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Use of circular permutation to assess six bulges and four loops of DNA-packaging pRNA of bacteriophage ϕ29

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ABSTRACT

A 120-base phage ϕ29 encoded RNA (pRNA) has a novel role in DNA packaging. This pRNA possesses five single-base bulges, one three-base bulge, one bifurcation bulge, one bulge loop, and two stem loops. Circularly permuted pRNAs (cpRNA) were constructed to examine the function of these bulges and loops as well as their adjacent sequences. Each of the five single-base bulges was nonessential. The bifurcation bulge could be deleted and replaced with a new opening to provide flexibility for maintaining an overall correct folding in three-way junction. All of these nonessential bulges or their adjacent bases could be used as new termini for cpRNAs. The three-base ($C_{18}C_{19}A_{20}$) bulge was dispensable for procapsid binding, but was indispensable for DNA packaging. The secondary structure around this CCA bulge and the phylogenetically conserved bases within or around it were investigated. Bases $A_{14}C_{15}U_{16}$ were confirmed, by compensatory modification, to pair with $U_{103}G_{102}A_{101}$. A_{99} was needed only to allow the proper folding of CCA bulge in the appropriate sequence order and distance constraints. Beyond these, the seemingly phylogenetic conservation of other bases has little role in pRNA activity. Each of the three stem loops was essential for procapsid binding, DNA packaging, and phage assembly. Disruption of the middle of any one of the loops resulted in dramatic reductions in procapsid binding, subsequent DNA packaging, and phage assembly activities. However, disruption of the loops at sequences that were close to double-stranded regions of the RNA did not interfere with pRNA activity significantly. Our results suggest that double-stranded helical regions near these loops were most likely not involved in interactions with components of the DNA-packaging machinery. Instead, these regions appear to be merely present to serve as a scaffolding to display the single-stranded loops that are important for pRNA tertiary structure or for interaction with the procapsid or other packaging components.

Keywords: circularly permuted RNA; cpRNA; nonessential bases of RNA; pRNA; RNA bulge; RNA–protein interaction; RNA secondary structure; viral DNA packaging; viral genome encapsidation

INTRODUCTION

The viral encoded 120-base pRNA ("p" for procapsid or packaging) of ϕ29 has been shown to be essential for encapsidation of viral genomic DNA (Guo et al., 1987b). This pRNA is transcribed from the left end of the ϕ29 genome (i.e., the end that is packaged first), binds to procapsids at the portal vertex (Guo et al., 1987a) (the site where DNA enters the procapsid), and is not present in the mature ϕ29 virion. Phylogenetic analysis of pRNAs from phages PZA, M2, NF, and SF5 (Bailey et al., 1990), as well as Cp-1 (Martin et al., 1996), shows very low sequence homology and few conserved bases, yet, the family of pRNAs appear to have similar predicted secondary structures. The requirement for pRNA in ϕ29 assembly appears to be very specific in that RNAs from other phages cannot replace the ϕ29 pRNA in in vitro packaging assays (Bailey et al., 1990), and a single-base mutation could render the inactive pRNA (Zhang et al., 1995b). Mg^{2+} induces a conformational change in the pRNA (Chen & Guo, 1997) that leads to its binding to the portal vertex. A detailed picture of pRNA function involves the solving of its structure and the determination of its specific interactions with the DNA-package machinery. ϕ29 is a beautiful system for the study of pRNA structure and function because, with the aid of the pRNA, 10^8 plaque-forming units per milliliter (pfu/mL) of infectious virions can be assembled in vitro with nine components presynthesized. Not a single background virion is detected when the pRNA is omitted (Lee & Guo, 1994, 1995b). The components involved in ϕ29 DNA packaging include the DNA-

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translocating vertex (connector or portal vertex), the DNA-packaging ATPase gp16, the DNA terminal protein gp3 (covalently linked to each 5′ end of the ϕ29 genome), and ATP. The capsid protein gp8 and the scaffolding protein gp7 may also be involved directly or indirectly in DNA packaging. The secondary structure of the pRNA has been proposed (Bailey et al., 1990) and partially confirmed (Wichitwechkarn et al., 1992; Reid et al., 1994a, 1994b, 1994c; Zhang et al., 1994, 1995b; Chen & Guo, 1997). Six copies of pRNA are needed to package one genomic DNA (Wichitwechkarn et al., 1989; Reid et al., 1994b; Trottier & Guo, 1997). DNA packaging is completely blocked when one of the six slots is occupied by one inactive pRNA with mutation at the 5′ or 3′ ends (Trottier et al., 1996; Trottier & Guo, 1997).

