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Modular assembly of chimeric phi29 packaging RNAs that support DNA packaging

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ABSTRACT

The bacteriophage phi29 DNA packaging motor is a protein/RNA complex that can produce strong force to condense the linear-double-stranded DNA genome into a pre-formed protein capsid. The RNA component, called the packaging RNA (pRNA), utilizes magnesium-dependent inter-molecular base-pairing interactions to form ring-shaped complexes. The pRNA is a class of non-coding RNA, interacting with phi29 motor proteins to enable DNA packaging. Here, we report a two-piece chimeric pRNA construct that is fully competent in interacting with partner pRNA to form ring-shaped complexes, in packaging DNA via the motor, and in assembling infectious phi29 virions *in vitro*. This is the first example of a fully functional pRNA assembled using two non-covalently interacting fragments. The results support the notion of modular pRNA architecture in the phi29 packaging motor.

During maturation of bacteriophage phi29, a ring-shaped protein/RNA complex acts as a motor to package the linear DNA genome inside a protein capsid (procapsid) [1–3]. The phi29 packaging motor is the strongest known biomolecular motor, capable of generating forces that are 2–8 times stronger than other motors such as myosin and RNA polymerase [4]. It is believed that phi29 shares the same DNA packaging mechanism as many other double-stranded DNA viruses, and several mechanistic models have been proposed for phi29 motor function [5–10].

The RNA component in the phi29 motor, called the packaging RNA (pRNA), is a unique class of non-coding RNA and is essential for motor function [11]. Understanding the structure/function relationship of pRNA is critical in dissecting the mechanism of packaging motor function, yet knowledge on pRNA structure and dynamics is limited [1–3]. In addition, pRNA has been utilized to construct artificial nano-structures and novel drug delivery vehicles [12], and information on pRNA is critical in these developments.

A functional pRNA monomer is ~120 nucleotides (nt) long and folds into two domains [2] (Fig. 1A). The DNA translocation domain is essential for DNA packaging yet dispensable for procapsid binding [13,14]. The procapsid binding domain is responsible for interacting with the procapsid [13], and has been reported to interact with ATP [15]. The procapsid binding domain contains two loops, called the R loop and the L loop, which are complementary to each other (Fig. 1). In the presence of Mg^{2+}, pRNA forms a ring-shaped oligomeric complex using multiple sets of inter-molecular R/L loop base-pairing interactions [16,17]. The R and L loop sequences can be varied, while motor function is preserved as long as inter-molecular R/L loop pairings are maintained [16,17]. The simplest pRNA ring-shaped complex is a closed dimer, which simultaneously contains two sets of R/L loop interactions [18]. The pRNA closed dimer can form in the absence of proteins [18–20], and has been used to obtain information on pRNA structure and function [2].

It was recently reported that the pRNA procapsid binding domain can be dissected into a two-piece chimeric construct, in which two individual RNA oligonucleotides, one encompassing the R loop and the other the L loop, are clamped together via a 6-nt duplex (Fig. 1B) [19]. This chimeric procapsid binding domain maintains a near wild-type ability to fold into the proper conformation to enable pRNA/pRNA interaction in the absence of proteins [19]. The results have led to the proposal that the procapsid binding domain contains two autonomous modules, which fold independently to form the active conformation for pRNA/pRNA interaction [19]. However, the chimeric procapsid binding domain has not been tested in DNA packaging, and it is not known whether

