JOURNAL OF VIROLOGY, May 1995, p. 2926–2931 Vol. 69, No. 5
0022-538X/95/$04.00+0

In Vitro Packaging of Individual Genomic Segments of Bacteriophage ϕ6 RNA: Serial Dependence Relationships

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Received 22 November 1994/Accepted 25 January 1995

Bacteriophage ϕ6 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. The packaging of the three segments shows a strong order of dependence in that segment S packages alone, but segment M requires S and segment L requires S and M for efficient packaging. Packaging of individual segments is dependent on unique packaging sequences of about 200 nucleotides near the 5′ ends of the segments. Deletions that invade these regions destroy packaging competence for the particular segment and for the dependent segments as well. In the presence of 2 mM phosphate and at magnesium ion concentrations above 4 mM, packaging becomes progressively more independent and ultimately nonspecific with respect to ϕ6 sequences.

Bacteriophage ϕ6 has a genome composed of three segments of double-stranded RNA (14). Empty procapsids are able to package the plus-strand transcripts of the genomic segments (7) and to use them as templates for minus-strand synthesis, resulting in double-stranded RNA within the procapsids (9). The efficiency of plating of the virus is close to unity, indicating that the distribution of the genomic segments is very precise (2). We have shown that preformed procapsids of ϕ6 are capable of packaging the three plus-strand precursors of the double-stranded genomic segments in vitro (9). One of the primary mechanisms responsible for this precise stoichiometry is the requirement for packaging of one representative of each of the three segments before minus-strand synthesis begins (4, 8).

Our earlier studies on in vitro packaging had found that individual plus strands could be packaged independently of the packaging of the other strands. In this study we find that there is indeed a dependent relationship in packaging. We have now found that, in the presence of phosphate, the concentration of magnesium ions profoundly affects the dependency and specificity of packaging. At concentrations above 4 mM, packaging is progressively more independent and nonspecific; at lower concentrations, packaging is dependent and specific.

Viral RNAs have been found to possess unique sequences that determine the specificity of packaging (5). In ϕ6 the packaging sequences have been localized to about 300 nucleotides near the 5′ ends of the plus strands (Fig. 1). Since minus-strand synthesis depends on the packaging of all three segments, we have used this reaction as an indicator of packaging (5). However, that test might involve additional requirements beyond packaging. In this study, we demonstrate that the same sequence requirements hold for both packaging and minus-strand synthesis, *cis* and *trans*.

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} ΔM15)] (17) was used for the propagation of all plasmids. Plasmid pLM450 contains a cDNA copy of genomic segment L of the virus ϕ6, encoding the four procapsid proteins (6). Plasmids pLM659, pLM656, and pLM682 contain cDNA copies of the genomic segments S, M, and L, respectively, in the pT7T3 19U vector (12). Plasmid pLM1157 contains a cDNA copy of segment L with the deletion of the *Xho*I fragment from positions 545 to 5366. Its transcript is about 1.5 kb in size and is packaged more efficiently than the normal L segment in vitro. Plasmids used for the production of plus strands with deletions near the boundaries of the packaging sequences are listed in Tables 1 and 2 and in reference 5.

Preparation of procapsids. Procapsids were isolated from *E. coli* JM109 containing plasmid pLM450. The procapsids were purified from French press lysates produced at 7,000 lb/in^2 by the method of Gottlieb et al. (6) except that the sucrose gradients contained 3 mM dithiothreitol in addition to the standard 10 mM phosphate and 1 mM magnesium. 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 derived from pLM656, pLM659, pLM1157, and pLM682 were cut with restriction endonuclease *Xba*I. The resulting 5′ overhang was removed with mung bean nuclease before transcription with T7 RNA polymerase (12). This procedure generated 3′ ends of the transcripts identical to that of the ϕ6 virus-produced mRNA. The polymerase reaction mixture contained a 2 mM concentration (each) of UTP, ATP, GTP, and CTP and 400 μCi of α-^{32}P-labeled UTP per ml. The RNA was purified by being filtered through G-50 Sephadex spin columns (Boehringer Mannheim).

