Nucleic Acid Synthesis in *Bacillus subtilis* Infected with Bacteriophage β 22

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Changes in the synthesis of host and phage nucleic acid after infection of *Bacillus subtilis* with virulent bacteriophage β 22 were analyzed by deoxyribonucleic acid (DNA)-DNA and ribonucleic acid (RNA)-DNA hybridization. Host DNA replication continued during the first third of the 55-min latent period and then ceased at approximately the time replication of the phage genome was initiated. Host-specific RNA was synthesized concurrently with phage RNA during the first half of the latent period but was repressed late in the infection. For much of the latent period, the population of phage-specific RNA changed continually as new species were transcribed and earlier species were repressed; detectable changes ceased coincidentally with the appearance of intracellular phage. Control over transcription of phage DNA was to some degree an intrinsic property of the interaction of *B. subtilis* DNA-dependent RNA polymerase and the phage genome, since only the early species of phage RNA were synthesized in vitro by *B. subtilis* polymerase and pure β 22 DNA. In vitro transcription of late functions was demonstrated by using the endogenous RNA polymerase activity of the nucleoprotein complex (nuclear fraction) from infected cells.

Bacillus subtilis is the host for a large number of virulent, temperate, and defective bacteriophages. This fact, coupled with the ability of this organism to produce spores, provides an opportunity to study the interaction of a number of parameters influencing the synthesis of macromolecules. These include the interaction of host-specific and phage-specific functions during phage infection, changes in the populations of host and phage ribonucleic acid (RNA) transcribed during the latent period, and the effects of sporulation on phage maturation.

Several investigators (9, 13) have noted that early and late phage-specific RNA species are produced by *B. subtilis* infected with SPO1 and β 22. The lytic cycles of these two phages permit a fine resolution of transcriptional events because relatively few RNA molecules synthesized in the early portions of the latent period are present in cells late in the infection.

Phage infections whose eclipse periods overlap the beginning of sporulation may be affected in one of two ways. In cells infected with β 3, PBS, or ϕ e, the maturation of the phages ceases during sporulation and phage-related functions become quiescent until spore germination (25, 28, 34). An opposite effect is seen in sporulating cells infected with β 22. In this instance, spore formation is inhibited after phage infection is

initiated, presumably due to suppression of host-related functions by the phage (34).

This report presents a detailed analysis of the synthesis of host-specific and phage-specific RNA and deoxyribonucleic acid (DNA) in *B. subtilis* infected with β 22 phage. In addition, two series of in vitro studies were made: first, the RNA transcribed by nuclear fractions isolated from β 22-infected cells was compared with RNA synthesized in vivo during the latent period; second, a study was made of the RNA synthesized by *B. subtilis* RNA polymerase and purified β 22 DNA.

MATERIALS AND METHODS

Bacterial strains and media. *B. subtilis* strain SB11 try⁻ was employed in these experiments. This strain is a derivative of *B. subtilis* W23 and was obtained from E. W. Nester. Bacteriophage β 22 was supplied by Roy Doi, SP82 by Charles Stewart, and SP02 by W. R. Romig.

Phage lysates were obtained from cells grown in M medium as described by Yehle and Doi (34). Cultures used for the preparation of nuclear fractions or for isotopic labeling were grown in a medium containing: 0.1% (NH₄)₂SO₄, 0.01% MgSO₄, 0.05% sodium citrate, 0.2% glucose, 0.1% Casamino Acids, 25 $\times$ 10⁻³ M tris(hydroxymethyl)aminomethane (Tris, pH 7.2), 10⁻³ M phosphate, 5 $\times$ 10⁻³ M CaCl₂, and 10⁻⁴ M MnCl₂.

Growth and infection of cultures. Cells were grown at 37 C on a rotary shaker. Optical density at 600 nm was measured in a Spectronic 20 Spectrophotometer with 1-cm circular cuvettes. Cultures to be infected with phage were grown to an optical density of 0.4 to 0.44 (2×10^8 to 2.5×10^8 cells/ml) and infected at a multiplicity of infection (MOI) of between 5 and 10 (discussed below). Approximately 95% of the cells were infected by 1 min as determined by measuring the number of surviving cells. Under these conditions, the cultures began to lyse about 55 to 60 min after infection.

Phage were isolated and purified from lysates by the procedure of Yehle and Doi (34), except that phage were purified by CsCl density centrifugation only when the phage were to be used as a source of DNA. $\beta 22$ stocks were stored at 4 C in 10% dimethyl sulfoxide (33).

Titer of phage and determination of multiplicity of infection. Initially, phage stocks were titered on M agar (34) with a 0.7% soft agar overlay. Plaques on this medium, however, were usually quite small, and repeated titring of the same stock showed marked fluctuations. Moreover, careful examination of the plates often revealed tiny plaques that could not be accurately counted.

An estimate of the phage titer was also made by comparing the number of surviving cells at various presumed MOI values with that expected on the basis of a Poisson distribution. This method indicated that the plaque counts measured only 10 to 50% of the particles capable of killing cells. Assuming that the latter were intact phages, the approximate titer of phage stock was estimated by multiplying the number of plaques by five. This figure was used to calculate the MOI.

Preparation of a nuclear fraction from phage-infected cells. The nuclear fraction from phage-infected cells was isolated by a modification of the method of Mizuno and Whiteley (16). Packed cells were thawed and suspended in 0.005 M phosphate buffer (pH 7.3) to give a concentration of 100 mg (wet wt/ml). The following solutions were added per milliliter of cell suspension: 4.5 ml of PLN (0.005 M phosphate buffer, pH 7.3, containing 26.7% lactose and 0.157 M NaCl) and 0.25 ml of a solution containing 2.4 mg of lysozyme per ml. This mixture was incubated at 37 C until the optical density at 420 nm of a 1:40 dilution of the preparations dropped below 0.08. A neutral detergent, Lubrol W (Imperial Chemical Industries, Great Britain), was then added to a final concentration of 0.25% to bring about complete lysis of any remaining cells or protoplasts, and the lysate was allowed to stand 15 min in ice. It should be noted that *B. subtilis* SB11, unlike strain 6 used by Mizuno and Whiteley (16), did not produce stable protoplasts after lysozyme treatment.

