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

I. Analysis of Viral-Specific Transcription by Deoxyribonucleic Acid-Ribonucleic Acid Competition Hybridization

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The ribonucleic acid (RNA) specified by bacteriophage $\phi 29$ was analyzed to determine its composition at various times in the viral lytic cycle. Although viral-specific RNA was detected immediately after infection, a large increase in the rate was observed at 10 min when DNA synthesis began. $\phi 29$ was found to resemble other viruses in that gene expression occurred in two stages which could be defined temporally as "early" and "late." Early RNA appeared before the onset of viral deoxyribonucleic acid (DNA) replication and accounted for approximately 40% of the viral genetic potential. This RNA was also present late in the infectious cycle because of the slow turnover rate of $\phi 29$ -specific RNA (approximately 10 min half-life) and the continued synthesis of much early viral RNA throughout infection. Late RNA was first detected at approximately the same time as viral DNA replication, although late transcription was not dependent upon DNA synthesis. This RNA was only partially displaced by early RNA in the appropriate competition experiments, suggesting that it contained sequences not present in the early class. Expression of viral genes was sensitive to rifamycin throughout the lytic cycle, the sensitivity resulting from a dependence upon the rifamycin phenotype of the host RNA polymerase.

Development of many bacteriophages is characterized by the temporal appearance or disappearance of viral-specific proteins. Numerous observations support the conclusion that regulation of protein synthesis during the life cycle of these viruses occurs mainly at the level of transcription, not at a subsequent step of ribonucleic acid (RNA) utilization (12). An understanding of transcription and its control is therefore basic to an understanding of the molecular events which regulate bacteriophage development.

The following observations make *Bacillus subtilis* bacteriophage $\phi 29$ (23) an attractive phage for analysis of viral transcription. Although this virus is genetically simple, its novel and complex morphology implies that much of its genetic information is devoted to the synthesis of capsid polypeptides which are readily characterized. This conclusion has been verified (D. J. Loskutoff, A. Ayers, and J. J. Pène, *Bacteriol. Proc.*, p. 171, 1971). $\phi 29$ particles are composed of seven polypeptides accounting

for approximately 60% of the coding capacity of $\phi 29$ deoxyribonucleic acid (DNA). These proteins have been assigned to specific morphological features of the virion (19).

The $\phi 29$ chromosome (11×10^6 daltons of double-stranded DNA) (3) probably consists of 15 to 20 genes rather than the 100 to 200 genes of the larger bacteriophages (12). Viral transcription, however, involves at least seven distinct transcripts (D. J. Loskutoff et al., *Bacteriol. Proc.*, p. 171, 1971) as well as a change in the transcribing strand as development proceeds (22). These observations indicate that $\phi 29$ transcription is a relatively simple, but highly regulated process.

In this communication we report the results of DNA-RNA competition-hybridization experiments designed to examine in greater detail the transcription of $\phi 29$ chromosomes. $\phi 29$ was found to resemble other viruses in that gene expression occurred in two stages which could be defined temporally as "early" and "late." Late RNA appeared in large amounts

concomitant with phage DNA synthesis but was not under the positive control of viral chromosome replication. In this respect the expression of ϕ 29 genes resembled simpler phages such as T7 (25) rather than complex viruses like T4 (6, 7, 12) and SP01 (11). In addition, RNA was not synthesized when infection occurred in the absence of protein synthesis. Some early genes also were not expressed under these conditions.

MATERIALS AND METHODS

Bacteria and bacteriophages. *B. subtilis* 60084, a nonmotile prototrophic derivative of strain 168, was isolated by Ernst Freese and used in all experiments. The efficiency of plating of ϕ 29 on this strain was 50% of that of its original host *Bacillus amyloliquefaciens* (27). *B. subtilis* 60084 was chosen as the host strain because ϕ 29 plaques were more easily enumerated on lawns of these bacteria. Bacteriophage ϕ 29 was kindly provided by K. Bott. The temperature-sensitive mutant of ϕ 29 TS35 blocked in DNA replication (15) was made available by D. Anderson.

Media. Bacteria were grown and infected in a medium containing, per liter, 4 g of nutrient broth (Difco, Detroit, Mich.), 1.5 g of yeast extract, 3.5 g of NaCl, and ϕ 29 ions (1 ml of a solution containing 1.5 M $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 1 M CaCl_2 , and 0.035 M $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$). Bacteria were maintained on tryptose blood agar plates (Difco). Tryptose blood agar was also used as the bottom layer for standard phage assays (1). The top agar soft overlay consisted of nutrient broth medium made 0.4% with agar. All media used for enumerating ϕ 29 contained ϕ 29 ions.

