VIROLOGY 207, 442–451 (1995)

Circularly Permuted Viral pRNA Active and Specific in the Packaging of Bacteriophage ϕ29 DNA

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Received October 25, 1994; accepted December 23, 1994

A viral-encoded 120-base pRNA has been shown to have an essential role in the packaging of bacteriophage ϕ29 DNA. The finding that both the 5′- and 3′-termini of the pRNA are proximate and crucial for biological function (C. Zhang, C. Lee, and P. Guo, 1994, *Virology*, 201, 77–85) prompted investigation of the activity of circularly permuted pRNAs (cpRNA) and of the expandability and essentiality of bases extending from the termini. A 117-base pRNA with a deletion of three bases downstream of the proximal terminus was active in DNA packaging. Concatemeric DNAs containing two tandem pRNA genes separated by a short or a long loop sequence were constructed. The cpRNAs from these DNA templates were transcribed *in vitro* and shown to be active in ϕ29 DNA packaging, with activity comparable to the parental (noncircularly permuted) pRNA, indicating that neither of the loops tested affected the activity and folding of the cpRNA. As few as four bases were sufficient to serve as a loop for the terminal 180° turn, and a loop as long as 27 bases did not affect the cpRNA structure and function. Eight cpRNAs were constructed to assess the effect of openings within the wild-type pRNA structure. Opening of the bulge at residue 38 did not affect cpRNA activity, but opening the bulge at residue 55 greatly reduced it. Although the sequence of the 5′,3′-terminal loop was not important for the folding and activity of the cpRNA, the activities of cpRNAs with openings at individual bulges or hairpins were different, indicating that each region plays a different role in pRNA folding and function. Our results indicate that it is possible to generate active circularly permuted pRNA by assigning internal sites of the pRNA as new 3′- and 5′-termini. The creation of new variable ends makes the labeling of internal bases of the pRNA molecule possible and will facilitate the analysis of pRNA secondary and tertiary structure.

INTRODUCTION

Common features have been found in the assembly of the dsDNA viruses, including adenovirus, herpesvirus, poxvirus, bacteriophages T1, T3, T4, T5, T7, P1, P2, P22, Mμ, ϕ21, ϕ29, SP01, SPP1, λ and their relatives (For review see Anderson and Bodley, 1990; Anderson and Reilly, 1993; Earnshaw and Casjens, 1980; Black, 1989; Bazinet and King, 1985; Guo, 1994; Guo and Trottier, 1994). Bacteriophage ϕ29 of *Bacillus subtilis* is typical of dsDNA viruses in that its genome is inserted into a preformed procapsid during maturation (Bjornsti *et al.*, 1981; Guo *et al.*, 1986, 1993). The viral encoded 120-base pRNA ("p" for packaging) of ϕ29 has been shown to have a novel and essential role in the DNA packaging process of this phage (Guo *et al.*, 1987b). The pRNA is transcribed from the left end of the ϕ29 genome (i.e., the end that is packaged first) (Guo *et al.*, 1987b), binds to procapsids at the portal vertex (Guo *et al.*, 1987a), the site where DNA enters the procapsid, and is released from the portal vertex after DNA-packaging is complete. The procapsid binding domain of the pRNA has been identified (Reid *et al.*, 1994b). Recent evidence has shown that mutagenesis of as few as 2 bases at the 5′-end can reduce the pRNA activity 10^5-fold, and a 4-base mutation at the 5′-end renders the molecule inactive (Zhang *et al.*, 1994). The 5′- and the 3′-ends have been proven to be proximate and compose one domain crucial for DNA packaging (Zhang *et al.*, 1994).

Currently, the exact role of this pRNA in DNA packaging is unclear. Bacteriophage ϕ29 is an ideal system to study such an intriguing molecule due to the availability of an efficient, defined *in vitro* packaging system (Guo *et al.*, 1986) as well as a highly sensitive system for the assay of pRNA activity (Lee and Guo, 1994). The assembly of infectious ϕ29 virions *in vitro* with protein components produced from cloned genes (Guo *et al.*, 1991a,b; Lee and Guo, 1994) and with pRNA transcribed *in vitro* (Reid *et al.* 1994b; Zhang *et al.*, 1994) greatly facilitates the study of pRNA.

A detailed picture of pRNA function involves the solution of its structure and the determination of its specific interactions. The secondary structure of the pRNA has been proposed (Bailey *et al.*, 1990). However, a defined tertiary structure has not been elucidated, although evidence for a pseudoknot structure has been reported

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0042-6822/95 $6.00

TABLE 1

PCR Primers

pRNA	Direction	Oligonucleotide	Size (bases)	Primer location at pRNA	Primer name
cpRNA38	Forward	5′ [a]ATTTAGGTGACACTATAGGGATTAAACCCTGATT 3′	34	38–54	P38
	Reverse	5′ CAACATACACATGACAA 3′	17	37–21	P37
cpRNA55	Forward	5′ [a]ATTTAGGTGACACTATAGAGTTCAGCCCACATAC 3′	34	55–71	P55
	Reverse	5′ AATCAGGGTTTAATCCC 3′	17	54–38	P54
cpRNA78	Forward	5′ [a]ATTTAGGTGACACTATAGATTGGTTGTCAATCAT 3′	34	78–94	P78
	Reverse	5′ AACAAAGTATGTGGGCT 3′	17	77–61	P77
cpRNA82	Forward	5′ [a]ATTTAGGTGACACTATAGGTTGTCAATCATGGCA 3′	34	82–98	P82
	Reverse	5′ AATCAACAAAGTATGTG 3′	17	81–65	P81
pRNA7/10	Forward	5′ [b]TAATACGACTCACTATAGCAATGGT 3′	25	1–8	P7
	Reverse	5′ GCAAAGTAGCGTGCACTTTTG 3′	21	117–96	P10
pRNA7/11	Reverse	5′ TTAGCAAAGTAGCGTGCACTTTTG 3′	24	120–96	P11
pRNA8/10	Forward	5′ [b]TAATACGACTCACTATAGGGGTGGTAC 3′	27	1–10	P8
pRNA19/11	Forward	5′ AAAGGAATGGTACGGTACTT 3′	20	1–17	P19

[a] SP_6 promoter.
[b] T_7 promoter.

