RNA (1999), *5*:805–818. Cambridge University Press. Printed in the USA.

Sequence requirement for hand-in-hand interaction in formation of RNA dimers and hexamers to gear ϕ29 DNA translocation motor

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ABSTRACT

Translocation of DNA or RNA is a ubiquitous phenomenon. One intricate translocation process is viral DNA packaging. During maturation, the lengthy genome of dsDNA viruses is translocated with remarkable velocity into a limited space within the procapsid. We have revealed that ϕ29 DNA packaging is accomplished by a mechanism similar to driving a bolt with a hex nut, which consists of six DNA-packaging pRNAs. Four bases in each of the two pRNA loops are involved in RNA/RNA interactions to form a hexagonal complex that gears the DNA translocating machine. Without considering the tertiary interaction, in some cases only two G/C pairs between the interacting loops could provide certain pRNAs with activity. When all four bases were paired, at least one G/C pair was required for DNA packaging. The maximum number of base pairings between the two loops to allow pRNA to retain wild-type activity was five, whereas the minimum number was five for one loop and three for the other. The findings were supported by phylogenetic analysis of seven pRNAs from different phages. A 75-base RNA segment, bases 23–97, was able to form dimer, to interlock into the hexamer, to compete with full-length pRNA for procapsid binding, and therefore to inhibit ϕ29 assembly in vitro. Our result suggests that segment 23–97 is a self-folded, independent domain involved in procapsid binding and RNA/RNA interaction in dimer and hexamer formation, whereas bases 1–22 and 98–120 are involved in DNA translocation but dispensable for RNA/RNA interaction. Therefore, this 75-base RNA could be a model for structural studies in RNA dimerization.

Keywords: DNA translocation; inter-RNA interaction; loop/loop interaction; phylogenetic analysis; RNA oligomers; RNA/RNA interaction; viral assembly

INTRODUCTION

Migration or translocation of DNA or RNA through barriers or cell membranes is a common process in biological systems. After transcription in the nucleus, mRNA and tRNA must pass the nuclear membrane to reach the translation machinery in the cytoplasm. After infection or transfection, most viral or plasmid DNA must pass the nuclear membrane to serve as a template for gene expression (Davis, 1995). The Rev protein of HIV helps in the translocation of viral mRNA from the nucleus to the cytoplasm through a nuclear pore (Pfeifer et al., 1991; Krug, 1993).

One of the most complex and intricate translocation processes is genomic DNA encapsidation of linear dsDNA viruses, including hepesviruses, poxviruses, adenoviruses, and the ds-DNA bacteriophages (Bazinet & King, 1985; Casjens & Hendrix, 1988; Black, 1989; Guo, 1994; Guo & Trottier, 1994). During replication, the lengthy genome of dsDNA viruses is translocated with remarkable velocity into a limited space within the procapsid. The question of how this fascinating task is accomplished has long been a puzzle. Bacteriophage ϕ29 encodes a 120-base RNA (pRNA) that plays a novel and essential role in its genomic DNA translocation (Guo et al. 1987a, 1987b, 1998). ATP is consumed to provide energy for the DNA translocating motor.

We revealed that six pRNAs form a hexagonal complex to gear the DNA translocating machine (Chen & Guo, 1997a; Guo et al., 1998; Zhang et al., 1998; for review, see Hendrix, 1998). These findings have brought about commonality between viral DNA packaging and other universal DNA/RNA-tracking/riding processes

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(Geiduschek, 1997) including DNA replication and RNA transcription (Young et al., 1994b; Doering et al., 1995; Guo et al., 1998). The DNA/RNA-tracking/riding enzymes, including helicases (Young et al., 1994a; Egelman, 1996; West, 1996; San Martin et al., 1997), enhancers (Herendeen et al., 1992), and terminator Rho (Geiselmann et al., 1993), Yeast PCNA, and DNA polymerase III holoenzyme (Geiduschek, 1997), also form a hexameric complex. Viral DNA packaging, cellular DNA replication, and RNA transcription all involve the relative movement of two components, one of which is nucleic acid. It is intriguing to show how ϕ29 pRNA could play a role that protein enzymes perform.

As a first step in DNA packaging, pRNA binds to the connector (the unique site on procapsids where DNA enters and exits) of procapsids in the presence of Mg^{2+} (Guo et al., 1986, 1987b, 1987c; Reid et al., 1994; Chen & Guo, 1997b). Procapsids with pRNA attached are competent to package DNA with the aid of the DNA packaging protein gp16 and ATP. We are able to package ϕ29 genomic DNA into procapsids in vitro with up to 90% efficiency using all purified proteins from cloned gene products (Guo et al., 1987c, 1991b; Lee & Guo, 1995a, 1995b). Furthermore, the DNA-filled capsids can be converted into infectious virions in vitro with the addition of tail protein (gp9), neck proteins (gp11 and gp12), and morphogenic factor (gp13), which were also produced from cloned genes. We can assemble up to 10^8 plaque forming units (pfu) per milliliter of infectious ϕ29 virions in vitro with nine purified protein components as well as pRNA and genomic DNA presynthesized in vitro (Lee & Guo, 1994). Omission of pRNA resulted in no plaque formation, thereby providing a system with a sensitivity of eight orders of magnitude for functional assay of the pRNA (Guo et al., 1991a; Lee & Guo, 1994, 1995b; Zhang et al., 1995a, 1995b, 1995c).

Quantitative method has been used to reveal that two single-stranded loops of pRNA are involved in inter-RNA interaction to form a pRNA hexamer for ϕ29 DNA transportation (Chen & Guo, 1997a; Trottier & Guo, 1997; Guo et al., 1998). These two loops interact alternately to generate interlocking chains. To facilitate the description, the two loops have been named the right- and left-hand loops (Fig. 1A). Although there is no chirality in pRNA, we arbitrarily refer to the loop close to the 5′ end as the right-hand loop and the one close to the 3′ end as the left-hand loop. Therefore intermolecular interaction of pRNA here is referred to as "hand-in-hand" interaction (see Discussion). In this article, we report the sequence requirement for the formation of a hexameric pRNA complex through base pairings between the right loop (bases 45–48) and the left loop (bases 82–85). We also define a pRNA domain responsible for inter-pRNA interaction and identify a 75-base pRNA fragment competent in dimer formation.

RESULTS

Formation of hexameric pRNA complex via right and left loop intermolecular interaction

Six pRNAs have been found to form a hexameric RNA complex as part of the ϕ29 DNA translocation machinery. Mixing of two, three, and six inactive mutant pRNAs resulted in full DNA packaging activity, as long as a hexameric ring could be predicted to form by the base pairing of the mutated loops (Guo et al., 1998). Here we provide additional information to show that this interlocking hexameric complex formation is accomplished by inter-pRNA interactions via bases 45–48 of the right loop and bases 82–85 of the left loop (Fig. 1A). We use uppercase and lowercase letters to represent the right and left loop sequences, respectively (Figs. 1B and 2). The same letter in upper- and lowercase symbolizes a pair of complementary sequences. For example, in pRNA D/d′, the right- and left-loop sequences are complementary, while in pRNA D/h′, the four bases of the right loop D are not complementary to those of the left loop h′. Mutant pRNAs with four base pairs between the two loops, for example, D/d′, H/h′, and J/j′, were as active as wild-type pRNA (I/i′) (Fig. 2). Mutant pRNAs with base mismatches between the two loops, for example, D/h′, H/j′, and J/d′, were all inactive when used alone (Fig. 2). Mixtures of any two of these three pRNAs were only partially active or strictly inactive when used in in vitro ϕ29 assembly. However, mixtures of all three pRNAs (D/h′, H/j′, and J/d′) in a 1:1:1 molar ratio restored pRNA activity in ϕ29 assembly (Fig. 2). These results strongly support our conclusions that pRNAs interact intermolecularly through base pairing of the right and left loops during DNA translocation.

