In Vitro Synthesis of Late Bacteriophage ϕ 29 RNA

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A crude P-100 fraction prepared from *Bacillus subtilis* 21 min after infection with wild-type phage ϕ 29 supported the in vitro synthesis of late ϕ 29 RNA by added RNA polymerase. Synthesis of late RNA was also detected when purified ϕ 29 DNA was transcribed by RNA polymerase in the presence of an S-150 fraction obtained by lysis of ϕ 29-infected cells in the presence of 1 M NaCl. Late ϕ 29 RNA was not synthesized when either the P-100 or the S-150 fraction was prepared from cultures infected with ϕ 29 having a mutation in gene 4.

Bacillus subtilis phage ϕ 29 contains linear double-stranded DNA having a molecular weight of approximately 12×10^6 (3). The genome consists of 18 cistrons, and the functions and products of most of the cistrons have been identified (28, 33). Immediately after infection, the host RNA polymerase transcribes the early genes, which account for approximately 40% of the coding capacity of the genome. As shown in Fig. 1, the early RNA is produced from the "light strand" of ϕ 29 DNA [i.e., the strand of lower density in CsCl-polyuridylic-polyguanylic acid [poly(UG)] gradients [31]] and is synthesized throughout infection (24, 36, 39). The late class of RNA, which represents the remaining 60% of the genome, is transcribed from the "heavy strand" (31, 39). Although the onset of late transcription occurs at approximately the same time that viral replication begins (36), late transcription is independent of viral DNA replication (24). Synthesis of the late class of RNA requires the product of an early ϕ 29 gene, cistron 4 (4, 5, 39); the mechanism by which the gene 4 product regulates transcription is not known. The locations of the early and late genes have been mapped and correlated with a restriction map of the genome (13, 19, 20, 44).

Transcription of purified ϕ 29 DNA by the *B. subtilis* RNA polymerase in vitro yields early RNA (7, 19, 20). The number and location of early ϕ 29 promoters have been determined (8), and the DNA sequences of three early promoters have been compared (32). Synthesis of late mRNA in vitro has not been reported and the location of the late promoters has not been ascertained, although two polymerase-binding sites were found in the late region of the genome by electron microscopy of DNA-RNA polymerase complexes (39).

Regulation of viral transcription through modification of *B. subtilis* RNA polymerase has been studied extensively during the development of

bacteriophages SP82 (23, 42) and SPO1 (13). These phages code for several small polypeptides which bind to and alter the transcriptional specificity of the host RNA polymerase. Earlier investigations (17) of RNA polymerase extracted from ϕ 29-infected cells showed that approximately 10% of the host polymerase contained a 30,000-dalton (30K) peptide which was not found in polymerase from uninfected cells. The transcriptional specificity of the 30K-containing polymerase with respect to ϕ 29 DNA was not determined. It is known that at least the β subunit of the host RNA polymerase is required for viral transcription since synthesis of late mRNA is inhibited by the addition of rifampin (24, 37).

In the present paper, we report that late ϕ 29 RNA was synthesized in vitro by purified rifampin-resistant RNA polymerase from uninfected *B. subtilis* in the presence of crude P-100 fractions from cells infected with wild-type ϕ 29 and treated with rifampin to inhibit the endogenous polymerase. We also report that synthesis of late mRNA was observed when reaction mixtures containing purified ϕ 29 DNA and purified RNA polymerase were supplemented with a supernatant fraction (S-150) obtained by lysing ϕ 29-infected cells in the presence of a high salt concentration. In both instances, i.e., in the presence of either a P-100 or the high-salt S-150 fraction, late mRNA was not produced if the cells were infected with phage having a mutation in gene 4.

MATERIALS AND METHODS

Strains and growth of phage-infected *B. subtilis*. *B. subtilis* SR22, an asporogenous mutant of *B. subtilis* 168, obtained from J. Spizizen (Department of Microbiology, University of Arizona, Tucson), was used as the host for wild-type ϕ 29 and as the nonpermissive host for ϕ 29 *sus4*. The suppressor strain, *B. subtilis* Su⁺⁴⁴, and the *sus4*(56) mutant were obtained from B. E. Reilly (School of Dentistry, University of Min-

nesota, Minneapolis). *B. subtilis* OSB420, a rifampin-resistant mutant of *B. subtilis* 168, was obtained from L. Brown (Department of Microbiology, Oregon State University, Corvallis).

Phage-infected *B. subtilis* was grown in 750-ml to 10-liter batches of M medium (1% tryptone, 0.5% yeast extract, 1% NaCl). After sterilization, this medium was supplemented with 0.4 mM MnCl_2 , 5 mM MgSO_4 , and 0.1% glucose added from sterile stock solutions. Cultures were grown at 37°C to a concentration of 2×10^8 cells per ml and infected with 5 to 10 $\phi 29$ particles per cell. After 21 min, sodium azide was added to a 0.01 M final concentration, the temperature was reduced to 0 to 5°C by the addition of ice, and the cells were harvested by centrifugation, frozen in a dry ice-ethanol bath, and stored at -70°C. When infected cells were treated with rifampin before harvesting, the inhibitor was added at a final concentration of 10 $\mu\text{g}/\text{ml}$ at 18 min after infection, incubation was continued to 21 min, and the cells were collected as described above. Lysates of $\phi 29$ were prepared using *B. subtilis* SR22 for wild-type phage and *B. subtilis* Su⁴⁴ for the *sus4* mutant, using the M medium described above. Lysis occurred at 36 to 39 min, yielding approximately 10^{11} phage per ml.