(Reid *et al.*, 1994b). Pinpointing specific interactions of this molecule is limited by the ability to uniquely label only the 5′- and 3′-ends with radioactivity or photoaffinity agents.

Utilizing 5′- to 3′-end ligation, Pan *et al.* (1991) were able to construct circular tRNA which was subsequently cleaved by limited alkaline hydrolysis to generate one random break per molecule. Correctly folded tRNAs have been identified by lead-catalyzed cleavage at neutral pH (Pan *et al.*, 1991). More recently, Nolan *et al.* (1993) reported the mapping of tRNA binding sites on RNase P by specific photoaffinity probes of tRNAs with native 5′- and 3′-ends linked by a synthetic loop and new termini in the interior of the native sequence (Nolan *et al.*, 1993). The feasibility of constructing circularly permuted RNAs rests on the close proximity of the native RNA 5′- and 3′-ends. The ϕ29 pRNA has such proximal 5′- and 3′-ends (Zhang *et al.*, 1994), hence a series of pRNA molecules with circular permutations were constructed. This report indicates that biologically active circularly permuted cpRNAs can be produced by *in vitro* synthesis with SP_6 RNA polymerase and concatemeric DNA substrates.

MATERIALS AND METHODS

Oligonucleotides

Thirteen oligonucleotides were used as PCR primers to amplify DNA fragments for synthesis of pRNAs in this study (Table 1). Primer P7 and P8 contain the T_7 promoter and primer P38, P55, P78, and P82 contain the SP_6 promoter.

Synthesis of mutant pRNAs

Mutant pRNAs were constructed as described previously (Zhang *et al.* 1994). Briefly, linear plasmid DNAs were used as templates to generate PCR DNA fragments (Guo *et al.*, 1994; Scholz *et al.*, 1995) with primer pairs P7/P11, P7/P10, P8/P10, and P8/P11 (Table 1), respectively. The purified PCR DNA fragments were used as templates to synthesize pRNA 7/11, 7/10, 8/10, and 8/11, respectively, by *in vitro* transcription with T_7 RNA polymerase (Milligan *et al.*, 1987; Rosenberg *et al.*, 1987). The pRNA from PCR template produced with primer pairs P7 and P11 was called mutant pRNA7/11, and other pRNAs derived from different primer pairs were named in a similar fashion. Mutant pRNA7/174 was transcribed from PCR DNA fragment produced with primer pairs P7/P174 on pRT71 template (Reid *et al.*, 1994a), with an additional digestion of the PCR fragment with *Bgl*II before transcription.

Construction of plasmid cpDNA3A and cpDNAT_7

To generate plasmid cpDNAT_7, a PCR DNA fragment was produced with primer pair P7/P11, using linearized plasmid pRT71 as a template. The amplified fragments were separated in 2%-equivalent agarose/synergel and purified by Qiaex (Qiagen), phosphorylated with T4 polynucleotide kinase, and subsequently repurified by Qiaex. The PCR fragment was then cloned into plasmid pRT71. Cloning was achieved by digesting pRT71 with *Bgl*II, converting the sticky ends to blunt ends by treatment with Mung bean nuclease, and treating the blunt ends with alkaline phosphatase prior to ligation. The resulting plasmid, cpDNAT_7, contained two pRNA genes separated by a T_7 promoter. Construction of plasmid cpDNA3A was accomplished with the same strategy as in the construction of plasmid cpDNAT_7, except the use of primer pair P19/P11 instead of P7/P11. The presence of the tandem pRNA gene was confirmed by DNA sequencing.