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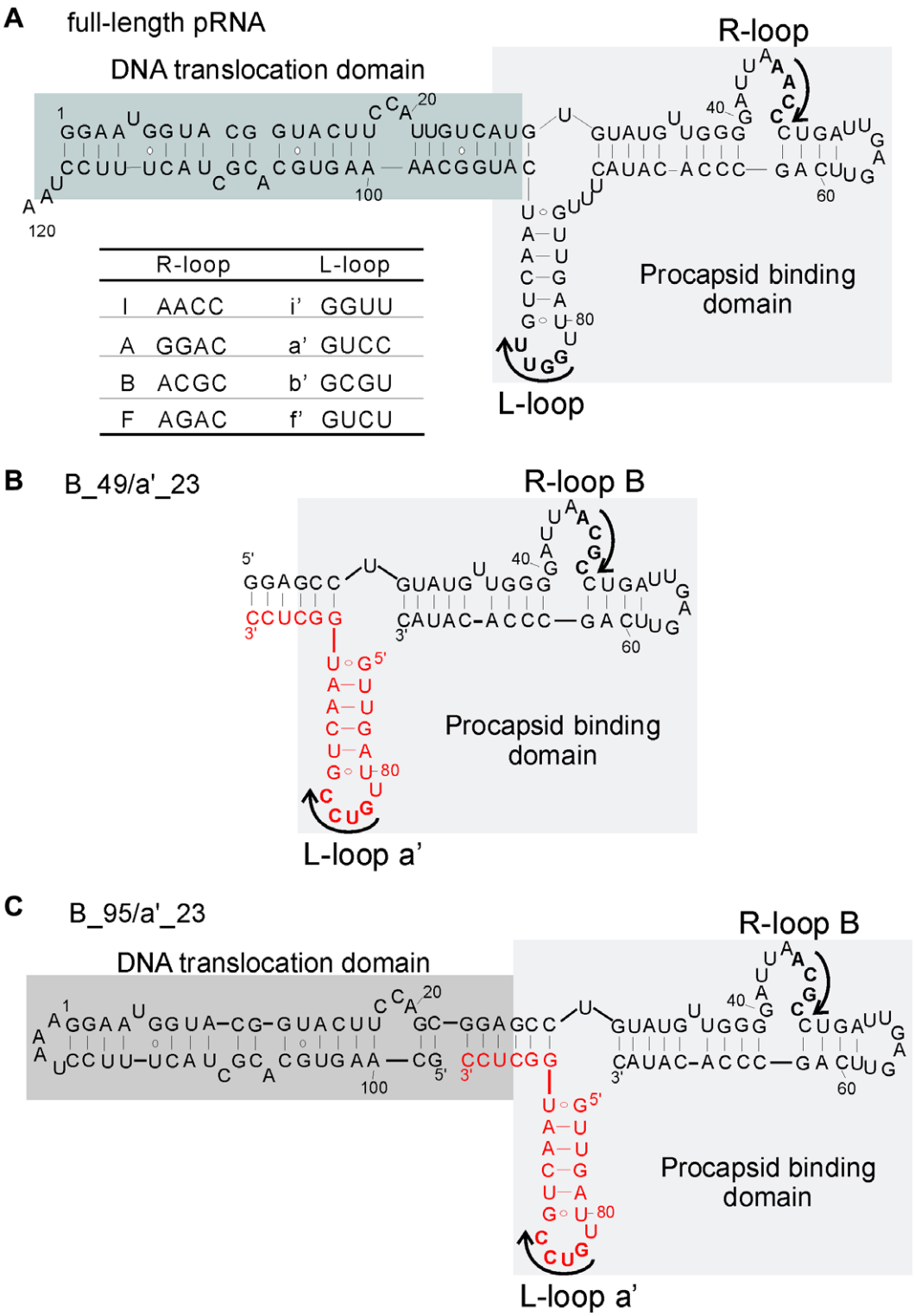

Fig. 1. Sequences and secondary structures of pRNA constructs. (A) The 120-nt intact pRNA, with the inset showing sequences and nomenclatures of the R and L loops used in this study. This 120-nt construct has very similar *in vitro* DNA packaging activity as compared to the 174-nt pRNA transcript produced in the phi29-infected cells. It is designated as the wild-type pRNA following previous literature [1,2]. (B) The chimeric procapsid binding domain [19]. (C) The chimeric construct containing both the procapsid binding domain and the DNA translocation domain.

the modular architecture model is applicable in the packaging motor, where both pRNA/pRNA and pRNA/protein interactions are present. Attempts were made previously to construct a chimeric full-length pRNA by simple extension of the 6-nt clamp to include the DNA translocation domain, but such a strategy failed due to RNA mis-folding arising from long stretches of single-stranded clamping sequences [19].

