

Packaging, replication and recombination of the segmented genomes of bacteriophage $\Phi 6$ and its relatives

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Abstract

The genomes of bacteriophage $\Phi 6$ and its relatives are packaged through a mechanism that involves the recognition and translocation of the three different plus strand transcripts of the segmented dsRNA genomes into preformed polyhedral structures called procapsids or inner cores. The packaging requires hydrolysis of NTPs and takes place in the order S:M:L. Minus strand synthesis begins after the completion of the plus strand packaging. The packaging and replication reactions can be studied *in vitro* with purified components. A model has been presented that proposes that the program of serially dependent packaging is determined by the conformational changes at the surface of the procapsid due to the amount of RNA packaged at each step. The *in vitro* packaging and replication system has facilitated the application of reverse genetics and the study of recombination in the family of Cystoviridae.

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1. Introduction

There are many strategies for packaging viral genomes. Among the options for genomic packaging are the filling of preformed capsid structures with previously synthesized nucleic acid or material that is being synthesized during packaging; the nucleation of capsid assembly around viral nucleic acid; or the association of genomic elements inside a membrane structure. Most RNA viruses chose the last two of these three options. Even among the dsRNA viruses, the yeast LA virus-like particle, the only dsRNA eukaryotic system where packaging has been approachable, appears to nucleate the capsid around a plus strand transcript.

Among the most intriguing packaging options are those where the genomic RNA or DNA is transported into an already assembled polyhedral particle. This process has been studied in the greatest detail with the double-stranded DNA bacteriophages (Catalano, 2000; Hendrix, 1998). It is likely that Herpes viruses use a strategy similar to that of the tailed dsDNA bacteriophages (Homa and Brown, 1997). Although many of the elements involved in these pathways have been worked out, there are still many unresolved questions even in those systems that are the most tractable. The nature of the

mechanism by which the ATPase motors move the nucleic acids into the particles has not been elucidated in any case. The packaging of the genomic RNA of the Reoviridae is a complete mystery although there is the possibility that the packaging of the RNA involves the filling of an assembled particle. There is no *in vitro* system to experiment with and there is no satisfactory model to consider. The Cystoviridae are a family of bacteriophages that have a number of striking similarities to the Reoviridae. They have genomes of three dsRNA segments packaged within an inner core composed primarily of 120 molecules of the major structural protein and 12 molecules of the RNA dependent RNA polymerase (RdRp). This is in comparison to the Reoviridae, which also have an inner core defined by 120 molecules of the major structural protein and genomes of 10, 11 or 12 dsRNA segments. The Cystoviridae are the only RNA viruses where it has been shown that the genomic RNA is acquired by an already assembled procapsid structure (Mindich, 1999).

Among the bacteriophages that package preformed dsDNA, the recognition and motor complex is called the terminase (Catalano, 2000). This is a structure organized at the unique portal vertex that is composed of a small number of proteins, and sometimes RNA molecules, that recognizes a sequence on the genomic DNA and through the expenditure of energy produced by hydrolysis of ATP moves the DNA into the preformed procapsid. Genomic packaging of

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the $\Phi 6$ genome shares some of these properties. However, the DNA phages generally package a single DNA molecule. The problem faced by $\Phi 6$ and the dsRNA viruses of the Reoviridae is that the genome is segmented and it appears that each virus particle contains one each of the genomic segments (Day and Mindich, 1980; Patton, 1990).

$\Phi 6$ is the first member of the Cystoviridae to be discovered and it has been studied in great detail (Vidaver et al., 1973). A number of relatives of $\Phi 6$ have been isolated recently (Mindich et al., 1999). Genomic packaging of these has been found to be similar to that of $\Phi 6$; but in some cases, less stringent (Sun et al., 2003). The inner core of $\Phi 6$ can be assembled in cells of *E. coli* carrying plasmids with cDNA copies of genomic segment L. The inner core particles can be purified and will package and replicate $\Phi 6$ plus strand transcripts in vitro. Using this system it has been possible to work out the basic elements of the packaging of this genome (Mindich, 1999).

There are a number of RNA viruses that have segmented genomes. The simplest cases are those like the bromoviridae that have each segment packaged in a separate particle. In this case it appears that the capsid structures form around the preformed plus strand RNA (Choi et al., 2000). Others, such as flock house virus have two plus strands that are packaged precisely within a capsid structure (Marshall and Schneemann, 2001). It appears that these viruses also form the capsid around the RNA. In the case of the influenza virus, the virion contains approximately twelve molecules of negative strand RNA in a nucleoprotein structure. The genome is comprised of eight different segments and the virions contain a random assortment of the genetic complement of the virus. In this case there is no capsid structure; simply a membrane envelope acting as a container (Hoffmann et al., 2002).

The virus-like particles of the killer system of yeast have a structural similarity to those of the Reoviridae and $\Phi 6$ in that they are composed of 120 molecules of the major structural

protein. However, the structure forms around the plus strand RNA and only a single species of RNA is found inside of each particle. Although the number of RNA molecules in the particle can increase if they are smaller than the normal size (Wickner, 1996).

