Dependence of Minus-Strand Synthesis on Complete Genomic Packaging in the Double-Stranded RNA Bacteriophage $\phi 6$

MIKKO FRILANDER,¹ PAUL GOTTLIEB,² JEFFREY STRASSMAN,² DENNIS H. BAMFORD,¹
AND LEONARD MINDICH^{2*}

*Department of Genetics, University of Helsinki, Helsinki, Finland,¹ and Department of Microbiology,
The Public Health Research Institute, New York, New York 10016²*

Received 13 April 1992/Accepted 8 May 1992

Bacteriophage $\phi 6$ has a segmented genome consisting of three pieces of double-stranded RNA (dsRNA). The viral procapsid is the structure that packages plus strands, synthesizes the complementary negative strands to form dsRNA, and then transcribes dsRNA to form plus-strand message. The minus-strand synthesis of a particular genomic segment is dependent on prior packaging of the other segments. The 5' end of the plus strand is necessary and sufficient for packaging, while the normal 3' end is necessary for synthesis of the negative strand. We have now investigated the ability of truncated RNA segments which lack the normal 3' end of the molecules to stimulate the synthesis of minus strands of the other segments. Fragments missing the normal 3' ends were able to stimulate the minus-strand synthesis of intact heterologous segments. Minus-strand synthesis of one intact segment could be stimulated by the presence of two truncated nonreplicating segments. The 5' fragments of each single-stranded genomic segment can compete with homologous full-length single-stranded genomic segments in minus-strand synthesis reactions, suggesting that there is a specific binding site in the procapsid for each segment.

Bacteriophage $\phi 6$ infects the plant pathogen *Pseudomonas syringae* pv. *phaseolicola* (*P. phaseolicola*) HB10Y (17). It has a double-stranded RNA (dsRNA) genome. Its genome is composed of three polycistronic segments that are designated L (6,374 bp), M (4,061 bp), and S (2,948 bp) (10). Genomic segments are localized in a polyhedral nucleocapsid composed of five protein species. P8 forms the outer shell of the nucleocapsid. The rest of the nucleocapsid proteins (P1, P2, P4, and P7) form the procapsid (11). The procapsid displays icosahedral symmetry and functions as a viral replicase and transcriptase (5, 6, 9, 14). Mature virions have a lipid-protein envelope that is exterior to the P8 shell.

The procapsid proteins P1, P2, P4, and P7 are encoded by genomic segment L. When a cDNA copy of segment L in an expression vector is introduced into *Escherichia coli* or *P. phaseolicola*, procapsid proteins which assemble into a polyhedral particle structurally identical to the natural viral procapsid are synthesized (6). Procapsids produced from cDNA are capable of packaging the positive-sense viral RNA and synthesizing the corresponding minus strands. Minus-strand synthesis has previously been referred to as replication. However, we suggest use of the word replication to describe the entire RNA duplication cycle starting from the transcription reaction and ending with minus-strand synthesis. The procapsids that have completed minus-strand synthesis to form dsRNA can also produce plus-strand transcripts from the dsRNA (9). This transcription reaction is in the late mode (1), where most of the transcripts are formed from the S and M segments. Procapsids that have formed dsRNA can be coated with purified P8 to form nucleocapsids that are infectious to spheroplasts of HB10Y, suggesting that procapsids produced in *E. coli* are identical to viral procapsids (13).

In a previous study, we have shown that the procapsid is

capable of packaging each genomic segment independently but that there is a strong dependence of minus-strand synthesis on packaging of other segments. In magnesium-containing buffers, only procapsids that have packaged all three segments are capable of synthesizing the minus strands efficiently (8). This regulation of minus-strand synthesis may ensure that only particles containing all three genomic segments are formed. In this report, we give further details on the activation mechanism of minus-strand synthesis.

