

## The Proximate 5' and 3' Ends of the 120-Base Viral RNA (pRNA) Are Crucial for the Packaging of Bacteriophage $\phi$ 29 DNA

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*In vitro* mutagenesis was performed to identify the DNA packaging domain of the 120-base pRNA essential and specific for DNA encapsidation by bacteriophage  $\phi$ 29 of *Bacillus subtilis*. All deletions and mutations targeted the 5' and 3' ends of the pRNA. DNA templates of a control or mutant pRNAs used for *in vitro* transcription with T7 RNA polymerase were generated by PCR. Fourteen mutant pRNA molecules were synthesized from DNA templates either directly after PCR or after cloning the PCR fragments into the pCR II vector. Ten of the mutant pRNA species were inactive in packaging of the  $\phi$ 29 genome. Mutation of base one at the 5' end did not affect the pRNA packaging activity. Mutation of the first two bases at the 5' end of the pRNA to noncomplementary bases in the predicted RNA secondary structure (U<sub>1</sub> C<sub>2</sub>/A<sub>117</sub>G<sub>118</sub> to G<sub>1</sub> G<sub>2</sub>/A<sub>117</sub>G<sub>118</sub>) resulted in a pRNA with no detectable DNA-gp3 packaging activity assayed by either sucrose gradient sedimentation or agarose gel electrophoresis, and 10<sup>5</sup>-fold reduction in activity was found when measured by plaque-forming units with a new highly sensitive assay system. Changing bases 116 and 117 so that they were complementary to the mutated bases, 1 and 2, from the previous mutant (G<sub>1</sub> G<sub>2</sub>/A<sub>117</sub>G<sub>118</sub> to G<sub>1</sub> G<sub>2</sub>/C<sub>117</sub>C<sub>116</sub>) generated an RNA molecule with restored DNA packaging ability. Our results show that, although not essential for procapsid binding, both the 5' and 3' ends of the pRNA were proximate and crucial for  $\phi$ 29 DNA packaging. © 1994 Academic Press, Inc.

### INTRODUCTION

One striking feature in the assembly of all linear dsDNA viruses is that the viral DNAs are packaged into a preformed procapsid during maturation. This group of viruses includes adenovirus, herpesvirus, poxvirus (perhaps), bacteriophages T1, T3, T4, T5, T7, P1, P2, P22, Mu,  $\phi$ 21,  $\phi$ 29, SP01, SPP1,  $\lambda$ , and their relatives (for review see Earnshaw and Casjens, 1980; Black, 1989; Bazinet and King, 1985; Serwer, 1988; Guo and Trottier, 1994; Guo, 1994). Studies of the mechanism of viral DNA packaging for the dsDNA phages document common features in this step of the phage life cycle. These common features include the use of a pair of noncapsid proteins and ATP to translocate the viral DNA into a procapsid, which has been preformed with the aid of scaffolding proteins that are removed before or during DNA packaging (Earnshaw and Casjens, 1980; Casjens, 1985; Black, 1988, 1989; Bazinet and King, 1985; Anderson and Reilly, 1993). The pair of noncapsid DNA packaging proteins can be classified into two groups. The smaller molecular weight proteins belong to group 1 and the larger ones to group 2 (Guo *et al.*, 1987c). Group 1 proteins are responsible for DNA binding; group 2 proteins are DNA-dependent ATPases and, as proven for

some phages, bind specifically to the portal vertex of the procapsid (Shibata *et al.*, 1987; Shinder and Gold, 1988; Sternberg and Coulby, 1987; Wu *et al.*, 1988; Pruss and Calendar, 1978; Bowden and Modrich, 1985; Backhaus, 1985; Poteete and Botstein, 1979; Strobel *et al.*, 1984; Son *et al.*, 1988; Bhattacharyya and Rao, 1993; Frackman *et al.*, 1984; Guo *et al.*, 1987d). DNA translocation is driven by the hydrolysis of ATP as demonstrated in bacteriophages  $\lambda$  (Becker *et al.*, 1988; Davidson and Gold, 1987; Higgins *et al.*, 1988), P22 (Eppler *et al.*, 1991),  $\phi$ 29 (Bjornsti *et al.*, 1983; Guo *et al.*, 1986; Guo *et al.*, 1987c), T3/T7 (Serwer, 1988; White and Richardson, 1987; Morita *et al.*, 1993), T4 (Franklin, 1992; Manne *et al.*, 1982; Rao and Black, 1988), and P2 (Linderoth *et al.*, 1991; Bowden and Modrich, 1985).

