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Transcription During the Development of Bacteriophage ϕ29: Definition of "Early" and "Late" ϕ29 Ribonucleic Acid

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Bacteriophage ϕ29 messenger ribonucleic acid (mRNA) production following infection of *Bacillus subtilis* has been analyzed. Early (*e*) ϕ29 RNA, made prior to the onset of phage deoxyribonucleic acid (DNA) replication and exclusively from the light (L) ϕ29 DNA strand, has been shown by RNA-DNA hybridization-competition experiments to be present throughout the phage latent period. No repression of *e* RNA production during ϕ29 development could be demonstrated. Unmodified host RNA polymerase molecules appear to be sufficient for the synthesis of *e* RNA since phage-specific RNA made in the presence of chloramphenicol hybridizes only to the L strand of ϕ29 DNA, and this RNA can be effectively competed during hybridization by *e* RNA. The appearance of late (*l*) ϕ29 RNA is coincident with the onset of viral DNA replication. This RNA consists of L DNA strand transcripts which are identical to *e* RNA and a new class of mRNAs made exclusively from the "heavy" (H) ϕ29 DNA strand (*l*H). Protein synthesis in infected cells is required for *l*H RNA production. Studies with the antibiotic rifamycin demonstrated that synthesis of the major ϕ29 structural proteins is dependent on production of *l*H RNA.

Detailed studies on transcription during development of *Escherichia coli* phage T4 (5, 6, 12, 20, 25) and *Bacillus subtilis* phage SPO1 (8, 9, 10, 11, 14) have revealed that the temporal control of ribonucleic acid (RNA) production by these viruses is complex. The transcripts produced during infection by these relatively large phages can be divided into a variety of classes and subclasses (5, 9, 11, 20). Regulation of production of these RNAs by a variety of mechanisms (7, 28) assures an appropriate dosage of phage proteins during viral development.

Phage ϕ29 is a small, virulent *B. subtilis* virus whose genome is approximately one-tenth the size of either T4 or SPO1. The double-stranded deoxyribonucleic acid (DNA) from ϕ29 has a molecular weight of only 1.1×10^7 (4) and can thus code for approximately 15 to 20 proteins (13, 16, 29). Since only four major and three minor species of ϕ29 RNA have been detected in ϕ29-infected cells (Loskutoff, Ayers, and Pène, Bacteriol. Proc., 1971, p. 197), it is likely that the transcriptional program of ϕ29 is far less complex than the programs for phages T4 or SPO1. In addition to the limited number of transcripts and gene products produced by ϕ29, there are other features of this phage which have encouraged us to begin a detailed analysis of transcription. It has been demonstrated that RNA is transcribed from both of the ϕ29 DNA strands during infection (17, 21). This asymmetry of transcription makes ϕ29 unique in comparison to other small virulent phages such as *E. coli* phage T7 (26) and *B. subtilis* phage SPP1 (19) where transcription is entirely from one of the complementary DNA strands. The observation that only one strand of ϕ29 DNA is transcribed early in infection and that both strands produce RNA late in infection implies a temporal control of ϕ29 transcription with at least one DNA strand switch during development (21). The findings that 45 to 60% of the coding capacity of the ϕ29 genome may be involved in the synthesis of structural proteins (1, 16, 21) and that a low number of phage genes are expressed prior to ϕ29 DNA replication (22, 27) indicate that the number of gene products directly involved in control of ϕ29 transcription may be low. The availability of conditionally lethal mutations in most of the ϕ29 genes (13) should eventually allow identification and analysis of the ϕ29 transcriptional control elements.

In this paper we demonstrate by use of

RNA-DNA hybridization-competition experiments that the ϕ29 transcripts can be clearly divided into two major classes based on their time of first appearance, the DNA strand from which they are transcribed, and dependency of their appearance on protein synthesis in infected cells. Production of early ϕ29 RNA is not repressed and continues throughout the latent period of phage development. Late ϕ29 RNA synthesis is required for production of the major ϕ29 structural proteins.