Requirements of at least one G/C pair when bases 45–48 were complementary to bases 85–82

To analyze the G/C pair requirement for the four base-pairing interactions of the right and left loops, 17 mutant pRNAs were constructed and their activities were determined with the in vitro ϕ29 assembly system (Table 1). In this study the change of the loop was applied to the left (L) and right (R) loops of the same pRNA, that is, the so called "one pRNA approach." Mutant pRNAs sud2/sud1, T/t′, and S2/sud1 did not contain any G/C pairs within this interacting region. All of these pRNAs were inactive in ϕ29 assembly (Table 1). Another mutant, P/p′, was designed to have one G/C pair and three A/U pairs within the interacting region. This pRNA showed an activity of 2.7×10^6 pfu/mL (Table 1). Furthermore, mutants C/c′, F/f′, S/s′, and L/l′ were designed to contain two G/C pairs among the interacting loops with four pairs. All of these mutants were as active as wild-type pRNA I/i′, which also contains two G/C pairs in the interacting loops (Table 1).

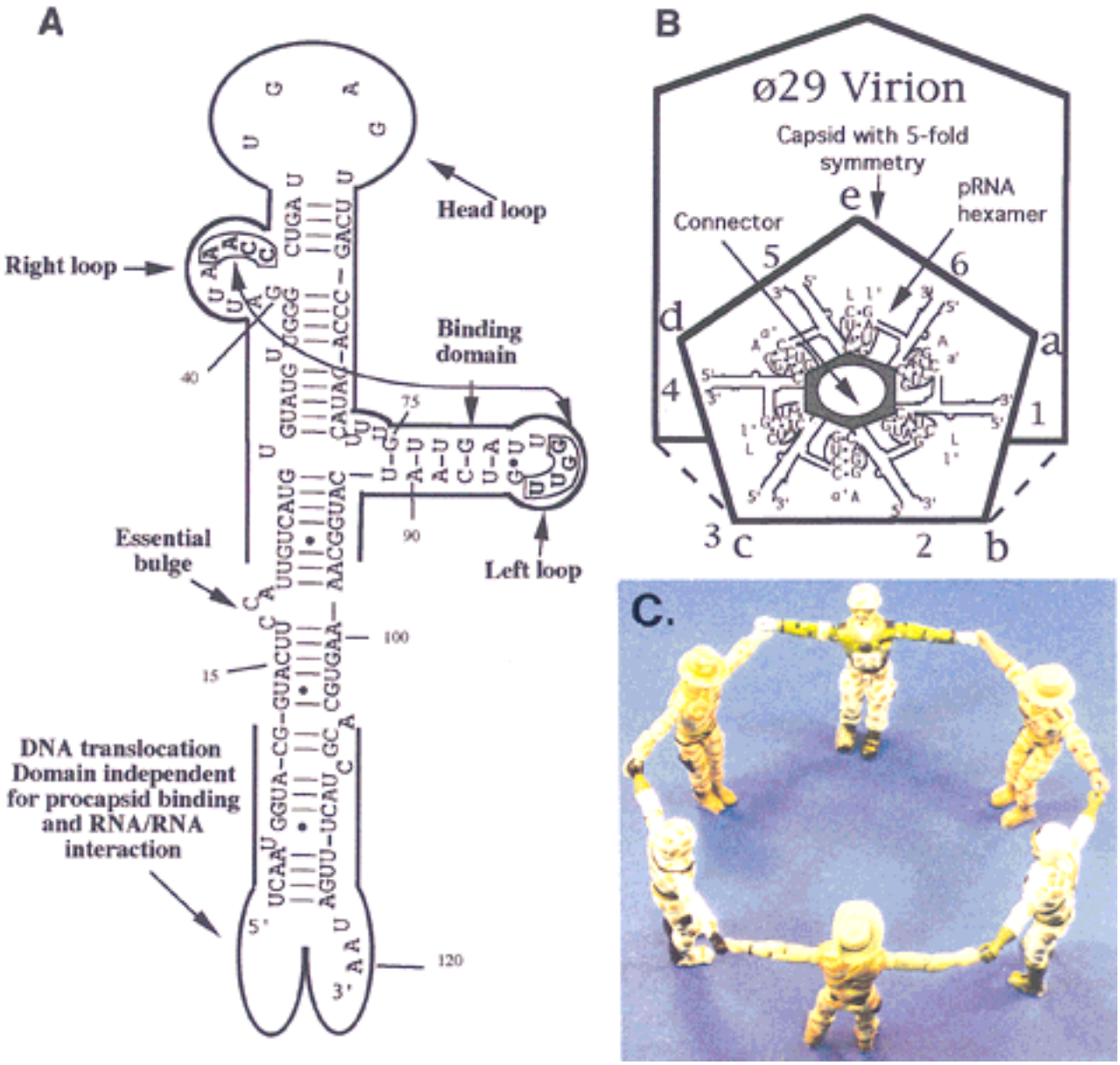


FIGURE 1. A: Secondary structure of wild-type pRNA. The binding domain and the DNA translocation domain are marked with bold lines. The four bases in the right and left loops, which are responsible for inter-RNA interactions, are bold and boxed. **B**: Diagrams depicting the formation of a pRNA hexameric ring and its location in the ϕ29 DNA packaging machine. Hexamer formation is via right- and left-loop sequence interactions. The shaded hexagon stands for the connector and the surrounding pentagon stands for the fivefold symmetrical capsid vertex, viewed as end-on with the virion as side-view. The six protrusions stand for six pRNAs with the central region bound to the connector and the 5′/3′ paired region extending outward. The RNA complex signifies a pRNA composed of an interlocking pair of A/I′ and L/a′. **C**: A cartoon depicting the formation of hexamer by hand-in-hand interaction.

Three G/C pairs were introduced into mutant J/j′, in which U_{81} was changed to A_{81} to prevent shifting in base pairing. This mutant pRNA was as active as wild-type pRNA (Table 1). Full activities were also observed with pRNAs A/a′, B/b′, D/d′, E/e′, and H/h′ (Table 1). Each of these pRNAs contained three G/C pairs in the interacting sequences of the two loops.

Additionally, a mutant, G/g′, with four G/C pairs between the right and left loops was designed. This mutant showed reduced activity (Table 1). Our results suggest that when four base pairs were predicted to form between the two loops, at least one G/C pair among these four base pairs is required to achieve pRNA activity, although this activity was reduced. Two or three G/C pairs among the four base pairs were required for full pRNA activity, and these pairs could be located at any position within the four base pairs. Four G/C pairs showed reduced activity, indicating that

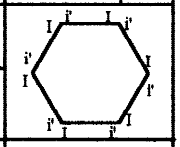
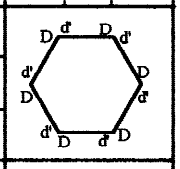
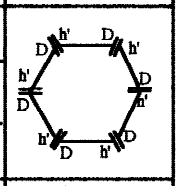
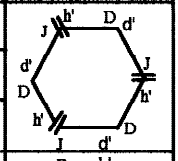
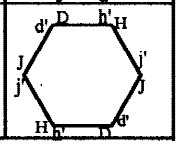
pRNAs	Interacting bases of R & L loops	number of pRNAs per reaction	pfu/ml	sketch of interaction of twoloops
- control		0	0	
I/i' (wild type)	5'-A45ACC-3' 3'-U85UGG-5'	1	10^7	
D/d'	5'-A45GGC-3' 3'-U85CCG-5'	1	10^7	
H/h'	5'-G45CGA-3' 3'-C85GCU-5'	1	10^7	
J/j'	5'-C45CGU-3' 3'-G85GCA-5'	1	10^7	
J/d'	5'-C45CGU-3' 3'-U85CCG-5'	1(unpaired)	0	
D/h'	5'-A45GGC-3' 3'-C85GCU-5'	1(unpaired)	0	
H/j'	5'-G45CGA-3' 3'-G85GCA-5'	1(unpaired)	0	
J/d'+ D/h'	miss one link	2	10^3	
D/h'+ H/j'	miss one link	2	10^4	
H/j'+ J/d'	miss one link	2	10^3	
D/h'+ H/j'+ J/d'	closed	3	10^7	

FIGURE 2. Mutant pRNA activity assay with a highly sensitive in vitro ϕ29 virion assembly system. The same letter in upper- and lowercase symbolizes the complementary right and left hand-in-hand loops, respectively. Two adjacent letters at the vertex signify the pairing of the right and left loops, while they signify the nonpairing of two loops if a double bar appears. For the nomenclature of mutant pRNAs, see the first paragraph in Results.

either the conformation of pRNA was altered or the interaction between two loops was too strong to provide any flexibility for normal pRNA function.

