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Role of the CCA Bulge of Prohead RNA of Bacteriophage ø29 in DNA Packaging

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Summary

The oligomeric ring of prohead RNA (pRNA) is an essential component of the ATP-driven DNA packaging motor of bacteriophage ø29. The A-helix of pRNA binds the DNA translocating ATPase gp16 (gene product 16) and the CCA bulge in this helix is essential for DNA packaging *in vitro*. Mutation of the bulge by base substitution or deletion showed that the size of the bulge, rather than its sequence, is primary in DNA packaging activity. Proheads reconstituted with CCA bulge mutant pRNAs bound the packaging ATPase gp16 and the packaging substrate DNA-gp3, although DNA translocation was not detected with several mutants. Prohead/bulge-mutant pRNA complexes with low packaging activity had a higher rate of ATP hydrolysis per base pair of DNA packaged than proheads with wild-type pRNA. CryoEM-3D reconstruction of proheads reconstituted with a CCA deletion pRNA showed that the protruding pRNA spokes of the motor occupy a different position relative to the head when compared to particles with wild-type pRNA. Therefore, the CCA bulge seems to dictate the orientation of the pRNA spokes. The conformational changes observed for this mutant pRNA may affect gp16 conformation and/or subsequent ATPase-DNA interaction and, consequently, explain the decreased packaging activity observed for CCA mutants.

Keywords

bacteriophage ø29; DNA packaging; prohead RNA; RNA structure; cryoEM-3D reconstruction

Introduction

Bacteriophage ø29 of *Bacillus subtilis* uses a powerful molecular motor to package its double-stranded DNA (dsDNA) into a precursor protein shell (prohead).¹ The motor is situated at a unique vertex in the prohead and is composed of the dodecameric head-tail connector (gene product 10, gp10),² a multimeric ring of prohead RNA (pRNA),^{2–4} and a ring of the packaging ATPase gp16.^{2,5,6} DNA is translocated through the axial channel of this tripartite motor by a mechanism that is as yet undefined. Although the dsDNA bacteriophages (ø29, lambda, T4, SPP1, etc.) likely share a common DNA packaging mechanism,⁷ an RNA component of the motor has only been demonstrated for ø29.

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pRNA is essential for DNA packaging, but is not a component of the mature virion.⁸ pRNA is a 174-base transcript from the extreme left end of the ϕ 29 genome, and a 120b form with full biological activity⁹ is commonly used in biological assays. Removal of pRNA from the prohead and subsequent reconstitution of RNA-free particles with pRNA results in loss and restoration of packaging activity, respectively, and this property allows the testing of pRNA mutants.^{10,11} pRNA homologs are found in relatives of ϕ 29, and although sequence similarity is low, these pRNAs are predicted to adopt a similar secondary structure.¹²

Bases 22–84 comprise the prohead binding domain of pRNA, as demonstrated by ribonuclease footprinting and mutational analysis (Figure 1, shaded region).^{13–15} This domain also includes bases 45–48 of the C–E loop and bases 82–85 of the D-loop that base pair to form the intermolecular interaction that links pRNA molecules to form the pentameric ring on the prohead observed in cryoEM-3D reconstructions^{2,6,16,17} that is needed for packaging activity.^{3,4} Bases 1–28 and 92–117 form the A-helix of pRNA, and mutations in this region affect DNA packaging, but not prohead binding (Figure 1).^{14,18,19} The ATPase gp16 binds to the A-helix of pRNA.^{5,6,20}

Although most of the single base bulges of the predicted pRNA structure are dispensable for DNA packaging activity, the CCA bulge (residues 18–20) of the A-helix is essential (Figure 1).^{14,15,19,21} A mutant pRNA lacking this bulge (Δ CCA) binds proheads, but no DNA packaging is observed.¹⁴ Consistent with the location of the bulge outside of the prohead binding domain, this mutant pRNA has competitor binding activity similar to that of wild-type pRNA. Additionally, proheads reconstituted with pRNA lacking the CCA bulge also bind the packaging ATPase gp16.^{5,20} Here, mutational analysis delineated the requirements for size and sequence of the CCA bulge necessary for biological activity. Additionally, cryoEM-3D reconstruction of the prohead reconstituted with the Δ CCA mutant pRNA showed that deletion of the bulge affects the conformation/orientation of the pRNA A-helix on the prohead. ATPase subunits of the packaging motor have been hypothesized to be gp16-pRNA heterodimers.²² Thus, the altered structure of pRNA in Δ CCA mutants may lead to an altered conformation of the ATPase gp16 ring or misalignment of the ring relative to other motor components, thereby preventing gp16 from properly interacting with DNA to drive translocation.

Results

Mutation of the CCA bulge of pRNA and the effect on DNA packaging

The CCA bulge (bases 18–20), along with other prominent secondary structural features of pRNA, is shown in Figure 1. Similar bulge structures are found in the pRNAs of ϕ 29 relatives.¹² These bulges vary in size from 3–4 bases, are flanked by A/U base pairs, and an adenine (A20 in ϕ 29) is conserved within the bulge in all ϕ 29 relatives. To assess the importance of the CCA sequence on the biological activity of ϕ 29 pRNA, base substitution and deletion mutants were made (Table 1). RNA-free proheads were reconstituted with each of the mutant pRNAs, and DNA packaging was assessed in the defined *in vitro* system (Figure 2 and Table 1). Previously, the residue in each of the three positions had been changed to one other base, with modest (C18G: 30% activity relative to wild-type) or no (C19A, A20G: 100% activity) effect on viral assembly.²¹ Here, to further probe the conserved A20, it was changed to C, U or G (Figure 2(a); Table 1). While the C or U substitutions had little or no effect on activity, the G substitution reduced packaging to 44% that of wild-type pRNA (Table 1). Next, alteration of position C18 showed a 5-fold drop in activity with a G substitution (GCA), consistent with previous work,²¹ whereas a U substitution (UCA) had activity 84% that of wild-type pRNA. Bulges with multiple substitutions were also constructed. The double mutant CAG had activity 49% that of the

wild-type pRNA, while the triple mutant AAG had 62% packaging activity. The mutants with the lowest packaging activity had G substitutions at positions 18 or 20.

