Gene Expression During the Development of *Bacillus subtilis* Bacteriophage $\phi 29$

II. Resolution of Viral-Specific Ribonucleic Acid Molecules

D. J. LOSKUTOFF AND J. J. PÈNE

*Department of Molecular, Cellular, and Developmental Biology, University of Colorado,
Boulder, Colorado 80302*

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The ribonucleic acid (RNA) specified by bacteriophage $\phi 29$ was isolated under conditions which minimized physical and enzymatic degradation, reduced aggregation, and enriched for completed molecules. This RNA was fractionated both by sedimentation through sucrose density gradients and electrophoresis through polyacrylamide gels to measure the size and relative amount of each component. Early RNA consisted of six components of molecular weight 0.75×10^6 , 0.44×10^6 , 0.37×10^6 , 0.25×10^6 , 0.09×10^6 , and 0.04×10^6 , accounting for 35% of the coding capacity of $\phi 29$ deoxyribonucleic acid (DNA). All of these components except the one at 0.44×10^6 were detected when infection occurred in the presence of chloramphenicol. Synthesis of the major early component (0.75×10^6) ceased shortly after the onset of viral DNA synthesis. The other species of early RNA were synthesized throughout the latent period. Three additional components, 1.75×10^6 , 0.93×10^6 , and 0.07×10^6 , appear at late times. The two large RNAs may be polycistronic messenger RNAs corresponding to the seven viral capsid proteins.

Characterization of individual species of messenger ribonucleic acid (mRNA) would contribute significantly to understanding mechanisms which affect gene expression during phage development. The isolation and resolution of intact mRNA molecules have been constrained both by technical problems associated with the purification of these metabolically unstable macromolecules and by the complexity of most systems judged attractive for genetic analysis. Thus, previous attempts to characterize transcription during bacteriophage development have relied primarily upon analysis of RNA by deoxyribonucleic acid (DNA)-RNA hybridization (4, 7), or upon detection of specific proteins by using standard biochemical techniques (4, 10). The technique of competition-hybridization is inadequate to detect single species of mRNA since this approach does not distinguish between intact and fragmented molecules. In addition, it lacks the sensitivity required to detect changes in single species of mRNA among complex populations of molecules. Examination of gene expression by direct measurement of proteins is similarly limiting, since in most cases only a few viral functions are known. Neither approach yields information about the number

of individual mRNAs or their size. In addition, information about the intramolecular organization of polycistronic mRNAs and the rates of initiation and termination of transcriptive units are difficult to obtain when utilizing these techniques. This paper summarizes the results of experiments employing polyacrylamide gel electrophoresis to fractionate the mRNA molecules specified by phage $\phi 29$. Their metabolism throughout the viral lytic cycle is described.

$\phi 29$ is a *Bacillus subtilis* bacteriophage which contains a single molecule of double-stranded DNA of molecular weight 11×10^6 (1, 9). Over 50% of the viral genetic information is devoted to the synthesis of seven capsid polypeptides (D. J. Loskutoff, A. R. Ayers, and J. J. Pène, *Bacteriol. Proc.*, p. 197, 1971; see also reference 14). If half of the DNA can be transcribed into mRNA, the sum total of potential viral-specific RNA should be 5.5×10^6 .

In a previous communication the transcription of phage $\phi 29$ chromosomes was examined by competition-hybridization (13). Two classes of $\phi 29$ -specific RNA were defined. "Early" genes were those activated before the onset of viral DNA replication, whereas "late" genes were those expressed at later times in

the viral lytic cycle, temporally at approximately the same time as the onset of DNA synthesis. Forty percent of the genome was activated early, an additional 60% after 10 min. Although late genes were expressed in conjunction with viral DNA replication, the replication of $\phi 29$ chromosomes was not necessary for their expression. Most of the early genome was found to be active throughout the viral latent period. No late transcripts and only 80% of the early ones could be detected when infection occurred in the presence of chloramphenicol.

