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Transcriptional Regulation of Three Double-Stranded RNA Segments of Bacteriophage φ6 In Vitro

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Three double-stranded RNA segments of bacteriophage φ6 (L, M, and S) were transcribed in vitro by a virion-associated RNA polymerase. Regulation of L transcription was distinct from regulation of M and S transcription. Transcription of the L segment, which codes for early proteins, required manganous ion and high concentrations of all four ribonucleoside triphosphates and was inhibited by polyamines such as spermine. Transcription of the M and S segments, which code for late proteins, required manganous or magnesium ion and relatively low concentrations of all ribonucleoside triphosphates except GTP and was enhanced by polyamines. Optimal conditions for L transcription were more stringent than those for M and S transcription. These two apparently different patterns produced in in vitro transcription presumably reflect the two distinct in vivo transcription patterns; i.e., (i) similar amounts of three single-stranded RNA species were transcribed from the three corresponding segments of double-stranded RNA (early pattern) and (ii) a much larger amount of single-stranded RNA species was transcribed from M and S segments than from the L segment (late pattern). The early transcription pattern may be changed into the late pattern by a change of environment, such as substrate concentration. This suggests that the different enzymatic properties under the different environmental conditions of the virion-associated transcriptase are responsible for the transcriptional regulation throughout the infection cycle of bacteriophage φ6.

Bacteriophage φ6 of *Pseudomonas phaseolicola* contains three segments of double-stranded (ds) RNA, with molecular weights of 4.8×10^6, 2.8×10^6, and 2.2×10^6 for segments L, M, and S, respectively (12). The early proteins are encoded by the largest segment, L, and late proteins are encoded by the two smaller segments, M and S (1, 3). The three segments have similar base compositions (18), and their terminal sequences are also similar at both ends (6).

The in vivo transcription of these three segments is regulated by an as yet unknown mechanism. In the early infection stage, φ6 penetrates the host cell, by means of a virion-associated lytic enzyme (5), as a partially uncoated particle called the fraction I particle. The fraction I particle consists of three dsRNA segments and proteins P1, P2, P4, and P7 (8); it is involved in the initial viral RNA synthesis. At this stage, similar amounts of three single-stranded RNA species, *l*, *m*, and *s* (corresponding to L, M, and S dsRNA segments), are synthesized (4). The main early proteins produced, however, are only from *l*, due to its high translational activity (1, 3).

On the other hand, at the late stage of the infection cycle in vivo, much larger amounts of *m* and *s* rather than *l* are synthesized by a morphogenetic intermediate named previrion II (4). Then the late-stage proteins encoded by *m* and *s* are assembled with previrion II to form the intact virion. Since the composition of previrion II seems to be the same as that of the fraction I particle (4), the question remains open as to what regulatory mechanism is involved in the early and late transcriptions of the φ6 infection cycle.

The in vitro synthesis of mRNA is catalyzed by a nucleocapsid, which is obtained by treating the φ6 virion with a detergent. The nucleocapsid is made of three dsRNA segments and proteins P1, P2, P4, P7, and P8. The products of the in vitro transcription by the nucleocapsid are similar to the products of in vivo transcription in the late infection cycle. Namely, *m* and *s* are made in a vast excess over *l*. However, the condition in vitro that will give rise to the early-stage products in vivo has yet to be identified (2, 11, 16).

To clarify the relationship between the transcriptional products and the regulatory factors of RNA polymerases, we investigated the enzymatic properties of transcriptases under various

