Interference with Bacteriophage $\phi 6$ Genomic RNA Packaging by Hairpin Structures

XUEYING QIAO, JIAN QIAO, AND LEONARD MINDICH*

Department of Microbiology, The Public Health Research Institute, New York, New York 10016

Received 6 April 1995/Accepted 30 May 1995

Bacteriophage $\phi 6$ has a genome of three segments of double-stranded RNA enclosed in a procapsid composed of four different proteins. The preformed procapsid is capable of packaging plus-strand transcripts of the genomic segments in an in vitro reaction. Minus-strand synthesis within the procapsid then results in the production of the double-stranded RNA genome. When plus-strand transcripts contain strong hairpin structures near the 3' ends, they are subject to heterologous recombination to remove the hairpins. We now find that the sequences bounded by the hairpins as well as those 3' to them are excluded from particles in packaging reactions. This finding implies that packaging occurs from the 5' end and that the explanation for the facilitation of recombination by the hairpin structures is the lack of entry of the 3' ends rather than a difficulty of progressing through the hairpin by the phage polymerase. Packaging of segment M is dependent on the packaging of segment S. An S segment containing a strong hairpin is able to facilitate the packaging of segment M. This result implies that there is more than one entry pore into the procapsid.

Bacteriophage $\phi 6$ has a genome composed of three segments of double-stranded RNA (15). Empty procapsids are able to package the plus-strand transcripts of the genomic segments in vitro (5) and to use them as templates for minus-strand synthesis, resulting in double-stranded RNA within the procapsids (6). The genome of $\phi 6$ can undergo heterologous recombination, which is the formation of genomic segments that have the 5' end of one of the normal segments and the 3' end of one of the other normal segments. The frequency of heterologous recombination is greatly increased when the 5' donor segment is packaged but has difficulty in serving as a template for minus-strand synthesis. Plus-strand transcripts that are missing their normal 3' ends are capable of being packaged by $\phi 6$ procapsids but do not serve as templates for minus-strand synthesis. They can be rescued by heterologous recombination. Plus-strand transcripts that contain very strong hairpin structures around engineered gene inserts are also poor templates for minus-strand synthesis. The inserts and their hairpin structures are removed at high frequency by heterologous recombination (9).

We have wondered why the hairpin structures were poor templates for minus-strand synthesis. Two alternative models are presented. In the first case, we propose that the hairpin structure cannot enter the procapsid during packaging. The 5' end, which contains the segment-specific packaging sequence (3), would be packaged, but the 3' end would remain outside. Thus, synthesis of minus strand on this template would be precluded because of the absence of the 3' end inside of the procapsid. The strand could be rescued by polymerase and nascent chain jumping from another strand onto this blocked template. The second model would entail the normal packaging of the strand with the hairpin structure. In this case, the polymerase would be unable to progress through the hairpin structure, and rescue would occur by polymerase jumping as in the first case. The first model can be tested by treating the

packaging complexes with RNase I. If the 3' end is outside the particle, it should be vulnerable to degradation.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Escherichia coli* JM109 [*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 λ^- Δ (lac-proAB)* (F' *traD36 proAB lacI^q Δ M15*)] (16) was used for the propagation of all plasmids. Plasmid pLM450 contains a cDNA copy of genomic segment L of the virus $\phi 6$, encoding the four procapsid proteins (4). Plasmids pLM658, pLM656, and pLM682 contain cDNA copies of the genomic segments S, M, and L, respectively, in the pT7F3 19U vector (10), and the other plasmids are derivative of these plasmids (Table 1). *lacH* is a longer version of *lac α* and results in much greater activity in pseudomonads. The *lacH* insert contains 427 bp, and the hairpin structures of pLM844 and pLM852 are produced by homopolymer tailing (12). The hairpin in pLM1286 was constructed by annealing oligonucleotide olm162 (GATATCCCCCCCCCCCCCCCCCCC) to a derivative of pLM659 in which the *ClaI* site was changed to *PstI* and then tailed with dGTP (14). The hairpin characteristics are listed in Table 1.