Preliminary experiments described the identification of four distinct and at least three less defined size classes of late viral RNA (D. J. Loskutoff, A. R. Ayers, and J. J. Pène, *Bacteriol. Proc.*, p. 197, 1971). This paper describes the results of further experiments to characterize the RNA molecules specified by $\phi 29$. Nine components, accounting for over 85% of the coding capacity of $\phi 29$ DNA, have been identified. Their time of appearance in the viral life cycle and the percentage of the genome devoted to their synthesis is in good agreement with the hybridization-competition data previously reported (13).

MATERIALS AND METHODS

Bacteria and bacteriophages, media, growth, and purification of $\phi 29$, isolation of viral DNA, and the procedures for DNA-RNA hybridization were described previously (13).

Labeling and purification of RNA. RNA from uninfected and infected bacteria was labeled by a 4-min exposure to uridine-5- 3H (New England Nuclear Corp.; 25.6 Ci/mmol) as described in the figures. The cells were treated simultaneously with chloramphenicol (CAP; Calbiochem, Los Angeles, Calif., 250 μ g/ml) to minimize normal intracellular degradation of RNA (11). This treatment was found to increase significantly the stability of RNA after addition of rifamycin (a gift of Ciba-Geigy Corp., Summit, N.J.) or actinomycin D (a gift of Merck and Co., Rahway, N.J.). RNA to be analyzed by polyacrylamide gel electrophoresis was prepared by a modification of the diethylpyrocarbonate (DEP) technique (20, 22). Bacteria were exposed to lysozyme (500 μ g/ml) for the final 30 sec of the labeling period, followed by the addition of sodium dodecyl sulfate (SDS) to 1.5% and DEP (Eastman Organic Chemicals, Rochester, N.Y.) to 3%. The clear, viscous lysate was incubated for an additional 3 min at 37 C and 0.5 volume of cold saturated NaCl was added. The chilled solution was centrifuged at $12,000 \times g$ for 10 min and the supernatant solution was filtered through a Nalgene 0.45- μ m membrane filter unit (Nalge Co., Rochester, N.Y.) and precipitated for 4 hr at -20 C with two volumes of ethanol. After centrifugation, the pellet was resuspended in water and dialyzed against 0.1X Loenings buffer (12) for 1 hr at 4 C. Loenings buffer contains 0.036 M tris-

(hydroxymethyl)aminomethane (Tris), 0.03 M NaH_2PO_4 , 0.001 M ethylenediaminetetraacetic acid (EDTA) and 0.2% sodium dodecyl sulfate, pH 7.7. The final RNA preparation was free of DNA (13) and had a specific activity of approximately 100,000 counts per min per μ g. All preparations for gel analysis were stored at -70 C until used. $\phi 29$ RNA processed and stored as described above was stable for weeks.

Sucrose gradients. Initial approximations of the size of $\phi 29$ RNA were obtained by subjecting total infected RNA to analysis by centrifugation through 15 to 30% linear sucrose density gradients containing 0.05 M sodium acetate, 0.01 M disodium EDTA, 0.01 M Tris (pH 7.4), and 0.2% sarkosyl (Ciba-Geigy Corp., Ardsley, N.Y.). Centrifugation was for 12 to 14 hr (or as indicated) at 4 C, in a Beckman SW41 rotor at 41,000 rev/min. After centrifugation, the contents of each tube were pumped through a flow cell and monitored at 260 nm to locate bacterial ribosomal and transfer RNAs; each tube was fractionated into 50 fractions containing 12 drops. Under these conditions, the 23S RNA moves approximately two-thirds of the way through the gradient.

The positions of 23, 16, and 4S RNA markers in the gradient were used to calculate the sedimentation coefficient of $\phi 29$ RNA. The amount of viral-specific RNA in each fraction was determined by hybridization to $\phi 29$ DNA (8, 13). Blank filters initially were included in each hybridization reaction to evaluate the extent of nonspecific binding (e.g., resulting from bacterial DNA in the preparation), but since the background was low and constant throughout the gradient, this step was subsequently eliminated.

