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“Push Through One-Way Valve”
Mechanism of Viral DNA Packaging

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Abstract

Double-stranded (ds)DNA viruses package their genomic DNA into a procapsid using a force-generating nanomotor powered by ATP hydrolysis. Viral DNA packaging motors are mainly composed of the connector channel and two DNA packaging enzymes. In 1998, it was proposed that viral DNA packaging motors exercise a mechanism similar to the action of AAA⁺ ATPases that assemble into ring-shaped oligomers, often hexamers, with a central channel (Guo et al. Molecular Cell, 2:149). This chapter focuses on the most recent findings in the bacteriophage φ29 DNA packaging nanomotor to address this intriguing notion. Almost all dsDNA viruses are composed entirely of protein, but in the unique case of φ29, packaging RNA (pRNA) plays an intermediate role in the packaging process. Evidence revealed that DNA packaging is accomplished via a “push through one-way valve” mechanism. The ATPase gp16 pushes dsDNA through the connector channel section by section into the procapsid. The dodecameric connector channel functions as a one-way valve that only allows dsDNA to enter but not exit the procapsid during DNA packaging. Although the roles of the ATPase gp16 and the motor connector channel are separate and independent, pRNA bridges these two components to ensure the coordination of an integrated motor. ATP induces a conformational change in gp16, leading to its stronger binding to dsDNA. Furthermore, ATP hydrolysis led to the departure of dsDNA from the ATPase/dsDNA complex, an action used to push dsDNA through the connector channel. It was found unexpectedly that by mutating the basic lysine rings of the connector channel or by changing the pH did not measurably impair DNA translocation or affect the one-way traffic property of the channel, suggesting that the positive charges in the lysine ring are not essential in gearing the dsDNA. The motor channel exercises three discrete, reversible, and controllable
steps of gating, with each step altering the channel size by 31% to control the direction of translocation of dsDNA. Many DNA packaging models have been contingent upon the number of base pairs packaged per ATP relative to helical turns for B-type DNA. Both 2 and 2.5 bp per ATP have been used to argue for four, five, or six discrete steps of DNA translocation. The “push through one-way valve” mechanism renews the perception of dsDNA packaging energy calculations and provides insight into the discrepancy between 2 and 2.5 bp per ATP. Application of the DNA packaging motor in nanotechnology and nanomedicine is also addressed. Comparison with nine other DNA packaging models revealed that the “push through one-way valve” is the most agreeable mechanism to interpret most of the findings that led to historical models. The application of viral DNA packaging motors is also discussed.

I. INTRODUCTION

DNA packaging into a preformed protein shell (procapsid) is characteristic of double-stranded (ds)DNA viruses, including bacteriophages, herpesviruses, and adenoviruses. Most viral procapsids are a few tens of nanometers in diameter, whereas viral genomes are several micrometers in length. Electron microscopy (EM) images revealed that packaged viral DNA inside the small procapsid can be condensed to 500 mg/ml, comparable to near-liquid crystalline density (Earnshaw and Casjens, 1980; Hohn, 1976). High internal pressure exists inside the procapsid during DNA packaging (Fuller et al., 2007a,b; Rickgauer et al., 2008; Smith et al., 2001); this energetically unfavorable process is accomplished by packaging motors powered by ATP hydrolysis through a DNA and procapsid-dependent ATPase (Guo et al., 1987d).

These natural, nanometer-scale DNA packaging motors are of special interest because of their inherent abilities to recognize specific viral genomes in a pool of DNAs and to package them into such a tiny space against a large internal force. Over the years, studies on the DNA packaging motor have focused primarily on fundamental aspects, including structure, biological/biochemical function, and mechanical or physical behaviors of the viral motor or its components for genome packaging. More recently, powerful biological motors have inspired novel biomimetic designs that have opened up possibilities for building artificial nanomotors operable outside their natural environment for use in nanodevices, nanomedicine, including the sensing of ions, chemicals, or DNA/RNA (Fang et al., 2012; Geng et al., 2011; Hess and Vogel, 2001; Jing et al., 2010a,b; Soong et al., 2000; Wendell et al., 2009), and targeted gene delivery or drug loading (Guo, 2010; Guo et al., 2005a, 2010; Khaled et al., 2005; Liu et al., 2007; Shu et al., 2011b; Zhou et al., 2011). A thorough
understanding of how the motor components interact with each other during the packaging process and how the energy from ATP hydrolysis is transferred into physical motion would provide valuable insights into fundamental phenomena and the development and application of nanomotor biomimetics. In addition, possible novel targets for antiviral therapy could be discovered based on studies of the viral DNA packaging mechanism (Bogner, 2002; Trottier et al., 1996; Visalli and van Zeijl, 2003; Zhang et al., 1995). Utilizing motor components in nanotechnology and/or disease treatments has also been pursued actively (Geng et al., 2011; Guo, 2010; Guo et al., 2005a, 2010; Jing et al., 2010a,b; Khaled et al., 2005; Liu et al., 2007; Shu et al., 2011b; Wendell et al., 2009; Zhou et al., 2011).

Various packaging mechanisms have been proposed throughout the years (Black, 1981; Chen and Guo, 1997; Grimes and Anderson, 1997; Guasch et al., 2002; Guo et al., 1998; Hendrix, 1978; Moffitt et al., 2009; Morita et al., 1995; Serwer, 2003, 2010; Sun et al., 2010; Yu et al., 2010). Several thorough reviews on the packaging of dsDNA viruses (Casjens, 2011; Guo and Lee, 2007; Rao and Feiss, 2008; Serwer, 2003, 2010; Sun et al., 2010) and φ29 (Grimes et al., 2002; Guo, 2002; Guo and Trottier, 1994; Lee et al., 2009; Yu et al., 2010) have been published. This chapter focuses primarily on recent publications of studies on the structure, function, and mechanism of the φ29 DNA packaging motor, but will also compare other phages to this unique system.

II. STRUCTURE OF VIRAL DNA PACKAGING MOTORS

The packaging motor of dsDNA viruses consists of a motor channel for dsDNA translocation (Fig. 1) and two DNA packaging components that are not fixed components in the purified procapsid. These components were classified in 1987 (Guo et al., 1987d) into two categories according to their role in DNA packaging: the first category is the large subunit responsible for binding to the procapsid and contains an ATP-binding consensus sequence for ATP binding and hydrolysis and the second category is the smaller subunit that interacts with DNA (Guo et al., 1987d). In bacteriophage φ29, the motor system uniquely involves an RNA ring (Guo et al., 1987b).

A. Motor channel as a one-way valve for unidirectional DNA translocation

The bacteriophage connector is embedded in the procapsid to connect the viral head to the tail. This essential motor component is composed of 12 copies of connector proteins. Detailed three-dimensional structures of
connectors in different phages have been studied and determined at high resolution through both cryo-EM and X-ray crystallography (Agirrezabala et al., 2005; Cingolani et al., 2002; Fokine et al., 2004; Guasch et al., 2002; Jiang et al., 2006; Jimenez et al., 1986; Lander et al., 2006; Lurz et al., 2001; Simpson et al., 2000; Trus et al., 2004). The connectors in various bacteriophages display similar morphology (Agirrezabala et al., 2005; Valpuesta and Carrascosa, 1994), although the sequence alignment showed a lack of homology among them.

The φ29 connector is assembled from 12 copies of connector protein gp10 and forms a truncated cone shape (Jimenez et al., 1986). The narrow end, with a diameter of 6.8 nm, extrudes out of the procapsid, while the wider end, with a diameter of 13.8 nm, is buried inside the procapsid (Fig. 1) (Guasch et al., 2002; Simpson et al., 2001). The N-terminal 14 amino acids...
acids of gp10 serve as the foothold for packaging RNA (pRNA) of φ29 (Sun et al., 2006; Xiao et al., 2005), as proved by cross-linking (Garver and Guo, 1997). Further point mutation studies of the 14 amino acids revealed that the three basic amino acids, Arg-Lys-Arg, at positions 3–5 of the N terminus of gp10 were responsible for pRNA binding. Mutation of any two of these three amino acids resulted in complete abolishment of pRNA binding to the DNA packaging motor (Atz et al., 2007). In the case of φ29, pRNA most likely serves as the foothold to which the packaging ATPase gp16 binds to exercise its force in pushing the DNA into the procapsid (Lee and Guo, 2006).