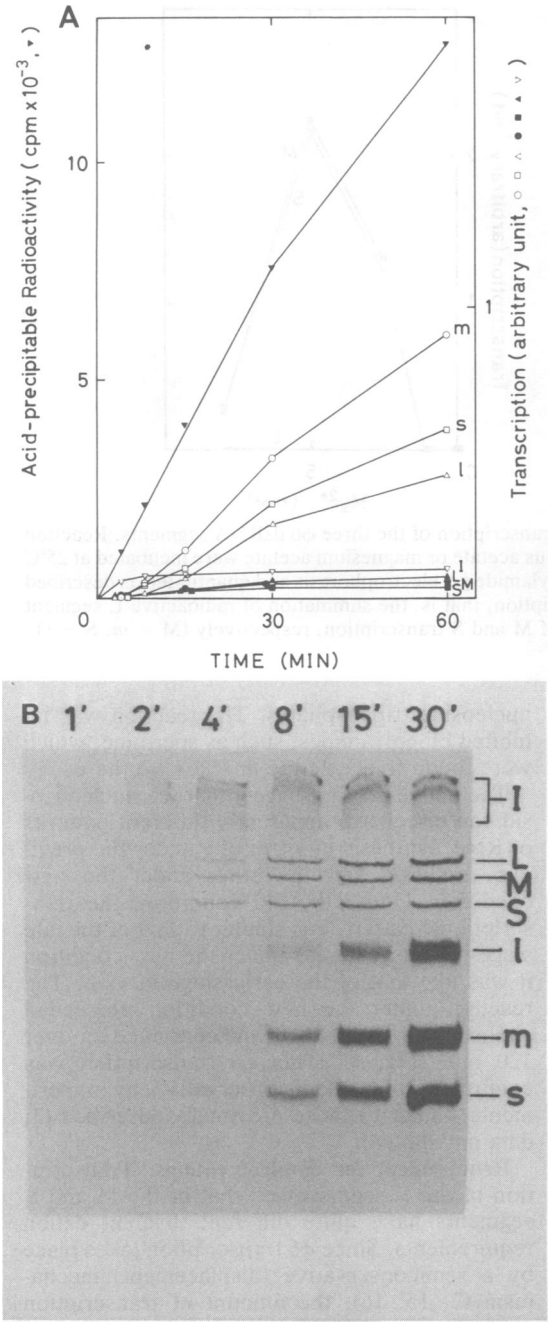

FIG. 1. Time course of in vitro transcription of the three segments. (A) Time course of incorporation of [^{3}H]UTP into acid-precipitable materials and each RNA species. (B) Electrophoresis profiles of the early period of the transcription products (1 to 30 min). A standard reaction mixture (0.2 ml) was incubated at 25°C. At the indicated time, a small portion (25 μl) was removed and counted for acid-precipitable radioactivity. Another portion was removed and analyzed by 2.3% polyacrylamide gel electrophoresis as described in the text. Each RNA species detected by fluorography was quantitated as described in the text. The letters I, L, M, S, l, m, and s represent intermediate RNA [dsRNA having a single-stranded RNA tail(s); see reference 2], L, M, and S segments of dsRNA, and *l*, *m*, and *s* species of single-stranded RNA, respectively.

conditions. We found the conditions in vitro that produced similar amounts of three single-stranded RNAs, either by a nucleocapsid or by previrion II. Then we showed that the scheduled transcriptional modes of φ6-infected cells in vivo may be explained in terms of those in vitro.

MATERIALS AND METHODS

Preparation of φ6 nucleocapsid. Bacteriophage φ6 was purified as previously described (7) and stored at −70°C in buffer A (10 mM potassium phosphate, 1 mM magnesium sulfate, pH 7.1). The φ6 nucleocapsid was obtained, in general, by treating the purified virion with 2% (final concentration) Triton X-100 in buffer A (17). However, the experimental results were essentially the same whether it was treated with sodium deoxycholate (0.15%) (2) or with other concentrations (1 to 4%) of Triton X-100 (16). The prepared nucleocapsid was suspended in 10 mM Tris-hydrochloride (pH 8.0) at a concentration of 1 mg of nucleocapsid protein per ml and used for RNA synthesis.

Preparation of previrion II-containing fractions from φ6-infected cells. Cell extracts were prepared from 50 to 300 ml of φ6-infected cultures (35 to 50 min after infection), and previrion II was purified from the cell extracts by 10 to 35% sucrose gradient centrifugation as described before (4).

In vitro transcription. RNA was synthesized in vitro at 25°C in a new standard reaction mixture (0.2 ml) containing 70 mM *N*,*N*-bis(2-hydroxyethyl)glycine (Bicine), pH 8.5, 3 mM manganese (II) acetate, 1 mM magnesium acetate, 125 mM ammonium chloride, 10 mM 2-mercaptoethanol, 140 μg of bentonite, 1 mM each ATP, CTP, GTP, and UTP, 2 μCi of [5-^{3}H]UTP (21.7 Ci/mmol), and 20 μg (as protein) of φ6 nucleocapsid. When previrion II was used as an enzyme, φ6 nucleocapsid was replaced by a previrion II-containing fraction and actinomycin D at a final concentration of 20 μg/ml. The reaction was terminated by sodium dodecyl sulfate (final concentration, 1%) at the indicated time, and the RNA was precipitated in ethanol (2 to 2.5 times the reaction mixture in volume).