Growth and purification of ϕ 29. Logarithmically growing bacteria (2×10^8 per ml) were infected with ϕ 29 at a multiplicity of 15. Under these conditions, lysis was complete 60 min after infection, resulting in a yield of 250 to 300 phages per bacterium. Cell debris was removed by centrifugation, and the phages were concentrated by the two-phase system of Albertson (2) followed by purification on cesium chloride gradients. Phage stocks were stored at -20°C in 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 7.4)-0.01 M MgCl_2 -10% dimethyl sulfoxide (w/v).

Isolation of DNA. Viral DNA was prepared by incubating ϕ 29 with Pronase (Calbiochem, Los Angeles, Calif., B grade, 1 mg/ml) in the presence of 0.5% sodium dodecyl sulfate (SDS) and $2 \times \text{SSC}$ (SSC, pH 6.8, is 0.15 M NaCl, 0.015 M sodium citrate) for 7 hr at 37°C . The DNA was then extracted with phenol saturated with SSC and containing 0.1% hydroxyquinoline (Matheson, Coleman, and Bell, Norwood, Ohio), dialyzed against 0.01 M Tris-0.01 M disodium ethylenediaminetetraacetate, pH 7.4, and stored at 4°C in the presence of chloroform. Viral DNA prepared as described above sedimented as a single homogenous component in neutral (24S) or in alkaline (27S) sucrose density gradients.

The technique of Marmur (18) was employed for the extraction of bacterial DNA. Radioactive DNA

was prepared with thymidine-methyl- ^3H (Schwartz BioResearch Inc., Orangeburg, N.Y.; 50 mCi/mM) as indicated in the legends to the figures. Uridine (200 $\mu\text{g}/\text{ml}$) was added with the ^3H -thymidine to suppress thymidine phosphorylase (9).

Labeling and purification of RNA. Radioactive RNA was prepared by exposing the bacteria to uridine-5- ^3H (New England Nuclear Corp.; 25.6 Ci/mM). In those experiments in which the RNA was to be used for competition hybridization, the cells were simultaneously treated with chloramphenicol (CAP) to minimize normal intracellular degradation of RNA (17). This treatment was found to increase significantly the stability of RNA after addition of rifamycin or actinomycin D.

The RNA was extracted in a manner similar to that described by Salser et al. (24). Infected or uninfected bacteria were poured on ice containing 0.01 M Tris-0.01 M MgCl_2 -0.01 M NaN_3 (pH 7.4), and 20 μg of electrophoretically purified deoxyribonuclease per ml (Worthington Biochemical Corp., Freehold, N.J.) was added. Lysozyme (500 $\mu\text{g}/\text{ml}$, Worthington Biochemical Corp., Freehold, N.J.) was added, and the suspension was incubated for 3 min at 37°C . Lysis was completed by the addition of SDS (1% final concentration). The clear lysate was extracted with water-saturated phenol at 67°C until denatured protein was absent at the phenol aqueous interphase. The aqueous phase was made 0.25 M with NaCl, two volumes of ethanol were added, and the nucleic acids were allowed to precipitate overnight at -20°C . After centrifugation, the RNA was resuspended in $2 \times \text{SSC}$ at a final concentration of 10 mg/ml and filtered through a $0.45\text{-}\mu\text{m}$ pore-size cellulose nitrate filter. The preparation was essentially free of DNA as shown by the absence of radioactivity on filters when heat-denatured samples were incubated with blank filters under hybridization conditions.

DNA-RNA hybridization. DNA-RNA hybridization was performed by the Gillespie and Spiegelman technique (14). DNA was diluted with water to 100 $\mu\text{g}/\text{ml}$ or less and denatured by the addition of 0.1 volume of 1 N NaOH. After 5 min at room temperature the solution was brought to pH 7.0 by rapid dilution with $6 \times \text{SSC}$ and filtered slowly through 24-mm nitrocellulose filters (Millipore Corp., type HA). The filters were dried for at least 4 hr at room temperature and baked for an additional 2 hr at 80°C . Hybridization was carried out in a final volume of 1 ml of $2 \times \text{SSC}$ at 63 to 65°C . After 16 hr, the filters were washed by repeated immersion in $2 \times \text{SSC}$, incubated in the presence of 5 μg of ribonuclease and 5 units of ribonuclease T₁ per ml (Calbiochem, Los Angeles, Calif.) for 60 min at 24°C , washed again with $2 \times \text{SSC}$, dried, and counted. Under these conditions, non-specific binding of RNA to blank filters (background) was reduced to less than 0.01% of the input. Binding of radioactive *B. subtilis* RNA to ϕ 29 DNA was less than 0.1%. The efficiency of the hybridization reaction under optimal conditions involving excess DNA was 48 to 52%.

