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Alanine Scanning and Fe-BABE Probing of the Bacteriophage ø29 Prohead RNA-Connector Interaction

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Abstract

The DNA packaging motor of the *Bacillus subtilis* bacteriophage ø29 prohead is comprised in part of an oligomeric ring of 174-base RNA molecules (pRNA) positioned near the N-termini of subunits of the dodecameric head-tail connector. Deletion and alanine substitution mutants in the connector protein (gp10) N-terminus were assembled into proheads in *Escherichia coli* and the particles tested for pRNA binding and DNA-gp3 packaging *in vitro*. The basic amino acid residues RKR at positions 3-5 of the gp10 N-terminus were central to pRNA binding during assembly of an active DNA packaging motor. Conjugation of Fe-BABE to residue S170C in the narrow end of the connector, near the N-terminus, permitted hydroxyl radical probing of bound [³²P]pRNA and identified two discrete sites proximal to this residue: the C-helix at the junction of the A, C and D helices, and the E helix and the CE loop/D loop of the intermolecular base pairing site.

Keywords

bacteriophage ø29; DNA packaging; RNA binding; packaging motor assembly; directed hydroxyl radical probing

INTRODUCTION

The packaging of the *B. subtilis* bacteriophage ø29 DNA-terminal protein complex (DNA-gp3) into the precursor capsid (prohead) is driven by a molecular motor that generates high force.

¹ This motor, situated at the unique vertex at the base of the prohead, is comprised of three components: the dodecameric head-tail connector (gene product 10, gp10) with a central channel through which the DNA is translocated during packaging, an oligomeric ring of the 174-base ø29-encoded prohead RNA (pRNA), and multiple copies of the packaging ATPase gp16 (reviewed in 2 and 3). In packaging motor assembly, the pRNA ring binds to the narrow end of the connector, and the ATPase gp16 binds to the pRNA. Fitting the crystal structure of the connector into the cryoEM-3D reconstruction of the prohead shows that residues near the N-termini of the gp10 subunits are in close proximity to the electron density of the ring of pRNA molecules.⁴

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The site of pRNA binding has been localized to the N-terminus of the gp10 connector protein. Removal of 14 residues from the N-termini of gp10 subunits by V8 protease reduces binding of the anti-lacZ mRNA⁵ or pRNA⁶ to free connectors. Xiao *et al.*⁶ have shown that V8 protease-treated connectors in proheads do not bind pRNA nor package DNA. Here we used site-directed mutagenesis of the cloned ϕ 29 connector gene, whose product was efficiently assembled into proheads in *E. coli*, to identify the residues in the N-terminus that are involved in pRNA binding. An electrophoretic mobility shift assay (EMSA) was used to assess the particle-pRNA interaction, and particles were tested for *in vitro* DNA packaging activity. The basic residues R3, K4 and R5 (Figures 1 and 2) at the N-termini of the ϕ 29 connector protein subunits constitute a pRNA binding sequence that resembles an arginine-rich motif (ARM).

Constraints for modeling the ϕ 29 pRNA structure^{7,8,9} were generated by prediction of the secondary structure by phylogenetic analysis,¹⁰ ribonuclease footprinting of the pRNA-prohead complex,¹¹ mutation of residues involved in intermolecular base pairing,^{7,12,13} covalent crosslinking studies,^{14,15,16} solution probing,^{16,17,18} and cryoEM-3D construction of the connector-pRNA complex.^{4,19,20} These studies place the pRNA E helix; the C/E, D and E loops; and the intersection of the A, C and D helices in contact with the prohead (Figure 1(a)). Further, the studies demonstrate that the pRNA intermolecular interaction involves base pairing between the CE and D loops. Finally, the studies direct the A helix obliquely away from the prohead (Figure 1(c)). Here we used site-directed hydroxyl radical probing with iron (S)-1-(p-bromoacetamidobenzyl) ethylenediaminetetraacetate (Fe-BABE) to extend these constraints to the orientation of pRNA structural elements relative to the twelve-fold symmetric connector. Conjugation of Fe-BABE to residue S170C of the narrow end of the connector was used to map this residue proximal to the intersection of the pRNA A, C and D helices and the CE loop/D loop dimerization site, providing new constraints. In addition, conjugation of Fe-BABE to the connector gp10 residues R3 and R5 inhibited the binding of pRNA, adding further evidence for the involvement of the gp10 N-termini in pRNA binding.

