* by *B. subtilis* RNA polymerase takes place mainly with GTP and to a lesser extent with ATP (26, 38). Table 3 also shows that *in vitro* RNA initiated with $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ (pppG-RNA) hybridized exclusively with the *EcoRI*-A and -C fragments but not with the *EcoRI*-B fragment. In addition, ^3H RNA made *in vitro* for 15 min at 30°C also hybridized preferentially to the *EcoRI*-A and -C fragments and to a lesser extent to the other *EcoRI* fragments of $\phi 29$ DNA. The amount of

FIG. 11. Size distribution of $\phi 29$ RNA synthesized in vitro with *B. subtilis* RNA polymerase in polyacrylamide gel electrophoresis. RNA was synthesized at 30°C in vitro by *B. subtilis* RNA polymerase as described in Fig. 10, except that the reaction mixture contained 100 μ g of RNA polymerase per ml, 160 μ g of $\phi 29$ DNA per ml, and 0.5 mCi of [3 H]ATP, [3 H]UTP, and [3 H]CTP per μ mol. At 1, 2, 4, 6, 8, and 10 min after the addition of $MgCl_2$ and rifampin, RNA was extracted and analyzed by 2.5% polyacrylamide gel electrophoresis as described in Materials and Methods. RNA prepared at 1 min (A), 2 min (B), 4 min (C), 6 min (D), 8 min (E), and 10 min (F).

labeled RNA synthesized in vitro that hybridized to the *Eco*RI-D fragment was reproducibly higher than the amount bound to the *Eco*RI-B fragment. Considering the size of the *Eco*RI-D fragment (4% of the genome), it appears that at least a part of the *Eco*RI-D fragment may be transcribed early in infection (Tables 1 and 2) as well as in vitro (Table 3).

To demonstrate that the RNAs synthesized in vitro by *B. subtilis* RNA polymerase were faithful transcripts of $\phi 29$ DNA, in vitro [3 H]RNA was subjected to hybridization competition with in vivo CAM-RNA. In vivo CAM-RNA competed with over 90% of in vitro [3 H]RNA synthesized for 30 s and competed with more than 70% of in vitro RNA synthesized for 15 min (Table 4).

The size distribution of RNA synthesized in the presence of either 3 H-labeled nucleotide triphosphates or [γ - 32 P]GTP by RNA polymerase for 15 min at 30°C was next analyzed by polyacrylamide gel electrophoresis (Fig. 12). It was

found that at least three major RNA products with molecular weights of 1.6×10^6 , 0.8×10^6 , and 0.5×10^6 were initiated with pppG (Fig. 12B and C). In vitro [3 H]RNA made during 15 min at 30°C was hybridized to the *Eco*RI-A, -B, and -C fragments of $\phi 29$ DNA. Hybrids were eluted and further analyzed by polyacrylamide gel electrophoresis. Figure 13 shows the electrophoretic patterns of in vitro [3 H]RNA hybridized and eluted from the *Eco*RI-A, -B, and -C fragments of $\phi 29$ DNA. It is evident that the largest RNA peak (1.6×10^6 daltons) hybridized mainly to the *Eco*RI-A fragment, whereas the RNA component with a molecular weight of 0.8×10^6 hybridized to the *Eco*RI-C and -A fragments. The lower-molecular-weight RNA components migrating near the 16S rRNA also hybridized to the *Eco*RI-A and -C fragments.

We also analyzed in vitro RNA synthesized in the presence of [γ - 32 P]ATP by polyacrylamide gel electrophoresis (Fig. 14). One major RNA peak was found to have a molecular

TABLE 3. Hybridization of [^3H]RNA and pppG-RNA synthesized in vitro by *B. subtilis* RNA polymerase with *EcoRI* fragments of $\phi 29$ DNA^a

DNA fragment (0.4 μg)	Hybridized cpm		
	30-s [^3H]RNA	30-s pppG-RNA	15-min [^3H]RNA
<i>EcoRI</i> -A	2,959	458	2,350
<i>EcoRI</i> -B	280	43	410
<i>EcoRI</i> -C	2,091	312	1,029
<i>EcoRI</i> -D	128		440
<i>EcoRI</i> -E	20		134