Polyacrylamide gel electrophoresis. RNA was analyzed by electrophoresis through columns (0.5 cm by 10 cm or 15 cm) of 2.5% polyacrylamide containing 0.07% *N,N'*-methylenebisacrylamide (Eastman Kodak, Rochester, N.Y.). The gels were polymerized for at least 12 hr, and electrophoresis was conducted for 4 hr at 5 v/cm prior to the addition of the sample. Gel tubes were completely submerged in electrophoresis buffer to minimize heating. Electrophoresis was conducted at room temperature at 10 v/cm (constant voltage) resulting in a current of 8 to 10 ma per gel. After electrophoresis, the gels were removed from the glass tubes and scanned at 260 nm using the linear transport attachment of a Gilford 2000 recording spectrophotometer. The gels were then frozen in an ethanol-dry ice bath and cut into 2-mm slices. The molecular weight of the RNA in each slice was estimated by comparing its relative position with that of 23, 16, 5, and 4S bacterial RNAs. Under these conditions a linear relationship exists between the distance traveled by an RNA molecule and the logarithm of its molecular weight (2). Each slice was incubated for 6 hr in 2 ml of $2 \times$ SSC at 65 C to elute the RNA. Viral-specific RNA was detected by DNA-RNA hybridization. Gel slices did not appear to interfere with the hybridization reaction.

RESULTS

Analysis of viral RNA by sedimentation

through sucrose gradients. The results of competition-hybridization experiments reported separately (13) indicated that gene expression during ϕ 29 development occurred in a highly regulated manner and involved two distinct stages. Viral RNA was analyzed by sedimentation through 15 to 30% sucrose gradients in an attempt to further define the transition from early to late transcription. Figure 1 shows the size distribution of ϕ 29 RNA labeled at progressively late intervals in the infectious cycle. In part A, the RNA was labeled from 2 to 6 min after infection and thus well before the onset of viral DNA replication at 10 min (9, 13). Three major components were resolved, migrating with sedimentation coefficients of 17, 12, and 9S. In addition a shoulder was present on the 9S component with a sedimentation coefficient of approximately 6S. Viral-specific RNA could not be detected in the region of the gradient corresponding to a size greater than 23S.

Figure 1B shows the sedimentation profile of RNA extracted 5.5 to 9.5 min after infection, a time spanning the onset of viral DNA replication. RNA greater than 23S begins to appear. In addition, a 14S component becomes prominent while the relative amount of the 17S component has decreased.

Figure 1C shows the profile of ϕ 29 RNA labeled from 21 to 25 min after infection, well after the onset of viral DNA replication. The first intracellular phages are detected at this time (*unpublished observation*). Data from DNA-RNA competition-hybridization experiments have shown that most of the sequences in early RNA were present in late RNA, but that late RNA contained unique species absent from early RNA (13). Furthermore, it was previously concluded that late RNA contained molecules of large molecular weight since experiments measuring the stability of late RNA after addition of rifamycin showed that phage RNA synthesis continued for 2 min after addition of the drug (13). It can be seen from Fig. 1C that the majority of viral-specific RNA late in ϕ 29 development migrated in the region of the gradient corresponding to material larger than 17S, the largest component found in early RNA. Distinct RNA components were recognized at 30, 26, 20, and 14S, as well as in the 6 to 9S region. The complexity of the pattern and the lack of resolution made it difficult to determine if the 12S and 17S components were still synthesized at this late time.

These experiments indicate that gene expression during phage ϕ 29 development occurs via the synthesis of at least eight components ranging in size from 6 to 30S. Material larger

than 30S was also present at the bottom of the centrifuge tube at late times after infection. An estimate of the molecular weight of RNA molecules was obtained by comparing sedimentation values of the viral RNAs with RNAs of known molecular weight (2). The size distribution is large, the smallest component being less than 10^5 daltons, whereas the largest one is approximately 2×10^6 daltons.

Figure 2 shows the result of a similar experiment analyzing the size distribution of ϕ 29 RNA extracted from bacteria which were infected in the presence of CAP. Under these conditions only 80% of the early RNA and no late RNA is detected (13). The pattern is very similar to the early patterns shown in Fig. 1 in that components were detected at 17, 12, 9, and 6S, and no material larger than 23S was detected.