RESULTS

Connector N-terminal amino acid sequence alignment and pRNA binding

When the crystal structure of the ϕ 29 head-tail connector is fitted into a cryoEM-3D reconstruction of the prohead, the N-terminal domain of the connector subunits (minus the first ten residues that are missing in the crystal structure) is in close proximity to the pRNA density (Figure 1(c)).^{4,19} This implicates the connector N-terminus as the site for pRNA binding and rationalizes prior reports that V8 protease treatment of the isolated ϕ 29 connector^{5,6} or prohead⁶ negates RNA binding.

To help identify the specific amino acids of the connector N-terminus that bind pRNA, N-terminal sequences from ϕ 29 and relatives^{21,22} that encode pRNAs¹⁰ were aligned (Figure 2). The closely related phages ϕ 29, PZA, and B103 have nearly identical sequences that include the R3•K4•R5 sequence. The more distantly related phage GA-1 that encodes a distinct pRNA has extensive connector protein N-terminal sequence homology to ϕ 29. However, negatively charged residues E3 and D4 that replace the RKR sequence present in ϕ 29 and additional downstream arginine residues imply a different mode of pRNA interaction. Connector proteins of the more distantly related “ ϕ 29-like” viruses, including Av-1, Cp-1, C1, phiP68, and P1 (International Committee on Taxonomy of Viruses Database), none of which has been shown biochemically or genetically to encode a pRNA, lack any discernable connector N-terminal sequence homology with ϕ 29 (data not shown).

pRNA binding to proheads with gp10 N-terminal mutations

To determine the role of specific connector protein N-terminal amino acids in pRNA binding, mutant connectors were assembled into proheads in *E. coli* from products of the cloned prohead genes (Table 1), and the particles were assessed for pRNA binding in electrophoretic mobility shift assays (EMSA) (Figure 3). Proheads with connectors lacking residues A2 through Y9 were unable to bind pRNA (data not shown). Next, proheads with connectors lacking residues A2 through R5 and particles containing connectors with either triple, double, or single alanine substitutions at residues R3, K4 and R5 were assayed in the EMSA (Figure 3). Proheads having connectors with the A2-R5 deletion, the triple substitution, or the three combinations of double substitutions were unable to bind the pRNA, which migrated to the position of the free pRNA (Figure 3, lanes 3 through 7). Proheads having connectors with single alanine substitutions (mutants R3A, K4A, or R5A) retained the pRNA at the particle position near the top of the gel, as did the wild-type particles (lanes 8 through 10; lane 13). Thus, proheads having any two residues of the RKR sequence retained the pRNA during electrophoresis. [The slight variations in particle mobility were due to the variable number of head fibers present on the different mutant particles.] Proheads having connectors with the downstream quadruple substitution S6A•N7A•T8A•Y9A bound pRNA as well as the wild-type proheads (lanes 11 and 13), and thus the RKR sequence appears to be the major determinant of pRNA binding.

DNA packaging by proheads with connector mutations

Proheads produced in *E. coli* and constituted with pRNA efficiently package the genomic DNA-terminal protein complex (DNA-gp3) in the *in vitro* DNA packaging system.²³ DNA-gp3 packaging is monitored by a DNase I protection assay that digests unpackaged DNA. pRNA is essential for DNA packaging, and connector mutations that reduce or negate pRNA binding will affect DNA packaging.

Proheads with connector mutations were assayed for DNA packaging activity (Figure 4). Proheads with connectors lacking residues A2 through R5 or having the triple alanine substitution R3A•K4A•R5A that did not bind pRNA in the EMSA (Figure 3) resulted in barely detectable or no DNA packaging (Figure 4, lanes 4 and 5). In contrast, proheads having connectors with single alanine substitutions (mutants R3A, K4A or R5A) and particles with the quadruple alanine substitution for the four downstream residues (mutant S6A•N7A•T8A•Y9A) that bound pRNA (Figure 3) packaged DNA (Figure 4, lanes 9 through 12). Surprisingly, however, particles with the three combinations of double alanine substitutions (mutants R3A•K4A, K4A•R5A, or R3A•R5A), all unable to bind pRNA in EMSA (Figure 3), packaged DNA-gp3 (Figure 4, lanes 6-8). Taken together, the results demonstrated that EMSA disrupted the interaction of pRNA with mutant connectors having only one residue of the RKR sequence, but this single basic residue at the N terminus of each connector subunit was sufficient for functional pRNA binding in the *in vitro* DNA packaging assay.