The RNA was analyzed by 2.3% polyacrylamide (5% cross-linking) slab gel electrophoresis, using a buffer system described by Loening (10). After electrophoresis, the gel was processed for fluorography (9) and exposed to a preflashed Kodak XAR film at −80°C for an appropriate period, which gave a linear response in optical density against the radioactivity. For quantitative analyses, the film was cut into strips and scanned by a Gilford gel scanner, model 250. The area under the peak was measured and expressed in arbitrary units.

Substrate and ion requirements in RNA synthesis. Samples of the transcriptase reaction mixture (0.2 ml per sample) containing different concentrations of substrates or ions as described in the figure legends were incubated at 25°C for the indicated period. For investigating the UTP requirement, [^{3}H]UTP (2 μCi) was

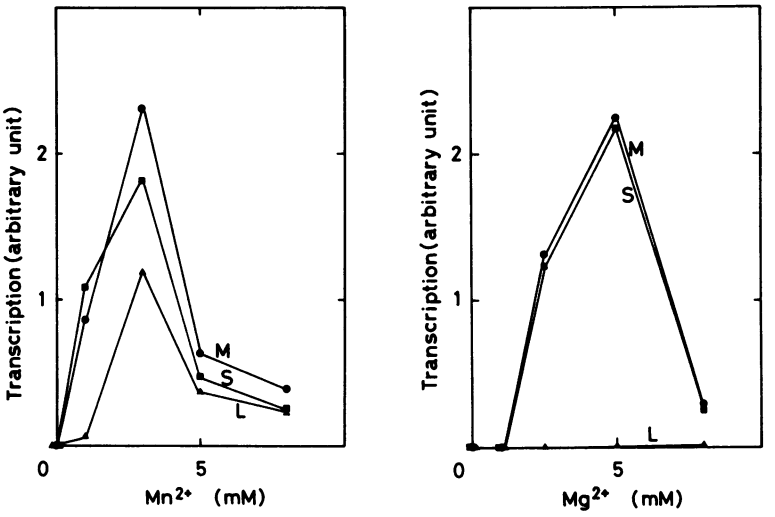

FIG. 2. Effect of Mg^{2+} and Mn^{2+} concentration on transcription of the three φ6 dsRNA segments. Reaction mixtures containing different concentrations of manganous acetate or magnesium acetate were incubated at 25°C for 30 min. The products were analyzed by 2.3% polyacrylamide gel electrophoresis and quantitated as described in the text. The letter L means the amount of L transcription, that is, the summation of radioactive L segment and *l* species. The letters M and S mean the amounts of M and S transcription, respectively (M + *m*, S + *s*).

replaced by [^{3}H]ATP (2 μCi; 23.3 Ci/mmol). The reaction was terminated by sodium dodecyl sulfate, and the RNA was precipitated and analyzed as described above.

Chemicals. All radioactive compounds were purchased from New England Nuclear Corp. Four nonradioactive ribonucleoside triphosphates were purchased from Boehringer Mannheim Corp., and actinomycin D was purchased from P-L Biochemicals, Inc.

RESULTS

RNA polymerase products in the new standard reaction mixture in vitro. The φ6 virion-associated RNA polymerase, nucleocapsid, synthesizes much larger amounts of *m* and *s* than of *l* (2, 16). This transcriptional pattern is similar to that of late-stage in vivo RNA synthesis. However, an in vitro condition for the nucleocapsid to give rise to the early-stage pattern of in vivo RNA synthesis had not been obtained. In the hope of finding this in vitro condition, we examined various experimental factors such as the requirement for cations, the effect of polyamines, and the dependence on substrate concentrations.

In the new standard reaction mixture, comparatively large amounts of *l* were transcribed (Fig. 1). Although it still amounted to little more than half the amount of *m* or *s*, it was much more than that obtained in the previous standard reaction mixture, where the amount of *l* synthesized was only about 1/20 of that for *m* or *s* (2).

The *l* synthesis in the new reaction mixture was dependent on the presence of manganese (II) ion and high concentrations of all four ribonucleoside triphosphates. The reaction was inhibited by polyamines, such as spermine, which were found to accelerate *m* and *s* syntheses.

The above results suggest that the nucleocapsid can direct two apparently different patterns of RNA synthesis in vitro: one under the previous condition and the other under the new condition. Under the old condition, the transcriptional pattern was similar to that of the late stage in vivo, whereas under the new condition it was like that of the early stage in vivo. The reaction under the new condition proceeded almost linearly for 60 min and continued for over 120 min (Fig. 1). This φ6 transcription was confirmed to be semiconservative by experiments similar to those previously described (3; data not shown).