MATERIALS AND METHODS

Bacteria, phage, and growth conditions. *B. subtilis* 12A (18, 21) was used as host for the preparation of RNA from infected bacteria. Phage ϕ29 was the wild-type virus described previously (3). Growth of cells and the conditions for phage infection have been described (4, 13, 23). For the preparation of unlabeled RNA, cells were grown and infected with phage in 2,800-ml Fernbach flasks (Bellco Glass, Vineland, N.J.) containing 1 liter of broth medium (18). Incubation was at 37 C in a model G25 shaker (New Brunswick Scientific Co., New Brunswick, N.J.). The eclipse period of phage ϕ29 under these conditions was the same (30 min) as in our previous studies (23), and the phage yield was approximately 400 per cell.

^{3}H-labeling and isolation of RNA. Pulse-labeling of infected cells with ^{3}H-uridine and conditions for RNA isolation were described previously (21).

Preparation of phage DNA. Phage DNA was prepared as described elsewhere (21). The heavy (H) and light (L) complementary strands of ϕ29 DNA were isolated (17) and fixed to filters for hybridization as described previously (21).

RNA-DNA hybridization. Hybridization of ^{3}H-labeled RNA to DNA immoblized on nitrocellulose filters was performed as described previously (21). Competition experiments were carried out at DNA excess over labeled RNA by the addition of varying amounts of unlabeled RNA. To reduce the amount of RNA required for these studies, annealing was performed in 10- by 75-mm test tubes with 7-mm filter pads containing known quantities of ϕ29 DNA. The final volume of the hybridization mixture was 0.2 ml, and the filters were washed at least three times before and after ribonuclease treatment by shaking with 4-ml volumes of 2× standard saline-citrate (21). Data on the conditions for these experiments is presented in detail below (see Results).

Labeling of proteins with ^{14}C-amino acids. Proteins in infected cells were labeled by the addition of a ^{14}C-amino acid mixture (2 μCi/ml, 230 μCi/mmole, New England Nuclear Corp., Boston, Mass.) at the time of infection. After 45 min at 37 C, egg white lysozyme (5 μg/ml, A grade, Calbiochem, Los Angeles, Calif.) and pancreatic deoxyribonuclease (2 μg/ml, Calbiochem) were added, and incubation was continued for 15 min. Trichloroacetic acid was added to a final concentration of 5% at 4 C. The insoluble proteins were collected by centrifugation (12,000 × g, 20 min, 4 C), gently washed with cold ether, and suspended in the sample buffer of Laemmli (15).

Gel electrophoresis and autoradiography. The system for sodium dodecyl sulfate (SDS) gel electrophoresis of ^{14}C-labeled proteins and the analysis of dried gels by autoradiography were described previously (21).

Radioisotope counting procedures. Radioactive samples were counted with a Packard 2425 Tri-Carb liquid scintillation spectrometer as described previously (24).

RESULTS

Conditions for RNA-DNA hybridization. The requirements for obtaining interpretable results from RNA-DNA hybridization-competition experiments have been discussed by Bolle et al. (5). The data presented below define the conditions for hybridization-competition analysis of ϕ29 RNAs by utilizing denatured ϕ29 DNA immobilized on membrane filters. The kinetics of annealing of ϕ29 mRNA to ϕ29 DNA is presented in Fig. 1. At the concentration of RNA employed in this experiment, at least 15 hr of incubation was required for maximum hybrid formation. Although the kinetics of hybrid formation varied with the ratio of DNA/RNA employed (data not shown), maximum annealing was obtained within 20 hr of incubation when the concentration of