Preparation of procapsids. Procapsids were isolated from *E. coli* JM109 containing plasmid pLM450. They were purified from French press lysates as described by Gottlieb et al. (4) except that the sucrose gradients contained 3 mM dithiothreitol. Purified procapsids were divided into aliquots and frozen at -70°C . Aliquots were thawed immediately prior to use.

In vitro synthesis of radioactive plus-sense transcripts by T7 polymerase. Plasmids were cut with restriction endonuclease *XbaI*. The resulting 5' overhang was removed with mung bean nuclease before transcription with T7 RNA polymerase (10). This procedure generated 3' ends of the transcripts identical to that of the $\phi 6$ virus-produced mRNA. The polymerase reaction mixture contained 2 mM each UTP, ATP, GTP, and CTP and 400 μCi of [α - ^{32}P]UTP per ml. The RNA was purified by filtration through Sephadex G-50 spin columns (Boehringer Mannheim).

RNA polymerase reaction conditions. Conditions for minus-strand synthesis were similar to those reported previously (5) except that the pH of the reaction buffer was 8.9 (2). Equimolar amounts of each segment were used, and the total RNA concentration was about 100 $\mu\text{g}/\text{ml}$ in a 25- μl reaction volume. Ten microliters of stop solution ($3\times$ sample buffer, 1 μg of carrier RNA, 25 mM EDTA) was added to 25- μl samples, and the mixture was heated at 85°C for 5 min. The reaction products were analyzed on a 1% agarose gel containing 0.1% sodium dodecyl sulfate in $0.5\times$ Tris-borate-EDTA buffer (7). Radioactivity in gel bands was determined by visualization and counting in a Packard Instant Imager electronic autoradiography device.

Packaging reaction conditions. Frozen purified procapsid preparations (6) produced in *E. coli* JM109 carrying plasmid pLM450 were thawed and incubated at 28°C in a 12.5- μl packaging reaction mixture consisting of 50 mM Tris-Cl (pH 8.9), 3 mM MgCl_2 , 100 mM ammonium acetate, 20 mM NaCl, 5 mM KCl, 5 mM dithiothreitol, 0.1 mM Na_2EDTA , 1 mM ATP, 0.1 mg of macaloid, 5% polyethylene glycol 4000, and about 150 ng of [^{32}P]UTP-labeled single-stranded $\phi 6$ RNA for each segment. Approximately 1 μg of procapsid was used per reaction. The samples were then treated with 10 U of RNase I (RNase One; Promega) (8) for 30 min at 28°C . Ten microliters of stop solution (see above) was added, and

* Corresponding author. Mailing address: Public Health Research Institute, 455 First Ave., New York, NY 10016. Phone: (212) 578-0845. Fax: (212) 578-0804. Electronic mail address: mindich@phri.nyu.edu.

TABLE 1. cDNA plasmids

Plasmid	Segment	Remarks	Source or reference
pT7T3 19U		Amp, P _{T7} , P _{T3} , <i>lacZ</i>	Pharmacia
pLM656 or -655	M	Amp, P _{T7} ; exact copy of segment M in pT7T3 19U	11
pLM658 or -659	S	Amp, P _{T7} ; exact copy of segment S in pT7T3 19U	2
pLM682	L	Amp, P _{T7} ; copy of segment L in pT7T3 19U	2
pLM844	M	pLM655 with <i>lacH</i> bounded by homopolymer arms in <i>PstI</i> site; 21 G's on the 5' side and 14 C's on the 3' side	12
pLM847	M	pLM655 with <i>lacH</i> bounded by <i>PstI</i> site	12
pLM852	S	pLM659 with <i>lacH</i> bounded by homopolymer arms in <i>PstI-ClaI</i> site; 21 G's on the 5' side and 27 C's on the 3' side	This study
pLM886	S	pLM659 with <i>lacH</i> bounded by <i>PstI-ClaI</i> site	This study
pLM1157	L	pLM682 with <i>XhoI</i> deletion; transcript is 1.5 kb	13
pLM1286	S	pLM659 with hairpin in <i>PstI-ClaI</i> site; 25 G's on the 5' side and 30 C's on the 3' side	This study

the samples were heated at 85°C for 5 min. The samples were then electrophoresed in 1.5% agarose gels. Note that segment L is the smallest segment in the packaging reactions. We use a segment with an internal deletion because its packaging efficiency is higher than that of the normal L segment. This transcript is produced by plasmid pLM1157 (Table 1).