^a [^3H]RNA was synthesized as described in Fig. 11. The reaction mixture for pppG-RNA synthesis contained 7.8 μM [$\gamma\text{-}^{32}\text{P}$]GTP (about 10^6 cpm/pmol), 0.1 mM concentrations of each of three other nucleoside triphosphates, and other components described in Fig. 11. After incubation for 3 min, 0.1 mM GTP, 10 mM MgCl_2 , and 10 μg of rifampin per ml were added (zero time), and incubation was continued. At indicated times, RNA was extracted with phenol. The aqueous layers were passed through a Sephadex G-75 column. RNA fractions eluted were treated with DNase I and then heated for 3 min at 95°C in the presence of 0.2% SDS. RNA was precipitated with ethanol and dissolved in 0.1% SDS. Hybridization experiments were carried out as described in Table 1. Hybridization of pppG-RNA was performed without treatment with RNases. Input radioactivities of 30-s [^3H]RNA, 15-min [^3H]RNA, and pppG-RNA were 25,000, 9,000, and 1,800 cpm, respectively. Radioactivity bound to membrane in the absence of DNA was about 17 cpm.

weight of 0.1×10^6 . Hybridization studies have indicated that pppA-RNA is a transcript of the *EcoRI*-A region of the $\phi 29$ genome (data not shown).

The results of these in vitro transcription studies are consistent with those obtained in vivo and indicate that synthesis of early RNA is initiated from at least two efficient promoter regions: one is located in the *EcoRI*-A region, and the other is located in the *EcoRI*-C region of the $\phi 29$ genome.

Synthesis of mRNA specific for *EcoRI*-A, -B, and -C fragments of $\phi 29$ DNA. Kinetics of mRNA synthesis during the lytic cycle were examined by using unique DNA fragments generated by cleavage with restriction endonuclease *EcoRI*. As shown above, the *EcoRI*-A and -C fragments contain early regions of the $\phi 29$ genome. The *EcoRI*-B and part of the *EcoRI*-A fragment contain late regions of the $\phi 29$ genome (Kawamura and Ito, unpublished data). Therefore, it is possible to determine the kinetics of transcription of the different regions of the $\phi 29$ genome by hybridization to these DNA segments. *B. subtilis* SR22 cells were infected with $\phi 29$ and pulse-labeled with [^3H]uridine for various times postinfection.

TABLE 4. Hybridization competition of in vitro [^3H]RNA with in vivo CAM-RNA^a

In vitro [^3H]RNA	Unlabeled RNA (mg)	Radioactivity retained (cpm)	Percent competition
30-s RNA	None	1,195	0
	0.07	827	31
	0.14	667	45
	0.28	413	80
	0.56	106	92
15-min RNA	None	3,122	0
	0.07	2,285	27
	0.28	528	84
	0.56	860	73

^a Hybridization of in vitro-synthesized [^3H]RNA to $\phi 29$ DNA was competed for by unlabeled CAM-RNA from $\phi 29$ -infected *B. subtilis*. Hybridization experiments were carried out as described in Table 1. $\phi 29$ DNA was immobilized at a concentration of 2.5 $\mu\text{g}/\text{filter}$. Input radioactivities were 2,950 and 9,000 cpm for 30-s [^3H]RNA and 15-min [^3H]RNA, respectively.

The ^3H -labeled RNA was isolated and hybridized to $\phi 29$ DNA and to the *EcoRI*-A, -B, and -C fragments (Fig. 15). Early mRNA's, specific for the *EcoRI*-A and -C fragments, were synthesized immediately after phage infection. RNA synthesis specific for the *EcoRI*-B fragment, on the other hand, was not initiated for at least several minutes after phage infection. These results confirm the previous results (Tables 1 and 2) showing that the *EcoRI*-A and -C fragments contain early regions of the $\phi 29$ chromosome, whereas the *EcoRI*-B fragment harbors the late region of the viral genome.