Bacteriophage  $\phi$ 29 of *Bacillus subtilis* is an ideal model for studying the mechanism of viral DNA packaging because its DNA can be packaged *in vitro*, with all components overproduced and purified (Guo *et al.*, 1987b,c,d, 1991a,b; Anderson and Bodley, 1990; Guo and Trottier, 1994). Recombinant procapsids produced from engineered structural genes of  $\phi$ 29 are competent for DNA packaging and the DNA-filled capsids prepared using this system can be converted into infectious viruses after the addition of the neck and tail proteins (Guo *et al.*, 1991a,b; Guo and Lee, unpublished data).

An exciting aspect in the study of  $\phi$ 29 DNA packaging is the discovery that a 120-base pRNA ("P" for packaging) of  $\phi$ 29 is indispensable for DNA packaging in extracts

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or in the defined *in vitro* DNA packaging system (Guo *et al.*, 1987a,b, 1991b). Involvement of a nonprotein factor in viral DNA packaging provides a new insight into DNA-protein interactions and extends previously demonstrated RNA functions. Studies of the interactions involving this pRNA molecule are likely to provide a model for a catalytic RNA molecule acting on DNA. Involvement of RNA in DNA packaging has been demonstrated in a phage  $\lambda/\phi 29$  chimeric system (Donate and Carrascosa, 1991; Valpuesta *et al.*, 1993) and in the Mu-like phage D108 (Burns *et al.*, 1990). Also, there is speculation of such RNA involvement in poxvirus (Parsons and Pickup, 1990) and adenovirus (Hatfield and Hearing, 1994). If involvement of such pRNAs in viral genome encapsidation is a general phenomenon, then the RNA can be a potential target for antiviral drug design.

Phylogenetic analysis of the pRNA has led to the development of a model for the secondary structure of the pRNA. This secondary structure has been found to be conserved rather highly among pRNAs isolated from several  $\phi 29$  relatives, even though primary sequences were not well conserved (Bailey *et al.*, 1990).

Although the pRNA is essential in DNA packaging, its exact function remains to be determined. The  $\phi 29$  pRNA is attached to the procapsid portal vertex (connector) and can be separated from purified procapsids in the presence of EDTA and reattached to the pRNA-free procapsids in the presence of  $Mg^{2+}$  (Guo *et al.*, 1987a,b). Nucleotides 22 to 84 are protected from ribonuclease digestion when bound to procapsid as determined in RNA footprinting assays (Reid *et al.*, 1993). This paper describes the mutation and/or deletion study of both the 5' and the 3' ends of the pRNA. The results show that both ends were essential for  $\phi 29$  DNA packaging, as a change of only two bases at the 5' end resulted in  $10^5$ -fold decrease in biological activity. It also strongly suggests that both ends become proximate in the formation of secondary structure, which agrees with predictions from computer and phylogenetic analysis (Bailey *et al.*, 1990).

## MATERIALS AND METHODS

### Oligodeoxynucleotides

Eleven oligonucleotides were used in this study. Oligonucleotide P1 (5'-TAATACGACTCACTATAGGTACTTCCATTGTCAT-3'), which contained the T7 promoter, was complementary to nucleotides 11 to 27 at the 5' end of the pRNA coding sequence; oligonucleotide P2 (5'-GGCACTTTTGCCATGATT-3') was homologous to nucleotides 107-89 at the 3' end of the pRNA coding sequence; oligonucleotide P3 (5'-TTGCCATGATTGACAAC-3') was homologous to nucleotides 99-83 at the 3' end of the pRNA coding sequence; oligonucleotide P4 (5'-TTATCAAAGTAGCGTGAC-3') was homologous to nu-

cleotides 120-102 of the 3' end of the wild-type pRNA sequence; oligonucleotide P5 (5'-TAATACGACTCACTATAGGTATGTTGGGGATTAAA-3'), which contained the T7 promoter, was complementary to nucleotides 28 to 46 at the 5' end of the pRNA coding sequence; oligonucleotide P6 (5'-ATTGACAACCAATCAAC-3') was homologous to nucleotides 91 to 75 at the 3' end of the pRNA coding sequence; oligonucleotides P7 (5'-TAATACGACTCACTATAGCAATGGT-3') and P8 (5'-TAATACGACTCACTATAGGGGTGGTAC-3'); which contained T7 promoter, were complementary to nucleotides 1 to 8 and 1 to 10 at the 5' end of the pRNA coding sequence; oligonucleotide P9 (5'-TTACCCCAGTAGCGTGC-3') was homologous to nucleotides 120 to 104 at the 3' end of the pRNA coding sequence; oligonucleotide FW (5'-GTAAAACGACGGCCAGTG-3') and oligonucleotide RV (5'-AACAGCTATGACCATG-3') were the M13 forward and reverse sequencing primers, respectively.