Requirement for divalent cations. Transcription of the L segment and that of the M and S segments have quite different divalent cation requirements. Since φ6 transcription takes place by a semiconservative (displacement) mechanism (2, 15, 16), the amount of transcription occurring on the three segments can be expressed as the sum of the radioactivities, L + *l*, M + *m*, S + *s*. Transcription of the M and S segments required magnesium or manganous ion (Fig. 2), and the maximal activities were nearly the same at their optimal concentrations. Transcription of the L segment, on the other hand, specifically required manganous ion. Only a trace amount of L transcription was detected after a 90-min reaction when magnesium ion was used (data not shown).

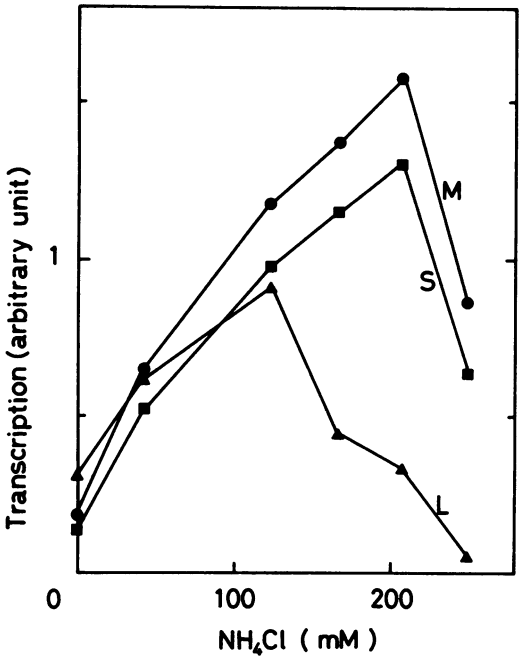

FIG. 3. Effect of NH_4^+ concentration on transcription of three φ6 dsRNA segments. Reaction mixtures containing different concentrations of ammonium chloride were incubated at 25°C for 30 min. The products were analyzed as described in the text. The letters L, M, and S represent the amounts of L, M, and S transcription, respectively, as described in the Fig. 2 legend.

When manganous ion was used at the optimal concentration (3 mM), the further addition of magnesium ion (1 mM) increased transcription of the three segments by approximately 30%. Consequently, magnesium ion (1 mM) was included in the new standard reaction mixture.

The above results show that manganous ion specifically regulates transcription of the L segment.

Requirement for monovalent cations. The ammonium ion requirement for L transcription was different from that for M and S transcriptions. However, the difference was not as drastic as for the divalent cation requirement. The optimal concentration of ammonium ion for L transcription was about 125 mM, whereas that for M and S transcription was about 200 mM (Fig. 3). The transcriptional pattern (i.e., the ratio of M/L or S/L transcriptions) was altered by the salt concentration (Table 1). Thus, we can change the transcriptional pattern by a minor change of salt concentration: high L transcription at 125 mM and low L transcription at 208 mM.

A similar result was obtained when K^+ or Na^+ was used as the monovalent ion. However, the maximal activity for K^+ or Na^+ ion was lower than that for NH_4^+ ion (data not shown).

Effect of polyamines. The effect of polyamines (spermine and spermidine) on L transcription was opposite that on M or S transcription. M or S transcription was stimulated by the addition of spermine (100 to 200 μM) or spermidine (100 to

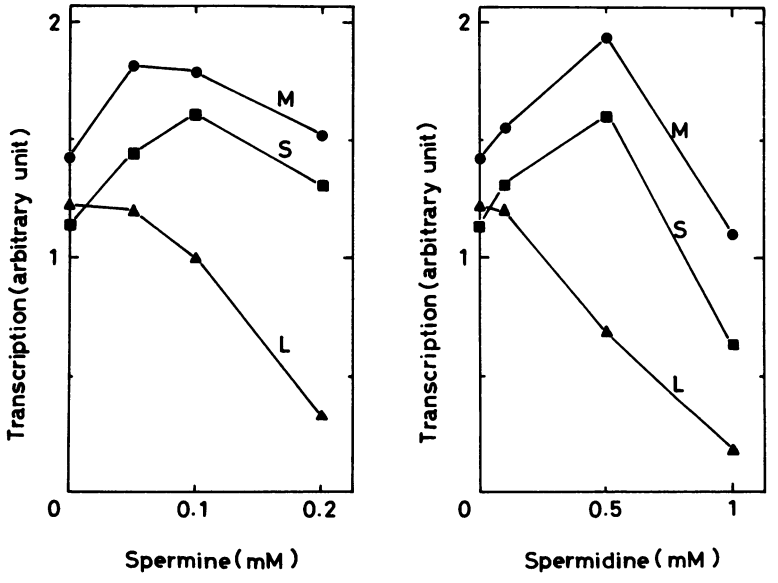

FIG. 4. Effect of polyamines on transcription of three φ6 dsRNA segments. Reaction mixtures containing different concentrations of spermine or spermidine were incubated at 25°C for 30 min. The products were analyzed as described in the text. The letters L, M, and S represent the amounts of L, M, and S transcription, respectively, as described in the Fig. 2 legend.