Structural analysis showed that the connector contains three layers consisting of two hydrophilic layers separated by a hydrophobic layer. Utilizing this characteristic, the φ29 connector was embedded successfully into a planar lipid membrane, and dsDNA translocation through the channel was studied by electrophysiological measurements (Fang et al., 2012; Jing et al., 2010a,b; Wendell et al., 2009) (Fig. 2). During translocation, DNA physically obstructs the connector channel, and the observed signal indicates a blockage of current. Measurements of dsDNA translocation through connectors in the membrane proved that translocation only occurred in one direction (Figs. 3 and 4). It was also found that the frequency of DNA translocation events changes in agreement with the different orientations

![FIGURE 2](image-url) Insertion of the re-engineered φ29 connector into lipid membrane and demonstration of robust conductivity property. Red trace: Discrete steps of current representing the insertion of one connector for each step. All these current steps are similar in conductance, indicating the homogeneity of the channel size. (Inserts) Fluorescence image of the fluorescently-labeled connector in the liposome membrane. Connector insertion into the bilayer only occurred when connector-reconstituted proteoliposomes were fused into the bilayer. Adapted with permission from Macmillan Publishers Ltd. (Wendall et al., 2009) ©2009.
of the connector when multiple connectors were fused in the membrane. DNA translocation frequency remained the same when additional connectors were inserted with an opposite orientation for DNA translocation or increased if the additional connector was inserted with the same orientation (Fig. 5). Using antibody or gold particles to bind to the tag-conjugated C-terminal, proper orientation of the connector for DNA translocation was probed to be from the N terminus to the C terminus, the same direction that DNA traverses during packaging. In addition, packaged viral dsDNA remained within the procapsid under the strong force of centrifugation (Shu and Guo, 2003b). All the facts strongly support the conclusion that the φ29 connector exercises a one-way traffic property for dsDNA packaging into procapsid from the N terminus to the C terminus of the connector (Jing et al., 2010a). Logistically, this brings up an intriguing question of how DNA is expelled from the virus during the infection process; further research revealed that the connector exercises three discrete steps of conformational changes in regulating the direction of DNA translocation (Geng et al., 2011).
B. Effect of pH, channel charge, and lysine ring mutations on DNA translocation

The \( \phi 29 \) connector channel was found to be robust and stable under even extreme pH conditions (Jing et al., 2010b). Single pore conductance assays of membrane-embedded connector showed that the connector remains open with uniform channel conductance under extreme pH and that both conductance and membrane insertion orientation of the channel are independent of the pH (Fang et al., 2012). While DNA translocation events were still observed at these extreme conditions, formation of apurinic acid at pH 2 led to shorter current blockade events. Overall, the connector retained its stable channel properties under strong acidic or alkaline conditions, despite the inherent effect on DNA structure.

The structural study of the \( \phi 29 \) connector showed that there are 48 positively charged lysine residues in the inner channel (four 12-lysine rings from the 12 gp10 subunits). It was believed that these positively charged rings could play an important role in DNA translocation through the channel in that they may interact with the negatively charged phosphate backbone of dsDNA during DNA translocation (Agirrezabala et al., 2005; Guasch et al., 2002). Different mutations were introduced to the

![FIGURE 4](#) One-way traffic of DNA translocation through the connector pore verified by switching polarity. (A) In the absence of DNA. (B–D) In the presence of DNA in both chambers where a single connector was inserted. Adapted with permission from Jing et al. (2010a) ©2010 American Chemical Society.
lysine residues (Fig. 6) in order to study the effect of lysine rings (Fang et al., 2012). The effect on channel size, procapsid assembly, dsDNA translocation, and connector outer surface charge distribution were assessed through their interaction with the lipid membrane by single pore conductance, direction of dsDNA translocation, efficiency of DNA packaging, and production of infectious virions. The channel size of the lysine mutant connectors can be deduced from the single channel conductance assays. Upon connector insertion into the lipid membrane, discrete current jumps were observed under an applied voltage, representing the open-pore current amplitude. When DNA passes through the channel, the capacity of the electrolyte ion passage is reduced, resulting in transient current blockade events. Because the diameter of dsDNA is 2 nm and the size of the narrowest region of the wild-type connector channel is 3.6 nm, the ratio of the cross-sectional area (represented by the ratio of the open-pore current and the current during the DNA blockade) can be used as a parameter to estimate the channel size at the narrowest point. Furthermore, these assays were performed in both acidic and basic environments to investigate the role of lysine residues in dsDNA translocation and the direction of dsDNA trafficking (Fig. 7). Results indicated that the four lysine rings within the \( \phi 29 \) connector channel are not involved

**FIGURE 5** Change of DNA translocation frequency in the presence of multiple connectors reflects the one-way traffic pattern of DNA translocation. (Insets a–d) DNA translocation frequency after insertion of each connector channel in the BLM for individual experiments. Adapted with permission from Jing et al. (2010a) © 2010 American Chemical Society.
in the active translocation of dsDNA (Fang et al., 2012) and supported the “push through a one-way valve” model of dsDNA packaging mechanism (Section IV).

It should be noted that mutation some of the lysine residues can lead to refolding of gp10 and change the charge of the external surface of the connector due to conformational change (Fang et al., 2012). Such change of the external surface charge has hindered the efficiency in serving as a nucleus in procapsid assembly (K200A, K209A, or both) in which the connector acts as a nucleating core (Fu and Prevelige, 2009; Guo et al., 1991; Lee and Guo, 1995b) and in insertion into lipid membrane for

FIGURE 6 Effect of mutations in lysine rings within the connector channel on motor activity. (a and b) Locations of mutations within the connector channel in side view (a) and top view (b). K200 (blue), K209 (red), and the border of tunnel loop N229 (cyan) and N246 (yellow). (c) Virus assembly and (d) DNA packaging activity of procapsids bearing the mutated connectors. Adapted from Fang et al. (2012) ©2011 with permission from Elsevier.
the measurement of single channel conductance (K200A/K209A) (Fang et al., 2012). Such low efficiency due to the change of external surface charge did not affect the conclusion related to the internal surface of the channel for DNA translocation (Fang et al., 2012).

C. DNA-pushing ATPase enzyme as a member of the AAA + family

When evidence of the pRNA hexamer was uncovered, it was proposed (Guo et al., 1998), and subsequently supported by other authors (de Haas et al., 1999; Guasch et al., 2002), that viral DNA packaging is similar to the mechanism in DNA replication and RNA transcription and that the mechanism responsible for those important phenomena can be correlated to the mechanism of viral DNA packaging. Moreover, like almost all DNA and RNA packaging motors, it was hypothesized that gp16, the ATPase in the φ29
packaging motor, belonged to the superfamily of AAA+ proteins (ATPases Associated with many cellular Activities) (Guo et al., 1998; Lee et al., 2006).

This large family of proteins is extremely diverse in function associated with a multitude of different cellular activities and implied in many others. However, the common characteristic of this family is their ability to convert chemical energy from the hydrolysis of the γ-phosphate bond of ATP into a conformational change inside the protein. This change of conformation generates a loss of affinity for the substrate and a mechanical movement, which is used to make or break contacts between macromolecules, resulting in local or global protein unfolding, assembly or disassembly of complexes, or transport of macromolecules relative to each other. These activities underlie processes critical to DNA replication and recombination, chromosome secretion, membrane sorting, cellular reorganization, and many others (Maurizi and Li, 2001).

Many biochemical and structural aspects of reactions catalyzed by AAA+ proteins have been elucidated, together with interesting allosteric phenomena that occur during ATP hydrolysis. For instance, the crystal structure of sliding clamp loader complex, a system (AAA+ DNA helicase) that helps polymerases overcome the problem of torque generated during the extension of helical dsDNA, has been useful in determining how ATP is required for binding to dsDNA and opening of the clamp (Oyama et al., 2001). The crystal model reveals a spiral structure in the clamp loader with a striking correlation to the grooves of helical dsDNA, suggesting a simple explanation for how the loader/DNA helix interaction triggers ATP hydrolysis and how DNA is released from the sliding clamp. This mechanism may provide hints for understanding the role of the DNA packaging ATPase in many phages.

Notably, structural studies have proven that AAA+ proteins often assemble into homohexameric complexes with a ring structure that acts in a coordinated fashion (Fig. 8) (Maurizi et al., 2001). In past years though, models based on EM reconstruction have suggested that gp16 exists as a pentameric structure (Moffitt et al., 2009; Morais et al., 2008).

D. Packaging RNA as a hinge to gear the motor

Different from other viral systems, an RNA molecule, named pRNA, was discovered to be an essential component in the φ29 DNA packaging motor (Guo et al., 1987b). This pRNA was demonstrated to be indispensable in DNA packaging and viral assembly. The detailed structure and
function studies of pRNA has been reviewed thoroughly (Guo, 2002). It has also been found that pRNA mediates binding of the motor ATPase gp16 to procapsid, indicating that the role of pRNA in motor function may be to serve as a hinge to connect motor components and thus gear the motor (Lee et al., 2006).