Assembly of proheads with cysteine substitutions in the connector

Site-directed hydroxyl radical probing with Fe-BABE was performed to probe the contact of the connector N-terminus with pRNA. This method utilizes cysteine residues as sites for conjugation. Two native, connector protein (gp10) cysteines (C76 and C265) were mutated to serines to first create proheads with cysteine-free connectors (cys-). These particles were found to have full biological activity. The other prohead proteins (scaffolding, major capsid, and head fiber) all lack cysteine. New cysteines were then introduced into specified sites for conjugation (Table 1). Eight cysteine substitutions were introduced, seven in the N-terminus (M1::C::A2 [an insertion between M1 and A2], A2C, R3C, K4C, R5C, S6C, and N7C) and one in the narrow end of the connector near the N-terminus (S170C). Sucrose gradient-purified mutant particles containing substitutions A2C, K4C, S6C, or N7C exhibited less than the full

complement of connector protein following SDS-PAGE (Figure 5, lanes 3, 5, 7 and 8, respectively), suggesting that these connectors were not stably incorporated into all particles; these particles were not studied further. Particles containing the substitutions M1: :C: :A2, R3C, R5C, or S170C had protein compositions identical to that of particles with cysteine-free connectors (Figure 5, compare lanes 2, 4 and 6 with lane 1).

Cysteine reactivity and Fe-BABE conjugation efficiency

Proheads containing the cysteine-substituted connectors M1: :C: :A2, R3C, R5C, or S170C were conjugated with Fe-BABE, and the occupation of reactive cysteine residues was assessed by a comparison of the ability to bind Cy3-maleimide before and after conjugation. The binding of Cy3-maleimide produced a fluorescent connector protein band in SDS-PAGE, and the connectors with the mutations R3C, R5C, and S170C showed a strong reactivity with Cy3 before Fe-BABE conjugation (Figure 6, lanes 5, 7 and 9, respectively). However, Cy3 labeling of the M1: :C: :A2 mutation was weak, suggesting that this cysteine residue was relatively unreactive (Figure 6, lane 3). After Fe-BABE conjugation, the mutants R3C, R5C, and S170C showed a marked reduction in the fluorescence of the connector band, indicating that most of the cysteine residues were blocked with Fe-BABE (lanes 6, 8 and 10, respectively). The fluorescent bands, other than gp10, seen before and after Fe-BABE are attributed to the labeling of minor contaminating host proteins.

Mapping the Fe-BABE-conjugated connector residue S170C onto the pRNA

The ability of the proheads conjugated with Fe-BABE to bind [³²P]pRNA was tested with filter binding assays that mimicked the site-directed hydroxyl radical probing reactions. Conjugation of Fe-BABE to the R3C or R5C connector mutants effectively removed the ability of these connectors to bind pRNA (Table 2). By comparison, binding was reduced to ~10 percent for the S170C connector mutant, while ~80 percent of the pRNA was bound by mock-conjugated particles containing cysteine-free connectors.

Proheads containing Fe-BABE conjugated to the R3C or R5C connector residues and incubated with [³²P]pRNA yielded cleavage profiles identical to control mock-conjugated particles containing cysteine-free connectors (Figure 7, lanes 1-3), reflecting the inability of these particles to bind pRNA. However, proheads containing Fe-BABE conjugated to the residue S170C showed cleavage mapped to two discrete groups of nucleotides. One of these includes a portion of the C helix (Figure 7, lane 4: nts 30-32 and 69-71) as well as nucleotides at the junction of the A, C and D helices (nts 28, 29 and 72). The second group includes a portion of the E helix (nts 49, 50 and 62) and the CE loop/D loop intermolecular base pairing site (nts 46-48 and 81-84). The results place these two discrete pRNA elements in proximity to the repeating twelve Fe-BABE-conjugated S170C residues around the perimeter of the connector.

DISCUSSION

The prohead RNA (pRNA) is an essential component of the ϕ 29 DNA packaging motor,²⁴ and the pRNAs of the ϕ 29 relatives PZA, M2, SF5 and GA-1¹⁰ are likely to be components of their packaging motors. In ϕ 29, the pRNA has been visualized in cryoEM-3D reconstructions to encircle the narrow end of the head-tail connector (Figure 1) and as a bridge between the connector and the packaging ATPase gp16.^{4,19} The predicted secondary structures of ϕ 29 pRNA and the pRNAs of its relatives are very similar, even though the sequence homology among these pRNAs is low.¹⁰ All of the pRNAs are encoded at the left ends of their genomes, and all of the genes are preceded by a strong promoter sequence.²⁵ For B103,²⁶ the pRNA is readily recognized by its position in the genome and by its promoter sequence.