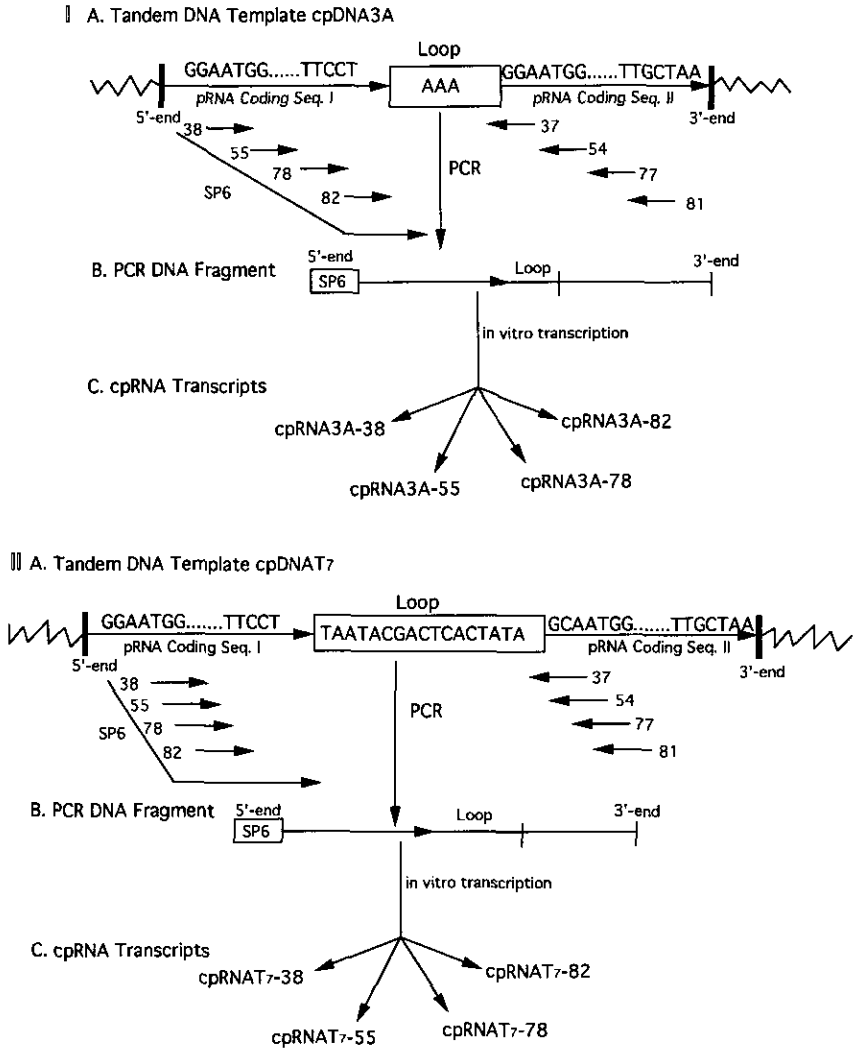

FIG. 1. Construction of tandem DNA templates for the synthesis of cpRNAs. Plasmids cpDNA3A (I) and cpDNAT$_7$ (II) containing a tandem pRNA coding sequence were connected by 3- or 17-nucleotide synthetic loops, respectively. Four forward primers P38, P55, P78, and P82, which consisted of a SP$_6$ promoter (17-nucleotide) followed by residues 38–54, 55–71, 78–94, and 82–98 of the pRNA gene, and four reverse primers complementary to residues 37–21, 54–38, 77–59, and 81–65 of the pRNA gene were used to generate PCR fragments. The PCR DNA fragments were directly used as templates for *in vitro* transcription with SP$_6$ RNA polymerase. Four cpRNAs, cpRNA3A-38, cpRNA3A-55, cpRNA3A-78, and cpRNA3A-82, were generated from the DNA template cpDNA3A. Four other cpRNAs, cpRNAT$_7$-38, cpRNAT$_7$-55, cpRNAT$_7$-78, and cpRNAT$_7$-82, were synthesized from the DNA template cpDNAT$_7$.

Synthesis of cpRNAs

Linear plasmids containing cpDNA3A and cpDNAT$_7$ were used as templates for the production of PCR DNA fragments with primer pairs P38/P37, P55/P54, P78/P77, and P82/P81 (Table 1), respectively. The purified PCR DNA fragments were used as templates to synthesize cpRNAs by *in vitro* transcription with SP$_6$RNA polymerase. Circularly permuted pRNAs, cpRNA3A-38, cpRNA3A-55, cpRNA3A-78, and cpRNA3A-82, were derived from the DNA template cpDNA3A (Fig. 1-I) containing the tandem RNA gene coding for parental pRNA pMbn (Zhang *et al.*, 1994). Circularly permuted pRNAs, cpRNAT$_7$-38, cpRNAT$_7$-55, cpRNAT$_7$-78, and cpRNAT$_7$-82, were derived from the DNA template, cpDNAT$_7$ (Fig. 1-II), containing the tandem RNA gene coding for parental pRNAP7/174. The pRNA from the PCR DNA fragment produced with primer pairs P38 and P37 on cpDNA3A template was called cpRNA3A-38, additional pRNAs were named in a similar manner.

Purification of the recombinant procapsid, DNA-gp3, and gp16

The purification of gp16 (Guo *et al.*, 1986) and DNA-gp3 (Lee and Guo, 1994) has been described previously. Procapsids were overproduced and purified from *Escherichia coli* with a procedure derived from previously reported methods (Guo and Moss, 1990; Guo *et al.* 1991a,b).

RNA activity assay with the ϕ29 assembly system

The construction of the highly sensitive ϕ29 assembly system has been reported (Lee and Guo, 1994). Briefly, extracts were prepared from *E. coli* HMS174 (DE3)/pLysS containing plasmid pAR8.5-9 and pAR11-12-13-14. Overnight cultures of these bacteria were diluted 1:100 in LB broth containing 50 μg/ml ampicillin and 25 μg/ml chloramphenicol. The diluted cultures were grown for 3 hr at 37° and the T_7 promoter in the plasmids was induced with IPTG at a final concentration of 0.5 m*M*. After incubating for another 3 hr at 37°, the cells were sedimented and resuspended in reaction buffer (10 m*M* ATP, 6 m*M* spermidine, 3 m*M* β-mercaptoethanol, 50 m*M* Tris–Cl, pH 7.8, 10 m*M* $MgCl_2$, and 100 m*M* NaCl) equal to 1/50 of original culture volume. A single freeze–thaw step lysed the suspended bacteria, as they contained lysozyme produced by the plasmid pLysS. These lysed extracts were used for biological assay of phage assembly (see below).