Analysis of viral RNA by polyacrylamide gel electrophoresis: chloramphenicol RNA. Figure 2 indicated that at least four size components were synthesized when infection occurred in the presence of CAP. The limited resolution of sucrose gradients suggested the possibility that other components might be present but not detectable. CAP RNA was, therefore, subjected to analysis by electrophoresis through polyacrylamide gels in an effort to define further its composition.

Figure 3 shows the size distribution of RNA extracted 29 min after infection from bacteria pretreated with CAP. Five components, superimposed on a heterogeneous background, were found. Although the characteristics of the background material vary slightly from experiment to experiment, these five components were reproducible. Since small variations arise from cutting or counting procedures, RNA peaks which consisted of less than two points on these graphs or which did not appear in all gels have been ignored. The molecular weights are presented as a fraction of 10^6 daltons.

The largest molecules migrate between the 23S and 16S ribosomal RNA and have a molecular weight of approximately 0.75×10^6 . Little or no viral RNA was detected in the area of the gel corresponding to sizes greater than 23S. Viral-specific material was also present at 0.37×10^6 , 0.25×10^6 , and 0.09×10^6 daltons, with a very prominent component migrating between the bromophenol blue marker dye and 5S RNA. This RNA has an estimated molecular weight of 0.04×10^6 . The five components have an accumulated molecular weight of about 1.5×10^6 , accounting for 27% of the transcriptional potential of the virus (assuming that only one strand of ϕ 29 DNA is transcribed

FIG. 1. Fractionation of $\phi 29$ RNA by centrifugation through sucrose gradients. Infected bacteria were exposed to CAP (250 $\mu\text{g/ml}$) and ^3H -uridine (40 $\mu\text{Ci/ml}$) at the indicated times. The RNA was extracted and fractionated as described in Materials and Methods. Approximately 100 μg of total RNA was layered on each 15 to 30% sucrose gradient. Centrifugation was for 14 hr using the SW41 rotor at maximum speed at 4 C. The ultraviolet-absorbing material was monitored (arrows indicate the position of bacterial 23, 16, and 4S RNA), and each fraction was hybridized to 10 μg of $\phi 29$ DNA to localize viral-specific RNA. A, 2 to 6 min after infection; B, 5.5 to 9.5 min after infection; C, 21 to 25 min after infection.

FIG. 2. Fractionation of CAP RNA by centrifugation through sucrose gradients. Logarithmically growing bacteria were treated with CAP (250 μ g/ml) at -5 min and infected with ϕ 29 at 0 min. The infected bacteria were labeled with 3 H-uridine (40 μ Ci/ml) from 25 to 29 min after infection, and the RNA was extracted by the DEP technique. Fractionation and detection of viral-specific material was the same as that described in the legend to Fig. 1.

and that each size class represents a distinct species of RNA and not a degradation product of a larger class). This number is, therefore, in good agreement with the estimation obtained from the DNA-RNA hybridization-competition data (13).

Analysis of early and late RNA. The number of viral RNA components and their changes during the lytic cycle were further characterized by examination of RNA prepared at various times after infection. Figure 4 shows the distribution of RNA labeled from 2 to 6 min after infection. The majority of the radioactivity was found to be associated with the 0.75×10^6 dalton component. Comparison of this gel profile and those in Fig. 1 and 2 indicates that this is the 17S RNA component resolved by sucrose. Viral-specific RNA was also found at 0.25×10^6 and 0.09×10^6 daltons. In addition, material hybridizing to ϕ 29 DNA

FIG. 3. Fractionation of CAP RNA by polyacrylamide gel electrophoresis. Logarithmically growing bacteria were treated with CAP (250 μ g/ml) 5 min prior to infection with ϕ 29. The infected bacteria were labeled with 3 H-uridine (40 μ Ci/ml) from 25 to 29 min after infection, and the RNA was extracted by the DEP technique. Between 20 and 30 μ g of total RNA was layered on a 15-cm, 2.5% acrylamide gel. Electrophoresis was for 2.5 hr at 10 v/cm (see Materials and Methods for details). The gel was analyzed at 260 nm to localize the 23, 16, 5, and 4S components (arrows) and cut into 2-mm slices. The RNA from each slice was hybridized to 5 μ g of ϕ 29 DNA to localize viral-specific material. The molecular weights of viral-specific RNAs are presented as a fraction of 10^6 daltons.