RNA polymerase reaction conditions. Conditions for minus-strand synthesis were similar to those reported previously (7), except that the pH of the reaction buffer was 8.9 (4). Equimolar amounts of each segment were used, and the total RNA concentration was about 100 μg/ml in a 25-μl reaction mixture volume. The reaction was stopped by adding 3× sample buffer (15) and EDTA to final concentrations of 10 mM. The reaction products were analyzed with 1 μg of carrier RNA on a 2% acrylamide–0.5% agarose composite gel (15) that was subsequently dried, and the ^{32}P-labeled RNA was visualized after autoradiography with a Cronex enhancing screen. In some experiments the RNA was analyzed on 1.5% agarose gels containing 0.1% sodium dodecyl sulfate in 0.5× Tris-borate-EDTA buffer (10). Radioactivity in gel bands was determined by visualization and counting in a Packard Instant Imager electronic autoradiograph device or by densitometry of the autoradiographic film with a Hewlett-Packard scanner with Adobe Photoshop and subsequent transfer to NIH Image 1.45.

Packaging reaction conditions. Frozen purified procapsid preparations produced in *E. coli* JM109 carrying plasmid pLM450 (9) were thawed and incubated for 90 min at 28°C in a 12.5-μl packaging reaction mixture consisting of 50 mM Tris-Cl (pH 8.9), 3 or 4 mM $MgCl_2$, 100 mM NH_4O acetate, 20 mM NaCl, 5 mM KCl, 5 mM dithiothreitol, 0.1 mM Na_2EDTA, 1 mM ATP, 100 ng of macaloid, 5% polyethylene glycol 4000, and about 150 ng of [^{32}P]UTP-labeled single-stranded ϕ6 RNA for each segment. Approximately 1 μg of procapsid was used per reaction mixture. The procapsid samples contribute 2 mM phosphate to the packaging reaction mixtures. The samples were then treated with 10 U of RNase I (RNase One; Promega) (11) and incubated for 30 min at 28°C. Ten microliters of stop solution (3× sample buffer [15], 1 μg of carrier RNA, 25 mM EDTA) was added, and the samples were heated at 85°C for 5 min. The samples were then electrophoresed in 1.5 or 2% agarose gels as described above. All of the genomic packaging results reported in this study were obtained by this method.

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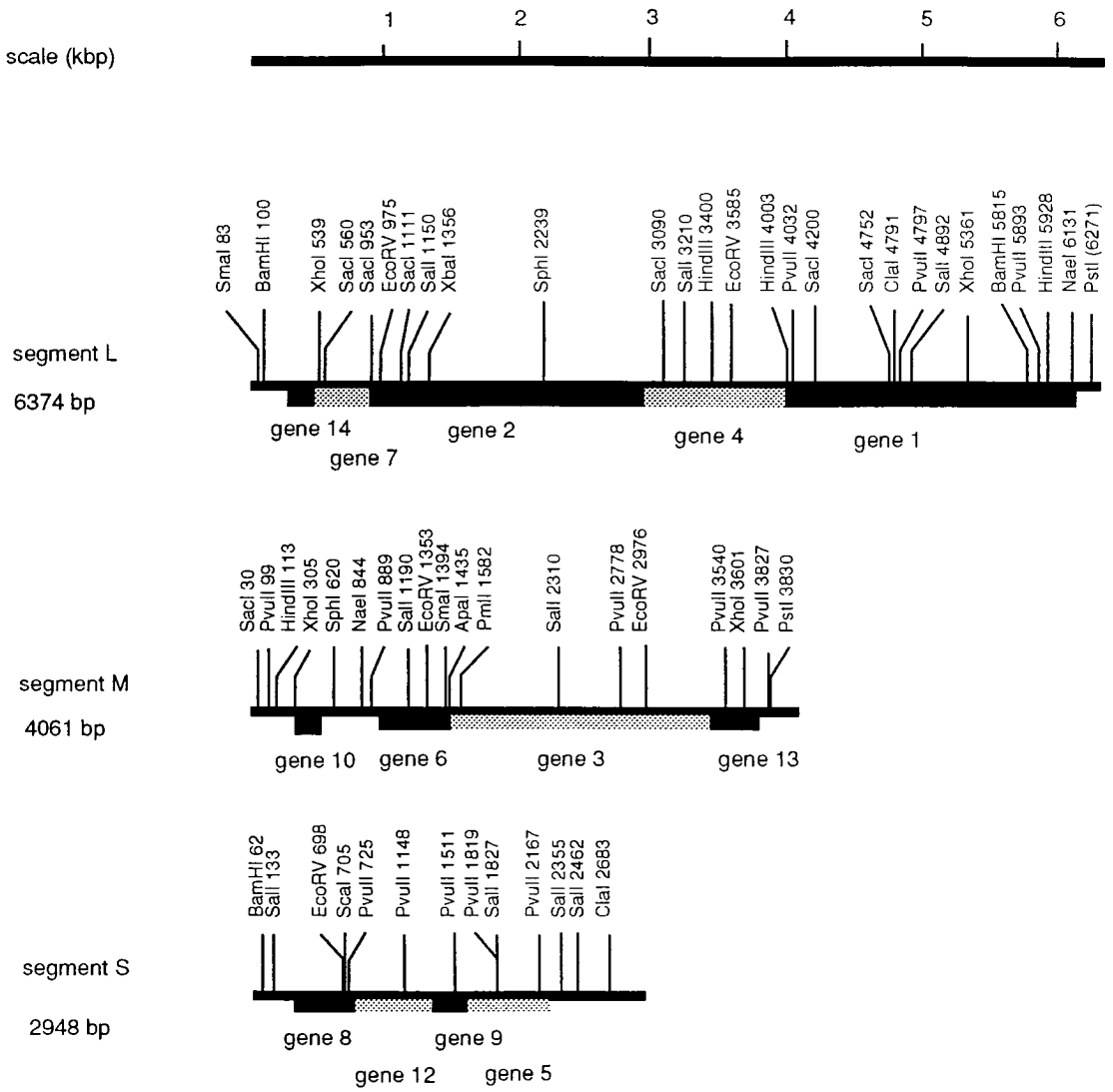