The effect of the bulge size on biological activity was also assessed. CCA deletion mutants were made (Table 1) and proheads containing these mutant pRNAs were tested for DNA packaging activity (Figure 2(b) and Table 1). A mutant pRNA with a deletion of the conserved A (ΔA), leaving CC as the bulge, had packaging activity comparable to that of wild-type (Table 1). In contrast, deletion of a C residue that leaves a CA bulge, showed a 3-fold reduction in packaging. Deletion of two bases (mutants ΔCA and ΔCC) or the entire bulge (mutant ΔCCA) showed no detectable DNA packaging.

gp16 binding to proheads reconstituted with bulge mutant pRNAs

To dissect the nature of packaging defects, select pRNAs with low or no packaging activity (CCG, GCA, ΔCCA and ΔC) or with high activity (CCC and ΔA) were bound to pRNA-free proheads and tested for binding of the ATPase gp16 (Figure 3). Proheads reconstituted with target pRNAs were incubated with gp16, sedimented in sucrose gradients to separate from unbound gp16, the prohead band isolated, and particles examined for gp16 content. Figure 3 shows that all proheads with CCA bulge mutant pRNAs were able to bind gp16 (lanes 4–9). Proheads with 71b pRNA, that lacks the A helix, did not bind gp16, and served as a control (lane 2). The amount of gp16 bound by proheads with either wild-type (lane 3) or mutant pRNAs (lanes 4–9) was about one-half that of the dodecameric head-tail connector (gp10), in agreement with the composition of the packaging motor reported previously.^{5,6}

Proheads/pRNA/gp16/DNA-gp3 complex formation

To initiate DNA packaging, the prohead/pRNA, gp16 and DNA-gp3 must interact to form a complex. Proheads reconstituted with CCA bulge mutant pRNAs were tested for the ability to form complexes with gp16 and [³H]DNA-gp3 (Table 2). The proheads were immobilized on magnetic beads (Materials and Methods) and incubated with gp16 and [³H]DNA-gp3, and complexes were isolated via a magnetic pull-down assay. Proheads with wild-type pRNA/gp16 retained only about 5% of the DNA-gp3. Addition of the non-hydrolyzable ATP analog γ -S-ATP increased DNA-gp3 binding to about 17%, demonstrating a nucleotide enhancement of this assembly event. All CCA bulge mutant pRNA/prohead/gp16 complexes tested were able to bind DNA-gp3 in the presence of γ -S-ATP. Combinations of DNA-gp3 with proheads or gp16 alone retained only about 1% of the [³H]DNA-gp3 on the beads, demonstrating that all the motor components are required for efficient DNA binding.

ATPase activity in DNA packaging

After initiation of DNA packaging, the motor engages in ATP-driven DNA translocation. The ATPase activity during packaging was quantified to assess the effect of the CCA pRNA mutants on this motor function (Table 3). Packaging reactions were prepared, an aliquot removed to measure ATP hydrolysis during the reaction, and the remaining sample used to determine the amount of DNA packaged. The observed hydrolysis of ATP in the reaction with proheads having wild-type pRNA was about one ATP per bp packaged; this ratio differs from the 2bp/ATP determined previously^{23,24} under different packaging and assay conditions (Materials and Methods). Bulge mutants CCC and ΔA , which showed packaging efficiencies similar to that of wild-type pRNA, resembled the wild-type pRNA in ATPase activity. No packaging was observed with ΔCCA pRNA, and ATP hydrolysis was at background levels (proheads plus gp16). Surprisingly, for the mutants CCG, GCA and ΔC that had packaging levels of 10–25% of wild-type pRNA, the ratio of bp packaged/ATP hydrolyzed was about one-half that of wild-type.

As some mutant pRNAs had packaging/hydrolysis ratios different from that of wild-type pRNA, DNA packaging was also assayed using [^3H]DNA-gp3 and sucrose density gradient centrifugation to ensure that partially packaged DNAs were detected (Figure 4).²³ To generate particles with partially-packaged DNA as a control, a reaction with wild-type pRNA was stalled at 1 min of packaging by the addition of γ -S-ATP (red). The unpackaged [^3H]DNA was then digested with DNase I, and the reaction mixture fractionated by sucrose density gradient centrifugation. The peak at fraction 9 in the gradient is characteristic of particles containing a partially packaged genome and marks the position of intermediates. Packaging reactions with proheads reconstituted with wild-type or CCA mutant pRNAs were prepared, and after 10 min incubation with ATP, γ -S-ATP was added to stall the motor, thus trapping any particles that had not completed packaging. Packaging with wild-type pRNA showed the expected profile with a peak at fraction 4, the gradient position of filled heads, and a peak at the top of the gradient that represents unpackaged DNA (black).²⁵ For ΔCCA pRNA (gray), the profile indicated that no detectable packaging occurred. With the $\underline{\text{GCA}}$, $\underline{\text{CCG}}$, and ΔC pRNA mutants (green, blue and orange, respectively), the profiles were nearly identical to that of wild-type pRNA, albeit with a reduced efficiency of packaging; no additional label representing partially packaged DNAs was observed, suggesting that packaging that initiated proceeded to completion.