RESULTS

Plasmid pLM847 is a template for an exact copy of the plus strand of genomic segment M with the insertion of a *lacH* gene in the *PstI* site, which is in the 3' noncoding region (Fig. 1). This transcript has been incorporated into phage by transfection, and the resulting virus forms blue plaques on a suitable indicator lawn (12). The construction is genetically stable. Plasmid pLM844 is similar to pLM847, but it has 21 G's on the 5' side of the *lac* insert and 14 C's on the 3' side. Phage that contain an M segment with the sequence of pLM844 are genetically unstable, with over half of the plaques having lost the *lac* insert (12). This loss is due to heterologous recombination. A similar set of observations was found for the pair of plasmids pLM886 and pLM852, which produce copies of segment S

(Table 1). These plasmids have *lac* inserts in the *ClaI* site of segment S. Plasmid pLM1286 has a hairpin with 25 G's on the 5' side and 30 C's on the 3' side of an *EcoRV* sequence. It therefore contains a strong hairpin without a large loop.

Plus-strand transcripts of normal genomic segments as well as those containing inserts were tested for the ability to support minus-strand synthesis in procapsids. Under normal conditions, minus-strand synthesis requires the packaging of plus-strand copies of all three genomic segments. There is some leakage in this control (Fig. 2, lanes 2 and 3); in comparison, all three normal segments were present in lane 4. A segment that has lost its 3' end can be packaged and can stimulate minus-strand synthesis of the other two segments while not itself serving as a template for minus-strand synthesis. We found that the plus strands containing simple *lac* inserts were able to serve their *trans* functions as well as to serve as templates for minus-strand synthesis (lanes 6 and 12, samples a and c). However, plus strands containing *lac* inserts bounded by the homopolymer tracts (lanes 8 and 14, samples b and d) were poor templates, although they performed their *trans* functions. In

FIG. 1. Map of the cDNA copies of the three genomic segments of ϕ 6. The three segments have 18 bases that are identical at the 5' end with the exception that the second base is U in segment L and is G in segments S and M. The packaging sequences are located near the 5' ends and reach to approximately position 270 in segment S, 305 in segment M, and 231 in segment L. The 75 nucleotides at the 3' ends are highly conserved but not identical. The *PstI* site in segment M and the *ClaI* site in segment S were the sites of the insertions of hairpin structures.

FIG. 2. Minus-strand synthesis on templates with and without hairpin structures. Transcripts of plasmids pLM659 (s), pLM656 (m), and pLM682 (l) are copies of $\phi 6$ genomic segments. Samples a, b, c, d, and e are transcripts of plasmids pLM847 (segment M with *lac* insert), pLM844 (segment M with *lac* insert bounded by hairpin), pLM886 (segment S with *lac* insert), pLM852 (segment S with *lac* insert bounded by hairpin), and pLM1286 (segment S with hairpin with six-base loop). High levels of minus-strand synthesis require packaging of all three segments, but there is some leakage, especially if segment L is present.

the case of the transcript of M with the hairpin-bounded *lac*, there is some minus-strand synthesis on the M template, consistent with this hairpin being weaker than those for segment S (Table 1). In the case of the transcript of S (sample d), there is no visible minus-strand synthesis on that template, but there is stimulation of minus-strand synthesis on the other two segments. The same is true for sample e (lane 16), which has a long hairpin but no *lac* insert.