2. The inner cores of the Cystoviridae

The inner core of $\Phi 6$ and its relatives is composed of four proteins, P1, P2, P4 and P7. P1 is the major structural protein and the core has 120 molecules of P1. P2 is the RNA dependent RNA polymerase and is responsible for both plus and minus strand synthesis. P4 is a hexameric NTPase that is necessary for genomic packaging. P7 is an accessory protein that seems to play a role in packaging and RNA synthesis. The empty inner core particle is dodecahedral in shape (Fig. 1). The P4 hexamers can be seen on the five-fold faces, which are pulled towards the interior of the particle (de Haas et al., 1999). Inner core particles that have packaged RNA have a more spherical shape (Butcher et al., 1997). Particles can be assembled without proteins P2, P4 or P7 and these proteins can be added back to the deficient structures (Casini et al., 1994; Juuti and Bamford, 1997; Paatero et al., 1998). Although earlier studies involved the assembly of particles in *E. coli* cells, it is now possible to assemble particles from purified components in vitro (Poranen et al., 2001; Poranen and Tuma, in press this issue).

When procapsids are incubated in simple buffers in the presence of PEG 4000, magnesium ions and a source of NTP they are able to package plus strand transcripts of the genomic segments. The packaging is serially dependent in that the transcript of S can be packaged alone, but that of M requires prior packaging of S and the packaging of L requires the prior packaging of M (Qiao et al., 1995a) (Fig. 2). The particles can be forced to act less rigorously but it appears that this dependence is the natural condition.

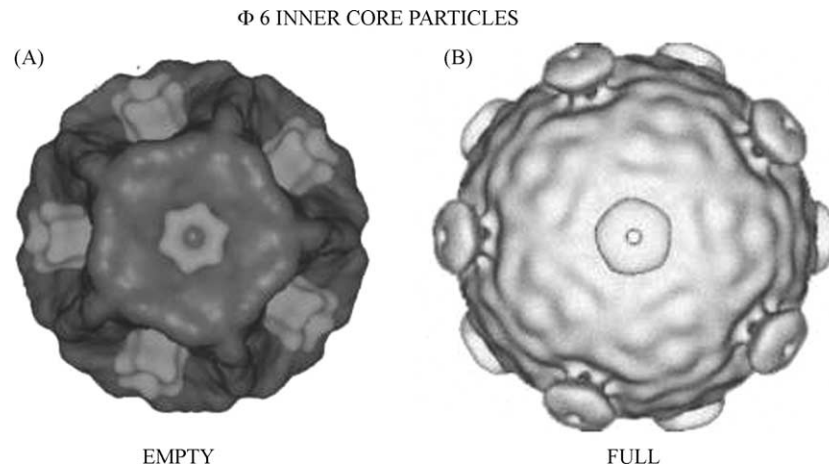


Fig. 1. Cryoelectron microscopy reconstructions of empty procapsids (de Haas et al., 1999) (A) and filled procapsids (Butcher et al., 1997) (B). The structures on the five-fold faces are the multimers of the NTPase, P4.

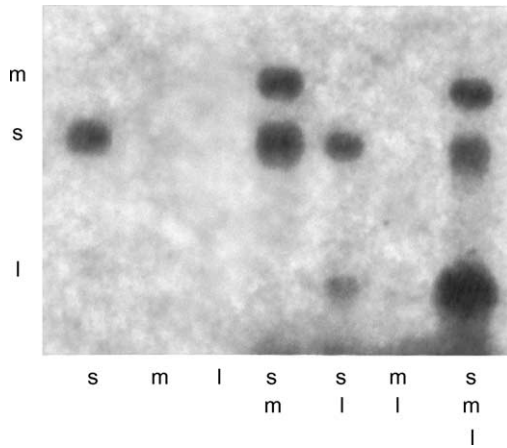


Fig. 2. Serial dependence of genomic packaging. Packaging of radioactive plus strand transcripts of exact copies of genomic segments S and M and of a truncated segment L of $\Phi 6$. Radioactive transcripts of plasmids were incubated with procapsids, treated with RNase I and applied to a 2% agarose gel.

Minus strand synthesis begins when all three segments have been packaged and plus strand synthesis begins when minus strand synthesis is completed (Frilander et al., 1992). Plus strand synthesis is semiconservative in contrast to the conservative synthesis found in the Reoviridae (Van Etten et al., 1980).

Normal packaging requires the four protein components of the inner core particles. However, packaging will occur in the absence of the polymerase P2. Minus strand synthesis does not occur in the absence of P2; however the protein can be added back to deficient particles and they then become competent to synthesize minus strands (Casini et al., 1994). Particles that are missing P7 can package at a reduced efficiency although with proper specificity. They also have defects in minus and plus strand synthesis (Juuti and Bamford, 1997). Particles without P4 cannot package RNA (Paatero et al., 1998). Packaging is dependent upon the presence and hydrolysis of nucleotide triphosphates. The specificity of the nucleotide requirement follows the specificity of the NTPase activity of protein P4. The associations of P2, P4 and P7 seem to be directly to P1, so that particles defective in one of these is not missing any of the others (Gottlieb et al., 1988).