Analysis of pRNA secondary structure reveals that it contains five single-base bulges, one three-base bulge, one bifurcation bulge, one bulge loop, and two stem loops (Fig. 1). The bulge and loop of RNA play vital roles in intra- and intermolecular interactions (Peattie et al., 1981; Haasnoot et al., 1986; Schroeder et al., 1991; Guenther et al., 1992; Zacharias & Hagerman, 1995). This paper reports functional analysis of each of these bulges and loops, as well as their adjacent sequences, using circularly permuted pRNAs (Pan et al., 1991; Nolan et al., 1993; Zhang et al., 1995c).

RESULTS

Each of the five single-base bulges was nonessential

It has been demonstrated previously that the individual deletion of the ϕ29 pRNA single-base bulges, U_5, U_{29}, U_{35}, A_{106}, and C_{109} (Fig. 1), did not affect pRNA activities significantly in ϕ29 DNA packaging, except for a 10-fold reduction in activity when U_5 was deleted (Reid et al., 1994c; Zhang et al., 1995b). pRNAs with double or triple deletion of the single-base bulges were further constructed to test the essentiality of these bulges. Our results showed that both A_{106} and C_{109} were nonessential and could be eliminated. However, all mutants with a U_5 deletion showed slightly re-

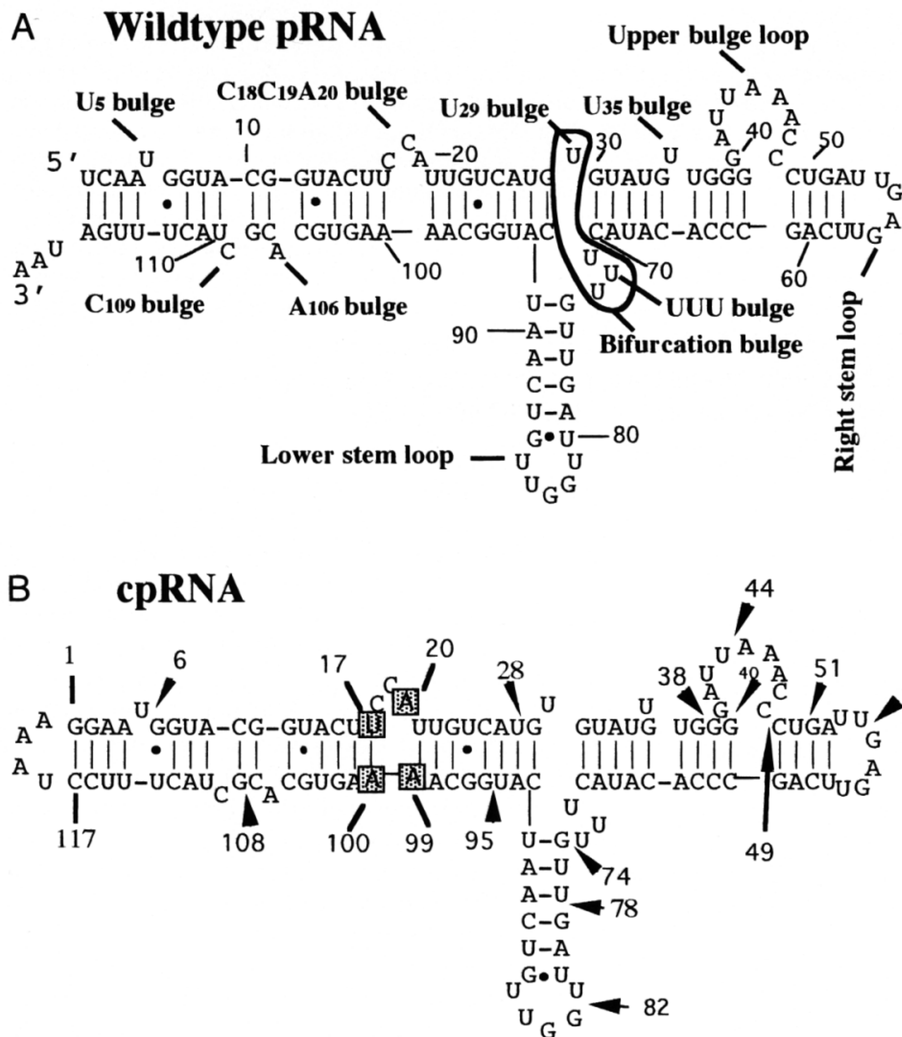

FIGURE 1. **A:** Secondary structure and sequence of wild-type pRNA indicating the location and nomenclature of the loops and bulges. **B:** Generalized circularly permuted pRNA structure with arrows indicating the location of new openings used in this paper. Numbers indicates the new 5′ end of the cpRNA. Original 5′ and 3′ ends were linked with an AAAU loop. Wild-type sequences of 5′-U_1C_2 and 3′-$A_{117}G_{116}$ were changed to G_1G_2 and $C_{116}C_{117}$, respectively, for cpRNA. Bases that seem to be conserved are shadowed.

duced activity, agreeing with our previous report (Zhang et al., 1995b) that U_5 played at least a minor role in pRNA structure and function. The locations of these nonessential bases were used as sites for new openings of active cpRNAs (see below).