Preparation of the P-100 fraction and the high-salt S-150 fraction. $\phi 29$ -infected cells which had been treated with rifampin and harvested at 21 min after infection as described above were used for the isolation of both fractions. Except when indicated otherwise, the following operations were carried out at 0°C. To obtain the P-100 fraction, frozen cells (ca. 0.2 g [wet weight]) were suspended in 4 ml of buffer A (0.05 M Tris-hydrochloride [pH 7.5], 0.1 M NaCl, 1 mM EDTA, 0.01 M 2-mercaptoethanol, 200 μg of phenylmethylsulfonyl fluoride per ml) supplemented with 30% glycerol, lysozyme was added (0.5 ml of a 5-mg/ml solution), and the suspension was incubated for 25 to 30 min at 10°C. The preparation was then centrifuged for 20 min at $100,000 \times g$ in a Beckman type 40 rotor at 4°C, the supernatant was removed carefully, and buffer A was added to the sediment to give a total volume of 1.5 ml. The thin, yellowish-brown sediment with a clear gelatinous layer above was resuspended by gentle homogenization with four or five strokes of a loosely fitted Dounce homogenizer and used immediately as a template for RNA synthesis. In some experiments, the preparations were centrifuged for 10 min at 10,000 rpm in a Sorvall centrifuge to remove any intact cells before high-speed centrifugation. In most experiments, this preliminary centrifugation was omitted, and if any intact cells were detected after the high-speed centrifugation (i.e., if a grey-white opaque center of any size could be seen in the yellowish sediment), the preparation was discarded. "Successful lysis" yielded P-100 fractions which had a negligible number of unlysed cells. Direct counts, by means of a Petroff-Hauser counter, showed that there were 2×10^6 to 5×10^5 cells per ml (mostly swollen and atypical in appearance) in our usual P-100 preparations and ca. 2×10^4 viable cells as determined by plate counts; the original 4 ml of suspension was calculated to have ca. 5×10^9 cells per ml.

To test for possible degradation of the DNA in the P-100 fraction, 0.5-ml amounts were diluted with 2 ml of proteinase K solution (50 $\mu\text{g}/\text{ml}$ in 0.01 M Tris-hydrochloride [pH 7.5]–0.01 M EDTA–0.5% sodium dode-

cyl sulfate [SDS]) and incubated for 2 h at 30°C. The samples were then extracted with phenol, dialyzed against 100 volumes of 0.1 M Tris-hydrochloride (pH 7.5)–1 mM EDTA for 24 h at 4°C, treated with 1 μg of RNase A per ml for 60 min at 22°C, and analyzed by electrophoresis through vertical agarose slab gels, using TBE buffer (0.089 M Tris base, 0.089 M boric acid, 0.0025 M EDTA).

A high-salt S-150 fraction was obtained by one of two methods. In the first, the procedure given above for isolation of the P-100 fraction was followed except that the treatment with lysozyme was reduced to 12 to 15 min at 10°C, NaCl was added from a 5 M stock solution to give a final concentration of 1 M, and the preparation was thoroughly mixed, cooled to 0°C, and centrifuged for 30 min at $150,000 \times g$ in a Beckman type 40 rotor at 4°C. The supernatant was removed and desalted by passage through a column of Sephadex G-25 equilibrated with buffer A, and the fraction having the highest absorbency at 280 nm ("high-salt S-150 fraction") was tested for its ability to stimulate late transcription from $\phi 29$ DNA. In the second method, two or more P-100 fractions isolated in the presence of 0.1 M NaCl were supplemented with 5 M NaCl to give a final concentration of 1 M, and the preparations were left on ice for 10 min, centrifuged, combined, and processed as described for the high-salt S-150 fraction. The second method also yielded preparations which stimulated the synthesis of late RNA, but they were considerably less effective and less stable than the high-salt S-150 fraction obtained by the first method.

Preparation of phage DNA. Phage were precipitated from 10-liter lysates by the addition of polyethylene glycol (43), dialyzed for 24 to 48 h against buffer B (0.01 M Tris-hydrochloride [pH 7.5], 0.1 M NaCl, 0.005 M MgCl_2), centrifuged through cesium chloride (31), and dialyzed for 12 to 18 h against buffer B containing EDTA and SDS at final concentrations of 0.01 M and 1%, respectively. After heating at 60°C for 2 min, an equal volume of proteinase K (100 $\mu\text{g}/\text{ml}$ in 0.01 M Tris-hydrochloride [pH 7.5]–0.01 M EDTA) was added, and the preparations were incubated for 4 to 6 h at 37°C and then extracted with phenol. Phage DNA was precipitated with ethanol, dissolved in 0.01 M Tris-hydrochloride (pH 7.5)–1 mM EDTA, and dialyzed against the latter solution for 24 h.

Digestion of DNA with restriction enzymes and electrophoresis. $\phi 29$ DNA was digested with *EcoRI* or *HindIII* obtained from New England Biolabs (Beverly, Mass.) according to methods described by the manufacturer. Fragments were separated by electrophoresis through a vertical 0.7 to 1.4% agarose slab gel (12 by 14 by 0.6 cm), as indicated, using TBE buffer, at 100 V. Staining of gels with ethidium bromide, visualization under UV light, and photography have been described previously (1).

Strand separation of *EcoRI* fragments of $\phi 29$ DNA. DNA was digested with *EcoRI* and complexed with poly(UG) by a modification of the method of Goldbach et al. (14). Specifically, 10 μg of digested DNA in 0.1 ml of 0.1 mM EDTA was mixed with 0.06 ml of partially hydrolyzed poly(UG) at a concentration of 1 mg/ml, and the mixture was then heated for 3 min at 100°C to denature the DNA and quickly cooled to 0°C. A critical step in this procedure was the preparation of partially hydrolyzed poly(UG). This was achieved by

mixing 1 volume of a 3-mg/ml solution of the polymer (Miles Laboratories, Inc., Elkhart, Ind.) with 0.1 volume of 0.5 M Na₂CO₃, incubating for 15 min at 67°C, and then cooling and adding 1 volume of 0.05 M HCl and 1 volume of 0.08 M Tris-hydrochloride (pH 7.7). After the addition of Ficoll and bromphenol blue, samples containing denatured fragments complexed with poly(UG) were subjected to electrophoresis on horizontal 0.45% agarose slab gels, using 0.04 M Tris-acetate (pH 7.7)–1 mM EDTA as the buffer (14). Electrophoresis was performed at 4°C at a constant current of 45 mA for 18 to 20 h, and the gels were stained with ethidium bromide.

In vitro synthesis of RNA. When preparations of the P-100 fraction were used as the template, reaction mixtures (total volume, 0.24 ml) contained 60 µl of the P-100 fraction, 10 to 20 µg of *B. subtilis* OSB420 RNA polymerase, 0.04 M Tris-hydrochloride (pH 7.9), 0.1 M NaCl, 0.015 M MgCl₂, 0.01 M 2-mercaptoethanol, 0.5 mg of bovine serum albumin per ml, and 10 µg of rifampin per ml. The preparations were maintained at 0°C and mixed thoroughly, and an aliquot of 0.1 ml was withdrawn and added to a 4-µl aliquot of a nucleotide solution containing 8 mM ATP, GTP, and CTP and 4 mM UTP plus 1 to 2 µCi of [α -³²P]UTP (30 to 50 mCi/mmol; New England Nuclear Corp., Boston, Mass.). The reaction mixtures were incubated for 10 min at 30°C or at the indicated temperature, and RNA was extracted for hybridization as described previously (41). When purified DNA was the template, the same procedure was used except that the P-100 fraction was replaced with 20 to 30 µg of ϕ 29 DNA per ml. Methods used to assay the activity of RNA polymerase have been described previously (1).