CryoEM-3D reconstruction of proheads with ΔCCA pRNA

A cryoEM-3D reconstruction of the prohead containing the ΔCCA pRNA showed that the A-helices are twisted and hooked compared to their position and orientation in particles with wild-type pRNA (Figure 5). Superposition of a reconstruction of the ΔCCA particle (Figure 5(b)) with a reconstruction of a particle reconstituted with wild-type pRNA (Figure 5(a)) indicated that the A-helices in the ΔCCA mutant were rotated in a clock-wise direction when looking at the pRNA along the long axis of the particle towards the interior of the capsid, thus giving the pRNA density a distinct handedness (Figure 5(c) and (d)). Furthermore, comparison of these two structures allowed the position of the CCA bulge to be approximated. The divergence of the A-helix in ΔCCA pRNA begins near the ring of pRNA that encircles the connector. This is consistent with the cryoEM reconstruction of a prohead containing 71b pRNA (bases 25–95), which lacks most of the A-helix and shows only this ring of density.⁶ Although the A-helices are clearly displaced, the features of individual domains within the pRNA appear to be maintained, as evidenced by the knob-like structure visible at the distal end of the A-helix in both the wild-type and mutant pRNA structures. Thus, the observed conformational changes likely result from rigid body motion originating from the CCA bulge.

Discussion

pRNA is an essential component of the $\phi 29$ DNA packaging motor.⁸ CryoEM-3D reconstructions of the prohead/gp16 motor complex show a pentamer of pRNA with gp16 molecules nested between the pRNA spokes.⁶ The A-helix of pRNA has been shown to be the site of gp16 binding, as a truncated 71b pRNA retains full prohead binding capacity but does not bind gp16.^{5,15,20} The CCA bulge on the pRNA A-helix is essential for packaging,^{14,21} but its exact role has been unknown.

The size of the CCA bulge of pRNA is shown here to be of primary importance in DNA packaging, with sequence having a secondary role. The conserved adenine (A20) was changed to U or C with little effect on packaging activity, and a moderate reduction in packaging was observed when A20 was changed to G (Table 1; Figure 2(a)). Although A20 is not required for packaging *in vitro*, the conservation of this adenine in $\phi 29$ relatives suggests that it may be important *in vivo*. Residue C18 was mutated to G or U, and to A in the triple mutant (see below), and sequence played a role as C18G had only 20% packaging

efficiency, while C18U had packaging activity of 84% (Table 1). The double mutant CAG had the reduced packaging activity seen in the CCG mutant, but an increased packaging activity was consistently observed for the triple mutant (AAG) (Figure 2(a); Table 1). While certain base changes affected packaging, no substitution abrogated packaging, and the fact that the entire bulge sequence could be replaced successfully suggested that the wild-type primary sequence of this region is not essential for *in vitro* packaging. In contrast, the size of the CCA bulge had a dramatic effect on packaging (Figure 2(b); Table 1). Deletion of one base had a variable effect on packaging, with ΔA near wild-type activity while ΔC had a reduction in packaging efficiency of ~70%. Deletion of two or more bases (ΔCC , ΔCA , ΔCCA) rendered the motor inactive.

To characterize the nature of defects in packaging caused by modification of the CCA bulge, and thereby explore the role of this pRNA substructure in motor function, four aspects of motor assembly and function were assessed: gp16 ATPase binding, DNA-gp3 substrate interaction, motor ATPase activity, and DNA translocation. All of the prohead/bulge mutant pRNA complexes tested here bound gp16 (Figure 3). In addition, all of these complexes demonstrated gp16-dependent binding of DNA-gp3 (Table 2), showing the co-assembly of all components required for DNA translocation. However, the nature of packaging initiation is not understood for any packaging system and may be more complex than simple co-assembly of components. For example, higher order DNA structure has been implicated in packaging.^{26,27} Interaction of DNA-gp3 with the ATPase gp16 yields supercoiled DNA, which is preferentially packaged, and it has been proposed that binding of the prohead to coiled loops of the DNA-gp3 helps localize the prohead to the end of the DNA to begin packaging.²⁷ Therefore, some aspect of initiation other than simple DNA binding, such as rearrangement of the DNA substrate after binding, may be defective or incomplete in proheads reconstituted with these pRNA CCA bulge mutants.

With motor assembly on proheads reconstituted with CCA bulge mutants confirmed, the base pairs of DNA packaged per ATP hydrolyzed was determined in the *in vitro* system. No packaging was detected with the ΔCCA mutant pRNA, and hydrolysis of ATP was at background levels (proheads plus gp16) (Table 3). Mutants that had high packaging activity (CCC and ΔA) had a bp/ATP ratio similar to that of wild-type pRNA. The three mutants with packaging activity about 10–25% that of wild-type pRNA (GCA, CCG and ΔC) had a bp/ATP ratio about one-half that of wild-type pRNA (Table 3). To test for a slower packaging rate by these mutants that would yield partially-packaged DNAs, packaging was assessed using [³H]DNA-gp3 and sucrose density gradient centrifugation, where heads containing partial genome lengths are easily identified by their slower sedimentation positions. The [³H]DNA sedimentation profiles of the GCA, CCG and ΔC mutants showed only filled heads, albeit fewer than with wild-type pRNA, and no partially packaged particles, demonstrating that DNA packaging, once initiated, went to completion. Taken together, the two-fold greater ATP hydrolysis observed in these mutants may result in part from abortive attempts to initiate packaging or from very early loss of packaged DNA.