Packaging is dependent upon sequences near the 5' ends of the plus strands. All three $\Phi 6$ transcripts have an 18 base identity at the 5' end, which is G(G/U)AAAAAACUUU-AUAUA. Each transcript has a sequence of about 200 nucleotides that is unique with virtually no similarity to that of the others and is placed about 50 nucleotides from the 5' end (Gottlieb et al., 1994). These sequences are called *pac* sequences (Fig. 3). The 5' end and the *pac* sequences are necessary and sufficient for packaging. The *pac* regions can be folded into stem loop structures (Mindich, 1999) and

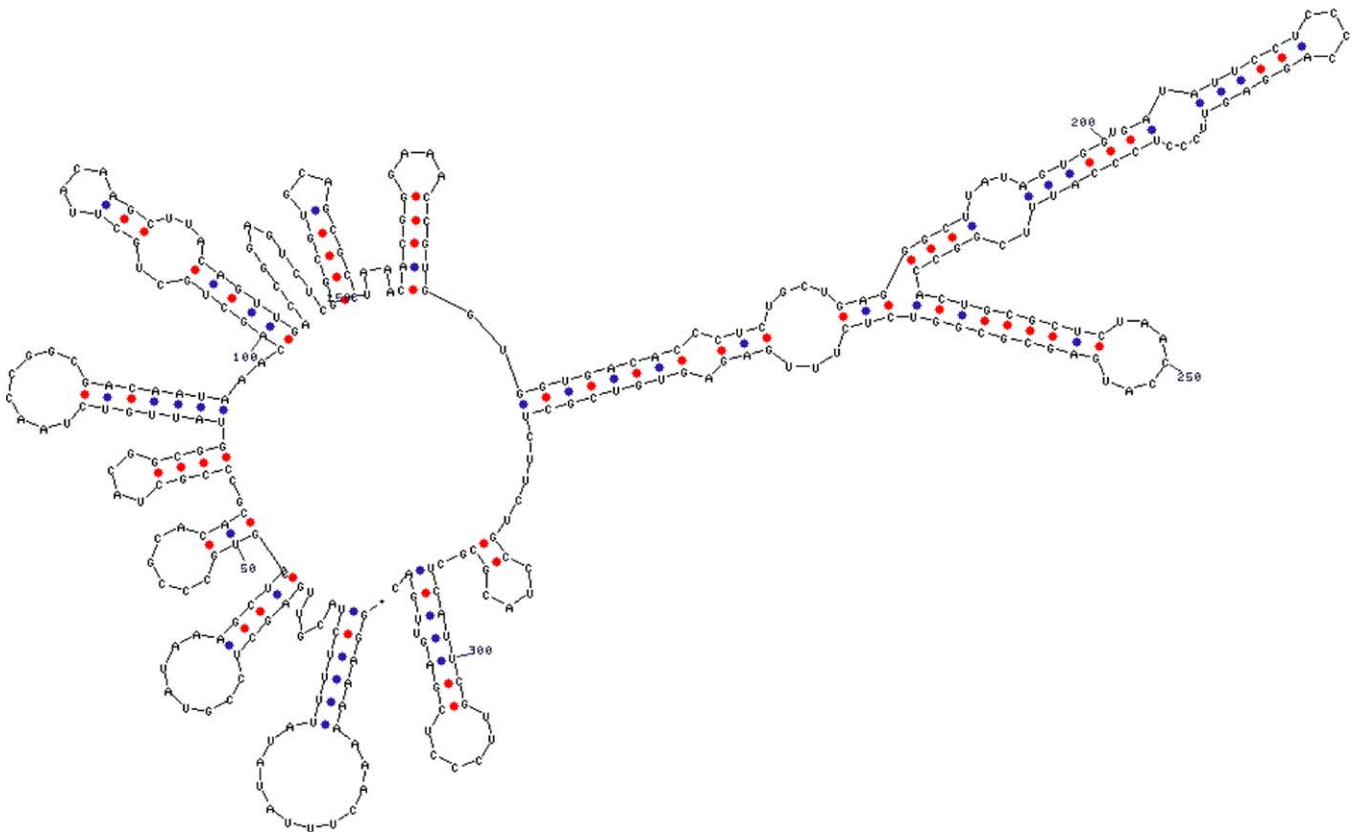


Fig. 3. 5' Sequence of the plus strand transcript of genomic segment M of $\Phi 6$. Secondary structure is predicted by the mfold program (Mathews et al., 1999).