FIG. 1. Restriction map of the cDNA copies of the three genomic segments of ϕ6. The three segments have 18 bases that are identical at the 5′ end, with the exception that the second base is U in segment L and is G in segments S and M. The packaging sequences are located near the 5′ ends and reach to 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.

RESULTS

Procapsids were isolated from cultures of *E. coli* JM109 that were carrying plasmid pLM450, which contains a cDNA copy of the entire L segment. These procapsids were used to study the packaging of the ϕ6 genomic segments. We had previously found that the packaging of each genomic segment was independent of the packaging of the other two segments (8). In these studies we have found that, in the presence of 2 mM phosphate, the concentration of magnesium ions in the packaging reaction mixture strongly influences the dependence of the packaging of segments M and L on the packaging of segment S. At magnesium concentrations below 4 mM, a strong dependent relationship exists. Figure 2 shows the packaging of radioactive plus strands of segments S and M and a reduced-size segment L, alone and in all combinations. The in vitro packaging of the normal-size L segment plus strand is usually very much less than that of S and M. It appears that only about 10% of the expected packaging is seen. It might be that the in

TABLE 1. cDNA plasmids used in this study

Plasmid	Segment	Remarks	Reference or source
pT7T3 19U		Amp P_{T7} P_{T3} *lacZ*	Pharmacia
pLM656	M	Amp P_{T7}. Exact copy of segment M in pT7T3 19U	13
pLM659	S	Amp P_{T7}. Exact copy of segment S in pT7T3 19U	4
pLM682	L	Amp P_{T7}. Copy of segment L in pT7T3 19U	4
pLM608	None	Amp P_{T7}. *lacZ* in pT7T3 19U. Transcript, 2 kb	This study

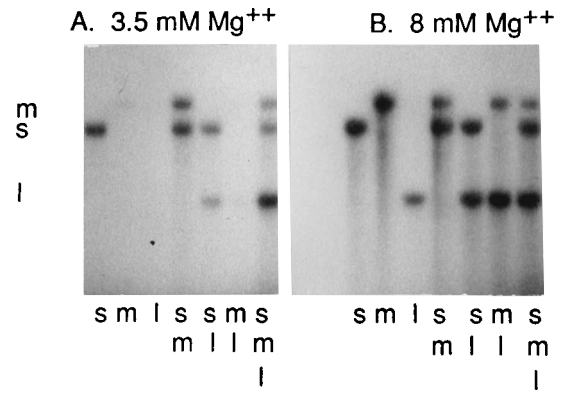

FIG. 2. Packaging of radioactive plus strands of exact copies of genomic segments S and M and of a truncated segment L. Radioactive transcripts of plasmids pLM659 (s), pLM656 (m), and pLM1157 (l) were incubated with procapsids as described in Materials and Methods and then treated with RNase I and applied to a 2% agarose gel. Samples on the left were incubated at 3.5 mM $MgCl_2$, and those on the right were incubated at 8 mM $MgCl_2$.

