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Bacterial Virus ϕ29 DNA-Packaging Motor and Its Potential Applications in Gene Therapy and Nanotechnology

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Summary

A controllable, 30-nm imitating DNA-packaging motor was constructed. The motor is driven by six synthetic adenosine triphosphate (ATP)–binding RNA (packaging RNA [pRNA]) monomers, similar to the driving of a bolt with a hex nut. Conformational change and sequential action of the RNA with fivefold (viral capsid)/sixfold (pRNA hexamer) mismatch could ensure continuous rotation of the motor with ATP as energy. In the presence of ATP and magnesium, a 5-μm synthetic DNA was packaged using this motor. On average, one ATP was used to translocate two bases of DNA. The DNA-filled capsids were subsequently converted into up to 10^9 PFU/mL of infectious virus. The three-dimensional structures of pRNA monomer, dimer, and hexamer have been probed by photoaffinity crosslinking, chemical modification interference, cryo–atomic force microscopy, and computer modeling. The pRNA's size and shape can be controlled and manipulated at will to form stable dimers and trimers. Cryo–atomic force microscopy revealed that monomers, dimers, and trimers displayed a checkmark outline, elongated shape, and triangular structure, respectively. The motor can be turned off by γ-S-ATP or EDTA and turned on again with the addition of ATP or magnesium, respectively. The formation of ordered structural arrays of the motor complex and its components, the retention of motor function after the 3'-end extension of the pRNA, and the ease of RNA dimer, trimer, and hexamer manipulation with desired shape and size make this RNA-containing motor a promising tool for drug and gene delivery and for use in nanodevices.

Key Words: pRNA; bacteriophage ϕ29; ribozyme; gene therapy; nanomotor; DNA packaging; hexamer; RNA structure and function; nanobiotechnology.

1. Introduction

During the life cycle, viral genomic DNA replication and structural protein synthesis are accomplished by two separate cell systems ***(1–5)***. After these two subprocesses are complete, the resultant components, protein and nucleic acid,

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must interact with one another in order to form a complete virion; this process is referred to as viral DNA packaging ***(6,8–14)***. Double-stranded DNA (dsDNA) viruses have their lengthy genomic DNA translocated with high velocity into a preformed shell (procapsid), and packaged into near-crystalline density ***(15,17,18)***. Because this is an energetically unfavorable motion task, a viral DNA-packaging motor, utilizing adenosine triphosphate (ATP) as energy, is employed ***(19,20,22–32)***. ϕ29, a bacterial dsDNA virus that infects *Bacillus subtilis*, possesses several characteristics common to linear dsDNA viruses, including the use of a pair of noncapsid proteins and ATP to translocate viral DNA into a procapsid, which has been preformed with the aid of scaffolding proteins that are removed before or during DNA packaging, with the presence of at least one component functioning in a manner similar to that of the adenosine triphosphatases (ATPases) ***(18,33–36)***; the presence of at least one component functioning in a manner similar to that of the adenosine triphosphatases (ATPases) ***(30)***. Examples of viruses with motors similar to ϕ29 include T4 ***(37)***, λ ***(38,39)***, T3 and T7 ***(40–42)***, phage 21 ***(43)***, P22 ***(44–46)***, P1 ***(47)***, psiM2 ***(48)***, Sfi21 ***(49)***, herpes simplex virus (HSV) ***(50–52)***, cytomegalovirus (CMV) ***(53)***, adenovirus ***(54–56)***, and poxvirus ***(57,58)***. Procapsids of these phages contain a portal vertex (connector or DNA-translocating vertex). Packaging of DNA at this vertex is directional and oriented ***(5,18,33–36)***. The pair of noncapsid DNA-packaging proteins can be classified into group 1 proteins, which are smaller and are responsible for DNA binding, and group 2 proteins, which are larger, DNA-dependent ATPases that, as has been proven in some phages, bind specifically to the portal vertex of the procapsid ***(3,30,41,59–74)***. As reported earlier ***(30)***, the nonstructural DNA-packaging components can be divided into two broad categories based on their role in DNA packaging: (1) procapsid binding, including gpA of λ ***(14,38)***, gp2ÄVof ϕ21 ***(43)***, gp19 of T3/T7 ***(28,76,77)***, and gp17 of T4 ***(37)***; and (2) DNA interaction, including gp18 of T3/T7, gpNu1 of λ, gp1 of ϕ21, and gp16 of T4 ***(30)***. In ϕ29, the nonstructural components involved in motor function include packaging RNA (pRNA), gp16, and gp3. pRNA and gp16 have been shown to bind procapsid and ATP ***(79,80)***, and gp3 binds DNA ***(81,82)***. ATP hydrolysis drives DNA translocation. Concatemeric DNA is used as a substrate for packaging except in the case of ϕ29, which is similar to adenovirus in that a protein is covalently attached to each 5' end of the viral genome ***(81,82)***.

Data from HSV ***(83)***, equine herpesvirus ***(84)***, CMV ***(85)***, and avian infectious laryngotracheitis virus ***(86)*** indicate that these viruses utilize a packaging pathway similar to that of the dsDNA bacteriophages in most, if not all, packaging stages. For instance, in these viruses, viral DNA is also packaged into a procapsid containing scaffolding protein ***(85–88)***. In addition, concatemeric DNA is formed and then cut into monomers during the maturation process ***(41,83,85,89)***. The nonstructural DNA-packaging enzyme contains a consen-

Table 1
Experimental Data for Stoichiometry and Slopes[a]

Components for ϕ29 virion assembly	Function	Total subunits/ virion	Mathematical quantification of stoichiometry	< of curve (°)	Slopes (tan) empirical	Slopes (tan) theoretical
DNA-gp3	Genome complex	1	1	45 ± 4	1	1
Procapsid	Hold DNA	1	1	47 ± 3	1	1
pRNA trimer	DNA packaging	6	2	56 ± 2	1	1.48
pRNA dimer	DNA packaging	6	3	64 ± 5	2	2.05
pRNA monomer	DNA packaging	6	6	72 ± 3	3	3.14
gp9	Tail-knob protein	10	9	77 ± 2	4	4.33
gp11	Lower collar	12	12	79 ± 1	5	5.67
gp12	Antireceptor	24	12	80 ± 2	6	5.67

[a]Adapted from **ref. *80*** with permission.

sus ATP-binding domain ***(88,90)***. Furthermore, DNA-filled capsids exhibit a fingerprint-like motif of finely spaced curvilinear striations, similar to some phages, when viewed with cryoelectron microscopy ***(88)***. Poxviruses also use concatemeric DNA as a substrate for packaging ***(91–93)***. The DNA-packaging enzyme of vaccinia virus has been found to be a potential ATPase ***(57)***. Adenovirus also shares similarities to dsDNA bacteriophages, including packing of DNA into preformed procapsids ***(12,55,94)***. Given the broad similarities found among different viruses, DNA packaging is an intriguing issue in virology, and it has relevance to many other biochemical processes such as DNA translocation, macromolecular interactions, and bioenergetics.

In vitro assembly of the linear double-stranded (dsDNA) viruses always proceeds in two steps: (1) motor-driven DNA packaging and (2) virion maturation ***(17,34,95–97)***. Bacteriophage ϕ29 of *B. subtilis* is a particularly ideal model for studying the function of viral DNA-packaging motor, because virtually every DNA molecule added can be efficiently packaged in vitro, with all components being overproduced and purified ***(5,9,30,79,98,99,101,102)***. Procapsids produced from cloned structural genes in ϕ29 are competent for DNA packaging, and the DNA-filled capsids prepared using this system can be converted into infectious virus although the addition of neck and tail proteins ***(101,102)***.