Labeling RNA in vivo. RNA was pulse-labeled at intervals during infection by the addition of 4 µCi of [³H]uridine (41.5 Ci/mmol; ICN, Irvine, Calif.) per ml and extracted according to previously described methods (41). Alternatively, phage-infected cultures were grown in a low-phosphate medium (1) and pulse-labeled with 100 µCi of ³²PO₄ (carrier-free; New England Nuclear) per ml.

Hybridization of [³²P]RNA to restriction fragments of ϕ 29 DNA. Fragments were transferred to nitrocellulose filters by the methods of Ketner and Kelly (21), and the nitrocellulose was cut into 3 to 4-mm-wide strips. Each strip was incubated with [³²P]RNA in 3 to 4 ml of buffer C (0.025 M Tris-hydrochloride [pH 7.5], 0.825 M NaCl) containing 0.1% SDS in a sealed polyethylene bag for 18 to 20 h at 67°C. The strips were washed in buffer C for 20 min at 60°C, treated with 10 µg of RNase A per ml in 0.3 M NaCl–0.03 M sodium citrate for 20 min at 37°C, and washed at 60°C for a total of 60 min with three changes of buffer C containing 0.1% SDS, 0.01 M sodium PP_i, 0.004 M disodium phosphate, and 1 to 2 µg of ATP per ml. The filters were dried and exposed to Kodak X-Omat film with an intensifying screen at –75°C.

RNA-DNA competition-hybridization. Anti-mRNAs may be synthesized by in vitro transcription of ϕ 29 DNA (19). These RNAs may self-anneal or hybridize with added competitor RNAs or both, thus leading to spurious results in competition-hybridization reactions. To minimize these effects, the competition-hybridization procedure (22) was modified to permit presaturation with competitor RNAs (26). In brief, nitrocellulose filters (5-mm diameter) containing 2 to 3

µg of denatured ϕ 29 DNA were incubated with 0 to 0.75 mg of unlabeled competitor RNA in 0.3 ml of hybridization buffer (0.01 M Tris-hydrochloride [pH 7.5], 0.33 M NaCl, 0.1% SDS) at 67°C for 16 to 20 h. The filters were washed to remove unhybridized RNA, and labeled RNA was added and incubated with the filters as described above. After washing, the filters were treated for 60 min at 20°C with a mixture of RNases A and T₁, washed, and dried, and the radioactivity was determined in a scintillation counter, using a toluene-based scintillant.

Miscellaneous methods. RNA polymerase was purified as described by Achberger and Whiteley (1). The procedure of Roberts (34) was used to purify rho factor from *Escherichia coli* MRE600; rho activity was assayed according to Lowery-Goldhammer and Richardson (25). Protein concentrations were determined by the method of Bradford (6), using gamma globulin as the standard.

RESULTS

Transcriptional map. As indicated in Fig. 1, early RNA is transcribed primarily from the *Eco*RI A and C fragments. One early transcript extends slightly into the *Eco*RI D fragment, and hybridization to fragment D has been reported by some investigators (20, 39) but not by others (19). RNA extracted from ϕ 29-infected cells late in infection hybridizes to *Eco*RI fragments B, D, and E in addition to fragments A and C. Symmetrical transcription of both the B and D fragments can occur (39). The in vitro transcription of ϕ 29 DNA by *B. subtilis* RNA polymerase yields several RNA species which hybridize predominantly to the *Eco*RI A and C fragments (19, 20, 39); in vitro transcription may also yield significant amounts of anti-late mRNA (19).

Strand separation. To study the in vitro synthesis of late ϕ 29 mRNA, we have determined the strand specificity of transcripts produced from a region of the genome containing late genes—i.e., the *Eco*RI A and B fragments. When *Eco*RI restriction fragments of ϕ 29 DNA were denatured, complexed with poly(UG), and subjected to electrophoresis, the complementary strands of the two largest fragments each yielded two rather diffuse, but separable, bands (Fig. 2, lanes a to d). The complementary strands of undigested ϕ 29 DNA and the strands of the *Eco*RI C fragment were not separable by this method, and the D and E fragments usually comigrated as a single large band.

Figure 3 compares autoradiograms obtained by hybridizing RNAs synthesized during infection to *Eco*RI and *Hind*III fragments and to strand-separated *Eco*RI fragments after electrophoresis and transfer of the fragments to nitrocellulose membrane filters. Control experiments (Fig. 3, lanes a, b, g, and h) indicated that early and late RNAs hybridized to the *Eco*RI and *Hind*III fragments in accordance with the map shown in Fig. 1. Early RNA hybridized to the

FIG. 1. Genetic, physical, and transcriptional maps of the $\phi 29$ genome.

EcoRI C fragment and to the slow-migrating band of the A fragment (Fig. 3, lane d). The latter has been designated "AL," since early RNA is transcribed from the light strand of $\phi 29$ DNA (31). The parallel experiment with late RNA (Fig. 3, lane e) demonstrated that in addition to AL, the fast-migrating band of the A fragment and the slow-migrating band of the B fragment were transcribed at late times after infection. These bands were designated "AH" and "BH," respectively, since late RNA is complementary to the heavy strand of $\phi 29$ DNA. These findings and strand assignments agree with those of Sogo et al. (39), who reported that the fast-moving band of all *EcoRI* fragments of $\phi 29$ DNA, except the A fragment, corresponded to the light strand and the slow-moving band corresponded to the heavy strand. Longer exposures of the autoradiograms presented in Fig. 3 also showed hybridization of late mRNA to the *EcoRI* D/E fragment region (data not shown). Occasionally, RNA extracted from cultures early in infection showed a small amount of hybridization in the BH region (faintly visible in lane d of Fig. 3 and in lane b of Fig. 5), but no hybridization was found to the *EcoRI* B fragment if the strands of the DNA were not complexed with poly(UG) and separated before transfer to nitrocellulose (Fig. 3, lane a). Because of this occasional spurious hybridization of early in vivo RNA to BH, hybridization to both AH and BH was considered to be the most reliable indicator of the presence of late RNA. The decreased hybridization of late RNA relative to the hybridization of early RNA to the *EcoRI* C fragment (Fig. 3, lanes a, b, d, and e) and to the *HindIII* C fragment (Fig. 3, lanes g and h) will be discussed separately (R. D. Holder and H. R. Whiteley, manuscript in preparation).