CryoEM-3D reconstruction of proheads reconstituted with ΔCCA pRNA showed an altered conformation of the pRNA A-helix spokes and a noticeable "handedness" to the pRNA ring that is absent in particles with wild-type pRNA (Figure 5). Although all of the CCA bulge mutants bound gp16 and DNA-gp3, the reconstruction of the particle with ΔCCA showed that the A-helices of the pRNA ring were oriented differently than in wild-type particles. Since gp16 binds the distal end of the pRNA spokes,^{5,6} and interacts directly with the DNA during translocation, docking onto misoriented pRNA spokes may result in altered or failed interactions with DNA during packaging. The variable effect of the different bulge mutants on packaging may reflect different degrees of change in alignment of the pRNA spokes and consequently the likelihood of gp16 contacting the DNA correctly. Alternatively, skewing of

the pRNA A-helices may result in a slight rotation of the oligomeric ring of ATPases that have been shown to bind to the A-helices.^{5,6} If packaging requires coordinated activity of the ATPase and another motor component, such as the connector, then such a rotation might cause a misalignment of the different motor components, thus interfering with synchronization of the motor. Also, the CCA bulge may provide flexibility to the pRNA structure that is required for proper motor function. Mutations in the CCA bulge might restrict motion of the pRNA needed for packaging.

Data suggesting that the position of the bulge on the stem is also important agrees with the results and interpretation described above. Specifically, the mutant pRNA A99G, predicted to form a new base pairing between C18 and the mutant G99 that effectively shifts the bulge one base closer to the prohead to yield a new bulge sequence of CAU (bases 19–21), did not support packaging.²¹ We confirmed the phenotype of the A99G mutation, as no DNA packaging was detected. However, replacing the CCA bulge sequence with the new predicted bulge sequence CAU resulted in DNA packaging activity at 79% that of wild-type levels. Therefore, the lack of packaging was not due to the bulge sequence change but rather to a shift in bulge position, where the displaced bulge would kink the A-helix differently than wild-type pRNA, thus adversely affecting the position of the gp16 and its alignment with the other components of the packaging motor.

Taken together, the data suggest a form-determining (morphopoietic) role for the pRNA in organizing the gp16 in assembly of a functional packaging motor. CryoEM reconstructions of proheads with Δ CCA/gp16 complexes and other mutant pRNA/gp16 motor combinations will help visualize how pRNA conformation dictates gp16 assembly and motor structure. NMR analysis of the A-helix containing the CCA bulge is underway to determine the structure of this region and to assess the flexibility of the pRNA. These studies will further provide a framework for interpreting the effects of CCA base substitutions and deletions and their variable effects on motor assembly and function.

Materials and Methods

Production of proheads, RNA-free proheads, gp16 and DNA-gp3

Proheads were purified from *B. subtilis* RD2 (*sup*[−]) cells infected with the mutant *sus16*(300)-*sus14*(1241) (defective for the packaging ATPase) as described,²⁷ with the following modifications: cells were harvested 75 min post-infection, concentrated 100X by centrifugation and lysed in TMS buffer (50 mM Tris-HCl (pH 7.8), 10 mM MgCl₂, 0.1 M NaCl) containing 1 mg/ml lysozyme and 20 U/ml RNase-free DNase I (Roche) and 60 U/ml RNase inhibitor. The proheads were purified by centrifugation in a 10 to 30% (w/v) linear sucrose density gradient in the SW55 rotor at 45,000 rpm for 1 h at 4°C. The prohead band was collected, and the particles were pelleted in the SW55 rotor at 35,000 rpm for 2 h at 4°C and then resuspended in TMS buffer overnight at 4°C.

To produce RNA-free proheads, purified proheads were treated with 0.4 µg/ml RNase A in the presence of 12.5 mM EDTA for 15 min at room temperature to remove pRNA, and the RNA-free proheads were isolated by centrifugation in a 5 to 20% (w/v) linear sucrose density gradient in the SW55 rotor at 35,000 rpm for 45 min at 4°C. The prohead band was collected, and the particles were pelleted in the SW55 rotor at 35,000 rpm for 2 h at 4°C and then resuspended in TMS buffer overnight at 4°C. Purified RNA-free proheads (5×10^{11} ; 83 nM) were reconstituted by incubation with either wild-type pRNA or mutant pRNA molecules (5×10^{12} ; 830 nM) in 10 µl of 0.5X TMS buffer for 10 min at room temperature prior to use.

gp16 was purified from *B. subtilis* (pSACB-gp16) as described previously^{5,27} with the following modifications: cells were harvested by centrifugation 2.5 h post-induction, resuspended in TMS buffer and lysed by two passes through a french press. The lysate was incubated for 15 min on ice and then clarified in the SS-34 rotor at 10,000 rpm for 30 min at 4°C. In the presence of Mg²⁺, gp16 associates with the pellet fraction. The pellet was resuspended in TE buffer (50 mM Tris-HCl (pH 7.8) and 10 mM EDTA) and incubated for 60 min on ice. The pellet fraction was then spun at 13,000 rpm for 30 min at 4°C. The supernatant now contained an enriched fraction of gp16, which was further purified by P11 chromatography. The supernatant fraction was incubated with P11 resin (prepared according to the manufacturer's instructions; Whatman) in 50 mM Tris (pH7.6), 100 mM NaCl buffer. The resin was then washed with 50 mM Tris with 0.2 M NaCl. gp16 was eluted in 50 mM Tris-HCl with 400 mM NaCl and stored at -70°C.