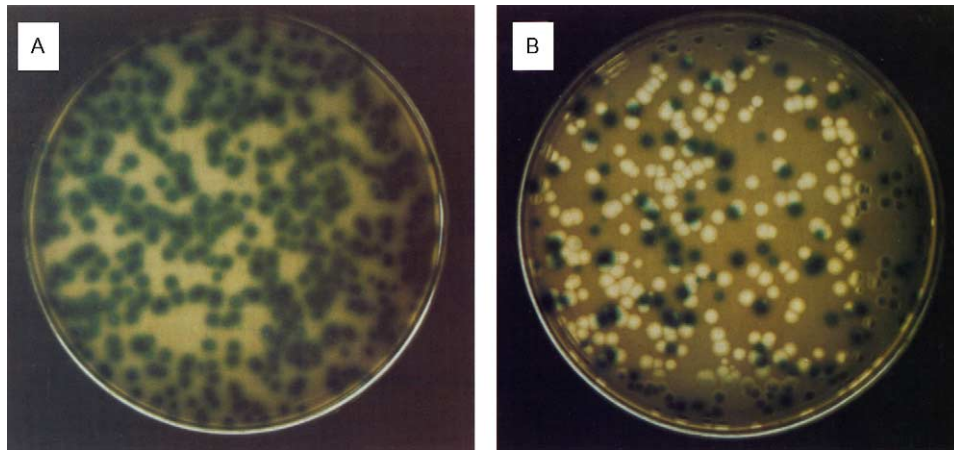


Fig. 5. Plaques of $\Phi 1817$, which has *lacH* stably inserted into the *PstI* site of segment M, on LM1034 in LB agar with Xgal (A); plaques of $\Phi 1819$, which is genetically unstable due to homopolymer arms at the position of the *lacH* insertion (B).

transcripts to yield virus with normal termini. The plasmids in this case, need not replicate in the host cells; the transcription is sufficient to produce phage. The plasmid electroporation can also be used to form carrier states in which virions replicate in cells without leading to cell death.

Using these techniques, it is possible to prepare live phages that have genomic segments that are less than normal size. When the RNA content of these phages is examined it is seen that segments that are approximately half the normal size are represented by the normal amount of RNA indicating that two molecules are maintained for that segment. It is also possible to have phages that carry deletions in one of their genomic segments acquire plasmid transcripts with the normal sequence of that segment. It is also possible to prepare a plasmid that produces a transcript with the *pac* sequence of another segment. These transcripts can be acquired rather efficiently; however when the sequence of the new genomic segment is determined it is found that a recombination occurred so as to place the *pac* sequence of the proper segment at the 5' end of the segment. It appears that the virus is able to determine the amount of RNA in each segment class for each particle (Onodera et al., 1995). Experiments with $\Phi 8$ have shown that the virus is able to acquire transcripts that completely lack *pac* sequences. This occurs at very low frequencies and depends upon powerful selection and recombination (Onodera et al., 2001). This offers a pathway to the acquisition of new genes from either other viruses or from cell transcripts.

RNA binds rather nonspecifically to the outside of the procapsids in the absence of NTP. Although there seems to be somewhat of a preference for phage transcripts, there is not a dramatic difference in the apparent affinities of the three plus strands under some conditions (Juuti and Bamford, 1995). We have found that specificity of binding can be demonstrated by constructing transcripts that are able to interfere with packaging, but that cannot be packaged themselves (Qiao et al., 1997b). Deletions were created in $\Phi 6$ plus strands in the space between the 5' 18 base identity and

the *pac* regions. If the deletion was too small, the molecules could be packaged and could compete with normal RNA. If the deletions were too large, the molecules would neither package nor compete. But deletions of about 20 nucleotides resulted in molecules that could not be packaged but could compete effectively with normal ones. This demonstrated that the specificity of packaging was determined on the outside of the particles (Qiao et al., 1997b). Moreover, it was found that binding to procapsids was specific when ATP was present (Qiao et al., 2003b). Older observations had indicated that the conformation of the nucleocapsid core particle could be altered in the presence of NTPs (Ojala et al., 1994). The deletion-containing plus strands were used for this demonstration because normal molecules would be packaged in the presence of ATP. Using deletion molecules that compete for packaging but are not themselves packaged, we have shown that empty particles have selective affinity for plus strands of segment S in the presence of ATP. Mutant particles that prefer to package M have greater affinity for plus strands of M. Cross-linking studies have shown that the RNA is bound to protein P1 between amino acids 98 and 155 (Qiao et al., 2003b). Some amino acid changes within this region have been found to drastically reduce packaging behavior while others have been found to suppress packaging dysfunction caused by changes in the *pac* sequences of viral RNA (Qiao et al., 2003a).

Another property of packaging was shown by experiments with plus strands that contained large stable hairpin structures (Qiao et al., 1995b). When hairpins with over twenty CG pairs were created near the 3' ends of plus strands it was found that these molecules could be packaged up to the point of the hairpin and that the part of the molecule inside the procapsid was able to move the packaging program to the next step. A plus strand of S with a hairpin could facilitate the packaging of M even though the material 3' to the hairpin was outside the procapsid and could be cut off by RNase I. This result suggests that more than one portal exists for the entry of RNA and that RNA entry is from the

5' end. Since there are 12 five-fold faces with NTPase P4 hexamers on each, it is possible that there are twelve entry portals. Experiments with procapsids that are deficient in P4 indicate that proper packaging might occur even when only one portal is functioning (Pirttimaa et al., 2002).