Cells harvested late in phage infection were often resistant to lysis by lysozyme. To effect complete lysis of these cells, the preparations were frozen in dry ice-ethanol, thawed at room temperature in water, and then incubated at 37 C.

The lysate was centrifuged at $78,000 \times g$ for 30 min, and the supernatant was decanted. The viscous pellet was homogenized in a Teflon homogenizer in a

small volume of TMm buffer (0.01 M Tris, pH 7.9; 0.01 M $MgCl_2$; 0.006 M 2-mercaptoethanol). The preparation was diluted with the homogenizing solution to one-half the volume of the original cell suspension and centrifuged at $27,000 \times g$ for 20 min. The supernatant from this centrifugation was carefully removed from the pellet of debris and was used as the "nuclear fraction" in most experiments.

In some experiments (e. g., those with nuclear fractions isolated 30 min after infection), 1-ml samples of nuclear fraction prepared as described above were layered on a step gradient (1.8 ml of 40% sucrose, 1.8 ml of 15% sucrose, both made in TMm buffer) and centrifuged at $73,449 \times g$ in an SW50L rotor. The nuclear fraction was then removed from the interface between the two sucrose layers with a pipette. Nuclear fractions were stored at -62 C until used.

The RNA transcribed *in vitro* by these latter preparations yielded the same results in competition-hybridization experiments as similar RNA synthesized *in vitro* by nuclear fractions not subjected to sucrose gradient centrifugation.

Partial purification of the DNA-dependent RNA polymerase. Partial purification of DNA-dependent RNA polymerase from *B. subtilis* strain 6 or SB11 was accomplished as described by Mizuno and Whiteley (16) with the addition of chromatography on diethylaminoethyl (DEAE)-Sephadex or sedimentation through a 10 to 30% glycerol gradient (Whiteley and Hemphill, unpublished data). The final preparations, representing a 300-fold purification, were free of ribonuclease and deoxyribonuclease activities. The latter were determined by incubating 3H -labeled *B. subtilis* RNA or DNA with the enzyme preparation in 0.05 M Tris, pH 7.9–0.05 M $MgCl_2$ for 2 to 16 hr and determining the radioactivity of the material precipitated by 5% trichloroacetic acid.

Conditions for *in vitro* RNA synthesis. *In vitro* synthesis of RNA by the endogenous activity of nuclear fractions, by combinations of nuclear fraction and added exogenous RNA polymerase, or by purified DNA and RNA polymerase were carried out in a reaction mixture containing: 5 μCi of 3H -adenosine triphosphate (ATP) (specific activity 5 to 20 mCi/mM); 0.8 $\mu moles$ each of ATP, guanosine triphosphate, uridine triphosphate, and cytosine triphosphate; 50 $\mu moles$ of Tris buffer (pH 7.9); 50 $\mu moles$ of $MgCl_2$; and 12 $\mu moles$ of 2-mercaptoethanol. Appropriate quantities of DNA, polymerase, and/or nuclear fractions and water were added to a total volume of 1 ml.

Nuclear fractions were added at a ratio of 0.2 ml/ml of reaction mixture. Purified DNA preparations and heated nuclear fractions were added at a concentration of 50 to 100 μg of DNA (or DNA equivalent in the nuclear fraction) per ml of reaction mixture. Partially purified RNA polymerase was added at a concentration of approximately 20 μg of protein/ml.

The RNA-synthesizing mixtures were incubated for 15 or 20 min at 37 C. At the end of the desired incubation time, the reaction was terminated by the addition of sodium lauryl sulfate (SLS) to give a final concentration of 0.2% and 60 C water-saturated

phenol. The RNA was purified by the procedure described below for RNA from pulse-labeled whole cells.

RNA synthesized in vitro from DEAE-Sephadex-purified RNA polymerase and purified DNA had specific activities of 643 to 2,500 counts per min per μg of RNA. RNA transcribed by the endogenous activity of nuclear fractions had specific activities in the range of 130 to 310 counts per min per μg of RNA, whereas that synthesized from heated nuclear fractions and exogenous polymerase ranged from 150 to 190 counts per min per μg .

Preparation of in vivo pulse-labeled RNA and DNA. Cultures were grown and infected as described above. At the desired time after infection, 1 μCi of ^3H -uridine per ml (5 to 10 Ci/mM) or 0.5 μCi of ^3H -thymidine per ml (15 Ci/mM) was added, and the cells were incubated with shaking for an additional 5 min in experiments with labeled uridine or for 10 min in experiments with radioactive thymidine. The cultures were then poured over an equal volume of ice, and the infected cells were harvested by centrifugation.

RNA was extracted by passing cell suspensions through a French pressure cell in the presence of 2% SLS and bentonite and then extracting the lysate with 60 C water-saturated phenol by a modification of the method of Scherrer and Darnell (24). After extracting the water layer with phenol, the supernatant was made 0.1 M with respect to NaCl, and the RNA was precipitated overnight at -20°C with three volumes of ethanol. The RNA was redissolved in 0.01 M sodium acetate–0.01 M magnesium acetate, pH 5.6, and treated with 10 μg of deoxyribonuclease (Worthington, ribonuclease-free) per ml for 1 hr at room temperature. The preparation was extracted a second time with 60 C water-saturated phenol and centrifuged in the cold, and the RNA was precipitated with three volumes of ethanol at -20°C . The precipitate was redissolved in 1/10 SSC (1 $\times$ SSC is 0.15 M NaCl–0.015 M sodium citrate) and dialyzed overnight against the same buffer. Unlabeled RNA for competition experiments was extracted as described above, and the final preparation was dialyzed against 2 $\times$ SSC.