Gel-shift assay also suggested that all mutant pRNAs that are active in in vitro assembly can form dimers in solution as long as the R- and L-loop sequence is paired intermolecularly (data not shown). The above results in both virion assay and gel-shift assay support the conclusion that, when four pair were present in the loops, at least one G/C pair within loop sequence 45–48/85–82 was required for DNA packaging activity.

Maximum number of base pairs between the right and left loops

To isolate RNA complexes, there is a desire for increased complex stability. It is logical to believe that the longer the sequence for pairing is, the more stable the complex is. The next question, therefore, is whether there is a limit to the number of base pairs for the pRNA intermolecular interaction. An extra U and A were added to the right and the left loops, respectively, to bring the number of base pairs from four to five. One U was inserted at position 86 to generate mutant pRNA I/5′ U, and one A was inserted at position 49 to generate mutant pRNA 3′ A/i′. Both of these mutants had five base pairs between the right and left loops. These two pRNAs were fully active (Table 2). The same results were observed for mutant pRNAs E/i′ and C/f′, which also have five base pairs predicted between the two loops (Table 2).

When the predicted base pair number was further increased to six as in mutant pRNA 3′A /5′ U, its assembly activity reduced four logs to 10^3 pfu/mL (Table 2). The results indicated that the maximum number of base pairs between the right and left loops to permit normal pRNA interaction is five.

The effect of the number of base pairings on dimer formation was investigated by gel-shift assay with the "two pRNA approach." In this study, the mixing of one pair of pRNAs with interlocking loops was carried out to test whether mutant RNAs were still able to form dimers through loop interaction. For example pRNA pair A/b′ and B/a′ had A/a′ intermolecular interlocking base pairing at one side and B/b′ at the other. As shown in Figure 3, dimer could form with three, four, and five base pairings between two loops (Fig. 3, lanes 4–15). However, dimer could not form when the base pairings between two loops was extended to six (Fig. 3. lanes 1–3). These results might explain why pRNAs were inactive in ϕ29 virion assembly when the base pairing between loops was extended to six.

TABLE 1. G/C pair requirement for pRNA activity.

pRNAs	Loop sequence	G/C pairs	Activity (pfu/mL)
No RNA			0
I/i′ (wt)	5′-AACC- | | | | $UUGGU_{81}$-5′	two	9.0×10^7
sud2/sud1	5′-AAAA- | | | | $UUUUU_{81}$-5′	none	0
T/t′	5′-UUUU- | | | | $AAAAU_{81}$-5′	none	0
S2/sud1	5′-GAGA- | | | | $UUUUU_{81}$-5′	none	0
P/p′	5′-AUAC- | | | | $UAUGU_{81}$-5′	one	2.7×10^6
C/c′	5′-GACA- | | | | $CUGUU_{81}$-5′	two	5.3×10^7
F/f′	5′-AGAC- | | | | $UCUGU_{81}$-5′	two	10^7
S/s′	5′-GGUU- | | | | $CCAAU_{81}$-5′	two	3.2×10^7
L/l′	5′-CUAC- | | | | $GAUGA_{81}$-5′	two	10^7
U/u′	5′-UUCC- | | | | $AAGGU_{81}$-5′	two	10^7
A/a′	5′-GGAC- | | | | $CCUGU_{81}$-5′	three	10^7
B/b′	5′-ACGC- | | | | $UGCGU_{81}$-5′	three	10^7
D/d′	5′-AGGC- | | | | $UCCGU_{81}$-5′	three	10^7
E/e′	5′-GCCA- | | | | $CGGUU_{81}$-5′	three	10^7
H/h′	5′-GCGA- | | | | $CGCUU_{81}$-5′	three	10^7
J/j′	5′-CCGU- | | | | $GGCAA_{81}$-5′	three	10^7
G/g′	5′-GGCC- | | | | $CCGGU_{81}$-5′	four	10^4

TABLE 2. The maximum number of pairs between the right and left loops required for pRNA activity.

pRNAs	Loop sequence	Insertion	Activity (pfu/mL)
None			0
I/i′ (wt)	5′-AACC- | | | | $UUGGU_{81}$-5′		9.0×10^7
I/5′ U	5′-$AAACC_{48}$- | | | | | $UUUGGU_{81}$-5′	U_{86}	7.8×10^7
3′ A/i′	5′-$AACCA_{48}$- | | | | | $UUGGU_{81}$-5′	A_{49}	8.0×10^7
E/i′	5′-$AGCCA_{48}$- | | | | | $UUGGU_{81}$-5′		1.1×10^7
C/f′	5′-$AGACA_{48}$- | | | | | $UUGGU_{81}$-5′		8.6×10^7
3′ A/5′ U	5′-$AAACCA_{49}$- | | | | | | $UUUGGU_{81}$-5′	A_{49} U_{86}	5.0×10^3

In some cases, only two G/C pairs in the interacting region were able to support pRNA activity

We found that mutant pRNAs U/i′, I/u′, and I/g′ had only two G/C pairs at $C_{47}C_{48}/G_{83}G_{82}$ while other bases in the interacting region were unpaired. These mutant pRNAs were almost fully active (Table 3). However, when the two G/C pairs were not located at bases 47–48/83–82, but at other locations in the right or left loops, these pRNAs were not active in ϕ29 DNA packaging (Table 3). For instance, mutant pRNAs J/b′, J/h′, K/e′, B/j′, E/k′, and H/j′ all have two G/C pairs within the base 45–48/85–82, but none showed activity in ϕ29 assembly (Table 3). Mutant pRNAs, including L/a′, L/f′, A/l′, F/l′, J/d′, B/a′, and D/l′, which have one G/C pair and one A/U or G/U, did not show any activity; neither did those mutant pRNAs, including T/s′, T/g′, and T′/i′, that have two A/U or G/U pairs, or one A/U and one G/U.

Nonrequirement for a purine in sequence 82–85 of the left loop

Studies on HIV RNA dimerization (Bender & Davidson, 1976; Prats et al., 1990) imply that purine quartets may be involved in dimer formation of dimerization linked sequences (DLS; Marquet et al., 1991; Sundquist & Heaphy, 1993). It would be worthwhile to inves-

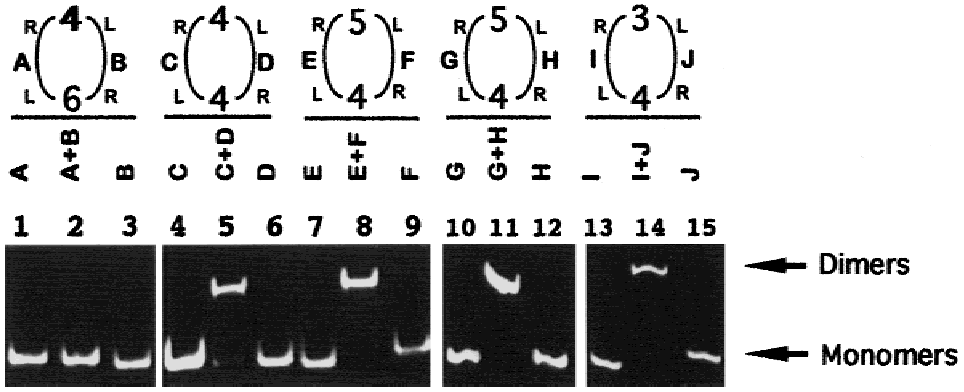


FIGURE 3. Native polyacrylamide gel showing the effect of the number of base pairings on dimer formation and minimum loop size requirement for dimer formation. A: pRNA A/5′ U; B: 3′ A/a′; C: A/b′, D: B/a′; E: E/a′; F: A/i′; G: C/b′; H: B/f′; I: M/a′; J: A/m′. The sequences of the pRNA can be found in Table 5. The numbers located between the letters R and L indicate the number of base pairings between the R and L loop from each pair of pRNA.

tigate whether purines are required for pRNA/pRNA interactions.