### Construction of DNA templates used for *in vitro* transcription

To generate mutant RNA transcripts, plasmid pBlu102Rev (Wichitwechkarn *et al.*, 1992), which contained DNA that encoded the  $\phi 29$  pRNA, was digested with *Xba*I and *Xho*I. The resulting fragment was used as a template to amplify four DNA segments by PCR with oligonucleotide pairs P1/P2, P1/P3, P1/P4, and P5/P6 as primers, respectively. Plasmid pRT71 (kindly provided by R. Reid and D. Anderson), containing the RNA coding sequence, was digested with *Bgl*II and then used as a template to amplify three DNA segments by PCR with oligonucleotide pairs FW/P2, FW/P3, and FW/P4 as primers, respectively. The entire plasmid pRT71 containing the RNA coding sequence was used as a template to amplify four DNA segments by PCR with oligonucleotide pairs P7/P4, P7/P9, P8/P4, and P8/P9 as primers, respectively. All amplified fragments were separated in 2%-equivalent agarose/synergel (Diversified Biotech) and purified by Qiaex (Qiagen). The purified fragments were then used directly as templates to generate mutant RNAs by *in vitro* transcription or cloned into the pCR II vector (Invitrogen) at the *Eco*RI site. The cloning was confirmed in each case by DNA sequencing. Inserts could be recovered from the plasmids by *Eco*RI digestion. Mutant RNAs were generated by *in vitro* transcription with T7 RNA polymerase (Promega). RNA pMbn was generated from DNA template after the Mung bean nuclease digestion of plasmid pRT71 linearized by *Bgl*II.

Another DNA segment for the transcription of pRNA FW/RV was amplified by PCR with oligonucleotide pair FW/RV as primers and plasmid pRT71 DNA as a template. After digestion with *Bgl*II, the PCR fragment was used as the template to generate pRNA FW/RV by *in vitro* transcription. The concentrations of all RNAs generated above were determined by spectrophotometry.

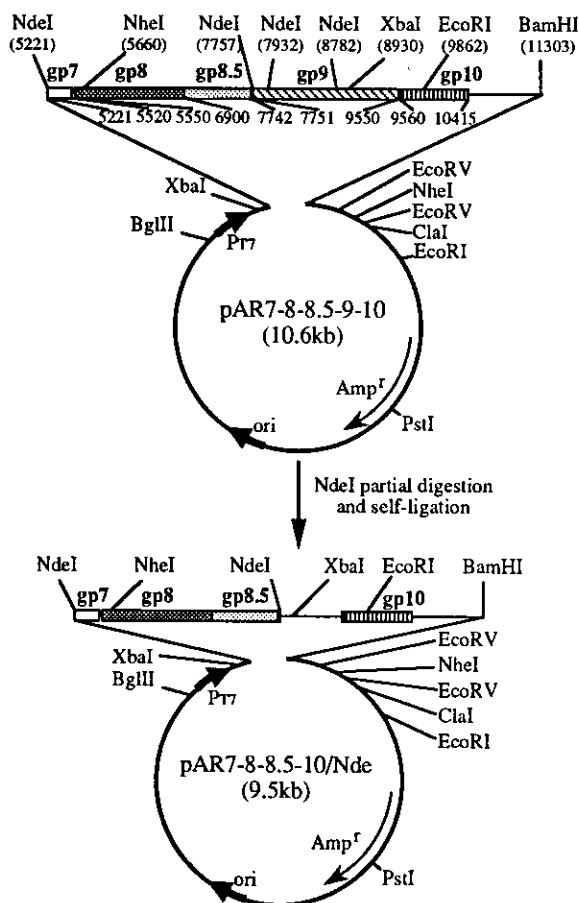


Fig. 1. Construction of plasmid pAR7-8-8.5-10/Nde.

### Construction of plasmid pAR7-8-8.5-10/Nde

Plasmid pAR7-8-8.5-10/Nde, indicating a plasmid that contains the  $\phi$ 29 structural genes 7, 8, 8.5, and 10, was a derivative of the previously reported plasmid pAR7-8-8.5-10 (Guo *et al.*, 1991a,b). Two *Nde*I DNA fragments (bases 7757–7934 and 7935–8782) containing the gene coding for gp9 (tail protein), located between gene 8 and gene 10 (Fig. 1) were removed by partial digestion and self-religation.