3. The genomic packaging model

We have proposed a model for the general outlines of the packaging process for the Cystoviridae (Qiao et al., 1997b) (Fig. 6). It takes into account many of the observations discussed above and makes predictions, which have been borne out. The model states that the empty procapsid has binding sites on the outside for only the plus strand of S. Plus strands of S would bind to specific sites on the particle and the 5' end would be positioned in the pore of one of the NTPase hexamers. ATP hydrolysis would power the entry of the RNA into the particle. An attractive model for the movement of the RNA would be a mechanism proposed for helicases which involves binding sites in the hexamer that sequentially change affinity and position due to ATP bind-

ing, hydrolysis and departure (Soultanas and Wigley, 2001). Once the S plus strand is packaged the particle would expand and the conformation of the shell would change so that the binding sites for S would be lost and those for M would appear. If the S molecule were half size, then the particle would not expand sufficiently to change and another molecule would be packaged. In this way, the particle would always package approximately the same weight of material for each segment class. After the M segment is packaged the particle would expand further so as to lose the M sites and expose those for L. After packaging of L the conformation would change again to affect a switch for the polymerase and minus strand synthesis would begin. Once minus strand synthesis has completed, the RNA content of the particle would have doubled and there would be another change that would activate the transcriptional activity of the polymerase.

A specific feature of this model is the idea that the progress through the packaging program is driven by the amount of RNA inside the particle and not by its sequence once it is inside. It was predicted that a molecule that was the size of the sum of S and M but had the *pac* sequence of S would enable the packaging of L. This was found to be true.

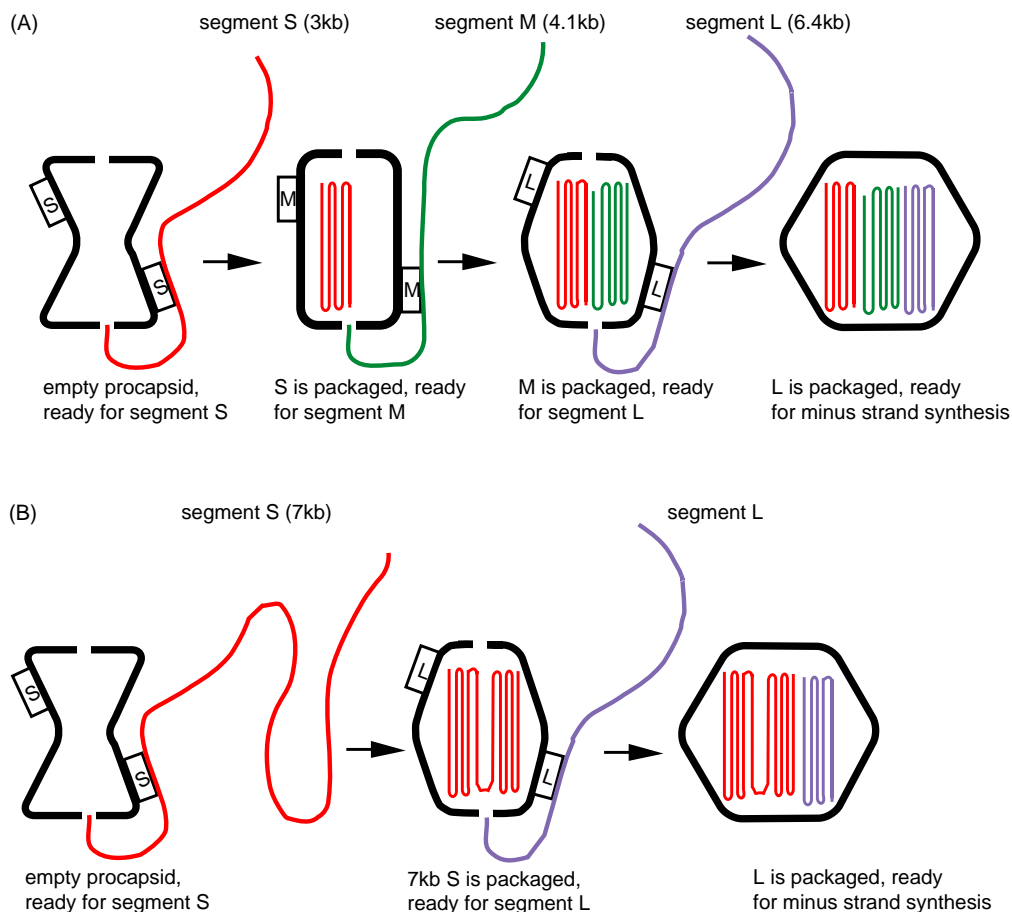


Fig. 6. (A) The packaging model. The empty procapsid shows only binding sites for S. After a full size S is packaged, the S sites disappear and M sites appear. After a full size M is packaged, the M sites disappear and L sites appear. After a full size L is packaged, minus strand synthesis commences. After minus strand synthesis is completed, plus strand synthesis commences. (B) If segment S is of the size equal to the sum of both S and M, the S sites will disappear and the L sites will appear and segment L will be packaged without segment M.