Here, we report successful design of new chimeric pRNA constructs that contain both the procapsid binding domain and the DNA translocation domain, and show that these constructs fully

support packaging motor function. In addition, we showed that the chimeric pRNAs can function with a variety of R loop and L loop sequences, thus maintaining a key feature of the wild-type pRNA. Overall, the data indicate that the modular architecture of pRNA is maintained in pRNA/protein interactions and in the packaging motor.

Materials and methods

Nomenclatures. The pRNA molecules were identified by the length and the R and/or L loop sequences. A particular R loop sequence is assigned an upper case letter (i.e., A, B, ...), and a particular L loop sequence is assigned a lower case letter with a prime (i.e., a′, b′, ...). The same set of letters (i.e., A and a′) designates complementary sequences, while different letters indicate non-complementary sequences. The loop sequences and their corresponding letter designations are listed in Fig. 1A inset. Specifically, the 120-nt intact full-length pRNA is designated as Rl′_120 (i.e., Ab′_120, Bf′_120, ...), the 95-nt fragment containing the R loop module and the DNA translocation domain is designated as R_95 (i.e., B_95, A_95, ...), and the 23-nt fragment containing the L loop module is designated as l′_23 (i.e., a′_23, i′_23, ...) (Fig. 1).

RNA synthesis. The 120-nt pRNAs with various R and L loop sequences (Fig. 1A) were synthesized by *in vitro* transcription following previously reported protocols [19].

The R_95 molecules (Fig. 1) were synthesized using template directed *in vitro* transcription [21]. The single-stranded DNA template has a sequence of 5′-GTATGTGGGCTGAACTCAATCAG**GCGT**TAATCCCCAACATACAGGCTCCGCTGGAAGTACCGTACCATTCCTTTAGGAAAGTAGCGTGCACTTGCtatagtgagtcgtattag, with the sequence corresponding to the R loop of the pRNA (in this case the sequence for B_95) shown in bold and underlined, and the lower case letters representing the T7 promoter segment. Transcription requires a 19-nt single-stranded DNA oligonucleotide (TS), which has a sequence of 5′-ctaatacgactcactatag (complementary to the T7 promoter). DNA templates and the TS oligonucleotide were synthesized using solid-phase synthesis (Integrated DNA Technologies, Coralville, IA), and were purified via denaturing gel electrophoresis. Transcriptions were carried out in a mixture containing 1.0 μM TS, 0.1 μM DNA template, 0.75 mM of each NTP, 0.375 U/μl of T7 RNA polymerase, 40 mM Tris–HCl (pH 7.5), 15 mM $MgCl_2$, 5 mM DTT, 2 mM spermidine, and 0.01% (v/v) Triton X-100. To initiate transcription, the DNA template and the TS oligonucleotide were mixed in water, heated at 95 °C for 1 min, and cooled at room temperature for 2 min. Salt, NTP, and T7 polymerase were then added, and the mixture was incubated at 37 °C for 3 h. Products were recovered by ethanol precipitation, and purified using denaturing gels. The RNA was quantitated according to absorbance at 260 nm, using an extinction coefficient of 10,000 $M^{-1}\,cm^{-1}$ per nucleotide.

The l′_23 molecules (Fig. 1B and C) and the B_49 molecule (Fig. 1B) were synthesized by solid-phase chemical synthesis (Integrated DNA Technologies, Coralville, IA). The oligonucleotides were purified using denaturing gels, and quantitated based on absorbance at 260 nm using extinction coefficients provided by the vendor.

Native gel detection of pRNA closed dimer formation. Native gel analysis of pRNA closed dimer formation was carried out following prior reported procedures [19]. Briefly, each RNA was pre-treated by heating at 95 °C for 1 min then cooling at room temperature for 2 min. If a two-piece pRNA construct was involved, the individual components were mixed during the 95 °C heating and treated as one RNA in subsequent steps. Following the pre-treatment, the RNAs were mixed together in the TBM buffer (89 mM Tris–HCl (pH 7.6), 0.2 M boric acid, and 5 mM $MgCl_2$), and incubated at 17 °C for 1 h. The pRNA monomer and dimer were then separated according to their mobilities on a 10% native polyacrylamide gel, which was prepared in the TBM buffer and run at 17 °C. The RNA bands were visualized by either ethidium bromide staining or autoradiography.