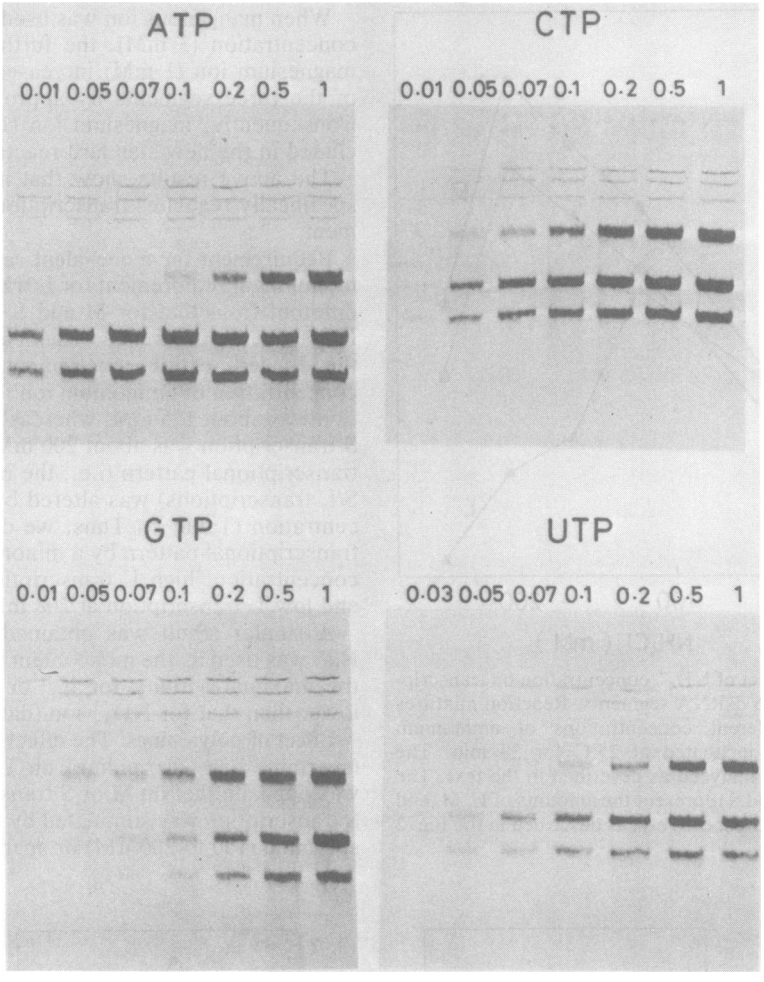

FIG. 5. Dependency on the concentration of four ribonucleoside triphosphate (NTP) for transcription of the three segments. Reaction mixtures which contained different concentrations (millimolar) of the NTP shown were incubated at 25°C for 60 min (the other NTPs were at 1 mM). The products were analzyed by 2.3% polyacrylamide gel electrophoresis as described in the text.

500 μM), but L transcription was severely inhibited at these concentration (Fig. 4). Thus, we can also change the transcriptional pattern by the addition of spermine or spermidine, although these polyamines are not required for RNA synthesis (Table 1).

Ribonucleoside triphosphate requirement. Studies of the substrate requirement revealed an interesting feature, which may help to elucidate the control mechanisms for ϕ6 transcription in vivo. A lower concentration of ATP or UTP, but not CTP or GTP, resulted in the specific inhibition of L transcription (Fig. 5; Table 1). The quantitative analysis of the gel clarified the enzymatic factors leading to the above phenomenon: transcription of the L segment requires high concentrations of all four ribonucleoside triphosphates, especially ATP and UTP, whereas transcription of M and S segments can occur at relatively low concentrations of all substrates except GTP (Fig. 6). The common requirement for a high concentration of GTP may stem from the limitation that the chain initiation of all three segments of dsRNA is expected to start with "G" (see sequences of the plus strands of ϕ6 dsRNA in reference 6).

Lowering the concentrations of all four ribonucleoside triphosphates also resulted in the specific inhibition of L transcription (Table 1).