The pRNA contains two structural domains, which could fold independently (Fig. 9). Its central region contains two interlocking loops responsible for its intermolecular interactions to form a hexameric ring (Figs. 9a and 9c) and binding to the procapsid, while its double-helical 5' / 3' paired region is essential in DNA packaging (Garver et al., 1997; Reid et al., 1994a,b,c; Zhang et al., 1994) and for the binding of the ATPase gp16 to bring it into proximity to the connector (Lee et al., 2006). A three-arms-around-a-hinge model for pRNA function was proposed based on the independent folding and function of the two structural domains (Fang et al., 2005). The two interlocking loop regions and the helical region of the pRNA were regarded as three individual arms connected through a hinge. It is believed that at functional magnesium concentration, the
FIGURE 9  Illustration of multivalent pRNA nanoparticle formation. (a) The concept of hand-in-hand interaction in pRNA hexamer formation. Adapted from Guo (2002) ©2002 with permission from Elsevier. (b) Illustration of the Φ29 packaging motor geared by six pRNAs with bottom view and side view. Adapted from Shu et al. (2003c) ©2003 with permission from Elsevier and from Macmillan Publishers Ltd. (Shu et al., 2011a) ©2011. (c) Schematic of pRNA hexamer ring. (d) Secondary structure of pRNA with green box indicating the central 3WJ domain. (e) The 3WJ domain composed of three RNA oligomers. Helical segments are represented as H1, H2, and H3. (f) Schematic of a trivalent RNA nanoparticle using the 3WJ-pRNA core sequence. (g) Atomic force microscopy images of (f). (h) Schematic of 3WJ-pRNA-siSur-Rz-FA nanoparticle using the 3WJ-pRNA core sequence. (i) Atomic force microscopy images of (h) (Chen et al., 1999; Shu et al., 2011a). Adapted with permission from Macmillan Publishers Ltd. (Shu et al., 2011a) ©2011.
three arms folded into a conformation in which pRNA would be fully active for motor function. It was also proposed that the addition of ATP would promote relative motion among the three arms of pRNA and thus package DNA (Fang et al., 2005; Shu and Guo, 2003a). This agrees with the hypothesis that the bifurcation polyuridine bulge (U72U73U74) at the helical junction may provide flexibility in orientation of the helices for pRNA to function correctly (Zhang et al., 1997). It has also been revealed that pRNA contains an ATP-binding motif (Shu et al., 2003a). However, pRNA itself does not display ATPase activity. It is expected that the ATPase active center is a complex of the entire motor and that ATP hydrolysis is a collective effort of all motor components. Indeed, in the absence of pRNA, the ATPase activity of the ATPase gp16 is extremely low (Grimes and Anderson, 1990; Guo et al., 1987d; Ibarra et al., 2001; Lee et al., 2008; Shu et al., 2003a).

In addition, the study on the bulged regions of pRNA showed that a C18C19A20 bulge in the double-helix domain is essential for DNA packaging, but dispensable for gp16 binding to the procapsid/pRNA complex (Lee et al., 2006; Reid et al., 1994b; Zhang et al., 1997). Further study on the role of the CCA bulge in DNA packaging revealed that the size and location, rather than the sequence of the bulge, are important for proper orientation of the double-helical domain, which could be critical for the correct contact between gp16 docked on pRNA and genomic DNA. The CCA bulge may also provide flexibility for pRNA to serve as a hinge to drive the motor (Lee et al., 2006; Zhao et al., 2008).

An unexpected finding has been reported regarding an unusual pRNA trifurcation motif (the 3WJ, or three-way junction core) domain. It was found that this pRNA 3WJ domain (Fig. 9d) can be assembled from three pieces of RNA oligonucleotides with remarkable thermodynamically stable properties (Shu et al., 2011a). The deep slope of the T_M melting curve from the three pieces is close to 90°, indicating extremely low free energy and unusually high affinity in 3WJ assembly. The self-assembled RNA nanoparticles with three or six pieces of RNA oligonucleotides guided by the 3WJ core are highly stable, resistant to 8 M urea denaturation, and do not dissociate even at extremely low concentrations (Figs. 10a–10c). In comparison with 36 other 3WJ motifs found in different biological RNAs, the 3WJ core of φ29 pRNA showed extraordinary stability demonstrated by T_M melting curves and slopes (Fig. 10d). Additionally, metal ions were found to be dispensable for nanoparticle assembly. Because the φ29 DNA packaging motor is one of the strongest biomotors discovered to date, the motor module should be stronger than the ordinary counterpart to perform such a special function as a hinge in motor gearing. This 3WJ core domain can serve as a potential platform to construct stable, multivalent nanoparticles for gene therapy purposes (Shu et al., 2011a)(see Section V).
E. Approaches in packaging RNA stoichiometry determinations that lead to hexamer conclusion

1. By concentration-dependent curve

An approach based on the concentration dependence of the dose–response curve was used to estimate the stoichiometry of pRNA (Lee and Guo, 1995a). The principle behind this approach is that the larger the stoichiometry of a component involved in the motor, the more dramatic the influence of the concentration on the motor activity. In comparison with a component of known stoichiometry, the copy number of both pRNA and gp16 can be estimated (Lee et al., 1995a).

FIGURE 10  The 3WJ-pRNA core motif showed unusual stability. (a) A 16% native gel of the 3WJ-pRNA core at 37°C. Fixed concentration of Cy3-labeled [ab*c]3WJ was incubated with various concentrations of unlabeled b3WJ at 37°C. (b) Urea-denaturing effects on the stability of [ab*c]3WJ evaluated by a 16% native gel. A fixed concentration of labeled [ab*c]3WJ was incubated with unlabeled b3WJ at ratios of 1:1 in the presence of 0–6 M urea at 25°C. (c) Dissociation assay for the [32P]3WJ-pRNA complex harboring three monomeric pRNA by twofold serial dilution (lanes 1–9). (d) Comparison of melting curves for different RNA 3WJ core motifs. Adapted with permission from Macmillan Publishers Ltd. (Shu et al., 2011a) ©2011.
2. By binomial distribution using mutant and wild-type pRNA
Mathematical approaches by statistical analysis based on binomial distribution were explored to determine the stoichiometry of pRNA (Chen et al., 1997; Trottier and Guo, 1997). Mutant pRNA with the ability to bind procapsid but not to package DNA were mixed with wild-type pRNA at various ratios in in vitro assembly assays. Thus, the pRNA ring on each procapsid would be a mixture of mutant and wild-type pRNA, and the probability could be determined by expansion of a binomial. Viral assembly activity was therefore simulated against different ratios between mutant and wild-type RNA for different stoichiometries of pRNA. By fitting the empirical data with predicted curves, it was determined that the stoichiometry of pRNA is either 5 or 6 (Trottier et al., 1997).

3. By the common factor of 2 and 3
Sequences of the two interlocking loops of pRNA (bases 45–48 of right-hand loop and bases 85–82 of left-hand loop) are complementary and allow pRNA to interact intermolecularly to form homo-oligomers. These sequences can be engineered so that pRNA can form hetero-oligomers. To simplify the description, uppercase and lowercase letters are used to represent the right- and left-hand loop sequences of the pRNA, respectively. The same letter in upper- and lowers cases symbolizes a pair of complementary sequences. For example, in pRNA Aa’, right loop A (5’GGAC48) and left loop a’ (3’CCUG82) are complementary, whereas in pRNA Ab’, the four bases of right loop A are not complementary to the sequence of left loop b’ (3’UGCG82). It was found that a mutant pRNA with complementary loop sequences (such as pRNA Aa’) was active in DNA packaging by itself, while a mutant with noncomplementary loops (such as pRNA Ab’) was inactive. However, when pRNA Ab’ is mixed with pRNA Ba’ at a 1:1 molar ratio, DNA packaging activity was restored through interaction of the loops. The stoichiometry of pRNA was therefore predicted to be a multiple of 2 and that dimeric pRNA is the building block of the pRNA ring (Chen et al., 2000; Guo et al., 1998). Similarly, mutant pRNAs J-p’, P-k’, and K-j’ were found to be inactive alone or in a mixture of any two, but regained activity when all three were mixed at a 1:1:1 molar ratio, suggesting that the stoichiometry of pRNA is also a multiple of 3. Combined with studies by mathematical approaches, stoichiometry was therefore concluded to be a common multiple of 2 and 3, and decisively determined to be 6 (Guo et al., 1998).

4. By single molecule photobleaching
A single molecule photobleaching assay in conjunction with statistical analysis was applied to elucidate the copy number of pRNA molecules on active motors (Fig. 11) (Shu et al., 2007). With the pRNA labeled singly
with Cy3 fluorophore and φ29 DNA labeled with Cy5, single active motors or the DNA packaging intermediates were isolated by dual-color fluorescence imaging (Shu et al., 2007). Utilizing the characteristic, quantized drop in intensity for single fluorophores, it was found that there are six photobleaching steps, representing six pRNA molecules on a single motor before or during DNA packaging, confirming the conclusion that stoichiometry of the pRNA on the motor is 6 (Fig. 11). Facilitated by the same single molecule photobleaching approach, a novel mechanism was also discovered in regards to RNA/protein interactions (Xiao et al., 2008).
It was demonstrated that the stable and specific binding of pRNA to the procapsid relies on formation of a closed RNA ring with correct ring size to fit on the connector rather than depending on specific sequences of RNA. Interruption of any one of the interlocking links in the closed RNA ring could impede the formation of the hexameric ring, resulting in abolishment of the pRNA to bind. Extension or reduction of the circumference of the RNA ring by modification of arms in the loop region also abolishes the binding of pRNA to the motor. In addition, an artificial RNA with a designed, proper ring size was found to be active in DNA packaging (Xiao et al., 2008). Formation of the closed pRNA ring from a pure dimer or pure trimer alone strongly supports the argument that the ring is a common multiple of two and three, which must be a hexamer.