was detected reproducibly in the region of the gel corresponding to 0.44×10^6 and 0.37×10^6 daltons. This RNA was different from CAP RNA in two respects, i.e., the component at 0.04×10^6 daltons was missing and a new one at 0.44×10^6 daltons was present. The RNA at 0.04×10^6 daltons was present at slightly later times but still well before the

FIG. 4. Fractionation of 2- to 7-min RNA by polyacrylamide gel electrophoresis. Infected bacteria were exposed to CAP (250 $\mu\text{g/ml}$) and ^3H -uridine (40 $\mu\text{Ci/ml}$) from 2 to 6 min after infection. The RNA was extracted and fractionated by the procedure outlined in Materials and Methods. The RNA from each fraction was hybridized to 5 μg of $\phi 29$ DNA to localize viral-specific material.

onset of viral DNA replication (*unpublished observation*). The accumulated molecular weight of the six early species is 1.94×10^6 or about 35% of the potential RNA.

The profile was very different when the RNA was labeled from 5.5 to 9.5 min after infection (Fig. 5). Synthesis of the components at 0.44×10^6 , 0.37×10^6 and 0.25×10^6 daltons was stimulated relative to the 0.75×10^6 dalton component and new components at 1.75×10^6 and 0.07×10^6 daltons were detected. This interval is thus a transition period characterized by the synthesis of new RNA components.

FIG. 5. Fractionation of 5.5- to 9.5-min RNA by polyacrylamide gel electrophoresis. Infected bacteria were exposed to CAP (250 $\mu\text{g/ml}$) and ^3H -uridine (40 $\mu\text{Ci/ml}$) from 5.5 to 9.5 min after infection. The RNA was extracted and fractionated by the procedure outlined in Materials and Methods. The RNA from each fraction was hybridized to 5 μg of $\phi 29$ DNA to localize viral-specific material.

DNA synthesis could not be detected under these conditions (CAP was added at 5.5 min), consistent with the conclusion that the transition from early to late RNA occurs independently of $\phi 29$ DNA replication (13).

Figure 6 shows an electropherogram of $\phi 29$ RNA molecules labeled from 25 to 29 min after infection. Distinct components were found at 1.75×10^6 and 0.93×10^6 daltons. Viral RNA was also located between these two components with an approximate molecular weight of 1.4×10^6 to 1.5×10^6 . The component at 0.07×10^6 daltons was clearly resolved at this time, whereas the major early component (0.75) was not detected.

Nine late RNA components were recognized

FIG. 6. Fractionation of 25- to 29-min RNA by polyacrylamide gel electrophoresis. Infected bacteria were exposed to CAP (250 μ g/ml) and 3 H-uridine (40 μ Ci/ml) from 25 to 29 min after infection. The RNA was extracted and fractionated by the procedure described in Materials and Methods. The RNA from each fraction was hybridized to 5 μ g of ϕ 29 DNA to localize viral-specific material.

in these gels, two migrating slower than the 23S RNA (1.75×10^6 and 1.4×10^6 daltons), one between the 23S and 16S (0.93×10^6 daltons) and six moving slower than the 16S ribosomal RNA component (0.44×10^6 , 0.37×10^6 , 0.25×10^6 , 0.09×10^6 , 0.07×10^6 , and 0.04×10^6 daltons). Again, much unresolved material was found in the middle of the gel, making it difficult to detect the component at 0.25 or to eliminate the possibility that others exist in this molecular-weight region. At these times, the rate of synthesis of all components relative to the one at 1.75×10^6 daltons was decreased, resulting in the tendency of this species to dominate the profile. Very little labeled RNA was found in that portion of the gel corresponding to 0.75×10^6 daltons. In addition, some material did not enter the

gels at these late times.

Analysis of the RNA specified in the absence of viral DNA replication. Previous experiments indicated that the transition from early to late RNA occurred independently of viral DNA replication (13). It was therefore important to confirm this result directly by gel analysis of the RNA components present. In addition, it was of interest to determine the effect of the restrictive temperature (45 C) on the RNA profile. Accordingly, bacteria were grown at 45 C and infected with wild-type ϕ 29 or TS35, a ϕ 29 mutant temperature-sensitive for DNA replication (9). The RNA was labeled with 3 H-uridine from 25 to 28 min after infection and extracted as described in Materials and Methods. The results are shown in Fig. 7.