To attach the pRNA to procapsids, 5 μl of purified procapsids were mixed with 1 μl pRNA (0.15 mg/ml) and dialyzed on a 0.025-μm type VS filter membrane (Millipore Corp.) against TBE (89 m*M* Tris–HCl, pH 8.3, 89 m*M* boric acid, 2.5 m*M* EDTA) for 15 min at ambient temperature and then against TMS for 30 min at ambient temperature. To package DNA-gp3, the pRNA-enriched procapsids were mixed with 3 μl reaction buffer, DNA-gp3 that had been dialyzed against TMS for 40 min at ambient temperature, and DNA-gp3 packaging protein, gp16, that had been dialyzed against 0.01 *M* Tris–Cl, pH 7.5, and 0.04 *M* KCl for 40 min on ice (Guo *et al.*, 1986). The final mixture was then incubated for 30 min at ambient temperature.

The DNA-filled procapsids were incubated with 18 μl of extract from *E. coli* HMS174(DE3)/pLysS containing plasmid pAR8.5-9 and 20 μl of extract from *E. coli* HMS174(DE3)/pLysS containing plasmid pAR11-12-13-14 for 2 hr at ambient temperature.

The activity of each RNA reported in this paper was measured by the number of plaque forming units per milliliter produced when the RNAs were used in the above assay. No experimental attempt was made to distinguish each RNAs procapsid binding ability from DNA-packaging function, etc. The activity is based solely on the number of plaques produced, in comparison to a control pRNA. RNA molecules which produced fewer PFU/ml than the control RNA, when used in the plaque forming assay, were said to have reduced activity.

Computer prediction of pRNA and cpRNA secondary structure

Secondary structures for the pRNAs and cpRNAs were predicted by the method of Zuker (1989) with the GCG program (Wisconsin), and only those structures with the lowest predicted energy were selected.

RESULTS

Mutagenesis of the 5′/3′ proximal region of the pRNA

Our previous work demonstrated that the 5′- and 3′-ends of the pRNA are proximate, and the 5′/3′ proximal region is crucial for ϕ29 DNA packaging (Zhang *et al.*, 1994). The pRNA secondary structure prediction reveals three additional unpaired bases, $U_{118}A_{119}A_{120}$, extending from the 3′-end of the paired region (Fig. 2). To construct circularly permuted pRNAs and design a loop connecting the 5′- and the 3′-ends, it was critical to determine if these three bases beyond the 5′/3′ proximal region were essential for pRNA function.

Mutants pRNA 7/11, 7/10, 8/10, 8/11, and 7/174 were synthesized by *in vitro* transcription with T_7 RNA polymerase and PCR DNA fragments, containing pRNA coding sequences, as templates. The biological activities of five mutant pRNAs were tested by a defined *in vitro* DNA packaging system (data not shown) and a highly sensitive phage assembly system (Lee and Guo, 1994). With mutant pRNA 7/11, the first base, U_1, at the 5′-end was changed to G_1, and the corresponding base at the 3′-end was changed from A_{117} to the complementary base, C_{117} (Fig. 2). Mutation of this base pair at the proximal region resulted in a pRNA with activity similar to control pRNA7/4 (Fig. 2).

The sequence of pRNA 7/10 was identical to pRNA 7/11 with the exception of the deletion of three bases extending from the 3′-end of the proximal region (Fig. 2). This 117-base pRNA was as active as the positive control pRNA 7/4 in phage assembly, indicating that the three bases beyond the proximal region are not essential (Fig. 2).

Mutations were further introduced into the 5′/3′ proximal region by the construction of pRNA8/11 (120-base) and pRNA8/10 (117-base). In comparison to pRNA7/11 and pRNA7/10, three additional base mutations, $C_2A_3A_4$ to $G_2G_3G_4$, were introduced into pRNA8/11 and pRNA8/10 (Fig. 2). These additional mutations resulted in two mutant pRNAs with no detectable activity in phage assembly (Fig. 2), supporting our previous conclusion that the 3′/5′ proximal region is crucial for viral DNA packaging and that the pRNA is highly specific.

Mutant pRNA7/174 was 124 bases in length and identical to pRT71 (Reid *et al.*, 1994a), which has been shown to be fully active in viral DNA packaging (Zhang *et al.*, 1994), except for the change of base G_{116} to C_{116}. One additional base mutation at this location resulted in a mutant pRNA with DNA-packaging activity reduced 10^4-fold in comparison to pRNA 7/11 (Figs. 2 and 3), again supporting our previous conclusion regarding the specificity of the pRNA and the necessity for the proximal 3′/5′ ends.