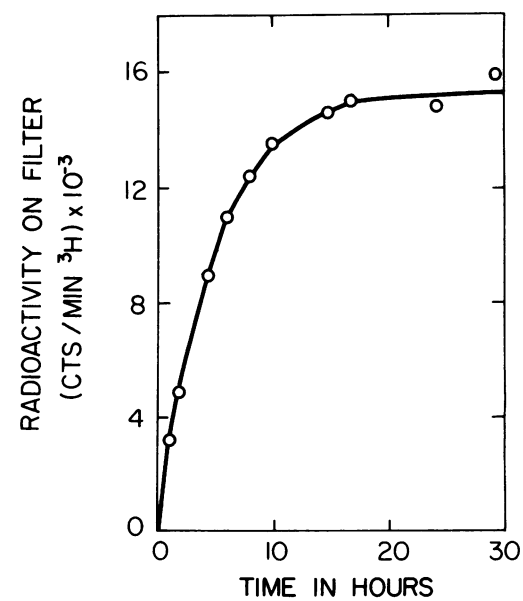

FIG. 1. *Kinetics of hybridization of ϕ29 mRNA to ϕ29 DNA. Each filter contained 2.0 μg of denatured ϕ29 DNA. The RNA was from cells pulse-labeled with ^{3}H-uridine 33 to 36 min after infection. Each tube contained 6.4 μg of RNA (33,550 counts per min per μg).*

DNA was 2 μg per filter and labeled RNA was varied from 0.5 to 50 μg. The addition of large quantities of nonhomologous RNA had little effect on the extent of hybridization with a given RNA sample (see Fig. 4), and the hybrids produced under our conditions were stable for at least 30 hr at 66 C (Fig. 1). At a DNA concentration of 2.0 μg per filter, 20 hr of annealing is suitable for the competition experiments presented below.

Figure 2 presents the results of a study to determine the amount of φ29 DNA required to ensure that this component would be in excess. Depending on the type of pulse-labeled RNA used, the amount of denatured DNA per filter required for DNA excess varied from 1 to 2 μg.

Figure 3 demonstrates that, at a concentration of 2 μg of DNA per filter, the binding of φ29 RNA labeled late in infection was directly proportional over a wide concentration range to the amount of RNA present. This is the expected result when DNA is present in excess (5). Unless specifically noted, all of the hybridization-competition experiments reported below were performed at a DNA concentration of 2 μg per filter.

Transcription prior to phage φ29 DNA replication. Previous studies had shown that, until the onset of phage DNA replication between 9 and 12 min after infection, the production of φ29 RNA was exclusively from the L strand of φ29 DNA (17, 21). For clarity and comparative purposes, we will designate phage RNA made prior to the onset of φ29 DNA replication as "early" (*e*). The slightly elevated production of RNA from the L strand late in φ29 infection (21) indicated that the synthesis of *e* φ29 RNA might continue throughout the lytic cycle. The hybridization-competition experiment presented in Fig. 4 demonstrates the presences of *e* φ29 RNA in RNA prepared from cells 27 min after infection. The ability of unlabeled 27-min RNA to compete with labeled *e* RNA at least as well as homologous RNA prepared 12 min after infection indicates that all of the viral transcript(s) synthesized between 9 and 12 min after infection are present in high concentration late in infection. Unlabeled RNA from uninfected cells was used as a control in this experiment, and this RNA does not compete to an appreciable extent with the *e* φ29 RNA. This confirms our previous observation that there is little RNA in uninfected *B. subtilis* which will anneal with φ29 DNA (21).

The earliest φ29-specific RNA detectable by hybridization is made between min 6 and 9