Bifurcation bulge was dispensable for procapsid binding and DNA packaging

The ϕ29 pRNA bifurcation bulge consists of a one-base bulge, U_{29}, and a three-base bulge, $U_{71}U_{72}U_{73}$, located at the junction of three helices (Fig. 1). The one-base bulge, U_{29}, was shown to be nonessential for pRNA function (see above). The three-base bulge $U_{71}U_{72}U_{73}$ was deleted without loss of procapsid binding or DNA-packaging activity.

Construction of cpRNA with new termini located at or near nonessential site

Previous studies have shown that active cpRNAs can be constructed, however, the activity of the nascent cpRNAs has been variable and was dependent on the location of the new 5′/3′ termini (Zhang et al., 1995c). The nonessential bases, bulges, and regions mentioned above were tested to address the question of whether the nonessential sites can be used for the assignment of new termini to obtain active cpRNAs. Our results indicated that nonessential single-base bulges, including the UUU bulge, could serve as new termini of cpRNAs without affecting their activity (data not shown).

CCA bulge was required for DNA packaging but dispensable for procapsid binding

Phylogenetic analysis of phages ϕ29, M2, SF5, PZA, NF, and GA1 (Bailey et al., 1990) revealed the presence of a three- or four-base bulge at analogous locations in each of the respective pRNAs. The last base of the bulge was an A in each RNA. A U/A pair and an A_{99} was also seemingly conserved (Fig. 1b). The function of the $C_{18}C_{19}A_{20}$ bulge was investigated by the construction of mutant pRNA 7/GGU by inserting a $U_{100}G_{101}G_{102}$ sequence to pair with the bulge sequence, $C_{18}C_{19}A_{20}$. Insertion of these three bases causes pairing of the CCA bulge in computer secondary structural predictions, possibly accounting for the observed loss of DNA-packaging activity seen with this mutant (Fig. 2a). However, it should be noted that this RNA retains the ability to bind to the procapsid at wild-type levels (data not shown), suggesting that the CCA bulge was essential for DNA packaging but dispensable for procapsid binding. The demonstration of the essentiality of this bulge is best illustrated by mutant 108/G99, which alters the $C_{18}C_{19}A_{20}$ to a $C_{18}A_{19}U_{20}$ (Fig. 2b). This mutant lost DNA-packaging activity while retaining procapsid binding ability. Therefore, mutant pRNA 108/G99 was able to compete with wild-type pRNA for procapsid binding and block DNA packaging in inhibition assays. The observed results from these mutant pRNAs led to further investigation into the specific base requirements within the $C_{18}C_{19}A_{20}$ bulge. Mutant pRNA 38/G18 contained a mutation that changed the bulge to $G_{18}C_{19}A_{20}$, resulting in a 10-fold reduction in DNA-packaging activity (Fig. 2c), suggesting that the identity of the base in position 18 is not critical for activity of the RNA. Mutation of position 19 from C to A resulted in no alteration of the activity of the pRNA in procapsid binding or DNA packaging (Fig. 2d), further suggesting that C in this position is not absolutely required for activity. Mutational analysis of position 20, by the exchange of the A in the wild-type sequence with a G, resulted in no loss of packaging activity, again arguing against a specific requirement at this position (Fig. 2e).