Utilizing pRNA's easily manipulable structure, a fully defined in vitro ϕ29 DNA-packaging system has been developed ***(9)***. With purified procapsid, gp16, pRNA, and ATP, up to 90% of the added DNA-gp3 can be packaged into the procapsid via the motor constructed with recombinant gene products. After DNA packaging, the in vitro assembly system can convert DNA-filled capsids into infectious ϕ29 virions when purified proteins gp9, gp11, gp12, and gp13 ***(103,104)*** are added. Many infectious virions have been assembled in vitro by extract complementation ***(1–4,74,105–114,116–118)***, and by the use of synthetic nucleic acid and purified recombinant protein components ***(103,104)***. Fully defined in vitro DNA-packaging systems have also been developed and constructed for T3 ***(8)***, T4 ***(37)***, and λ ***(119)*** and ϕ29 ***(9)***.

2. Components of the ϕ29 DNA-Packaging Motor

2.1. Procapsid and DNA-Packaging Motor

Procapsids play an active role in the DNA-packaging process of dsDNA viruses. Phage procapsids are largely composed of three structural proteins: (1) the capsid protein, (2) the scaffolding protein, and (3) the connector protein ***(36,95,120)***. The three-dimensional (3D) structure of ϕ29's empty procapsid precursor has been obtained by reconstruction of cryo–electron microscopy images ***(121)***. The icosahedral procapsid of ϕ29 consists of 235 copies of the major capsid protein (gp8), 26 copies of scaffolding protein (gp7), and 12 cop-

ies of the head-tail connector protein (gp10) ***(260)***. Studying sequential interactions of these three proteins, ***(122)*** found that ϕ29 procapsid assembly differs considerably from the single-assembly pathway, and that the coexistence of scaffolding protein, major capsid protein, and connector over a threshold concentration is required to form ϕ29 procapsid. During such a process, scaffolding protein links major capsid protein and connector. The three components interact so quickly that no clear intermediates have been observed.

2.2. Capsid Proteins

The capsid proteins are the major structural components for the formation of viral protein shells. Examples include gpE for bacteriophage λ ***(123,124)***, gene 23 (major capsid subunit) or gene 24 (pentamer subunit) product for bacteriophage T4 ***(37)***, and gp8 for bacteriophage ϕ29 ***(122)***. Besides playing a structural role, it has been found that capsid proteins can play a role in packaging. For example, missense mutations in the capsid proteins of bacteriophage λ can block packaging in proheads that are still morphologically normal ***(126–128)***. The procapsid might be involved in an ATP-binding process that has been reported in dsDNA virus ϕ6 ***(129)***.

2.3. Scaffolding Proteins

Scaffolding proteins are structural proteins that are required for successful procapsid assembly, but are not part of the mature virion; they are released from procapsids at the initiation of or during DNA packaging. For ϕ29, the scaffolding protein (gp7) is not required for the competence of procapsids in DNA packaging ***(4,36)***. Scaffolding proteins of phage P22 are catalytic and can be utilized in the assembly of a second procapsid once they are released from the first ***(109)***. The exact function of scaffolding proteins, however, is not yet clear, and multiple roles are possible. For example, the scaffolding protein may form a core structure around which capsid proteins assemble ***(130–135)***. In some cases, they may serve as chaperones to promote the correct folding of capsid proteins. Additionally, they may be involved in mediating a putative capsid protein/connector protein interaction, excluding cellular proteins from the inside of the procapsid, or facilitating the early stages of DNA entrance into the procapsid ***(97)***.

2.4. Connector

One of the essential components of the motor is the connector complex, a dodecameric structure with a central channel (30–60 Å) through which viral DNA is packaged into the capsid and exits during infection ***(50,136–141)***. ϕ29 connector is a trapezoid-shaped cylinder with a 3.6-nm central channel through which DNA enters the capsid. Although the individual portal

proteins of different viruses share little sequence homology and exhibit large variations in molecular weight, the portal complexes possess a significant amount of morphological similarity ***(35)***. In the case of ϕ29, the structure of one phage portal protein has been determined at atomic resolution ***(136,137)***. The connector ring consists of a 12 α-helical subunit, with the central channel being formed by three long helices of each subunit. The ring is 138 Å across at its wide end and 66 Å at the narrow end. The internal channel is 60 Å at the top and 36 Å at the bottom. The wide end of the connector is located in the prohead, and its narrow end partially protrudes out of the capsid. The connector is located at the fivefold vertex of the viral capsid, which leads to a symmetry mismatch between capsid and portal ***(35,142)***. It has been proposed that such a mismatch is required for the smooth rotation of the portal protein during DNA packaging ***(142,143)***.

2.5. Genomic DNA

A virus-encoded protein is covalently linked to each 5' end of the ϕ29 genome ***(144–146)***. The terminal protein gp3 functions both as a primer for the initiation of DNA replication ***(147)*** and as an essential protein for DNA packaging ***(5,99)***. Typically, ϕ29 DNA-gp3 is purified from cells infected by a 4^- 8^- suppressor-sensitive mutant (amber mutation) ***(98)***. In this mutant, late proteins are not produced when the phage grows in a nonpermissive host, and large quantities of phage DNA-gp3 can be isolated. gp3 has also been purified from cloned gene products ***(82)***. Infectious DNA-gp3 can be synthesized in vitro ***(82,103,145–149)***.

2.6. gp16

The two noncapsid DNA-packaging proteins of various bacteriophages apparently cooperate to link the procapsid to the DNA-packaging substrate, cut monomers of DNA from concatemers, and serve as a DNA-translocation enzyme and/or ATPase. It was found that gp16 of ϕ29 contains both A-type and B-type consensus ATP-binding sequences, and the predicted secondary structure for ATP binding ***(30)***. The A-type sequence of gp16 is "basic-hydrophobic region-G-X_2-G-X-G-K-S-X_7 hydrophobic." One of the nonstructural DNA-packaging proteins of all dsDNA phages likely functions as an ATPase ***(30)***. This prediction has been supported by subsequent studies involving bacteriophages T4 ***(150)***, λ ***(38,152,153)***, T3 ***(154)***, and P22 ***(155)***, as well as herpesviruses ***(88,90)*** and the vaccinia virus ***(57)***.

gp16 is quite hydrophobic, and when overproduced in *E. coli*, it agglutinates into lumps and loses biological activity. A very simple procedure has been developed to purify gp16 to homogeneity in a soluble, active form, in a single purification step ***(9)***. The lumps (inclusion bodies) are collected by differential centrifugation, and the protein, denatured and dissolved with guanidine chloride, is passed through a Sephadex column in the presence of 7.5 *M* urea. gp16

is eluted as a discrete peak with >90% purity. The purified gp16 is then renatured by dialysis against potassium chloride ***(9)***. It has been shown that two unique cysteine residues of gp16 are important for biological activity, and, therefore, the reducing agent dithiothreitol is strictly required when guanidine chloride is used for denaturation.

gp16 has been found to bind ATP ***(19,30,156,157)***, but the role of gp16 in the ϕ29 DNA-packaging motor is still a mystery. The hydrophobicity, low solubility, and self-aggregation of ϕ29 gp16 have long hindered further refinement of the current understanding of its packaging mechanism. Contradictory data regarding ATPase activity, binding location, and the stoichiometry of gp16 have been published ***(19,30)***. In the traditional method ***(30)***, gp16 was purified in a denatured condition, and active gp16 was obtained by dialysis against 4 m*M* KCl buffer for 40 min for renaturation, but the renatured gp16 aggregated again within 15 min after renaturation. Recently, it was reported that gp16 was made soluble in the cell by coexpression with groE ***(157)***. Indeed, active gp16 was purified through use of this overexpression system. However, although coexpression with groE solved the problem of aggregation within the cell, it could not solve the problem of self-aggregation after purification. PEG and acetone have been used to aid in the purification of gp16 in a homogenous and highly active form ***(19,259)***.