RNA isolated from pulse-labeled cells had specific activities ranging from 330 to 2,440 counts per min per μg of RNA. RNA from cells pulsed early in the latent period (when host stable RNA was still being synthesized) had higher specific activities than RNA from cells pulsed late in the lytic cycle.

DNA from infected, uninfected, and ^3H -thymidine-labeled cultures was obtained from lysozyme-treated cells by the procedure of Marmur (15). Iso-propanol-precipitated material, collected by spooling on a glass rod, was dissolved in 1/10 SSC, quantitated from absorbancy at 260 nm, and used without further purification for in vitro RNA synthesis. Phage DNA was extracted with cold, buffered (pH 7.2) redistilled phenol, spooled by the addition of ethanol, carefully redissolved in 1/10 SSC, dialyzed against 1/10 SSC, and used without further purification as a template for RNA synthesis.

Host and phage DNA species used in hybridiza-

tion experiments were further purified by extraction at 60 C with buffered phenol.

DNA isolated from ^3H -thymidine pulse-labeled cells had specific activities ranging from 785 to 2,952 counts per min per μg of DNA.

Preparation of radioactive phage DNA. Radioactive $\beta 22$ DNA was prepared by growing a culture of *B. subtilis* cells to an optical density (600 nm) of 0.33 and infecting with $\beta 22$. Twenty minutes after infection, 1 μCi of ^3H -thymidine per ml was added, and phage infection was allowed to proceed to lysis. Phage were isolated by differential centrifugation, and the DNA was isolated by a cold phenol extraction as described above. The DNA had a specific activity of 5,800 counts per min per μg .

Uridine incorporation in phage-infected cells. Studies of the incorporation of uridine after phage infection were made by growing the cells in the medium described above, supplemented with 100 μg of uridine, cytidine, and thymidine per ml and 2 μCi of ^3H -uridine per ml. At appropriate time intervals before and after infection, duplicate 0.1-ml samples were removed, precipitated with cold 5% trichloroacetic acid, and collected on membrane filters. The filters were dried and counted in the scintillation counter by using a toluene-based scintillation solution.

DNA synthesis in phage-infected cells. The amount of DNA present in cells before and after phage infection was assayed as follows: at various time intervals, duplicate 10-ml samples of the cultures were removed and precipitated with 0.25 N HClO_4 . The cells were collected by centrifugation, resuspended in 0.25 N HClO_4 , and centrifuged a second time. The rinsed cells were resuspended in 0.5 N HClO_4 (3 ml) and heated at 70 C for 30 min. The extracted cells were collected by centrifugation and extracted a second time with 2 ml of 0.5 N HClO_4 , and the supernatants from the two extractions were pooled. Samples (1 ml) of the pooled extractions were assayed for DNA by the method of Burton (6).

DNA-DNA and RNA-DNA hybridizations. RNA-DNA hybridization studies were done by the method of Gillespie and Spiegelman (11). The relative amounts of host RNA and phage-specific RNA present in various preparations were determined by incubating increasing quantities of the desired radioactive RNA with host DNA or phage DNA. Duplicate sets of reaction vials were prepared containing various amounts of the radioactive RNA, unlabeled competitor RNA if required, and 10 to 15 μg of host or phage DNA adsorbed to two or three 4-mm membrane filters (B-6 Bac-T-Flex; Carl Schleicher and Schuell Co., Keene, N.H.) mounted on a stainless-steel pin. The salt concentration was adjusted to 2 $\times$ SSC; the final volume in the reaction vial was 0.5 ml. The vials were incubated for 18 hr at 63 C. At the completion of the incubation period, the pins with the DNA filters were removed, rinsed in three 15-ml volumes of 2 $\times$ SSC, dried at 80 C, and counted in a scintillation counter, by using a toluene-based scintillant.

DNA-DNA hybridizations were done approximately as described for RNA-DNA hybridizations except that the filters containing denatured DNA

were treated to reduce nonspecific binding (8). Radioactive DNA to be hybridized to the filter-bound DNA was heat-denatured by boiling for 5 min and then rapidly cooled in ice.

Sucrose gradient density sedimentation. The sedimentation properties of various RNA preparations were analyzed by layering 0.1-ml samples of RNA preparations on 5-ml linear sucrose gradients (20 to 5% in 0.01 M Tris-0.001 M ethylenediaminetetraacetic acid-0.1 M NaCl, pH 7.0). The gradients were centrifuged in a Spinco (model L) centrifuge with an SW50L rotor.

Molecular weight determinations of DNA. The molecular weight of DNA templates was determined from sedimentation velocities in the Spinco model E analytical ultracentrifuge fitted with ultraviolet absorption optics. A 2- μ g amount of DNA was sedimented in a cell equipped with a band-forming centerpiece, by using 0.9 M NaCl-0.1 M NaOH as the solvent by the method of Studier (26). Sedimentation was at 33,450 rev/min; photographs were taken at 2- or 4-min intervals. The molecular weight of the β 22 genome was estimated from the rate of annealing of heat denatured DNA by the method of Britten and Kohne (2).

RESULTS

Host range of β 22. Yehle and Doi (34) demonstrated that β 22 bacteriophage infects *B. subtilis* strains 168 and W23. We have found that β 22 was able to lyse *B. subtilis* strains 168, SB11, 6, a 168M strain carrying SPO2 prophage [*B. subtilis* 168M (SPO2)], and *B. amyloliquefaciens*, but did not lyse *B. megaterium*. The lytic cycle and the synthesis of macromolecules have been examined in greatest detail in strains 168 and SB11. The length of the lytic cycle (55 to 60 min), the burst size (100 to 125), and changes in the synthesis of RNA and DNA in these strains appeared to be identical (13, 34).