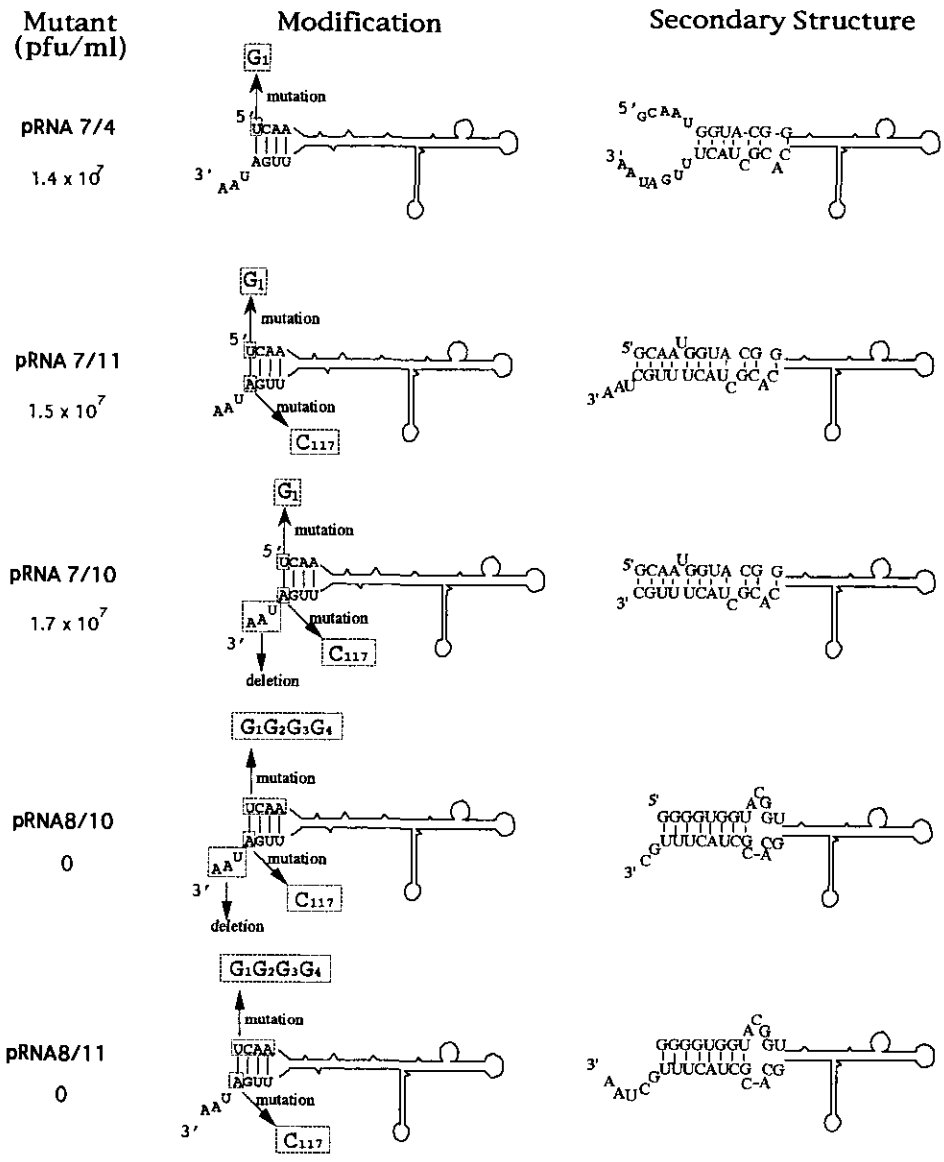

FIG. 2. The modification, secondary structure, and biological activity of five mutant pRNAs.

Synthesis of cpRNAs active in ϕ29 DNA packaging

As mentioned previously in this paper, the activity of mutant pRNA7/174 was reduced 10^4-fold, and as reported previously (Zhang *et al.*, 1994), mutant pMbn was fully active in ϕ29 DNA packaging. These two molecules were used as template pRNA genes for the construction of circularly permuted pRNA and for the analysis of factors affecting cpRNA activity. The fully active pMbn was selected for the identification of cpRNAs with reduced activity, paying particular attention to bulge or loop structures within the pRNA. The partially active mutant pRNA7/174 was selected for testing whether the connecting loop sequences can improve the cpRNA activity.

The sequences of DNA templates cpDNA3A and $cpDNAT_7$, containing tandem pRNA coding sequences, were confirmed by DNA sequencing before analysis of pRNA activity. Four cpRNAs, cpRNA3A-38, cpRNA3A-55, cpRNA3A-78, and cpRNA3A-82, were synthesized by *in vitro* transcription with SP_6 RNA polymerase and PCR fragments from the tandem DNA template, cpDNA3A (Fig. 1-I), with primer pairs of P38/P37, P55/P54, P78/P77, and P82/P81, respectively. The size of each of these four cpRNAs was 121 bases, which included 120 bases from mutant pMbn and one additional base A. The additional A, together with the three nonessential bases $U_{118}A_{119}A_{120}$ extending from the 5′/3′ proximal region, served as a loop in the cpRNA, connecting the original 5′- and 3′-ends of pRNA. Of the four cpRNAs tested, cpRNA3A-38 was unique in that it possessed full biological activity, as