The same transcripts were tested for sensitivity to RNase I after packaging. It should be noted that under the conditions of packaging used here, segment S can be packaged alone but the packaging of segment M requires simultaneous packaging of segment S (13). The RNA molecules that contained simple *lac* inserts (samples a and c) were not susceptible to RNase I after packaging (Fig. 3, lanes 10, 15, and 16). Their sizes were the same as those of untreated samples (asterisks, lanes 8 and 14). However, the RNA molecules that contained *lac* inserts with homopolymer tracts bounding them (samples b and d) were truncated by the RNase (lanes 13, 18, and 19). The truncated segment in lane 13 is difficult to discern, but it is easily seen in lanes 18, 19, 21, and 22. The same was true of sample e in lanes 20, 21, and 22, which has a hairpin without a

FIG. 3. Packaging of radioactive plus strands of copies of genomic segments. Radioactive transcripts of plasmids pLM659 (s), pLM656 (m), and pLM1157 (truncated homolog of l) were incubated with procapsids as described in Materials and Methods, then treated with RNase I, and applied to a 2% agarose gel. Samples a, b, c, d, and e are transcripts of plasmids pLM847 (segment M with *lac* insert), pLM844 (segment M with *lac* insert bounded by hairpin), pLM886 (segment S with *lac* insert), pLM852 (segment S with *lac* insert bounded by hairpin), and pLM1286 (segment S with hairpin with six-base loop). The lanes with asterisks are untreated samples that have not been packaged or treated with RNase I.

FIG. 4. Diagram of the packaging of normal RNA and a hairpin structure by procapsids. The diagram is not to scale, and the small secondary structure is the normal 3' end of the genomic segments. The upper pathway is that of a normal segment, and the lower pathway is that of a hairpin structure.

lac insert. The sizes of the packaged RNA were consistent with the entire hairpin and material 3' to it being degraded by the RNase treatment. Note that the truncated S segment has the same size in lanes 18, 19, 21, and 22, indicating that the digestion removes the entire hairpin, including the *lac* insert. It should be noted that the hairpin in segment M is weaker than those in segment S. This difference is manifested in the ability of the M construct to serve as a template for minus-strand synthesis. The M construct is also the one that yields normal phage as well as recombinants (12). The constructions in S yield only recombinants.

DISCUSSION

Complementary homopolymer tracts bounding either a kanamycin resistance gene or a *lacH* gene were found to increase the frequency of heterologous recombination in $\phi 6$ by over 1,000-fold (9, 12). Deletion of one of the arms led to stabilization of the inserts, suggesting that strong hairpin structures were the cause of the increase in frequency. Two very different models could be proposed to explain these findings. The first would be that the hairpin cannot be taken through the pores through which packaging occurs in the viral procapsid. This would result in the packaging of the 5' end of the plus strand. The 3' end would be on the outside of the procapsid and subject to degradation. The second possibility would be that the entire segment is packaged but that the polymerase cannot move through the strong hairpin structure. In either case, minus-strand synthesis on a heterologous segment could jump to the 5' side of the hairpin, resulting in a heterologous recombination.

In this study, we have found that the first model is validated. The second is also possible, but the first is sufficient to explain our results. RNase treatment of packaging complexes results in molecules truncated at the position of the hairpin structures (Fig. 4). Molecules of the same size but lacking the hairpins are packaged intact. The loop material does not seem to be of consequence, since we find that a hairpin structure without an appreciable loop is truncated similarly to that found with the *lac* inserts. The size, and therefore the strength, of the hairpin structure is an important factor in whether it enters the procapsid. The transcript of pLM852 has 21 G's and 27 C's and does not enter at all. The same is true for the transcript of pLM1286, which does not have an insert but has 25 G's and 30 C's. The transcript of pLM844 is only partially truncated, and this RNA does produce live, albeit unstable phage (12), which

means that some proportion of the RNA must enter procapsids.

The results of this experiment answer several other questions as well. Since the 5' ends of the segments are protected, it seems that packaging must proceed from the 5' end. This would tend to argue against packaging models in which the 5' end would bind to the exterior of the procapsid and a loop of the RNA would enter. Such a model has been proposed for DNA packaging in bacteriophages (1). Since the packaging of segment S supports the packaging of segment M, even though the complete entry of S is blocked by the hairpin, there must be more than one portal through which packaging can occur. The results of this study also add support to the contention that genomic packaging in $\phi 6$ is via a pore in a preformed procapsid (6). The current view of $\phi 6$ procapsid structure is a dodecahedron with 12 pentagonal faces, each of which could contain a packaging pore. The dimensions of the hairpins prepared in this study, either with or without their inserts, are such that they might very well be visible in electron micrographs.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants GM-31709 and GM-34352 awarded to L.M.