Physical properties of β 22. β 22 is a large bacteriophage with a head width of about 100 nm and a tail length of 300 nm (L. Brown and R. Doi, *personal communication*). Most other physical properties of this phage and phage DNA have been reviewed by Yehle and Doi (34). One anachronism is that the molecular weight of the phage DNA as determined by sedimentation velocity (3.3×10^7) seems unusually low relative to the large size of β 22 (34). We have determined the molecular weight of the phage DNA by reassociation kinetics to be 90×10^6 , utilizing the procedure of Britten and Kohne (2). The reason for the discrepancy between these values and sedimentation results is not known, but it is possible that the phage genome is in more than one piece.

Because the size of β 22 is very close to that of *B. subtilis* phages 2C, ϕ e, and SP82, an attempt was made to determine whether β 22 showed any

DNA homology with a representative of this group. DNA-DNA hybridization with 3 H-labeled DNA from β 22 showed that 46% of the input counts bound to β 22 DNA but none bound to SP82 DNA. On this basis, there appeared to be no homology between the DNA of β 22 and SP82 phages.

Synthesis of RNA and DNA during the lytic cycle. Infection of *Bacillus subtilis* with β 22 bacteriophage did not bring about the precipitous changes in host nucleic acid synthesis that have been documented in coliphage infections of *Escherichia coli*. The immediate effects of adding β 22 appeared to be relatively benign (Fig. 1). The curves for growth and the synthesis of DNA and RNA did not differ from uninfected controls for the first third of the latent period. Between 20 and 25 min after phage addition, the accumulation of RNA as measured by uridine incorporation ceased, and the growth rate of the culture diminished until a plateau was reached about 30 min after infection. DNA synthesis, as measured by the diphenylamine reaction, also paralleled uninfected controls until 25 min after infection but then increased dramatically. At the time of lysis, the cells contained three times as much DNA as controls and eight times the quantity of DNA present at the time the culture was infected.

A quantitative analysis of nucleic acid syntheses which distinguished between the contributions of host and phage confirmed that host syntheses continued for a considerable time into the latent period. In these studies, radioactive DNA or RNA isolated from cells pulsed with 3 H-thymidine or 3 H-uridine at various intervals after infection were bound to denatured β 22 or *B. subtilis* DNA adsorbed to nitrocellulose filters. The results (Table 1) demonstrated that the replication of host DNA continued for the first 10 to 20 min of the latent period and then ceased completely. Similarly, host RNA transcription (Table 2) persisted for the first half of the lytic cycle during which it changed in two phases. First there was a rapid change in the proportions of host and phage RNA synthesized until about half was synthesized from each genome. About 25 min after infection, there was a more general suppression of host RNA synthesis until only residual amounts (possibly due to the presence of uninfected cells) were synthesized late in the infection.

The data obtained by the above RNA-DNA hybridization studies probably underestimated the extent of transcription of host DNA, since the sites specific for ribosomal and transfer RNA species would be quickly saturated. Figure 2 indicates that a considerable amount of RNA

sedimenting in the 4S, 16S and 23S regions of a sucrose gradient were synthesized 15 to 20 min after infection. Moreover, the accumulation of ^3H -uridine by infected cells (Fig. 1) also meas-

FIG. 1. Change in absorbance (A), uridine incorporation (B), and DNA synthesis (C) in *B. subtilis* cells infected with $\beta 22$. In all of the figures, (●) represents uninfected control and (○) culture infected with $\beta 22$ at time zero. Absorbance was measured at 600 nm with a 1-cm cuvette. Uridine incorporation was measured as counts/min of ^3H -uridine incorporated per 0.1 ml of culture. DNA was quantitated by the diphenylamine reaction as μg of DNA per 10 ml of culture.

TABLE 1. Comparison of quantities of host and phage DNA synthesized at various times after infection as measured by DNA-DNA hybridization

Time after infection cells were pulsed	Counts binding to host DNA	Counts binding to $\beta 22$ DNA
min	%	%
10-20	77	23
20-30	5	95
30-40	4	96

TABLE 2. Comparison of quantities of host and phage RNA synthesized at various times after infection as measured by RNA-DNA hybridization

Time after infection cells were pulsed	Counts binding to host DNA	Counts binding to $\beta 22$ DNA
min	%	%
5-10	95	5
10-15	38	62
15-20	55	45
20-25	33	67
25-30	8	92
30-	5	95

FIG. 2. Sedimentation pattern of RNA isolated from *B. subtilis* pulse-labeled 15 to 20 min after infection with $\beta 22$. One μCi of ^3H -uridine/ml was added to a culture 15 min after infection, 500 μg of unlabeled uridine per ml was added at 20 min, and the culture was harvested 10 min later. RNA, extracted as described in Materials and Methods, had a specific activity of 1,025 counts per min per μg . RNA (1.27 μg ; 0.2/ml) was layered on a 4.5-ml sucrose gradient and centrifuged at 49,000 rev/min in a Spinco model L centrifuge for 2.5 hr in an SW50 rotor. Solid line, optical density at 260 nm; broken line, radioactivity.

ures synthesis of stable RNA species and, as noted previously, radioactive uptake in phage-infected cultures closely paralleled that in uninfected controls for the first third of the latent period. In toto, these data suggest that the syn-

thesis of host-specific RNA probably exceeded the transcription of phage-specific RNA until 20 to 25 min after infection.