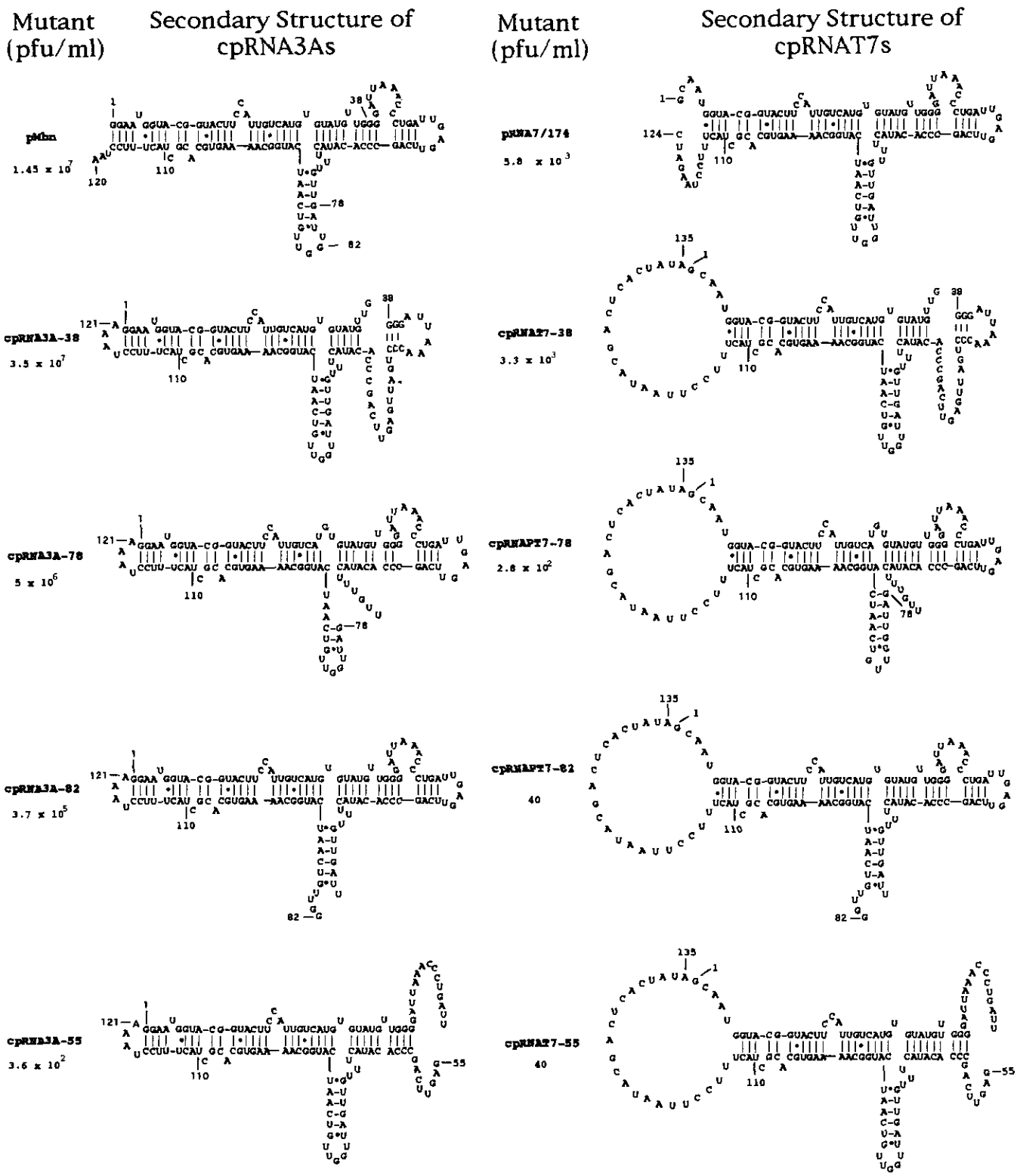

FIG. 3. The biological activity and secondary structure of the cpRNAs. The left column shows the cpRNAs from the plasmid cpDNA 3A, and the right column shows the cpRNAs from the plasmid cpDNAT$_7$. Each cpRNA pair in the same row as produced with the same primer pair, but with a different cpDNA template.

TABLE 2

Activity Assay of cpRNA in ϕ29 Assembly with the Sensitive System

Tadem DNA template	cpRNAs	PFU/ml (n = 2)[a]
cpDNA-T_7	cpRNAT_7-38	3.4×10^3
	cpRNAT_7-55	40
	cpRNAT_7-78	2.8×10^2
	cpRNAT_7-82	40
cpDNA-3A	cpRNA3A-38	3.5×10^7
	cpRNA3A-55	3.6×10^2
	cpRNA3A-78	5×10^6
	cpRNA3A-82	3.7×10^5

[a] *n, number of experiments.*

compared to the parental pRNA7/174 (Table 2). The other three cpRNAs had greatly reduced activities, ranging from an almost 10-fold reduction to 10^5-fold, depending upon where the new 5′ and 3′ termini were located (Table 2) (Fig. 3). The 5′/3′ loop of the cpRNA in this group consisted of 4 bases. The fact that one of the cpRNAs, cpRNA3A-38, was as active as its parental pRNA, pMbn (Fig. 3), indicates that the loop has no disruptive effect in pRNA activity, and that it is possible to construct cpRNA with full biological activity.

Another four cpRNAs, cpRNAT_7-38, cpRNAT_7-55, cpRNAT_7-78, and cpRNAT_7-82 (Fig. 3), were synthesized by *in vitro* transcription with SP_6 RNA polymerase and PCR fragments from the tandem DNA template cpDNAT_7 (Fig. 1-II). These four cpRNAs were transcribed using DNA templates generated with primer pairs P38/P37, P55/P54, P78/P77, and P82/P81 (Table 1), respectively. The size of each of these four cpRNAs was 135 bases, which included 118 base pairs of pRNA coding sequence and 17 base pairs of T_7 promoter coding sequence, which was used to create a loop to connect the 5′- and 3′-ends of cpRNA. The 5′/3′ loop of the cpRNA in this group was 27 bases long (Fig. 1-II). The parental pRNA for this group was pRNA7/174 with the deletion of two nonessential bases, $A_{119}A_{120}$, extending from the 5′/3′ proximal region. One of these cpRNAs, cpRNAT_7-38, had biological activity that was comparable to the parental pRNA (Fig. 3), suggesting that the 27-base loop had no deleterious effect on the folding or activity of the RNA. The other three cpRNAs, however, had significantly reduced activities (Table 2), ranging from a 10 to a 100-fold reduction, depending upon where the new 5′ and 3′ termini were located.