Mutant pRNA S2/s′2 contains no purines in sequences 82–85 of the left loop. This pRNA was fully active in in vitro ϕ29 assembly. This result suggests that no purines were required in the left loop.

Minimum loop size requirement

To study the effect of surrounding sequences on hand-in-hand interaction, certain bases in the right- and left-hand loops, which were nine and five bases, respectively, were deleted. Two bases, U_{81} and U_{85}, in the left loop were deleted to generate mutant I/m′. Three bases, $A_{41}U_{42}U_{43}$ in the right loop, were deleted to generate mutants M/i′. Each of these mutants was fully active (Table 4). The bases $A_{41}U_{42}U_{43}$ in the right loop and U_{81} and U_{85} in the left loop were deleted to generate a mutant M/m′, which was predicted to form only three base pairs between the two loops. Interestingly, this mutant had activity similar to wild-type pRNA (Table 4). When an additional base (U_{84}) was deleted from the left loop resulting in mutant I/q′, this mutant was almost inactive (Table 4). These results indicated that two bases, U_{81} and U_{85}, could be deleted from the left loop with little effect on pRNA function. The resulting mutant pRNA with both the right and left loop deletion was able to form interlocking dimers in polyacrylamide gel as well (Fig. 3, lanes 13–15). This also demonstrated that the number of bases in the left loop could be as few as three.

When four bases ($G_{40}A_{41}U_{42}U_{43}$) were deleted from the right loop to generate mutant R2/i′, the activity of this mutant was 5.3×10^6 pfu/mL (Table 4). However, when five bases were deleted to generate mutant R1/i′, the activity of R1/i′ was greatly reduced to 10^3 pfu/mL (Table 4). These results suggested that four bases, $G_{40}A_{41}U_{42}U_{43}$, not five, in the right loop could be deleted with little effect on pRNA function. The fact that the minimum number of bases in the right loop was five was also demonstrated.

Minimum size requirement for dimer formation

As pRNA can interact intermolecularly to form a hexamer, it is beneficial to understand the structure base for dimer and hexamer formation. To solve pRNA structure by NMR or crystallography, it would be desirable to identify a smallest RNA molecular that would be able to accomplish the same function. We attempted to determine the boundary of pRNA domain responsible for dimer and hexamer formation. This information would allow us to isolate a minimal size pRNA domain or fragment competent in dimer and hexamer formation.

Mutant pRNAs with different truncation from the 5′/3′ end were constructed using A/i′ and I/a′ as a dimer pair. pRNA 23–99 A/i′ and 23–99 I/a′, with the deletion of bases 1–22 and 100–120, were able to form stable dimers in the gel (Fig. 5, lanes 4–6). Ultracentrifugation of pRNA bands isolated from the gel confirmed that the band with the lower migration rate was dimer, whereas the one with a fast migration rate was monomer. Further deletion of an additional five bases at the 3′ end resulted in a pRNA 23–94 A/i′ that was not able to form stable dimers with 23–99 I/a′ (Fig. 5, lanes 6–8). The ill-defined low density band in lane 7 indicates that heterogeneous complexes were produced by 23–99 I/a′ and 23–94 A/i′ interaction. Mixing of 28–91 A/i′ and 28–91 I/a′ could not generate detectable dimers in the gels. Again, the ill-defined low density band in Figure 5, lane 13, suggests the production of heterogeneous complexes when 28–91 A/i′ and 28–91 I/a′ were mixed. Truncation on another pair of dimers, B/w′ and W/b′, supported that bases 23–97 are the minimum fragment that can form stable dimers in polyacrylamide gels (Fig. 5, lanes 9–11).

TABLE 3. Only two G/C pairs were able to support pRNA activity.

pRNAs	Loop sequence	Activity (pfu/mL)
I/i′ (wt)	5′-AACC- | | | | UUGGU$_{81}$-5′	9.0×10^7
I/u′	5′-AACC- | | AAGGU$_{81}$-5′	5.2×10^6
U/I′	5′-UUCC- | | UUGGU$_{81}$-5′	2.1×10^6
I/g′	5′-AACC- | | CCGGU$_{81}$-5′	6.9×10^3
J/b′	5′-CCGU- | | | UGCGU$_{81}$-5′	0
J/h′	5′-CCGU- | | CGCUU$_{81}$-5′	0
K/e′	5′-UCCU- | | CGGUU$_{81}$-5′	0
B/j′	5′-ACGC- | | GGCAA$_{81}$-5′	0
E/k′	5′-GCCA- | | AGGAG$_{81}$-5′	0
H/j′	5′-GCGA- | | GGCAA$_{81}$-5′	0
L/a′	5′-CUAC- | | CCUGU$_{81}$-5′	0
L/f′	5′-CUAC- | | UCUGU$_{81}$-5′	0
A/l′	5′-GGAC- | | GAUGA$_{81}$-5′	0
F/l′	5′-AGAC- | | GAUGA$_{81}$-5′	0
J/d′	5′-CCGU- | | UCCGU$_{81}$-5′	0
B/a′	5′-ACGC- | | CCUGU$_{81}$-5′	0
D/l′	5′-AGGC- | | GAUGA$_{81}$-5′	0
T/s′	5′-UUUU- | | CCAAU$_{81}$-5′	0
T/g′	5′-UUUU- | | CCGGU$_{81}$-5′	0
T/i′	5′-UUUU- | | UUGGU$_{81}$-5′	0

TABLE 4. The minimum loop size required for pRNA activity.

pRNAs	Loop sequence	Deletion	Activity (pfu/mL)
None			0
I/i′ (wt)	5′-AACC- | | | | UUGGU$_{81}$-5′		9.0×10^7
I/m′	5′-AACC- | | | UGGU$_{80}$-5′	U$_{81}$, U$_{85}$	6.9×10^6
I/q′	5′-AACC- | | G$_{86}$GGU$_{80}$-5′	U$_{81}$, U$_{84}$U$_{85}$	0
M/i′	5′-AACC- | | | | UUGGU$_{81}$-5′	A$_{41}$U$_{42}$U$_{43}$	1.1×10^7
R2/i′	5′-AACC- | | | | UUGGU$_{81}$-5′	G$_{40}$A$_{41}$U$_{42}$U$_{43}$	5.3×10^6
R1/i′	5′-AACC- | | | | UUGGU$_{81}$-5′	G$_{40}$A$_{41}$U$_{42}$U$_{43}$A$_{44}$	10^3
M/m′	5′-AACC- | | | UGG-5′	A$_{41}$U$_{42}$U$_{43}$ U$_{81}$, U$_{85}$	1.0×10^7
R2/m′	5′-AACC- | | | UGGU$_{80}$-5′	G$_{40}$A$_{41}$U$_{42}$U$_{43}$ U$_{81}$, U$_{85}$	0

Phylogenetic analysis

Phylogenetic analysis of pRNAs from *Bacillus subtilis* phages SF5, B103 (Pecenkova et al., 1997), ϕ29, PZA, M2, NF, and GA1 (Bailey et al., 1990) shows very low sequence identity and few conserved bases, yet, the family of pRNAs appears to have similar predicted secondary structures (Zuker, 1989) (Fig. 4). All seven pRNAs of these phages contain both the right and left loops. Complementary sequences within the two loops were found in each of these pRNAs (Fig. 4). The numbers of paired bases were from five (5′-GUUUU/CAAAA-5′) for SF5 to four (5′-AACC/UUGG-5′) for ϕ29/PZA and B103, to three (5′-AUC/UAG-5′) for M2/NF, and two (5′-CC/GG-5′) for GA1. Phylogenetic analysis agrees with our conclusion deduced from experimental data. The conclusion of the requirement of at least one G/C pair was supported by the finding that all predicted paired sequences in the right and left loops contain at least one G/C pair. Two G/C pairs were found in ϕ29 PZA and GA1 pRNAs, and only one G/C pair was found in pRNAs of B103, M2/NF, and SF5 (Fig. 4). The conclusion that two G/C pairs are able to initiate pRNA activity was supported by the finding that the paired loop sequences in GA1 contained only two G/C pairs (Fig. 4).