REFERENCES

1. Black, L. W. 1988. DNA packaging in dsDNA bacteriophages, p. 321–373. In R. Calendar (ed.), *The bacteriophages*, vol. 2. Plenum Publishing Corporation, New York.
2. Frilander, M., P. Gottlieb, J. Strassman, D. H. Bamford, and L. Mindich. 1992. Dependence of minus-strand synthesis upon complete genomic packaging in the double-stranded RNA bacteriophage $\phi 6$. *J. Virol.* **66**:5013–5017.
3. Gottlieb, P., X. Qiao, J. Strassman, M. Frilander, and L. Mindich. 1994. Identification of the packaging regions within the genomic RNA segments of bacteriophage $\phi 6$. *Virology* **200**:42–47.
4. Gottlieb, P., J. Strassman, D. H. Bamford, and L. Mindich. 1988. Production of a polyhedral particle in *Escherichia coli* from a cDNA copy of the large genomic segment of bacteriophage $\phi 6$. *J. Virol.* **62**:181–187.
5. Gottlieb, P., J. Strassman, A. Frucht, X. Qiao, and L. Mindich. 1991. In vitro packaging of the bacteriophage $\phi 6$ ssRNA genomic precursors. *Virology* **181**:589–594.
6. Gottlieb, P., J. Strassman, X. Qiao, A. Frucht, and L. Mindich. 1990. In vitro replication, packaging, and transcription of the segmented double-stranded RNA genome of bacteriophage $\phi 6$: studies with procapsids assembled from plasmid-encoded proteins. *J. Bacteriol.* **172**:5774–5782.
7. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
8. Meador, J., B. Cannon, V. J. Cannistraro, and D. Kennell. 1990. Purification and characterization of *Escherichia coli* RNase I: comparisons with RNase M. *Eur. J. Biochem.* **187**:549–553.
9. Mindich, L., X. Qiao, S. Onodera, P. Gottlieb, and J. Strassman. 1992. Heterologous recombination in the double-stranded RNA bacteriophage $\phi 6$. *J. Virol.* **66**:2605–2610.
10. Olkkonen, V. M., P. Gottlieb, J. Strassman, X. Qiao, D. H. Bamford, and L. Mindich. 1990. In vitro assembly of infectious nucleocapsids of bacteriophage $\phi 6$: formation of a recombinant double-stranded RNA virus. *Proc. Natl. Acad. Sci. USA* **87**:9173–9177.
11. Onodera, S., V. M. Olkkonen, P. Gottlieb, J. Strassman, X. Qiao, D. H. Bamford, and L. Mindich. 1992. Construction of a transducing virus from double-stranded RNA bacteriophage $\phi 6$: establishment of carrier states in host cells. *J. Virol.* **66**:190–196.
12. Onodera, S., X. Qiao, P. Gottlieb, J. Strassman, M. Frilander, and L. Mindich. 1993. RNA structure and heterologous recombination in the double-stranded RNA bacteriophage $\phi 6$. *J. Virol.* **67**:4914–4922.
13. Qiao, X., G. Casini, J. Qiao, and L. Mindich. 1995. In vitro packaging of individual genomic segments of bacteriophage $\phi 6$ RNA: serial dependence relationships. *J. Virol.* **69**:2926–2931.
14. Roychoudhury, R., E. Jay, and R. Wu. 1976. Terminal labeling and addition of homopolymer tracts to duplex DNA fragments by terminal deoxynucleotidyl transferase. *Nucleic Acids Res.* **3**:101–116.
15. Semancik, J. S., A. K. Vidaver, and J. L. Van Etten. 1973. Characterization of a segmented double-helical RNA from bacteriophage $\phi 6$. *J. Mol. Biol.* **78**:617–625.
16. Yanisch-Perron, C., J. Vieira, and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**:103–119.