### Purification of the recombinant procapsid, DNA–gp3, and gp16

The purification of gp16 and DNA–gp3 has been described previously (Guo *et al.*, 1986). Procapsids were overproduced and purified with a procedure derived from previously reported methods (Guo and Moss, 1990; Guo *et al.*, 1987a,d, 1991b). A 5-ml overnight culture of *Escherichia coli*/HMS174(DE3) harboring plasmid pAR7-8-8.5-10/Nde was diluted into 500 ml Terrific Broth (Guo and Moss, 1990) with 50  $\mu$ g/ml ampicillin and grown at 37° with vigorous shaking at 250 rpm for 2 hr. The T7 promoter was induced with 0.5 mM IPTG and the cells were incubated for 2 more hr at 37°. Cells were collected by centrifugation at 7000 rpm for 15 min in the Beckman JS

7.5 rotor, resuspended in 16 ml Buffer A (50 mM Tris–HCl, pH 8.0, 50 mM NaCl, 1 mM EDTA, 2 mM DTT, 1 mM PMSF, 1  $\mu$ l RNase-free DNase (50  $\mu$ g/ml), 10  $\mu$ g/ml lysozyme), and incubated on ice for 30 min. Cells were then subjected to lysis by passing the French pressure cell twice at 15,000 psi. The lysate was clarified for 15 min at 10,000 rpm at 4° in a Beckman JA21 rotor. The supernatant was loaded onto a 10–30% sucrose gradient in TBE buffer (89 mM Tris–borate, 2 mM EDTA, pH 8.0). The gradient was spun at 25,000 rpm for 5 hr at 4° in the Beckman SW28 rotor. The upper opalescent band was removed and the lower procapsid band was collected from the gradient, pelleted at 35,000 rpm for 5 hr at 6° in a Beckman Type 45 rotor, and resuspended by soaking overnight in 100–200  $\mu$ l of TBE buffer. The procapsid was then aliquoted and some aliquots were stored at 4° for use within 2 weeks and others at –70° for later use.

### Assay of pRNA activity

The activity of pRNA was assayed with the *in vitro* DNA packaging system (Guo *et al.*, 1986, 1991b), or with the highly sensitive system for the determination of the formation of infectious  $\phi$ 29 virions (Lee and Guo, 1994). DNA packaging efficiency was determined by both sucrose gradient sedimentation (Guo *et al.*, 1986, 1987d) and agarose gel electrophoresis (Grimes and Anderson, 1989).

### Computer prediction of pRNA secondary structure

Secondary structures for the mutant pRNAs tested in this paper 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

### Purification of recombinant procapsids

We have previously reported the purification of active  $\phi$ 29 procapsid from *E. coli* harboring plasmid pAR7-8-8.5-10 (Guo *et al.*, 1991a,b). This plasmid also contains the genes for the head fiber (gp8.5) and tail protein (gp9). Deletion of two *Nde*I fragments from this plasmid removed the gp9 gene. The yield of recombinant procapsid from plasmid pAR7-8-8.5-10/Nde was higher than from plasmid pAR7-8-8.5-9-10. However, specific activity of the procapsid in DNA packaging remained unchanged (data not shown).

### Synthesis of $\phi$ 29 pRNA transcribed *in vitro* with T7 RNA polymerase and DNA templates generated by PCR

To facilitate mutation, deletion, and truncation studies of  $\phi$ 29 pRNA, T7 RNA polymerase was used to synthesize 14 mutant pRNAs (Fig. 2) in large quantities with the

*in vitro* transcription system. The DNA fragments for *in vitro* transcription were generated by PCR using a DNA template containing the pRNA coding sequence and primers containing T7 promoter. To further confirm the sequences of the DNA templates from PCR, purified PCR DNA fragments were cloned into vector pCR II and their identity was confirmed by DNA sequencing. Results from DNA sequencing indicated that the PCR DNA fragments contained sequences in accordance with primers and the original template for PCR (data not shown), as well as the junction areas. Biologically active  $\phi 29$  pRNA was successfully synthesized with the PCR DNA template. DNA packaging assays showed that the pRNA generated directly from PCR DNA templates possessed activity that was identical to that obtained with pRNA from DNA templates cloned into the plasmid vector (data not shown).