Compensatory modification of bases 14–17/100–103

The 5′/3′ ends of the pRNA have been shown to exist as a helical stretch with eight base pairs (Zhang et al., 1995b). It would be interesting to know the secondary structure of the region between the helix and the CCA bulge. Is this region an extension of the helix, or a loose structure to facilitate the expansion of the bulge? A series of 12 mutant pRNAs was constructed to analyze the secondary structure in the predicted four-base pair helical region comprising bases 14–17/100–103. The wild-type sequence in this region is $A_{14}C_{15}U_{16}U_{17}/A_{100}A_{101}G_{102}U_{103}$. The first mutant constructed, pRNA 14–17/10, was a helix-disrupting mutation by changing $A_{14}C_{15}U_{16}U_{17}$ to $U_{14}G_{15}A_{16}A_{17}$. This mutant was found to have no DNA-packaging activity and a reduction in procapsid binding activity (Fig. 2f). A serious reduction in DNA-packaging activity was also observed with mutant 7/100–103, which also contained a helix disruption via a change of bases $A_{100}A_{101}G_{102}U_{103}$ to $U_{100}U_{101}C_{102}A_{103}$ (Fig. 2g). Two compensatory rescue mutants, pRNAs 14–17/100–103 and 14–16/101–103 (Fig. 2h,i), were found to restore DNA-packaging activity. Two other mutants, with changes of bases $A_{14}C_{15}U_{16}$ to $U_{14}G_{15}A_{16}$ (Fig. 2j), and $A_{101}G_{102}U_{103}$ to $U_{101}C_{102}A_{103}$ (Fig. 2k), resulted in an almost complete loss of activity for mutant pRNA14–16/10 and a 10^4-fold reduction in activity for mutant pRNA 7/101–103. These results indicate that this region was indeed helical.

It was noticeable to find that the activity of mutant 14–17/100–103 (Fig. 2h) was 100-fold lower than 14–16/101–103 (Fig. 2i). The sequences of these two mutants were the same except that, in 14–16/101–103, the U_{17}/A_{100} pair was not altered. This suggests that more than simple helical interactions were occurring in the