In vitro synthesis of late RNA. P-100 fractions were prepared from $\phi 29$ -infected cultures 21 min after infection as described above and used as

templates for the synthesis of RNA. Since DNA replication begins at 8 to 10 min after infection, the presence of large amounts of $\phi 29$ DNA was expected. Electrophoresis of complexes after digestion with proteinase K showed two bands on agarose gels. One band corresponded in molecular weight to mature $\phi 29$ DNA, and the other, having an approximate molecular weight of 30×10^6 , probably contained sheared *B. subtilis* DNA. The samples also contained some high-molecular-weight DNA which did not enter the gel. There was no evidence of degradation of either the host or phage DNA during the incubation of the complexes with nucleotides and added polymerase as judged by electrophoresis. The

FIG. 2. Strand separation of *EcoRI* fragments of $\phi 29$ DNA by gel electrophoresis (photograph of a 0.45% agarose gel stained with ethidium bromide). (a) *EcoRI*-digested $\phi 29$ DNA; (b) heat-denatured *EcoRI*-digested $\phi 29$ DNA; (c) *EcoRI* fragments of DNA which were heat denatured and complexed with poly(UG) before electrophoresis as described in the text; (d) purified *EcoRI* A fragment denatured and complexed with poly(UG) before electrophoresis; (e) purified *EcoRI* B fragment denatured and complexed with poly(UG) before electrophoresis.

FIG. 3. Autoradiograms showing the hybridization of early and late in vivo [^{32}P]RNAs to restriction fragments of $\phi 29$ DNA. $\phi 29$ -infected cells were labeled with $^{32}\text{PO}_4$, and RNAs were extracted and hybridized to *Eco*RI- and *Hind*III-digested $\phi 29$ DNA as described in the text. (a) Early RNA (pulse-labeled for 2 to 7 min) hybridized to *Eco*RI fragments; (b) late RNA (pulse-labeled for 25 to 30 min) hybridized to *Eco*RI fragments; (c) photograph of ethidium bromide-stained 0.7% agarose gel of *Eco*RI fragments; (d) early RNA hybridized to separated strands of *Eco*RI fragments; (e) late RNA hybridized to separated strands of *Eco*RI fragments; (f) photograph of ethidium bromide-stained 0.45% agarose gel of separated strands of *Eco*RI fragments; (g) early RNA hybridized to *Hind*III fragments; (h) late RNA hybridized to *Hind*III fragments; (i) photograph of ethidium bromide-stained 1.2% agarose gel of *Hind*III fragments.

P-100 fractions also contained many proteins, including RNA polymerase and probably ribosomes, since ribosomes were detected in earlier experiments with similar preparations (30). Sedimentation of the P-100 preparations in 10 to 30% glycerol gradients showed that RNA polymerase and additional peptides cosedimented with DNA, suggesting that the preparations contained nucleoprotein complexes.

Because the P-100 fractions contained endogenous polymerase, transcription was performed in the presence of rifampin by using rifampin-resistant polymerase isolated from uninfected *B. subtilis* OSB420. To ensure that RNA synthesis by preparations containing the P-100 fraction resulted from de novo initiation by the added rifampin-resistant polymerase and did not result merely from elongation of transcripts initiated in vivo, cultures were treated with rifampin for 3 min before harvesting the cells. The data in Fig. 4 show that this treatment totally inhibited RNA synthesis by the endogenous polymerase present in the P-100 fractions. It should be noted that

all in vitro transcriptions were performed using RNA polymerase preparations containing the delta peptide (2).

Two types of P-100 fractions were compared: those obtained from cells infected with wild-type $\phi 29$ and those prepared from nonpermissive cells infected with $\phi 29$ carrying a nonsense mutation in cistron 4. Mutations in this cistron have previously been shown to prevent expression of late genes in vivo (4, 5, 39). RNAs synthesized from such complexes by exogenously added rifampin-resistant polymerase were analyzed by hybridization to blots of strand-separated *Eco*RI fragments of $\phi 29$ DNA as in Fig. 3. Hybridization to fragments AH and BH was defined as indicating the synthesis of late mRNA.

Transcription of the P-100 fraction derived from cells infected with wild-type $\phi 29$ yielded RNAs which hybridized strongly to fragments AL, AH, and BH and weakly to fragment C (Fig. 5, lane a). The same fragments were hybridized by RNAs synthesized in vivo 25 to 30

FIG. 4. RNA synthesis from the P-100 fraction in the presence and absence of added RNA polymerase. Reaction mixtures contained 150 μ l of P-100 fraction, 10 g of rifampin per ml, 50 μ g of rifampin-resistant RNA polymerase ($\circ$ or Δ) or no added polymerase ($\bullet$ or $\blacktriangle$), buffer, and nucleotides including [^{32} P]UTP in a total volume of 0.75. The reactions were incubated at 30°C, and at the indicated times, 50- μ l samples were withdrawn and the incorporation of radioactivity into trichloroacetic acid-insoluble material was measured. ($\circ$, $\bullet$) P-100 fraction isolated from $\phi 29$ -infected cells which had not been treated with rifampin before harvesting; (Δ , $\blacktriangle$) P-100 fraction isolated from $\phi 29$ -infected cells which had been treated with rifampin before harvesting.

min after infection (Fig. 3, lane e) except that there was a relatively weaker hybridization to AL in the latter experiments. Transcription of P-100 obtained from cells infected with the gene 4 mutant did not yield late RNA (Fig. 5, lane b). Hybridization was found predominantly to fragment AL and more weakly to fragment BH. Since hybridization to fragment AH could not be detected and there was no hybridization to the B fragment if the poly(UG) complexing step was omitted (data not shown), the hybridization to BH was considered to be an artifact. The absence of hybridization to the *Eco*RI C fragment in lane b of Fig. 5 will be discussed elsewhere (Holder and Whiteley, in preparation). For comparison, lane c of Fig. 5 presents the results of a separate experiment in which purified $\phi 29$ DNA was used as the template in place of the P-100 fraction. Late RNA was not synthesized from this template, as evidenced by the absence of hybridization to AH and BH. Hybridization was observed to fragments AL, C, and D/E, indicating the synthesis of early RNA (19, 20). Hybridization was also found to fragment BL, indicat-

ing the synthesis of anti-late RNA. This observation is discussed in a later section. Parallel experiments in which the S-100 fraction obtained from cells infected with wild-type $\phi 29$ was used for the synthesis of RNA by rifampin-resistant polymerase yielded only trace amounts of RNA, probably because the S-100 fraction contained relatively low concentrations of DNA. The hybridization of this RNA was not investigated.