2.7. pRNA

Even considering its similarities to other dsDNA viruses regarding DNA, several aspects of the specific structure of ϕ29 are novel and unique, and it is therefore a particularly promising choice for further study. A 120 nt RNA (discovered in 1987 ***[98]***) is vital to proper functioning of the motor. This pRNA binds ATP ***(80)*** and enhances the ATPase activity of gp16 ***(156)***. A computer model of the pRNA/connector complex has been reported ***(158)***. Notably, pRNA binds the connector and participates actively in DNA translocation ***(79,159)***, but it leaves the capsid after DNA packaging is completed ***(160)***; ϕ29-encoded pRNA is not present in the mature virion ***(98)***. Similar but distinct pRNAs have been revealed through phylogenetic analyses of other phages; there are few conserved bases, but the observed secondary structures are quite similar ***(161,162)***. pRNAs from other phages can never replace ϕ29 pRNA in its DNA packaging ***(161)***.

2.7.1. Two Domains of pRNA

The two functional domains of pRNA are the procapsid-binding domain, located at its central region ***(159,163–165)***, bases 23 to 97, and the DNA translocation domain, located at the 5'/3' paired ends ***(166)***. A number of different approaches have been employed to confirm this result, including base deletion

and mutation ***(166–169)***, ribonuclease probing ***(160,161,163)***, oligo targeting ***(170)***, competition assays to inhibit phage assembly ***(171–173)***, ultraviolet crosslinking to portal protein ***(159)***, psoralen crosslinking ***(160)***, and computer modeling ***(158)***. It has been reported that the $C^{18}C^{19}A^{20}$ bulge of pRNA is solvent exposed when pRNA is bound to procapsid ***(174)*** and is critical for DNA translocation ***(143,162,169) (163)***. The $C^{18}C^{19}A^{20}$ bulge might be directly involved in interacting with ATP, gp16, DNA-gp3, or capsid components.

2.7.2. Formation of Dimers, Trimers, and Hexamers

pRNA dimer is the building block and dimer → tetramer → hexamer is the pathway to hexamer assembly. Six individual ϕ29 pRNAs form a ring through intermolecular base pairing between the right loop (bases 45–48) and the left loop (bases 82–85) ***(143)***. Cryo–atomic force microscopy (cryo-AFM) has been used to directly visualize the tertiary structure of pRNA monomers, native and covalently linked dimers, and native trimers. The pRNA monomers fold into a checkmark-shaped structure, the dimers have an elongated shape, and the trimers exhibit a triangular shape. Mg^{2+} induces appropriate folding of pRNA for dimerization ***(160,174)***. pRNA dimers bind to the portal vertex (connector) and serve as the building blocks for hexamer assembly ***(164,174)***. The DNA-translocating machine is geared by the formation of a hexameric complex; this suggests that the mechanism employed is similar to that of the consecutive firing of six cylinders of a car engine. Base pairing is demonstrated through an experimental procedure in which pRNAs with mutated loop sequences are constructed. Uppercase and lowercase letters have been used in the literature to indicate the right- and left-hand loops of the pRNA, respectively. Complementary sequences are delineated by the same letter in uppercase and lowercase. For example, in pRNA A-a', right loop A ($5'GGAC_{48}$) and left loop a' ($3'CCUG_{82}$) are complementary, whereas in pRNA A-b', the four bases of the right loop A are not complementary to the sequence of the left loop b' ($3'UGCG_{82}$). Mutant pRNAs with complementary loop sequences (such as pRNA I/i') are active in ϕ29 DNA packaging, whereas mutants with non-complementary loops (such as pRNA A/b') are inactive ***(175,176)***. It was found that pRNAs A-i' and I-a' were inactive in DNA packaging alone, but when A-i' and I-a' were mixed together, DNA-packaging activity was restored. This result is explained by the transcomplementarity of pRNA loops; the right-hand loop A of pRNA A-i' pairs with the left-hand loop a' of pRNA I-a' ***(175,176)***.

2.7.3. Stoichiometric Determination of pRNA

As a first step in the determination of pRNA's role in DNA packaging, it is important to conclusively determine how many copies of pRNA are used in

each DNA-packaging event. A number of methods have been proven useful in determining the stoichiometry of pRNA and of the other components necessary for motor function ***(163,172,175,177,178)***. These various approaches have indicated that six pRNAs are present in the ϕ29 DNA-translocation motor. Such methods include binomial distribution ***(172,178)***; comparing slopes of concentration dependence ***(103,172)***; and finding the common multiples of 2, 3, and 6 by using sets of two interlocking pRNAs, three interlocking pRNAs, and six interlocking pRNAs ***(175)***.

2.7.3.1. Binomial Distribution ***(172,178)***

pRNAs mutated at the 5'/3' paired region retain procapsid-binding capacity but lose DNA-packaging function. When mutant pRNA and wild-type (WT) (**Fig. 1**) pRNA are mixed at various ratios for in vitro assembly assays (e.g., 30% mutant and 70% WT), the probability of procapsids possessing a certain amount of mutant and a certain amount of WT pRNA can be determined by expanding the binomial as follows:

$$(p+q)^Z = \binom{Z}{0}p^Z + \binom{Z}{1}p^{Z-1}q + \binom{Z}{2}p^{Z-2}q^2 + \ldots \binom{Z}{Z-1}pq^{Z-1} + \binom{Z}{Z}q^Z = \sum_{M=0}^{Z}\binom{Z}{M}p^{Z-M}q^M$$

in which $\binom{Z}{M} = [Z!/M!(Z-M)!]$; Z is the total number of pRNAs per procapsid; and p and q are the percentage of mutant and WT pRNA, respectively, used in the reaction mixture. For example, if the total number of pRNA per procapsid required for DNA packaging (Z) is 3, then the probability of all combinations of mutant (M) and WT (N) pRNAs on a given procapsid can be determined by expansion of the binomial: $(p+q)^3 = p^3 + 3p^2q + 3pq^2 + q^3 = 100\%$. That is, in the procapsid population, it is possible to determine the probability of a procapsid possessing either three copies of mutant pRNA, two copies of mutant and one copy of WT, one copy of mutant and two copies of WT, or three copies of WT, represented by p^3, $3p^2q$, $3pq^2$, and q^3, respectively. Suppose that there are 70% mutant and 30% WT pRNA in a reaction mixture. The percentage of procapsids that possess one copy of mutant and two copies of WT would then be $3pq^2$, i.e., $3(0.7)(0.3)^2 = 19\%$. If only one mutant pRNA per procapsid is sufficient to render the procapsid unable to package DNA, then only those procapsids with three bound WT pRNAs (zero mutant pRNA, q^3) would package DNA. The yield of virions from the empirical data was plotted and compared to a series of predicted curves to find a best fit. It was found that the experimental inhibition curve most closely resembled, in both slope and magnitude, the predicted curves in which $Z = 5$ or 6 ***(172)***.