Inhibitors. Rifamycin (a gift of Ciba-Geigy Corp., Summit, N.J.) was suspended in distilled water and used at 5 $\mu\text{g}/\text{ml}$. The inhibitor of bacterial DNA

synthesis (5), 6-(hydroxyphenylazo)-uracil (HPAU), was a gift of Imperial Chemical Industries, Ltd., Macclesfield, England. HPAU was added at a final concentration of 100 $\mu\text{g/ml}$, 5 min before infection. Uridine (200 $\mu\text{g/ml}$) was added at the same time as HPAU.

RESULTS

ϕ 29 DNA synthesis. Since the expression of viral genes is often coupled to viral DNA replication (7, 12), experiments were carried out to determine the time in the lytic cycle when ϕ 29 DNA replication begins. The nucleotide analogue HPAU has been shown to inhibit DNA replication in certain gram-positive bacteria (5) but does not affect the replication of a number of phages which infect them (4). In addition, Brown found that the synthesis of protein, RNA, lipid, or cell wall material was not affected by this compound (4). The rate of bacterial DNA synthesis does not appear to be altered after infection by ϕ 29 (*unpublished observation*). The effects of HPAU on DNA synthesis in ϕ 29-infected and uninfected bacteria are shown in Fig. 1.

Incorporation of tritiated thymidine into acid-precipitable material was reduced by more than 99% when uninfected bacteria were treated with this inhibitor. Bacteria infected with ϕ 29, on the other hand, lysed at the expected time and showed a rapid increase in incorporation beginning 8 to 10 min after infection. In ϕ 29-infected *B. subtilis* treated with HPAU, tritiated thymidine was incorporated into viral DNA. This was shown by annealing labeled, denatured DNA extracted from these bacteria to Millipore filters containing viral or bacterial DNA (*unpublished data*).

Selective inhibition of host DNA synthesis with HPAU revealed that, in *B. subtilis* 60084, ϕ 29 DNA synthesis is initiated about 8 to 10 min after infection, somewhat earlier than previously detected (C. R. Schachtele, and D. L. Anderson, *Bacteriol. Proc.*, p. 173, 1969). Figure 1 also shows that, when infection occurred in the presence of CAP, incorporation of radioactive precursor into viral DNA was not observed.

Figure 2 illustrates the properties of ϕ 29-TS35, a temperature-sensitive mutant blocked in DNA replication (15). In this experiment, bacteria were infected at a multiplicity of 10 to 15 with ϕ 29 TS35, and viral DNA synthesis was measured by the uptake of ^3H -thymidine in the presence of HPAU. Incorporation of ^3H -thymidine into acid-precipitable material was not detected when infection with this mutant was carried out at the restrictive temperature,

i.e., 45 C. Figure 2 also illustrates the resumption of viral DNA synthesis following a shift-down from 45 to 37 C. ϕ 29 DNA synthesis resumed within 10 min.

ϕ 29 RNA synthesis. Infection by ϕ 29 does not inhibit bacterial RNA synthesis (*unpublished observation*). The rate of synthesis of viral RNA throughout the latent period was, therefore, determined by measuring the amount of pulse-labeled material hybridizing to viral DNA at various times after infection. Figure 3 indicates that RNA synthesis begins immediately after infection although at a very low rate. A large increase in the rate was observed at 10 min when DNA synthesis begins. The maximum rate of ϕ 29-specific RNA synthesis occurred 25 to 30 min after infection. These observations are in good agreement with those obtained previously by using a different strain of *B. subtilis* (C. F. Schachtele, and D. L. Anderson, *Bacteriol. Proc.*, p. 173, 1969).