Confirmation that the P-100 fraction from cells infected with wild-type $\phi 29$ supported the synthesis of late RNA in vitro came from RNA-DNA competition-hybridization experiments (Fig. 6). The differences in hybridization in the presence of early and late competitor RNAs were similar to those obtained with pulse-labeled in vivo late RNA. In contrast, the data obtained with RNAs produced from purified $\phi 29$ DNA indicate that only early RNA was synthesized; the ability of late RNA to compete in this hybridization was due to the fact that early RNA is produced throughout infection (7, 24, 37). Hybridization of RNA synthesized from P-100 to saturating amounts of $\phi 29$ DNA indicated that approximately 25% of the transcripts were $\phi 29$ specific (data not shown), and the relative intensities of the bands in lane a of Fig. 5 and

FIG. 5. Autoradiogram showing the hybridization of separated strands of *Eco*RI fragments of $\phi 29$ DNA with RNAs synthesized in vitro. RNA was synthesized as described in the text during a 10-min incubation at 30°C, using rifampin-resistant RNA polymerase, rifampin, and: (a) a P-100 fraction isolated from cells infected with wild-type $\phi 29$, (b) a P-100 fraction isolated from cells infected with a gene 4 mutant of $\phi 29$, and (c) purified $\phi 29$ DNA. Note that two sets of filters were used in this experiment: one set for lanes a and b and the other set for lane c.

FIG. 6. Competition-hybridization experiments in which RNAs synthesized in vivo and in vitro were used. (A) RNA extracted from $\phi 29$ -infected cells labeled with $^{32}\text{PO}_4$ at 2 to 7 min after infection and competed with 7-min unlabeled RNA ($\bullet$) or 30-min unlabeled RNA ($\circ$); RNA extracted from $\phi 29$ -infected cells labeled with $^{32}\text{PO}_4$ at 25 to 30 min after infection and competed with 7-min unlabeled RNA ($\blacktriangle$) or 30-min unlabeled RNA ($\triangle$). (B) $[^{32}\text{P}]$ RNA synthesized in vitro as described in the text from purified $\phi 29$ DNA and competed with 7-min unlabeled RNA ($\bullet$) or 30-min unlabeled RNA ($\circ$); $[^{32}\text{P}]$ RNA synthesized in vitro as described in the text from a P-100 fraction isolated from $\phi 29$ -infected cells and competed with 7-min unlabeled RNA ($\blacktriangle$) or 30-min unlabeled RNA ($\triangle$).

subsequent figures indicate that significant amounts of late RNA were synthesized.

Effects of temperature, dilution, and NaCl. The capacity of the P-100 fraction isolated from cells infected with wild-type $\phi 29$ to support the in vitro synthesis of late RNA was highly dependent upon the temperature at which transcription was performed. Figure 7 presents data on the hybridization between strand-separated *Eco*RI fragments of $\phi 29$ DNA and RNAs syn-

thesized by preparations containing P-100 preparations at 20, 25, 30, 37, and 42°C. Two overall trends were apparent from these autoradiograms. First, late RNA was not synthesized at the two higher temperatures as judged by hybridization to *Eco*RI fragments AH and BH (Fig. 7, lanes d and e), and synthesis of anti-late RNA was observed (hybridization to fragment BL). Temperature shift experiments (data not shown) indicated that the factor or factors required for late transcription were irreversibly inactivated in vitro at temperatures higher than 30°C. The second trend evident in Fig. 7 was a progressive inhibition in the synthesis of early RNA as the temperature was decreased. RNA complementary to the *Eco*RI C fragment was not synthesized at 25°C (Fig. 7, lane b), and at 20°C there was little detectable transcription from the *Eco*RI A fragment (Fig. 7, lane a). Thus, at lower temperatures, virtually all transcription by reaction mixtures containing the P-100 fraction came from late sequences. Since these assays were performed with crude preparations, it is not known whether the apparent temperature lability of the factor(s) is due to degradation by proteases or to some other mechanism.

The efficient synthesis of late RNA in vitro required relatively high concentrations of the P-100 fraction (50 to 100 μl per 240- μl reaction mixture; the reaction mixtures also contained 0.5 mg of bovine serum albumin per ml). When low concentrations were used (30 μl per 240- μl reaction mixture), there was little or no hybridization to AH and BH, and hybridization to BL was detected, indicating the synthesis of anti-

FIG. 7. Autoradiogram showing the hybridization of separated strands of *Eco*RI fragments of $\phi 29$ DNA with RNAs synthesized at different temperatures from a P-100 fraction. $[^{32}\text{P}]$ RNAs were extracted from reaction mixtures containing rifampin-resistant polymerase and a P-100 fraction isolated from $\phi 29$ -infected cells after 10 min of incubation at: (a) 20, (b) 25, (c) 30, (d) 37, and (e) 42°C.

late RNA. It may be speculated that the gene 4 product was present in limiting amounts or that it was readily inactivated by dilution or both.

The addition of 0.05 to 0.15 M NaCl had no effect on the synthesis of late RNA, although some inhibition was noted at a concentration of 0.2 M NaCl. This finding is of interest because of the effect of NaCl in limiting the formation of nonspecific complexes between RNA polymerase and DNA. Detailed studies on the binding of *B. subtilis* RNA polymerase to phage SP82 DNA (1) have shown that in the presence of 0.1 to 0.2 M NaCl, *B. subtilis* RNA polymerase preparations which contain the delta peptide form complexes only with strong early phage promoters—i.e., there is little or no nonspecific complex formation. The formation of complexes by SP82-modified forms of RNA polymerase with middle and late SP82 promoters was even more sensitive to increasing concentrations of NaCl than the interaction of unmodified polymerase with early SP82 promoters. If the interaction of polymerase with late ϕ 29 promoters resembles the interaction of this enzyme with SP82 promoters, then the observation that late ϕ 29 RNA was produced in the presence of 0.15 M NaCl suggests that this synthesis did not result from a nonspecific interaction between polymerase and ϕ 29 DNA.