DNA-gp3 was isolated from *B. subtilis* RD2 infected with the mutant *sus* 4(369)-*sus* 8(22) that is defective for late transcription and the major shell protein as described previously²⁷ with the following modifications: infected cells were collected 90 min post-infection and lysed in TE buffer with 1 mg/ml lysozyme. DNA-gp3 was isolated by CsCl isopycnic gradient centrifugation in TE buffer in the SW55 rotor at 30,000 rpm for ~40 h at 20°C. The gradient was fractionated and DNA-gp3 identified by agarose gel electrophoresis. The DNA was dialyzed on a 0.025 µm membrane filter (Millipore) against 20 mM Tris-HCl (pH 7.6) for 45 minutes just prior to use. Alternatively, DNA-gp3 was isolated from purified phage as described.^{27,28}

Production of pRNA and pRNA mutants

Mutant pRNAs were constructed in the plasmid pRT72 encoding the wild-type ø29 pRNA gene.¹³ All proposed changes in pRNA were folded by use of the RNA folding program mfold (version 3.2)^{29,30} to confirm the predicted secondary structure. Mutants were created by either the Transformer site-directed mutagenesis kit (Clontech) or by inverse PCR based on Wang and Wilkinson.³¹ Plasmid DNA was sequenced to verify the presence of the desired mutation. Wild-type and mutant pRNAs were produced by *in vitro* transcription using T7 RNA polymerase and purified by denaturing-urea PAGE.¹³

DNA packaging assay

The *in vitro* DNA packaging assay was performed as described by Grimes and Anderson.²⁷ Briefly, reconstituted proheads (1×10^{11} ; 8.3 nM) were mixed with purified DNA-gp3 molecules (5×10^{10} ; 4.2 nM) and gp16 molecules ($1.2\text{--}1.5 \times 10^{12}$; 100–125 nM) in 0.5X TMS buffer in 20 µl and incubated for 5 min at room temperature. ATP was then added to 0.5 mM to initiate packaging and the mixture incubated for 15 min. Unpackaged DNA was digested with 1 µg/ml DNase I for 10 min. The reaction mixture was then treated with 25 mM EDTA and 500 µg/ml proteinase K for 30 min at 65°C to inactivate the DNase I and release the packaged DNA from particles. The packaged DNA was analyzed by agarose gel electrophoresis. DNA packaging efficiency was quantified by densitometry using a UVP Gel Documentation System.

For analysis of packaging by sucrose density gradient centrifugation, [³H]DNA-gp3 was used as the packaging substrate. After packaging for 10 min, γ-S-ATP was added to 250 µM to stall the motor, and then treated with 10 µg/ml DNase I for 5 min. The sample was diluted with 100 µl 0.5X TMS buffer, loaded onto a 5 to 20% linear sucrose gradient in 0.5X TMS buffer containing 1 µM γ-S-ATP and centrifuged in the SW55 rotor at 35,000 rpm for 30 min at 20°C. The gradient was fractionated and the [³H] in each fraction quantified by liquid scintillation counting.

gp16 binding assay

Proheads reconstituted with either wild-type pRNA or mutant pRNA molecules (6.25×10^{11} ; 5.2 nM) were incubated with gp16 molecules (1.9×10^{13} ; 158 nM) in 200 μ l of TM buffer (25 mM Tris-HCl (pH 7.8), 5 mM MgCl_2) at room temperature for 20 min. The sample was layered on a 5% to 20% (w/v) linear sucrose density gradient and centrifuged in the SW55 rotor at 35,000 rpm for 30 min at 4°C. 150 μ l of the prohead band was collected, concentrated in the Speedvac and the protein composition determined by NuPAGE (10% Bis-Tris Gel, Invitrogen) in MOPS-SDS buffer.

Prohead/pRNA/gp16/DNA-gp3 complex formation

Magnetic beads (2.8 μ m) coated with protein G (Dynabeads®, Invitrogen) were concentrated using a magnet, washed with PBS buffer and then incubated with purified IgG from ϕ 29 antiserum for 30 min at room temperature. The antiserum does not contain antibodies against gp16. The beads were then blocked with BSA (1.5 mg/ml) for 1h at room temperature, concentrated using a magnet and washed 2X with PBS buffer followed by 3 washes with 0.5X TMS. The beads were suspended in the original volume in 0.5X TMS buffer with 400U/ml of RNasin (Promega). 20 μ l beads were incubated with reconstituted proheads (2×10^{11} ; 1.6 nM), incubated at room temperature for 30 min, the beads collected on the magnet and the unbound proheads removed by washing in 0.5X TMS buffer. Prohead-bound beads containing $\sim 1 \times 10^{11}$ (4.2 nM) proheads were incubated with gp16 molecules (2.7×10^{12} ; 113 nM) and [^3H]DNA-gp3 (5×10^{10} ; 2.1 nM) in 40 μ l in 0.5X TMS buffer with 250 μ M γ -S-ATP for 15 min. The ratio of DNA-gp3 to proheads on beads was $\sim 1:2$. The beads were concentrated using a magnet and the supernatant containing the unbound [^3H]DNA-gp3 removed. The beads were washed 2X with 0.5X TMS buffer with 100 μ M γ -S-ATP and then resuspended in 0.5X TMS. The [^3H]DNA-gp3 in the unbound fraction, each wash and in the bead fraction was quantified by liquid scintillation counting.