Activity assay of cpRNAs with new termini to assess the structure and function of pRNA bulges and hairpins

Bulged nucleotides might play a role in ribozyme function (Schroeder *et al.*, 1991). The cpRNAs were designed so as to create new termini at different bulges or hairpins to assess the importance of these regions in pRNA structure and function. The secondary structure of eight cpRNAs was predicted by the method of Zuker (1989) (Fig. 3). The group of cpRNAs from the cpDNA3A template were predicted to contain a small 5′/3′ loop of four bases. The group of cpRNAs from the cpDNAT_7 template were predicted to contain a large 5′/3′ loop of 27 bases. The predicted secondary structures of the cpRNAs differed at the sites of the new 5′ and 3′ termini, as can be seen, for example, by comparing cpRNA3A-38 and cpRNA3A-78. (Fig. 3). Changing the 5′/3′ loop, however, did not appear to alter the folding of the cpRNAs. For instance, cpRNA3A-38 has an identical predicted secondary structure as cpRNAT_7-38, with the exception of the loop. The similarity and variation in secondary structure was concomitant with the biological activity of the cpRNAs as described below.

The biological activities of eight different cpRNAs were assayed with the ϕ29 assembly system (Table 2 and Fig. 4). In the cpDNA3A group, the cpRNA with highest activity, cpRNA3A-38, was as active as, but not better than, its parental pRNA pMbn (Fig. 4A). The activity of the cpRNA3A-55 was 10^5-fold lower than that of the cpRNA3A-38. In this group, the order of activity, sequentially, from highest to lowest activity, was cpRNA3A-38 → cpRNA3A-78 → cpRNA3A-82 → cpRNA3A-55. Similarly, in the cpDNAT_7 group, the cpRNA with highest activity, cpRNAT_7-38, was as active as, but not better than, its parental pRNA P7/174 (Fig. 4B). In this group, the order of activity, sequentially, from highest to lowest activity, was cpRNAT_7-38 → cpRNAT_7-78 → cpRNAT_7-82 ≥ cpRNAT_7-55. As can be seen, there is perfect agreement between the two groups with respect to the order of activity, indicating that the opening of the bulge at residue 38 did not affect the pRNA activity, but opening of the bulge at residue 55 greatly reduced the pRNA activity.

DISCUSSION

In the construction of circularly permuted RNA, the first consideration is the sequence of the loop connecting the 5′- and 3′-ends. We have used A/T rich sequences for this purpose to reduce the potential of forming alternative secondary structures. The phage T_7 promoter was suitable for serving as a connecting loop due to its high A/T content and the potential of DNA strand separation for the binding of transcription factors during the initiation of mRNA polymerization. Our results show that for ϕ29 cpRNAs, the predicted folding was not deleteriously affected when either a loop of 4 or 27 bases was used to connect the natural 5′ and 3′ termini. The fact that in each group, the presence of one of the cpRNAs yielding a plaque titer as high as its parental pRNA suggests that neither loop hindered cpRNA activity.

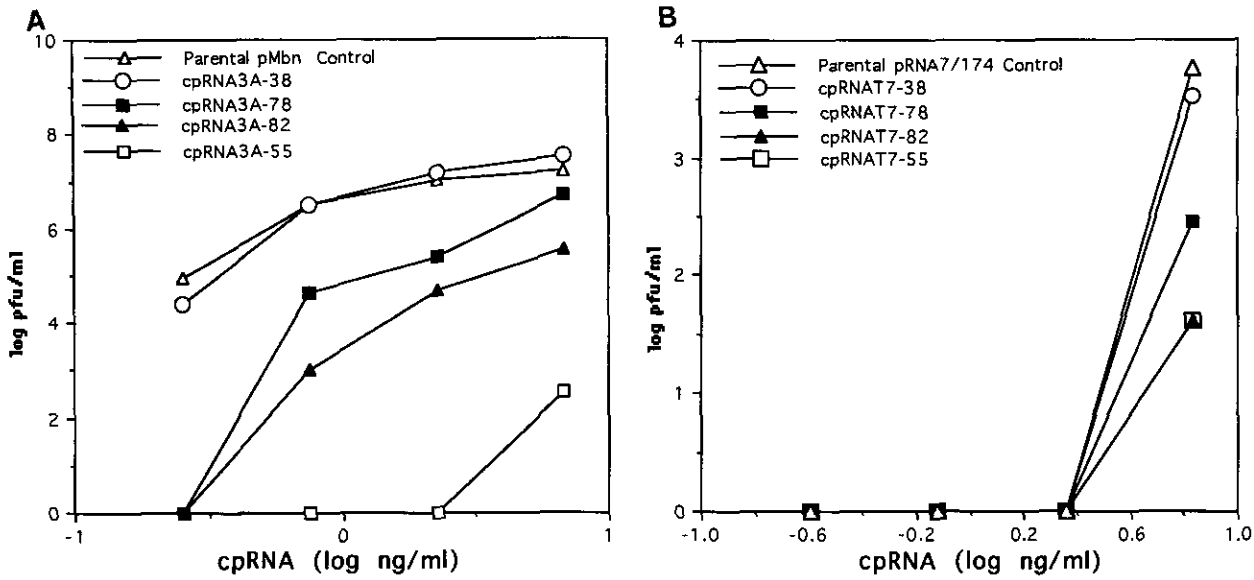

FIG. 4. Log/log plot of cpRNA activities with plaque forming units per milliliter obtained in the highly sensitive ϕ29 assembly assay as a function of ng/ml cpRNA. *In vitro* ϕ29 assembly was performed and varying concentrations of cpRNA from the templates of cpDNA3A (A) and cpDNAT$_7$ (B), respectively, were tested.