Sensitivity of ϕ 29 development to rifamycin

FIG. 1. Viral DNA synthesis. Bacteria were exposed to uridine (200 $\mu\text{g/ml}$) and 6-(hydroxyphenylazo)-uracil (100 $\mu\text{g/ml}$) at -5 min. ^3H -thymidine (1 $\mu\text{Ci/ml}$), thymidine (0.05 $\mu\text{g/ml}$), and ϕ 29 were added at 0 min. At the indicated times, samples were removed and precipitated with cold trichloroacetic acid for 30 min and filtered through Whatman GFA filters, and the counts were determined. Symbols: O, uninfected, and infected in the presence of 250 μg of CAP per ml; ●, infected.

FIG. 2. DNA synthesis after infection with TS35. Bacteria growing at 37 or 45 C were infected with TS35 at a multiplicity of infection of 10 to 15. DNA synthesis was measured by the uptake of ^3H -thymidine (1 $\mu\text{Ci/ml}$) in the presence of 100 μg of HPAU per ml and 200 μg of uridine per ml. Symbols: ●, uninfected; ○, infected at 37 C; ▲, infected at 45 C; ■, infected at 45 C, shifted to 37 C at the time indicated by the arrow.

cin. Gene expression during the development of bacteriophage T7 is initially sensitive to rifamycin but undergoes a change to become resistant to this antibiotic (8). The change has been shown to reflect the synthesis of a viral-specified RNA polymerase. It was therefore interesting to determine the sensitivity of ϕ 29 transcription to rifamycin. The amount of viral-specific RNA made at various times after infection in the presence or absence of this antibiotic was examined. The ability of infected bacteria to synthesize ϕ 29-specific RNA after 10 min in the presence of rifamycin was tested by exposing them to radioactive uridine and determining the amount of labeled RNA hybridizing to ϕ 29 DNA. It is clear from Table 1 that viral transcription remains sensitive to the antibiotic at all times examined. ϕ 29 RNA synthesis was normal in the presence of rifamycin in a strain selected for its resistance to rifamycin.

FIG. 3. Viral RNA synthesis. Bacteria were infected with ϕ 29 at 0 min. Samples were removed at the indicated times and exposed to ^3H -uridine (2 $\mu\text{Ci/ml}$) for 1-min intervals. The RNA was extracted and hybridized to ϕ 29 DNA. The ratio of DNA to RNA was 25:1. The results are expressed as the percentage of the total labeled input RNA binding to the filters after ribonuclease treatment.

TABLE 1. Effects of rifamycin on ϕ 29-specific RNA synthesis in *B. subtilis* 60084^a

Min after infection	Amount of RNA per hybridization reaction (μg)	ϕ 29-specific counts/min	
		- Rifamycin	+ Rifamycin
-2	30	4,238	3
+6	30	7,478	17
+26	30	2,436	5

^a Logarithmically growing cultures of *B. subtilis* 60084 were infected with ϕ 29 at 0 min. Rifamycin (5 $\mu\text{g/ml}$) was added at -2, 6, and 26 min after infection. The infected bacteria were grown for an additional 10 min in the presence of the antibiotic and then exposed to 5 μCi of ^3H -uridine per ml for 4 min. Total RNA from rifamycin-treated and untreated bacteria was extracted and hybridized to excess ϕ 29 DNA.

cin (*unpublished observation*). These results support the idea that $\phi 29$ development requires the continuous activity of the bacterial RNA polymerase and, in this sense, resembles the larger phages like T4 (16) and SP01 (13) and not phage T7 (8, 26).

Stability of viral RNA. The stability of messenger RNA in uninfected and infected bacteria, at early and late times in the lytic cycle, was measured. Logarithmically growing bacteria were exposed to radioactive uridine for a brief interval. The labeling period was terminated by the addition of uridine and rifamycin.

Figure 4 shows the results of experiments

utilizing DNA-RNA hybridization in the presence of excess stable bacterial RNA to follow the metabolism of bacterial and phage-specific messenger RNA (mRNA). Under these conditions, bacterial mRNA loses its ability to hybridize with a half-life of approximately 2 min. The rate of decay of mRNA appears to be the same in uninfected and infected bacteria, at early and late times in the lytic cycle. Infection with $\phi 29$ does not seem to alter the stability of host mRNA. Viral-specific RNA continued to form ribonuclease-resistant hybrids with $\phi 29$ DNA for a much longer period with a half-life of about 10 min, suggesting that viral RNA is four to five times more stable than bacterial RNA. A similar conclusion was reached when actinomycin D was employed to inhibit RNA synthesis (*unpublished observation*).