The present results clearly demonstrated that the R3•K4•R5 sequence at the ϕ 29 connector protein N-terminus is essential for pRNA binding (Figures 2 and 3). Particles with the double alanine substitutions R3A•K4A, K4A•R5A or R3A•R5A and particles having Fe-BABE conjugated to R3 or R5 did not show pRNA binding in the EMSA (Figure 3) and the double filter binding assay (Table 2), respectively, while the triple substitution R3A•K4A•R5A negated stable pRNA binding that led to DNA packaging (Figure 4). Since pRNA is an essential component of the DNA packaging motor, the discrepancy in the pRNA binding and the DNA packaging results likely reflects the different assays used. In the EMSA and the filter binding assays, once pRNA dissociates from a particle containing a mutant connector, it is physically separated from the particle. In contrast, in the packaging assay, dissociated pRNA can re-bind the particle, and its interaction with the prohead may be modeled and strengthened by the binding of the packaging ATPase gp16 and by the engagement of the motor with DNA in packaging.

The arginine and lysine residues essential for ϕ 29 pRNA binding are frequently found in interactions with the RNA phosphate backbone in resolved protein-RNA structures.^{27,28} However, in the connector protein N-terminus of phage GA-1, a phage of *Bacillus* but not *B. subtilis*, the acidic residues E3 and D4 replace the K3 and R4 residues in gp10 of ϕ 29, while having substantial N-terminal sequence homology to gp10 over 27 residues (Figure 2). Aspartate and glutamate residues are seldom involved in RNA binding; however, these residues can interact with RNA bases, as in the U1A spliceosome.²⁹ Differences in RNA binding structures have been discovered in closely related proteins that bind related target RNAs. For example, HIV Tat is thought to use an extended conformation to bind the HIV TAR RNA,³⁰ while the BIV Tat uses a β -hairpin to bind the BIV TAR RNA.^{31,32} When the ϕ 29 connector N-terminus (residues A2-T8) was replaced with the GA-1 N-terminus (residues L2-S7; Figure 2), the chimeric connector could no longer bind ϕ 29 pRNA nor package ϕ 29 DNA (data not shown), confirming that ϕ 29 and GA-1 use different modes of pRNA binding. The phages Cp-1, P1, Av-1, phiP68, and C1 (“ ϕ 29-like” phages) have not been shown to encode a pRNA. Accordingly, there is great variation in their connector N-terminal sequences, all of which are distinct from ϕ 29 (data not shown).

The present site-directed hydroxyl radical probing study yielded several new pRNA modeling constraints, placing two discrete groups of nucleotides in proximity to the S170 residues at the narrow end of the connector. These hydroxyl radical cleavage sites fall within or next to nucleotides previously identified as being protected by the prohead from ribonuclease digestion¹¹ (Figure 7) and chemical solution probing.¹⁷ On the pRNA secondary structure map, the separation between the two discrete cleavage groups (first, the C-helix and the junction of the A, C and D helices, and second, the E helix and the CE loop/D loop intermolecular base pairing site; Figures 1 and 7) suggests that each pRNA molecule in the oligomeric ring may span two S170C residues of adjacent subunits of the connector dodecamer. Modeling of the pRNA oligomer using constraints generated by mutagenesis of intermolecular base pairing sites^{7,12,13} predicts that a separation between the two groups is needed for the pRNAs to encircle the connector. Equally spaced S170 residues seemingly align with cleavage sites in the multimeric pRNA ring. Coaxial stacking of helices to form a single group of cleavage sites is contrary to the distribution of density of pRNA in cryo-EM 3D reconstructions.¹⁹ Unfortunately, the inability to effect cleavage by particles with Fe-BABE conjugated to either of the mutants R3C or R5C prevented triangulation of the cleaved nucleotides with other connector residues and thereby precluded the estimation of distances between the probe and these nucleotides using gel band intensity-to-distance correlations.^{33,34}

Our method for site-directed hydroxyl radical probing could not separate the Fe-BABE-conjugated particles bound with pRNA from the Fe-BABE-conjugated particles without pRNA. These latter particles with probes but without pRNA might possibly encounter and

cleave free, unbound pRNA present in the reaction. However, extraneous cleavage was not observed. In reaction mixtures of [^{32}P]pRNA with either R3C or R5C Fe-BABE-conjugated particles that were unable to bind pRNA (Table 2), cleavage of the pRNA was identical to mock-conjugated control particles (Figure 7, lanes 1-3) and therefore constituted background. This demonstrated that Fe-BABE conjugated to the S170C residue had cleaved the bound pRNA (lane 4).