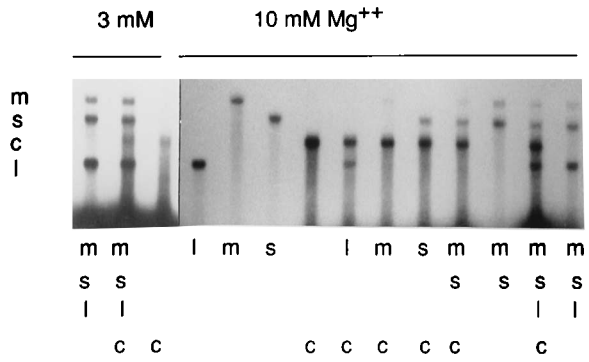

FIG. 4. Packaging of radioactive plus strands. Radioactive transcripts of plasmids pLM659 (s), pLM656 (m), and pLM1157 (l) were incubated with procapsid in various combinations with and without the transcript of pLM608 (c), which contains no ϕ6 sequences. The concentration of the pLM608 transcript is five times that of the specific transcripts. The autoradiogram for the samples incubated at 3 mM magnesium was incubated for 15 times as long as that for the samples incubated at 10 mM magnesium.

vitro system has difficulty in packaging such a large molecule. We therefore used a truncated homolog of segment L (pLM1157) in Fig. 2 and 3. At 3.5 mM magnesium, it appears that segment S can be packaged alone, whereas segment M requires copackaging of segment S and segment L requires copackaging of segments S and M. At a magnesium concentration of 8 mM the packaging of segment M and that of segment L are independent. The dependent relationship in packaging is demonstrable at RNA concentrations 10-fold lower than that used in Fig. 2 (results not shown). This suggests that the dependent packaging is highly efficient in packaging the three segments together.

The response of packaging behavior to magnesium concentration is not discontinuous; rather, it is progressive. The packaging of segments M and L at various magnesium concentrations is shown in Fig. 3. There is little packaging of L at 3 and 4 mM, but at 6 mM some independent packaging of L is seen. At 8 mM magnesium, L is packaged better independently than with the other segments. Segment M shows progressively more and more independent packaging with increasing magnesium concentration. At 8 mM magnesium, M is also packaged better independently than with the other segments. We have also examined the packaging behavior of RNA molecules with partial packaging sequences or lacking any ϕ6 sequence at all. We found that there is progressively more and more packaging of nonspecific RNA as the magnesium concentration rises; at concentrations of 10 mM there is as much packaging of nonspecific RNA as there is of specific RNA (Fig. 4). The transcript of pLM608, which is 2 kb in size and has no ϕ6 sequence, as well as the ϕ6 segments, was packaged at 10 mM magnesium (Fig. 4). It competed effectively with the specific segments at the high magnesium concentration. It was packaged at less than 10% that of segment S at 3 mM magnesium and didn't show appreciable competition with normal packaging. The packaging of the pLM608 transcript as well as that of the independent packaging of segment M at high magnesium concentrations was ATP dependent. This precludes arguments that the RNase protection is due to a condition other than packaging. In Fig. 5, we show the packaging of the three normal segments at 4 mM magnesium. The dependence of M on S can be seen very clearly. There is some packaging of L in the presence of S alone, but the packaging of L is much greater when M is also packaged. The packaging of the normal L segment is barely discernible in Fig. 5 but is unambiguous in the original autoradiogram. The packaging of the normal-size segment L can be seen to be much less than that observed with the smaller homolog of segment L in Fig. 2, 3, and 4.