DNA-RNA competition experiments: competition between early and late RNA for binding sites on $\phi 29$ DNA. For many bacterial and animal viruses, the expression of large numbers of genes is apparently coupled to viral DNA replication (7, 12). In phage T4, the appearance of late proteins and RNAs requires the continuous synthesis of viral DNA (7), suggesting that parental DNA is not adequate for late transcription but must be modified to express this portion of the genome. DNA-RNA competition experiments were conducted to determine if $\phi 29$ transcription had a similar requirement for phage DNA synthesis or if parental DNA was sufficient for complete transcrip-

FIG. 4. Stability of bacterial and viral RNA. Logarithmically growing bacteria were infected with $\phi 29$ at a multiplicity of 10 to 15 phages per bacterium. ^3H -uridine ($5 \mu\text{Ci/ml}$) was added to the cultures at 5 and 25 min after infection. Rifamycin ($5 \mu\text{g/ml}$) and uridine (1 mg/ml) were added at 7 and 27 min. Samples were removed at intervals after the addition of the rifamycin. The RNA was extracted and hybridized to bacterial (O) and viral (●) DNA. The ratio of DNA to ^3H -RNA was 250:1, whereas the ratio of stable, actinomycin D-chased RNA (i.e., transfer and ribosomal RNA; assuming that 1 optical density unit at 260 nm is equivalent to $40 \mu\text{g}$ of RNA) to pulse-labeled RNA was 100:1. A, RNA extracted from uninfected bacteria. The 100% value represents 2,800 counts per min. B, RNA from infected bacteria exposed to rifamycin at 7 min after infection. The 100% values are 6,100 counts per min for bacterial RNA and 3,946 counts per min for viral RNA. C, RNA from infected bacteria exposed to rifamycin at 27 min after infection. The 100% values are 3,300 counts per min and 6,763 counts per min for the bacterial and viral RNAs, respectively. In all panels, the ordinate represents the percentage of hybrid counts obtained at the start of the chase period.

tion of the phage genome. The relatedness of phage-specific RNA molecules made before and after DNA replication was tested by their ability to compete for sites on ϕ 29 DNA. It is

FIG. 5. Competition between early and late RNA for binding sites on ϕ 29 DNA. Radioactive RNA was prepared from bacteria exposed to ^3H -uridine ($5 \mu\text{Ci/ml}$) from 3 to 7 and 23 to 27 min after infection. Unlabeled competitor RNA was prepared from uninfected bacteria as well as from infected bacteria at 7 and 27 min after infection. For each point, hybridization was carried out by using $0.1 \mu\text{g}$ of ϕ 29 DNA, a saturating amount of ^3H -labeled RNA, and the indicated amount of unlabeled competitor RNA. Each point is the average of at least four separate determinations. Details of the hybridization reaction are given in Materials and Methods. A, RNA labeled at times early in the infectious cycle (3–7 min) was incubated with increasing amounts of unlabeled RNAs. The 100% value was 1,527 counts per min. B, ^3H -labeled late RNA (23 to 27 min after infection) was competed with increasing amounts of unlabeled RNAs. The 100% value was 2,811 counts per min. Symbols: Δ , uninfected; $\circ$, 7 min after infection; $\bullet$, 27 min after infection.

apparent from Fig. 5, part A, that sequences present 7 min after infection were also found at 29 min. Part B shows that RNA made 7 min after infection was able to compete with only 40% of the sequences labeled at 23 to 27 min.

It appears therefore that ϕ 29 RNA, present after DNA replication, consists of both early RNA molecules and sequences in addition to those made at early times after viral infection. Since early viral RNA is relatively stable (30% of 7-min ϕ 29 RNA was still able to form RNA hybrids with ϕ 29 DNA even after a 20-min chase period; data not shown; see also Fig. 4), it is difficult to conclude that all early sequences are synthesized late after infection. The observation that late RNA competes with that made at early times could simply reflect the inherent stability of early sequences.

These experiments do not indicate if the differences between early and late viral RNA depend upon viral DNA replication or result from other regulatory processes. The following experiment was conducted to determine if late ϕ 29 transcription has an obligate requirement for viral DNA replication: viral RNA was isolated at times in the infective cycle normally devoted to late transcription but under conditions which would not allow viral DNA replication. Figure 6 shows the results of competition experiments between ^3H -RNA labeled from 23 to 27 min after infection, and 27-min unlabeled RNA extracted from bacteria infected with wild-type ϕ 29 or with ϕ 29 TS35 at 45 C. RNA formed in the absence of viral DNA replication was similar to that formed if DNA synthesis occurred. Viral DNA replication is therefore not required for expression of temporally late genes.

