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In Vitro Packaging and Replication of Individual Genomic Segments of Bacteriophage φ6 RNA

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The genome of bacteriophage φ6 contains three segments of double-stranded RNA. Procapsid structures whose formation was directed by cDNA copies of the large genomic segment are capable of packaging the three viral message sense RNAs in the presence of ATP. Addition of UTP, CTP, and GTP results in the synthesis of minus strands to form double-stranded RNA. In this report, we show that procapsids are capable of taking up any of the three plus-strand single-stranded RNA segments independently of the others. In manganese-containing buffers, synthesis of the corresponding minus strand takes place. In magnesium-containing buffers, individual message sense viral RNA segments were packaged, but minus-strand replication did not take place unless all three viral single-stranded RNA segments were packaged. Since the conditions of packaging in magnesium buffer more closely resemble those in vivo, these results indicated that there is no specific order or dependence in packaging and that replication is regulated so that it does not begin until all segments are in place.

Bacteriophage φ6 infects the plant pathogen *Pseudomonas phaseolicola* (*Pseudomonas syringae* pv. phaseolicola) HB10Y (23). Its genome is composed of three separate pieces of double-stranded RNA (dsRNA) encapsidated within a polyhedral nucleocapsid (19). The segments are designated L (6,374 bp), M (4,061 bp), and S (2,948 bp) (11). The virus particles contain one copy of each segment (2). The nucleocapsid has RNA polymerase activity (3, 17). Transcription of the dsRNA is semiconservative (21, 22). In the assembly pathway, a polyhedral procapsid composed of proteins P1, P2, P4, and P7 is constructed and is then filled with one copy each of the three single-stranded RNA (ssRNA) genomic segments (2, 13). The ssRNA serves as template for the synthesis of minus strands to form dsRNA (4). Filled procapsids are then covered with a shell of protein P8, and this resulting nucleocapsid is subsequently enveloped within a lipid-containing membrane (12).

Cloned cDNA copies of the genomic segment L have been used to direct the synthesis of proteins P1, P2, P4, and P7, the components of the φ6 procapsid (5). These proteins assemble in both *Escherichia coli* and *P. phaseolicola* to form polyhedral particles that appear structurally identical to the natural viral procapsids. The procapsids are capable of in vitro packaging of plus-sense ssRNA derived from viral transcription reactions. In the presence of a single nucleotide triphosphate (NTP), the RNA is packaged but not replicated (6). In the presence of all four NTPs, the packaged RNA serves as template for minus-strand synthesis to form dsRNA. The procapsids that have packaged and replicated viral RNA can be coated with purified viral protein P8 to form nucleocapsids that are infectious to spheroplasts of HB10Y (16).

In this report, we further probe the mechanisms of viral packaging and replication. The virus must be able to package RNA almost perfectly, since the efficiency of plating is 1 and it contains one each of the three genomic segments (2). Accurate packaging could be ensured by the procapsids having independent packaging sites for each of the three segments, or it could have a single site for one segment whose packaging would form a site for the second segment, which would form a site for the third. This model might entail a specific order of packaging of the genomic segments. If that were so, one might expect that the packaging of particular segments would be dependent upon the prior packaging of others. The mechanism for the precise genomic packaging in segmented dsRNA viruses is unknown at the present time.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* JM109 [*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* λ⁻ Δ(*lac-proAB*) (F′ *traD36 proAB lacI*^{q} ΔM15)] (24) was used for the propagation of all plasmids. pLM450 contains a cDNA copy of the L genomic segment of φ6 encoding the four procapsid proteins (7). pLM656 contains a cDNA copy of the M segment of φ6 within the vector pT7T3 19U (16). pLM682 and pLM658 contain cDNA copies of the L and S genomic segments, respectively, within the pT7T3 19U vector.

Construction of plasmids pLM658 and pLM682. Plasmid pLM658 contains an exact cDNA copy of genomic segment S in the T7 promoter plasmid pT7T3 19U. The cDNA was taken from plasmid pLM279 (14). The cDNA copy of the genomic segment was modified through the polymerase chain reaction to remove the homopolymer ends that were present in pLM279, and the copy was inserted so that the first nucleotide of the T7 promoter transcript was identical to the first nucleotide of genomic segment S. The insert was cloned into the vector with an *Xba*I site positioned at the 3′ end so that after cutting and digestion with mung bean nuclease, the runoff transcript would have a 3′ end identical to that of the genomic segment. The transcript has been shown to have the complete biological activity of the natural segment (data not shown). Details of plasmid construction are available upon request.