It is also evident that the synthesis of early RNA, specific for the *EcoRI*-C fragment, continues throughout the infectious cycle. Since the *EcoRI*-A fragment also contains late genes of the $\phi 29$ genome, it is difficult to conclude that the early mRNA, specific for the *EcoRI*-A fragment, is also produced continuously late after phage infection. However, our hybridization competition experiments have shown that in vivo pulse-labeled [^3H]RNA (14 to 16 min) hybridized to the *EcoRI*-A fragment, and the hybridization was competed with CAM-RNA (not shown). We conclude that the early mRNA made from the *EcoRI*-A region of the genome, and possibly all early mRNA synthesis, is produced continuously throughout the lytic cycle.

DISCUSSION

The present study demonstrates that there are at least two efficient initiation sites for early transcription on the bacteriophage $\phi 29$ chromosome. By hybridization to unique DNA

FIG. 12. Electropherogram of $\phi 29$ RNA synthesized in vitro by RNA polymerase. $[^3\text{H}]\text{RNA}$ and pppG-RNA were synthesized in vitro by *B. subtilis* RNA polymerase for 15 min at 30°C and purified as described in Fig. 11 and Table 3, respectively. Purified RNA was analyzed by 2.5% polyacrylamide gel electrophoresis for 3 h. The gels were cut into 0.1-mm slices, and the radioactivity was determined. (A) In vitro 15-min $[^3\text{H}]\text{RNA}$ ($\circ$), ^{14}C -labeled rRNA ($\bullet$); (B) in vitro 15-min RNA labeled by $[\gamma\text{-}^{32}\text{P}]\text{GTP}$; (C) in vitro 15-min $[^3\text{H}]\text{RNA}$ ($\circ$), in vitro 15-min RNA labeled by $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ ($\bullet$).

fragments generated by the restriction endonuclease *EcoRI* (24), two major early RNAs synthesized in the cells infected with $\phi 29$ were assigned to the *EcoRI*-A and -C fragments of this viral genome.

It has been shown that $\phi 29$ early mRNA is transcribed from the light strand and the late

mRNA is transcribed from the heavy strand of $\phi 29$ DNA (36, 42, 43). Jiménez et al. (26) have described the initiation of $\phi 29$ DNA transcription with *B. subtilis* RNA polymerase in vitro as taking place mainly with GTP and to a lesser extent with ATP. We have confirmed their results and in addition have found that $[\gamma\text{-}$

FIG. 13. Analysis of *in vitro* $\phi 29$ RNA specific for *EcoRI*-A, -B, and -C fragments by polyacrylamide gel electrophoresis. *In vitro* [^3H]RNA, obtained as described in Fig. 12, was hybridized with *EcoRI*-A, -B, and -C fragments of $\phi 29$ DNA, eluted, and analyzed by polyacrylamide gel electrophoresis as described in Fig. 12. (A) *EcoRI*-A-specific *in vitro* $\phi 29$ RNA; (B) *EcoRI*-B-specific *in vitro* $\phi 29$ RNA; (C) *EcoRI*-C-specific *in vitro* $\phi 29$ RNA. Symbols: (●) ^3H -labeled $\phi 29$ RNA; (○) ^{14}C -labeled rRNA.

^{32}P]GTP-labeled RNA transcribed from $\phi 29$ DNA *in vitro* with *B. subtilis* RNA polymerase hybridized specifically to the *EcoRI*-A and -C fragments of $\phi 29$ DNA.