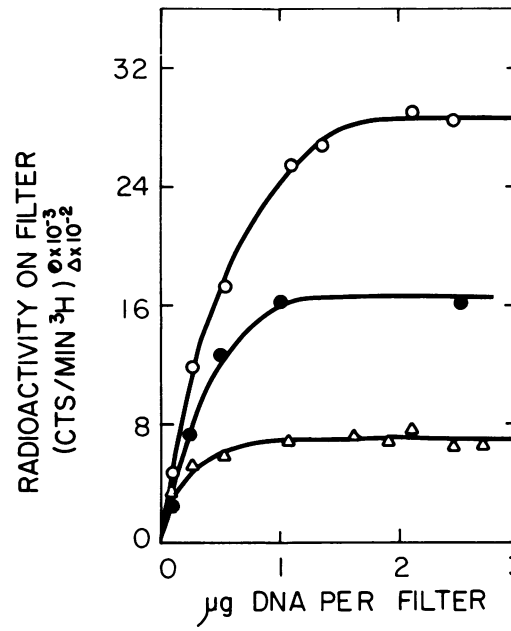

FIG. 2. *Hybridization of 9- to 12-min and 33- to 36-min pulse-labeled φ29 RNA to increasing quantities of φ29 DNA. The 9- to 12-min RNA was used at a concentration of 5.5 μg (9,315 counts per min per μg) and the 33- to 36-min RNA at a concentration of 12.8 μg (33,550 counts per min per μg). Symbols: Δ, 9- to 12-min RNA and denatured, unfractionated φ29 DNA; O, 33- to 36-min RNA and denatured, unfractionated φ29 DNA; ●, 33- to 36-min RNA and the purified H strand of φ29 DNA. The conditions of hybridization for this last curve were described previously (21).*

after infection (21). Table 1 demonstrates that this RNA can be effectively competed by unlabeled RNA extracted at various times throughout the phage lytic cycle. This result, in combination with the data in Fig. 4, indicates that all of the nucleotide sequences transcribed from the L strand of φ29 DNA prior to φ29 DNA replication are present at all subsequent times after infection. Since these findings could result from continued production of *e* RNA throughout infection or lack of degradation of this RNA fraction during φ29 development, we analyzed the φ29 RNA made after the initiation of phage DNA replication.

Transcription during phage φ29 DNA replication. It was demonstrated previously that, coincident with the onset of φ29 DNA replication, φ29-specific RNA began to be synthesized in large quantities from the H strand of φ29 DNA (21). Continued production of φ29 RNA at a slightly elevated rate from the L strand was also demonstrated (21). Figure 5

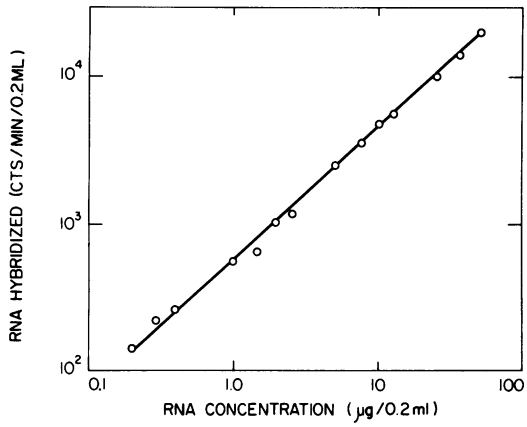

FIG. 3. *Proportionality of hybridization to RNA concentration. RNA pulse-labeled with ^{3}H-uridine from 33 to 36 min after φ29 infection was hybridized to φ29 DNA (2 μg per filter). The average hybridization efficiency was 1.4%, and all of the data have been corrected for the background of counts bound to filters without DNA.*

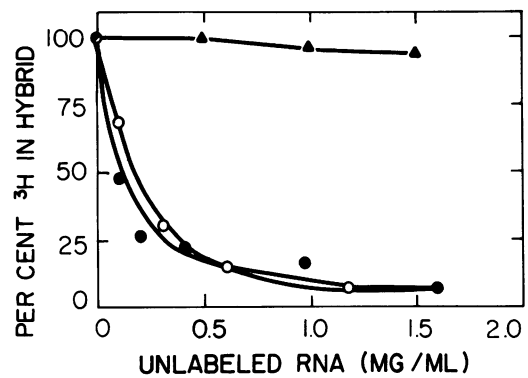

FIG. 4. *Hybridization competition of ^{3}H-labeled, 9- to 12-min RNA from φ29-infected cells with unlabeled RNA extracted from uninfected cells and cells infected for 12 and 27 min. Labeled RNA was used at a concentration of 15.2 μg per tube. Hybridization at 100% corresponded to 629 counts per min, and this represented 0.30% of the total radioactivity present. Maximum background hybridization in the absence of DNA was 27 counts per min. Symbols: ▲, RNA from uninfected cells; O, RNA from cells infected for 12 min; ●, RNA from cells infected for 27 min.*