Properties of RNA polymerase activity associated with a morphogenetic intermediate, previrion II. Previously we reported that previrion II had RNA polymerase activity comparable to that of the nucleocapsid. Then we suggested that the activity of previrion II could be responsible for late RNA synthesis in vivo. Here we examined

TABLE 1. Changes of transcriptional patterns at various concentrations of monovalent cation, polyamines, and substrates

Reagent(s)	Concn (mM)	Transcription ratio[a]	
		M/L	S/L
Monovalent cation	125	1.3	1.1
(NH_4Cl)	208	4.8	3.9
Polyamine (no addition)		1.2	1.0
Spermine	200 μM	4.3	3.7
Spermidine	500 μM	2.8	2.4
Substrate(s)			
4 NTPs[b]	1	1.1	1.0
ATP	0.1[c]	8.8	5.6
UTP	0.1[c]	3.1	2.1
CTP	0.1[c]	1.6	1.0
GTP	0.1[c]	0.5	0.3
4 NTPs	0.3	1.1	1.0
4 NTPs	0.05	7.5	5.4

[a] Transcription ratios were calculated from quantitative analyses as shown in Fig. 3 to 6.
[b] NTPs, Nucleoside triphosphates.
[c] The other three NTPs were at 1 mM each.

the enzymatic properties in detail to determine whether the RNA polymerase activity of previrion II is also dependent on the presence of manganous ion or on the concentrations of ribonucleoside triphosphates. As far as the requirements of the divalent cation and ribonucleoside triphosphates are concerned, previrion II showed results similar to those for the nucleocapsid (Fig. 7).

Despite the fact that previrion II mainly directs late-stage RNA synthesis in vivo, as shown before (4), it is also possible that previrion II can direct early-stage RNA transcription under the conditions optimized for L transcription. Then, it may well be the case that the low L transcription in vivo by previrion II is caused by the unfavorable environmental conditions for L transcription of previrion II.

DISCUSSION

During the infection cycle of bacteriophage φ6, the pattern of mRNA synthesis changes from the early-stage pattern, $l \sim m \sim s$, to the late-stage pattern, $l \ll m \sim s$. In the transcriptional experiments in vitro, however, only the late-stage pattern had been observed (2, 16) in the past. In this report, we show that early-stage transcription could be obtained in vitro, using either the nucleocapsid or previrion II. The change in the transcriptional pattern from the early type to the late type was obtained by the addition of polyamines or ammonium ion or by a drastic depletion of manganous ion or ribonucleoside triphosphates (Fig. 2 to 5; Table 1). Consequently we could obtain the two types of transcriptional activity in vitro, which corresponded to those in vivo, using either the nucleocapsid or the previrion II. Since no other factors were involved in the switch of transcriptional patterns, it is conceivable that the enzymatic characters of the virion-associated RNA polymerase

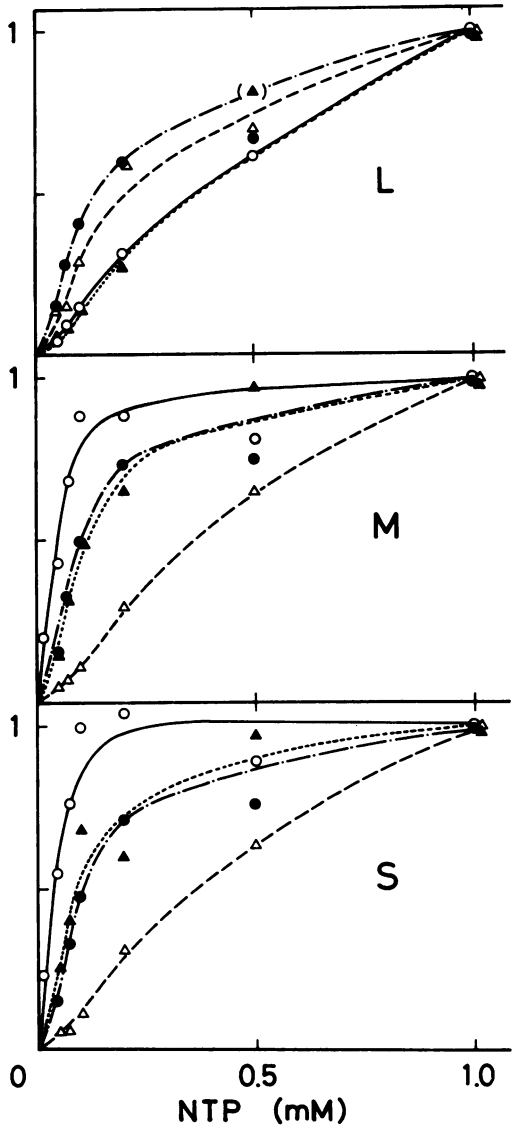

FIG. 6. Quantitative analysis of substrate requirement. Samples identical to those shown in Fig. 5 were quantitated as described in the text. Relative amounts of three transcription segments (L, M, S) were plotted against the values shown at 1 mM. NTP, Nucleoside triphosphate. Symbols: ○, ATP; ●, CTP; △, GTP; ▲, UTP.