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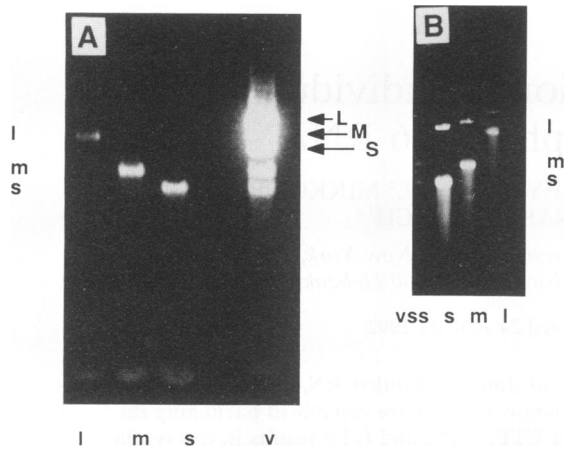

FIG. 1. (A) Isolation of ssRNA from a ϕ6 nucleocapsid transcription reaction. A 1% agarose gel stained with ethidium bromide shows single-stranded ϕ6 viral RNA message segments resolved by sucrose gradient fractionation. Lanes: 1, segment l; m, segment m; s, segment s; v, unfractionated viral transcript containing both dsRNA (L, M, and S) and ssRNA (l, m, and s). (B) ssRNA produced by runoff transcription of cDNA with T7 polymerase. ssRNA was produced by runoff transcription with T7 polymerase from cDNA-containing plasmids. A 1% agarose gel stained with ethidium bromide shows these segments. The plasmid DNA was not hydrolyzed and remains in the preparation above the RNA. Lanes: vss, single-stranded ϕ6 viral RNA; s, segment s; m, segment m; l, segment l.

Plasmid pLM682 contains an essentially exact cDNA copy of genomic segment L in plasmid pT7T3 19U. The cDNA was taken from plasmid pLM362 (5). The cDNA insert was prepared similarly to that described above for pLM658. This insert differs from the natural genomic segment L in that the second nucleotide is G instead of U. This was done to improve the ability of the insert to be transcribed by T7 RNA polymerase. Although we have not yet prepared viable phage from this transcript, we know that it can be packaged and replicated and that its genes code for functional proteins. Details of plasmid construction are available upon request.

Plasmid pLM656 contains an exact cDNA copy of genomic segment M (16).

Preparation of the ϕ6 ssRNA. Nucleocapsids were prepared from purified preparations of ϕ6 (2). In vitro transcription was performed by the conditions specified by Emori et al. (3). The reactions were carried out in a volume of 5 to 10 ml. After the reaction, the RNA products were isolated by phenol/chloroform-isoamyl alcohol extraction and concentrated fivefold by ethanol precipitation. The samples were applied to a 5 to 20% sucrose gradient (50 mM Tris-HCl [pH 7.5] with 0.3 M NaCl) and centrifuged in an SW41 rotor at 21,000 rpm at 4°C for 17 h. Fractions were collected, and aliquots were analyzed on composite gels of 2% acrylamide and 0.5% agarose (20) (Fig. 1A). Fractions containing l, m, or s ssRNA segment were combined, ethanol precipitated, and resuspended in glass-distilled water.

When prelabeled ϕ6 ssRNA was synthesized, the transcription reaction was carried out in a volume of 1 ml with 200 μCi of [α-^{32}P]UTP included. Gradient fractionation of the ssRNA was done as described above; however, the composite gels were dried after electrophoresis and exposed to Kodak XAR film with a Dupont Lightning-Plus intensifier screen to visualize the radiolabeled RNA species.