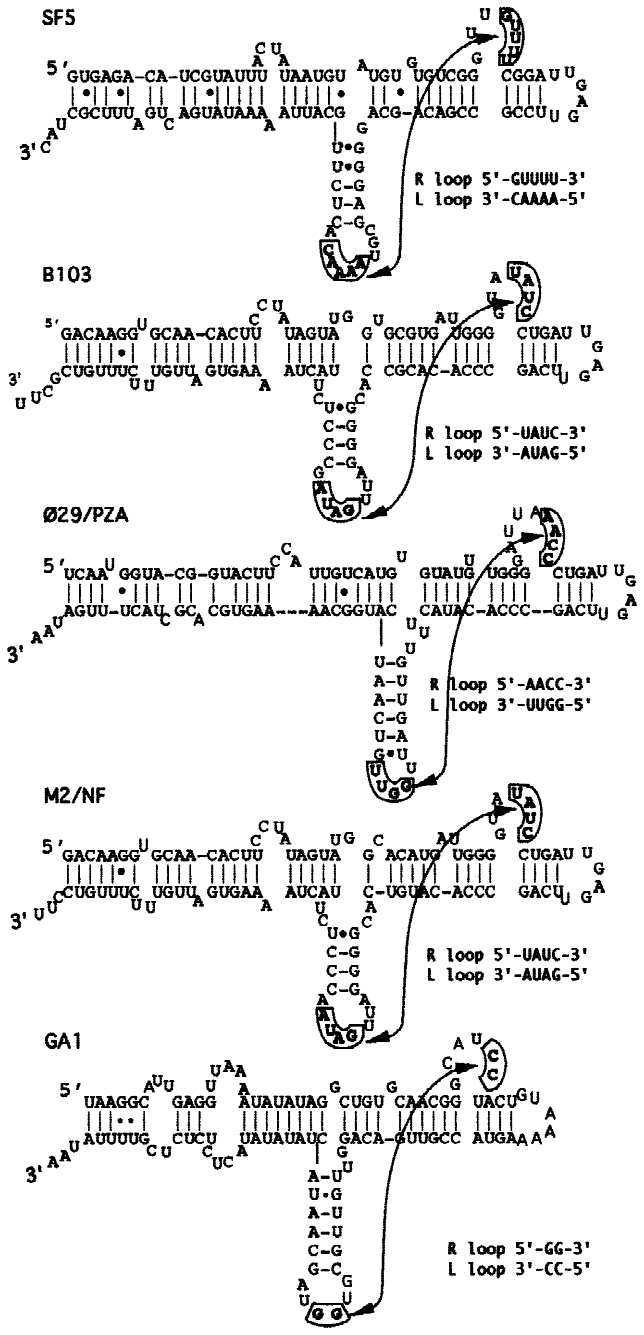


FIGURE 4. Phylogenetic analysis and predicted secondary structures of pRNAs in phages SF5′ B103, ϕ29, PZA, M2, NF, and GA1 of *Bacillus subtilis*. The bases in the right and left loops responsible for inter-RNA interaction are boxed and in bold.

A 75-base pRNA segment was able to compete with full-length pRNA form procapsid binding and to inhibit ϕ29 assembly in vitro

As mentioned above, we have defined the boundary of pRNA domain responsible for dimer formation in native polyacrylamide gel. To know whether the minimum domain was able to incorporate into the hexamer with equal affinity as full-length pRNAs, we carried out competition inhibition assay. The basis for such competition inhibition study is as follows. If a component of a complex biological system contains two functional domains, one for binding of the component to other target, and the other for other biological activity but dispensable for binding, then a lethal mutant of this component could be constructed by introducing a mutation into the functional domain while keeping the binding domain intact. If this mutant retains the wild-type binding affinity, then its stoichiometry, Z, could be determined by mixing a known amount of mutant and wild type in the reaction. The probability of the distribution of the mutant and wild type per receptor could be determined via the expansion of the binomial

$$(p+q)^z = \binom{z}{0}p^z + \binom{z}{1}p^{z-1}q + \binom{z}{2}p^{z-2}q^2 + \cdots$$

$$+ \binom{z}{z-1}pq^{z-1} + \binom{z}{z}q^z = \sum_{x=0}^{z}\binom{Z}{M}p^{z-x}q^x,$$

$$\binom{Z}{M} \text{ is equal to } \left(\frac{Z!}{M!(Z-M)!}\right),$$

where p and q are the percentage (ratio) of mutant and wild type in reaction mixture. Using various ratios of mutant to wild type in experiments, percentage mutant versus the yield of reactions could be plotted and compared to a series of curves deduced from binomial to find a best-fit.

For example, if the stoichiometry is 3, the probability of all combinations of mutant (M) and wild type (W) participating in one single reaction can be determined by the expansion of the binomial: $(p+q)^3 = p^3 + 3p^2q + 3pq^2 + q^3 = 100\%$. That is, in the population of the reaction mixture, the probability of receptor possessing three copies of mutant is p^3; two copies of mutant and one copy of wild type is $3p^2q$; one copy of mutant and two copies of wild type is $3pq^2$; and three copies of wild type is q^3. Suppose that there were 70% ($p = 0.7$) mutant and 30% ($q = 0.3$) wild type in the reaction mixture; then the percentage of receptors that possessed at least two copies of wild type would be the sum of those possessing one copy of mutant and two copies of wild type, $3pq^2$, and those possessing three copies of wild type, q^3. The probability of the population that possesses at least two copies of wild type is $3pq^2 + q^3 = 3(0.7)(0.3)^2 + (0.3)^3 = 0.216 = 21.6\%$.

Previously, we used competition and inhibition methods to reveal that six copies of pRNA are needed for the packaging of one viral genome (Trottier & Guo, 1997; Guo et al., 1998). Here we use the competition inhibition method to test whether pRNAs 23–97 A/b′ could be incorporated into the hexamer complex with equal affinity as wild-type pRNA. The mutant used as inactive pRNA control was pRNAs B/a′ CCA, which has been proven to be incompetent in DNA translocation while it retained the full competency in hexamer