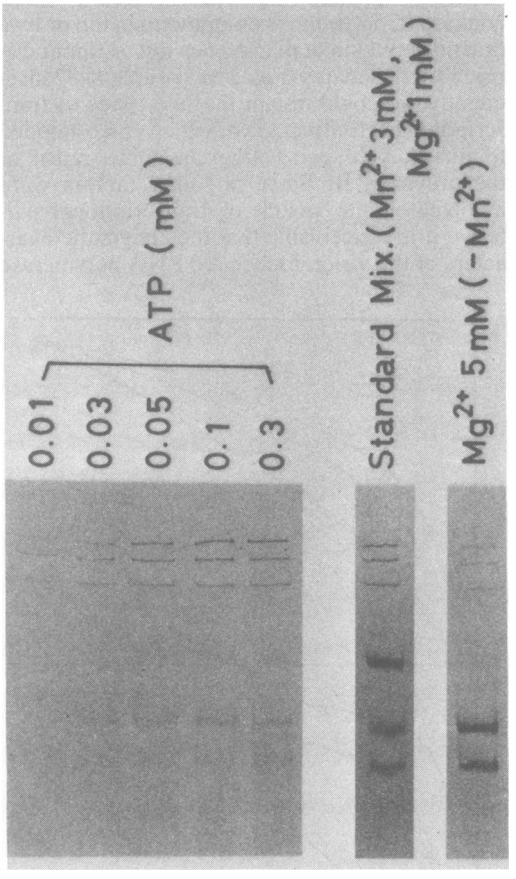

FIG. 7. Properties of the transcriptase activity found in previrion II. Fractions containing previrion II were prepared and assayed for their transcriptase activity as described in the text. Reaction conditions were the same as the standard reaction mixture except as otherwise noted.

alone are responsible for the change in transcriptional pattern in the infection cycle of phage ϕ6.

Although we have no experimental evidence for the physiological transition from the early stage to the late stage as yet in ϕ6-infected cells, it is easy to speculate that changes in the physiological environment of the RNA polymerase will alter the transcription pattern in vivo. At the time of penetration of a partially uncoated ϕ6 particle into the cell, the physiological condition of the bacterial cell is expected to be suitable for the early transcription type. After about 30 min, however, the amount of RNA synthesis will increase with the accumulation of previrion II (4), and the ribonucleotide concentration in the cell may decrease. Consequently, the environment of previrion II becomes suitable for the late-stage transcription. Since the optimal condition of L transcription is more stringent than those of M and S transcription, even a small change in the environment could easily inhibit the L transcription and thus result in the switching of the transcription from the early to the late type.

What might be the mechanism for the transcriptional regulation? Considering that the favorable transcriptional conditions are widely different between the L segment and the M and S segments, there may be a distinct difference among the three dsRNAs in the primary structures that are involved in transcriptional control. In fact, this might be the case, since the second nucleotide from the 5′ end of the plus strand differs between the L segment and the M and S segments. This difference is located within the region of 18 nucleotides from the 5′ end, where the recognition sites of the RNA polymerase are probably located. However, we have no experimental results as yet on the structure and species of the RNA polymerase or on the roles of the individual protein components in the ϕ6 particle. The control mechanism is far from elucidated.

In an influenza virus, interesting results were obtained by the technique of cross-linking the primer and the RNA polymerase (14). In the cases of reovirus (19) and cytoplasmic polyhedrosis virus of *Bombyx mori* (13), an abortive initiation process is considered to be a possible mechanism of transcriptional control. For dsRNA viruses, however, little is known about transcriptional regulation. It is our hope that the studies of drastically different patterns of ϕ6 transcription in vivo and in vitro described in this report may lead to a better understanding of the control mechanism for segmented dsRNA transcription in general.