The structure of the Fe-BABE-conjugated connector is presently viewed as having the gp10 N-termini protruding from the crystal surface of the connector and the Fe-BABE probes on residue S170C projecting from recesses between the N-termini into the area occupied by the pRNA. Molecular modeling suggests the N-terminal residues, disordered in the crystal structure, extend 10-15 Å from the current surface of the connector crystal structure (Shuhua Ma, unpublished data). Here, the N-termini may behave like the extensions of ribosomal proteins that make direct contact with the 16S rRNA but are not visible in protein structures in the absence of RNA.³⁶ Filter binding assays find the conjugated probes interfered with pRNA binding (Table 2). Particles containing either cysteine-free, mock-conjugated cysteine-free, or S170C connectors bound [^{32}P]pRNA equally well, while particles containing Fe-BABE conjugated to residue S170C bound [^{32}P]pRNA approximately one-eighth as well (data not shown). Recesses between the N-termini are envisioned as confines for the Fe-BABE probes and the observation that the pRNA cleavage is narrowly restricted to two discrete groups of nucleotides reinforces the notion that the probes are confined. Expansion of pRNA cleavage to other, additional nucleotides is presumably blocked by the binding of pRNA to the residues R3, K4, and R5 of gp10.

The symmetry of the pRNA bound to the prohead (six-fold versus five-fold) is controversial. Genetic studies determined a six-fold structure,^{7,12} and recent single-fluorophore quantification of labeled pRNA oligomers inferred the number of pRNAs to be six.³⁷ Cryo-EM 3D reconstructions found both a five-fold^{4,19,20} or six-fold structure.³⁸ A pentameric pRNA ring fits well to the circumference of the connector crystal structure that has disordered N-termini.⁴ But molecular modeling of the N-termini (Figure 1B) suggests that these residues increase the diameter of the connector, possibly making a fit for a hexameric pRNA⁷ (unpublished data). The observed cleavage of pRNA at two discrete sites by Fe-BABE conjugated to connector residue S170C is consistent with the symmetry match of a six-fold pRNA bound to a twelve-fold connector, while a more diverse cleavage pattern would be anticipated from the mismatch symmetry of a five-fold pRNA bound to the dodecameric connector.

An immediate goal is to expand the use of site-directed hydroxyl radical probing by conjugating Fe-BABE to new connector sites to produce a more detailed picture of the orientation of the pRNA on the connector. The method is also applicable to study of the pRNA-gp16 ATPase complex. In addition, a new attempt to model the pRNA oligomer by the use of all available constraints, including those presented here, is warranted. The ultimate goal is to produce an atomic resolution structure of the connector-pRNA-gp16 complex that constitutes the powerful ϕ 29 DNA packaging motor.

MATERIALS AND METHODS

Connector sequence alignment

Head-tail connector sequences for ϕ 29 relatives and ϕ 29-like phages were identified by BLAST,³⁹ using the ϕ 29 connector protein sequence, and aligned with T-coffee.⁴⁰ Proteins used in the alignment were the ϕ 29 upper collar gp10 (GenBank accession no. **P04332**), PZA upper collar (GenBank accession no. **NP 040728**), B103 upper collar gene 10 (GenBank accession no. **NP 690644**), GA-1 head-tail connector gene 10 (GenBank accession no.

CAC21531), *Staphylococcus* phage phiP68 upper collar orf19 (GenBank accession no. NP 817335), *Streptococcus* phage C1 head-tail connector orf15 (GenBank accession no. NP 852021), *Streptococcus* phage Cp-1 connector orf10 (GenBank accession no. CAA87731), *Mycoplasma* virus P1 P38.4 protein (GenBank accession no. NP 064644), and *Actinomyces* phage Av-1 upper collar orf 9 (Allan Delisle, personal communication). The N-terminal connector protein sequence of phiP68 orf 19 was extended by 77 amino acids to an upstream methionine to reflect the homology found in the N-terminus of orf 15 of its closest relative phage C1. *Mycoplasma* virus P1 protein 38.4 was identified as a connector protein by the use of PSIPRED⁴¹ to predict secondary structures for all open reading frames and align conserved residues (P183, K234, E242, and G273 in the ø29 gp10 sequence) found in the ø29 relatives and the ø29-like phages.

Protein expression and purification of proheads

The ø29 prohead scaffold, major capsid, head fiber and connector genes were co-expressed in *E. coli* (pAR7-8-8.5-10)²³ after induction with 0.5 mM IPTG. After two hr, cells were concentrated by centrifugation and incubated in TES buffer (20 mM Tris-HCl [pH 8.0], 2 mM EDTA and 100 mM NaCl) containing 10 mg/ml lysozyme for 20 min at 37°C. Cells were lysed by the addition of 0.2% sodium deoxycholate, 3 mM MgCl₂ and 1.5 µg/ml DNase I (final concentrations) and the lysate incubated for an additional 20 min. Particles were isolated by centrifugation in 10 to 40% (w/v) sucrose density gradients in TMS buffer (50 mM Tris-HCl [pH 7.8], 10 mM MgCl₂, and 100 mM NaCl) containing 2 mM dithiothreitol (DTT) in the Beckman SW28 rotor at 15K rpm for 16.5 hr. The particles were pelleted in the Beckman Ti-50.2 rotor at 35K rpm for 5 hr., resuspended in TMS buffer containing 2 mM DTT, further purified in a 0.42% (w/v) CsCl isopycnic density gradient, and concentrated in a Centricon YM-30 (Amicon) in TMS buffer containing 2 mM DTT.