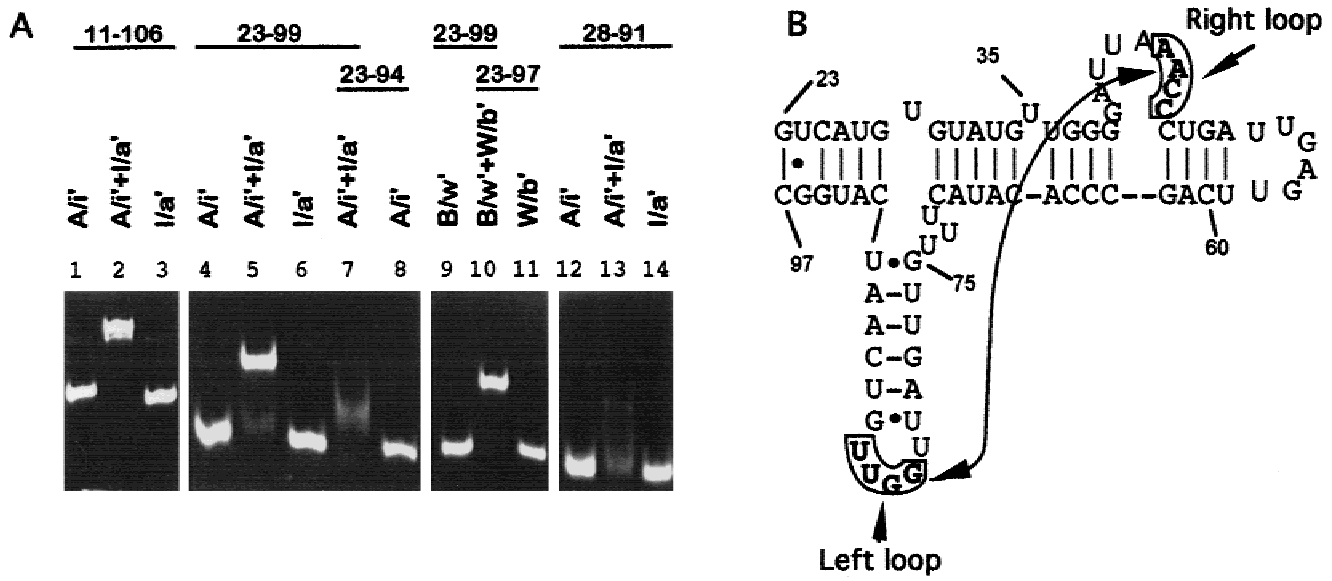


FIGURE 5. Native polyacrylamide gel showing minimum pRNA size requirement for dimer formation. The numbers, such as "11–106" in **A**, indicate that the size of the pRNA is from base 11 to 106. The minimal size pRNA 23–97 I/i′ is shown in **B** with the numbers indicating base location in wild-type pRNA.

formation and the wild-type procapsid binding affinity (Zhang et al., 1997; Guo et al., 1998). When variable amounts of pRNA 23–97 A/b′ were mixed with pRNAs A/b′ and B/a′ (A/b′ and B/a′ were present in equal amounts) and used in in vitro ϕ29 assembly assays (i.e., 10% 23–97 A/b′, 40% A/b′, and 50% B/a′), the probability of procapsids that possess a certain amount of 23–97 A/b′ and a certain amount of normal A/b′ was predicted with the binomial (see Material and Methods and Trottier & Guo, 1997). The yield of virions from empirical data was plotted and compared to a series of predicted curves to find a best-fit (Fig. 6). The rationale behind this experimental design is that, if 23–97 A/b′ could be incorporated into the hexamer with an equal affinity as wild-type pRNA, each procapsid should contain three copies of B/a′ and three copies of A/b′ with a certain number of A/b′ replaced by 23–97 A/b′, where the copy number of 23–97 A/b′ incorporated into each procapsid can be predicted based on the percentage of 23–97 A/b′ presented in the reaction mixture. Two theoretical curves were predicted, one with the assumption that the pRNA ring is composed of six copies of pRNA, the other with the assumption that the pRNA ring is composed of 12 copies of pRNA. Our results show that the empirical competition inhibition curve of 23–97 A/b′ overlaps with the competition inhibition curve of B/a′ CCA. Both curves most closely matched, in both slope and magnitude, the curve predicted with six copies, not 12 copies (Fig. 6), of pRNA per procapsid. These results suggest that pRNA fragment 23–97 A/b′ was incorporated into the hexamer with the same affinity as the pRNA B/a′ CCA that has a procapsid binding affinity equal to wild-type full-length pRNA.

DISCUSSION

The loop/loop interaction of pRNA is different from pseudoknots (Studnicka et al., 1978; Pleij & Bosch, 1989) and kissing loops (Bender & Davidson, 1976;

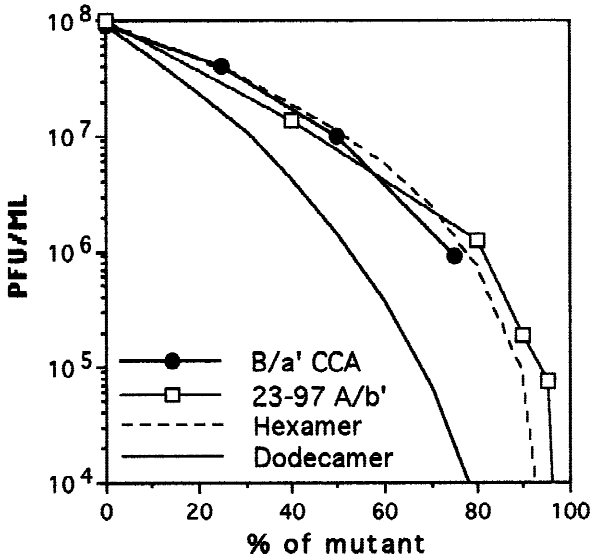


FIGURE 6. Semi-log plot of predicted and empirical curves showing the yield of virion production (PFU/mL) versus percentage of mutant pRNA to determine procapsid binding affinity and inhibition efficiency of pRNA fragment 23–97 A/b′ (open square). pRNA B/a′ was mixed with a varied ratio of mutant 23–97 A/b′ versus A/b′. Competition curve from mutant B/a′ CCA and B/a′ plus A/b′ served as a control (solid circle). Theoretical curves were predicted from binomial distribution (see text), assuming that the total pRNA number per procapsid, *Z*, is equal to 6 (dashed line) or 12 (thin line).

Prats et al., 1990; Sundquist & Heaphy, 1993; Paillart et al., 1996; Chang & Tinoco, 1997; Laughrea et al., 1997). Pseudoknots involve the intramolecular interaction existing in one single molecule. Kissing loops involve the interaction of two self-complementary loops in dimer formation (Homann et al., 1993; Clever et al., 1996). However, pRNA multimers involve multiple RNAs to form closed rings. The intermolecular interaction of pRNA here requires that each RNA molecule contributes one loop to pair with the alternate loop of the next pRNA. The key feature of hand-in-hand interactions is that multiple RNAs interact via alternate interlocking loops to form a closed ring, whereas interaction of kissing loops refers to formation of dimers, not rings. Such atypical hand-in-hand loop/loop interaction might not be alone. RNA–RNA interaction via alternative loops has also been reported for *bicoid* mRNA in *Drosophila* embryo (Ferrandon et al., 1997). Formation of *bicoid* mRNA dimers via alternate loops in vitro was clearly documented. We feel that the mechanism of *bicoid* mRNA interaction and translocation might be similar to that of ϕ29 pRNA. We would not exclude the possibility of forming a *bicoid* mRNA hexameric or polymer ring to ride, track, or rotate along Staufen protein during *bicoid* mRNA transportation. Indeed, the formation of *bicoid* mRNA multimer is evidenced (Fig. 3 of Ferrandon et al., 1997) and postulated by the authors. These two systems might provide models for the study of the requirement of three-dimensional structure for RNA/RNA interactions involving a mechanism of hand-in-hand interaction.

The interaction of the right and left loops in wild-type pRNA involves four base pairs. We showed that only two G/C pairs in a certain location within the loops were able to provide the pRNA with assembly activity. This was supported by phylogenetic analysis, showing that only two G/C pairs were predicted between the right and left loops of bacteriophage GA1 pRNA. Although the presented data are convincing, it is reasonable to believe that the interaction of two base pairs might not be sufficient for the formation of a stable RNA/RNA complex. We expect that the tertiary structure of the environment around the loops might contribute to inter-RNA interactions and to the stability of the hexameric complex. This expectation is also supported by the evidence that some incorrect pairs were able to program up to four logs of phage assembly, albeit several logs less than correct pairing (Guo et al., 1998). Though the residual activity might be interpreted by noncanonical interaction or tolerated mismatches, the pairing of only four bases or mismatch pairing may not be strong enough to hold a stable RNA complex without the help of the three-dimensional neighboring environment. This speculation can be exemplified by the following analogy. To assemble a new desk with packed parts from the department store or factory, nails or screws are needed to hold the parts. The two or four base pairs between the right and left loops might play a role similar to nails or screws to hold six pRNAs together.