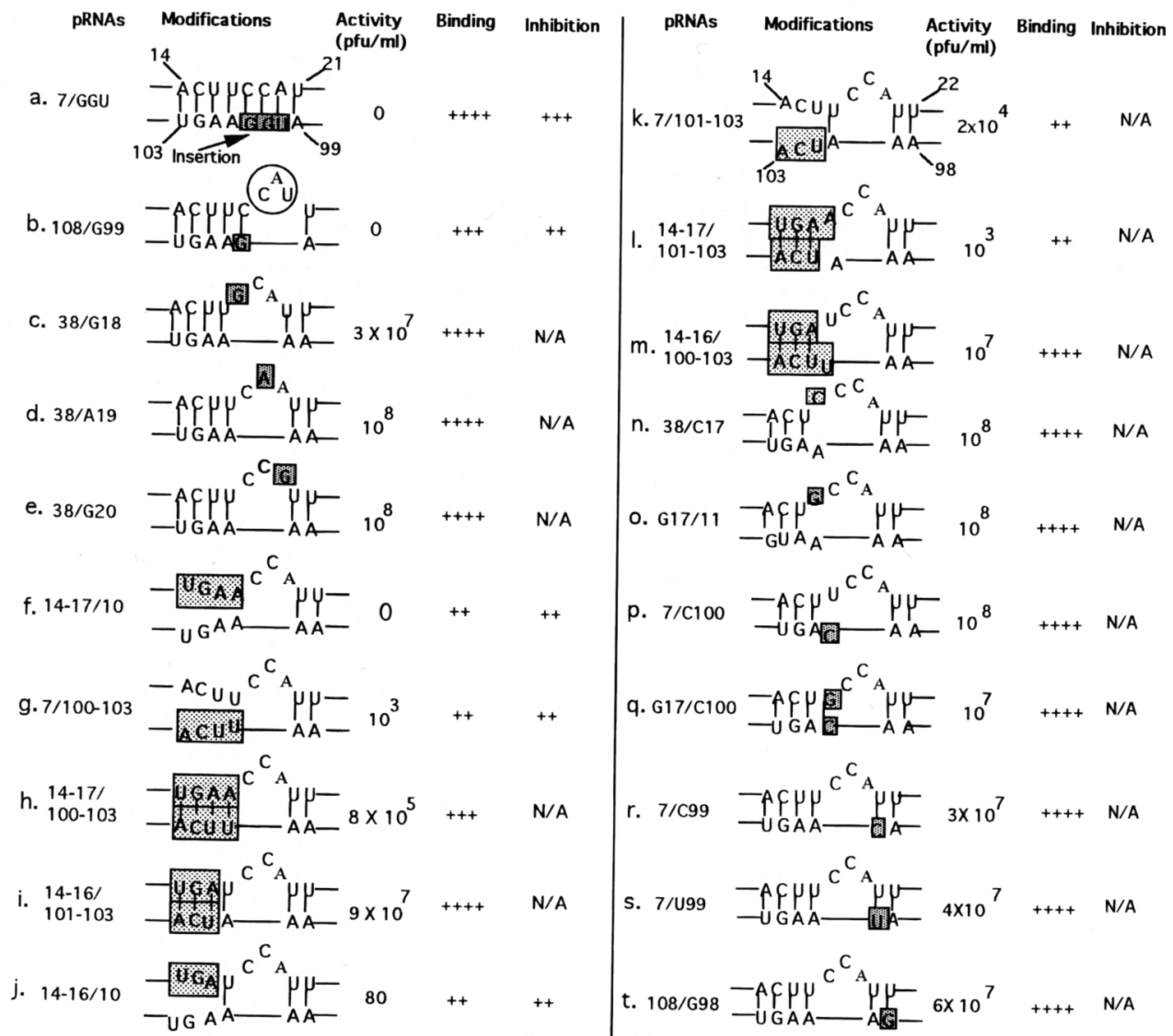

FIGURE 2. Construction of pRNAs to demonstrate the function of the CCA bugle and the seemingly conserved U_{17}/A_{100} pair. Activity of each pRNA was determined as its capacity to package ϕ29 DNA in vitro in the defined system, and the subsequent conversion of the DNA-filled capsids to infectious virions, expressed as pfu/mL. Mutated bases are placed in boxes and shaded. Relative binding indexes are displayed quantitatively with + and − signs to indicate the presence and absence of binding, respectively. The intensity of binding, when present, was graded with a series of increasing + symbols with increased binding. "++++" indicates that the procapsid binding activity of the cpRNA was very close to or equal to wild-type pRNA. "Inhibition" indicates whether the mutant cpRNA could inhibit ϕ29 assembly in vitro when mixed with wild-type pRNA. Mutant RNAs with partial or full activity could not be tested for inhibition and are labeled N/A. Shifted CCA bulge of cpRNA 108/G99 (**b**), as predicted by computer folding, was placed in a circle.

wild-type pRNA sequence. Eight additional mutant pRNAs were constructed to investigate whether the alteration of the U_{17}/A_{100} pair, which was predicted to be conserved by phylogenetic analysis, might account for this activity reduction. The sequence and secondary structure of mutants 14–17/101–103 and 14–16/100–103 (Fig. 2l,m) were similar except that U_{17} in the former was altered, whereas it was unchanged in the latter. The activity of 14–17/101–103 was reduced 10^4-fold in comparison with 14–16/100–103. These data, combined with the poor activity of pRNA 14–17/100–103 (Fig. 2h) and the high activity of mutant 14–16/101–103 (Fig. 2i), led to the observation that uridine in position 16 or 17 was preferable to adenine. Analysis of the activity of 38/C17, G17/11, 7/C100, and G_{17}/C_{100} (Fig. 2n,o,p,q) led to the conclusion that pairing of bases 17/100 was not essential for DNA-packaging activity. Substitution of U_{17} with either guanine (pRNA G17/11) or cytosine (pRNA 38/C17) did not interfere with pRNA activity, indicating that the U at this position was not conserved as predicted previously.

Analysis of the predicted phylogenetically conserved bases A98 and A99

The potential roles of A_{98} and A_{99} were investigated because an adenine has been found to be present in

similar locations of all members of the pRNA family (Fig. 1b) (Bailey et al., 1990). Substitution of A_{99} (Fig. 2r,s) with either C (pRNA 7/C99) or U (pRNA 7/U99) did not interfere with pRNA activity, indicating that the base at this position was not conserved. Interestingly, substitution of A_{99} with G (pRNA 108/G99) (Fig. 2b) resulted in a mutant pRNA that was completely inactive in ϕ29 assembly, although the procapsid binding activity was close to that of wild-type pRNA. G could not serve as a substitute for $_{A99}$, perhaps due to a disruption of local folding of this region, as seen in computer predictions, leading to a reorganization of the CCA bulge to a CAU bulge (pRNA 108/G99 in Fig. 2b). These data strongly suggests that A_{99} plays no special role in the RNA function, other than perhaps to pair with U_{21}. The conservation of A_{99} appears to be needed for maintaining the $C_{18}C_{19}A_{20}$ bulge only, because active cpRNAs, with A_{99} changed to U or C, have been obtained. Based on the data obtained from mutants pRNA 108/G98 (Fig. 2t), along with computer-predicted secondary structures, the conservation of A_{98} also appears to be important in maintaining the $C_{18}C_{19}A_{20}$ bulge only, because an active cpRNA, with A_{98} changed to G, has been obtained. The predicted secondary structure of this mutant is similar to that of wild-type pRNA.