In vitro transcription of plasmids with T7 polymerase. Recombinant plasmids of the cDNA plasmids were treated with endonuclease *Xba*I. This introduces a cut 3′ to each cDNA insert. The 5′ overhanging four nucleotides were removed by treatment with mung bean nuclease (Pharmacia), leaving a 3′ end identical to that of viral ssRNA. A 250-μl transcription reaction was performed by using the Stratagene T7 RNA polymerase transcription reagent and buffer kit. The reaction products were isolated by phenol/chloroform-isoamyl alcohol extraction and concentrated 10-fold by ethanol precipitation. Aliquots of each reaction mixture were analyzed on 1% agarose gels in Tris-borate-EDTA buffer (Fig. 1B).

Preparation of procapsids. *E. coli* JM109 carrying plasmid pLM450 was used to generate procapsid particles (7). Procapsids were purified by sucrose gradient fractionation. Isolated particles were frozen, and aliquots were thawed at the time of the reaction.

RNA polymerase reaction conditions and packaging assay. RNA polymerase reaction mixtures contained 50 mM Tris-HCl (pH 8.2), 3 mM magnesium chloride or 2 mM manganese chloride, 100 mM ammonium acetate, 20 mM NaCl, 5 mM KCl, 5 mM dithiothreitol, 0.1 mM EDTA, 1 mM each ATP, GTP, and CTP, 0.1 mM UTP, 5% polyethylene glycol 4000, 1 mg of macaloid, and 100 μCi of [α-^{32}P]UTP. About 10 μg of procapsid and about 2 μg of each RNA species were used for the reaction in a volume of 125 μl. This would result in an approximately twofold excess of each RNA species with respect to procapsid particles. The packaging and replication reactions were not saturating for either RNA or procapsids (7). Immediately after the reaction of 90 min at 27°C, the material was applied to a 10 to 20% sucrose gradient in a mixture containing 50 mM Tris-HCl (pH 8.2), 0.3 M NH_4Cl, 3 mM $MgCl_2$, 20 mM NaCl, 5 mM KCl, 5 mM dithiothreitol, and 0.1 mM Na_2EDTA.

The samples were centrifuged in an SW41 rotor at 23,000 rpm at 23°C for 75 min and fractionated. The fractions were precipitated with ethanol together with about 1 μg of an unfractionated mixture of unlabeled ϕ6 ssRNA and dsRNA, after which the RNA was resuspended in 20 μl of sample buffer (20). Electrophoresis of fractions containing labeled RNA was performed on composite gels, which were then analyzed by autoradiography.

Packaging assays performed with [α-^{32}P]UTP-prelabeled ϕ6 ssRNA segments were carried out with the standard reaction conditions except that 1 mM ATP was the only nucleotide included. The gradient conditions were the same as described above. Reactions that utilized T7 polymerase transcripts were done in a 25-μl volume.

RESULTS

Packaging of individual ssRNA segments with magnesium ions. We have shown previously that ssRNA segments can be packaged by procapsids in the presence of either ATP or dATP without concomitant synthesis of minus-strand RNA (6). We now examine whether individual plus strands can be packaged independently of each other. Labeled viral ssRNA was prepared by in vitro transcription of viral nucleocapsids and fractionated in sucrose gradients to obtain pure segments s and m. The purified RNAs were added to procapsids alone or in combination and the particles were then fractionated on sucrose gradients. RNA was extracted from individual fractions, separated by gel electrophoresis, and subjected to autoradiography (Fig. 2). The individual s and m segments were packaged as well as was the mixture of s and m. There is no evidence for the dependence of either on the