ϕ 29 RNA made in the presence of CAP. It has been shown with other bacteriophages that gene expression in the absence of DNA synthesis can be divided into additional subclasses, the immediate and delayed early RNAs of T4 (20, 21, 24) and the viral polymerase-independent and -dependent classes of T7 (8, 26), largely on the basis of studies employing CAP. It was, therefore, important to determine in ϕ 29-infected cells if viral RNA made in the presence of CAP was equivalent to early RNA or a subclass of it, and to see if late RNA could be synthesized under these conditions. Figure 7 shows the results of such an experiment. Part A indicates that all sequences synthesized when infection was carried out in the presence of CAP were also present in early and late RNA. On the other hand, CAP RNA was capable of excluding less than 30% of the

FIG. 6. RNA specified at late times in the absence of DNA synthesis. Bacteria were grown at 45 C to a density of 2×10^8 cells per ml and infected with $\phi 29$ or TS35. Unlabeled competitor RNAs were prepared at 25 min after infection. Radioactive RNA (5 μ Ci of uridine per ml) (23–27 min) was isolated from bacteria grown at 37 C and infected with wild-type $\phi 29$. The 100% value was 2,800 counts per min. Symbols: O, unlabeled competitor RNA extracted from bacteria infected with wild-type $\phi 29$; ●, unlabeled competitor RNA extracted from bacteria infected with TS35; Δ , unlabeled competitor RNA extracted from uninfected bacteria.

late sequences from $\phi 29$ DNA (part B). Early, 7-min RNA excluded about 40% of these same late RNA sequences (see Fig. 5).

These results suggested that some early genes were not transcribed when infection occurred in the presence of CAP. This conclusion is supported by the results of experiments shown in Fig. 8. $\phi 29$ -specific RNA made in the presence of CAP could not compete all early sequences. Approximately 20% of RNA labeled from 3 to 7 min after infection hybridized with $\phi 29$ DNA even in the presence of large amounts of unlabeled CAP RNA.

DISCUSSION

The results presented in this communication suggest that gene expression during the infective cycle of bacteriophage $\phi 29$ is a relatively complex process, a surprising conclusion in view of the limited genetic potential of this virus. Our data indicate that $\phi 29$ transcription, unlike that of phage T7, is dependent on the rifamycin phenotype of the host RNA polym-

FIG. 7. RNA formed in bacteria infected in the presence of CAP. Logarithmically growing bacteria were treated with CAP (250 μ g/ml) at -5 min and infected with $\phi 29$ at 0 min. Unlabeled competitor RNA was prepared at 29 min. Radioactive RNA was extracted from infected bacteria exposed to 3 H-uridine (5 μ Ci/ml) from 25 to 29 min after infection. A, Competition between 3 H-CAP RNA, and 7- and 27-min unlabeled RNA for sites on $\phi 29$ DNA. The 100% value is 445 counts per min. Symbols: Δ , uninfected RNA; $\square$, 27-min CAP RNA; $\circ$, 7-min RNA; $\bullet$, 27-min RNA. B, Competition between 23- and 27-min 3 H-RNA and 27-min, unlabeled CAP RNA. The 100% value is 2,811 counts per min. Symbols: Δ , uninfected RNA; $\bullet$, 27-min CAP RNA.

erase throughout the latent period. On the other hand, the effects of CAP and HPAU on $\phi 29$ DNA replication support the conclusion that this virus synthesizes at least one of the components involved in the replication of its DNA rather than depending entirely on enzymes present in uninfected bacteria.

Gene expression during $\phi 29$ development was found to occur in a highly regulated way with only a portion of the genome expressed

FIG. 8. Competition between 3- and 7-min ^3H -RNA and unlabeled CAP RNA from infected bacteria. The 100% value is 1,492 counts per min. Symbols: Δ , uninfected RNA; $\circ$, 7-min RNA; $\bullet$, 27-min CAP RNA.