ATPase assay

DNA packaging reactions containing proheads reconstituted with wild-type or mutant pRNAs were assembled as described above. At 10 min packaging, 10 μ l of sample was removed, and ATP hydrolysis during the reaction was measured by quantification of inorganic phosphate by the malachite green Lanzetta assay³² and by reference to a standard curve. The remaining reaction was processed for DNA packaging by agarose gel electrophoresis as described above. DNA packaging efficiency was quantified by densitometry using a UVP Gel Documentation System.

Packaging experiments utilized a two-fold excess of proheads over DNA, an incubation time of 10 min to allow packaging to go to completion, and quantification of DNA packaged by gel electrophoresis. The conditions employed here maximize packaging and facilitate analysis of pRNA mutants with low packaging efficiencies, although there is potentially less control of background ATPase levels which would be present in all samples. In contrast, in the recently published determination of 2bp packaged/ATP hydrolyzed,²³ proheads were made limiting and buffer conditions altered to suppress background ATPase activity from non-packaging prohead-pRNA/gp16 complexes. Also, in the previous work the packaging reaction was stalled by the addition of γ -S-ATP before termination of packaging to avoid potential futile ATP hydrolysis post-packaging, and bp packaged were measured by isolation of filled heads containing radiolabeled DNA²³. These conditions, designed to minimize background ATPase levels, result in reduced packaging efficiency since proheads are limiting. The present result of 1 bp/ATP may be due to any or all of these experimental differences.

Electron microscopy

Prohead particles reconstituted with Δ CCA pRNA were flash-frozen on holey grids in liquid ethane. Images were recorded at $50,000\times$ magnification with a JEM2200 FS FEG microscope equipped with an in-column energy filter. The electron dose for each micrograph was approximately $20\text{ e}^-/\text{\AA}^2$. All micrographs were collected on a CCD camera with a step size of $2.0\text{ \AA}$ per pixel.

Individual particle images were boxed, floated, and preprocessed to normalize mean intensities and variances and to remove linear background gradients. Structure factor phases were modified as indicated by the parameters of the contrast transfer function. Reference projections of a previously published prohead reconstruction¹⁷ were used to initially classify particles for three-dimensional reconstruction. The resulting model was used to recalculate reference projections for better particle classification. Several cycles of iterative particle classification and reconstruction were performed until convergence had been reached. Five-fold symmetry was assumed in all stages of the reconstruction procedure. The resolution of the final reconstruction, which included 1174 particles, was $26\text{ \AA}$, as determined by the Fourier Shell correlation method using a correlation of 0.5 between independent half data sets as the cut-off criterion. All steps of the reconstruction process, including determination of the contrast transfer function parameters, were performed with the program EMAN.³³

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References

1. Smith DE, Tans SJ, Smith SB, Grimes S, Anderson DL, Bustamante C. The bacteriophage ϕ 29 portal motor can package DNA against a large internal force. *Nature*. 2001; 413:748–752. [PubMed: 11607035]
2. Simpson AA, Tao Y, Leiman PG, Badasso MO, He Y, Jardine PJ, Olson NH, Morais MC, Grimes S, Anderson DL, Baker TS, Rossmann MG. Structure of the bacteriophage ϕ 29 DNA packaging motor. *Nature*. 2000; 408:745–750. [PubMed: 11130079]
3. Zhang F, Lemieux S, Wu X, St-Arnaud D, McMurray CT, Major F, Anderson D. Function of hexameric RNA in packaging of bacteriophage ϕ 29 DNA *in vitro*. *Mol. Cell*. 1998; 2:141–147. [PubMed: 9702201]
4. Guo P, Zhang C, Chen C, Garver K, Trottier M. Inter-RNA interaction of phage ϕ 29 pRNA to form a hexameric complex for viral DNA transportation. *Mol. Cell*. 1998; 2:149–155. [PubMed: 9702202]
5. Koti JS, Morais MC, Rajagopal R, Owen BAL, McMurray CT, Anderson DL. DNA packaging motor assembly intermediate of bacteriophage ϕ 29. *J. Mol. Biol.* 2008; 381:1114–1132. [PubMed: 18674782]
6. Morais MC, Koti JS, Bowman VD, Reyes-Aldrete E, Anderson DL, Rossmann MG. Defining molecular and domain boundaries in the bacteriophage ϕ 29 DNA packaging motor. *Structure*. 2008; 16:1267–1274. [PubMed: 18682228]
7. Jardine, PJ.; Anderson, D. DNA packaging in double-stranded DNA bacteriophage. chapt. 6. In: Calendar, R., editor. *The Bacteriophages*. Oxford Press; 2006. p. 49-65.
8. Guo P, Erickson S, Anderson D. A small viral RNA is required for *in vitro* packaging of bacteriophage ϕ 29 DNA. *Science*. 1987; 236:690–694. [PubMed: 3107124]
9. Wichitwechkarn J, Bailey S, Bodley JW, Anderson D. Prohead RNA of bacteriophage ϕ 29: size, stoichiometry and biological activity. *Nucleic Acids Res.* 1989; 17:3459–3468. [PubMed: 2498842]