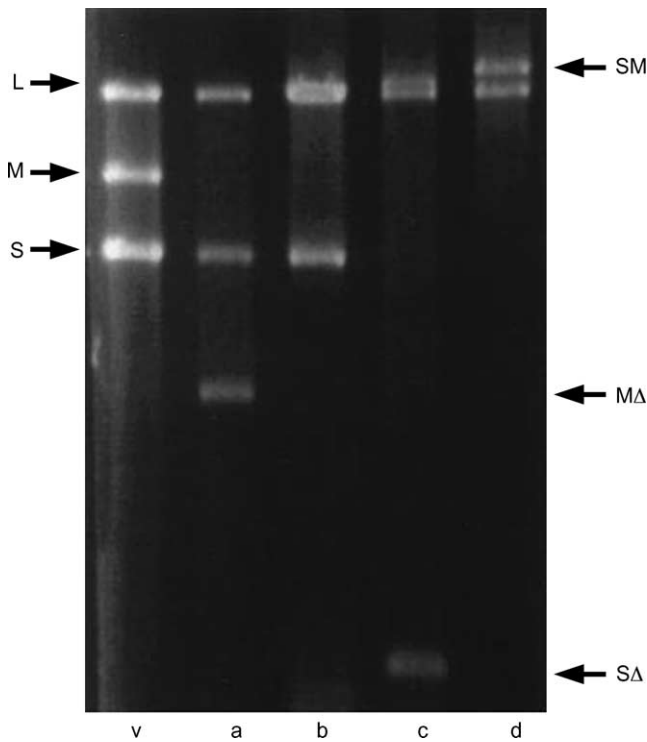


Fig. 7. Agarose gel electrophoresis of dsRNA isolated from virions. Lane V shows the distribution of normal segments L, M and S. Lane a shows dsRNA from bacteriophage Φ 2007, which has a deletion in segment M. Lane b shows RNA from Φ 2064 which has normal L, an MS chimera picked up from pLM1114 and a normal segment S. Lane c shows RNA from Φ 2323, which contains normal L, the MS chimera shown in b and a deleted segment S that contains no genes and is only 798 bp. Lane d shows RNA from Φ 2361, which contains normal L, a chimera of S and M, but no normal segment M or S.

Similarly a molecule with the *pac* sequence of M but the size of the sum of M and L would turn on minus strand synthesis after the packaging of S. This also was confirmed. In fact, it was possible to prepare transcripts that were the size of the complete genome in one piece with the *pac* sequence of S and these were able to be packaged and they turned on minus and then plus strand synthesis in vitro.

A number of experiments with in vivo constructions are consistent with the model (Fig. 7). It is possible to prepare chimeric molecules with the genes of both segments S and M. If the *pac* region of S is at the 5' end of the transcript, a virus can be formed that has only two segments; the chimera and a normal L segment. If the *pac* region of M is at the 5' end then an S segment must also be present. The S segment can be very small and have no genes, but viable virus is not formed without the S segment. A one-segment virus can also be created by preparing a transcript that has all the genes of the virus and the *pac* sequence of S at the 5' end (Fig. 8). This has been accomplished for Φ 6 and Φ 13 (Qiao et al., 2000).

Φ 6 and its relatives can establish carrier states in host cells (Cuppels et al., 1979). If the genome contains a reporter group or a selective marker it is possible to maintain