Virion assembly activity assay. The packaging activity of pRNAs was assayed using the *in vitro* phi29 assembly system reported previously [22]. Briefly, 1 μl of pRNA was mixed with 10 μl of purified procapsid (0.3 mg/ml), dialyzed using a 0.025 μm pore size membrane filter (type VS; Millipore Corp.) for 15 min against the TBE buffer (89 mM Tris–borate 2.5 mM EDTA, pH 8.3). The buffer was then switched to TMS (50 mM Tris, 100 mM NaCl, 10 mM $MgCl_2$, pH 8.0) and dialysis continued for 30 min. The pRNA procapsid solution was then mixed with 6 μl gp16 (10 μg/ml), 1 μl DNA-gp3 (0.2 mg/ml), and 3 μl reaction buffer (10 mM ATP, 50 mM Tris–HCl, 10 mM $MgCl_2$, 100 mM NaCl, pH 8.0) to complete the DNA packaging reaction (total reaction volume 21 μl). After 30-min incubation at ambient temperature, excess amount of neck, tail, and morphogenic proteins were added to the DNA packaging reaction to complete infectious virion assembly. The assembled virions were plated with *Bacillus subtilis* $su^{+44}(sup^{+})$ to determine their plaque forming units (pfu).

Results

The B_49/a′_23 chimeric construct inhibits DNA packaging

A chimeric construct, B_49/a′_23 (Fig. 1B), has been shown to fold properly and maintain the capability to interact with its pRNA partners in the absence of motor proteins [19]. The B_49/a′_23 construct mimics the pRNA procapsid binding domain but lacks the DNA translocation domain, and is inactive in DNA packaging (Table 1, row 1). Therefore, the ability of the B_49/a′_23 construct to fold properly to interact with the motor was tested indirectly by its inhibition effects on *in vitro* virion assembly. The virion assembly assay [22] has been established as an effective tool to examine pRNA function in the packaging motor [2,16]. Infectious virion formation depends directly on viral DNA packaging, which requires active pRNAs [2,16,18,22]. Omitting the pRNA gives zero virion, while the presence of inactive pRNAs eliminates or severely diminishes virion formation [2,16,18,22].

In this experiment, infectious virion formation was assayed using a pair of full-length pRNAs, Ab′_120 and Ba′_120, which fully mimic functional pRNAs in the virus [16]. In the absence of B_49/a′_23, 7.0×10^7 pfu/ml of infectious virion was formed, representing a normal level of packaging motor activity (Table 1, row 2). In the presence of 250 ng of B_49/a′_23, only 2.6×10^4 pfu/ml of virion was formed, representing a 0.037% activity (Table 1, row 2). This indicates that B_49/a′_23 inhibits DNA packaging and infectious virion formation.

The inhibition data indicate that B_49/a′_23 is capable of occupying the position of the corresponding full-length pRNA (i.e., Ba′_120), acting as a competitive inhibitor of DNA packaging and infectious virion assembly. Similar results were reported for a one-piece 75-nt construct (Ba′_75) that encompasses the procapsid binding domain but lacks the DNA translocation domain [18]. The B_49/a′_23 chimeric construct mimics Ba′_75 in both sequence and secondary structure, and shows a similar inhibition of DNA packaging and infectious virion assembly. This suggests that the two-piece B_49/a′_23 construct is active in interacting with motor proteins. The data also showed that at a lower B_49/a′_23 concentration (25 ng), inhibition was not observed (Table 1, row 3), indicating that the chimeric construct may have a lower affinity to the motor than that of an intact Ba′_120.