#### Loss of DNA packaging activity after deletion at the 5' and 3' proximate ends of the pRNA

Computer and phylogenetic studies suggest that the 5' and 3' ends of the pRNA become proximate in the formation of the secondary structure (Bailey *et al.*, 1990). Deletion was performed at both ends to study the DNA packaging domain and to obtain the shortest pRNA that is functional in  $\phi 29$  DNA-gp3 packaging (Fig. 2). Mutant P1/P2, which indicates a pRNA produced from a PCR product generated with primers P1 and P2, contained a deletion of 24 bases: 10 bases at the 5' end and 14 bases at the 3' end; P1/P3 contained a deletion of 31 bases: 10 bases at the 5' end and 21 bases at the 3' end; P1/P4 contained a deletion of 10 bases at the 5' end of the pRNA; P5/P6 contained a deletion of 56 bases: 27 bases at the 5' end and 29 bases at the 3' end; FW/P2 contained a deletion of 14 bases at the 3' end; and FW/P3 contained two mutations at the 5' end and a deletion of 21 bases at the 3' end. All deletions on these mutant pRNAs were focused at the proximate 5' and 3' ends of the pRNA molecule. When these mutant RNAs were added to the *in vitro* DNA packaging system with purified recombinant procapsid, DNA-gp3, DNA packaging protein gp16, and ATP, no DNA packaging activity was detected via 1% agarose gel (Fig. 3) or sucrose gradient sedimentation (data not shown). These six mutant RNAs were further tested with the highly sensitive system recently developed in our lab (Lee and Guo, 1994). It was found that all six of the mutant RNAs were inactive in the assembly of infectious  $\phi 29$ , as measured by plaque-forming units (PFU) (Table 1), which is in agreement with the DNA packaging assay. The results indicated that the 5' and/or the 3' end of the pRNA were

essential for DNA packaging, either on their own or in interaction with each other.

#### Change of two or four complementary bases to noncomplementary bases at the 5' end of the pRNA resulted in the dramatic reduction or loss of the DNA packaging activity but change of one base did not

To further define the RNA domain essential for DNA packaging, the bases at the 5' and 3' ends of the pRNA were modified (Fig. 2). For P7/P4 the first base at its 5' end was changed from  $U_1$ , which was complementary with  $A_{117}$  located at the 3' end of the pRNA, to  $G_1$ . For FW/P4 the first and the second bases at its 5' end were changed from  $U_1C_2$ , which were complementary with  $A_{117}G_{116}$  located at the 3' end of the pRNA, to  $G_1G_2$ . For P8/P4 four bases at its 5' end were changed from  $U_1C_2A_3A_4$ , which were complementary with  $A_{117}G_{116}U_{115}U_{114}$  located at the 3' end of the pRNA, to  $G_1G_2G_3G_4$ . When these mutant pRNAs were tested in the *in vitro* packaging system, no DNA packaging activity was detected (Fig. 3, lanes D and M), except for P7/P4 (Fig. 3, lane B), by any assay procedure reported previously, e.g., sucrose gradient sedimentation (data not shown) or agarose gel electrophoresis (Fig. 3). These mutant RNAs were further tested with the highly sensitive system mentioned above (Lee and Guo, 1994). P7/P4 was biologically active and P8/P4 was inactive in the assembly of infectious  $\phi 29$  measured with PFU (Table 1). FW/P4 (with a two-base mutation) had decreased  $10^5$ -fold ( $1.6 \times 10^2$  PFU/ml) compared with that of P7/P4 ( $1.4 \times 10^7$  PFU/ml) which had only a one-base mutation (Table 1). The results of the phage assembly assay agreed with that of the DNA packaging assay and indicated that the 5' end was important enough that the mutation of only two bases at this end of the pRNA to bases noncomplementary to the 3' end as proposed in the secondary structure, ( $U_1C_2/A_{117}G_{116}$  to  $G_1G_2/A_{117}G_{116}$ ), resulted in a dramatic reduction in activity for DNA-gp3 packaging.