5. By gold and ferritin labeling to pRNA
Gold and ferritin conjugation (Fig. 12) was used to observe the number of pRNA on each motor. EM images of motors with single gold- or ferritin-conjugated pRNA revealed that six particles were attached to the vertex of the procapsid (Moll and Guo, 2007). It was found that procapsid particles

![Figure 12](image.png)

**FIGURE 12** Demonstration of the presence of six pRNA on each procapsid by conjugation of one ferritin particle (a) or one gold nanoparticle (b) to each pRNA. Adapted from Xiao et al. (2008) by permission of Oxford University Press and Macmillan Publishers Ltd. (Shu et al., 2007) ©2007.
harbored six gold particles or no gold at all (Xiao et al., 2008) (Fig. 12). The result supports the finding that stable and specific binding of pRNA to the procapsid relies on formation of a closed RNA ring with correct ring size to fit on the connector rather than depending on specific sequences of RNA. Interruption of any one of the interlocking links in the closed RNA ring could impede formation of the hexameric ring, resulting in abolishment of the pRNA to bind.

F. Challenge and discrepancy in determination of stoichiometry of motor packaging RNA

To elucidate the role of pRNA in DNA packaging, it is critical to know how many copies of the pRNA are involved in each DNA packaging event. The approaches described earlier strongly demonstrated the pRNA ring as a hexamer. However, a discrepancy in pRNA stoichiometry has been reported. In 1998, the formation of hexameric pRNA ring was demonstrated by two back-to-back papers (Guo et al., 1998; Zhang et al., 1998) and featured in Cell by Hendrix (1998). In cryo-EM studies, contradictory results of a sixfold (Ibarra B et al., 2000) or fivefold (Morais et al., 2001, 2008; Simpson et al., 2000) symmetry of the pRNA ring were reported by two separate groups. Traditionally, because cryo-EM was developed to study protein complexes with symmetrical structural arrangement, this technology is very useful to study the viral capsid structure with defined, symmetrical arrangement. The application of cryo-EM to study RNA structure is very intriguing but very challenging for the following reasons. (1) Compared to protein, the chemically unmodified RNA is unstable and very sensitive to RNase, especially the single-stranded region such as the pRNA C18C19C20 bulge and the hand and head loops (Bailey et al., 1990; Chen et al., 1999; Guo et al., 1987a). In cryo-EM image reconstruction, data are averaged from the computation pool of many viral particles. This average will be smaller due to RNA partial degradation or cleavage. (2) Compared to protein, RNA is structurally flexible due to its dynamic nature and kinetic and thermodynamic characteristics (Bothe et al., 2011). Solution-state nuclear magnetic resonance studies revealed that many noncoding RNAs do not fold into a single native conformation but fold into many different conformations along their free-energy landscape. The dynamic motion of RNA spans from the pico-second to second timescale. (3) The RNA structure depends on many ions, including ATP, ADP, and Pi. Alteration of sampling conditions leads to a variety of structures. (4) RNA folding relies on sequences, ligands, and environments. Any alteration in sequences and substrate binding can lead to alterations in RNA structure. (5) It is challenging to use technology for detection of a stationary image to assess a machine in motion undergoing a dynamic process without structural symmetry (Chen et al., 1997; Moffitt
et al., 2009). In the meantime, a plethora of studies using biochemical, mathematical, and genetic approaches (Chen et al., 1997; Shu et al., 2007; Trottier et al., 1997; Xiao et al., 2008) were carried out to study the stoichiometry of pRNA on the motor and have supported the hexamer observation (Guo et al., 1998; Shu et al., 2007; Xiao et al., 2008; Zhang et al., 1998). The conclusion of a hexameric ring has also been observed in the T3 system showing that the DNA packaging protein gp19 forms six subunits on the T3 DNA packaging motor (Fujisawa et al., 1991).

The group who asserted the model of a pentameric pRNA ring on the motor argues that pRNA binds to the fivefold procapsid and a pRNA hexamer is formed initially, but one of the pRNA molecules dissociates afterward due to a conformational change (Simpson et al., 2000). However, cross-linking approaches revealed that pRNA did not bind to the procapsid protein but to the connector (Garver et al., 1997). Moreover, another paper (Xiao et al., 2005) provided solid evidence that pRNA binds to the N terminus of the connector protein gp10 via the interaction of the positively charged residues in protein and the negatively charged phosphate backbone of RNA. Further mutation analysis revealed that the three basic amino acids, Arg-Lys-Arg, at the N terminus are responsible for pRNA binding (Atz et al., 2007). Furthermore, it would be difficult to account for how a fivefold pRNA ring docks at the sixfold symmetric connector. In addition, single molecule photobleaching studies of purified DNA packaging intermediates showed six copies of pRNA on active motors filled partially with DNA, disputing the explanation that one pRNA molecule would leave the motor after binding (Shu et al., 2007). Chen and Guo (2000) demonstrated that the pRNA dimer is the building block in hexamer formation. Other literature (Ding et al., 2011; Fang et al., 2005, 2008; Gu and Schroeder, 2011; Robinson et al., 2006) all support the long-standing finding (Chen et al., 2000; Shu et al., 2007; Xiao et al., 2008) that the pRNA dimer is the building block and that the sequential action in hexamer assembly is $2 \rightarrow 4 \rightarrow 6$. A study with RNA alone in the absence of the procapsid, a form of tetramer, was revealed by X-ray crystallography (Ding et al., 2011). However, the authors used the tetramer-based crystal structure to compute a pRNA pentamer on the procapsid by docking the monomeric RNA fragment abstracted from the tetramer by cryo-EM computation. As noted earlier, the RNA structure is flexible, and multiple conformations can result from one RNA sequence, depending on the energy of the landscape, the ligand, and the environment. RNA could change its structure after binding to the procapsid; thus, the pRNA on the procapsid might not be identical to the free RNA in solution that grew into the crystal structure (Ding et al., 2011). In addition, the RNA fragment used in crystallization is not a full pRNA molecule but is a truncated RNA fragment, and several bases were changed in this truncated version (Ding et al., 2011). The truncated and mutated RNA fragment might not display
the same conformation as that in the integrated pRNA ring sheathing to
the viral motor (Hoeprich and Guo, 2002). Indeed a publication on struc-
tural study of the pRNA 3WJ motif using spin labeling established a very
different global structure with significant variation from that in the crystal
structure (Zhang et al., 2012). Whether such structures and conformations
are the real depiction of pRNA on the motor remain to be elucidated.

III. ENERGY SOURCE AND CONVERSION MECHANISM

A. Sequential interaction of motor ATPase with DNA and ATP

Viral DNA packaging has long been recognized to require energy (Hohn,
1976; Kellenberger, 1976; Riemer and Bloomfield, 1978) or ATP (Masker,
1982). In 1986, it was discovered that the larger subunit of the two DNA
packaging enzymes of dsDNA bacteriophage is a DNA and procapsid-
dependent ATPase that contains both A- and B-type ATP-binding con-
sensus sequences (Guo et al., 1987d). In the past, several studies were
aimed at understanding the function of the φ29 motor ATPase, gp16, and
how it catalyzes ATP hydrolysis in combination with dsDNA.

A nonhydrolyzable ATP analog, γ-s-ATP, was used to inhibit φ29 DNA
packaging (Chemla et al., 2005; Guo et al., 1987c; Llorca et al., 1997;
Schwartz et al., 2012; Shu et al., 2003b; Smith et al., 2001). The γ-s-ATP
was also used to isolate dsDNA packaging intermediates through sucrose
gradient sedimentation (Shu et al., 2003b). These intermediates were then
converted into functional phages and infectious viruses by inputting
additional ATP and gp16 as demonstrated by phage assembly assay
(Shu et al., 2003b). These experiments suggested that the packaging pro-
cess is strongly dependent on the energy produced by ATP, and blocking
the production of energy mediated by gp16 can easily stall packaging of
the DNA into the procapsid. The stalling is, however, reversible, as the
intermediates can be transformed into functional phages by adding gp16
and ATP, thus restoring the production of energy (Shu et al., 2003b).

The γ-s-ATP was also used to block the departure of the ATPase gp16
from the dsDNA/ATPase complex (Schwartz et al., 2012). Electrophoretic
mobility shift assays (EMSA) and fluorescence resonance energy transfer
(FRET) studies were used to study the binding affinity of gp16 to dsDNA.
The nonhydrolyzable ATP analog, γ-s-ATP, was used to stabilize the
binding between gp16 and dsDNA, indicating that gp16 assumes a
high-affinity state for DNA when it is complexed with the nucleotide
triphosphatate. Addition of ATP was capable of releasing the protein
from dsDNA (Schwartz et al., 2012). A scheme of reaction, similar to
other AAA+ proteins, can be drawn from these experiments in which
gp16 alternatively assumes a high- and a low-affinity state for DNA.
before and after ATP hydrolysis occurs, respectively, and the hydrolysis of ATP induces a power stroke for DNA translocation in which the ATPase pushes the DNA away from itself (Schwartz et al., 2012).

Whether gp16 preferred to bind to dsDNA or ATP first during motor action was also assayed. The EMSA was again employed and dsDNA or γ-s-ATP was applied initially and the missing component subsequently added. The complex was stabilized better when γ-s-ATP was added first, suggesting that the ATPase first binds to ATP, promoting a conformational change to bind tightly to dsDNA (Schwartz et al., 2012).