Genetic studies of $\phi 29$ mutants by Mellado et al. (34) have established a genetic map of this bacteriophage in which 17 genes have been or-

dered. Through extensive genetic and biochemical studies (3, 5, 6, 33, 35, 40, 41), a great deal of information as to the function of each cistron of $\phi 29$ has currently become available. Furthermore, restriction enzyme cleavage maps of the $\phi 29$ genome have been constructed (22-24). These have recently been correlated with a ge-

FIG. 14. Electropherogram of $\phi 29$ $^{32}\text{pppA}$ -RNA synthesized *in vitro* by *B. subtilis* RNA polymerase. The reaction mixture for synthesizing pppA-RNA contained 25 M $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (about 3×10^4 cpm/pmol), 0.1 mM concentrations of each of three other nucleotide triphosphates (GTP, CTP, and UTP), and other components, as described in Fig. 10. After incubation for 3 min at 30°C, 0.1 mM ATP, 10 mM MgCl_2 , and 10 μg of rifampin per ml were added, and incubation was continued for 15 min at 30°C. RNA was purified as described in Materials and Methods and analyzed by polyacrylamide gel electrophoresis. Symbols: (●) *in vitro* RNA labeled with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$; (○) ^{14}C -labeled rRNA.

FIG. 15. Sequential synthesis of mRNA in $\phi 29$ -infected cells. *B. subtilis* SR22 cells were grown to 10^8 cells/ml in Min-CH medium at 37°C and infected with $\phi 29$ at a multiplicity of infection of 10. At various times after infection, cultures were pulse-labeled with ^3H uridine (50 $\mu\text{Ci}/\text{ml}$). ^3H -labeled RNA was isolated and hybridized to $\phi 29$ DNA (2 $\mu\text{g}/\text{filter}$) and to the EcoRI-A, -B, and -C fragments (0.4 $\mu\text{g}/\text{ml}$), respectively, as described in Materials and Methods. Input radioactivities were adjusted to 50,000 cpm.

netic map, using marker rescue techniques (22, 23).

Combining the present *in vivo* and *in vitro* transcription studies with the current detailed knowledge of the gene function of $\phi 29$ (4, 49), we now propose a transcription map of bacteriophage $\phi 29$ (Fig. 16). The direction of the early $\phi 29$ transcription is right to left with orientation to the genetic map (Fig. 16). This is based on the following observations: (i) $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ -

labeled RNA and ^3H -labeled RNA made *in vitro* for 30 s at 30°C hybridized only to EcoRI-A and -C fragments; (ii) *in vitro* RNA made for 15 min at 30°C hybridized to the EcoRI-D fragment of $\phi 29$ DNA; and (iii) CAM-RNA prepared from infected cells also hybridized to the EcoRI-D fragment.

Genetic as well as biochemical studies (3, 35, 40) have shown that a nonsense mutation in gene 10 causes a polar effect on the expression of gene 11 and gene 12, suggesting that the direction of late transcription is from left to right. In addition, we have observed that large late mRNA isolated from infected cells hybridized to EcoRI-A and -B fragments but not to the EcoRI-C fragment (Kawamura and Ito, unpublished data). These findings are consistent with the transcription map proposed here.

The present study does not allow us to make any statement about the precise location of the initiation sites of $\phi 29$ transcription. Since the largest of the five EcoRI fragments (the EcoRI-A fragment) comprises over 50% of the entire $\phi 29$ genome (22, 24), utilization of additional cleavage fragments generated by other restriction endonucleases is required to unambiguously localize the early and late mRNA sequences on the $\phi 29$ chromosome.

Recently, it has been observed by electron microscopy that *B. subtilis* RNA polymerase binds to the $\phi 29$ DNA at three different regions: the EcoRI-A, -B, and -C fragments (Salas, personal communication). In our *in vivo* and *in*

FIG. 16. Transcriptional map of the $\phi 29$ genome. The order and relative size of the *EcoRI* cleavage fragments are from Ito et al. (24). Correlation between the physical and genetic maps is based on the studies by F. Kawamura, J. Ito, and B. E. Reilly (in preparation) and by Inciarte et al. (22). Gene order and function are based on the studies by Mellado et al. (34), Anderson and Reilly (3), and Carrascosa et al. (5). Cistrons are not drawn to scale. The exact locations and numbers of the initiation sites for the late mRNA are not known.

vitro studies, however, we have been unable to show that the *EcoRI*-B fragment contains initiation sites for early $\phi 29$ transcription. It is possible that a low-molecular-weight early RNA is transcribed from the *EcoRI*-B fragment of $\phi 29$ DNA and has escaped our detection.