10. Guo P, Bailey S, Bodley JW, Anderson D. Characterization of the small RNA of the bacteriophage ϕ 29 DNA packaging machine. *Nucleic Acids Res.* 1987; 15:7081–7090. [PubMed: 3116499]
11. Wichitwechkarn J, Johnson D, Anderson D. Mutant prohead RNAs in the *in vitro* packaging of bacteriophage ϕ 29 DNA-gp3. *J. Mol. Biol.* 1992; 223:991–998. [PubMed: 1538407]
12. Bailey S, Wichitwechkarn J, Johnson D, Reilly BE, Anderson DL, Bodley JW. Phylogenetic analysis and secondary structure of the *Bacillus subtilis* bacteriophage RNA required for DNA packaging. *J. Biol. Chem.* 1990; 265:22365–22370. [PubMed: 2125049]
13. Reid RJD, Bodley JW, Anderson D. Characterization of the prohead-pRNA interaction of bacteriophage ϕ 29. *J. Biol. Chem.* 1994; 269:5157–5162. [PubMed: 8106496]
14. Reid RJD, Bodley JW, Anderson D. Identification of bacteriophage ϕ 29 prohead RNA domains necessary for *in vitro* DNA-gp3 packaging. *J. Biol. Chem.* 1994; 269:9084–9089. [PubMed: 8132646]
15. Reid RJD, Zhang F, Benson S, Anderson D. Probing the structure of bacteriophage ϕ 29 prohead RNA with specific mutations. *J. Biol. Chem.* 1994; 269:18656–18661. [PubMed: 8034614]
16. Morais MC, Tao Y, Olson NH, Grimes S, Jardine PJ, Anderson DL, Baker TS, Rossmann MG. Cryo-EM image reconstruction of symmetry mismatches in bacteriophage ϕ 29. *J. Struct. Biol.* 2001; 135:38–46. [PubMed: 11562164]
17. Morais MC, Choi KH, Koti JS, Chipman PR, Anderson DL, Rossmann MG. Conservation of the capsid structure in tailed dsDNA bacteriophages: the pseudoatomic structure of phi29. *Mol. Cell.* 2005; 18:149–159. [PubMed: 15837419]
18. Zhang CL, Lee C-S, Guo P. The proximate 5' and 3' ends of the 120-base viral RNA (pRNA) are crucial for the packaging of bacteriophage phi29 DNA. *Virology.* 1994; 201:77–85. [PubMed: 8178491]
19. Zhang CL, Tellinghuisen T, Guo P. Confirmation of the helical structure of the 5'/3' termini of the essential DNA packaging pRNA of phage phi29. *RNA.* 1995; 1:1041–1050. [PubMed: 8595559]
20. Lee T, Guo P. Interaction of gp16 with pRNA and DNA for genome packaging by the motor of bacterial virus phi29. *J. Mol. Biol.* 2006; 356:589–599. [PubMed: 16376938]
21. Zhang CL, Tellinghuisen T, Guo P. Use of circular permutation to assess six bulges and four loops of DNA-packaging pRNA of bacteriophage phi29. *RNA.* 1997; 3:315–322. [PubMed: 9056768]
22. Grimes S, Jardine PJ, Anderson D. Bacteriophage ϕ 29 DNA packaging. *Adv. Virus Res.* 2002; 58:255–294. [PubMed: 12205781]
23. Chemla YR, Aathavan K, Michaelis J, Grimes S, Jardine PJ, Anderson DL, Bustamante C. Mechanism of force generation of a viral DNA packaging motor. *Cell.* 2005; 122:683–692. [PubMed: 16143101]
24. Guo P, Peterson C, Anderson D. Prohead and DNA-gp3-dependent ATPase activity of the DNA packaging protein gp16 of bacteriophage ϕ 29. *J. Mol. Biol.* 1987; 197:229–236. [PubMed: 2960820]
25. Bjornsti MA, Reilly BE, Anderson DL. Morphogenesis of bacteriophage ϕ 29 of *Bacillus subtilis*: oriented and quantized *in vitro* packaging of DNA-gp3. *J. Virol.* 1983; 45:383–396. [PubMed: 6185695]
26. Turnquist S, Simon M, Egelman E, Anderson D. Supercoiled DNA wraps around the bacteriophage ϕ 29 head-tail connector. *Proc. Natl. Acad. Sci. U.S.A.* 1992; 89:10479–10483. [PubMed: 1438237]
27. Grimes S, Anderson D. The bacteriophage phi29 packaging proteins supercoil the DNA ends. *J. Mol. Biol.* 1997; 266:901–914. [PubMed: 9086269]
28. Grimes S, Anderson D. *In vitro* packaging of bacteriophage ϕ 29 DNA restriction fragments and the role of the terminal protein gp3. *J. Mol. Biol.* 1989; 209:91–100. [PubMed: 2530357]
29. Zuker M. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res.* 2003; 31:3406–3415. [PubMed: 12824337]
30. Mathews DH, Sabina J, Zuker M, Turner DH. Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. *J. Mol. Biol.* 1999; 288:911–940. [PubMed: 10329189]
31. Wang J, Wilkinson MF. Site-directed mutagenesis of large (13-kb) plasmids in a single-PCR procedure. *Biotechniques.* 2000; 29:976–978. [PubMed: 11084857]

32. Lanzetta PA, Alvarez LJ, Reinach PS, Candia OA. An improved assay for nanomole amounts of inorganic phosphate. *Anal. Biochem.* 1979; 100:95–97. [PubMed: 161695]
33. Ludtke SJ, Baldwin PR, Chiu W. EMAN: semiautomated software for high-resolution single-particle reconstructions. *J. Struct. Biol.* 1999; 128:82–97. [PubMed: 10600563]

Figure 1.

120 base form of pRNA. The secondary structure was determined by phylogenetic analysis using pRNAs from $\phi 29$ relatives.¹² Conserved bases are shown in bold. The bases involved in the intermolecular pseudoknot that forms the pRNA multimer are boxed. Bases 1–28 and 92–117 form the A-helix. The CCA bulge is circled in red; the adenine residue is conserved among the $\phi 29$ relatives.