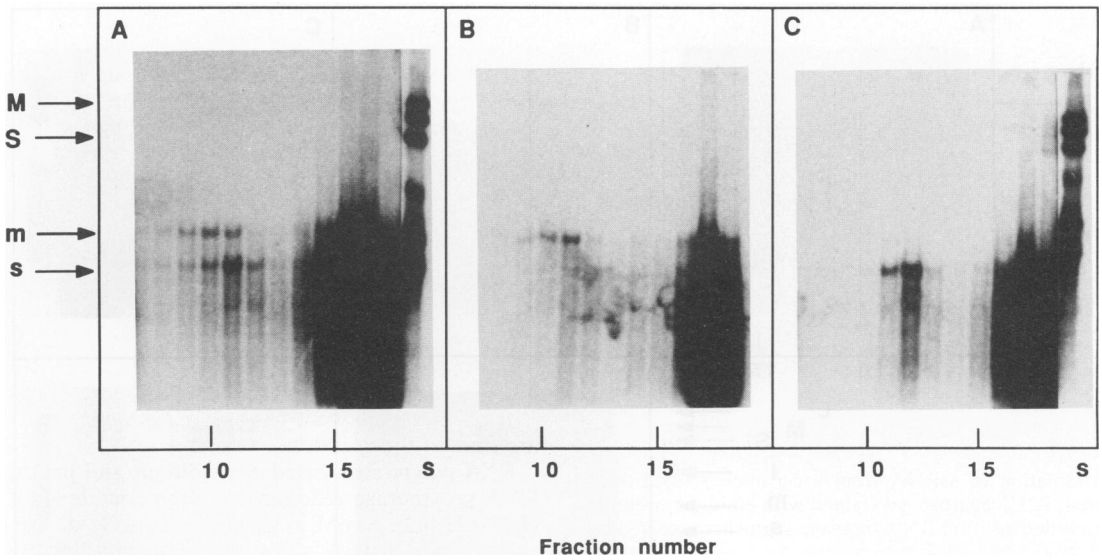

FIG. 2. Packaging of individual prelabeled segments of φ6 ssRNA. Procapsids were incubated with individual prelabeled ssRNA segments isolated from a sucrose gradient. The reaction mixture contained magnesium ions and 1 mM ATP as the only nucleotide. Fractions were precipitated with ethanol and dissolved in sample buffer. Aliquots were applied to a composite gel, which was subsequently dried and autoradiographed. Sedimentation is from right to left, and 18 fractions were collected from each gradient. (A) Reaction with both m and s ssRNA segments; (B) reaction with the m ssRNA segment; (C) reaction with the s ssRNA segment.

prior packaging of the other. A mixture of all three segments packaged to the same extent as the single segments or the mixture of two (data not shown). The packaged segments remain single stranded as expected. RNA that was not packaged is seen at the top of the gradients, and degradation of this RNA is evident.

The replication/packaging reaction. If the ssRNA segments can be packaged independently, perhaps they can replicate independently as well. The combined replication/packaging reaction uses unlabeled ssRNA and a mixture of the four NTPs, with UTP having radioactive label. The φ6 ssRNA segments were viral transcripts isolated from sucrose gradient fractions as described above (Fig. 1). Reaction mixtures contained either all three viral RNA segments or only one of each. The reaction products were fractionated on sucrose gradients. Replicated dsRNA is evident in particles when all three substrate ssRNA segments are present in the reaction (Fig. 3A). However, when individual segments are supplied, the incorporation of label into dsRNA in particles is enormously reduced (Fig. 3B to D). When the gel containing the sucrose gradient fractions is exposed to XAR film for longer periods, a small degree of label incorporation into L, M, and S dsRNA is observed. This could be the result of the imperfect isolation of the ssRNA segments from the preparative sucrose gradient causing each individual segment to be partly contaminated by the other two (Fig. 1). This residual incorporation is at least 100-fold less than incorporation with all three RNA segments.

The replication/packaging reaction in manganese-containing buffers. Procapsid particles in a buffer containing manganese ions instead of magnesium were incubated with combinations of purified φ6 ssRNA as described in Materials and Methods. The particles were then fractionated on sucrose gradients, and the RNA was analyzed. It can be seen in Fig. 4A to C that individual viral ssRNA molecules may be incorporated into the procapsid and replicated under these reaction conditions. There is less radioactivity in the segment L preparation because of the low yield in the original