Several models can explain the dependence of minus-strand synthesis on the presence of all three segments. The polymerase might be dependent on the amount of RNA inside the procapsid, it might require three origins of minus-strand synthesis, or it might require that all the packaging sites inside the procapsid be occupied.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* JM109 *recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 λ^- $\Delta(lac-proAB)$ [F' *traD36 proAB lacI^q Δ M15]* (18) was used for propagation of all plasmids. pLM450 contains a cDNA copy of genomic segment L of $\phi 6$, which encoded the four procapsid proteins (6). Plasmids pLM658, pLM656, and pLM682 contain cDNA copies of the genomic segments S, M, and L, respectively, in pT7T3 19U (12, 13). The 5' end of the L segment in pLM682 starts with nucleotides GG instead of GU, which are found in the natural segment L. This change was necessary because T7 RNA polymerase prefers two Gs at the start of the RNA transcript.*

Preparation of procapsids. Procapsids were produced in *E. coli* JM109 containing plasmid pLM450. They were purified according to Gottlieb et al. (6). Purified procapsids were divided into aliquots and frozen at -70°C . Aliquots were thawed just prior to use.

In vitro synthesis of positive-sense transcripts by T7 polymerase. Plasmid pLM658, pLM656, or pLM682 was cut with

* Corresponding author.

endonuclease *Xba*I, and the resulting 5' overhang was removed with mung bean nuclease before transcription with T7 RNA polymerase (13). Truncated segments were produced by cutting the plasmids with endonuclease *Eco*RV before the transcription reaction (see Fig. 1). RNA was purified by extracting once with phenol-chloroform-isoamyl alcohol and then with chloroform-isoamyl alcohol.

RNA polymerase reaction conditions. RNA polymerase reaction conditions were similar to those reported previously (7) except that the pH of the reaction buffer was 8.9. Non-radioactive nucleoside triphosphates as well as [α - 32 P]UTP were included. Equimolar amounts of each segment were used so that the total RNA concentration was about 100 μ g/ml in a 25- μ l volume. The reaction was stopped by adding EDTA and sodium dodecyl sulfate to final concentrations of 5 mM and 0.07%, respectively. The reaction products were purified by phenol-chloroform-isoamyl alcohol extraction and concentrated by ethanol precipitation. Aliquots of each reaction were analyzed in either 1% agarose strand-separating gels after heat denaturation (4, 5) or in 2% acrylamide-0.5% agarose composite gels (16) that were subsequently dried and autoradiographed with a Cronex enhancing screen. The radioactivity of each individual band was quantitated with a Betagen Betascope model 603 blot analyzer.

Neither the procapsids nor the RNA is saturating in the minus-strand synthesis reaction (9). Since the reaction cocktail contains polyethylene glycol, it is not possible to assess true binding constants. The calculated concentrations of procapsid and viral RNAs in infected cells are approximately 10-fold higher than those used in these reactions. It is impractical for us to achieve these concentrations, but the fact that we do see competition in our reactions (see Results) indicates that we are near saturation conditions for RNA.

Preparation of plus-strand transcripts from the ϕ 6 nucleocapsid. Nucleocapsids were prepared from purified preparations of ϕ 6 (2). In vitro transcription was performed according to the conditions specified by Emori et al. (3). The reactions proceeded in a volume of 5 to 10 ml containing 1 mg of phage equivalent per ml. The RNA products were purified by phenol-chloroform-isoamyl alcohol extraction, concentrated with ethanol precipitation, and resuspended in distilled water.

RESULTS

Inhibition of minus-strand synthesis of a specific segment by competing nonreplicating fragment. Our earlier studies (5a) indicated that positive-sense single-stranded molecules that are missing their normal 3' ends can be packaged but that minus strands corresponding to the truncated fragments are not usually formed. If there is a specific packaging sequence on each single-stranded RNA (ssRNA) genomic segment, then these fragments should be capable of competing with a corresponding intact ssRNA genomic segment in a packaging reaction. When saturating concentrations of the complete genomic segments are used, the addition of a segment that is missing the 3' end should inhibit the minus-strand synthesis of the corresponding complete segment. If there is a specific binding site in the procapsid for each genomic segment, then the addition of a 5' fragment of one segment should not inhibit the minus-strand synthesis of the other two segments.