Figure 2.

In vitro DNA packaging by proheads containing pRNA with CCA base substitution or deletion mutations. Packaged DNA is protected from DNase digestion, and DNA extracted from the particles is shown following agarose gel electrophoresis. (a) Base substitutions. The lanes contain: 1) input DNA added to the packaging reaction; 2–9) packaging with proheads reconstituted with various pRNAs, including 2) wild-type 120b, 3–5) substitutions of A20, 6–7) substitutions of C18, 8) double substitution, and 9) triple substitution; 10) no ATP control. (b) Deletions. The lanes contain: 1) input DNA; 2–7) packaging with proheads reconstituted with various pRNAs, including 2) wild-type 120b, 3–4) single deletion of A or

C, respectively, 5–6) double deletion of CA or CC, respectively, and 7) triple deletion; 8) no ATP control. The lanes have been rearranged for clarity in presentation.

Figure 3.

gp16 binding to proheads containing pRNA with mutant CCA bulges. Prohead-gp16 complexes were isolated by sucrose density gradient centrifugation and the protein content determined by SDS-PAGE. The lanes contain: 1) gp16 control; 2–9) prohead/gp16 complexes from proheads reconstituted with various pRNAs, including 2) 71b, 3) wild-type 120b, 4) ΔCCA, 5) ΔC, 6) ΔA, 7) GCA, 8) CCC and 9) CCG; 10) prohead control.

Figure 4.

Lack of partially packaged DNAs by proheads reconstituted with CCA bulge mutants. Packaging of [^3H]DNA-gp3 was initiated with ATP and then stalled at 10 min with $\gamma\text{-S-ATP}$ (or at 1 min for the wild-type packaging intermediate control). The reactions were treated with DNase to digest the unpackaged DNA, and the particles containing DNA fractionated by sucrose density gradient centrifugation. Packaging with proheads reconstituted with various pRNAs included wild-type 120b (black), wild-type stalled at 1 min (red), ΔCCA (gray), GCA (green), CCG (blue) and ΔC (orange). Sedimentation is from right to left. Filled heads sediment at fraction 4, and unpackaged DNA is found at the top of the gradient. Packaging stalled at 1 min generated heads with partially packaged genomes, and this peak centered on fraction 9. The total dpm in the gradient for each mutant profile has been normalized to the wild-type. The packaging efficiency of proheads with GCA , CCG and ΔC pRNAs compared to wild-type was 10%, 19% and 22%, respectively.

Figure 5.

CryoEM-3D reconstruction of proheads containing wild-type versus Δ CCA pRNA. (a) wild-type 120b pRNA, low pass filtered at 26 Å and (b) Δ CCA pRNA at 26 Å resolution, respectively. (c) and (d) superposition of density due to pRNA for wild-type (teal) and Δ CCA (purple) from side-view (c) and end-on view (d).

Table 1

pRNA CCA bulge mutants and DNA packaging

	Sequence	% DNA packaged* mean & SD, n=3
	CCA (wt)	100
Base Substitutions	<u>CCC</u>	92 ± 7
	<u>CCU</u>	101 ± 7
	<u>CCG</u>	44 ± 3
	<u>GCA</u>	20 ± 4
	<u>UCA</u>	84 ± 6
	<u>CAG</u>	49 ± 5
	<u>AAG</u>	62 ± 8
Deletions	CC - (ΔA)	101 ± 6
	- CA or C - A (ΔC)	32 ± 6
	- C - or C - - (ΔCA)	nd**
	- - A (ΔCC)	nd
	- - - (ΔCCA)	nd

* % packaging is calculated relative to wild-type pRNA

** not detected

Table 2**[³H]DNA-gp3 binding to gp16/pRNA/prohead/bead complexes**

pRNA	% [³ H]DNA-gp3 bound mean & SD, n=3
wt CCA (no γ-S ATP)	4.6 ± 1.4
wt CCA	16.5 ± 1.9
ΔCCA	11.9 ± 4.5
ΔC	23.8 ± 3.1
ΔA	18.3 ± 2.2
<u>G</u> CA	16.6 ± 1.8
CCC <u>U</u>	22.1 ± 0.7
CC <u>G</u>	20.3 ± 1.2
Prohead/wt pRNA + DNA *	1.3 ± 0.2
gp16 + DNA	1.3 ± 0.4

* Prohead/mutant pRNA + DNA mixtures gave comparable values.

Table 3

ATPase activity during DNA packaging

RNA	bp packaged $\times 10^{14}$ mean & SD, n=3	ATP hydrolyzed $\times 10^{14}$ mean & SD, n=3	bp/ATP
wt	2.57 $\pm$ 0.46	2.96 $\pm$ 0.30	0.87
Δ CCA	nd*	0.51 $\pm$ 0.03	--
Δ C	0.58 $\pm$ 0.17	1.42 $\pm$ 0.16	0.41
Δ A	2.44 $\pm$ 0.59	2.54 $\pm$ 0.40	0.96
<u>G</u> CA	0.26 $\pm$ 0.11	0.61 $\pm$ 0.11	0.43
CC <u>C</u>	2.15 $\pm$ 0.34	2.72 $\pm$ 0.11	0.79
CC <u>G</u>	0.58 $\pm$ 0.19	1.32 $\pm$ 0.20	0.44
Prohead + gp16	na**	0.31 $\pm$ 0.21	--

*
not detected**
not applicable: no DNA added