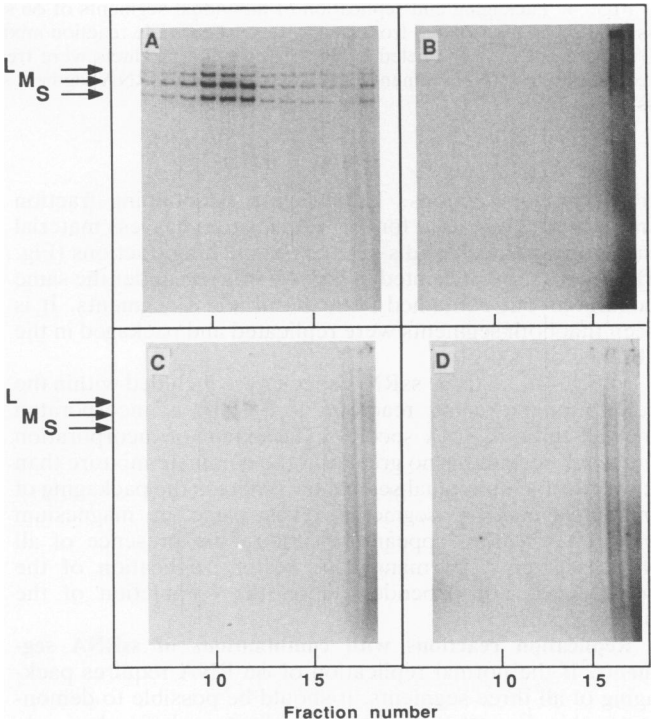

FIG. 3. Packaging and replication of individual segments of φ6 ssRNA. Procapsids were incubated with individual ssRNA segments isolated from a sucrose gradient. The reaction mixture contained magnesium ions, [α-^{32}P]UTP, 1 mM each ATP, CTP, and GTP, and 0.1 mM unlabeled UTP. The reaction products were treated as described for Fig. 2. (A) Reaction with all three ssRNA segments; (B) reaction with ssRNA segment l; (C) reaction with ssRNA segment m; (D) reaction with ssRNA segment s. The labeled material above band L in panel A is replicative intermediate.

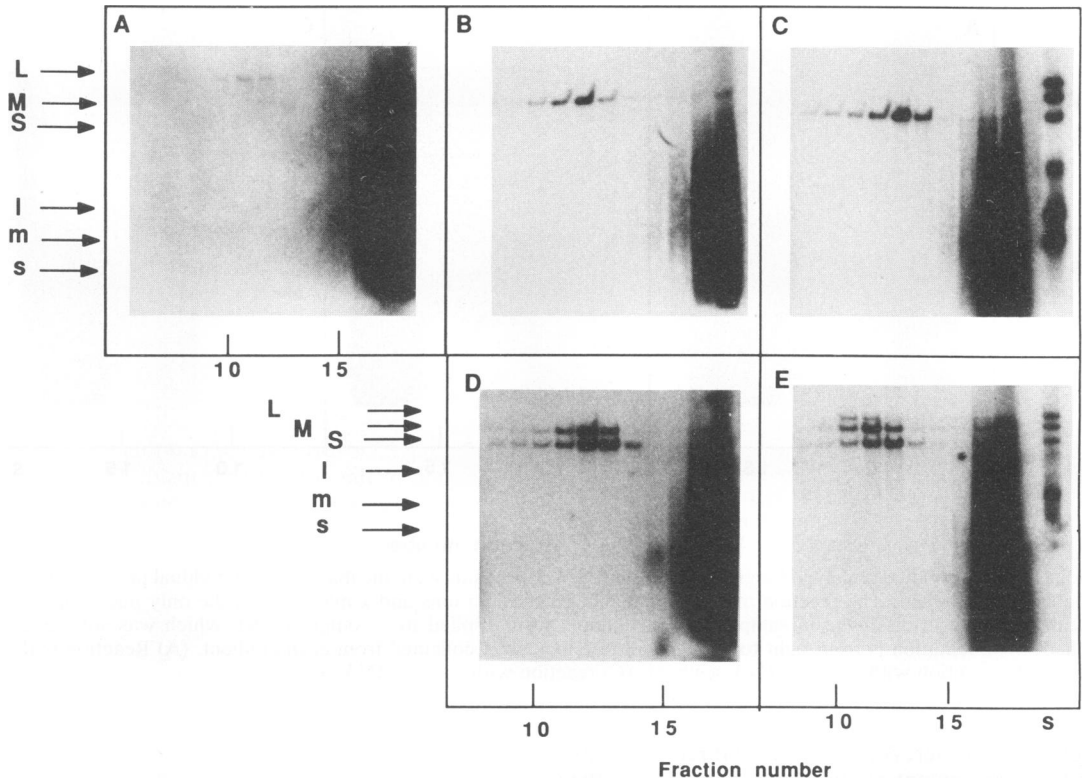

FIG. 4. Packaging and replication of individual segments of ϕ6 ssRNA in manganese buffer. Procapsids were incubated with individual ssRNA segments isolated from a sucrose gradient. The reaction mixture contained manganese ions, [α-^{32}P]UTP, 1 mM each ATP, CTP, and GTP, and 0.1 mM unlabeled UTP. The reaction products were treated as described for Fig. 2. (A) Reaction with ssRNA segment l; (B) reaction with ssRNA segment m; (C) reaction with ssRNA segment s; (D) reaction with ssRNA segments m and s; (E) reaction with all three ssRNA segments.