B. Packaging force and velocity

Many biomotors, found in several different environments (cellular trafficking, ATP-dependent protein degradation, helicases), are powered by breaking down an energy-rich bond of ATP (Dittrich and Schulten, 2006; Grigoriev et al., 2004; Hua et al., 1997; Ishijima et al., 1998; Stock et al., 1999; Walker et al., 1982), similar to combustion engines breaking down molecules in petroleum distillates. Other motors are driven by the flow of protons along a proton gradient, an electrochemical potential across a membrane, just as electrical motors are powered by the flow of electrons along an applied voltage; an example of such can be found in bacterial flagella (Berry, 2001) or in ATP-generating machinery (F\textsubscript{0}/F\textsubscript{1} ATP synthase)(Sambongi et al., 1999). Viral packaging motors are powered by chemical energy derived from the hydrolysis of ATP. The active sites of motor ATPases bind ATP and catalyze its degradation to ADP and inorganic phosphate (Pi), thereby releasing a significant quantity of energy and inducing conformational changes in the motor structure that are then converted into mechanical force for the motor to function. Such conformational changes are most likely related to the release of inorganic phosphate to generate the power stroke on the DNA rather than the ATP-binding step. This catalytic process is then restarted by the next ATP molecule, and the motor continues to function in a processive and sequential manner (Chen et al., 1997) until the DNA is completely packaged. Studies have shown that energy converted from the hydrolysis of one ATP molecule corresponds to the packaging of every 2 (Guo et al., 1987d) or 2.5 (Moffitt et al., 2009) bp of DNA in the φ29 system, comparable to that of T3 with 1.8 bp per ATP (Morita et al., 1993).

All viral packaging enzymes, including gp16 of φ29, possess ATPase activity and contain a conserved Walker-A ATP-binding motif (G/A) X\textsubscript{4}GK(S/T), also called the P-loop, and a Walker-B motif (h\textsubscript{4}DE) in which the two negatively charged residues, aspartate and glutamate, are involved in γ-phosphate bond hydrolysis (Guo et al., 1987d). Mutations of a single amino acid in the Walker-A or Walker-B regions are capable of abolishing ATP binding or hydrolysis, thereby completely inhibiting
DNA packaging (Huang and Guo, 2003a,b). In φ29, gp16 itself was found to be a slow ATPase with low affinity to ATP, while it exhibited much higher activity in ATP hydrolysis when ssDNA, dsDNA, or RNA were bound (Guo et al., 1987d; Shu et al., 2003a). Interestingly, the strength of ATPase stimulation is dependent on the structure and chemistry of the nucleic acids, with an order of pRNA ~ poly d(pyrimidine) > dsDNA > poly d(purine) (Lee et al., 2008). Similar DNA-dependent ATPase activities were also found in other viral systems, including λ (Hwang et al., 1996) and T3 (Morita et al., 1993), and in many AAA+ helicases where ATP hydrolysis is stimulated from 10- to 100-folds.

Procapsids with pRNA also stimulate the ATPase activity of gp16 by 10-fold, compared to the lack of stimulation by procapsids alone, in agreement with the finding that gp16 binds to procapsid through interaction with pRNA on the motor (Lee et al., 2006). ATPase activity was stimulated maximally only when all packaging motor components, including pRNA, procapsid, gp16, and DNA-gp3, were present (Grimes et al., 1990; Guo et al., 1987c,d; Huang et al., 2003a,b; Ibarra et al., 2001; Lee et al., 2006, 2008; Shu et al., 2003a). It was also found in φ29 that both the DNA packaging protein gp16 and pRNA possess higher binding affinity for ATP than for ADP or AMP (Shu et al., 2003a), and the stimulation of gp16 to kick away from dsDNA has a stronger effect by ATP than by ADP or AMP (Schwartz et al., 2012).

The coupling of energy production to DNA translocation is a complex task that needs to be fine-tuned by viral packaging motors. Regardless of stoichiometry, packaging motors have the necessities to coordinate the work performed by each single subunit in order to obtain a linear unidirectional translocation of DNA from the cellular environment into the viral capsid. Evidence of such coordination can be found in the negative cooperativity in ATPase activity observed in many members of the viral packaging motors subfamily and the AAA+ proteins in general, as well as the ability of a single inactive subunit in the oligomer of a packaging motor to completely impair the translocation ability of the entire packaging machinery (Chen et al., 1997; Maurizi et al., 2001; Miyagishi et al., 2004; Trottier et al., 1996, 1997). Mechanical and physical explanations of such cooperation are still lacking for the viral packaging motor, albeit some hypotheses have been produced for other subfamilies of AAA+ proteins, such as the Glutamate switch for helicases (Ionel et al., 2011; Zhang and Wigley, 2008) or particular forms of steric hindrance proposed for proteolysis-associated chaperones (Kravats et al., 2011). All the models share the fact that every single subunit of a AAA+ oligomer is able to “sense” the nucleotide state of the others and adjust consequently in order to contribute to the overall directional translocation (Black, 1981; Casjens, 2011; Chen et al., 1997; Grimes et al., 1997; Guasch et al., 2002; Hendrix, 1978; Moffitt et al., 2009; Morita et al., 1995; Serwer, 2003, 2010; Sun et al.,
For gp16 of φ29, mechanisms of cooperative function have been deduced from studies demonstrating that DNA translocation is performed in a discrete stepwise manner (10 bp per step), corresponding to the hydrolysis of three to four ATP per step most likely in a coordinated manner (Moffitt et al., 2009; Yu et al., 2010).

To package viral DNA to near-crystalline density inside the procapsid, the packaging motor has to overcome the entropic energy associated with the constrained size and function against the large internal force from the filled procapsid. Single molecule studies with tweezers demonstrated that φ29 packaging motors are, to date, the strongest biological motors, generating about 57–110 pN of force acting on beads linked to dsDNA (Casjens, 2011; Rickgauer et al., 2008; Smith et al., 2001). Phage λ (＞50 pN) and T4 (＞60 pN) package viral DNA with high velocities and high processivity. The initial packaging rate was found to be 165 bp/s for φ29 DNA packaging (Rickgauer et al., 2008; Smith et al., 2001), comparable to that of λ, with an average velocity of about 600 bp/s (Fuller et al., 2007b). The packaging rate for T4 was found to be strikingly higher than φ29 and λ, with the average rate ranging up to about 2000 bp/s, which is in accordance to the much longer length of the T4 genome (Fuller et al., 2007a). The speed of translocation appears to be dependent on the size of the internal capsid cavity. The velocity in packaging varies but tends to decrease after the procapsid is partially filled due to internal pressure. Occasional pauses and slips were observed for all three motors (Fuller et al., 2007a,b; Rickgauer et al., 2008; Smith et al., 2001). Slow speeds are desirable for development of a high-throughput single pore genomic DNA sequencing apparatus, as the current bottleneck for this technology is that the DNA translocation speed is too fast for any available sensing equipment to detect at single base sensitivity. By attaching a bead to the end of DNA and using a magnet, the speed of φ29 DNA packaging can be as slow as 20–50 bp per second (Chang et al., 2008).

C. Rationale for the discrepancy between 2 and 2.5 bp of double-stranded DNA per ATP

It has been reported (Guo et al., 1987d) that packaging of 2 bp of DNA requires the hydrolysis of one ATP. This stoichiometry was subsequently supported by the T3 system showing the consumption of one ATP for 1.8 bp (Morita et al., 1993). Subsequently, this rate has been used extensively by biochemists and biophysicists to interpret the mechanism of motor function (Chemla et al., 2005; Mancini et al., 2004; Rao et al., 2008; Roos et al., 2007; Serwer, 2003; Sun et al., 2008). However, it was subsequently reported in φ29 that 2.5 bp of DNA are translocated per ATP by single molecule studies using optical tweezer (Moffitt et al., 2009). The newly proposed “push through one-way valve” mechanism for DNA packaging
motor (Schwartz et al., 2012) might have reconciled the discrepancy between 2 and 2.5 bp per ATP. Currently, the motion mechanism is interpreted based on structural properties of a connector channel with 12-fold symmetry and dsDNA with 10.5 bp per turn of 360° for the common B-type DNA. The 2 or 2.5 bp per ATP has been used as evidence to argue for four, five, or six discrete steps of DNA translocation. It is logical to believe that an integral number of ATP is required to translocate a definitive number of base pairs of dsDNA if gp16 and connector are an integrated concrete component similar to that in a metal engine. Calculating the ratio of ATP and the number of base pairs packaged might be useful in interpreting the motor mechanism if there is only one functional motor protein or the multiple components act as one module or are synchronized. However, biological machines are more intricate and do not follow simple physical and engineering principles. It has been found that the motor is operated by two distinct unsynchronized components: gp16 for active pushing or driving and the channel as a one-way valve to prevent reverse motion (Fang et al., 2012; Geng et al., 2011; Jing et al., 2010a; Schwartz et al., 2012). Although the pushing or driving force is from ATPase gp16, the connector channel also affects the speed of DNA translocation, as pause or DNA slipping events during translocation through the connector channel have been reported (Aathavan et al., 2009; Chemla et al., 2005; Yu et al., 2010). The two uncoordinated force-generating factors, gp16 and connector, make it impossible to obtain a definite and reproducible number of base pairs per ATP utilized.