Table 1
Inhibition of infectious virion formation by B_49/a′_23

Ab′_120 (ng)	Ba′_120 (ng)	B_49/a′_23 (ng)	Virion assembled (pfu/ml)	% Activity
50	0	50	0	–
50	25	0	7.0×10^7	100
50	25	25	7.0×10^7	100
50	25	250	2.6×10^4	0.037

Assembly of a two-piece chimeric construct that contains the DNA translocation domain

Building on the B_49/a′_23 inhibition studies, we explored various designs of chimeric constructs that retain the DNA translocation domain and therefore may support DNA packaging. After testing a large number of sequences using the "mfold" program [23], a 95-nt RNA was found to maintain the secondary structures of the respective DNA translocation domain and the R loop domain (Fig. 1C). This RNA, called B_95 (with B indicating the R loop sequence), has the DNA translocation domain linked to the R loop domain via a 6-nt single-stranded region. The a′_23 molecule could clamp at this 6-nt linker to complete the procapsid binding domain (Fig. 1C).

The closed dimer formation assay was used to test the ability of the B_95 molecule to interact with a′_23 to assemble a properly folded pRNA (Fig. 2). To form a pRNA closed dimer requires simultaneous inter-molecular pairing between two sets of R and L loops [19]. This requires the proper folding of the procapsid binding domain of each pRNA monomer, including the two-piece chimeric construct (B_95/a′_23). It also necessitates that the DNA translocation domain, which accounts for ~50% of B_95 (45 out of 95 nucleotides), does not interfere with the assembly and proper folding of the procapsid binding domain.

On native gels, B_95 interacted with a′_23 to form a single band that migrates with the same mobility as that of an intact 120-nt pRNA (Fig. 2B). In the presence of Mg^{2+}, the B_95/a′_23 chimeric pRNA interacted with the Ab′_120 pRNA to form a single band that