transcription reactions. The l-segment-containing fraction from the gradient used for ssRNA isolation has less material in it than do the m- and s-segment-containing fractions (Fig. 1). The reaction presented in Fig. 4D was run under the same conditions but contained both the m and s segments. It is seen that both segments were replicated and packaged in the absence of l ssRNA.

In Fig. 4E, all three ssRNA species are included within the replication/packaging reaction. [^{32}P]UMP is incorporated into all three dsRNA species. The extent of incorporation into each segment is no greater in the complete mixture than it was for the individual segments. Whereas the packaging of individual ssRNA segments takes place in magnesium buffer, replication appears to require the presence of all three segments. In manganese buffer, replication of the segments is not dependent upon the completion of the packaging process.

Replication reactions with combinations of ssRNA segments. If the normal replication of ϕ6 RNA requires packaging of all three segments, it should be possible to demonstrate this dependence without gradient analysis. A simple measurement of replication should be a measure of both packaging and replication. It should also be possible to use T7 promoter transcripts of cDNA copies of the segments instead of gradient-purified viral transcripts.

Replication reactions were run in the presence of magnesium, four nucleotides (UTP at 0.1 mM), and 20 μCi of [^{32}P]UTP. Each reaction contained a different combination of ssRNA segments. These segments were produced in vitro by runoff transcription with T7 polymerase. The M- and S-segment transcripts were identical to those produced by viral transcription. The synthetic L segment was identical to viral L except that G was substituted into position 2, which is normally occupied by U. This change was necessary because of the requirements of T7 RNA polymerase. These transcripts contained some plasmid DNA but were otherwise pure (Fig. 1B). The reaction products were treated as described in Materials and Methods and applied directly to a composite gel (Fig. 5).

No incorporation of radioactivity is seen when segment l or m was provided as a substrate for a reaction (Fig. 5, lanes 2 and 3). Segment s is seen to replicate to a small degree (lane 4), far less then that observed in a reaction containing all three transcripts (lane 8). The degree of replication was enhanced when the reaction contained two RNA segments (lanes 5 to 7), with the s segment apparently the most sensitive. Replication appears most efficient with all three segments included within the reaction (lane 8). A reaction in which the concentration of the m segment was tripled was analyzed to control for the possibility that this replication enhancement is a function of RNA concentration and not RNA segment identity.

DISCUSSION

ϕ6 shares with the members of the family *Reoviridae* the characteristic of a segmented dsRNA genome. The virion contains one each of its three genomic segments, and the

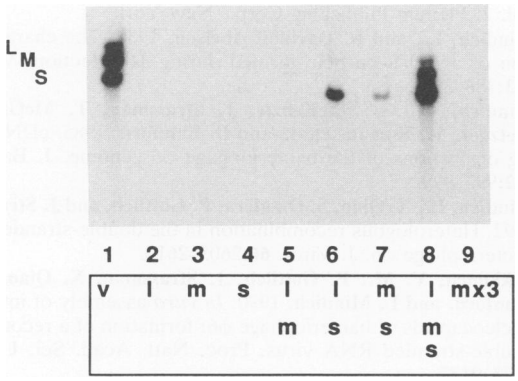

FIG. 5. Replication of individual segments of message sense RNA that had been produced by runoff transcription. Procapsids were incubated with individual ssRNA segments produced by runoff transcription of cDNA. The reaction mixture contained magnesium ions, [α-^{32}P]UTP, 1 mM each ATP, CTP, and GTP, and 0.1 mM unlabeled UTP. Aliquots of each reaction mixture were applied to a composite gel, which was subsequently dried and autoradiographed. Lanes: 1, reaction with all three viral ssRNA segments produced by nucleocapsid transcription; 2, reaction with T7-produced transcript of pLM682 (segment L); 3, reaction with T7-produced transcript of pLM656 (segment M); 4, reaction with T7-produced transcript of pLM658 (segment S); 5, reaction with both T7 transcripts of segments L and M; 6, reaction with both T7 transcripts of segments L and S; 7, reaction with both T7 transcripts of segments M and S; 8, reaction with all three T7-produced transcripts; 9, reaction with three times the concentration of T7-produced M segment as in the reaction in lane 3.