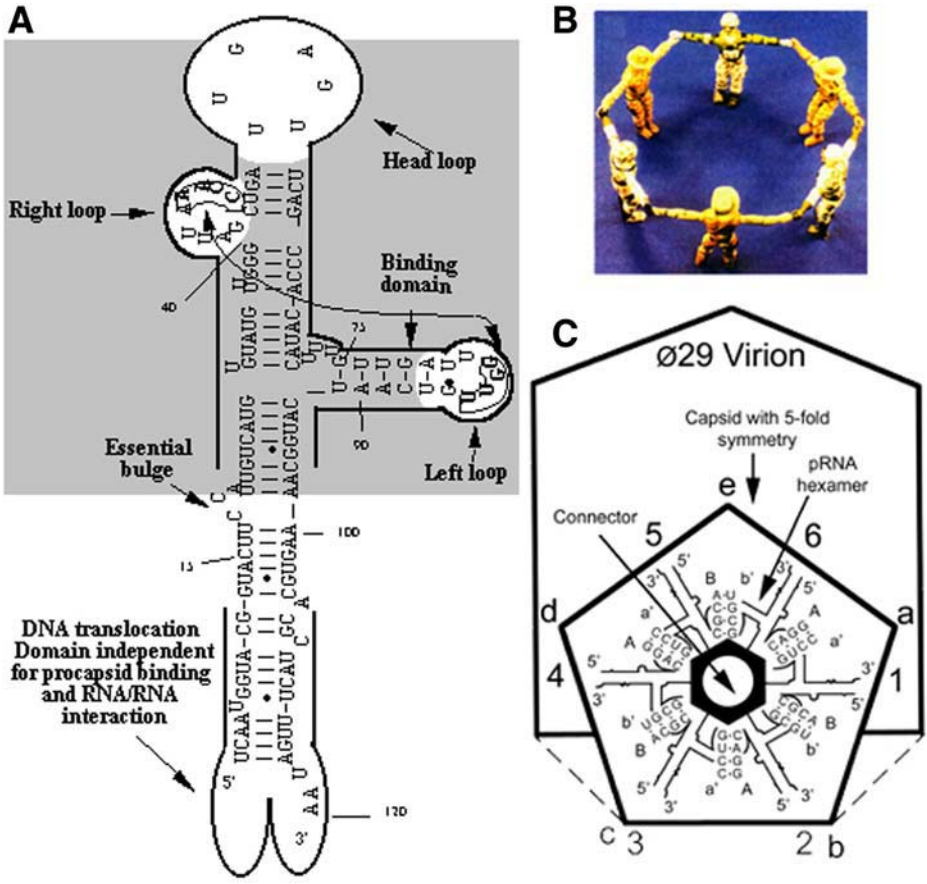

Fig. 1. Structure, domain and location of pRNA on ϕ29 viral particle. **(A)**, Secondary structure of pRNA(A-b'). A and b' are the left and right loops responsible for intermolecular interaction. The intermolecular binding domain (shaded area) and the reactive DNA translocation domain are marked with bold lines. The four bases in the right and left loops, which are responsible for inter-molecule interactions, are placed in boxes. **(B)**, Power Rangers to depict pRNA hexamer by hand-in-hand interaction. **(C)**, ϕ29 particle showing the DNA-packaging motor with pRNA hexamer formed via the interaction of pRNA (A-b') and (B-a'). The surrounding pentagon stands for the fivefold symmetrical capsid vertex, viewed as end-on with the virion as a side view. The central region of pRNA binds to the connector and the 5'/3' paired region extends outward (From **ref.** ***162*** with permission.)

2.7.3.2. Slopes of Concentration Dependence ***(103,172,179)***

The dose response curves of in vitro ϕ29 assembly vs concentration of various assembly components have been used to approximate the stoichiometry of pRNA. ϕ29 assembly components with known stoichiometry serve as standard controls. In these curves, the larger the stoichiometry of the component, the more dramatic the influence of the dilution factor on the reaction. A slope of one indicates that one copy of the component is involved in the assembly of one virion, as is the case for genomic DNA in ϕ29 assembly. A slope larger than one indicates multiple-copy involvement. Using this method, the stoichiometries of DNA-gp3, gp11, gp12, and gp16 have been compared. The result is consistent with other approaches in that the stoichiometry of gp11 and gp12 is greater than the stoichiometry of gp16 and pRNA. It is known that the stoichiometry of gp11 and gp12 in the ϕ29 assembly is 12, whereas the stoichiometry of pRNA is between 5 and 6. Interestingly, the curves for gp16 and pRNA do not overlap. Whether such a difference is owing to the possible reusability of pRNA or gp16 remains to be elucidated.

2.7.3.3. Common Multiples of 3 and 6

This stoichiometric method begins with mixing two inactive mutant pRNAs intermolecularly with complementary loops, and then assaying the activity of such mixtures in DNA packaging ***(175,176)***. The predicted secondary structure of the pRNA reveals two loops, known as the left- and right-hand loops ***(162)***. Sequences of these two loops (bases 45–48 of the right-hand loop and bases 85–82 of the left-hand loop) are complementary and were originally proposed to form a pseudoknot ***(165)***. However, numerous studies have revealed that these two sequences interact intermolecularly, allowing the formation of pRNA multimers. Several pRNAs with mutated left- and right-hand loop sequences have been constructed.

2.7.3.3.1. A Set of Two Interlocking pRNAs ***(175)***

An important finding in pRNA research was that mixing two mutant pRNAs with transcomplementary loops restores full DNA-packaging activity. pRNAs A/b' and B/a' are inactive in packaging when alone, but when they are mixed together, DNA-packaging activity is restored; the right-hand loop A of pRNA A/b' pairs with the left-hand loop a' of pRNA B/a'. Since mixing two inactive pRNAs with interlocking loops—such as when pRNA I-j' and J-i' are mixed in a 1:1 molar ratio—results in the production of infectious virions, the stoichiometry of the pRNA is predicted to be a multiple of two. This, taken together with the results from binomial distribution and serial dilution analyses, strongly indicates that the stoichiometry of pRNA is six.

2.7.3.3.2. A Set of Three Interlocking pRNAs ***(175)***

A similar procedure was used to confirm the formation of trimers through hand-in-hand interaction. Another set of mutants was composed using three pRNAs: A-b', B-c', and C-a'. This set is expected geometrically to form a 3, 6, 9, or 12mer ring that carries each of the three mutants. When tested alone, each individual pRNA exhibited little or no activity. When any two of the three mutants were mixed, again little or no activity was detected. However, when all three pRNAs were mixed in a 1:1:1 ratio, DNA-packaging activity was fully restored. The lack of activity in mixtures of only two mutant pRNAs and the restored activity in mixtures of three mutant pRNAs is to be expected, since the mutations in each RNA are engineered such that only the presence of all three RNAs will produce a closed ring. Since three inactive pRNAs are fully active when mixed together, this suggests that the number of pRNAs in the DNA-packaging complex is a multiple of 3, in addition to being a multiple of 2. Thus, the number of pRNAs required for DNA packaging must be a common multiple of 2 and 3, likely 6 (or 12, but this number has been conclusively excluded by binomial distribution and serial dilution analyses ***(172)***, which revealed pRNA's stoichiometry to be between five and six, confirming that the stoichiometry of pRNA is six).

2.7.3.3.3. A Set of Six Interlocking pRNAs ***(175)***

DNA-packaging activity can also be achieved by mixing six mutant pRNAs, each of which is inactive when used alone. Thus, an interlocking hexameric ring is predicted to form by base pairing of the interlocking loops.