Changes in the population of phage-specific RNA during the latent period. The first two-thirds of the lytic cycle was marked by a rapid succession of changes in the populations of phage-specific RNA present in the infected cells. Figure 3 illustrates a series of hybridization-competition

FIG. 3. Competition between phage-specific RNA isolated from β 22-infected *B. subtilis* pulse-labeled with ^3H -uridine at various times in the latent period and unlabeled RNA from 15-min (●) and 40-min (○) infected cells. Conditions for binding were as described in Materials and Methods. Input of radioactive RNA and per cent of total acid-precipitable counts binding to β 22 DNA with zero competitor (100%) were: 5 to 10 min RNA = 16 μg of RNA (6,200 counts per min per μg) with 1% binding; 10 to 15 min RNA = 4.1 μg of RNA (2,020 counts per min per μg) and 15% binding; 15 to 20 min RNA = 7 μg of RNA (1,200 counts per min per μg) and 17% binding; 20 to 25 min RNA = 3.2 μg of RNA (1,165 counts per min per μg) and 45% binding; 25 to 30 min RNA = 7.8 μg of RNA (670 counts per min per μg) and 32% binding; and 35 to 40 min RNA = 6.0 μg of RNA (330 counts per min per μg) and 59% binding. β 22 DNA was present at a concentration of 15 μg per reaction.

curves in which the binding of RNA pulse-labeled at 5-min intervals from the 5th to the 40th min of the latent period was measured in the presence of increasing amounts of unlabeled RNA isolated from cells, 15 and 40 min after infection. The competition curves for 5 to 10- and 35 to 40-min pulse-labeled RNA demonstrate a dramatic difference between the RNA species present early and late in the infection. The same data reveal that the shift from early to late transcription was achieved by a continuum of small changes in the RNA population. As the infection progressed, there was a steady increase in the ability of RNA extracted 40 min after infection to compete in the binding of different pulse-labeled preparations. Conversely, the unlabeled RNA extracted 15 min after infection decreased in effectiveness as a competitor indicating that RNA species transcribed late in the infection contained fewer species homologous with the early competitor.

Figures 4A and 4B present data relevant to an examination of phage-specific RNA in the last third of the latent period. As expected from previous results with 40-min unlabeled RNA, Fig. 4A demonstrates that an RNA isolated 50 min after infection showed marked differences in its ability to compete in the binding of RNA from cells pulse-labeled at early and late times in the

FIG. 4. Competition between phage-specific RNA isolated from β 22-infected *B. subtilis* cells pulse-labeled with ^3H -uridine at various times in the latent period with unlabeled RNA from (A) 50-min and (B) 40-min infected cells; (●) 15 to 20 min pulse-labeled RNA; (○) 35 to 40 min pulse-labeled RNA; (△) 45 to 50 min pulse-labeled RNA. The amount of labeled RNA, the specific activity, and the per cent of acid precipitable radioactivity binding at zero competitor were: 15 to 20 min RNA = 7 μg of RNA (1,200 counts per min per μg) with 19% (Fig. A) and 18% (Fig. B) binding; 35 to 40 min pulse-labeled RNA = 6 μg of RNA (330 counts per min per μg) with 70% (Fig. A) and 55% (Fig. B) binding; 45 to 50 min pulse-labeled RNA = 11.3 μg of RNA (207 counts per min per μg) with 42% (Fig. A) and 36% (Fig. B) binding. β 22 DNA (10 μg) immobilized on membrane filters was present in each hybridization.

latent period. Special note, however, should be taken of the competition with RNA species obtained by pulse-labeling 35 to 40 and 45 to 50 min after infection. These preparations showed virtually no differences in the isotope dilution curves, indicating that the unlabeled RNA extracted 50 min after infection could not distinguish between these two pulse-labeled RNA species. Therefore, all species of RNA molecules transcribed 35 to 40 min after infection were still present in the competitor RNA isolated 10 min later in infection.

Figure 4B illustrates data on the reciprocal experiment in which the same pulse-labeled RNA species were incubated with increasing amounts of unlabeled competitor RNA obtained from cells in the 40th min of the latent period. In this study, the unlabeled RNA was actually slightly less effective as a competitor with the homologous 35- to 40-min pulse-labeled RNA than with the 45- to 50-min RNA. These results indicate that all the species of RNA synthesized 45 to 50 min after infection were present in the unlabeled RNA extracted 40 min after infection, i.e., no new species were synthesized. The slightly greater competition observed with the 45- to 50-min unlabeled RNA probably reflects a quantitative difference in the RNA populations present in the two radioactive preparations; it should be noted that the curves approach the same asymptote.

The results of these two experiments indicate that there were few if any qualitative changes in RNA synthesis in the last third of the latent period; a conclusion supported by previous experiments with rifampin (13).

Changes in populations of host-specific RNA after phage infection. We also examined the populations of RNA molecules transcribed from the host genome during those portions of the latent period when substantial host transcription was still taking place. Figure 5A demonstrates that host-specific RNA synthesized in the interval 15 to 20 min of the latent period was competed at least as well by RNA from uninfected cells as by RNA from 15-min infected cells. Apparently no new species of host-specific RNA were synthesized after infection.

Figure 5B demonstrates that RNA extracted from infected cells gradually lost its ability to compete RNA from uninfected cells. By 40 min after the addition of $\beta 22$, the cells apparently no longer contained significant quantities of host RNA. This latter observation suggests that not only did host transcription cease late in infection (as noted earlier), but the host-specific RNA already present was lost.