#### Restoration of DNA packaging activity by introducing two complementary bases at the 3' end to the mutant FW/P4

To further investigate whether loss or reduction of DNA packaging activity that resulted from the change of two bases was due to sequence specificity or due to change in the pRNA secondary structure, mutant pMbn with additional base modifications was synthesized and analyzed. This mutant contained two mutated bases at each end. Two corresponding complementary bases,  $A_{117}G_{116}$  at the

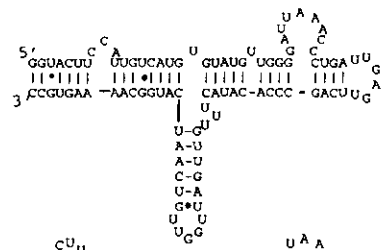
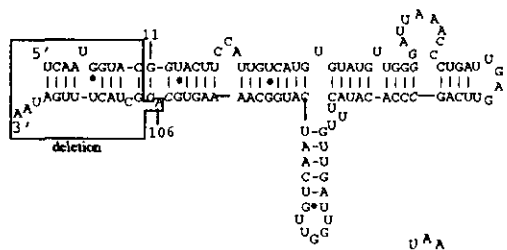
Fig. 2. Deletions and mutations of 14 mutant pRNAs as well as their predicted secondary structure. The left lane represents the secondary structure of the wild-type pRNA.

Mutant

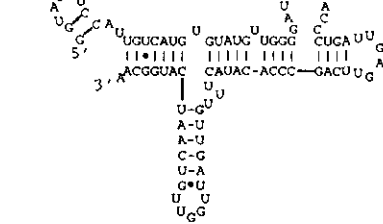
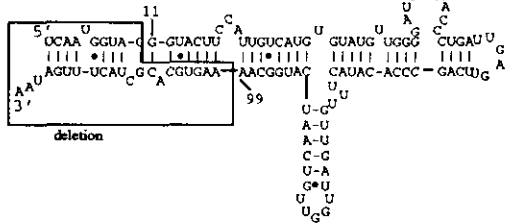
Modification

Secondary Structure

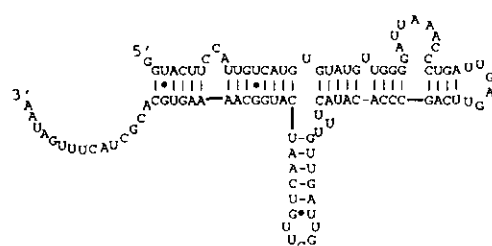
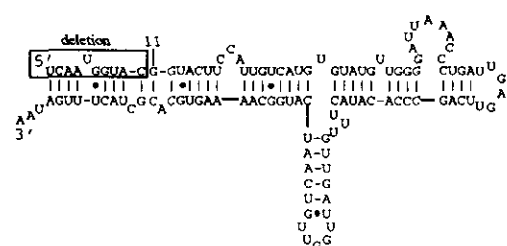
P1/P2



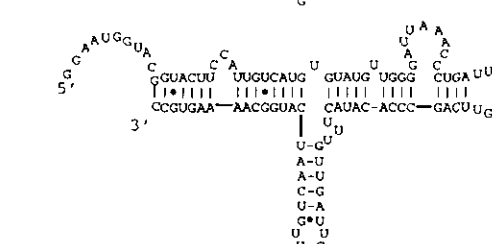
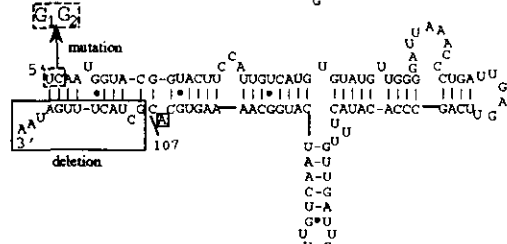
P1/P3



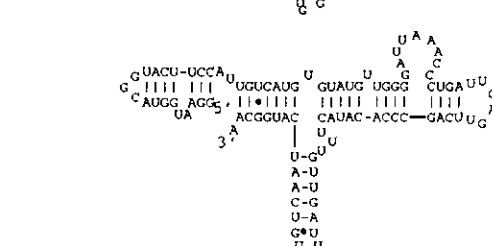
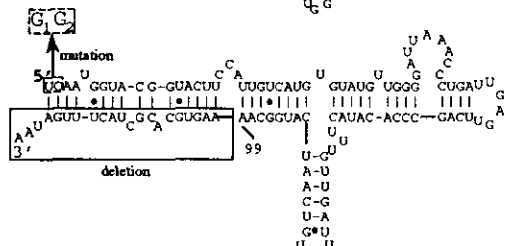
P1/P4