2.7.3.3.4. Stoichiometry of Other ϕ29 Components

The highly sensitive in vitro ϕ29 assembly system has been utilized to determine the stoichiometry of each of the components needed for assembly ***(103,104,172,173,178)***. The log/log plot method incorporates only the functional unit (oligo or complex), not the copy number of the subunits. The procapsid, despite containing ***(26)*** copies of scaffolding protein gp7, 235 copies of capsid protein gp8, and 12 copies of the portal protein gp10, is a complex that is regarded as one component. DNA-gp3 is also treated as one component since the DNA and terminal protein gp3 are covalently linked. The log/log plot has revealed that the slope of the log/log concentration-dependent curve for procapsid is one; therefore, one copy of procapsid is needed for the assembly of one virion ***(103,104,172,173,178)***. The genomic DNA-gp3 acts in the same way. The stoichiometry for pRNA, gp11, and gp12 has been determined to be 6, 12, and 12, respectively, using log/log slope assay ***(178)***, binomial distribution ***(172,178)***, common multiples of two and three ***(175,180)***, migration rates in gel ***(181)***, and electron microscopy (EM) ***(182,183)***. Although it has been found that six pRNAs form a hexamer as a vital part of the DNA-translocating

motor, the hexamer can be assembled in vitro from individual pRNA molecules or complexes. If the hexamer is assembled from one A-a' or B-b' monomer or six monomers A-b', B-c', C-d', D-e', E-f', F-a' ***(175)***, the monomer is regarded as one component and the stoichiometry of the pRNA monomer is six ***(162)***. If the hexamer is assembled from purified dimer composed of (I-a')/(A-i'), the dimer is regarded as one component, and the stoichiometry for dimer RNA is three ***(164,175)***. If the hexamer is assembled from purified (A-b')/(B-c')/(C-a') trimer, the trimer is regarded as one component and the stoichiometry of trimer-RNA is two ***(162,175)***. It was found that gp12 formed a dimer before being packaged into a ϕ29 assembly intermediate ***(184,185)***, and each dimeric gp12 is regarded as one component. Therefore, the stoichiometry of gp12 is 12, instead of 24, disregarding the fact that there are 24 copies of gp12 in each ϕ29 virion. Purified tail protein gp9, expressed in *Escherichia coli*, has been shown to be active in in vitro ϕ29 assembly. The absolute copy number of gp9 in one virion has been reported to be nine ***(181)***.

2.8. ATP: The Source of Motor Energy

Translocation of DNA into the procapsid is energetically quite unfavorable. The DNA arrangement within the capsid is very compact, and the packaged DNA undergoes about a 30- to 100-fold decrease in DNA volume compared with its volume before packaging ***(15,186)***. The conclusion that the unfavorable reaction is driven by ATP hydrolysis is derived from studies involving bacteriophages λ ***(2,152,153,187–189)***, P22 ***(155)***, ϕ29 ***(5,9,30)***, T3/T7 ***(8,10,28,29,62,63,154,190)***, T4 ***(37,150)***, and P2 ***(69,70)***. The first quantification of ATP consumption in DNA packaging was performed in the ϕ29 system with purified components ***(30)***. In the defined in vitro DNA-packaging system, ATP was hydrolyzed to adenosine 5'-diphosphate and P_i. Approximately one ATP was required to package 2 bp of ϕ29 DNA. Similarly, with bacteriophage T3, it was found that, on average, one ATP molecule was required to package 1.8 bp of T3 DNA ***(28)***. In T3, however, it was found ***(28)*** that two separate ATPase activities existed. One of these, called *pac*-ATPase, required viral DNA, and the other, called *non-pac*-ATPase, was stimulated by nonpackagable DNA (i.e., single-stranded or circular) or RNA (nonspecific). However, the non-pac ATPase activity continued, after initial activation, in the absence of viral DNA. These individual ATPase activities can be stimulated by mixing with other components. For example, the ATPase activity of prohead was stimulated by the addition of ϕ29 DNA, and an additional fivefold increase in ATPase activity was found in the complete packaging reaction ***(30)***. Recent results indicate that pRNA has an effect on the ATPase activity of gp16, stimulating it fourfold. This ATPase activity continues only when pRNA is present, suggesting the formation of a gp16-pRNA complex. Similarly, procapsids with pRNA

stimulate the ATPase activity of gp16 10-fold, as compared with the lack of stimulation by procapsids alone ***(156)***. The results from both the ϕ29 and T3 systems suggest that the consumption of ATP in the DNA-packaging system is extremely complicated. ATP might be used for the initiation of DNA packaging and translocation. In ϕ29, all components in the packaging system, including pRNA, procapsid, gp16, and DNA-gp3, are involved in the generation of maximal ATPase activity.

3. Mechanisms of Motor Motion to Stuff Viral DNA

3.1. Experimental Attempts to Elucidate Motor Mechanism of ϕ29

3.1.1. Establishment of a Highly Sensitive In Vitro Packaging System

Utilizing pRNA's readily manipulable structure, a defined in vitro ϕ29 DNA-packaging system has been developed ***(9)***. With purified procapsid, gp16, pRNA, and ATP, up to 90% of the added DNA-gp3 can be packaged into the procapsid via the motor constructed with recombinant gene products. After DNA packaging, the in vitro assembly system can convert a DNA-filled capsid into infectious ϕ29 virions with the addition of purified proteins gp9, gp11, gp12, and gp13 ***(103,104)***. With this system, up to 5×10^9 infectious virions/mL can be obtained in the presence of all required components, yet not a single infectious virion is detected when even one essential component is absent. This system, with a sensitivity of eight orders of magnitude, has been used to assay the function of the DNA-packaging motor ***(180,192)***.

3.1.2. Circularly Permuted pRNA

An important approach in analyzing pRNA function has been the construction of circularly permuted pRNAs (cp-pRNAs), in which any internal base of the pRNA could be reassigned to serve as new 5'- or 3'-termini ***(169,193)***. Two tandem pRNA coding sequences separated by a 3- or 17-base loop sequence were cloned into a plasmid. Polymerase chain reaction (PCR) primer pairs such as P6/P5, complementary to various locations within the tandem pRNA coding sequences, were designed to synthesize PCR fragments for the transcription of cp-pRNAs. Results indicated that neither the small nor the large loop interferes with biological activity of the molecule. It has been demonstrated that nonessential bases or ones adjacent to them can be used as new termini for constructing active cp-pRNA; this system greatly facilitates the construction of mutant pRNA via PCR and enables the labeling of any specific internal base by radioisotopes, fluorescence ***(194)***, or photoaffinity agents.

3.1.3. Computer Modeling

The goal of modeling pRNA structure is to organize collections of structural data from crosslinking, chemical or ribonuclease probing, chemical modifica-

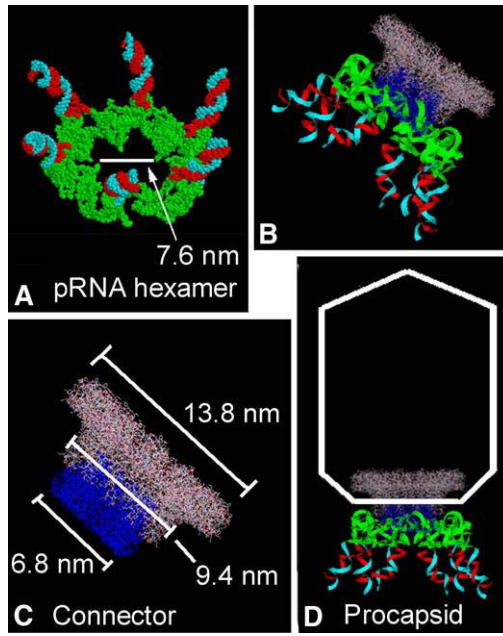

Fig. 2. Computer models of pRNA hexamer and the motor complex of bacterial virus ϕ29. **(A)**, Hexamer shows the connector binding domain in green, as well as the DNA translocating domain colored red (5'-end) and cyan (3'-end). The DNA translocating domain of the 5'/3'-paired region points up. **(B)**, The crystal structure of the connector (From refs. Valle, Kremer et al., 1999; Valpuesta, Fernandez et al., 1999). The RNA Recognition Motif is blue. **(C)**, Docking of the pRNA hexamer to the RNA binding domain of the connector. **(D)**, Procapsid Bottom view. Adapted from (Shu, Huang et al., 2003) with permission of *J. Biol. Chemistry*.

tion interference, cryo-AFM, and other genetic data into a 3D form. Because a large number of structural constraints are available, computer programs can successfully construct 3D structures ***(158,195,196)*** (**Fig. 2**).