Although all cpRNAs reported here were active in ϕ29 DNA packaging, the activity varied with the location of new termini. Our results show that the opening of a bulge at a certain location could change the folding of the cpRNA and concomitantly reduce the activity of the cpRNA. The fact that all cpRNA molecules yielded plaques when tested in the plaque-forming assay, regardless of the varying reduction in titer, indicates that in the cpRNA population, there was a certain number of cpRNA molecules with a folding that was comparable to wild-type pRNA, and a certain number of cpRNA molecules which were misfolded. This is not surprising since each RNA molecule can fold into several forms with variable energy (Cech *et al.*, 1983; Zuker, 1989). Although the folding with the lowest energy is the most stable one, the active RNA molecule is not necessarily the one with the lowest folding energy (H. L. Weith, personal communications). Due to the variation of cpRNA activity, it is necessary to test the biological activity of each cpRNA before the use of these cpRNA for labeling or modification in the analysis of pRNA tertiary structure. The ϕ29 assembly system provides an ideal system for such an assay of cpRNA activity.

Several cpRNAs were synthesized in order to assess the effects of the location of the new termini on RNA activity. Two sets of cpRNAs were constructed, one with a 5′/3′ connecting loop of 4 bases, one with a connecting loop of 27 bases (Fig. 3). Because in each set, there was one cpRNA molecule which showed an activity that was comparable to the parental RNA for that set, it was concluded that the 5′/3′ loop, whether 4 or 27 bases long, had no negative effect on ϕ29 pRNA folding or function. This result seems reasonable since the 5′ and 3′ termini of the wild-type molecule are proximate (which facilitates circle formation (Nolan *et al.*, 1993)) and because the 3′ extended bases, 118–120, of the pRNA are dispensable (Fig. 2).

Within each set of cpRNAs, it was also observed that the activity of each RNA varied drastically depending on where the new 5′ and 3′ termini were located. For example, when an opening was made between bases 37 and 38 of the molecule, the RNA had an activity that was similar to its parental RNA, indicating that an opening in the molecule at this site had no disruptive effect on pRNA activity (Fig. 3). This result may be of concern since RNase footprinting studies have localized a procapsid binding region of the pRNA to residues 22–84 (Reid *et al.*, 1994a), and the opening at residue 38 is within this binding region. However, RNase V1 footprinting studies have shown an enhanced cleavage at residues 37–40 of the molecule when the pRNA is bound to the procapsid (Reid *et al.*, 1994a), indicating that this region of the molecule is exposed to solution and not interacting with the procapsid.

A second opening was positioned between residues 54 and 55, disrupting a bulge and a stem loop structure, according to the secondary structure prediction (Fig. 3). The cpRNAs with 5′/3′ termini at this location had a greatly reduced activity in the phage assembly assay. Reid *et al.* (1994a) have shown that residues 51–82 are protected from RNase T1 when pRNA is bound to procapsids. It was also observed that pRNAs with mutations in residues 54–57 competed poorly with wild-type pRNA for procapsid binding (Reid *et al.*, 1994b). One explanation for the se-

verely reduced activity of these cpRNAs may be that the opening between residues 54 and 55 disrupted the folding of the molecule so as to lessen its affinity for procapsid binding.

The last two cpRNAs had openings located in the stem–loop structure at the bottom of the molecule (as pictured in Fig. 3). The opening between residues 77 and 78 was located in the stem of the stem loop, and led to a 10-fold reduction in RNA activity. As can be seen in Fig. 3, this opening does not alter the secondary structure drastically, which may be the reason for the RNAs comparatively low reduction in activity. However, a structural alteration is evident and it is located in the procapsid-protected region of the pRNA (Reid *et al.*, 1994a). Opening at this region might affect the procapsid binding efficiency and might account for the 10-fold reduction in activity. A fourth 5′ and 3′ termini location, positioned between residues 81 and 82, produced RNA with a 100-fold reduction in activity (Fig. 3). The activity of these RNAs is considerably lower than the activity of RNAs with openings between residues 77 and 78 (just four bases away). Recent studies (Reid *et al.*, 1994c) have provided evidence for a pseudoknot structure in the pRNA which forms by the base-pairing of residues 45, 46, 47, and 48 with residues 85, 84, 83, and 82, respectively. It was shown that the complementarity of these bases is critical for pRNA activity, and thus any modification of the structure at or around this region might cause the change of pRNA secondary structure that might affect the procapsid binding activity.

With the synthesis of biologically active ϕ29 cpRNA, the analysis of specific interactions of unique internal bases of the pRNA with components of the ϕ29 DNA packaging complex is now possible. By labeling the new termini with 5′- or 3′-end photoaffinity cross-linking agents, specific interactions of the modified bases with protein, DNA, or RNA can be elucidated (Nolan *et al.*, 1993). The goal of our research is to determine the overall tertiary structure of the ϕ29 pRNA and to ascertain the molecule's specific role in DNA-packaging.

ACKNOWLEDGMENTS

We thank Drs. Norman R. Pace and James M. Nolan at Indiana University for their comments and suggestions, Professor Qingyun Huang for DNA sequencing of the plasmids, and Tim Tellinghuisen for comments on the manuscript. This work was supported by NIH Grant GM48159 to P.G.

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