In vitro synthesis of RNA from nuclear fractions. The preceding in vivo studies demonstrated two

FIG. 5. RNA-DNA hybridization analysis of host-specific RNA in *B. subtilis* cells after infection with $\beta 22$. (A) Competition between the host-specific RNA isolated from cells pulsed 15 to 20 min after infection and unlabeled RNA from (●) 15-min infected cells and (□) uninfected cells. Radioactive RNA (14 μ g; 1,200 counts per min per μ g) was added to each vial and 15% of the acid-precipitable counts were bound with zero competitor (100%). (B) *B. subtilis* DNA (10.5 μ g) was added to each reaction. (B) Competition between pulse-labeled RNA from uninfected cells and unlabeled RNA from (□) uninfected cells, (●) 15-min infected cells, and (○) 40-min infected cells. Radioactive RNA (8.5 μ g; 1,370 counts per min per μ g) was added to each reaction, and 10.5% of the acid-precipitable counts were bound with zero competitor (100%). *B. subtilis* DNA (10.5 μ g) was added to each reaction. Conditions for RNA-DNA hybridizations were as described in Materials and Methods.

clearly defined parameters in the transcription of RNA in $\beta 22$ infected cells: first, a series of alterations in the proportions of RNA synthesized from the host and phage genomes, and second, a dynamic population of phage-specific RNA whose profile rapidly changed through the first two-thirds of the latent period.

To investigate whether these changes could be detected in RNA synthesized in vitro, studies were made with nucleoprotein complexes ("nuclear fractions") isolated from $\beta 22$ -infected *B. subtilis*. As shown previously (16), such nuclear fractions contain considerable endogenous polymerase activity and, if placed in an appropriate reaction mixture containing the four nucleoside triphosphates, synthesized RNA in vitro. The RNA can then be isolated and subjected to the same analysis as RNA from infected cells. Details of the procedure are given above. Table 3 establishes that the ratio of host to phage RNA transcribed in vitro by nuclear fractions prepared from cells harvested at different times after $\beta 22$ infection was almost precisely that measured in infected cells pulsed at equivalent times.

Figures 6A and 6B present competition-hybridization curves of RNA synthesized by nuclear fractions isolated from cells 11 and 30 min after infection. Figure 6A indicates that RNA synthesized by an "11-min nuclear fraction" was

TABLE 3. Comparison of hybridization properties of *in vivo* pulsed RNA and RNA synthesized *in vitro* from nuclear fractions

Time of pulse	Counts binding to $\beta 22$ DNA	Time after infection nuclear fraction was isolated	Counts binding to $\beta 22$ DNA
min	%	min	%
5-10	5	5	11
10-15	65	11	65
15-20	45	16	50
25-30	93	30	95

FIG. 6. RNA-DNA hybridization analysis of phage-specific RNA synthesized *in vitro* by the endogenous RNA polymerase activity of nuclear fractions from $\beta 22$ infected cells. (A) Competition between unlabeled RNA isolated from cells 15 min (●) and 40 min (○) after $\beta 22$ infection and radioactive RNA synthesized *in vitro* by the endogenous polymerase activity of a nuclear fraction from cells harvested in the 11th min of the latent period. Radioactive RNA (10.8 μ g; 175 counts per min per μ g) was added to each vial, and 33% of the acid-precipitable counts bound in the absence of competitor. $\beta 22$ DNA (21 μ g) was added to each reaction. (B) Radioactive RNA synthesized *in vitro* by a nuclear fraction from cells harvested in the 30th min of the latent period competed with unlabeled RNA from 11-min (□) and 40-min (○) infected *B. subtilis*. In the third curve (△), increasing quantities of 40-min unlabeled RNA were added to a constant amount (0.76 mg) of 11-min RNA. Radioactive RNA (9.2 μ g; 180 counts per min per μ g) was added to each vial, and 26% of the acid-precipitable counts bound with zero competitor. $\beta 22$ DNA (12.2 μ g) was added to each reaction.

predominantly composed of early species, i.e., unlabeled RNA isolated from cells 15 min after infection was a better competitor than unlabeled RNA extracted from cells in the 40th min of the latent period. Figure 6B, showing competition curves obtained with labeled RNA synthesized *in vitro* by a nuclear fraction extracted 30 min after infection, demonstrates that the unlabeled 40-min RNA was a more effective competitor than 11-min RNA, indicating that the late sequences were being transcribed. Furthermore,

addition of increasing quantities of unlabeled 40-min RNA, together with a saturating amount of 11-min RNA, resulted in a decrease in binding approximating that obtained with 40-min RNA alone.

Together, these results indicate that *in vitro* transcription from the nuclear fraction closely resembled that occurring in whole cells.

Transcription of purified DNA *in vitro* and studies on the mechanism of control of transcription. Three hundred-fold purified DNA-dependent RNA polymerase of *B. subtilis* was used in a series of studies on possible mechanisms involved in determining the ratios of phage- and host-specific RNA species produced during the latent period. One mechanism which has been suggested for the shut-off of host RNA synthesis in T4-infected *E. coli* is based on differences in the apparent K_m obtained *in vitro* with T4 and *E. coli* DNA (27). According to this hypothesis, a lower K_m value for T4 DNA results in a preferential binding of the host polymerase to the virus DNA followed by a rapid reduction in the amount of host DNA transcribed.

In light of these experiments, we analyzed the ratios of RNA transcribed by *B. subtilis* polymerase *in vitro* when 1:1 mixtures of several DNA species were used as templates (Table 4). Our results with mixtures of $\beta 22$ and host DNA seemed to indicate that the polymerase did preferentially bind to phage DNA and the product RNA was 86% rather than 50% phage. However, it seemed unlikely that such a slight preference could account for the results *in vivo*, when a small input of phage DNA accounted for 50 to 90% of the RNA synthesized. Moreover, we have noted that the DNA isolated from whole cells is usually inferior in templating capacity to that obtained from purified phage, and it is doubtful that the two DNA species can fairly be compared *in vitro* (Hemphill and Whiteley, unpublished observations).