The change in packaging specificity seen with higher magnesium concentrations is dependent on the presence of phosphate in the packaging reaction mixture. The threshold for the effect is about 1.5 mM phosphate; our reaction mixtures contain about 2 mM phosphate. In the absence of phosphate, the

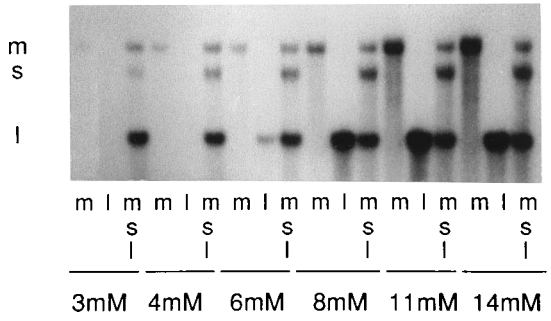

FIG. 3. Packaging of plus strands at increasing magnesium ion concentrations. Radioactive transcripts as described in the legend to Fig. 2 were incubated with procapsids at the magnesium concentrations shown below the lanes.

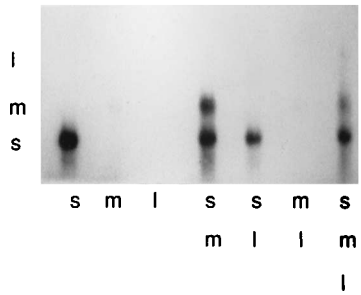

FIG. 5. Packaging of radioactive plus strands of exact copies of genomic segments. Radioactive transcripts of plasmids pLM659 (s), pLM656 (m), and pLM682 (l) were incubated with procapsids as described in Materials and Methods and then treated with RNase I and applied to a 1.5% agarose gel.

TABLE 2. Deletion plasmids used in this study

Deletion plasmid[a]	Segment	Position of deletion	Size (bases) of transcript	Minus-strand synthesis
pLM922	S	150–1160	1937	−
pLM919	S	234–1214	1967	−
pLM914	S	270–1181	2036	+/−
pLM918	S	356–1198	2105	+
pLM1299	S	63–66	2944	−
pLM794	M	11–43	4030	−
pLM786	M	23–70	4015	−
pLM723	M	34–170	3926	−
pLM722	M	104–123	4043	−
pLM1166	M	113–122	4053	−
pLM1297	M	114–117	4057	−
pLM861	M	237–860	3439	−
pLM868	M	268–328	4002	−
pLM1157	L	545–5366	1554	+

[a] See reference 5.

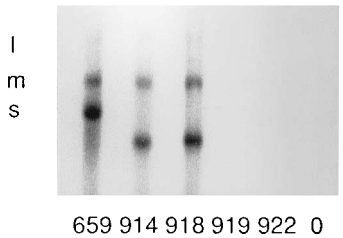

FIG. 6. Packaging of radioactive plus strands with transcripts of segment S that have deletions near the 5′ end. All the lanes contain plus strands of segments M (m) and L (l). The numbers below the lanes refer to the plasmid template for preparation of segment S. pLM659 is the normal S (s) transcript. pLM914 contains a deletion from bases 270 to 1181. pLM918 contains a deletion from bases 356 to 1198, while plasmid pLM919 has a deletion starting at position 234, and pLM922 has a deletion starting at position 150. Magnesium concentration, 4 mM.

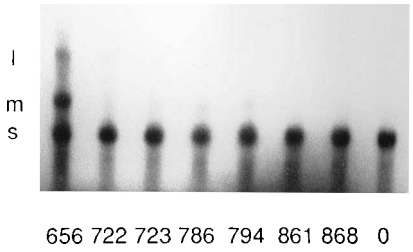

FIG. 7. Packaging of radioactive plus strands with transcripts of segment M (m) that have deletions near the 5′ ends. All the lanes contain plus strands of normal segments S (s) and L (l). The numbers below the lanes refer to the plasmid template for preparation of segment M. pLM656 is the normal M transcript. pLM722 contains a deletion from bases 104 to 123. pLM723 contains a deletion from bases 34 to 170, while plasmid pLM786 has a deletion from positions 23 to 70, and plasmid pLM794 has a deletion from positions 11 to 43. These transcripts all have deletions coming in from the 5′ side of the packaging sequence. Plasmid pLM861 has a deletion from positions 237 to 860, and pLM868 has a deletion from positions 268 to 328. Magnesium concentration, 4 mM.

packaging specificity does not change with increasing magnesium concentration (results not shown).