immediately after infection. Competition-hybridization experiments indicated that transcription occurred in two distinct stages, defined temporally as early and late. Early RNA consisted of sequences specified prior to the onset of viral DNA replication (at 10 min after infection). Approximately 40% of the potential RNA sequences were present in this class. The data shown in Fig. 5A indicate that this RNA is also present at late times in the viral lytic cycle, much of it being synthesized at that time (Fig. 5B). Viral DNA replication, therefore, does not signal termination of expression of most of the early genome as occurs during the development of bacteriophages T4 (7, 12) and SP01 (11). On the other hand, since early ϕ 29 RNA was found to be relatively stable (Fig. 4), it is difficult to conclude from Fig. 5A that all early sequences are actually synthesized throughout the latent period. Since the data in Fig. 4 show that degradation of early ϕ 29 RNA is not delayed after the addition of rifamycin, an antibiotic which blocks initiation in termination of RNA chains (10), we conclude that early viral RNA is similar in size to that of bacterial RNA. For early viral RNA, therefore, we cannot argue that increased size would contribute significantly to its stability to exonucleolytic digestion. However, since late ϕ 29 RNA syn-

thesis continued in the presence of rifamycin for up to 2 min, we cannot conclude that late viral RNA is also more inherently stable than bacterial RNA since it appears to be larger and thus might take considerably longer to degrade exonucleolytically. In a subsequent communication, we confirm the conclusion that most early sequences are synthesized late in the infectious cycle. In addition, we show that one mRNA species, the major early component, is not synthesized after the onset of viral DNA replication.

Late viral RNA was defined as those transcripts which were not present immediately after infection but were detected only after a significant delay. Their appearance occurred at approximately the same time as viral DNA replication in the lytic cycle. The data in Fig. 5B indicate that 60% of the genome is activated at this time. The data of Fig. 4 also indicate that the addition of 1 mg of uridine per ml was not sufficient to eliminate immediately the uptake of the radioactive precursor. The appearance of late RNA during ϕ 29 development does not seem to depend upon viral DNA replication (Fig. 6). In this respect, ϕ 29 resembles bacteriophage T7 in which the expression of late genes occurs independently of viral DNA replication (8, 25, 26). In that case, however, it has been shown that the expression of late genes is under the positive control of the gene 1 product (26) which has been identified as a rifamycin-resistant, viral-coded RNA polymerase (8). In ϕ 29 development, however, transcription of viral chromosomes appears to be completely dependent on the rifamycin phenotype of the host RNA polymerase. It is possible that a viral product(s) may modify the host RNA polymerase. We have found by hybridization-competition analysis that the RNA synthesized in vitro with *B. subtilis* RNA polymerase extracted from uninfected bacteria is analogous to in vivo phage RNA synthesized in the presence of CAP (*unpublished observation*).

ϕ 29 transcription was dissected further with the aid of CAP, an inhibitor of protein synthesis. Only 30% of the RNA sequences were transcribed from ϕ 29 DNA when infection occurred in the presence of this antibiotic (Fig. 7B).

It is difficult to interpret the early-late transition in ϕ 29 RNA synthesis entirely in terms of a single, passive model of regulation in which the time of appearance of late RNA molecules is dependent only upon the proximity of these sequences to a specific promoter. Although the data are not conclusive at this point, we will

present evidence in a subsequent communication which favors a model involving active, viral-mediated transition from early to late RNA. The further restriction placed on transcription by CAP (i.e., the inability to detect all early sequences) is not understood at present. The RNA of the distal portion of the tryptophan operon also cannot be detected in the presence of CAP, and it has been claimed that the presence of this antibiotic may cause accelerated breakdown of the distal portions of some operons (21).