It is clear that distinct components are resolved in both cases and that these are similar to those seen at late times at 37 C (see Fig. 6). The presence of distinct species at 45 C supports the conclusion that ϕ 29 RNA is relatively stable (13). The RNAs extracted from wild-type-infected (Fig. 7A) and TS35-infected (Fig. 7B) bacteria are very similar. The absence of the 0.75×10^6 dalton component is apparent in both cases, and both contain the large-molecular-weight material which is characteristic of late RNA.

Effects of CAP on late profile. Pulitzer (18) and Cascino (3) have shown that expression of late T4 genes depends both upon viral DNA replication and the products of genes 55 and 33. Continuous protein synthesis was required for the continued expression of late genes. It was interesting, therefore, to determine whether late ϕ 29 RNA continues to be made in the absence of protein synthesis, or whether gene expression reverts back to a pattern which is characteristic of early development. These questions were answered directly by gel analysis.

CAP was added to infected bacteria at 21 min after infection, a time when late RNA is being synthesized. Radioactive uridine was then added for 4-min intervals at 21 (Fig. 8A) or 35 (Fig. 8B) min after infection, and the RNA was extracted and analyzed. The results are shown in Fig. 8. It can be seen that the RNA components present in the control (Fig. 8A) are also present after 18 min of additional exposure to CAP. ϕ 29 transcription is thus very different from that of T4 in that expression of late ϕ 29 genes continues even after prolonged periods in the absence of protein synthesis.

FIG. 7. Fractionation of the RNA specified by TS35 at the nonpermissive temperature. *B. subtilis* 60084 was grown at 45 C to 2×10^8 cells per ml and infected with wild-type phage or mutant TS35 (multiplicity of infection = 10–15). CAP (250 μ g/ml) and 3 H-uridine (20 μ Ci/ml) were added at 25 min after infection. The RNA was extracted at 28 min and fractionated by the procedure described in Materials and Methods. The RNA from each fraction was hybridized to 5 μ g of ϕ 29 DNA to localize viral-specific material. A, ϕ 29 wild type; B, TS35.

DISCUSSION

In the discussion of the experiments reported in this communication we have assumed that in vivo transcription of ϕ 29 chromosomes is asymmetric and that the maximum amount of RNA which can be copied from ϕ 29 DNA

would represent half of the molecular weight of the DNA. We therefore expect, as a maximum value, to account for 5.5×10^6 daltons of RNA sequences. As shown by electrophoresis on acrylamide gels, early ϕ 29 RNA is composed of six components: 0.75×10^6 , 0.44×10^6 , 0.37×10^6 , 0.25×10^6 , 0.09×10^6 , and $0.04 \times$

FIG. 8. Viral RNA synthesis after prolonged exposure to CAP. Logarithmically growing bacteria were infected with ϕ 29 at 0 min and exposed to CAP (250 μ g/ml) at 21 min. A, 3 H-uridine (20 μ Ci/ml) added from 21 to 25 min; B, 3 H-uridine added from 35 to 39 min. The RNA was extracted and analyzed by electrophoresis through 2.5% acrylamide gels. The RNA from each fraction was hybridized to 5 μ g of ϕ 29 DNA to localize viral-specific material.

10^6 daltons. All of these viral-specific molecules except the component at 0.44 were detected when infection occurs in the presence of CAP and, therefore, probably represent transcription of viral chromosomes by the host RNA polymerase. Although the ϕ 29 RNA species at 0.04×10^6 daltons was always detected as

one of the members of CAP RNA (Fig. 3), its appearance does not coincide with that of the other species of early RNA (see Fig. 4). The following two possibilities arise from this observation. (i) The RNA at 0.04×10^6 daltons could represent an example of delayed transcription in the absence of protein synthesis.