ϕ29 pRNA is 174 bases when transcribed from ϕ29 genome. Fifty bases at the 3′ end have been found not to be involved in DNA packaging activity. Removal of a 74-base RNA fragment from its 3′ end resulted in a 120-base pRNA that is fully active in ϕ29 DNA packaging (Guo et al., 1987a, 1987b; Wichitwechkarn et al., 1989). We report here that base 23–97 was able to form dimer, which competes with wild-type pRNA for procapsid binding, and therefore to inhibit ϕ29 assembly in vitro. The predicted inhibition curve using binomial distribution shows that pRNA 23–97 was able to interlock into the hexameric pRNA complex with an efficiency equal to pRNA B/a′ CCA or wild-type pRNA. Our result suggests that pRNA base 23–97 is a domain involved in procapsid binding and RNA/RNA interaction in dimer and hexamer formation, while bases 1–22 and 98–120 were involved in DNA translocation, but dispensable for RNA/RNA interaction. Therefore, we have identified a minimal size pRNA of 75 bases that could be used as a model for study of the structural requirements in RNA dimerization.

Though current and previous data clearly indicate that six pRNAs form a hexamer attached to the DNA translocating connector, the circumstances for pRNA oligomerization in solution is not clear. We can detect the presence of monomers, dimers, trimers, tetramers, pentamers, and hexamers in solution with certain pRNAs (Guo et al., 1998) or mutants in a certain physical and chemical environment. However, we can only detect the presence of monomers or dimers with certain mutant pRNAs or their mixture in solution, as presented in this report. The conditions that lead to different numbers of oligomerization are under investigation.

MATERIALS AND METHODS

Synthesis and nomenclature of mutant pRNAs

Fifty-three oligonucleotides were used as PCR primers (Tables 5 and 6). The DNA fragment of pRNA75/71 was used as a PCR template to amplify DNA fragments for in vitro synthesis of mutant pRNAs to study the loop sequence requirement for pRNA interaction as described previously (Zhang et al., 1994, 1995c). The mutant pRNA produced from the PCR template using primer pairs 3′PD and 5′Pd′ was called mutant pRNA D/d′. The uppercase letter represents mutations in the right loop and the lowercase letter symbolizes mutations in the left loop. To study the minimum size of pRNA for interaction, 5′/3′ A/b′ DNA fragment was used to amplify a truncated pRNA template as desired.

Methods for pRNA synthesis in vitro have been described previously (Zhang et al., 1994, 1995c). The sequences of mutant pRNAs were confirmed by primer extension.

TABLE 5. Primers used for constructing mutant pRNA with loop alteration.

pRNAs	Loop sequence	Primer sequences	Oligo name
A/a′	5′-GGAC$_{48}$	5′-TAATACGACTCACTATAGTTGATT**GTCC**GTCAATCATG-3′	P5′a′
	$_{85}$CCUG-5′	5′-GTATGTGGGCTGAACTCAATCAG**GTCC**TAATC-3′	P3′A
B/b′	5′-ACGC$_{48}$	5′-TAATACGACTCACTATAGTTGATT**GCGT**GTCAATCATG-3′	P5′b′
	$_{85}$UGCG-5′	5′-GTATGTGGGCTGAACTCAATCAG**GCGT**TAATC-3′	P3′B
C/c′	5′-GACA$_{48}$	5′-TAATACGACTCACTATAGTTGATT**TGTC**GTCAATCATG-3′	P5′c′
	$_{85}$CUGU-5′	5′-GTATGTGGGCTGAACTCAATCAG**TGTC**TAATC-3′	P3′C
D/d′	5′-AGGC$_{48}$	5′-TAATACGACTCACTATAGTTGATT**GCCT**GTCAATCATG-3′	P5′d′
	$_{85}$UCCG-5′	5′-GTATGTGGGCTGAACTCAATCAG**GCCT**TAATC-3′	P3′D
E/e′	5′-GCCA$_{48}$	5′-TAATACGACTCACTATAGTTGATT**TGGC**GTCAATCATG-3′	P5′e′
	$_{85}$CGGU-5′	5′-GTATGTGGGCTGAACTCAATCAG**TGGC**TAATC-3′	P3′E
F/f′	5′-AGAC$_{48}$	5′-TAATACGACTCACTATAGTTGATT**GTCT**GTCAATCATG-3′	P5′f′
	$_{85}$UCUG-5′	5′-GTATGTGGGCTGAACTCAATCAG**GTCT**TAATC-3′	P3′F
G/g′	5′-GGCC$_{48}$	5′-TAATACGACTCACTATAGTTGATT**GGCC**GTC-3′	P5′g′
	$_{85}$CCGG-5′	5′-GTATGTGGGCTGAACTCAATCAG**GGCC**TAATC-3′	P3′G
H/h′	5′-GCGA$_{48}$	5′-TAATACGACTCACTATAGTTGATT**TCGC**GTCAATCATGG-3′	P5′h′
	$_{85}$CGCU-5′	5′-GTATGTGGGCTGAACTCAATCAG**TCGC**TAATC-3′	P3′H
I/i′	5′-AACC$_{48}$	5′-TAATACGACTCACTATAGTTGATT**GGTT**GTCAAT-3′	P5′i′
	$_{85}$UUGG-5′	5′-GTATGTGGGCTGAACTCAATCAG**GG**-3′	P3′I
J/j′	5′-CCGU$_{48}$	5′-TAATACGACTCACTATAGTTGAT**AACGG**GTCAATC-3′	P5′j′
	$_{85}$GGCA-5′	5′-GTATGTGGGCTGAACTCAATCAG**ACGG**TAATC-3′	P3′J
K/k′	5′-UCCU$_{48}$	5′-TAATACGACTCACTATAGTTGAT**GAGGA**GTCAATC-3′	P5′k′
	$_{85}$AGGA-5′	5′-GTATGTGGGCTGAACTCAATCAG**AGGA**TAATC-3′	P3′K
L/l′	5′-CUAC$_{48}$	5′-TAATACGACTCACTATAGGTTGAT**AGTAG**GTCAATCATG-3′	P5′l′
	$_{85}$GAUG-5′	5′-GTATGTGGGCTGAACTCAATCAG**GTAG**TAATC-3′	P3′L
N/n′	5′-GGCC$_{48}$	5′-TAATACGACTCACTATAGTTGAT**GGCC**GTCAATC-3′	P5′n′
	$_{85}$CCGG-5′	5′-GTATGTGGGCTGAACTCAATCAG**GGCC**TCCC-3′	P3′N
P/p′	5′-AUAC$_{48}$	5′-TAATACGACTCACTATAGTTGATT**GTAT**GTCAATC-3′	P5′p′
	$_{85}$UAUG-5′	5′-GTATGTGGGCTGAACTCAATCAG**GTAT**TAATC-3′	P3′P
S/s′	5′-GGUU$_{48}$	5′-TAATACGACTCACTATAGTTGATT**AACC**GTCA-3′	P5′s′
	$_{85}$CCAA-5′	5′-GTATGTGGGCTGAACTCAATCAG**AACC**TAATC-3′	P3′S
S2/s′2	5′-GAGA$_{48}$	5′-TAATACGACTCACTATAGGTTGATT**TCTC**GTCA-3′	P5′s′2
	$_{85}$CUCU-5′	5′-GTATGTGGGCTGAACTCAATCAG**TCTC**TAATC-3′	P3′S2
T/t′	5′-TTTT$_{48}$	5′-TAATACGACTCACTATAGGTTGATT**AAAA**TGTCAATC-3′	P5′t′
	$_{85}$AAAA-5′	5′-GTATGTGGGCTGAACTCAATCAG**AAAA**TAATCC-3′	P3′T
sud1/sud2	5′-AAAA$_{48}$	5′-TAATACGACTCACTATAGTTGATT**TTTT**GTCAATCATGGC-3′	P5′sud1
	$_{85}$T TT T-5′	5′-GTATGTGGGCTGAACTCAATCAG**TTTT**TAATCCC-3′	P3′sud2
3′ A/5′ U	5′-AACC$_{48}$	5′-TAATACGACTCACTATAGTTGATT**GGTTT**GTCAAT-3′	P5′ U
	$_{85}$UUGG-5′	5′-GTATGTGGGCTGAACTCAATCAG**TGGTT**TAATCC-3′	P3′ A
U/u′	5′-UUCC$_{48}$	5′-TAATACGACTCACTATAGGTTGATT**GGAA**GTCAAT-3′	P5′u′
	$_{85}$AAGG-5′	5′-GTATGTGGGCTGAACTCAATCAG**GGAA**TAATCC-3′	P3′U