CpRNAs with any one of the three large loops disrupted or truncated were defective in both procapsid binding and DNA packaging

The three remaining large loops were also analyzed by mutagenesis. The "upper" bulge loop, consisting of the single-stranded region of the pRNA from bases 40 to 48, is the largest loop in the pRNA and has been proposed to be involved in a pseudoknot with the lower loop comprising bases 82–85 (Reid et al., 1994c). This loop is also part of the pRNA molecule that is known to interact with the procapsid based on RNAse footprinting (Reid et al., 1994b), and should therefore have a significant role in procapsid binding, and thus packaging activity, of the pRNA. A cpRNA with a deletion of the entire loop, mutant 49/39 (Fig. 3g), results in a complete loss of procapsid binding and DNA-packaging activity. Two mutant pRNAs, which have disruptions in the upper bulge loop by either generating a new opening within the loop or deleting or mutating bases in the interior of the loop, pRNAs 40/37 and 44/43 (Fig. 3e,f), showed complete losses in procapsid binding and DNA-packaging activities. Mutant pRNA 55/54 (Fig. 3l), which contained a disruption of the interior of the "right" loop, at positions 53–58, by generating an opening at position 54, showed a significant drop in packaging activity (10^5-fold) and procapsid binding ability. It appears that the sequence of the loop region is important, as is the continuity of the interior of the loop, because new openings within loops were not tolerated.

The lower loop of the pRNA, consisting of a five-base loop (positions 81–85) at the end of a six-base helical stem, was also analyzed. Mutant cpRNA 82/81 (Fig. 4a), in which the lower loop was disrupted, showed a reduction of procapsid binding and DNA-packaging activity. CpRNA 95/71 (Fig. 4c), which completely deleted the entire lower stem and loop structure as well as the $U_{71}U_{72}U_{73}$ bulge, showed a complete loss of DNA-packaging and procapsid binding activities. A second mutant cpRNA, 95/74 (Fig. 4d), contained a deletion of the entire stem loop, but retained the $U_{71}U_{72}U_{73}$ bulge. This pRNA was also inactive in DNA packaging. A third mutant, pRNA 95/81 (Fig. 4e), had a deletion of the lower bulge and the 3′ portion of the helical stem spanning positions 82–94. This RNA was also found to be inactive in DNA packaging. A mutant RNA, 82–71, was generated that had deletion of one base of the lower loop, the 5′ portion of the helical stem, and the $U_{71}U_{72}U_{73}$ bulge. RNA 82–71 was found to have no DNA-packaging activity (Fig. 4f). The final mutant generated in this region, 82/77 (Fig. 4g), contained a deletion of a small portion of the helical stem on the 5′ end, as well as a deletion of the first U of the lower loop. This mutant was found to function in DNA packaging at a 10^4-fold reduction in activity. Procapsid binding of this mutant was also reduced.

CpRNAs with termini located adjacent to the three large loops were competent for procapsid binding and DNA packaging

A series of mutant cpRNAs, 38/37, Ud38/37, 40/39, 49/48, 51/50 (Fig. 3b,c,d,i,j), and 78/77 (Fig. 4b), was generated with openings or mutations in the paired regions adjacent to the loop structures. All of these RNAs were active, although some had slightly reduced DNA-packaging activity. Toleration of disruption at the end of the loops (the "foundation") suggests that the openings at the base of the loops have little affect in the overall tertiary structure of the pRNA, probably by still providing a single-stranded loop region to form pseudoknots or to interact with portal protein gp10. It also suggests that the double-stranded stem(s) around these loops were not involved in external or internal molecular interactions (aside from being part of the helix), but merely serve to assist the loop to maintain its single-stranded status.

Analysis of bases involved in the predicted pseudoknot formation

Three cpRNAs, 38/37, Ud38/37, and 40/39 (Fig. 3b,c,d), with new termini located at different locations, were constructed. Comparison of the predicted secondary structures showed that the pairing around the upper

cpRNAs	Modifications	Description	Activity (pfu/ml)	Binding	Inhibition
a. pRNA		wild type	10^8	++++	N/A
b. 38/37		Opening at the stem left to upper loop	10^8	++++	N/A
c. Ud38/37		Same as above except with C39C40G47G48 mutation	4×10^7	++++	N/A
d. 40/39		Opening at left margin of upper loop with deletion of G38G39	10^8	++++	N/A
e. 40/37		Same as above except deletion of G38G39	0	-	-
f. 44/43		Disruping upper loop	0	-	-
g 49/39		Same as above except with deletions of G40A41U42U43	0	-	-
h. 44/39		Delete upper loop	0	-	-
i. 49/48		Opening at right margin of upper loop with G49 and C62 mutation	3×10^4	++	N/A
j. 51/50		Opening at stem right to upper loop	2×10^5	+	-
k. 51/48		Deletion of C49U50	0	-	-
l. 55/54		Disrupting right loop	10^3	+	N/A

FIGURE 3. Construction of cpRNA for functional analysis of both the upper and right loops. "5′- and 3′-" marks the location of the new termini of each cpRNA. Activity, binding, and inhibition data are as described in Figure 2. "−" indicates negative results. Construction of mutant pRNA is summarized in the Description column. The Modification column indicates the predicted computer-predicted folding of the mutant pRNAs.