IV. MECHANISM FOR DNA PACKAGING MOTOR

A. Historical models

Historically, many DNA packaging models have been proposed for motor action, including (1) five-fold/six-fold mismatch and connector rotating thread (Guasch et al., 2002; Hendrix, 1978; Simpson et al., 2000), (2) electrodipole within central channel (Guasch et al., 2002), (3) force from osmotic pressure (Serwer, 1988), (4) Brownian motion (Astumian, 1997), (5) connector contraction hypothesis (Morita et al., 1995), (6) sequential action of motor components (Chen et al., 1997; Moffitt et al., 2009), (7) ratchet mechanism (Fujisawa and Morita, 1997), (8) supercoiled DNA wrapping (Grimes et al., 1997), and (9) DNA compression and relaxation (Oram et al., 2008; Ray et al., 2010a,b; Sabanayagam et al., 2007). There are cases where some models have been validated in one viral system but disproved in other systems. None of these models have been supported conclusively by experimental data.

Models of the fivefold/sixfold mismatch connector rotation model (Guasch et al., 2002; Hendrix, 1978; Simpson et al., 2000) and the electrodipole
within the central channel (Guasch et al., 2002) all portrayed rotation of the connector. However, all of these connector rotation models have been invalidated by single molecule studies using a fluorescently labeled connector (Hugel et al., 2007) and have been argued against through the use of a connector cross-linking assay (Baumann et al., 2006; Maluf and Feiss, 2006).

The negatively charged interior channel wall of the motor is decorated with a total of 48 positively charged lysine residues displayed as four 12-lysine rings from the 12 gp10 subunits that enclose the channel (Badasso et al., 2000; Guasch et al., 1998, 2002; Jimenez et al., 1986; Simpson et al., 2000, 2001). The electrodipole central channel model was also proposed for φ29 (Guasch et al., 2002). In this model, positively charged lysine residues inside the highly negative charged connector channel were thought to interact with the negatively charged phosphate backbone of DNA during translocation, and the connector rotates in relation to the dsDNA (Guasch et al., 2002). After contact has been established, the connector rotates by 6°, powered by ATP hydrolysis, dissociating the lysine–phosphate bond, and ultimately leads to packaging of a single base pair. This model fails to explain that an ATP-binding motif has never been discovered on either the connector or DNA, but ATP-binding motifs are clearly present on gp16 (Guo et al., 1987d) and pRNA (Shu et al., 2003a). Furthermore, it was shown that portal rotation is not likely to occur in both T4 and φ29 (Baumann et al., 2006; Hugel et al., 2007). To test this hypothesis, a mutation was introduced to change the charge of the lysine ring (Fang et al., 2012). It was demonstrated that the interior channel lysine residues K200, K209, and K234 were not essential for φ29 DNA translocation. The conclusion that lysine rings do not play an active role in DNA translocation is also supported by manipulation of the pH. Translocation was retained even at high pH (basic) environments (Fig. 7), where the lysine residues were deprotonated. Furthermore, at a low pH, a state that would reduce the overall net negative charge of the channel, DNA translocation was also not affected (Fig. 7). All data argue against a rotating connector based on charge/charge interaction of dsDNA with the lysine rings of the connector. As mentioned earlier, the connector rotation implied in this model (Guasch et al., 2002) was also invalidated by single molecule studies of a fluorescently labeled connector (Hugel et al., 2007) and testing with a connector cross-linking experiment (Baumann et al., 2006; Maluf et al., 2006).

**B. Recently revealed “push through one-way valve” mechanism**

A new mechanism of “push through one-way valve” was discovered in the φ29 DNA packaging motor (Fang et al., 2012; Geng et al., 2011; Jing et al., 2010a; Schwartz et al., 2012) (Fig. 13). The discovery of this mechanism can
interpret and explain models based on compression and relaxation (Khan et al., 1995; Oram et al., 2008; Sabanayagam et al., 2007), the ratchet mechanism (Fujisawa et al., 1997), supercoiled DNA wrapping (Grimes et al., 1997), and sequential action of motor components (Chen et al., 1997; Moffitt et al., 2009), and solves many of the puzzles surrounding the investigation of viral DNA packaging throughout the decades (see next section).

The AAA⁺ family possesses a common adenine nucleotide-binding fold with a ubiquitous characteristic of coupling chemical energy from ATP hydrolysis to mechanical motion. AAA⁺ ATPases assemble into oligomers, often hexamers, which form ring-shaped structures with a central channel. In 1998, Guo and colleagues proposed that viral DNA packaging motors implement a mechanism similar to the action of the hexameric AAA⁺ protein acting on dsDNA. A pump-and-valve mechanism was also proposed for the bacteriophage φ29 DNA packaging motor (Guo et al., 2007). More and more evidence has emerged to support this mechanism. It has been demonstrated that φ29 DNA packaging is accomplished via a “push through one-way valve” mechanism (Fang et al., 2012; Geng et al., 2011; Jing et al., 2010a; Schwartz et al., 2012). The ATPase gp16 forms a hexameric complex geared by the hexameric pRNA ring to push dsDNA through the unrotating connector section by section into the procapsid (Schwartz et al., 2012). The dodecameric connector channel remains unrotating and functions merely as a one-way valve that only allows dsDNA to pass the channel from the narrower N-terminal entrance to the wider C-terminal but not to exit the procapsid during DNA packaging (Fang et al., 2012; Geng et al., 2011; Jing et al., 2010a,b; Wendell et al., 2009).
Although the roles of the ATPase gp16 and the motor connector channel are separate and independent, bridging by the pRNA to link these two components promotes coordination between the two units to become functional modules of an integrated motor. It was found that interaction of ATP with gp16 induced a conformational change of gp16 ATPase that led to the high affinity of binding to dsDNA. ATP hydrolysis led to the departure of dsDNA from the ATPase/dsDNA complex, an action to push dsDNA to pass the connector channel. It was found unexpectedly that neither mutation of the basic residues nor changing of pH to 4 or 10 could impair DNA translocation measurably or affect the one-way traffic property of the channel (Fang et al., 2012), suggesting that positive charges in the lysine ring of the channel are not essential in interacting with the negatively charged DNA phosphate backbone. This also supports the finding that the connector serves only as a valve rather than a rotating machine. The motor channel exercises three discrete, reversible, and controllable steps of gating with each step altering the channel size by 31% to control the direction of motion of dsDNA (Geng et al., 2011). This finding may in fact appear to be quite unique, but is consistent in many facets with data from other phages and is not contradictory to many models proposed previously.

C. Using “push through one-way valve” mechanism to interpret historical DNA packaging models

1. Using “push through one-way valve” to interpret T4 compression and relaxation model (Khan et al., 1995; Oram et al., 2008; Sabanayagam et al., 2007)

Ray and coworkers found that in T4, the portal protein compressed upon DNA entry, resulting in a DNA “crunching” phenomenon (Ray et al., 2010b) (Fig. 14a). Although the authors did not define the role of the channel and interpret that the compression was a result of torsional force from coiling of upstream DNA, the model is not contradictory with the “push through one-way valve” mechanism which suggests that the external force for compression of dsDNA is a result of pushing by the ATPase. The procapsid in their model can also be related to the understanding that the portal acts as a one-way, nonrotating valve through conformational changes associated with the internal loop of the connector protein (Geng et al., 2011; Guasch et al., 2002; Jing et al., 2010a,b; Wendell et al., 2009). Furthermore, the torsion and coiling of dsDNA in this proposal are also in concert with the speculation that φ29 DNA packaging protein gp16 is a member of the AAA+ family that tracks along helical dsDNA to generate torsional force (Guo et al., 1998; Lee et al., 2006).
2. Using “push through one-way valve” mechanism to interpret the T3 ratchet model (Fujisawa et al., 1997)

A “ratchet” model (Fig. 14c) has been used to elucidate the packaging of T3 DNA. In this model, it was proposed that one packaging ATPase gp19 is bound to each component of the six domains of the connector. The other
domain of one of the gp19 ATPase molecules contacts the sugar-phosphate backbone of the DNA duplex. When ATP is hydrolyzed, a conformational change of gp19 is induced to generate a force to translocate DNA into the procapsid. When a new ATP binds, the ATPase dissociates from the DNA and returns to the original conformation. At the same time, the next gp19 binds to the neighboring sugar-phosphate backbone. When this alternating function processes six total cycles, six molecules of ATP have been hydrolyzed to complete the translocation of one helical turn of dsDNA. Thus, one ATP molecule is utilized to translocate 1.7 bp DNA.

Similar to the “push through one-way valve” mechanism, this model describes the control of dsDNA translocation by motor components for the control of the direction of DNA translocation; albeit it fails to elucidate how the motor prevents DNA exit from the high pressure inside the capsid during each hydrolysis transition. The difference between the ratchet model and the “push through one-way valve” mechanism is that, in the ratchet model, the component to control the direction of dsDNA translocation is ATPase, not the connector as in the “push through one-way valve” mechanism (Fang et al., 2012; Geng et al., 2011; Jing et al., 2010a; Schwartz et al., 2012).