Production of connector protein N-terminus mutants

N-terminal deletion mutants of the ø29 connector protein (gp10) were made in *E. coli* (pAR7-8-8.5-10), which assembles proheads,²³ by inverse PCR, following the protocol of Wang and Wilkinson.⁴² Alanine substitution mutations were created by first introducing unique NcoI and AgeI sites into the 5' end of the gene 10 of ø29 in *E. coli* (pAR7-8-8.5-10) by site-directed mutagenesis.⁴³ 8 pmol of complimentary oligonucleotides (Sigma-Genosys) coding for the desired alanine substitutions and flanked by overhanging NcoI and AgeI sequences were combined in 20 µl of annealing buffer (10 mM Tris-HCl [pH 8.0] and 10 mM MgCl₂), heated to 95°C for 2 min., and cooled to 25°C over 140 min. The modified pAR7-8-8.5-10 plasmid was digested with NcoI and AgeI and the annealed oligonucleotides ligated into the plasmid. The genes encoding gp7, gp8 and the mutagenized gp10 were sequenced in each resulting plasmid. For the Fe-EDTA footprinting studies, the two native cysteines C76 and C265 in the connector were mutated to serine by site-directed mutagenesis in the above plasmid. Single cysteine substitutions were introduced by ligation of annealed oligonucleotides (Sigma-Genosys) into the NcoI and AgeI sites of gene 10 of the plasmid. The S170C mutant was generated by site-directed mutagenesis. Plasmids were transformed into *E. coli* HMS174(DE3) and mutants selected on plates containing 100 µg/ml ampicillin. The mutants are given in Table 1.

DNA-gp3 packaging

ø29 pRNA-free proheads (5×10^{11}) produced in *E. coli* (pAR7-8-8.5-10) were incubated with 120nt pRNA (5×10^{12} molecules) in a final volume of 10 µl in 0.5x TMS for 10 min at ambient temperature. Packaging in the defined *in vitro* system was performed as described by Grimes and Anderson.⁴⁴ Briefly, DNA-gp3 (5×10^{10} molecules), particles reconstituted with pRNA (1×10^{11}), the packaging ATPase gp16 (1.2×10^{12} molecules) and 500 µM ATP were mixed in

a final volume of 20 μ l in 0.5x TMS buffer to initiate packaging. After 10 minutes, unpackaged DNA-gp3 was digested with DNase I (1 μ g/ml) during incubation for 10 min. The packaged, protected DNA-gp3 was extracted from the particles by the use of 25 mM EDTA and 500 μ g/ml Proteinase K for 30 min at 65°C. Packaging was determined following agarose gel electrophoresis.

Electrophoretic mobility shift assay

120nt pRNA was dephosphorylated with Shrimp Alkaline Phosphatase and 5'-end-labeled with γ [³²P]-ATP by the use of OptiKinase™ (USB Corporation). Excess label was removed with a MicroSpin™ G-50 column (Amersham Biosciences). Proheads having connectors with a gp10 N-terminal deletion or alanine substitution (9×10^{12}) and 5'-end-labeled pRNA (1.8×10^{13} molecules) were incubated in a 20 μ l volume of TMS buffer containing 2 mM DTT for 20 min at ambient temperature. pRNA likely forms multimers in solution, and therefore particles were present in an excess of about 3-fold. Glycerol was added to each reaction to 5% and EMSA run in a 2% GQA agarose (ISC BioExpress) gel in TB buffer (0.89M Tris-HCl [pH8.3] and 0.89 boric acid) containing 2mM DTT and 5% glycerol for 4 hr at 4°C. Bromophenol blue was added to a separate lane to monitor migration of pRNA. Wet gels were exposed to X-ray film for autoradiography.