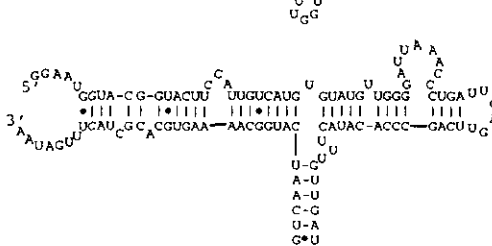
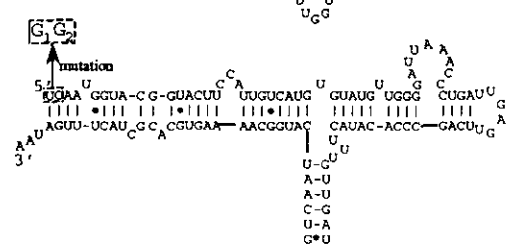
FW/P2



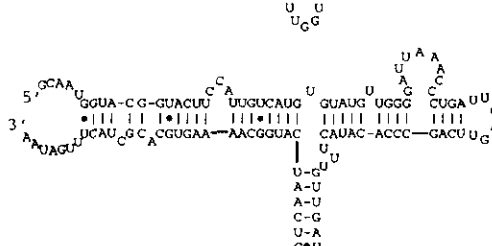
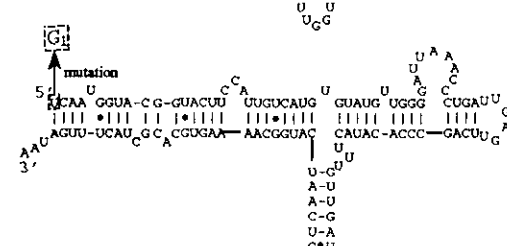
FW/P3



FW/P4



P7/P4

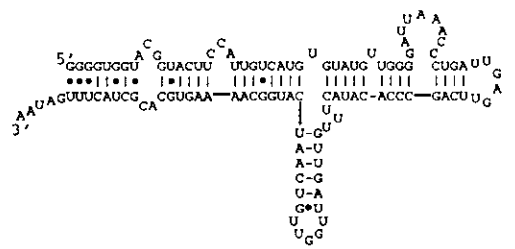
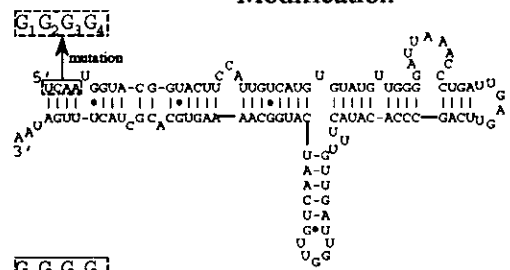


Mutant

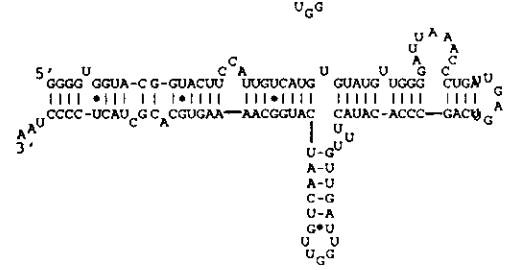
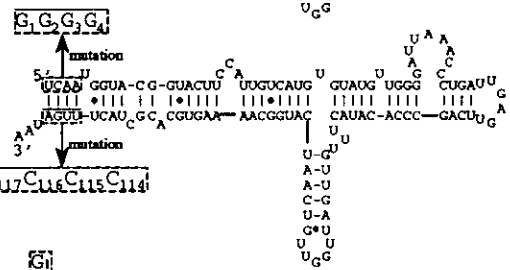
Modification

Secondary Structure

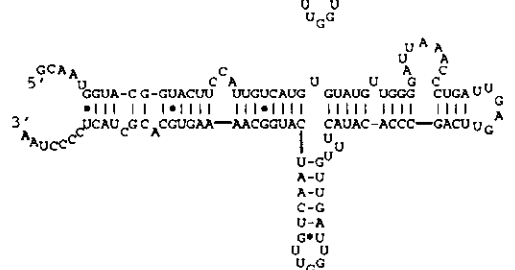
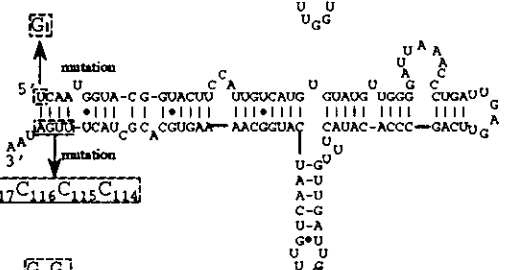
P8/P4