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LITERATURE CITED

- Adams, M. H. 1959. Bacteriophages. Interscience Publishers, New York.
- Albertson, P. 1967. Two-phase separation of viruses. In K. Maramorosch and H. Koprowski (ed.), *Methods in virology*. Academic Press Inc., New York.
- Anderson, D. L., D. D. Hickman, and B. E. Reilly. 1966. Structure of bacteriophage ϕ 29 and the length of ϕ 29 deoxyribonucleic acid. *J. Bacteriol.* **91**:2081-2089.
- Brown, N. C. 1970. 6-(hydroxyphenylazo)-uracil: A selective inhibitor of host DNA replication in phage-infected *Bacillus subtilis*. *Proc. Nat. Acad. Sci. U.S.A.* **67**:1454-1461.
- Brown, N. C., and R. E. Handshumacher. 1966. Inhibition of the synthesis of deoxyribonucleic acid in bacteria by 6-(p-hydroxyphenylazo)-2,4-dihydroxypyrimidine. I. Metabolic studies in *Streptococcus fecalis*. *J. Biol. Chem.* **241**:3083-3089.
- Calendar, R. 1970. The regulation of phage development. *Annu. Rev. Microbiol.* **24**:241-296.
- Cascino, A., S. Riva, and E. P. Geiduschek. 1970. DNA ligation and the coupling of T4 late transcription to replication. *Cold Spring Harbor Symp. Quant. Biol.* **35**:213-220.
- Chamberlin, M., J. McGrath, and L. Waskell. 1970. New RNA polymerase from *Escherichia coli* infected with bacteriophage T7. *Nature (London)* **228**:227-231.
- Denhardt, D. T. 1969. Formation of ribosylthymine in *Escherichia coli*: studies on pulse-labeling with thymine and thymidine. *J. Biol. Chem.* **244**:2710-2715.
- DiMauro, E., L. Snyder, P. Marine, A. Lamberti, A. Coppo, and G. P. Tocchini-Valentini. 1969. Rifampicin sensitivity of the components of DNA-dependent RNA polymerase. *Nature (London)* **222**:533-536.
- Gage, L. P., and E. P. Geiduschek. 1971. RNA synthesis during bacteriophage SP01 development. II. Modulations and prerequisites of the transcription program. *Virology* **44**:200-210.
- Geiduschek, E. P., and R. Haselkorn. 1969. Messenger RNA. *Annu. Rev. Biochem.* **38**:647-676.
- Geiduschek, E. P., and J. Sklar. 1969. Role of host RNA polymerase in phage development. *Nature (London)* **221**:833-836.
- Gillespie, D., and S. Spiegelman. 1965. A quantitative assay for DNA-RNA hybrids with DNA immobilized on a membrane. *J. Mol. Biol.* **12**:829-842.
- Hagen, E., V. Zeece, and D. L. Anderson. 1971. A genetic study of temperature-sensitive mutants of the *Bacillus subtilis* bacteriophage ϕ 29. *Virology* **43**:561-568.
- Haselkorn, R., M. Vogel, and R. D. Brown. 1969. Conservation of the rifamycin sensitivity of transcription during bacteriophage T4 development. *Nature (London)* **221**:836-838.
- Levinthal, C., A. Keynan, and A. Higa. 1962. Messenger RNA turnover and protein synthesis in *Bacillus subtilis* inhibited by actinomycin D. *Proc. Nat. Acad. Sci. U.S.A.* **48**:1631-1638.
- Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. *J. Mol. Biol.* **3**:208-218.
- Mendez, E., G. Ramirez, M. Salas, and E. Vinuela. 1971. Structural proteins of bacteriophage ϕ 29. *Virology* **45**:567-576.
- Milanesi, G., E. N. Brody, O. Grau, and E. P. Geiduschek. 1970. Transcription of the bacteriophage T4 genome *in vitro*: separation of "delayed early" from "immediate early" transcription. *Proc. Nat. Acad. Sci. U.S.A.* **66**:181-188.
- Morse, D. E. 1970. "Delayed-early" mRNA for the tryptophan operon. An effect of chloramphenicol? *Cold Spring Harbor Symp. Quant. Biol.* **35**:495-496.
- Mosharrafa, E. T., C. F. Schachtele, B. E. Reilly, and D. L. Anderson. 1970. Complementary strands of bacteriophage ϕ 29 deoxyribonucleic acid: preparative separation and transcription studies. *J. Virol.* **6**:855-864.
- Reilly, B. E., and J. Spizizen. 1964. Bacteriophage deoxyribonucleate infection of competent *Bacillus subtilis*. *J. Bacteriol.* **89**:782-890.
- Salser, W., A. Bolle, and R. H. Epstein. 1970. Transcription during bacteriophage T4 development: a demonstration that distinct subclasses of the "early" RNA appear at different times and that some are "turned off" at late times. *J. Mol. Biol.* **49**:271-295.
- Siegel, R. B., and W. C. Summers. 1970. The process of infection with coliphage T7. III. Control of phage-specific RNA synthesis *in vivo* by an early phage gene. *J. Mol. Biol.* **49**:115-123.
- Summers, W. C., and R. B. Siegel. 1969. Control of template specificity of *E. coli* RNA polymerase by a phage-coded protein. *Nature (London)* **223**:1111-1113.
- Welker, N. E., and L. L. Campbell. 1967. Unrelatedness of *Bacillus amyloliquefaciens* and *Bacillus subtilis*. *J. Bacteriol.* **94**:1124-1130.