In order to test this, we prepared positive strands that were lacking normal 3' ends. The truncated segments were prepared by transcribing cDNA plasmids that had been cut

FIG. 1. Restriction maps of the cDNA copies of the genomes of ϕ 6 S, M, and L segments. Selected restriction sites are indicated. The corresponding *Eco*RV5' fragment is indicated below each segment.

with endonuclease *Eco*RV, yielding the ssRNA segments s(*Eco*RV5'), m(*Eco*RV5'), and l(*Eco*RV5'). The *Eco*RV5' fragments are 677, 1,349, and 698 bp, respectively (Fig. 1). Twofold-increasing amounts of the *Eco*RV5' fragments were added to reaction mixtures that contained approximately equimolar amounts of all three intact segments. The starting concentration of the *Eco*RV5' fragments was approximately equal to that of the intact ssRNA genomic segments within a factor of 2.

The results of the combined packaging and minus-strand synthesis reactions are shown in Fig. 2. All three *Eco*RV5' fragments behaved as segment-specific inhibitors when they were added to a reaction mixture containing three complete plus strands. This indicates that they were competing only with their corresponding intact strands. The fragments inhibited only the minus-strand synthesis of their homologous segment, even when increasing concentrations of *Eco*RV5' fragments were used. No minus-strand synthesis of the *Eco*RV5' fragments was observed, indicating that the packaging sequence is located in the 5' end of the molecule and that the normal 3' end is necessary for minus-strand synthesis.

The results shown in Fig. 2 were analyzed quantitatively by determining the radioactivity in the individual bands (data not shown). The quantitation confirms the information obtained from the gel picture: the *Eco*RV5' fragments inhibit minus-strand synthesis of the homologous genomic segments and do not inhibit minus-strand synthesis of the two heterologous segments. There is a clear, almost twofold stimulation of the minus-strand synthesis of the two heterologous segments (Fig. 2, lanes f and g). The same is also true to a lesser extent in lanes h, i, and j. This indicates that the concentration of the intact plus strands was not saturating in the packaging reaction. For this reason, the *Eco*RV5' fragment could act as an inhibitor of the minus-strand synthesis of one segment, but because the RNA concentration was nonsaturating, they could stimulate the minus-

FIG. 2. Segment-specific inhibition of minus-strand synthesis by EcoRV5' fragments. In vitro reactions containing all three intact positive-strand segments were performed. Increasing amounts of EcoRV5' fragments were added to the reactions, as indicated below the gel. The reaction products were separated in a 1% agarose strand separation gel that was subsequently dried and autoradiographed. Locations of the EcoRV5' fragments were deduced from ethidium bromide-stained gels.

strand synthesis of the other two heterologous fragments in those particles (see below).

Effect of replacing a single segment with its truncated counterpart. We have previously shown that minus-strand synthesis is dependent on the packaging of all three positive strands inside the procapsid (8). We next tested whether the minus-strand synthesis of one segment is dependent on the minus-strand synthesis of the other two segments.

In vitro reactions were performed such that one of each plus strand, s, m, or l, was replaced by the corresponding EcoRV5' fragment. Approximately equimolar amounts of the two ssRNA genomic segments and a twofold molar excess of the EcoRV5' fragments were used in the reactions. The reactions were run in pairs. The first contained only the two intact plus strands; the second contained the same pair plus the EcoRV5' fragment of the third segment.

The results are shown in Fig. 3A. No minus-strand synthesis was observed in reactions that contained only segments s and m (lane a) or s and l (lane c). When EcoRV5' fragments of the third segment, l (lane b) or m (lane d), were added to the reaction, the level of minus-strand synthesis of the two complete segments was similar to that found in the control reaction that contained all three complete segments. Some minus-strand synthesis can be observed in a reaction that contains only segments m and l (lane e). However, in this case, addition of the EcoRV5' fragment of s enhanced the synthesis by a factor of 2 (lane f). In overexposed autoradiograms, it is possible to see a low level of minus-strand synthesis in Fig. 3A, lanes a and c. No minus-strand synthesis of the EcoRV5' fragments can be seen. Although

FIG. 3. (A) Effect of substituting an EcoRV5' fragment for a single genomic segment. (B) Effect of substituting two EcoRV5' fragments for their corresponding complete genomic segments. The contents of individual reactions are indicated below the gels. In both panels, reaction products were separated in a 1% agarose strand separation gel that was subsequently dried and autoradiographed. Locations of the EcoRV5' fragments were deduced from ethidium bromide-stained gels.

the stimulation of replication is seen in all repetitions of this experiment, we do see occasional differences in the residual synthesis found with only two segments. Thus, we have found some synthesis with a mixture of segments s and l (8).