Location of the promoter(s) for late transcription. As noted above, the location and number of late promoters on ϕ 29 DNA have not been determined. To localize the site(s) at which late transcription was initiated in vitro in the presence of the P-100 fraction, transcription was terminated after increasing intervals of incubation and the RNAs were then hybridized to blots of *Hind*III restriction fragments of ϕ 29 DNA (Fig. 8, lanes a to f). Lanes g and h of Fig. 3 show, respectively, the hybridization of RNAs pulse-labeled at early and late times of infection.

RNAs synthesized in vivo 2 to 8 min after infection hybridized to *Hind*III fragments B, C, G, H, and K, whereas RNAs produce 25 to 30 min after infection hybridized to the same fragments as early RNA plus *Hind*III fragments A, D, E, F, I, and J (Fig. 3, lane g). Hybridization to the two smallest fragments, *Hind*III-L and -M, was not detected in any experiments; a similar lack of hybridization to small restriction fragments was noted originally by Southern (40).

RNAs synthesized in vitro during the first 30 s of incubation in the presence of the P-100 fraction were complementary to the *Hind*III B, C, H, and I fragments (Fig. 8, lane a). With increasing times of incubation (Fig. 8, lanes b to f), additional regions of the genome were transcribed in a sequence which is consistent with the transcriptional map shown in Fig. 1, if one allows for the fact that hybridization to frag-

FIG. 8. Autoradiogram showing the hybridization of *Hind*III fragments of ϕ 29 DNA with RNAs synthesized after increasing intervals of incubation from a P-100 fraction. [^{32} P]RNAs were extracted from reaction mixtures containing rifampin-resistant polymerase and a P-100 fraction isolated from ϕ 29-infected cells and incubated at 30°C for: (a) 30 s, (b) 60 s, (c) 90 s, (d) 2 min, (e) 4 min, and (f) 8 min.

ments L and M was not detected. The results of the experiment presented in Fig. 8 suggest that early RNA synthesis in vitro is initiated on the *Hind*III B, C, H, and I fragments and proceeds from right to left on the physical map. In vitro transcription of the late regions was evidently initiated in the region near or within the *Hind*III-H-I region, and the sequential appearance of RNA complementary to fragments E, J (faintly hybridized), D, A, and F indicates that the direction of transcription was from left to right. Although the *Hind*III I fragment was the first exclusively late fragment to which hybridization was detected, the exact position of the late promoter could not be determined since the H and I fragments are separated by the M fragment which did not hybridize. It is evident, however, from Fig. 8 that transcription comparable to that of the H-I region was not initiated in the middle of the late region or at the B1 and B2 sites (39). The latter two sites, at positions 59.4 and 79.3 from the left end of the genome, were identified as binding sites for *B. subtilis* RNA polymerase by electron microscopic examination of polymerase-DNA complexes.

Elution of the factor(s) from the P-100 fraction. Treatment of the P-100 fraction with 1 M NaCl, followed by centrifugation or lysis of phage-

infected cells in the presence of 1 M NaCl, apparently released the factor(s) required for transcription of late sequences from the material sedimented by high-speed centrifugation. Lanes a to e of Fig. 9 indicate that the addition of increasing amounts of the high-salt S-150 fraction (after decreasing the concentration of NaCl to 0.1 M) to transcription reactions in which purified $\phi 29$ DNA and purified RNA polymerase were employed not only stimulated the synthesis of late RNA (hybridization to AH and BH) but also progressively reduced the synthesis of early RNA (hybridization to AL and C) and anti-late RNA (hybridization to BL). Presumably, as discussed below, RNAs complementary to the *EcoRI* D and E fragments and to BL were produced by read through from an initiation site in *EcoRI*-C. The P-150 fraction resulting from the high-salt treatment was significantly less effective in stimulating late transcription than was a P-100 fraction (data not shown). When the same experiments were performed using cells infected with $\phi 29$ carrying a nonsense mutation in cistron 4, the high-salt S-150 preparations (Fig. 9, lane g) did not stimulate transcription of late sequences in vitro, and synthesis of RNAs complementary to the C and D/E fragments was not detected. The lack of synthesis from the C fragment in this experiment agrees with results

FIG. 9. Autoradiogram showing the hybridization of separated strands of *EcoRI* fragments of $\phi 29$ DNA with RNAs synthesized in the presence of a high-salt S-150 fraction isolated from cells infected with wild-type $\phi 29$ and from cells infected with a gene 4 mutant of $\phi 29$. [^{32} P]RNAs were synthesized at 30°C for 10 min as described in the text in reaction mixtures (total volume, 0.1 ml) containing 3 μ g of $\phi 29$ DNA, 4 μ g of rifampin-resistant RNA polymerase, 10 g of rifampin per ml, buffer, nucleotides, and the following amounts of the high-salt S-150 fraction from cells infected with wild-type $\phi 29$: (a) none, (b) 6 μ l, (c) 12 μ l, (d) 20 μ l, (e) 32 μ l, (f) none, and (g) 32 μ l of a high-salt S-150 fraction isolated from cells infected with a gene 4 mutant of $\phi 29$.

obtained in two earlier experiments (Fig. 3, lanes b, e, and h, and Fig. 5, lane b).

Since the $\phi 29$ DNA used in the above experiments was purified using proteinase K treatment, the template lacked the $\phi 29$ gene 3 protein (16, 35). Synthesis of late RNA in vitro, therefore, does not depend on this protein. Previous studies have demonstrated that the gene 3 product does not affect either RNA polymerase binding (39) or in vitro synthesis of early RNA (8, 20).

The following preliminary observations have been made with respect to the properties of the high-salt S-150 preparation. (i) Electrophoresis on SDS-polyacrylamide gels and on agarose gels showed that these fractions contained a complex mixture of peptides and small amounts of nucleic acids (some rRNA and trace amounts of DNA). (ii) As stated earlier, the S-150 fractions prepared by either of two methods did not provide a template for added RNA polymerase—i.e., the addition of purified $\phi 29$ DNA was required for RNA synthesis and late transcription. (iii) No rifampin-resistant incorporation of [^{3} H]UTP into RNA could be detected in the presence of $\phi 29$ DNA when the high-salt S-150 fraction was used as the source of RNA polymerase in the standard RNA assay procedure (the concentration of NaCl in the eluate was reduced to 0.1 M before assay). (iv) The addition of the high-salt S-150 fraction obtained from cells infected with wild-type $\phi 29$ or with the cistron 4 mutant resulted in a two- to threefold stimulation in the activity of the rifampin-resistant RNA polymerase. This increase was noted in assays in which two DNA templates ($\phi 29$ and SP82 DNAs) were employed and probably can be attributed to the release of the sigma subunit from endogenous RNA polymerase. (v) Treatment with 1 M NaCl was required to release the active factor(s) from P-100 fractions. (vi) The factor(s) responsible for synthesis of late RNA was rapidly inactivated by dilution and exposure to temperatures greater than 30°C. (vii) To date, attempts to fractionate the factor(s) by sedimentation in glycerol gradients or by chromatography at 4°C on DEAE-cellulose, phosphocellulose, Biorex 70, and DNA-cellulose have been unsuccessful.