TABLE 4. RNA-DNA hybridization analysis of RNA synthesized from 1:1 mixtures of various DNA species^a

Mixture of DNA used in the <i>in vitro</i> transcription	In <i>in vitro</i> -synthesized RNA binding to $\beta 22$ DNA
	%
$\beta 22$ / <i>Bacillus subtilis</i>	84
$\beta 22$ /SPO2.....	56
$\beta 22$ /SP82.....	38

^a Molecular weights as determined by sedimentation at an alkaline pH (26) for the phage DNA species were 2.88×10^7 or greater; the molecular weight of *B. subtilis* DNA was 2.14×10^8 .

To circumvent this problem, we took advantage of the observation that $\beta 22$ was able to suppress the in vivo transcription of either phage SPO2 or SP82, even if $\beta 22$ was added 5 min after infection with one of the latter phages (P. E. Kolenbrander, Hemphill, and Whiteley, *Bacteriol. Proc.*, p. 175, 1970; Kolenbrander, Hemphill, and Whiteley, *unpublished observations*). If such template preference exists, it should be possible to demonstrate it between appropriate phage DNA species as well as between phage and host DNA. Preferential synthesis of $\beta 22$ -specific RNA did not occur in vitro when 1:1 mixtures of $\beta 22$ and SPO2 DNA species or $\beta 22$ and SP82 DNA species were used as templates (Table 4). These results suggest that the RNA polymerase isolated from uninfected *B. subtilis* did not have a markedly greater affinity for $\beta 22$ DNA. This does not, of course, preclude the possibility that the polymerase may be altered during infection and that the altered polymerase may transcribe $\beta 22$ DNA preferentially.

Preferential transcription of $\beta 22$ DNA could be brought about by degradation or modification of the *B. subtilis* DNA so that it cannot serve as an effective template. Although it is difficult to exclude all possible in vivo permutations of this hypothesis, several lines of evidence indicate that in other phage infections, host DNA does not undergo extensive degradation (18, 22). The following experiment suggests that *B. subtilis* DNA also does not undergo marked alterations after phage infection.

A nuclear fraction isolated from cells 15 min after the addition of $\beta 22$ was divided into two samples. One sample was used to synthesize RNA in vitro by using the endogenous polymerase activity; 50% of the produced RNA annealed to $\beta 22$ DNA and 50% to *B. subtilis* DNA. The second sample was heated to 65°C for 15 min to destroy the endogenous polymerase activity and was then used as a template for exogenously added polymerase. An equal amount of RNA was synthesized, but only 15% of this RNA annealed to $\beta 22$ DNA and 85% annealed to the host DNA. This percentage of phage-specific RNA was close to the amount of phage DNA calculated to be present on the basis of input of $\beta 22$ phage.

The time interval beyond 25 min after infection was of particular interest because virtually all RNA transcription in vivo and in nuclear fractions was phage-specific. However, experiments with nuclear fractions or with DNA isolated from this stage of the infections were complicated because, as shown in Fig. 1, large quantities of phage DNA have already been synthesized. Simply on the basis of the quantity of DNA

present, phage transcription should be favored even if the host DNA was unaltered. However, DNA isolated from cells as late as 30 min after infection, at a time when the cells were synthesizing 90 to 95% phage-specific RNA, yielded 50% phage-specific and 50% host-specific RNA when incubated in an in vitro reaction mixture with *B. subtilis* RNA polymerase.

Collectively, these experiments suggest that large quantities of host DNA remained in the cells late into the infection and that this DNA was not altered in a manner that prevented its transcription in vitro.

Populations of phage-specific RNA synthesized in vitro from purified $\beta 22$ DNA. In light of the well-documented evidence that the in vitro synthesis of RNA by *E. coli* RNA polymerase incubated with T4 DNA yields "early" messenger RNA (10), similar experiments were performed to characterize the RNA transcribed in vitro by *B. subtilis* polymerase from $\beta 22$ DNA. This RNA was asymmetric, bound specifically to $\beta 22$ DNA in hybridization experiments, and (Fig. 7) the in vitro RNA was more homologous to 15-min than to 40-min RNA. As in T4 infections, control over the transcription of the "early" messenger RNA was apparently an intrinsic property of the interaction of RNA polymerase with phage DNA.

FIG. 7. Competition between radioactive RNA synthesized in vitro using $\beta 22$ DNA and 300-fold purified *B. subtilis* RNA polymerase and unlabeled RNA from *B. subtilis* cells infected 15 min ($\circ$) and 40 min ($\bullet$) after infection. The third curve ($\triangle$) shows competition achieved with a 1:1 mixture of the 15- and 40-min unlabeled RNA species. Conditions for RNA/DNA hybridization were as described in Materials and Methods. Radioactive RNA (1.02 μ g; 2,500 counts per min per μ g) was added to each vial and 52% of the trichloroacetic acid-precipitable counts bound with zero competitor. $\beta 22$ DNA (10 μ g) was added to each vial.

The fact that the combinations of 15- and 40-min RNA did not compete better than 15-min RNA alone emphasizes, first, that no detectable late sequences were transcribed at all and, second, even the small amount of competition achieved with 40-min RNA alone was due to RNA sequences transcribed both at 15- and 40-min after infection. The existence of such RNA species in vivo may be inferred from Fig. 3.

DISCUSSION

The present study with virulent bacteriophage $\beta 22$, in common with similar investigations with phages 2C and SP82 (18; S. Neubart and J. Marmur, *Bacteriol. Proc.*, p. 184, 1970), indicates that the sequence of events after viral infection of *B. subtilis* is different from those observed in the infection of *E. coli* with T2 and T4. In the latter, host macromolecular syntheses are totally suppressed within a few minutes of the addition of phage (7, 30); in $\beta 22$ infections, however, host RNA and DNA synthesis continued well into the latent period. Final suppression of host DNA replication occurred about 20 min after the addition of $\beta 22$, whereas RNA transcription was perturbed in two phases. First, there was a rapid change in the ratio of hybridizable RNA (largely unstable RNA species) transcribed so that by 10 to 15 min after infection about half of this RNA was phage-specific. At this time, however, only a slight decrease in the rate of synthesis of stable host RNA species was observed. Second, at the midpoint of the latent period, transcription of virtually all host RNA species was suppressed.