X-ray crystallography studies have revealed that the ϕ29 connector contains three sections: a narrow end, a central section, and a wider end, with diameters of 6.6, 9.4, and 13.8 nm, respectively ***(136,137,197)***. The hexameric pRNA model by Hoeprich and Guo ***(158)*** describes a central channel with a diameter of 7.6 nm that perhaps can sheath onto the narrow end of the connector to be anchored by the central section of the connector, which is wider than the central channel of the pRNA hexamer.

The connector-binding domain is located in the middle of the pRNA primary sequence (i.e., bases 23–97), and the DNA-translocation domain is located at the 5′/3′ paired ends. It has been predicted that the connector protein (gp10) contains a conserved RNA recognition motif (RRM), located between residues 148 to 214 of each gp10 monomer. This region of gp10 is located at the narrow end of the dodecameric connector that protrudes from the procapsid ***(156,198)***. The hexamer model by Hoeprich and Guo ***(158)*** agrees with the aforementioned data, showing that pRNA bases 23 to 97 within the connector-binding domain interact with the predicted RRM motifs of the connector, and the 5′/3′ paired region, comprising the DNA-translocation domain, extends away from the connector.

3.1.4. Use of Tweezers to Determine Force of Motor

Optical tweezers offer an interesting method by which to measure packaging force and speed ***(199)***. In this procedure, partly packaged motor complex has a microsphere attached to it via unpackaged DNA. This microsphere is caught in an optical trap and tethered to a second bead. When ATP is introduced and packaging initiates, the two beads move closer together. The amount of DNA tension is monitored, and from this, the bead displacement can be calculated; various measurements of packaging dynamics are then possible, including an examination of the presence of packaging "slips" and "pauses," where irregularities occur in packaging speed. With additional load, the force needed to prevent the DNA from being inserted is about 57 pN, indicating that the ϕ29 DNA-packaging motor is the strongest biomotor studied to date. Using this method, it has been possible to determine the speed of ϕ29 DNA packaging, which is initially about 100 bases/s, gradually slowing to a halt as the capsid is filled ***(199)***. The fivefold/sixfold symmetry mismatch between the procapsid and pRNA has been cited to help explain the mechanism of motor rotation ***(142,143,180)***, but the precise mechanism by which DNA moves into the capsid during packaging has yet to be elucidated.

3.2. Models for Packaging of DNA by ϕ29

It is generally understood that pRNA is part of an ATPase and that it possesses at least two conformations: relaxed and contracted. Driven by ATP hydrolysis, which causes an alternation between relaxed and contracted states, the individual parts of the hexameric pRNA complex help rotate the translocation machinery. gp16, a protein that binds ATP, is also involved in ATP hydrolysis.

The requirement of an intermolecular loop/loop interaction between individual pRNA molecules during DNA packaging has led to the belief that pRNA forms a hexamer ***(175,176)*** and supports a model incorporating the sequential action of pRNA. Individual pRNAs likely need to communicate with each other during DNA packaging to ensure that motion is consecutive. Inter-pRNA

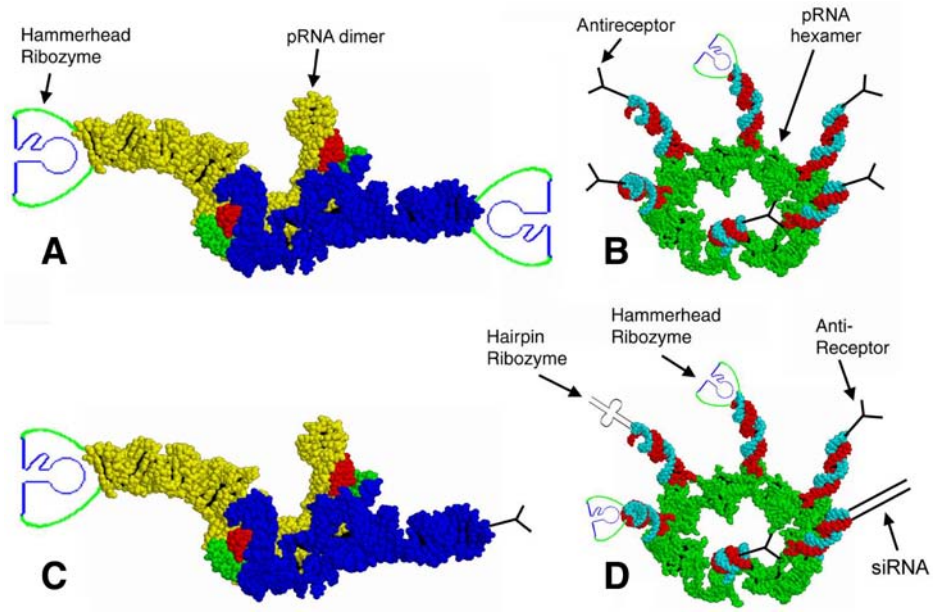

Fig. 3. ϕ29 pRNA as polyvalent vehicles for delivery genes in therapy. pRNA multimers could be used to target a cell receptor and to co-deliver therapeutic siRNA, ribozymes or other biologically active molecules. Adapted from (Shu, Huang et al., 2003) with permission of *J. Biol. Chemistry*.

interactions via loops might serve as a link to pass signals to adjacent pRNAs, regulating sequential conformational changes and/or interactions. Thus, base pairing between the right- and left-hand loops might be necessary to transfer a conformational change from one pRNA to an adjacent one.

Several models for DNA translocation into the ϕ29 procapsid have been proposed ***(137,143,200–202)***. It is generally believed that DNA is translocated through the axial hole of the portal vertex, much like a threaded rod moving along a nut. Another model hypothesizes that supercoiled DNA wraps around the portal vertex and that its rotation allows DNA to pass into the procapsid via the outside of the vertex ***(202)***. In a model proposed in 1997 ***(143)***, sequential action of multiple pRNAs, in conjunction with other components, is vital to DNA packaging. Six copies of pRNA form a hexamer that interacts with the capsid pentamer and moves in discrete 12° steps (**Fig. 3**). Thirty ATPs are needed for each completed cycle of motor rotation. This model is in good agreement with existing 3D structural data obtained recently by crystallography ***(137)***, complementary modification, photoaffinity crosslinking, chemical modification, chemical modification interference, nuclease probing, AFM, and computer-modeling ***(158)*** methods.

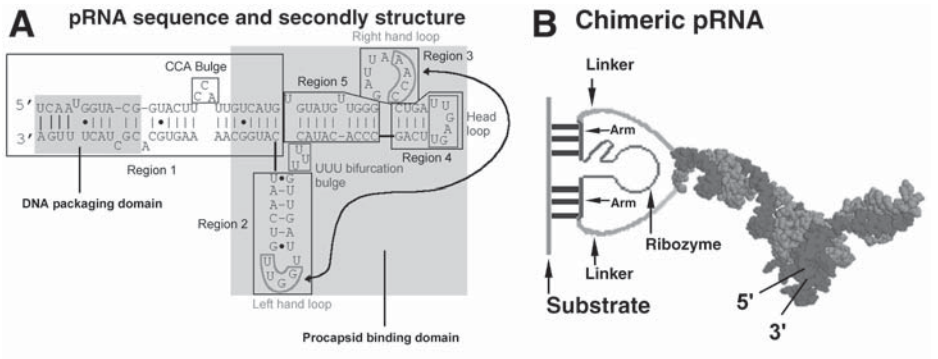

Fig. 4. Sketch showing chimeric pRNA harboring a ribozyme. **(A),** The sequence and secondary structure of pRNA. The curved line points to the two interacting loops. **(B),** Structural outline of the pRNA/ribozyme chimera. A ribozyme targeting at the Hepatitis B virus mRNA is connected to both the 5' and 3'-end of pRNA. Adapted from (Hoeprich, Zhou et al., 2003) with permission of Nature Publisher.