Fe-BABE conjugation

Proheads assembled with connectors having cysteine-free or cysteine-substituted gp10 (1.8×10^{13}) were exchanged into TMS buffer lacking DTT by the use of a MicroSpin™ G-50 column (Amersham Biosciences), with the flow-through mixing directly with 10 nmol of iron (S)-1-(p-bromoacetamidobenzyl) ethylenediaminetetraacetate (Fe-BABE, Pierce) to yield a final volume of 100 μ l, and the mixture was incubated at 37°C for 60 minutes. To assay cysteine reactivity, a like number of proheads with connectors having cysteine-free or cysteine-substituted gp10 were exchanged into TMS lacking DTT, mixed directly with 1.5 nmol of Cy3-maleimide (Amersham BioScience), and incubated at 37°C for 60 minutes. To assess the efficiency of Fe-BABE conjugation, an aliquot of particles reacted with Fe-BABE (1.2×10^{12}) were immediately incubated with 600 pmol of Cy3-maleimide in a final volume of 40 μ l at 37°C for 60 minutes. Unreacted Fe-BABE was removed from reactions by the use of Centricon YM-30 (Amicon) filtration, and the proheads were washed and concentrated in TMS buffer containing 2 mM DTT. Proteins of proheads containing Cy3-labeled gp10 were resolved by SDS-PAGE, and the fluorescence in gp10 was quantified with a fluorometer (Fuji FLA-5100).

Double filter pRNA binding assay

The binding of [³²P]pRNA to proheads complexed with Fe-BABE was measured by a double filter binding assay.⁴⁵ 5'-end-labeled [³²P]pRNA was purified in a 6% acrylamide/8M urea gel, eluted from excised gel pieces in TEN buffer (10 mM Tris-HCl [pH 8.0], 1 mM Na₂EDTA, 100 mM NaCl), and precipitated with 150 mM sodium acetate [pH 5.2], 5 mM MgCl₂, 32 μ g/ml glycogen, and 50% isopropanol. Pellets were washed with 70% ethanol and dissolved in water. Proheads with connectors having gp10 complexed to Fe-BABE (9×10^{11}) were incubated with 5'-end-labeled [³²P]pRNA (1.8×10^{12} molecules) in TMS containing 2 mM DTT in a final volume of 20 μ l for 30 minutes on ice. Complexes were diluted with 180 μ l of TMS containing DTT and immediately applied to stacked Protran® BA85 (Whatman) and Hybond™-N+ (Amersham Biosciences) filters under vacuum. Filters were washed three times under vacuum with 3 ml of TMS containing DTT, air dried, and the radiolabel quantified by scintillation counting.

Fe-BABE mapping

Site-directed hydroxyl radical probing followed methods used in ribosomal study.³⁵ Proheads having connectors with gp10 complexed with Fe-BABE (4.5×10^{11}) were incubated with gel-purified, 5' end-labeled [^{32}P]pRNA (9×10^{11} molecules) in TMS containing 2 mM DTT, in a final volume of 10 μl , on ice for 30 minutes. Particles were not repurified as it was not possible to separate pRNA-containing from pRNA-free proheads. Cleavage was initiated by adding 1 μl of 60 mM ascorbate and 1 μl of 0.3% H_2O_2 , and the mixture was incubated on ice for 10 minutes. Reactions were terminated by the addition of 2 μl of 150 mM Na2EDTA/25mM thiourea. pRNA fragments were resolved by electrophoresis on a 10% acrylamide/8M urea sequencing gel buffered in TBE, and the cleavage pattern of pRNA was determined by exposing the wet gel to X-ray film or by the use of phosphor-imaging (Bio-Rad Molecular Imager® FX).

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Figure 1.

(a) ø29 pRNA secondary structure determined by mfold with stem/loop letter designations¹⁰ (blue). Residues involved in intermolecular base pairing are boxed. (b) N-terminal residues (multi-colors), disordered in the connector crystal structure, modeled onto the connector structure (gray). Residues R3, K4, and R5 are implicated in the binding of pRNA. Residue S170 conjugated with Fe-BABE was used in the mapping of the connector-pRNA interaction. (c) Cryo-EM density fitted with the crystal structure of the dodecameric connector¹⁹ (yellow) displays the spatial relationship of the N-termini of the connector, density interpreted as a pentameric pRNA (green) forming a basket around the connector, and density interpreted as capsid (gray) appearing to contact the pRNA. The circular inset shows the close proximity of the N-termini of connector subunits (beginning with residue 11 and denoted by orange stars) and residues S170 (marked by blue dots) to the density of the pRNA. Ribbon model of the pRNA (magenta) is superimposed onto spokes of density interpreted as the pRNA A helix.⁴

Figure 2.

Connector protein N-terminus sequence alignments of ϕ 29 and relatives by T-coffee. Magenta dots indicate residues that are disordered in the N-termini of subunits of the ϕ 29 connector crystal structure, and the helix denotes amino acids resolved in helix 1 of the ϕ 29 connector.

⁴ Identical residues are indicated by a star and semi-conserved residues by a colon.