cpRNAs	New ends	Deletions	Activity (pfu/ml)	Binding	Inhibition
a. 82/81	5' G82 / 3' U81	No deletion	4×10^6	+++	N/A
b. 78/77	5' G78 / 3' U77	No deletion	5×10^7	++++	N/A
c. 95/71	5'G95 / 3'C71	U72 - U94	0	-	-
d. 95/74	5'G95 / 3'U74	G75 - U94	0	-	-
e. 95/81	5'G95 / 3'U81	G82 - U94	0	-	-
f. 82/71	5'G82 / 3'C71	U72 - U81	0	-	-
g. 82/77	5'G82 / 3'U77	G78 - U81	$5\mathrm{X}10^3$	+	N/A

FIGURE 4. Construction of cpRNA for functional analysis of the lower stem loop. Activity, binding, and inhibition are the same as defined in Figure 2.

loop and the right loop were reorganized, and the folding of these three cpRNA were predicted to be different from that of wild-type pRNA. However, all of these cpRNAs had procapsid binding and DNA-packaging activities close to that of wild type.

The upper loop is believed to interact with the lower loop to form a pseudoknot as predicted by phylogenetic analysis and shown by compensatory mutations (Reid et al., 1994c). Alignment of the upper loop sequences with the lower loop sequences of these cpRNAs (Fig. 5) revealed that, although the sequences were altered, base pairing was still available in each of these cpRNAs. This indicated that the potential for pseudoknot formation still existed. The original sequence of the upper loop that is believed to be involved in pseudoknot formation is AACC. In cpRNA Ud38/37 (Fig. 3c), the sequence $A_{45}A_{46}C_{47}C_{48}$ was changed to $A_{45}A_{46}G_{47}G_{48}$. Pseudoknot formation should have been disturbed because 5′-AAGG cannot pair with 3′-$U_{85}U_{84}G_{83}G_{82}$ of the lower loop. However, this mutant was as active as wild-type pRNA. Sequence analysis revealed that the sequence 3′-$U_{84}G_{83}G_{82}U_{81}$ of the lower loop could pair with either 5′-GCCA or AUUA of the upper loop of Ud38/37 (Fig. 5c). If this was the case, it would suggest that $A_{45}A_{46}C_{47}C_{48}$ was not critical for base pairing in this pseudoknot, because the upper loop potentially could refold to provide different complementary sequences that can still form a pseudoknot. Although all active mutant cpRNA fulfilled the base pairing requirement for pseudoknot formation, it appears that base pairing to form the pseudoknot was not the only factor required for the activity of cpRNA. As shown in Figure 3e, mutant 40/37 also possesses the loop sequence needed for base pairing, but was inactive in procapsid binding and DNA packaging. Whether the inactivity was due to the additional deletion present in this mutant remains to be investigated.

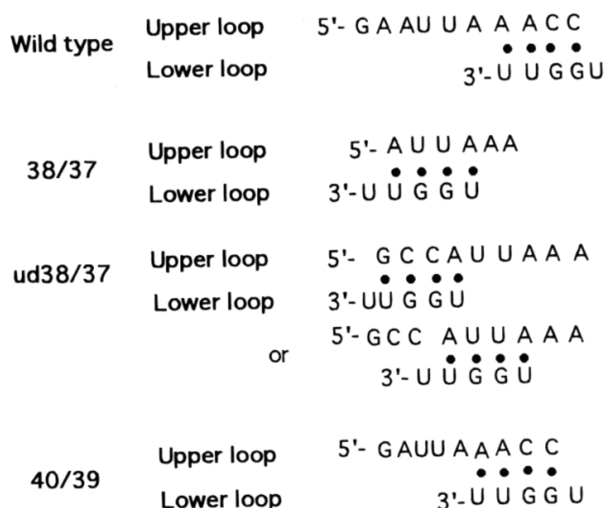

FIGURE 5. Proposed base pairing of the two potential pseudoknot-forming loops of cpRNA.

DISCUSSION

Deletion mutagenesis has been a popular technique for the study of RNA structure and function concerning the essentiality of certain bases, regions, or domains. Generally, mutant RNA is constructed with the deletion of the area to be investigated, without alteration of the original 5′ and 3′ termini. Although mutagenesis is a powerful technique, the ability to generate RNA mutants far from the ends of a sequence has been difficult using standard PCR-based mutagenesis systems, and has led to the development of cpRNA with openings in new areas. The first consideration in cpRNA construction is the location of the new termini. To use cpRNAs for the study of RNA structure and function, the new opening should not interfere with cpRNA folding and biological activity. This report showed that cpRNAs with new termini located at nonessential sites or its adjacent bases were active in ϕ29 DNA packaging, whereas cpRNAs with new termini located near essential sites were inactive or had greatly reduced activity.