presents the results of an experiment where RNA, pulse-labeled late in the phage eclipse period (24–27 min), was analyzed by competition with unlabeled RNA prepared early (9 min) and late (27 min) in infection. As expected, in comparison to the extensive competition in the self-competition control (Fig. 5, open circles), it is clear that *e* RNA does not contain all of the transcripts present late in infection (Fig. 5, closed circles). However, *e* φ29 RNA does compete with pulse-labeled late RNA to a level which indicates that approximately 30% of the nucleotide sequences transcribed between min 24 and 27 after infection are present in the *e* RNA preparation. This result supports the observation made above that *e* φ29 RNA is present late in the phage latent period and demonstrates that this RNA is actually being synthesized late in infection.

Our previous data reference (21; Fig. 3) demonstrated that φ29 RNA made between min 24 and 27 after infection could be annealed to both the H and L strands of φ29 DNA. Approximately 25% of the total hybridizable φ29 RNA was specific for the L φ29 DNA strand. This value is comparable to the level of *e* RNA which was shown in the

TABLE 1. *Hybridization-competition analysis of φ29 mRNA produced 6 to 9 min after infection*[a]

Competing RNA (min)	Concentration of competing RNA (mg/ml)	Counts retained on filter	Percent competition
None		234	0
9	1.92	47	80.0
12	2.04	28	88.0
15	1.96	15	93.6
18	2.15	30	87.2
27	1.92	20	91.5
36	1.92	7	97.0

[a] The 6- to 9-min mRNA was used at a concentration of 8.1 μg per tube. The hybridization efficiency in the absence of competitor was 0.3%.

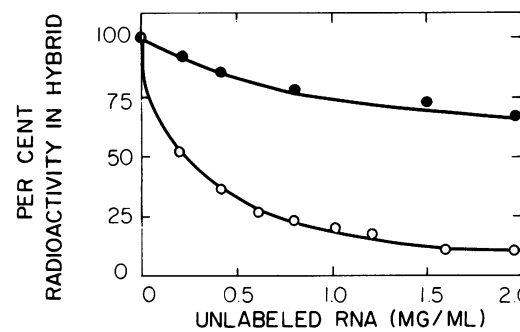

FIG. 5. *Hybridization competition of ^{3}H-labeled, 24- to 27-min RNA from φ29-infected cells with unlabeled 9-min (●) and 27-min (O) RNA. Labeled RNA was used at a concentration of 1.2 μg per tube. Hybridization at 100% corresponded to 2,734 counts per min; this represented 18.0% of the total radioactivity present. Maximum background hybridization was 14 counts per min.*

competition experiment in Fig. 5 to be present in the 24- to 27-min pulse-labeled RNA. Thus, it was possible that the partial competition of late ϕ29 RNA by *e* ϕ29 RNA represented only those nucleotide sequences which were transcribed from the L DNA strand. The experiment presented in Table 2 tests this possibility. The ability of unlabeled *e* (9 min) and late (27 min) ϕ29 RNA to compete with ϕ29 RNA labeled late in infection for sites on the isolated H and L strands of ϕ29 was compared. It is clear that, in contrast to the self-competition control (Table 2, line 3), *e* ϕ29 RNA competes effectively only with the late transcripts specific for the L DNA strand (Table 2, line 2). The almost complete competition of late RNA on the L DNA strand by unlabeled *e* RNA indicates that if there is any RNA with new nucleotide sequences made from this DNA strand late in infection, it must represent only a very small fraction of the total RNA synthesized from the L DNA strand. The low level of competition between the unlabeled *e* RNA and labeled late RNA on the H DNA strand was not greater (less than 10%) than what might be expected using an equivalent concentration of unlabeled RNA from uninfected cells (see Fig. 4, closed triangles). Thus, ϕ29 RNA synthesized after the onset of ϕ29 DNA replication consists of transcripts from the L DNA strand which are identical to *e* ϕ29 messengers and transcripts from the H DNA strand (to be designated *l*H) which are not present prior to ϕ29 DNA replication.