Genomic packaging in φ6 is dependent on specific sequences near the 5′ ends of the plus strands (5). These sequences had been identified primarily on the basis of an assay of minus-strand synthesis. φ6 procapsids normally support minus-strand synthesis on the plus-strand templates only when all three segments are packaged (8). That is to say, a segment that is incapable of being packaged because of the lack of a packaging sequence will not stimulate minus-strand synthesis on the heterologous templates. In this study we have examined the packaging of the sets of deletion constructions in segments S and M by assaying the RNase protection of these molecules as well as that of the heterologous segments. The packaging sequence of segment S had been defined as having its 3′ limit near nucleotide 270 (5) (Table 2). A transcript having the 5′ region up to nucleotide 270 (pLM914) gave stimulation of minus-strand synthesis, but one with sequence up to nucleotide 234 (pLM919) did not (5). When assayed by RNase protection, the transcript of plasmid pLM914 was seen to have been protected, whereas the transcript of plasmid pLM919 was not protected. Moreover, the packaging of normal segment M did not occur when segment S had only 234 nucleotides of the 5′ packaging sequence (Fig. 6). A deletion of only four nucleotides in the *Bam*HI site of S at position 62 also resulted in the absence of packaging of the transcript as well as that of normal middle and large segments (not shown).

Similarly, with transcripts homologous to segment M, but having deletions reaching nucleotide 268, the segment M homologs were not packaged, nor was normal segment L (Fig. 7). This result is consistent with our previous finding that this deletion did not support minus-strand synthesis. The packaging sequence in M was defined as reaching nucleotide 305 on the basis of minus-strand synthesis (5). In addition, deletions on the 5′ side of the packaging sequence that did not support minus-strand synthesis (5) also did not show packaging of themselves or of segment L (Fig. 7; Table 2). The transcript of pLM786 which is missing nucleotides 23 to 70 in segment M does not support minus-strand synthesis and is not itself packaged. Deletions that are internal to the packaging sequence have also been examined. The transcript of pLM722 is missing nucleotides 104 to 127 and shows very poor packaging of itself and intact segment L. The transcripts of pLM1166 and pLM1297 which are missing eight nucleotides from 114 to 121 or four nucleotides from 115 to 118 also show poor packaging of themselves and intact segment L (results not shown). It appears that the packaging sequences are highly specific despite the fact that they include several hundred nucleotides. This suggests that there are many contacts between the RNA and procapsid and/or with other RNA molecules.

DISCUSSION

Earlier studies on the genomic packaging of φ6 had shown that individual segments could be packaged independently of each other (8). These results were somewhat puzzling in that they implied that there existed independent binding sites for the three genomic segments. Yet, there was the suggestion of some sort of interaction, because minus-strand synthesis did not begin until all three segments were packaged. We also found that the stringency of the packaging pattern was variable. The laboratory of Dennis Bamford has found a pattern of partial dependence in packaging in which S and M are packaged independently but L is dependent on M (3).

We now find that a strong dependent relationship exists in packaging, with segment S packaging alone, packaging of segment M dependent on segment S, and that of segment L dependent on both S and M. We can reconcile our new findings with our previous findings as well as with those of the Bamford laboratory. In a collaborative study done in our laboratory with the participation of Mikko Frilander of the Bamford laboratory, we have found that the differences in their

procapsid preparations and ours were not great and that their preparations do show a dependence of M packaging on that of S, although to a lesser extent than seen in ours. Two conditions distinguish our preparations from those of the Bamford laboratory. Our preparations are made in low-salt buffers, whereas those of the Bamford laboratory are in 150 mM NaCl. The difference in the extent of dependence seems to be due primarily to their use of a Triton X-114 treatment after the French press lysis of the cells containing the procapsids. This results in procapsids with less dependence in the packaging of M on the packaging of S. Exposure to Triton X-114 is not sufficient to change the behavior of the procapsids. In addition, the procapsids in our laboratory are purified through sucrose gradients containing phosphate. The phosphate in the packaging assays results in a condition under which packaging becomes more and more nonspecific as the magnesium concentration increases. At magnesium concentrations higher than 4 mM, we find the results that were reported previously (8). At concentrations below 4 mM, we find that packaging shows strong dependence. Segment S packages alone, packaging of segment M depends on segment S, and that of segment L depends on both S and M. At the higher magnesium concentrations, segment M and segment L are packaged independently. At the higher magnesium concentrations even nonspecific RNA is packaged, and this packaging is dependent on ATP.