Underlined sequences represent T7 promoter. The four bases involved in loop/loop interactions are underlined twice and in bold.

RNA activity assay with the highly sensitive ϕ29 assembly system

The activity of pRNAs was assayed with the highly sensitive ϕ29 in vitro assembly system reported previously (Lee & Guo, 1994, 1995a, 1995b). The purification of procapsids (Guo et al., 1991a, 1991b), gp16 (Guo et al., 1986), DNA-gp3, and the preparation of neck and tail protein extracts has been described previously (Lee & Guo, 1994, 1995a, 1995b). In vitro ϕ29 assembly was performed also as described previously (Lee & Guo, 1994). The activity of each RNA reported in this paper was measured as the number of plaque-forming units per milliliter produced when the total of 50 ng of pRNAs was used in the viral-assembly assay (Zhang et al., 1995b), as it is well documented that the pRNA only participates in the DNA packaging step (Guo et al., 1987c, 1991b). No experimental attempt was made to distinguish each RNA procapsid's binding ability from its DNA-packaging function. The activity is based solely on the number of plaques produced. When used in the plaque-forming assay, pRNA molecules, which produced fewer plaque-forming units per milliliter than the wild-type control pRNA, were said to have reduced activity.

TABLE 6. Primers for deletion and truncation of pRNA.

Primers used for loop deletions		
Name	Deletions	Primer sequences
P5′ m′	U_{81}, U_{85}	5′-TAATACGACTCACTATAGGTTGA**TGG**TGTCAATC-3′
P5′ q′	U_{81}, $U_{84}U_{85}$	5′-TAATACGACTCACTATAGGTTGAT**GG**GTCAATC-3′
P3′ M	$A_{41}U_{42}U_{43}$	5′-GTATGTGGGCTGAACTCAATCAG**TTGG**TCCC-3′
P3′ R1	$G_{40}A_{41}U_{42}U_{43}A_{44}$	5′-GTATGTGGGCTGAACTCAATCAG**GGTT**CCCAA-3′
P3′ R2	$G_{40}A_{41}U_{42}U_{43}$	5′-GTATGTGGGCTGAACTCAATCAG**GGTT**TCCCAA-3′
Primers for minimum size of pRNA for interaction		
Name	Bases	Primer sequences
5′ P1	11–27	5′-TAATACGACTCACTATAGGTACTTCCATTGTCAT-3′
5′ P G23	23–39	5′-TAATACGACTCACTATAGGTCATGTGTATGTTGGG-3′
3′ P2	106–89	5′-GGCACTTTTGCCATGATT-3′
3′P i′99	99–83	5′-TTGCCATGATTGAC**AAC**-3′
3′P a′99	99–75	5′-TTGCCATGATTGAC**GGAC**AATCAAC-3′
3′Pw′99	99–75	5′-TTGCCATGATTGAC**GGTT**AATCAAC-3′
3′P b′97	97–78	5′-GCCATGATTGAC**ACGC**AATC-3′
3′P i′94	94–78	5′-ATGATTGAC**AACC**AATC-3′

Underlined sequences represent T7 promoter. The four bases involved in loop/loop interactions are underlined twice and in bold.

Native TBM PAGE for dimer detection

Ten percent native polyacrylamide gels were prepared in TBM buffer (Tris, 89 mM; Boric acid, 200 mM; $MgCl_2$, 5 mM, pH 7.6). About 0.5 to 0.6 μg of total mutant RNAs in 10 μL of TBM were used for each lane. Equal molar ratios of each pRNA was applied to study the formation of dimers, while keeping the total amount of pRNA constant. After running at 4 °C for 3 h, the RNA was visualized by ethidium-bromide staining. Images were captured by an Eagle Eye II system (Stratagene).

Using binomial distribution to predict competitive inhibition

The procedure for using binomial distribution to predict competitive inhibition and determine pRNA stoichiometry in the ϕ29 in vitro assembly system with mutant pRNA has been described (Trottier & Guo, 1997; Chen & Guo, 1997a; Chen et al., 1997; Guo et al., 1998). In this work, in vitro ϕ29 assembly was performed in the presence of various ratios of active and inactive mutant pRNAs. Here "active" is defined as the competency of ϕ29 pRNA in genomic DNA packaging that subsequently leads to the formation of plaques in the in vitro assembly system. Both active and inactive mutant pRNAs should possess equal binding affinity to procapsid. To study inter-pRNA interaction, the pRNA pair A/b′ and B/a′ was used. The inactive pRNA was either pRNA A/b′ or B/a′ containing a mutation or truncation that impairs the essential packaging domain of the pRNA. As reported previously (Trottier & Guo, 1997; Zhang et al., 1997; Guo et al., 1998), pRNA B/a′ CCA, with the elimination of the essential $C_{18}C_{19}A_{20}$ bulge, would be a competitor in DNA packaging by competing with pRNA B/a′ for a procapsid binding site and served as a control for pRNA 23–97 A/b′, with bases 1–22 and 98–120 eliminated.

Since both active and inactive pRNA have equal binding affinity, distribution of procapsids, containing a certain number of each type of active pRNA and a certain number of inactive pRNA, could be calculated using the binomial

$$(p+q)^T = \binom{T}{0}p^T + \binom{T}{1}p^{T-1}q + \binom{T}{2}p^{T-2}q^2 + \cdots$$

$$+ \binom{T}{T-1}pq^{T-1} + \binom{T}{T}q^T = \sum_{M=0}^{T}\binom{T}{M}p^{T-M}q^M,$$

where p and q are the percentage of B/a′ CCA and B/a′, respectively; T is half of the total number, Z, of pRNA per procapsid (as each procapsid is proposed to have 50% A/b′ and 50% B/a′); and M is the number of B/a′ CCA bound to one procapsid. The binding of pRNA A/b′ was ignored in this calculation, as it was supposed that its binding was unaffected by the presence of pRNA B/a′ CCA. The probability calculation was extrapolated to predict the yield of plaque-forming units per milliliter produced in each in vitro ϕ29 assembly reaction. The yield of virions from empirical data was plotted and compared to a series of predicted curves to find a best fit. Predicted curves were generated by varying values for p and q. Z is presumed to be either 6 or 12.

Computer prediction of pRNA secondary structure

Secondary structures for the pRNAs were predicted by the method of Zuker (Jaeger et al., 1989; Zuker, 1989) with the Mulfold computer program retrieved from Indiana University. Only those structures with the lowest predicted energy were selected.

ACKNOWLEDGMENTS

This work was supported by grant MCB-9723923 from the National Science Foundation and GM48159 from the National Institutes of Health, USA to P. Guo.

Received February 22, 1999; returned for revision March 17, 1999; revised manuscript received March 30, 1999

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