The ratios of host and phage RNA transcribed in vivo at various times after the addition of $\beta 22$ were also synthesized in vitro by the endogenous polymerase activity of nuclear fractions isolated from infected cells at corresponding times in the latent period. Moreover, the species of phage-specific RNA synthesized in vitro were similar to those found in the infected cells. While other explanations are possible, we interpret these results to mean that the nucleoprotein complex contained RNA polymerase molecules bound to DNA at the sites which they were transcribing at the time the infected cells were harvested. It appears likely, therefore, that part of the RNA synthesized in vitro represented the completion of chains initiated in vivo. Since the nuclear fractions appeared to retain the integrity of the in vivo transcription complex, these preparations were used to determine if the eventual cessation in the synthesis of host-specific RNA was due to modification or degradation of host DNA.

A number of investigators have noted that there

is little if any degradation of host DNA after infection with *B. subtilis* phages 2C and ϕ e (18, 22). In the present experiment, the in vitro templating properties of nuclear fractions (heat-treated to inactivate endogenous polymerase) and DNA isolated from cells at various times in the latent period were investigated. We found that the host DNA present in the infected cells retains the ability to serve as a template for RNA synthesis at least up to the 30th min of the latent period. Furthermore, under these conditions, the relative amounts of host and phage-specific RNA species synthesized were roughly proportional to the amounts of each DNA in the reaction mixture, suggesting that the polymerase does not have a significantly greater affinity for $\beta 22$ DNA. Other evidence that the *B. subtilis* RNA polymerase does not have a template preference was obtained from in vitro experiments by using polymerase and 1:1 mixtures of *B. subtilis*, various *B. subtilis* phage DNA species, or both. If the host polymerase does not bind preferentially to $\beta 22$ DNA in vivo and the host DNA is not degraded, possibly modification of the enzyme plays a role in the shut-off of host-specific RNA.

The kaleidoscopic pattern of phage-specific RNA observed during the course of the lytic cycle is one of the notable features of *B. subtilis* phage infections (9, 13). As the latent period progressed, de novo transcription of early $\beta 22$ RNA species ceased, and the copies of early RNA already synthesized were destroyed. Concurrently, new species of RNA (late RNA) were synthesized until, by 35 to 40 min after infection, the population of phage-specific RNA present was almost totally different from that observed during the first quarter of the latent period. This sequence of changes is different from that observed in T-even phage infections where many of the early RNA species apparently are still synthesized or at least are present in the cells late in the infection (12).

The mechanisms controlling the transcription of $\beta 22$ have not been investigated, but there are both similarities and differences from transcription of T4 in *E. coli*. The current status of T4 transcription may be summarized as follows. Three classes of RNA have been detected in T4 infection: pre-early, early, and late (4, 23, 29). Synthesis of pre-early RNA does not require protein synthesis and apparently involves unmodified host polymerase (4, 23). Correct transcription of some of these RNA classes may also involve a specific termination factor (20). Production of early RNA requires the synthesis of a specific T4 sigma factor which can function with host core enzyme or modified core enzyme

(29). Synthesis of late RNA requires phage DNA replication (1) and involves a modified core enzyme (31), but specific sigma factors have not yet been identified.

Similar studies of the role of sigma factors and core enzyme have not yet been made for the infection of *B. subtilis* by phages. The present investigation has shown that the *in vitro* synthesis of RNA by *B. subtilis* RNA polymerase yielded early RNA from a purified $\beta 22$ DNA template. This observation is probably sufficient to explain why the late sequences of phage-specific RNA were not synthesized early in the $\beta 22$ infection. Pene and Marmur (19) have demonstrated that, in phage 2C infections of *B. subtilis*, the expression of late functions required replication of phage DNA. Although similar experiments have not been made with $\beta 22$, the present studies show that the synthesis of late RNA was correlated in time with the beginning of phage DNA replication. The possibility that $\beta 22$ infection is accompanied by changes in sigma factors, core polymerase, or both has not been explored. Such changes may be required for the synthesis of several classes of RNA or for the synthesis of late RNA and may explain the failure of the polymerase late in infection to transcribe apparently unaltered host DNA. In addition, evidence has been presented by Wilson and Geiduschek (32) that a repressor is produced during the infection of *B. subtilis* by phage SPO1 which may function to shut off the synthesis of early RNA.

Finally, it should be noted that $\beta 22$ differs markedly in several respects from other well-characterized virulent *B. subtilis* phages such as ϕ e, 2C and SP82. Whereas the latter phages appear to be closely related on the basis of antigenicity, DNA homology, and the presence of hydroxymethyl uracil (3, 14, 21; and J. Marmur, Preliminary classification of *Bacillus subtilis* phages, Informational Exchange Group no. 7, Memo 317), $\beta 22$ DNA does not contain hydroxymethyl uracil (34) and has no homology with the DNA of SP82. Moreover, $\beta 22$, alone among all *B. subtilis* phages, is reported to be able to infect and lyse cells committed to sporulation (34). We have also observed that $\beta 22$ has the capacity to suppress the synthesis of SP82 in mixed infections, even if added in the 5th min of the SP82 latent period (Kolenbrander, Hemphill, and Whiteley, unpublished observations). This last result may mean that some $\beta 22$ functions are quite different from those noted in the hydroxymethyl uracil-containing phages.

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