Figure 3.

EMSA analysis of pRNA binding to proheads containing mutations of the ϕ 29 connector protein N-terminus. [32 P] 5' end-labeled 120nt pRNA was incubated with proheads containing wild-type, deletion, and alanine-substituted mutations of the connector N-terminus, and the complexes were detected by agarose gel electrophoresis, followed by autoradiography. [32 P] pRNA bound to particles is observed near the top of the gel, whereas free [32 P] pRNA is found near the bottom of the gel. The first letter of mutant designations indicates the wild-type amino acid residue that was mutated to alanine, and the number denotes its position in the connector protein N-terminal amino acid sequence. Lanes 1 and 12 show the position of free pRNA in the absence of particles.

Figure 4.

In vitro DNA packaging by ϕ 29 proheads containing wild-type, deletion, or alanine-substituted mutations in the connector protein N-terminus. The first letter indicates the wild-type amino acid residue that was mutated to alanine, and the number denotes its position in the amino acid sequence. Lanes 1 and 2 show 50% and 100% of the input DNA added to the reaction, respectively, for quantification of DNA packaging. DNA in lanes 3-12 represent packaged DNA that is protected from DNaseI treatment (see Materials and Methods). Negative controls in which ATP is omitted from the packaging reaction were done for each particle type. One such control is shown in lane 13.

Figure 5. SDS-PAGE showing the amount of gp10 assembled into cys- proheads versus proheads with an N-terminal cysteine insertion or with N-terminal cysteine substitutions. The other prohead constituents are major head protein (gp8), head-fiber protein (gp8.5) and scaffolding protein (gp7).

Figure 6.

SDS-PAGE of proheads showing Cy3-maleimide labeling of particles containing gp10 without the native cysteines, particles having gp10 with a cysteine insertion, or particles having gp10 with substitutions before (-) and after (+) conjugation of the connectors with Fe-BABE.

Figure 7.

Hydroxyl radical cleavage pattern of [^{32}P]120nt pRNA bound to proheads with Fe-BABE-conjugated connectors having the R3C, R5C or S170C mutations, compared to cleavage by mock-conjugated cys- proheads. Arrows point to the cleavage locations in the pRNA secondary structure, and residues shown in magenta are the specific sites of cleavage. Nucleotides protected by the prohead from ribonuclease digestion¹¹ are enclosed in the gray boxes. Bases 1 through 25 showed no distinct cleavage patterns (data not shown).

Table 1
Mutants of the ø29 connector protein assembled into proheads

Mutant	Amino acid sequence									
wild-type (aa 1-10)	M	A	R	K	R	S	N	T	Y	R...
ΔA2-R5					M	S	N	T	Y	R...
R3A•K4A•R5A	M	A	A	A	A	S	N	T	Y	R...
R3A•K4A	M	A	A	A	R	S	N	T	Y	R...
K4A•R5A	M	A	R	A	A	S	N	T	Y	R...
R3A•R5A	M	A	A	K	A	S	N	T	Y	R...
R3A	M	A	A	K	R	S	N	T	Y	R...
K4A	M	A	R	A	R	S	N	T	Y	R...
R5A	M	A	R	K	A	S	N	T	Y	R...
S6A•N7A•T8A•Y9A	M	A	R	K	R	A	A	A	A	R...
M1::C::A2	M	C	A	R	K	R	S	N	T	Y
A2C	M	C	R	K	R	S	N	T	Y	R...
R3C	M	A	C	K	R	S	N	T	Y	R...
K4C	M	A	R	C	R	S	N	T	Y	R...
R5C	M	A	R	K	C	S	N	T	Y	R...
S6C	M	A	R	K	R	C	N	T	Y	R...
N7A	M	A	R	K	R	S	C	T	Y	R...
wild-type (aa 167-174)		...	N	Q	L	S	L	K	Q	V...
S170C		...	N	Q	L	C	L	K	Q	V...

Table 2

Percent of [^{32}P] 120nt pRNA radiolabel retained on protein-binding membranes by proheads containing Fe-BABE conjugated connectors in double filter binding assays

Conjugation site		Experiment 1	Experiment 2
mock-conjugated, cysteine-free	batch 1	79.4%	80.7%
	batch 2	78.5%	76.0%
R3C•Fe-BABE	batch 1	3.2%	4.1%
	batch 2	0.7%	0.7%
R5C•Fe-BABE	batch 1	1.5%	2.3%
	batch 2	0.5%	0.5%
S170C•Fe-BABE	batch 1	14.1%	15.1%
	batch 2	8.8%	9.9%
no protein control	batch 1	0.1%	0.1%
	batch 2	0.3%	0.3%