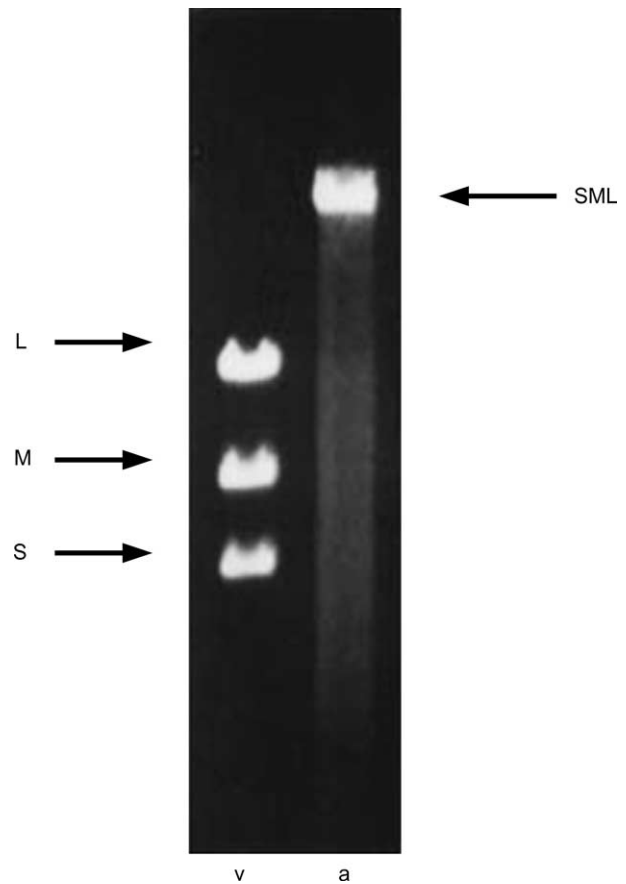


Fig. 8. Agarose gel electrophoresis of dsRNA isolated from virions. Lane v shows the distribution of normal segments L, M and S. Lane a shows dsRNA from Φ 2515, which contains the entire genome of Φ 6 in one segment. The migration of the RNA indicates a size of about 14 kbp.

the carrier state efficiently (Onodera et al., 1992). In some cases where a gene for kanamycin resistance was placed in segment M of Φ 6, it was found that segment S could be lost, leading to a two segment phage that could replicate in the host cell but could not form infectious viral particles (Onodera et al., 1998). Sequencing of gene 1 showed that a mutation had occurred leading to an amino acid change R14G. Procapsids could be produced in *E. coli* carrying plasmids whose transcripts contained the genes of segment L with the mutant gene 1. These procapsids were able to package segment M and L without segment S and, in fact, excluded segment S when the other two RNA molecules were present (Fig. 9). This showed that the packaging program could be changed, but that the normal condition required the prior packaging of S. It also showed that changes in the packaging behavior were determined by protein P1, the major structural protein of the inner core. Recent experiments have shown that carrier states can be achieved by the electroporation of cDNA containing non replicating plasmids into host cells. In this way, carrier states can be achieved with just segments L and M in Φ 6; however this requires a mutation in gene 1 (Sun et al., 2004). Additional experiments have demonstrated that directed changes in the *pac*

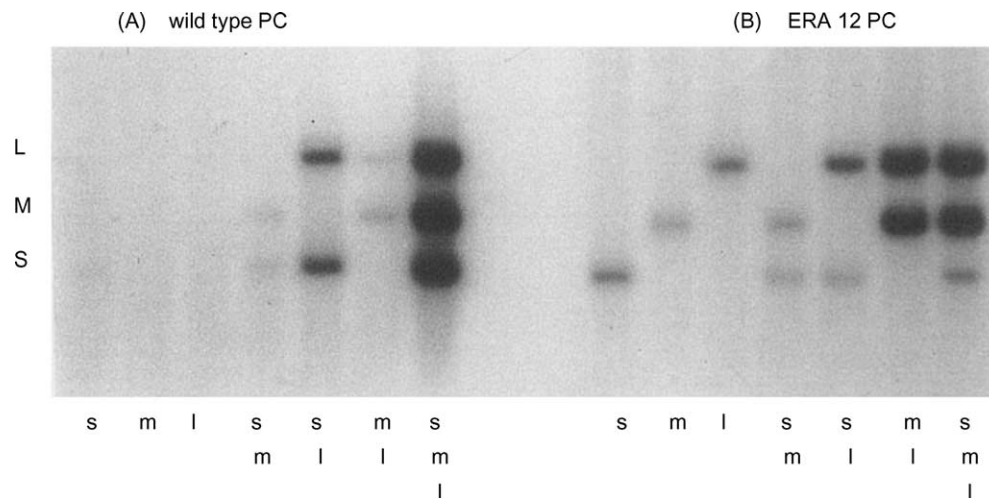


Fig. 9. In vitro minus strand synthesis with procapsids containing wild type (A) and mutant (B) protein P1. Wild type procapsids require all three plus strands for minus strand synthesis while ERA12 procapsids need only L and M. ERA12 excludes the plus strand of S.

sequences of $\Phi 6$ and $\Phi 8$ can be compensated by changes in P1 (Qiao et al., 2003a). Cross-linking studies have indicated interaction between plus strands and P1 before packaging (Qiao et al., 2003b).

4. Recombination in the Cystoviridae

The ability to package and replicate RNA in vitro has made it possible to investigate some of the parameters of RNA recombination in the Cystoviridae. Recombination is due to template switching and usually involves very little sequence identity at the crossover point. The average identity is about three bases but can range from 0 to 12 nucleotides. There does not seem to be any preference for either the takeoff or the landing points with respect to their positions on the respective templates (Qiao et al., 1997a). The recombination appears to take place during minus strand synthesis. Homologous recombination can take place if the identity is of the order of 500 nucleotides or more, but these viruses do not normally contain regions of such extensive identity (Onodera et al., 2001).