We do not know, yet, how the packaging of S facilitates the packaging of M or how the packaging of L is facilitated. There might be an interaction between the RNA molecules or there might be a conformational change in the procapsid, or both. An important question is whether the situation in vivo is similar to that found in vitro with high or low magnesium concentrations. Since minus-strand synthesis requires packaging of all three segments, either condition would result in the proper stoichiometry in mature particles as long as packaging is reversible. A procapsid that had packaged nonspecific RNA at high magnesium concentrations could lose this RNA if specific RNA ultimately bound to the specific sites. A more telling argument in favor of the low-magnesium condition being the more accurate one is the finding that segments which have deletions in the packaging sequences that preclude packaging at 3 mM magnesium do package at 8 mM magnesium. However, minus-strand synthesis does not take place in *cis* or *trans* in such cases at 8 mM magnesium. This again implies that much of the packaging at high magnesium concentrations is not the true specific packaging.

Although it is not yet clear whether the high- or low-magnesium condition reflects the in vivo situation, we can suggest a benefit of the dependent packaging for the economy of infection. If the concentration of viral RNA in the cell is low relative to the number of procapsids, the distribution of RNA in procapsids would be random in the case of independent packaging. The onset of minus-strand synthesis requires packaging of all three segments in a particle; particles without a complete set of segments would not start minus-strand synthesis. In the dependent condition, packaging would result in the association of segments S, M, and L in the same particles, resulting in minus-strand synthesis at lower RNA concentrations.

We have previously described an interesting carrier state isolate that had lost segment S (13). In the carrier state, the genes of segment S are not necessary, since their products are not needed for RNA synthesis. However, the fact that segment S can be lost suggests that the packaging function of S can be dispensed with for the packaging of M and L and/or the need for packaging of all three segments for minus-strand synthesis can be dispensed with. It is likely that the cells with this unique carrier state have incurred mutations in the viral genome that would enable this loss of stringency, but the mutation(s) or its nature has not been characterized.

Our working model for the packaging of genomic segments proposes that entry into the particle is ATP dependent but nonselective. This is a major distinction between genomic packaging in ϕ6 and large double-stranded DNA-containing bacteriophages (1). In the latter case, the specificity mechanism is located at the outside of the procapsid, and selectivity is exerted at the level of entry. We propose that selectivity in ϕ6 is determined inside the procapsid. At low magnesium concentrations, segment S binds to a protein site in the procapsid with a high affinity. Segments M and L do not bind and do not remain in the particles when packaged alone; neither do nonspecific RNA molecules. If segment S is packaged, then segment M can bind to it or to a combination of S RNA and a protein site. If S and M are packaged, then L can bind as well. Nonspecific RNA is not retained even when specific RNAs are present. At higher magnesium concentrations, in the presence of phosphate, segments S, M, and L can be retained in procapsids, but this is not specific, and only segment S is bound at a high affinity if present alone. If segment S is present, then segment M binds at a high affinity as well as being retained nonspecifically. The binding of segment L is dependent on both segment S and segment M in the same way. Its retention at high magnesium concentrations is also a combination of nonspecific retention as well as specific binding. Nonspecific RNA should be able to compete for the packaging of M and L when alone at high magnesium concentrations but not when packaged in the presence of S. This situation should result in proper packaging of the three segments even at high magnesium concentrations. It is suggested that there is some procapsid binding site activity for each of the segments but that high-affinity binding of M involves binding to S RNA as well and high-affinity binding of L involves binding to M RNA and perhaps to S RNA as well.

We do not know why the combination of 2 mM phosphate and a high magnesium concentration leads to the retention of nonspecific RNA in the procapsids. We consider two primary possibilities. The first is that this combination leads to a change in the conformation of the RNA inside the procapsid that prevents its exit. The second is that the combination results in a specific inhibition of the protein machinery involved in the efflux of RNA from procapsids. We are currently trying to distinguish between the two possibilities. In any case, we believe that the effect is a useful one in that it facilitates the investigation of the nonspecific entry of RNA into the procapsids. The concentration of phosphate that we have used is within the normal range of phosphate concentrations in bacteria. The phosphate concentration in *E. coli* has been estimated to be 4 mM (16).

ACKNOWLEDGMENTS

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

We are indebted to Mikko Frilander and Dennis Bamford for communicating their results to us before publication and for showing us that the packaging assay with RNase I works so well. We also appreciate their direct collaboration in resolving the differences between the preparations of the two laboratories.

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