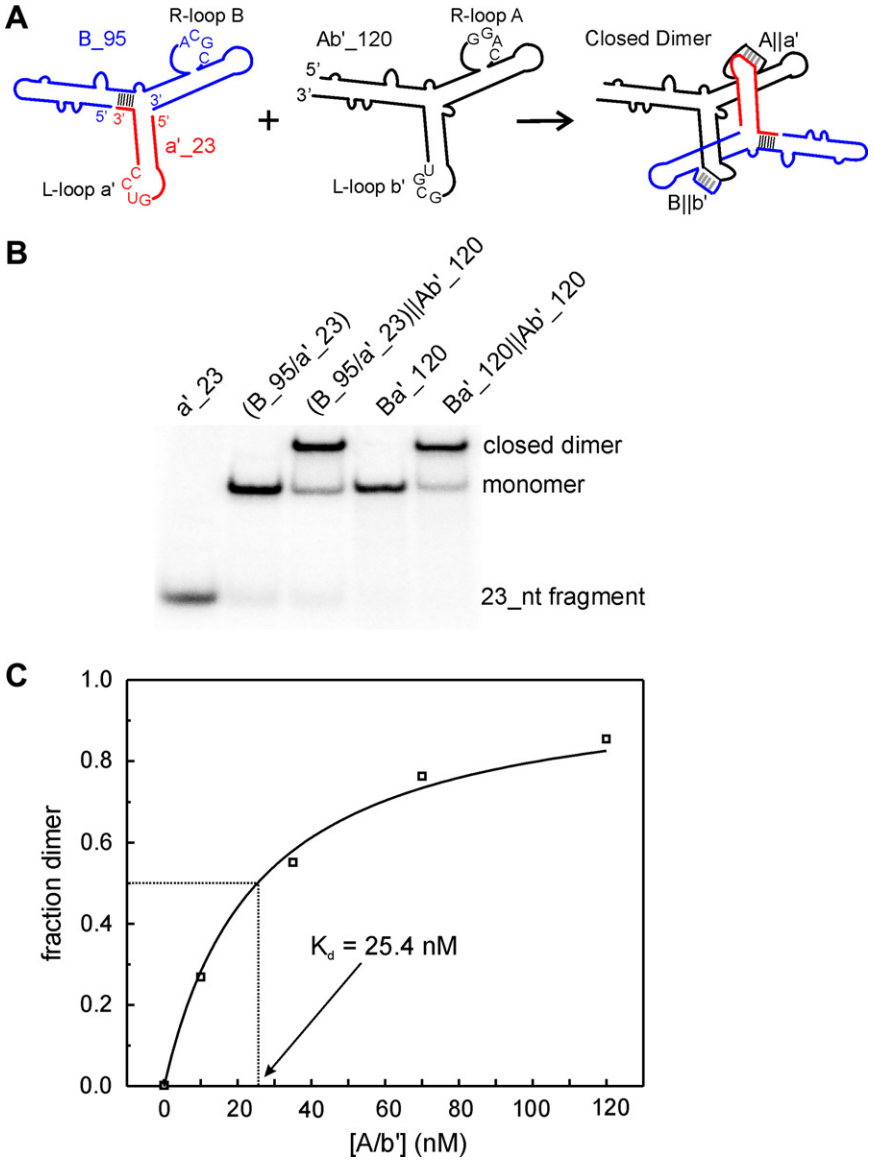

Fig. 2. *In vitro* pRNA/pRNA interaction activity of B_95/a′_23. (A) A schematic of closed dimer formation between B_95/a′_23 and Ab′_120. (B) A native gel showing the pRNA monomer and closed dimer formed using B_95/a′_23. pRNA species were visualized with a 5′ ^{32}P labeled a′_23 oligonucleotide. All non-radioactive species were at 100 nM. (C) Quantitation of K_d between (B_95/a′_23) and Ab′_120. K_d = 25.4 nM in this data set.

migrates with the same mobility as that of a closed dimer formed by the intact Ba′_120 and Ab′_120 molecules (Fig. 2B), and no interaction between B_95/a′_23 and Ba′_120 was detected (Supplemental data). These results demonstrated that B_95/a′_23 supports pRNA closed dimer formation. Furthermore, the average dissociation constant (K_d) of a closed dimer between B_95/a′_23 and Ab′_120 was measured to be 23.4 ± 2.8 nM (Table 2 and Fig. 2C). This is very close to that between the intact Ba′_120 and Ab′_120 molecules (21.7 ± 8.2 nM) [19]. The K_d measurement is a quantitative indicator of proper folding of the procapsid binding domain [19]. The similarity between these measured K_d values strongly indicates that the B_95/a′_23 chimeric construct possesses a properly folded procapsid binding domain, thus maintaining its ability for pRNA/pRNA interactions.

The two-piece B_95/a′_23 construct is active in DNA packaging

The *in vitro* virion assembly assay was used to assess the activity of the B_95/a′_23 construct in DNA packaging. When 50 ng of the B_95/a′_23 RNA was presented together with the Ab′_120 partner, $(2.95 \pm 0.64) \times 10^7$ pfu/ml of virions were assembled, a result very similar to that obtained using an intact Ba′_120 pRNA ($(3.01 \pm 1.00) \times 10^7$ pfu/ml, Fig. 3A). This indicates that the B_95/a′_23 construct maintains a native-like folding of both the procapsid binding domain and the DNA translocation domain, and properly interfaces with motor proteins to assemble a fully functional DNA packaging motor.

When lower concentrations of B_95/a′_23 were tested, DNA packaging was observed, although the activity is reduced as compared to that of intact Ba′_120 (Fig. 3A). The chimeric construct may be inherently more flexible than that of the intact pRNA, which may affect its ability to function in DNA packaging.