In the model proposed in **ref.** ***137***, DNA located in the central channel interacts with one subunit of the portal. A 12° rotation of the narrow end of the connector leads to lengthwise expansion of the connector via a slight change in the angle of the long helices, and the wide end of the connector follows the narrow end. Such a "following" allows the structure to relax and contract while translating 2 bp of DNA into the capsid ***(137)***. In addition, the "motor-ratchet" model of DNA packaging has been proposed and reviewed recently ***(17)***. In this model, the connector rectifies DNA motion via either thermal, biased thermal, or oscillating processes. The motor-ratchet hypothesis has been widely used to interpret data pertaining to biochemistry, genetics, energetics, structure, and dynamics in packaging. The structure of both the connector and pRNA ***(158)*** exhibits orientational tilting toward one direction.

At least four distinct models can be proposed to describe the specific mechanisms underlying the translocation of DNA resulting from such motors, which are discussed next.

3.2.1. Model 1

The force of DNA movement is Brownian motion ***(17,203,204)***. The motor is a ratchet ***(17)*** used simply to ensure that DNA moves in one direction, but not the opposite.

3.2.2. Model 2

DNA is translocated through the axial hole of the portal vertex, much like a threaded rod processing through a nut, and thus DNA packaging is achieved by

utilizing the "threaded" helical nature of dsDNA ***(142)***. The motor, composed of the pRNA and connector, operates like a hex nut to drive a bolt ***(143)***. Evidence for this model would include establishing that DNA translocates regularly at a fixed ratio of distance moved to the number of motor turns.

3.2.3. Model 3

The fivefold/sixfold symmetry mismatch and sequential contraction and relaxation of motor components generates the force required for motor rotation ***(142,143)***. If this model is correct, discrete steps in 12° intervals in component rotation will be found.

3.2.4. Model 4

DNA located in the central channel interacts with one subunit of the portal ***(137)***. A 12° rotation of the narrow end of the connector leads to lengthwise expansion of the connector via a slight change in the angle of the long helices; the wide end of the connector follows the narrow end. Such a "following" allows the structure to relax and contract while translating 2 bp of DNA into the capsid ***(137)***. This model implies that one complete turn of the connector transfers 60 bp of DNA into the procapsid.

4. Applications of the ϕ29 Nanomotor in Research and Nanotechnology

4.1. A Nanomotor With Potential to Be Incorporated Into Nanodevices

The ability to harness and utilize nanomotors has the potential to expand and revolutionize the field of nanotechnology ***(205–207)***. One viable option that has been pursued for the development of mechanical parts for nanotechnology is to incorporate the ϕ29 motor and other motors and their constituent parts into nanodevices. Techniques generally involve the characterization, manipulation, modification, control, creation, and/or assembly of organized materials on the nanoscale level ***(208–210)***. These materials can then be used as building blocks for the construction of larger devices and systems ***(211–214)***. Nanotechnological endeavors are expected to play critical roles in many scientific disciplines, including chemistry, physics, biology, medicine, materials science, engineering, and computer technology.

Several factors combine to make ϕ29 pRNA an ideal choice for applications in nanotechnology and other fields. It is readily reproducible, as evidenced by the recent development of a defined in vitro ϕ29 DNA-packaging system, whereby with purified procapsid, gp16, and pRNA, up to 90% of DNA-gp3 can be packaged ***(78)***. This process has been utilized to quantify the forces involved in DNA translocation with an optical trap ***(199,215)***. The ϕ29 DNA-packaging motor has, through this method, been confirmed as the strongest

known nanomotor, with a stalling force of 57 pN ***(199)***. Perhaps most importantly, however, ϕ29 pRNA is readily maneuverable and controllable, tending strongly toward interactions resulting in the formation of dimers, trimers, and hexamers. Complementary loop sequence production procedures allow for the formation of predefined pRNA multimers, modifiable at the earliest possible stage of the construction of larger structures and systems. pRNA's two domains are independent and tightly self-folded. As many as 120 nucleotides can be extended from the 3' end of pRNA without hindering its procapsid-binding or DNA-packaging activity ***(216,217)***.

4.2. Polyvalent Gene Delivery System Using Motor pRNA

Antigen assays and Southern blot analyses have demonstrated that ϕ29 pRNA can chaperone and escort the hammerhead ribozyme to function in the cell, enhancing cleavage efficiency and the inhibition effect of the ribozyme on hepatitis B virus. Such an increase in ribozymal activity likely results from pRNA's ability to prevent the ribozyme from misfolding and to protect it from degradation by exonucleases. It has been successfully demonstrated that exogenous RNA can be connected to the end of pRNA without affecting pRNA folding. At least 120 nonspecific bases can be extended from the 3' end of aptRNA without hindering folding or function of the pRNA, which indicates that the 117-base pRNA was folded independently of bases extended from its 3' end. These findings suggest that therapeutic RNA molecules could be placed between the 3' and 5' ends of the pRNA and would be able to fold without being influenced by the original pRNA sequence.

ϕ29 pRNA tends strongly toward the formation of dimers ***(158,162, 174,218)***, and dimers are the primary building blocks of hexamers ***(164,175, 176,200)***. The formation of monomers and dimers can be controlled through manipulation and control of the sequences of the two interacting loops ***(158,162,174,176,218)***. The formation of pRNA dimers may also assist in stabilizing pRNA/ribozyme chimeric molecules. As long as the openings of the circularly permutated pRNAs are close enough to an area of dimer formation, the tertiary structure can help prevent exonucleases from accessing the ends of the RNA molecules.

The formation of dimers and hexamers also facilitates the delivery of multiple therapeutic agents. For example, one of the pRNA subunits of a hexamer could carry the hammerhead ribozyme, and the other pRNA subunit could carry a hairpin ribozyme or an antisense RNA. Applications of multiple therapeutic agents might enhance the efficiency of in vivo therapy. The multivalent subunits of pRNA show strong potential to facilitate specific targeting and delivery of ribozymes. One of the subunits could, for example, be utilized to carry an RNA molecule that interacts with a cell-surface receptor. Binding of dimer

or hexamer pRNA to the specific receptor would enable specific delivery of the RNA complex to the cell via endocytosis. Specific receptor-binding RNA molecules can be isolated through systematic evolution of ligands by exponential enrichment (SELEX) ***(219,220)***. Starting from a library containing random RNA sequences, in vitro evolution techniques allow for the selection of RNA molecules that are able to bind a specific ligand or receptor ***(221–224)***. The chimeric RNA carrying the hammerhead ribozyme and the chimeric RNA carrying the antireceptor can be mixed to form dimers via inter-RNA loop/loop interaction, as reported previously ***(164,175,176,200)***.

4.3. DNA-Packaging Motor as a DNA-Sequencing Apparatus or Molecular Sorter

Another burgeoning area of interest in biology is the development of efficient and sensitive analytical tools that can be used to probe and manipulate single molecules. Given recent projects mapping bacterial, human, and other mammalian genomes, new types of arrays and methodologies will be required to analyze, interpret, and utilize genomic information efficiently and inexpensively. Scientists are working to develop a nanopore-based DNA-sequencing device ***(225)***. Such a device will recognize a single base pair, based on the electrical signals generated through the interaction of the bases of the DNA with a pore. A similar concept may be useful for single-molecule analysis of other biological molecules. The ϕ29 DNA-packaging motor has the potential to be developed into a DNA-sequencing apparatus, since the DNA-packaging process involves movement of the DNA through a 3.6-nm pore surrounded by six RNAs that can be modified to accept chemical or electrical signals.