Phage transcription in the absence of protein synthesis. Chloramphenicol at a concentration of 100 μg/ml has been shown to inhibit protein synthesis in ϕ29-infected *B. subtilis* by greater than 95% (data not shown). Figure 6 demonstrates the production of ϕ29-specific RNA in the presence of this concentration of chloramphenicol. It would appear that *e* RNA (synthesized prior to 12 min after infection) can be made in reduced amounts in the absence of protein synthesis. The acceleration of phage RNA production associated with the onset of *l* RNA synthesis does not occur in the presence of chloramphenicol. Table 3 demonstrates that the phage RNA made in the presence of chloramphenicol will anneal only to the L strand of ϕ29 DNA. The competion experiment presented in Table 4 demonstrates that phage RNA made in the presence of chloramphenicol contains nucleotide sequences similar to the sequences found in *e* RNA. Thus, the production of *e* RNA from the L strand of ϕ29 DNA does not require the synthesis of any new protein in an uninfected cell. In contrast, the switch in transcription to the H DNA strand required for the production of *l*H RNA would

TABLE 2. *Hybridization-competition analysis of DNA strand specificity of late ϕ29 RNA*[a]

Competing RNA	Concentration of competing RNA (mg/ml)	Counts retained on filter[b]	
		Strand L	Strand H
None		391	934
12 min	2.9	29	841
36 min	1.5	53	87

[a] Late ϕ29 RNA was isolated from cells pulse-labeled with ^{3}H-uridine 33 to 36 min after infection.

[b] Labeled RNA was used at a concentration of 12.8 μg (66,000 counts per min per μg). The DNA concentration was 0.15 μg for both the L and H strands.

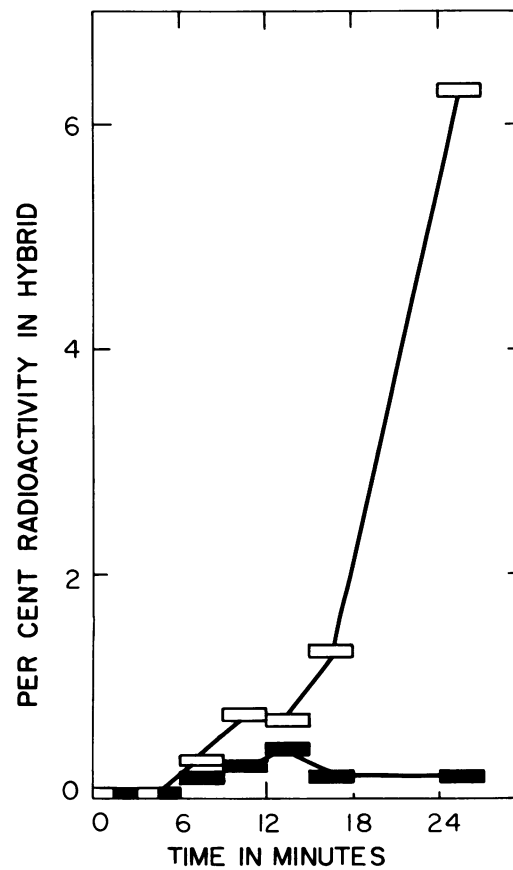

FIG. 6. *Hybridization to denatured ϕ29 DNA of pulse-labeled RNA from cells infected in the presence (closed bars) and absence (open bars) of chloramphenicol. Labeled RNA was used at a concentration of 3.9 to 5.6 μg per tube.*

TABLE 3. *Hybridization of phage φ29 RNA made in the presence of chloramphenicol to the isolated φ29 DNA strands*

Pulse-labeled RNA	Counts retained on filter[a]	
	Strand L	Strand H
24–27 min (7.2 μg, 50,949 counts per min per μg) ...	478	1,119
24–27 min plus chloramphenicol (5.6 μg, 14,087 counts per min per μg)	149	4

[a] The DNA concentration was 0.15 μg for both the L and H strands. The data have been corrected for the background of counts bound to filters without DNA.