In lanes e, f, and g in Fig. 3A, a low level of transcription (plus strand) of segment M can be seen as a band below the minus-strand segment M. However, this band is missing in lane b. This effect will be discussed below.

Effect of replacing two segments with their truncated counterparts. We tested the ability of a single intact segment to act as a template for minus-strand synthesis in a particle that had packaged two truncated heterologous segments. Three reactions were analyzed for each segment. Two reactions contained one intact plus strand and one of the two remaining EcoRV5' fragments. The third reaction contained one intact plus strand and the EcoRV5' fragments of the other two.

The results are shown in Fig. 3B. All three plus strands can serve alone as templates for minus-strand synthesis in reactions that contain the EcoRV5' fragments of the other two segments. (Fig. 3B, lanes d, g, and j). This indicates that the minus-strand synthesis of one segment is not dependent on the minus-strand synthesis of other two segments.

The minus-strand synthesis of segment s requires that the EcoRV5' fragments of both m and l be packaged into the procapsid. No minus-strand synthesis can be observed (Fig. 3B, lanes b and c) if only one of these is packaged. When EcoRV5' fragments of both m and l are present in the reaction mixture with complete s, the level of minus-strand synthesis of s is roughly the same as in a control reaction in which all three intact segments are present.

As shown in Fig. 3A, segments m and l do not absolutely require the packaging of the EcoRV5' fragment of s for minus-strand synthesis. A low level of synthesis of m is observed when the EcoRV5' fragment of l is present and the EcoRV5' fragment of s is absent (Fig. 3B, lane f). Similarly, minus-strand synthesis of l is observed when the EcoRV5' fragment of m is packaged by the procapsid (Fig. 3B, lane i). However, addition of the EcoRV5' fragment of s to these reactions enhances the extent of minus-strand synthesis (Fig. 3A, lanes e and f).

Effect of the truncated fragments on transcription. The presence of labeled plus strands in the reactions indicated transcription. A low level of transcription of segment M was observed in some but not all of the reactions (Fig. 2 and 3A). The transcripts of S and L are difficult to see in the gel because the plus and minus strands are not well separated. The transcription of M seems to be dependent on the presence of an intact L segment. When the minus-strand synthesis of L was inhibited by adding an excess of the l(EcoRV5') fragment (Fig. 2, lanes h, i, and j) or when the l(EcoRV5') fragment was used instead of an intact l segment (Fig. 3A, lane b) in RNA polymerase reactions, no transcription of M was observed.

The l segment used in the RNA polymerase reactions is identical to the natural l segment except that the second nucleotide is changed from U to G. The reactions were repeated with natural ssRNA so as to eliminate the possibility that the observed effect was due to the sequence difference.

Nucleocapsids were used as a source of viral transcripts. The resulting mixture of dsRNA and positive-strand ssRNA was used as a template in RNA polymerase reactions. Three reactions were carried out, wherein minus-strand synthesis of intact s, m, or l was inhibited by the corresponding EcoRV5' fragment. The control reaction contained no com-

FIG. 4. Effect of minus-strand synthesis on the transcription reaction. The reactions were run in the presence of viral RNA and [32 P]UTP. A molar excess of EcoRV5' fragments was added to the reactions. The reaction products were separated in a composite gel (2% acrylamide, 0.5% agarose). The contents of the reactions were as follows: lane a, control reaction containing only viral RNA; lane b, viral RNA and the EcoRV5' fragment of m; lane c, viral RNA and the EcoRV5' fragment of s; lane d, viral RNA and the EcoRV5' fragment of l. The minor band at the bottom of lane c is artifactual and does not correspond in size to any of the reaction components. Locations of the EcoRV5' fragments were deduced from ethidium bromide-stained gels.

petitor. The reactions were analyzed in 2% acrylamide–0.5% agarose composite gels (16) so that the individual plus-strand RNAs would be separated from the dsRNA.