In view of the earlier observation (17) that a small part of the RNA polymerase isolated from $\phi 29$ -infected cells contained a 30K peptide, it was of interest to determine whether the synthesis of late $\phi 29$ RNA could be correlated with the presence of this peptide in the high-salt S-150 preparation. Electrophoretic comparisons of such fractions obtained from cells infected with wild-type and gene 4 mutant phages showed no significant differences in the amounts of peptides in the 25,000- to 35,000-dalton range (R. D.

Holder, Ph.D. thesis, University of Washington, Seattle, 1981). Furthermore, when extracts of cells infected with wild-type ϕ 29 were precipitated with antibody to the core subunits of RNA polymerase and the antibody complexes were analyzed by electrophoresis on SDS-polyacrylamide gels, no additional peptides were found associated with the core subunits. However, when the same antibody preparations were used in parallel experiments with extracts of SP82-infected cells, the SP82-coded peptides were found in association with the core subunits (D. L. Linemeyer, Ph.D. thesis, University of Washington, Seattle 1977). These observations, coupled with the fact that a reproducible isolation of a 30K-containing form of RNA polymerase has not been achieved, suggest that the 30K peptide is not involved in transcription of late genes. Alternatively, the 30K peptide may have a low affinity for the core subunits and therefore is not precipitated by antibodies to the core or retained with the core subunits during purification.

In vitro synthesis of anti-late RNA. Inspection of the restriction map of ϕ 29 DNA (Fig. 1) indicates that the in vitro synthesis of anti-late RNA (RNA which hybridizes to BL; Fig. 5, lane c and Fig. 7, lanes d and e) results from read through of transcription beyond a termination site near the junction of the *Eco*RI C and D fragments. Davison et al. (8) have shown that although RNA polymerase will terminate transcription at this site in vitro, termination is not efficient and RNA of high molecular weight is produced by read through beyond this site. Perhaps such read through may occur in vivo as well, since hybridization of early RNA to the *Eco*RI D fragment has been demonstrated (20, 39). Inciarte et al. (19) reported that the addition of a crude extract of *B. subtilis* decreased transcription through this site and suggested that a termination factor may be involved. We attempted to isolate a *B. subtilis* rho factor by using methods described for the isolation (34) and assay (25) of *E. coli* rho but did not succeed (Holder, Ph.D. thesis); subsequently, Hwang and Doi (18) isolated a rho factor from *B. subtilis* by using other methods.

However, we found that the addition of the *E. coli* rho factor decreased the incorporation of [^{3} H]UTP into RNA by *B. subtilis* RNA polymerase by approximately 30% when ϕ 29 DNA was the template and that *E. coli* rho had no effect when polydeoxyadenylic acid-polydeoxythymidylic acid was the template (data not shown). Figure 10 presents data on the hybridization of RNAs synthesized in the presence and absence of *E. coli* rho. The addition of this protein inhibited the synthesis of anti-late RNA—i.e., RNA that hybridized to BL (Fig. 10, lanes a and b). Lanes c and d of Fig. 10 provide evidence

FIG. 10. Autoradiograms showing the hybridization of *Eco*RI fragments of ϕ 29 DNA with RNAs synthesized from ϕ 29 DNA in the presence and absence of *Eco*RI rho factor. [^{32} P]RNAs were synthesized as described in the legend to Fig. 8 in the absence (lanes a and c) or presence (lanes b and d) of 3 μ g of *Eco*RI rho factor. Lanes a and b show the hybridization to separated strands of *Eco*RI fragments, and lanes c and d show the hybridization to *Eco*RI fragments of ϕ 29 DNA.

that rho prevented synthesis from the E fragment as well as from the B fragment. *E. coli* rho-induced termination occurred, therefore, near the junction of the *Eco*RI D and E fragments. However, hybridization of early and late in vivo RNAs (Fig. 3) demonstrated that early transcription did not extend into the *Eco*RI-D region; in our experiments, the B, D, and E fragments represent exclusively late regions. Therefore, although the *E. coli* rho factor was able to terminate in vitro transcription of ϕ 29 DNA by *B. subtilis* RNA polymerase and prevent the synthesis of at least a part of the anti-late RNA, it seems that this factor does not recognize the termination site utilized in vivo by ϕ 29-infected *B. subtilis*. It should be noted also that the pattern of hybridization in lane c of Fig. 10 is identical to that observed with late in vivo RNA (Fig. 3, lane b). Use of separated strands, however, allows discrimination between late and anti-late RNA and clearly shows that hybridization of the B fragment in lane c of Fig. 10 represents anti-late transcription.

DISCUSSION

The present investigation demonstrated that both early and late ϕ 29 RNAs were synthesized in vitro by RNA polymerase from DNA provided by a crude P-100 fraction obtained from ϕ 29-infected *B. subtilis* at a late stage of infection. Rifampin was added to the cultures just before extraction of the P-100 fraction to inhibit endog-

enous polymerase; transcription was performed in the presence of rifampin, using a purified rifampin-resistant RNA polymerase. The pattern of RNA synthesis by this system, as detected by hybridization to restriction fragments of $\phi 29$ DNA, was very similar to that obtained with RNA synthesized *in vivo* late in infection. A similar *in vitro* system employing a crude lysate of T4-infected *E. coli* has recently been reported to synthesize middle T4 RNA (9, 10). As in the present studies, rifampin was used to block transcription by endogenous polymerase, and RNA synthesis was catalyzed by added rifampin-resistant *E. coli* RNA polymerase in the presence of this inhibitor. It was proposed that the binding of rifampin-inhibited polymerase to immediate early promoters prevented chain elongation to the middle genes, thereby permitting detection of middle gene expression under the control of the *mot* gene product. It is not known what effect, if any, the binding of rifampin-inhibited polymerase to early promoters had on the $\phi 29$ *in vitro* system. Possibly, such binding may have decreased transcription from early promoters, thus allowing a more active synthesis of late RNA.