4.4. Model for Study of RNA Dimers and Trimers in Other Living Systems

Stable pRNA dimers and trimers in the absence of protein have already been isolated ***(164)***. Dimerization/trimerization assays provide simple and stable systems for studying structural solutions to pRNA complexes and for investigating the mechanisms of RNA/RNA interactions. RNA dimerization and oligomerization have been found to play an important role in many living systems. Dimerization of retroviral genomic RNA is critical to many stages in the retroviral life cycle, including translation, reverse transcription, RNA encapsidation, and virion assembly ***(226–228)***. For instance, RNA-RNA intermolecular interactions are required for the formation of a specific ribonucleoprotein particle, *bicoid* mRNA 3' UTR-STAUFEN, that determines the formation of the anterior pattern of the *Drosophila* embryo ***(229)***. RNA-RNA interaction is also an essential step in the cleavage reaction of RNase P on tRNA ***(230–232)***. Replication of plasmid ColE1 is regulated by plasmid-speci-

fied small RNAs (RNA I and RNA II) that form complexes through complementary RNA stem-loop interactions ***(233)***.

4.5. A Model for Design of New Antiviral Strategies

Extensive investigation into DNA packaging of the dsDNA viruses documents certain common features in this step of the viral life cycle. Commonalities include the use of a pair of noncapsid enzymes to translocate viral DNA into a procapsid coupled with the hydrolysis of ATP ***(28–30,38,59, 143,152,191,234–236)***. Similarities in DNA packaging between dsDNA bacteriophages and herpes viruses, adenovirus, parvovirus, and pox viruses (as mentioned in **Subheading 1.**) justify the use of ϕ29 as a model system for the design of new antiviral strategies. Using ϕ29 pRNA as a target, several methods have been modeled ***(170,171,173)*** for the inhibition of viral assembly.

4.5.1. Inhibition of ϕ29 Assembly by Antisense DNA Targeting pRNA In Vitro

Antisense oligos include antisense RNA and antisense DNA. They are small, regulatory, single-stranded polynucleotides that bind to complementary regions on a specific target in order to control their biological function. Antisense DNA could bind to pRNA and cause a change in pRNA electrophoretic mobility ***(170)***. Antisense DNA oligos were shown to hybridize to ϕ29 pRNA by gel shift assays ***(170)***.

ϕ29 pRNA contains two functional domains. One domain is located at the central region and is required for procapsid binding. The other domain, consisting of paired 5' and 3' ends, is needed for DNA translocation into procapsids but is not required for procapsid binding. Oligo P6 targeting the left-hand loop of the procapsid-binding domain could block the binding of pRNA to procapsids, resulting in the inhibition of ϕ29 assembly in vitro. Oligos P11 and P15 targeting either the 5' or the 3' end of the pRNA, respectively, do not prevent pRNA from binding to procapsid, but strongly inhibit DNA packaging ***(166,170)***.

4.5.2. Complete Inhibition of ϕ29 Assembly With Mutant pRNA In Vitro

ϕ29 is being used as a model to explore new avenues in antiviral research beyond in vitro inhibition with antisense oligos targeting pRNA. As already noted, pRNA contains two functional domains. Sequence alterations that disrupt base pairing at the 5'/3' ends of the DNA-translocating domain result in mutant pRNAs that are completely inactive in phage assembly in vitro but still retain WT procapsid-binding affinity ***(159,166,172,173)***. These mutant pRNAs are able to compete with WT pRNAs for procapsid binding and efficiently inhibit viral assembly both in vitro and in vivo ***(173)***.

For in vivo studies, a plasmid expressing a pRNA with a 4-base mutation at the 3' end was constructed and transformed into host cells. Cells harboring this plasmid were completely resistant to plaque formation by WT ϕ29. The interesting result here is that "complete" inhibition was gained, which indicates that factors involved in viral assembly can be utilized for efficient and target-specific antiviral treatment. This high level of efficiency has resulted from two pivotal features. First, since the two domains are quite distinct and separate, mutation of the packaging domain results in pRNA with no DNA-packaging activity, but procapsid-binding competence remains intact. Second, six pRNAs are involved in the packaging of one genome. This high-order dependence of pRNA results in a correspondingly higher order of inhibitive effects, since blocking only one of the six positions could result in complete cessation of the assembly of virions ***(172)***. The principle of using molecules containing two functional domains and requiring multiple-copy involvement as inhibitors could be applied to gene therapy, intracellular immunization, antiviral drug design, and the construction of transgenic plants resistant to viral infection, using certain viral structural proteins, enzymes, and other RNAs involved in the viral life cycle.

4.6. Similar Mechanism Between Viral DNA Translocation and Other Nucleic Acid Sliding/Riding Processes

There is a group of nucleic acid-binding proteins that plays a similar role in DNA or RNA riding or sliding related to DNA replication, translocation, recombination, RNA packaging, and RNA transcription ***(175,237–240)***. This group can be subdivided into two subsets: proteins that bind nucleic acid and proteins that act on nucleic acid. The chief feature common to both subsets is that they interact with RNA or DNA in a polymer conformation and with a ring-shaped morphology. Most of the proteins in this group are also hexameric.

The subset that acts on nucleic acid includes helicase ***(241,242)***; *E. coli* transcription termination protein Rho ***(243,244)***; the yeast DNA polymerase processivity factor ***(245)***; bovine papillomavirus E1 replication initiator ***(246)***; and the *E. coli* DNA polymerase III holoenzyme ***(247,248)*** and dsRNA-packaging motor ***(239,240)***, which exist as hexamers ***(241,249–252)***. Although the mechanisms of this kind of DNA–protein interaction remain to be elucidated, the finding that six copies of pRNA are attached to a sixfold symmetrical connector during DNA translocation indicates that it might have something in common with these hexamers. The processes of both viral DNA packaging and DNA (or RNA) replication (or RNA transcription) involve the relative motion of two components, one of which is nucleic acid. It would be intriguing to show how pRNA may play a role similar to that of the protein enzymes, such as DNAS-helicase or the termination factor Rho.

4.7. Insight Into Mechanisms of Macromolecular Translocation Through Cellular Membrane Wall

Migration or transportation of macromolecules, such as proteins or nucleic acids, through a barrier or cell membrane is a common process in life systems. After transcription in the nucleus, mRNA and tRNA must pass the nuclear membrane to reach the translation machinery in the cytoplasm. Similarly, nuclear proteins must pass from the cytoplasm, where they are synthesized, to the nucleus, where they function. After infection or transfection, most viral or plasmid DNA must pass through the nuclear membrane in order to serve as a template for gene expression ***(253)***. The Rev protein of human immunodeficiency virus assists in the translocation of viral mRNA from the nucleus to the cytoplasm through a nuclear pore ***(254,255)***. Varieties of proteins and other elements migrate into and out of the cell and nucleus to perform their respective functions. Molecular migration or translocation is also manifest in the tracking and rail riding of enhancers or transcription factors along DNA ***(251)***, the translocation of the transcription termination protein Rho along RNA ***(252)***, and the migration of helicases along single-stranded DNA during DNA replication ***(241,256–258)***.

One of the most complex and intricate translocation processes is viral DNA encapsidation. The study of DNA encapsidation could provide hints for macromolecular translocation through cell membranes.

5. Conclusion

The ϕ29 DNA-packaging motor serves as an effective model for the study of dsDNA viral packaging. The development of a defined in vitro packaging system utilizing purified ϕ29 components will aid further research into both the specific attributes of ϕ29 packaging and DNA packaging in general. This will provide for a number of technological advances, including the targeted delivery of therapeutic agents to cells, precise single-molecule sequencing techniques, and direct attachment of various motor parts to nanodevices. In addition, ϕ29's strong tendency to form dimers, trimers, and hexamers can potentially be harnessed and utilized to construct self-assembling arrays that have far-ranging technological possibilities, including the development of ultrahigh-density memory storage systems and the isolation and separation of multiple pathogens in medical diagnoses. Ongoing research into specific motor parts and overall motor function is making such important goals increasingly realizable.

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