To characterize $\phi 29$ early RNA synthesized in vivo, RNA was fractionated by hybridization to and elution from *EcoRI* digestion fragments. The eluted RNA was analyzed by acrylamide gel electrophoresis. At least 10 RNA bands with molecular weights of 0.05×10^6 to 1.0×10^6 were found for early RNA specific to the *EcoRI*-A fragment. Three major RNAs specific to the *EcoRI*-C fragment were similarly resolved. Thus, a total of at least 13 early $\phi 29$ RNA species could be identified by acrylamide gel electrophoresis. The sum of the molecular weights of these early RNAs, relative to those of rRNA, is about 5.8×10^6 .

Considering that early RNA is transcribed from only one strand (the light strand) of the $\phi 29$ genome, having a molecular weight of 11×10^6 to 12×10^6 (1, 2), and that about half of the *EcoRI*-A fragment contains early genes, this value is in excess. This indicates that some RNA species contain the same nucleotide sequences. We have obtained evidence suggesting that high-molecular-weight RNAs (e-I and e-II RNAs) are unstable (Kawamura and Ito, manuscript in preparation). In the present study, we show that the e-I RNA consists of two RNA species, one that hybridizes to the *EcoRI*-A fragment and one that hybridizes to the *EcoRI*-C fragment. The sum of the molecular weights of these three RNAs [$2 \times (\text{e-I}) + \text{e-II}$] is 2.44×10^6 , which accounts for approximately 40% of the information contained in the viral genome. This value is in general agreement with the results of $\phi 29$ -induced protein synthe-

sis: several lines of evidence have indicated that the early proteins are specified by 35 to 40% of the $\phi 29$ genome region (7, 17). If these computations are valid, then it is likely that the smaller, more stable RNAs have been derived from the higher-molecular-weight RNA molecules, possibly by processing with specific RNases. Such a situation would be similar to the case of T7 and T3 mRNA synthesis (12, 20), where it is known that host *E. coli* RNA polymerase transcribes the early genes into a polycistronic RNA, which is subsequently cleaved into five monocistronic mRNAs by host RNase III (12, 20). Alternatively, these smaller RNAs may arise as unique transcription products and not from cleavage of larger RNA species, since some of the low-molecular-weight RNAs are also synthesized in vitro (Fig. 11). Our present studies also showed that larger $\phi 29$ RNA than in vivo RNA can be synthesized in vitro. This indicates that some specific termination factors are involved in $\phi 29$ transcription in vivo.

It is also possible that low-molecular-weight mRNA is chemically stable but functionally inactive. T7 mRNA's are stable in terms of acid solubility and hybridizability, but decay rapidly in terms of translational ability (32, 50). Thus, it remains to be determined whether $\phi 29$ stable mRNA's are functionally stable.

Our results could also be explained if overlapping transcription similar to the phage T7 system occurs. Pachl and Young have shown (37) that some of the late T7 proteins are encoded by multiple mRNA species differing in molecular weight. They have suggested that the mode of overlapping transcription plays an important role in regulating the levels of T7 proteins. Since no evidence has so far been reported for the existence of translational control in the $\phi 29$ system, the overlapping tran-

scription becomes an attractive model for the regulation of gene activity during the life cycle of this virus.

These possibilities can be resolved by analysis of mRNA species for their functional integrity, using an *in vitro* translational system (6), and for their origin, using specific fragments of the $\phi 29$ genome generated with several different restriction endonucleases (23). Now that a detailed physical map of the $\phi 29$ genome is available (23), it should be possible to conduct these studies.

ACKNOWLEDGMENTS

We are grateful to Gayle Mildner for her excellent assistance. We also thank C. Bieger and J. Ashwell for their help in preparing this manuscript. We further thank E. P. Geiduschek and J. J. Duffy for critical reading of the manuscript.

This research was supported in part by Public Health Service grant GM-20055-01 from the National Institute of General Medical Sciences, National Science Foundation grant GB-43590, and American Cancer Society grant NP-203.

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