Recombination is rare in normal cystovirus constructs. However, there are several conditions that promote recombination. The first demonstration of heterologous recombination involved reporter genes inserted into the 3' non-coding regions of segment M of $\Phi 6$. In the case of either kanamycin resistance or *lac α* genes that were bounded by sequences that could form hairpin stems, recombinants were observed that had lost the reporter genes and had gained 3' sequences of either segments S or L (Onodera et al., 1993) (Fig. 5). The stronger the hairpin stems, the higher the frequency of recombination. In vitro packaging and replication studies showed that the strong hairpins did not enter the procapsids and the RNA molecules with the strong hairpins were not templates for minus strand synthesis except under conditions of template switching. It was also found that removing

the 3' end of a plus strand RNA could destroy its ability to serve as a template for minus strand synthesis, depending upon the sequence that remained at the terminus. The normal sequence at the 3' end of the $\Phi 6$ segments is CUCUCUCUCU. A sequence of CCC could support minus strand synthesis but GAGCAGC would not, nor would several other sequences. Purified polymerase is less stringent in its template choices (Makeyev and Bamford, 2001), but the polymerase in the intact core is very choosy. Such plus strand molecules could be packaged and could serve to promote the packaging program, but would not serve as templates for minus strand synthesis unless involved in heterologous recombination (Qiao et al., 1997a). The frequency of rescue by recombination was of the order of 1% of the number of plaque forming units obtained with intact segments (Qiao et al., 1997a). This brings us back to the question of the recombination promoted by the hairpin structures. It is possible that the RNA distal to the hairpin and inside the procapsid is able to serve as template after accepting a nascent minus strand coming from another template. It is also possible that the hairpin is eventually digested outside the particle and that the resulting 3' end of the plus strand can now load into a polymerase molecule. The structure of the $\Phi 6$ polymerase has been solved (Butcher et al., 2001), and it appears that the only access for an RNA molecule is to enter at its 3' end. This would mean that a molecule with its 3' end outside the procapsid should not be able to interact with the polymerase.

Another way to promote recombination is to remove secondary structure from the 3' ends of the plus strands (Mindich et al., 1994). All plus strands have secondary structure composed of stem-loops near the 3' end. The RNA continues to serve as a template for minus strand synthesis if structures are removed but the terminal sequences remain. However, phages that have incorporated these constructs yield recombinants at very high frequencies. It appears that the secondary structure acts to protect the 3' ends from nuclease activity in the host cells. In the absence of the

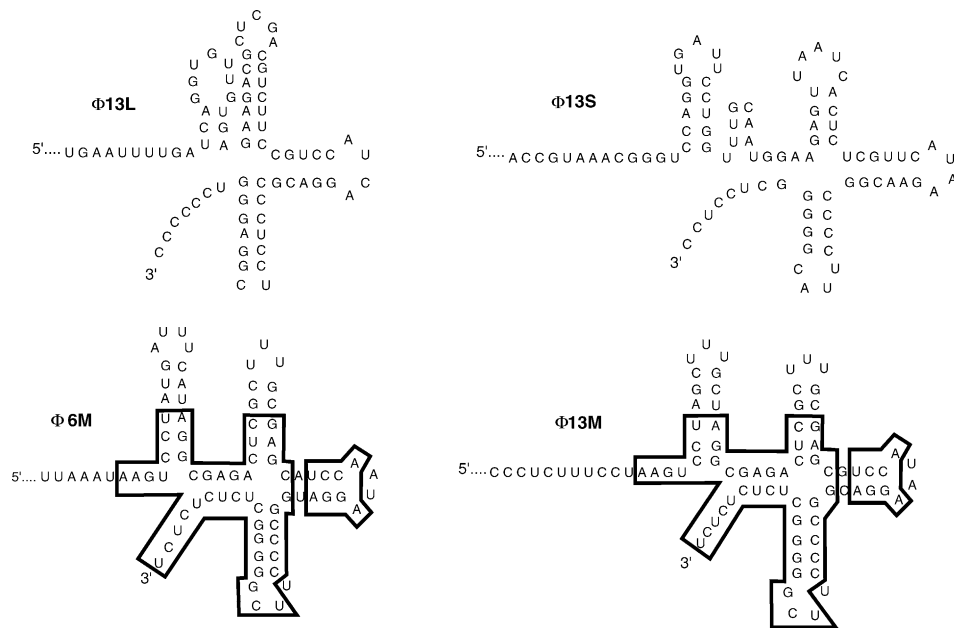


Fig. 10. The secondary structure at the 3' end of the $\Phi 13M$ plus strand is strikingly similar to that of $\Phi 6M$. It is proposed that it was acquired by recombination.

structures the molecules are nibbled and then do not serve as templates unless rescued by recombination.

Although most examples of recombination are the result of laboratory manipulations, the structure of the $\Phi 13M$ segment appears to be the product of recombination in the wild. The sequence at the 3' end is identical to that of the $\Phi 6M$ segment while being quite dissimilar to that of the other two $\Phi 13$ segments (Fig. 10).

Acknowledgements

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