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LITERATURE CITED

1. **Cuppels, D. A., J. L. Van Etten, D. E. Burbank, L. C. Lane, and A. K. Vidaver.** 1980. In vitro translation of the three bacteriophage ϕ6 RNAs. J. Virol. **35**:249–251.
2. **Emori, Y., H. Iba, and Y. Okada.** 1980. Semi-conservative transcription of double-stranded RNA catalyzed by bacteriophage ϕ6 RNA polymerase. J. Biochem. **88**:1569–1575.
3. **Emori, Y., H. Iba, and Y. Okada.** 1980. Assignment of viral proteins to the three double-stranded RNA segments of bacteriophage ϕ6 genome: translation of ϕ6 messenger RNA transcribed *in vitro*. Mol. Gen. Genet. **180**:385–389.
4. **Emori, Y., H. Iba, and Y. Okada.** 1982. Morphogenetic pathway of bacteriophage ϕ6: a flow analysis of subviral and viral particles in infected cells. J. Mol. Biol. **154**:287–310.
5. **Iba, H., M. Nanno, and Y. Okada.** 1979. Identification and partial purification of a lytic enzyme in bacteriophage ϕ6 virion. FEBS Lett. **103**:234–237.
6. **Iba, H., T. Watanabe, Y. Emori, and Y. Okada.** 1982. Three double-stranded RNA genome segments of bacteriophage ϕ6 have homologous terminal sequences. FEBS Lett. **141**:111–115.

7. **Kakitani, H., Y. Emori, H. Iba, and Y. Okada.** 1978. Lytic enzyme activity associated with double-stranded RNA bacteriophage ϕ6. Proc. Jpn. Acad. **54A**:337–340.
8. **Kakitani, H., H. Iba, and Y. Okada.** 1980. Penetration and partial uncoating of bacteriophage ϕ6 particle. Virology **101**:475–483.
9. **Laskey, R. K., and A. D. Mills.** 1975. Quantitative film detection of ^{3}H and ^{14}C in polyacrylamide gels by fluorography. Eur. J. Biochem. **56**:335–341.
10. **Loening, U. E.** 1967. The fractionation of high-molecular-weight ribonucleic acids by polyacrylamide gel electrophoresis. Biochem. J. **103**:251–257.
11. **Partridge, J. E., J. L. Van Etten, D. E. Burbank, and A. K. Vidaver.** 1979. RNA polymerase activity associated with bacteriophage ϕ6 nucleocapsid. J. Gen. Virol. **43**:299–307.
12. **Semancik, J. S., A. K. Vidaver, and J. L. Van Etten.** 1973. Characterization of a segmented double-stranded RNA from bacteriophage ϕ6. J. Mol. Biol. **78**:615–625.
13. **Smith, R. E., and Y. Furuichi.** 1982. A unique class of compound, guanocine nucleotide tetraphosphate G(5′)pppp(5′)N, synthesized during the *in vitro* transcription of cytoplasmic polyhedrosis virus of *Bombyx mori*. J. Biol. Chem. **257**:485–494.
14. **Ulmanen, I., B. A. Broni, and R. M. Krug.** 1981. Role of two of the influenza virus core P proteins in recognizing cap 1 structures (m^{7} GpppNm) on RNAs and in initiating viral RNA transcription. Proc. Natl. Acad. Sci. U.S.A. **78**:7355–7359.
15. **Usala, S. J., B. H. Brownstein, and R. Haselkorn.** 1980. Desplacement of parental RNA strands during *in vitro* transcription by bacteriophage ϕ6b nucleocapside. Cell **19**:855–862.
16. **Van Etten, J. L., D. E. Burbank, D. A. Cuppels, L. C. Lane, and A. K. Vidaver.** 1980. Semiconservative synthesis of single-stranded RNA by bacteriophage ϕ6 RNA polymerase. J. Virol. **33**:769–773.
17. **Van Etten, J. L., L. Lane, C. Gonzalez, J. Partridge, and A. K. Vidaver.** 1976. Comparative properties of bacteriophage ϕ6 and ϕ6 nucleocapsid. J. Virol. **18**:652–658.
18. **Van Etten J. L., A. K. Vidaver, R. K. Koshki, and J. P. Burnett.** 1974. Base composition and hybridization studies of the three double-stranded RNA segments of bacteriophage ϕ6. J. Virol. **13**:1254–1262.
19. **Yamakawa, M., Y. Furuichi, K. Nakashima, A. J. LaFiandra, and A. J. Shatkin.** 1981. Excess synthesis of viral mRNA 5′-terminal oligonucleotides by reovirus transcriptase. J. Biol. Chem. **256**:6507–6514.