Under optimal conditions of temperature and concentration of the P-100 fraction, there was no synthesis of anti-late $\phi 29$ mRNA in our *in vitro* reactions. Read through of a termination site near the *EcoRI* C and D fragments to produce anti-late mRNA was observed when purified $\phi 29$ DNA was used as a template in place of the P-100 fraction. Addition of the *E. coli* rho factor prevented synthesis of some of the anti-late RNA, but termination did not occur at precisely the same site as termination in $\phi 29$ -infected *B. subtilis*. Possibly, a *B. subtilis* rho factor (18) functions both *in vivo* and in the *in vitro* system to prevent read through.

Previous studies have shown that the product of cistron 4 is required for the synthesis of the late class of $\phi 29$ RNA *in vivo* (4, 5, 13, 39). We found that late RNA was not synthesized *in vitro* when the P-100 fraction was derived from a *sus4* mutant, suggesting that late transcription *in vitro* requires an active gene 4 protein. Earlier studies (5) suggested that peptide LM 3B may be the gene 4 product; more recently (11, 29) gene 4 has been cloned and sequenced, and a peptide of 12,500 daltons has been identified as its product.

By stopping transcription from the P-100 fraction after increasing intervals of incubation and then hybridizing the RNAs to blots of *HindIII* fragments of $\phi 29$ DNA, we localized the region from which *in vitro* late transcription was initiated. These experiments indicated that late RNA synthesis begins in the *HindIII*-H-I region of the genome and that transcription proceeds sequentially from left to right, consistent with the *in*

vivo transcriptional map presented in Fig. 1. Electron microscopy of polymerase-DNA complexes (39) revealed that a polymerase binding site (A3) was located in this region and that there were two additional binding sites (B1 and B2) in the *HindIII* A and F fragments, respectively. Although the results of the present studies do not rule out the possibility that there was inefficient or weak initiation from the B1 and B2 sites concomitantly with stronger or more efficient initiation from the *HindIII*-H-I region, the simplest explanation for our results is initiation near the A3 binding site followed by rightward transcription.

By treating the P-100 fraction with 1 M NaCl or by lysing phage-infected cells in the presence of 1 M NaCl, the factor(s) required for late transcription was released into the supernatant. *In vitro* synthesis of late RNA was observed when such supernatant fractions (high-salt S-150 fraction) were prepared from cells infected with wild-type $\phi 29$ and added to transcription reactions containing purified $\phi 29$ DNA and RNA polymerase from uninfected cells. When the high-salt S-150 fraction was obtained from non-permissive cells infected with $\phi 29$ carrying a nonsense mutation in cistron 4, late transcription was not detected.

Several possible mechanisms could be proposed to account for the effect of the high-salt S-150 fractions in promoting the synthesis of late RNA by unmodified RNA polymerase from purified $\phi 29$ DNA. One possibility is that these fractions contain the gene 4 product and that this protein is similar to the phage-coded peptides which replace sigma in the modification of RNA polymerase in SP82- or SP01-infected *B. subtilis* (13, 23, 42). Thus, it could be imagined that the gene 4 product binds to the free core subunits which are present in most polymerase preparations or that it displaces the sigma subunit from the holoenzyme, thereby providing the specificity required for the recognition of late promoter sequences. However, if this mechanism operates in the transcription of $\phi 29$ DNA, the *in vitro* properties of the factor(s) are significantly different from those of the SP82-coded specificity-determining peptides. The latter bind sufficiently tightly to the core subunits to be retained throughout the purification of RNA polymerase, they are precipitated with the core by antibodies to the core, and they do not display any unusual sensitivity to temperature or dilution (12, 13, 23, 42).

Another possibility is that the gene 4 product functions in antitermination. An antiterminator mechanism for late transcription of $\phi 29$ has been suggested (39) based on the observation that when large amounts of early RNA and late chloramphenicol RNA were used, hybridization

was detected to the heavy strand of the *EcoRI* A fragment but not to the heavy strand of the *EcoRI* B fragment. Sogo et al. (39) proposed that the *EcoRI* A fragment may contain a late promoter and that transcription of the B fragment requires an antiterminator protein which is not produced early in infection or in the presence of chloramphenicol.

The lability of the factor(s) which stimulates the in vitro synthesis of late $\phi 29$ RNA is of particular interest. We found that the capacity of the P-100 fraction to support the synthesis of late RNA decreased as the temperature of incubation of the reaction mixtures was increased and that at temperatures above 30°C, little or no late RNA was produced. The factor(s) required for late in vitro synthesis was irreversibly inactivated at moderate temperatures and by dilution. Moreover, as the temperature of synthesis was decreased, synthesis of early RNA was inhibited so that at 20°C virtually all RNA synthesized from the P-100 fraction represented transcription from late sequences. It appears, therefore, that at low temperatures, *B. subtilis* RNA polymerase may initiate synthesis in vitro at a late promoter(s) but is unable to do so at early $\phi 29$ promoters. If the same factor(s) is involved in late transcription in vivo, it must either be stabilized in some way or be synthesized continuously, since raising the temperature to 42 to 45°C decreases the burst size of wild-type $\phi 29$ by only 50% (38). It is known that the antiterminator N protein is unstable in vivo at 37°C (15). Apparently, continuous synthesis is required to maintain N in lambda-infected *E. coli*, since in the presence of chloramphenicol the protein has a half-life of only 2 min (15).

Mangel and Chamberlin (27) have shown that the interaction between *E. coli* RNA polymerase and phage T7 promoters is temperature dependent and that polymerase is unable to form open promoter complexes at temperatures lower than 15 to 20°C. Similar observations have been made with *B. subtilis* RNA polymerase and early SP82 promoters except that the delta-less form of RNA polymerase was able to initiate some RNA synthesis at temperatures below 20°C (2). The finding that delta-containing *B. subtilis* RNA polymerase is unable to transcribe early $\phi 29$ sequences at 20°C probably reflects the inherent inability of polymerase to separate the DNA strands at low temperatures. Since polymerase was able to initiate synthesis of late RNA at 20°C, however, possibly the $\phi 29$ gene 4 product, a basic protein (11, 29), can act as a DNA "melting protein" and assist in separating the DNA strands to form open complexes at late promoter sites. A similar role has been proposed for the *mot* gene product, which controls middle T4 RNA synthesis in vitro (9, 10). Alternatively,

it may be speculated that the gene 4 product interacts with RNA polymerase to permit binding at late promoter sites.

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