According to the interpretation, this RNA would be located distal to the promoter of one of the other early species and would originate by "run-through" of the host RNA polymerase. (ii) The RNA at 0.04×10^6 daltons could be a breakdown product of one of the larger early species. Our data, at this point, cannot support either of these possibilities. The major obstacle in obtaining additional information about the origin of this species is the fact that very little early RNA is made immediately after infection. The sum of the molecular weight of early RNA molecules is 1.94×10^6 or 35% of the maximum amount of RNA which could be transcribed from $\phi 29$ DNA. This value is in good agreement with the 40% value obtained from competition-hybridization experiments (13).

A difference between CAP RNA and early RNA was the absence of the 0.44 component in the former case. We cannot conclude from this observation that a viral protein is required as a positive control element for the expression of this messenger RNA since it has been demonstrated that promoter distal sequences in the tryptophan operon are not detected in the presence of CAP (16, 17). Thus, the distinction

which has been made between "immediate early" from "delayed early" functions in bacteriophage T4 (15) or SPO1 (5, 6) may reflect an effect of CAP on promoter distal transcripts.

The mechanism by which a drug inhibiting protein synthesis can affect transcription is not understood, although it has been suggested that this antibiotic may affect the stability of the distal portion of the tryptophan operon (17). In vitro transcription of $\phi 29$ DNA molecules by purified RNA polymerase extracted from uninfected *B. subtilis* gives a product which is homologous by competition-hybridization analysis to CAP RNA isolated in vivo (*unpublished observation*).

Continued transcription of late $\phi 29$ RNA occurred even after prolonged exposure to CAP (Fig. 8). The large-molecular-weight components were still synthesized 18 min after addition of CAP, and synthesis of the 0.75×10^6 early component did not resume. These observations suggest that CAP, when added before infection, may in fact restrict transcription to 30% of the genome by inhibiting the synthesis of an early viral protein. If restriction resulted from an uncoupling of transcrip-

FIG. 9. Summary of $\phi 29$ transcription. Each arrow represents a size class of $\phi 29$ -specific mRNA. The initiation and termination points (heads and tails of the arrows) were determined both from the data in this communication as well as from the hybridization-competition data previously reported (13). The numbers above each arrow are the molecular weights of the components, expressed as a fraction of 10^6 daltons. The star after the early species at 0.04×10^6 daltons represents an uncertainty about its exact time of appearance (see Discussion).

tion and translation (21) or from the decreased stability of operator distal portions of viral transcripts (17), the profile should revert back to the one characteristic of the RNA synthesized in the presence of CAP. This does not happen.

ϕ 29 late RNA is composed of at least three and possibly four RNA molecules. These molecules were detected in bacteria infected with wild-type ϕ 29 or with ϕ 29 TS35, a temperature-sensitive mutant blocked in DNA replication. It therefore appears that viral DNA replication is not a requirement for the expression of late genes in ϕ 29. ϕ 29 late RNA contains components of molecular weight 1.75×10^6 , 0.93×10^6 , and 0.07×10^6 . In addition, RNA with molecular weight of approximately 1.4×10^6 was often detected. We are not certain at this time that the component at 1.4×10^6 represents a unique RNA species since it was detected very late in the infectious cycle, after the appearance of infective ϕ 29 particles. Further work is in progress to determine whether or not this RNA represents a specific cleavage product of the larger-molecular-weight RNA. With the omission of the RNA at 1.4×10^6 daltons, the sum of the class III RNA is 2.75×10^6 daltons or 50% of the total potential RNA. This value agrees with the value determined by competition-hybridization analysis (13). It should be noted that synthesis of the major early component, 0.75×10^6 daltons, appears to cease at approximately the time in infection when late RNA begins to be synthesized. The cessation of synthesis of this molecule does not appear to require viral DNA replication since shut-off can be detected in bacteria infected with ϕ 29 TS35 at the restrictive temperature.

Figure 9 is a representation of the ϕ 29 transcription program as we understand it. We have also detected late in infection ϕ 29 RNA with a molecular weight of 2.5×10^6 (see Fig. 7) both in bacteria infected with wild-type and with TS35. The appearance of this large RNA molecule after the production of intracellular virus suggest that it may represent the failure of RNA polymerase to terminate properly at these late times.

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