3. Using “push through one-way valve” mechanism to interpret supercoiled DNA wrapping model (Grimes and Anderson, 1997)
In 1997, Grimes and Anderson proposed that φ29 DNA-gp3 is supercoiled and wrapped around the connector and that translocation is powered by rotation of the connector relative to the viral capsid with the aid of ATP hydrolysis (Fig. 14b). The group observed the formation of DNA–gp3 lariats by EM. Treatment with topoisomerase I shifted fast-sedimenting complexes toward the uncoiled lariat position in sucrose density gradients. They proposed that the packaging proteins gp3 and gp16 supercoil the DNA ends as a prerequisite for efficient interaction with the procapsid. The shared feature of this model with the T4 compression model and the “push through one-way valve” mechanism is that the DNA packaging enzyme twists the dsDNA outside the connector to generate a torsional force for dsDNA translocation. It is very possible that gp16 interacts with gp3 to form a complex for the initiation of dsDNA packaging in φ29. However, the proposal that the connector rotates and that dsDNA is wrapped outside the connector channel are contradictory to the “push through one-way valve” mechanism.

4. Using “push through one-way valve” mechanism to interpret sequential action of motor components (Chen et al., 1997; Moffitt et al., 2009)
In 1997, Chen and Guo reported the sequential action of six motor components (Chen et al., 1997; Moffitt et al., 2009). It was proposed that the procapsid contains a sixfold symmetrical connector surrounded by a ring
of the packaging component. The relative motion of two rings could provide a driving force for DNA translocation. Analogous to a car engine, the sequential action model was a popular proposal regarding turning of the motor. The finding that a hexameric pRNA complex binds to the connector and that six pRNAs work sequentially, as evidenced from mathematical computations and modeling, lends support to the sequential action model. It was proposed that pRNA contains two domains: one for connector binding and the other 5′/3′ domain, which is free to interact with other components, such as gp16. Furthermore, it was theorized that pRNA is part of the ATPase complex and possesses at least two conformations—a relaxed and a contracted form. Alternating between contraction and relaxation, each member of the hexameric RNA complex powered by ATP hydrolysis helps generate torque to drive the DNA translocation machine. The proposal that pRNA bridges the connector and the ATPase gp16 was proved later (Koti et al., 2008; Lee et al., 2006; Zhao et al., 2008), and the sequential action was also confirmed to regulate motor action by proving coordination between subunits (Chen et al., 1997; Moffitt et al., 2009). The “push through one-way valve” mechanism agrees with the sequential action model in that both describe the action of the ATPase by sequential steps, as many members of the AAA+ family do during dsDNA tracking or translocating (Ammelburg et al., 2006; Frickey and Lupas, 2004; Hanson and Whiteheart, 2005; Iyer et al., 2004a,b; Pyle, 2008; Singleton et al., 2007; Wang, 2004).

Except for the connector rotating model (Simpson et al., 2000, 2001), which has been invalidated, and the osmotic pressure model or the Brownian motion model, which are not supported by the most recent data, all other models, such as sequential action, ratchet mechanism, supercoiled DNA wrapping, and DNA compression, can be interpreted by the “push through one-way valve” mechanism in some aspects. More importantly, most experimental data that led to these historical models can be well interpreted by the “push through one-way valve” mechanism. This comment is reflected in a recent thorough and comprehensive review (Serwer, 2010). Overall, the “push through one-way valve” is the most agreeable mechanism in concert with most models throughout history.

D. Three reversible discrete steps of conformational change and gating of connector channel in controlling direction of DNA translocation

The mechanism for viral motor channel in one-way traffic control raises a question of how dsDNA is ejected during infection if the channel only allows dsDNA to travel in one inward direction. It has been proposed that viral procapsids and motor connectors adopt conformational changes...
during procapsid maturation and the DNA packaging process. We have proposed that the direction of DNA is controlled by conformational changes of the channel; such conformational changes render the channel to allow dsDNA to travel in one direction. Substantial conformational change upon completion of DNA packaging has been reported for the φ29 connector (Gonzalez-Huici et al., 2004; Tang et al., 2008; Tao et al., 1998). A similar significant rearrangement of the connector after DNA packaging has also been reported in other phage systems (Kemp et al., 2004; Lebedev et al., 2007; Lhuillier et al., 2009), indicating that the structural change is a common feature for phages. Such change may reversely favor DNA exit during infection.

A study on the direct observation of the φ29 connector with single channel recordings showed that the channel experiences conformational changes induced by molecule binding to the C-terminal located within the capsid, or by a high electrical voltage shift (Geng et al., 2011) (Fig. 15). The conformational change exhibited three discrete steps in conductance, with each step reducing the channel size by 31%. It is therefore possible that the interaction of dsDNA or φ29 terminal protein gp3 during DNA packaging can induce a conformational change of the connector that distorts the shape of the channel proteins. This shift may lead to the opening or closing of the channel, which will help control the packaging or release of the viral genome during infection.

The gating properties of the φ29 connector under external potentials were also studied (Geng et al., 2011). The significantly different gating

![Figure 15](image-url)

**FIGURE 15** Three discrete steps in φ29 connector channel gating, with each step representing a reduction of channel size by 31%. Adapted from Geng et al. (2011) ©2011 with permission from Elsevier.
behaviors of the internal flexible loop-cleaved connector suggests that flexible loops may play a key role in voltage gating (Geng et al., 2011). After removal of these loops, both the occurrence and the extent of gating were greatly reduced. The gating mechanism can be explained by conformational changes of α helices in voltage-gated sodium and calcium channels. Based on the crystal structure of the φ29 connector, it is therefore possible that the applied potential can induce a conformational change in the protein channel embedded in a membrane. The single chain internal loops possess the flexibility to create a conformational change in response to environmental stimuli. The stepwise conformational change of the connector protein is also reversible and controllable, making it an ideal nanovalve for constructing a nanomachine with potential applications in nanotechnology and nanomedicine.

V. APPLICATION OF VIRAL PACKAGING MOTORS

Inspired by the ingenious configuration of natural biomotors at nanometer precision and better understanding of the assembly and function of these motors, the application of biomotors in nanotechnology and nanomedicine has continued to become a more critical scientific approach (Baneyx and Vogel, 1999; Fang et al., 2012; Geng et al., 2011; Grigoriev et al., 2004; Hess et al., 2001; Jing et al., 2010a,b; Lee et al., 2003; Park et al., 2007; Soong et al., 2000; Wendell et al., 2009). The potential application of biomotors and their modules include integration into nanodevices, such as actuators, molecular sorters, molecular sensors, nanoelectromechanical machines, intricate arrays for diagnostics or computation, electronic or optical devices, and reconstruction as nanocarriers for the delivery of drugs or therapeutic RNA and DNA for the treatment of diseases. One of the most important potential applications is the use of the nanomotor as a high-throughput single pore sequencing apparatus for sequencing of the dsDNA genome of human, plants, and yeast for disease control and health improvement. Due to the strong power of the viral DNA packaging motors, phage designs have, in turn, become stronger and more robust due to natural selection and evolution (Jing et al., 2010b; Shu et al., 2011a). The robust properties of the viral motor and its modules make its application in nanotechnology feasible.

A. Biomimetic machinery

Highly efficient in vitro DNA packaging has been accomplished in defined packaging systems for many viral packaging motors (Fujisawa et al., 1991; Guo et al., 1986; Hwang and Feiss, 1997; Oliveira et al., 2005; Rao and Black, 1988). Precise control to turn on and off the motors can also be
achieved using ATP analogs, metal ion-chelating reagents, or other regulatory aptamers (Guo et al., 1987c; Ko et al., 2008; Shu et al., 2003b; Smith et al., 2001). It is therefore possible to engineer a controllable packaging motor into nanodevices. To incorporate the motor into nanodevices, techniques generally involve the characterization, manipulation, modification, control, creation, and/or assembly of organized materials on the nanoscale level (Guo, 2005; Niemeyer, 2002; Schmidt and Eberl, 2001). These materials will then be used as building blocks for the construction of larger devices and systems. Nanoporous anodic aluminum oxide (AAO) membranes were fabricated to capture and align the φ29 DNA packaging motor (Moon et al., 2009). The pore size of the membranes was controlled precisely by using atomic layer deposition of aluminum oxide. In addition, the AAO membrane was found capable of sorting DNA-filled φ29 procapsids from empty procapsids. This nanoporous AAO membrane may be used in the future to integrate the φ29 motor with other artificial nanostructures.