packaging must be very precise because the efficiency of plating is 1. It is not definite that the packaging of the reoviridae is precise, but there is a consensus that it is. The molar amounts of the individual dsRNA genomic segments in both reovirus and rotavirus are essentially equal (18). This is consistent with the findings that dsRNA is found only within particles and that mature particles have a complete set of genomic segments. It had been previously proposed that the coordination of rotavirus replication could be fostered by a requirement for complete packaging before the onset of replication (18). How does the packaging particle ensure that one representative of each genomic segment is packaged? One way would be to have independent packaging sites for each segment. This would require three sites in the case of ϕ6 and 11 in the case of rotavirus. Another possibility would have one primary site and require that the packaging of subsequent segments be dependent upon the prior packaging of others. In this study, we present evidence that the genomic segments of ϕ6 package independently of each other, suggesting that three different binding sites must exist in each particle and that each genomic segment has its own packaging sequence.

Although we have not established the number of sites in the procapsid for each segment, we assume that there is only one since the mature particles contain only one of each segment. It is possible, however, that there are more than one and that the selection of the segments that remain associated with the procapsid is determined by another mechanism. If there is only one site for each segment, then an intriguing question presents itself. How does a symmetrical particle such as the ϕ6 procapsid generate three unique sites? The yeast viruslike particle (VLP) appears to contain only one molecule of its read-through *gag-pol* protein. It has been suggested that the VLP forms by first associating the *gag-pol* protein with ssRNA and subsequently nucleating the assembly of the capsid around it (9). The large DNA phages establish a unique site by nucleating their capsid assembly around a specific structure called the portal vertex (1). The genome packaging elements associate with this structure. Capsids of DNA phages can form in the absence of the portal vertex, but they are heterogeneous in size in some cases (8).

In the case of ϕ6, procapsidlike structures can be formed from the major structural proteins P1 and P4. These structures appear similar to complete procapsids that contain P1, P4, P7, and P2. Protein P2 is present in about 10 to 20 copies per virion (2). It has sequence similarity to other viral RNA polymerases (10), it is necessary for RNA synthesis (7), and we assume that it contains the active site for polymerization. It is also necessary for packaging of genomic segments (6). We propose that under normal conditions, a single structure containing P2 is formed, and that this structure nucleates the formation of the normal procapsid. The P2 structure would be the determinant of the three binding sites, and these sites would interact with each other.

We found a strong mutual dependence in the replication of the genomic segments. The synthesis of negative strands is dependent upon all three plus strands being present in the particle. It is tempting to propose teleological reasons for such a regulation. One reason would be to ensure that particles with replicated RNA have complete sets so as to preclude premature maturation of procapsids, or uncontrolled transcription, since transcription follows replication. Another possibility would be to foster recombinational repair. We have found that ϕ6 is capable of heterologous recombination, a process in which the 3′ end of one segment is replaced by that of another in a nonreciprocal manner (15). We have found that this process can serve to rescue damaged segments (unpublished results). Since the recombination probably involves copy-choice replication, a particle that packages a segment that is missing its normal 3′ end would be able to rescue it only if the other segments had not yet replicated.

How does the replication machinery know that all three binding sites are occupied? How do the binding sites communicate with each other? There are examples of global conformational changes in procapsids of the large DNA phages (1), but we would again suggest that a single structure containing P2 and having all three sites for binding would be an efficient way to couple packaging and the signal for replication.

The observation that individually packaged segments can replicate independently in manganese buffers is consistent with many instances in which polymerase specificity is broadened in the presence of manganese ions. For example, ϕ6 polymerase specificity is stringent in magnesium buffers but not so in the presence of manganese. We found that 16S rRNA is replicated but not packaged in manganese buffers and is not replicated at all in magnesium buffers (7).

The dependence of replication upon the packaging of all three segments is demonstrable even without the gradient isolation of procapsid particles. In Fig. 5, we see that this relationship holds in the direct assay of replication.

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