TABLE 4. *Hybridization-competition analysis of φ29 RNA made in the presence of chloramphenicol*[a]

Competing RNA (min)	Concentration of competing RNA (mg/ml)	Counts retained on filter	Percent competition
None		186	0
12	2.04	10	94.6
18	2.15	23	87.6
27	1.92	14	92.5
36	1.92	8	95.7

[a] The RNA employed was the same as in Table 3. The hybridization efficiency in the absence of competitor was 0.22%.

appear to be dependent on the synthesis of protein(s) after phage infection.

Late φ29 RNA and φ29 structural proteins. Previous studies have shown that φ29 DNA replication can first be detected between min 9 and 12 after phage infection (23) and that φ29 *l*H RNA production commences within the identical time interval (21). Blockage of φ29 transcription 9 min after infection should therefore inhibit the synthesis of proteins coded for by the H φ29 DNA strand. Since rifamycin has been shown to rapidly block transcription throughout the φ29 latent period (21), this antibiotic was added to a phage-infected culture at various times, and production of the major φ29 structural proteins was evaluated by SDS-gel electrophoresis. The microdensitometer tracings in Fig. 7 demonstrate that the addition of rifamycin at 9 min after infection blocked the subsequent production of φ29 structural proteins. Addition of rifamycin at later times revealed that φ29 structural proteins could first be detected at 10 min after infection. Thus, appearance of the major φ29 structural proteins can be directly correlated with the production of *l*H φ29 RNA.

DISCUSSION

Previous studies of transcription in phage φ29-infected cells demonstrated that host RNA synthesis continued throughout the latent period of phage development (21). Our observations that *e* φ29 RNA is continuously made from the L φ29 DNA strand during phage development (Fig. 5, Tables 1 and 2) and that production of this RNA does not require protein synthesis in an infected cell (Tables 3 and 4) indicate that the RNA polymerase molecules which continue to make host RNA during infection are also responsible for the synthesis of *e* φ29 RNA. Support of this proposal has been obtained in experiments which demonstrated that the RNA produced from φ29 DNA in vitro, utilizing a purified RNA polymerase from uninfected *B. subtilis*, was made only from the L φ29 DNA strand and that this RNA had nucleotide sequences similar to *e* φ29 RNA (Schachtele et al., *manuscript in preparation*).

The continued production of *e* φ29 RNA throughout infection makes this phage different from other phages where a detailed analysis of transcription has been performed. Early RNA produced within the first few minutes after infection with *B. subtilis* phage SPO1 is made by an unmodified host RNA polymerase (9, 11). This RNA can be divided into two classes (*e* and *em*). Production of both of these classes of RNA is interrupted early in infection by a mechanism that requires protein synthesis in the infected cell (11). A repressor-like protein (TF1) which may be involved in the blockage of *e* and *em* RNA synthesis in SPO1-infected cells has been studied in detail (14, 30). Studies with *E. coli* phage T4 have demonstrated that there are distinct subclasses of early T4 RNA and that production of some of these classes is terminated during phage development (20, 25). As discussed previously (21), the finding that phage φ29 does not appear to code for transcriptional control elements which work in a negative manner could reflect the limited amount of genetic information carried by this virus. It is not clear at this time whether the continued production of *e* φ29 RNA during development is essential for normal phage maturation.

The data presented in this paper demonstrate that the *e* φ29 RNA made exclusively from the L φ29 DNA strand is the only class of RNA made from this DNA strand. All of the