The results are shown in Fig. 4. Negative-strand synthesis results in the incorporation of label into dsRNA. The transcription reaction also results in the labeling of dsRNA, but because of the strand displacement mechanism of transcription, labeled ssRNA transcripts are produced during the second round of transcription. The addition of the EcoRV5' fragments of the m or s segments inhibited both negative- and positive-strand synthesis (transcription) of the corresponding segment but did not have any effect on the minus-strand synthesis or transcription of the other segments (Fig. 4, lanes b and c). When minus-strand synthesis of the intact segment l was inhibited by adding the EcoRV5' fragment of l, the transcription of both S and M was also inhibited (Fig. 4, lane d). This indicates that the minus-strand synthesis of segment L is required for the transcription of the other segments.

DISCUSSION

There is a strong dependence of the minus-strand synthesis of any one genomic segment on the packaging of all three genomic segments in bacteriophage $\phi 6$. The polymerase requires that all positive strands be packaged before the minus-strand synthesis of any one can take place (8). It has been found that the genomic segments of rotavirus are present in equimolar amounts, and it has been proposed that the initiation of replication (minus-strand synthesis) in rotavirus requires the prior packaging of all the genomic segments (15).

The packaging region and the origin of minus-strand synthesis are located at different ends of the $\phi 6$ RNA molecules. The packaging region is at the 5' end, and the origin of minus-strand synthesis is at the 3' end. It has been proposed that a double-stranded structure wherein the 5' and

the 3' ends of the RNA molecule are base paired is necessary for both packaging and replication in reovirus (19). This conclusion was based on the analysis of the defective interfering RNA. Our results show that in the case of $\phi 6$, the 5' region is necessary and sufficient for packaging but not sufficient for minus-strand synthesis.

Our results show that in addition to being necessary for packaging, the 5' end of the genomic segment is a sufficient signal for activating the viral RNA polymerase. The 5' fragments that lack the origin of minus-strand synthesis support efficient minus-strand synthesis of the other segments. We believe that the packaging site in the procapsid is involved in this activation of the polymerase, but our results do not rule out the possibility that, in addition to the packaging region, there is a separate region at the 5' end of the RNA that would be responsible for the initiation signal for the polymerase. The identification of the packaging sequence should answer this question.

Whereas the 5' end of the plus strand is a sufficient signal for the activation of minus-strand synthesis, the same does not hold true for transcription. The initiation of transcription is controlled by the minus-strand synthesis of segment L so that a prior synthesis of L minus strand is required before the transcription of any segment can be initiated. Together, these two regulatory processes result in strict control over RNA polymerization in the procapsid.

We assume that there is only one packaging site in the procapsid for each genomic segment. The packaging sites might form one complex, or they might be located in different places in the procapsid structure. If the packaging sites are separated from each other, a global conformational change could lead to activation of minus-strand synthesis. If the sites are clustered into one complex, perhaps a structure analogous to the portal vertex in many bacteriophages but containing RNA polymerase functions, the signaling between packaging and minus-strand synthesis could be transmitted by local conformational changes. Since protein P2 is involved in both RNA packaging and minus-strand synthesis (7, 9), the signal might not have far to travel.

The system described in this paper allows us to set up a simple assay for the packaging of synthetic RNA and therefore makes possible the determination of the packaging sequence of each genomic segment. For example, deletions of a possible packaging region within the 5' end of the genome can be prepared and assayed for either stimulation of heterologous segment minus-strand synthesis or inhibition of homologous-segment minus-strand synthesis. If there is only one site for both packaging and stimulation, then a segment that is packaged should stimulate the minus-strand synthesis of the other two segments and should inhibit the minus-strand synthesis of its corresponding wild-type segment. If there are separate sites for stimulation and packaging, then a fragment that is able to inhibit the minus-strand synthesis of its corresponding segment may not necessarily be able to stimulate the minus-strand synthesis of the two other segments.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant GM-34352 awarded to L.M. Mikko Frilander was supported by a grant from the Academy of Finland awarded to Dennis Bamford.

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