Functional chimeric constructs with variable R loop and L loop

A unique feature of the pRNA is that the R and L loops co-vary [16,17]. To further assess the similarity between the chimeric construct and the intact 120_nt pRNA, loop variability in the chimeric construct was studied. First, DNA packaging and infectious virion formation was tested for a B_95/i′_23 variant, where the B_95 RNA is re-constituted with a different L loop module, i′_23 (Fig. 3B). The B_95/i′_23 construct, together with its proper partner Ib′_120, produced similar amount of infectious virions as compared to the intact pRNA Ii′_120 ($(1.50 \pm 0.71) \times 10^8$ pfu/ml vs. $(2.05 \pm 0.07) \times 10^8$ pfu/ml, Fig. 3B), again indicating that the chimeric construct is active in DNA packaging. B_95 or i′_23 alone showed no packaging activity (Fig. 3B). Individually either the Ib′_120 pRNA or the B_95/i′_23 pRNA showed greatly reduced virion production ($(3.75 \pm 0.35) \times 10^4$ pfu/ml and $(8.38 \pm 1.12) \times 10^6$ pfu/ml, respectively, Fig. 3B), indicating that neither pRNA alone is sufficient to support DNA packaging. This is consistent with the known property of pRNA, that its function requires inter-molecular pairing between the R and L loops [16,17].

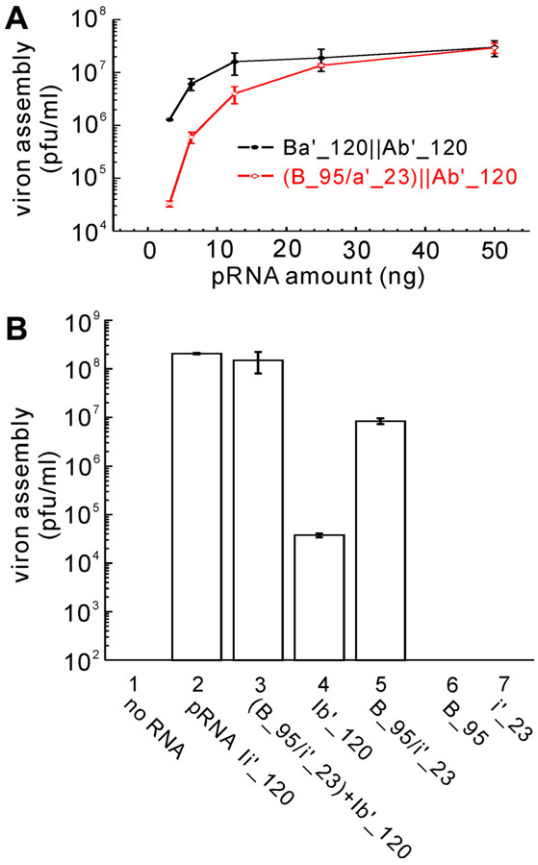

Fig. 3. Infectious virion formation with chimeric pRNAs. Shown are average values from replicates measured within the given experiments, with error bars representing the standard deviations. (A) Virion assembly with B_95/a′_23. (B) Virion assembly with B_95/i′_23. The amount of each pRNA was 50 ng in each measurement. Controls shown in columns 1, 6, and 7 gave measured values of zero. pRNA Ii′_120 (column 2) has self-complementary R and L loops and is able to function by itself. Individual Ib′_120 and B_95/i′_23 RNAs adopt cryptic R/L pairing of 2- and 3-bp, respectively, giving rise to background activities of 0.018% and 4.1%, respectively.

In addition, the closed dimer formation assay was used to test R_95/l′_23 constructs with three sets of loop sequences (B||b′, A||a′, and F||f′, Fig. 1A inset) that minimally perturb the pRNA secondary structure (Fig. 4 and Table 2). Besides the B_95/a′_23 construct, closed dimer bands can be observed for A_95/b′_23, A_95/

Table 2
Closed dimer formation with chimeric pRNAs of variable loop sequences[a]

Chimeric pRNA	Partner pRNA[b]	Average K_d (nM)[c]	$\Delta\Delta G^0$ (kcal/mol)[d]
Ba′_120 (intact pRNA control)	Ab′_120	21.7 ± 8.2[e]	–
B_95/a′_23	Ab′_120	23.4 ± 2.8	0.0
F_95/b′_23	Bf′_120	21.8 ± 0.1	0.0
F_95/a′_23	Af′_120	76.0 ± 7.6	0.7

[a] Loop sequences shown in Fig. 1A inset.
[b] Intact 120-nt pRNA.
[c] K_d measured at 17 °C and 5 mM $MgCl_2$. Errors were standard deviations between multiple measurements.
[d] $\Delta\Delta G^0 = RT\ln\frac{K_d^{mutant}}{K_d^{wild\text{-}type}}$, with R = 0.001987 kcal/mol and T = 290 K.
[e] From [19].