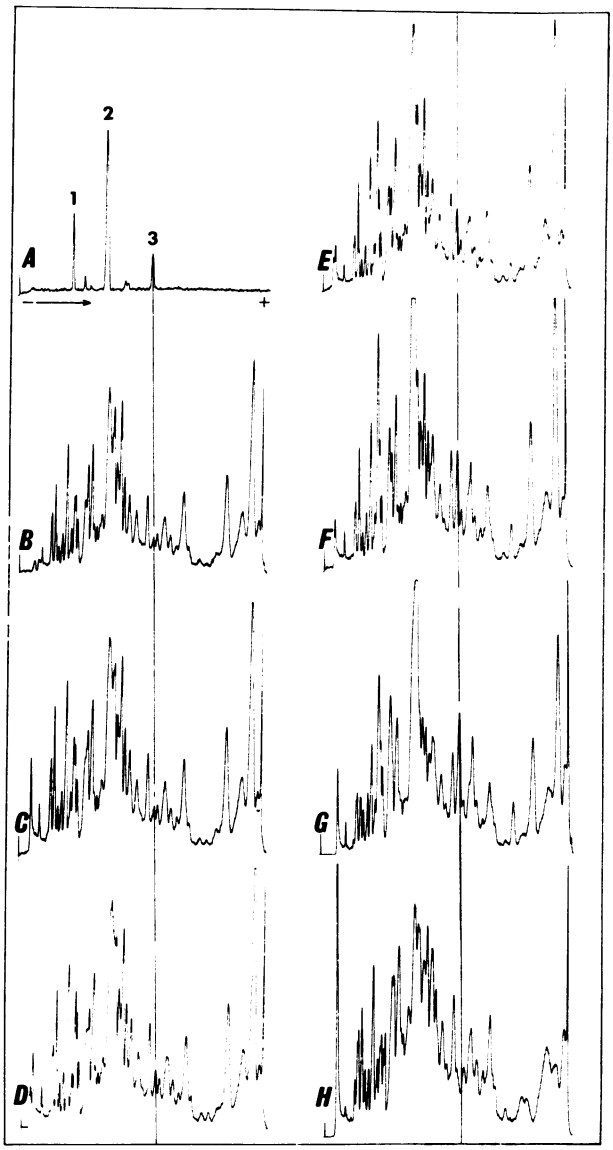

FIG. 7. *Effect of time of addition of rifamycin on φ29 structural protein production. B. subtilis 12A was infected with phage φ29 in broth supplemented with ^{14}C-amino acids. Rifamycin (2 μg/ml) was added to portions of the culture at various times, and incubation was continued until 45 min after infection. ^{14}C-labeled proteins were analyzed by SDS-gel electrophoresis. A, Purified φ29 labeled with ^{14}C-amino acids; B, C, D, E, and F, rifamycin added at 9, 10, 11, 13, and 15 min, respectively; G, no rifamycin added; h, uninfected control cells. Peaks 1, 2, and 3 correspond to the appendage, major head, and horn φ29 structural proteins (21). A guide line was drawn through the horn peak to allow comparison of the amount of this protein present in each gel.*

new transcripts made at the time of initiation of phage φ29 DNA replication are made exclusively from the H φ29 DNA strand. These observations are very different from the findings of Gage and Geiduschek (11) with phage SPO1. These workers demonstrated that the *e* SPO1 transcripts are made from both strands of SPO1 DNA. Transcription from the light SPO1 DNA fraction decreased during infection, and light DNA strand transcripts could not be detected 14 min after infection. RNA made late in SPO1 infection was made exclusively from the heavy fraction of SPO1 DNA. The work of Guha et al. (12) with *E. coli*

phage T4 indicated that reading of the DNA strands during development of this phage was similar to transcription of the ϕ29 DNA strands. It was demonstrated that the immediate-early and delayed-early T4 RNA classes were coded for by one of the T4 DNA strands (*l* strand). A portion of the quasi-late RNA class also appeared to be made from this strand. T4 RNA made in the presence of chloramphenicol hybridized exclusively to the *l* T4 DNA strand. Thus, protein synthesis in both phage ϕ29- and phage T4-infected cells is required for the switch in transcription from one viral DNA strand to the other. We are currently performing studies to determine if the switch in DNA strand transcription during ϕ29 development can be correlated with the production of a ϕ29-induced sigma factor (see Holland and Whiteley, Abstr. Annu. Meet. Amer. Soc. Microbiol., 1972, p. 244).

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