B. Single molecule sensing

One emerging field in biology is the development of efficient, inexpensive, and highly sensitive analytical tools that can be used to probe, analyze, interpret, and manipulate single molecules, such as single channel ion transportation pores or nanopore-based DNA-sequencing devices (Deamer and Akeson, 2000). The essential component of the DNA packaging motor, the connector, provides a nanometer-sized channel for DNA’s entry and exit of the viral procapsid. It has been demonstrated that the φ29 connector can be incorporated into planar lipid membranes and produce a robust single molecule-sensing system (Geng et al., 2011; Jing et al., 2010a,b; Wendell et al., 2009; Haque et al., 2012). Translocation of dsDNA through the membrane-embedded connector pore can be detected by electrophysiological measurements. The structure and length of the dsDNA can be recognized by the characteristic dwell times and current blockades (Wendell et al., 2009). The system is also highly sensitive to ion species and is stable even at extreme pH environments and high salt conditions (Fang et al., 2012; Jing et al., 2010b). As the crystal structure of the connector has been well elucidated, it is possible to reengineer the connector for a wide range of applications, including single molecule sensing, DNA sequencing, gene delivery, drug loading, and bioreactors. It could also be used to provide insights into the mechanism of DNA translocation in viral systems (Jing et al., 2010a). As the connectors in different viral systems share similar morphologies, it is likely that the connector channels of phages other than φ29 could be integrated into nanodevices in a similar approach.
C. Bioreactor

Insertion of the motor connector into a lipid membrane has generated new interest in nanobiotechnology (Fang et al., 2012; Geng et al., 2011; Jing et al., 2010a,b; Wendell et al., 2009). Liposomes containing the connector channel are similar to the construction of an active viral DNA packaging motor. This essentially creates an active bioreactor for observing reactions when analytes enter into the reaction chamber gradually through the channel. This will serve as a prototype for other biomimetic DNA packaging motors for future development. It can serve as an active drug or DNA-loading machine against the concentration gradient for drug or gene delivery in vivo.

The connector channel-containing liposome can be used for vesicle encapsulation (Okumus et al., 2004; Rhoades et al., 2003) (Fig. 16). The reaction mixture will be encapsulated within this small unilamellar vesicle bioreactor for a variety of reactions such as total internal reflection fluorescence imaging. Vesicles provide an environment close to the native condition of the sample. Vesicles with a diameter of 100–200 nm are smaller than optical resolution and thus lateral motions of the samples within the vesicles will be negligible. α-Hemolysin-containing liposomes have been used to study helicase function and RNA folding by single molecule FRET (Cisse et al., 2007; Okumus et al., 2009). The artificial channel allows the exchange of ions, metabolites, or other small molecules such as ATP, which pass through the channel to reach the encapsulated environment. The φ29 connector with a 3.6-nm-wide channel has been inserted successfully into a liposome (Wendell et al., 2009) and its transportation characteristics have been well studied. Similarly, the connector embedded in the liposome can be applied to single molecule observation of a variety of DNA packaging motors, including that of φ29 (Fig. 16).
The size of the liposomes can be controlled by traditional extrusion through filters of a defined pore size.

For example, to initiate the reaction, buffer containing ATP could be infused into the chamber. Real-time videos would then be recorded by a single molecule fluorescence imaging system (Shu et al., 2007; Zhang et al., 2007, 2010). As the porous liposome allows the exchange of buffers, ATP would diffuse into the liposome through the connector channel. The gp16 ring fixed to the dsDNA chain by γ-s-ATP could then resume its motion by hydrolyzing ATP. Using fluorescently labeled protein and DNA, eGFP-gp16 and Cy3-dsDNA, respectively, changes in FRET efficiency could be observed if the gp16 ring is moving along the chain until it moves out of the FRET range or eventually falls off the chain. Due to the limited volume of the liposome, gp16 may rebind to the DNA chain quickly and start another cycle. Results will provide direct evidence that the ATPase gp16 slides along the dsDNA chain. Other topics concerning motor motion (Balci et al., 2005), interaction of motor components (Wang et al., 2005), or kinetics related to ATP concentration (Lee et al., 2008; Moffitt et al., 2009) can also be examined.

D. Gene therapy in nanomedicine

One possible application of viral packaging motors or its module in nanomedicine is to use the motor to inject therapeutic DNA into targeted cells for disease treatment. The motor, along with its essential components, can be fused into the targeted cell membrane. The membrane-integrated motor may be able to insert functional genes for therapy into the targeted cells, utilizing the energy from ATP hydrolysis, or electrical force, as demonstrated (Fang et al., 2012; Geng et al., 2011; Jing et al., 2010a,b; Wendell et al., 2009).

Additionally, the building blocks of viral packaging motors possess the ability of oligomer formation and thus are ideal candidates in bottom-up assembly to produce functional nanomaterials (Figs. 17 and 18) (Guo, 2005; Khaled et al., 2005; Shu et al., 2004, 2011b; Xiao et al., 2009a,b). The pRNA of the φ29 motor has a strong tendency to form dimers, trimers, and hexamers via interlocking loop/loop interactions. A variety of structures and shapes, such as twins, tetramers, rods, triangles, and arrays, several micrometers in size, can be produced using reconstructed φ29 pRNA containing a palindrome sequence (Fig. 18). These pRNA nanoparticles were found to be stable and resistant to a wide range of temperatures, salt concentrations, and pH (Shu et al., 2003d, 2004). Utilizing the property of controllable self-assembly of pRNA, RNA nanoparticles can be constructed to carry different therapeutic agents, such as siRNA (Guo et al., 2005a; Khaled et al., 2005), ribozyme (Liu et al., 2007), drugs, and fluorescent or radioactive markers, as well as the targeting ligands of
folate (Guo et al., 2005a, 2006) or RNA aptamer (Guo et al., 2005a; Khaled et al., 2005; Zhou et al., 2011) for targeted delivery to treat cancer, viral infection, and genetic disease (Fig. 19). Moreover, using the pRNA-based nanoparticle for delivery in nanomedicine has an advantage in that the size of the pRNA particles can be controlled to fall in the range of 10–100 nm, the optimal size to enter the cell while avoiding kidney filtration (Guo, 2010; Guo et al., 2010; Shu et al., 2011b).

RNA holds the advantage over DNA in that it can be manipulated as easily as DNA, but it possesses the versatile structure and catalytic activity similar to that of proteins. RNA is therefore a particularly attractive building block for the bottom-up fabrication of nanostructures (Guo, 2010). As mentioned previously, pRNA of the φ29 DNA packaging motor has been used as a carrier for delivery of therapeutics for the treatment of diseases (Guo et al., 2005a, 2006; Hoeprich et al., 2003; Khaled et al., 2005; Zhou et al., 2011). However, in vivo dissociation of RNA nanoparticles without covalent modifications or cross-linking has been the main negative

**FIGURE 17** Formation of nanostructures by φ29 motor connector. (a–c) Formation of massive sheets of connector mediated by lipid bilayers. (a) Scheme of the lipid directed formation of single layer connector array. (b) EM image and (c) Atomic force microscopy image of single layer connector array. (d) Ellipsoid nanoparticles self-assembled from connector. Adapted with permission from Xiao et al. (2009b) and Xiao et al. (2009a) ©2009 American Chemical Society.
factor that has reduced the delivery efficacy of RNA nanoparticles in vivo. The authors discovered the unusual stability of the pRNA three-way junction (3WJ, the pRNA trifurcate domain) of the φ29 DNA packaging motor. This pRNA 3WJ (Figs. 9 and 10) has been utilized for fabricating highly stable, multivalent RNA nanoparticles to deliver siRNA to specific
cancer cells (Shu et al., 2011a). Thermodynamically stable pRNA from three to six pieces of RNA oligomers were assembled without the use of metal salts to form stable multifunctional nanoparticles. Each RNA oligomer contains a receptor-binding ligand, aptamer, siRNA, or ribozyme functional module. When mixed together, they self-assemble into tristar nanoparticles using the 3WJ as a core, as demonstrated by both atomic force microscopy (Fig. 9) and EM imaging (unpublished results). Nanoparticles are resistant to 8 M urea denaturation and remain intact at extremely low concentrations in vitro or in vivo. With a simple chemical modification at the 2’ hydroxyl, these RNA nanoparticles are also resistant to degradation by serum (Shu et al., 2011a). All modules within the nanostructure exhibited independent functionalities for specific cell binding, cell entry, gene silencing, catalytic function, and cancer targeting both in vitro and in animal trials (Fig. 20). Modules remain functional in vitro and in vivo, suggesting that the 3WJ core can be used as a platform for building a variety of multifunctional nanoparticles.

VI. PROSPECTIVE

Studying the mechanism of viral DNA packaging not only promotes understanding of how these strong and powerful motors function, but may also elucidate the mechanism of DNA or RNA translocation through
FIGURE 20  *In vitro* and *in vivo* binding and entry of 3WJ-pRNA nanoparticles into targeted cells. Flow cytometry (a) and confocal images (b) revealed binding and specific entry of fluorescent-[3WJ-pRNA-siSur-rZ-FA] nanoparticles into folate receptor-positive (FA⁺) cells. Target gene knockdown effects shown by (c) quantitative reverse transcriptase polymerase chain reaction with GADPH as endogenous control and by (d) Western blot assay with β-actin as endogenous control. (e) 3WJ-pRNA nanoparticles target FA⁺ tumor xenografts upon systemic administration in nude mice. (Lv, liver; K, kidney; H, heart; L, lung; S, spleen; I, intestine; M, muscle; T, tumor). Adapted with permission from Macmillan Publishers Ltd. *(Shu et al., 2011a)* ©2011.
cell membranes, nucleic acid repair, and DNA replication or RNA transcription. Viral motors also possess the potential to be applied to nanotechnology and nanomedicine, including targeted delivery, precise single molecule sensing, high-throughput dsDNA sequencing, or direct incorporation into nanodevices. Further understanding of the mechanism of viral motor function would facilitate the realization of these applications.

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