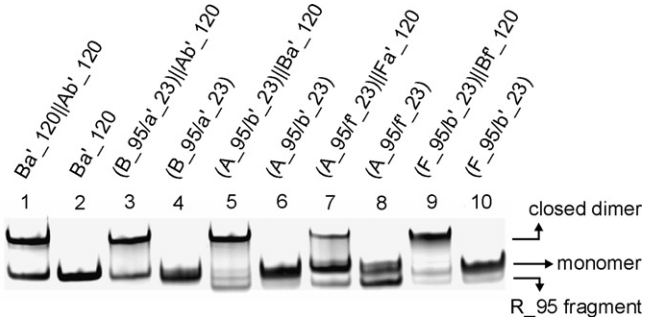

Fig. 4. A native gel showing closed dimer formation with chimeric constructs containing variable R/L loops. In each lane pRNA identities are indicated above, and the concentration of each pRNA monomer was 1.5 μM. The pRNA species were visualized using ethidium bromide staining.

f′_23, and F_95/b′_23 (Fig. 4, lanes 5, 7, and 9). In quantitative measurements of selected R_95/l′_23 constructs, the K_d values were all similar to that of the intact pRNA, with only a small reduction in the standard state free energy of binding observed in F_95/a′_23 (Table 2). This demonstrates that co-varied R/L loops can be accommodated in the R_95/l′_23 construct to maintain pRNA/pRNA interaction.

On the other hand, the R and/or L loop sequences did influence the assembly of the chimeric pRNA. For example, multiple species were observed when the monomeric A_95/f′_23 is assembled (Fig. 4, lane 8). The A/f′ sequences may base-pair with each other (5′GGAC3′/3′ucug5′, with a G/u wobble pair), which may compete with the function of the 6-nt clamp (Fig. 1). This led to mis-folding of the monomeric A_95/f′_23, and interfered with dimer formation.

All in all, the data showed that R_95/l′_23 constructs retain the ability to accommodate variable R and L loop sequences, which is a key feature of pRNA.

Discussion

Data presented here demonstrated a new class of chimeric two-piece pRNAs, R_95/l′_23. These constructs maintain the characteristic co-varying R/L loop feature, are competent in interacting with partner pRNAs, and support DNA packaging and infectious virion formation. Unlike previous chimeric constructs that are hampered by RNA mis-folding [19], the R_95/l′_23 design successfully maintains the architectures of the individual pRNA modules (i.e., the DNA translocation domain, the R loop domain, and the L loop domain). This strategy has been generalized to design additional functional chimeric pRNAs (data not shown).

Previous studies of a chimeric procapsid binding domain (i.e., B_49/a′_23), which were carried out in the absence of proteins, suggested a modular assembly model of the pRNA [19]. It was proposed that the pRNA conformation can be described as three rigid (helical) sub-modules spanning around a hinge, and relative movements between these sub-modules enable DNA packaging [19]. Data reported here established that the modular architecture of pRNA is maintained within the functional motor complex. This represents an important step forward in testing the rigid-body-motion hypothesis for pRNA function in DNA packaging. Further studies will map the conformations of the sub-modules and investigate relative sub-module movements during DNA packaging.

Using the R_95/l′_23 constructs, it is now possible to manipulate specific functional groups within the procapsid-binding domain using solid-phase chemical synthesis, and to assess the roles of these groups in motor function. This provides opportunities for a wide range of biophysical and biochemical studies in further investigations of pRNA and packaging motor functions. These chimeric constructs also expand our ability to design artificial pRNA-like molecules, which may aid in development of pRNA-based nano-technology and nano-medicine [12].

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.05.094.

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