By using cpRNAs with openings at nonessential sites, we have been able to examine the role of the five single-base bulges, the bifurcation bulge, the CCA bulge, the bulge loop, and two stem loops in the pRNA predicted secondary structure. All five single-base bulges have been found, in this publication and previous work (Reid et al., 1994c; Zhang et al., 1995b), to be nonessential for activity. The three large loops, the bulge loop and two stem loops, were shown to be absolutely required for procapsid binding and DNA-packaging activity, in agreement with the finding reported previously (Wichitwechkarn et al., 1992; Reid et al., 1994a, 1994c). Interestingly, the sequences and helical nature of the stems

and the foundation of these loops were not required for activity, suggesting that the specific bases of the loop can maintain the appropriate tertiary arrangement without these regions, and that the stems were only needed to ensure the correct folding of the loops.

The CCA bulge has been shown to be required for packaging activity, but not for procapsid binding, in agreement with the previous finding with alternative approach (Reid et al., 1994a). This observation leads us to speculate a role for this loop in contacting a yet to be determined component of the DNA-packaging machinery.

The bifurcation polyuridine loop was also found to be of interest due to the unusual and conflicting results obtained by previous deletion mutagenesis and our cpRNA-based deletion mutants. Previously, D. Anderson's group has shown that deletions of the UUU bulge have led to RNAs that were inactive for packaging activity and binding (Reid et al., 1994c). The data presented here, however, show that the UUU bulge can be deleted with little affect on RNA activity. Our current hypothesis to explain this discrepancy is that the earlier deletion mutants did not alter the location of the 5′ and 3′ ends of the RNA, whereas our cpRNAs had new termini at the site of the deletion. The bifurcation UUU loop is present as a small bulge at the point of a predicated three-way helix junction, and may provide additional flexibility in the orientation of the helices into the correct spatial orientation. Earlier work (Reid et al., 1994c) has postulated the role of the UUU loop to be similar to the unpaired bases at the I, II, and V helix junction in 5S rRNA (Baudin et al., 1996). We feel that the deletion of this hinge region led to a loss of activity in earlier mutants due to the loss of the correct spatial arrangement of the helices in the junction region, not by the loss of tertiary interactions of these bases with other sequences. It is our belief that the placing of new termini in the region of our deletion has provided the same flexibility, through discontinuity of the phosphodiester bond, as was provided by the small hinge-like UUU bulge. Our results suggest that the location of the new cpRNA opening possibly provides additional flexibility in this region that is lacking in previous deletion studies. It would appear that a loop that imparts RNA flexibility is important at this location, but a new break in the phosphodiester backbone of the RNA can substitute for this flexible loop region. This hypothesis, if valid, leads to an interesting caveat for deletion mutagenesis and cpRNA based mutagenesis, that is, that the location of new openings, although not altering the sequence of the RNA, may provide the required flexibility to maintain an overall correct folding.

MATERIALS AND METHODS

Methods for pRNA synthesis (Zhang et al., 1994, 1995c), circularly permuted pRNA construction (Zhang et al., 1995c), binding assay (Trottier & Guo, 1997), inhibition assay (Zhang et al., 1995a; Trottier et al., 1996; Trottier & Guo, 1997), and secondary structure prediction (Zuker, 1989) have been described previously. The activity of pRNAs and cpRNAs was assayed with the highly sensitive ϕ29 in vitro assembly system reported previously (Lee & Guo, 1994, 1995a, 1995b). The sequences of mutant cpRNAs were confirmed by primer extension. We have been able to assemble infectious ϕ29 in vitro using cloned gene products as well as synthetic pRNA and genomic DNA-gp3 (Salas, 1991; Blanco et al., 1994). The pRNA transcribed in vitro was attached to purified procapsids (Guo et al., 1987a, 1991a,b; Chen & Guo, 1997) in the prescience of Mg^{2+}. Genomic DNA was packaged subsequently into the RNA-enriched procapsids with the aid of ATP and the purified DNA-packaging enzyme (gp16) (Guo et al., 1986). The DNA-filled capsids were converted into infectious virions in vitro with the addition of neck and tail protein gp9, gp11, and gp12, as well as the morphogenic factor (gp13), which were also produced from cloned genes. Typically, 10^7–10^8 pfu/mL were obtained with wild-type pRNA, and omission of any one of the components resulted in no plaque formation.

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