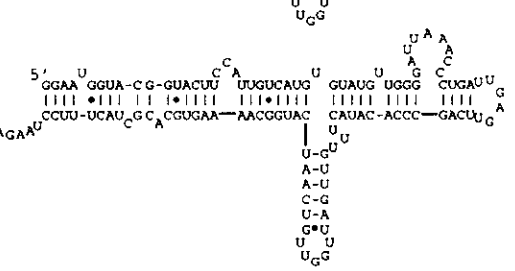
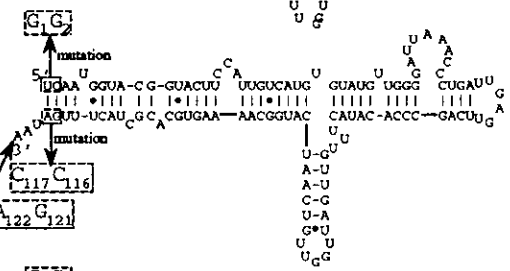
P8/P9



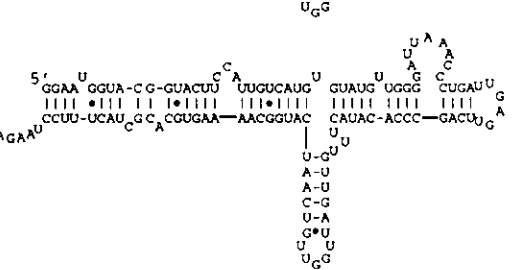
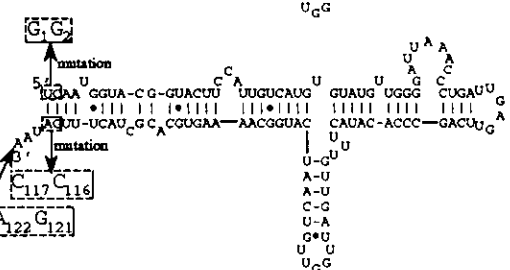
P7/P9



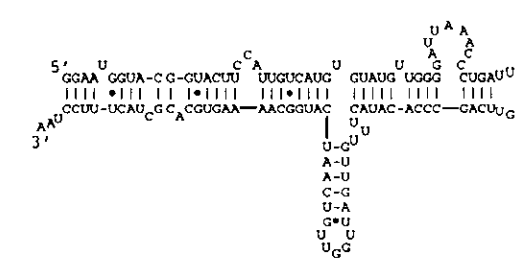
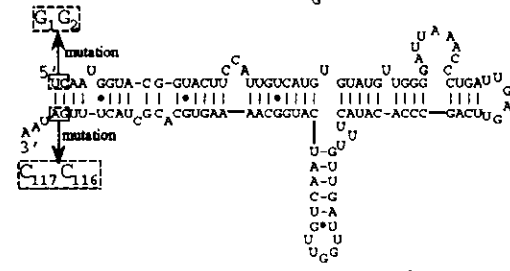
FW/RV



pRT71



pMbn



P5/P6

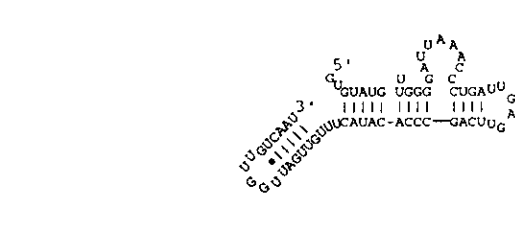
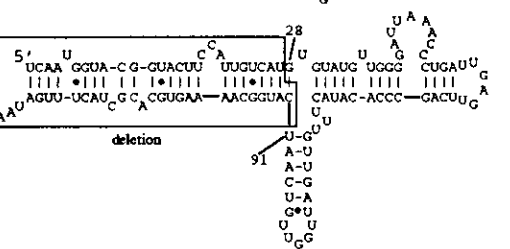


FIG. 2 — Continued

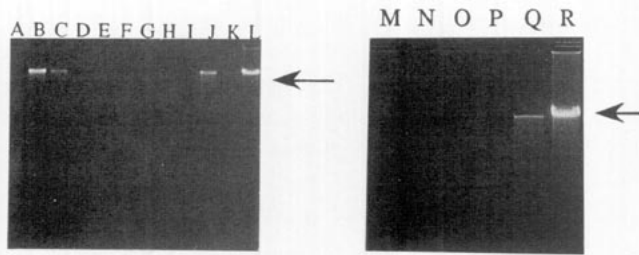


Fig. 3. Agarose gel demonstrating DNA packaging activity of mutant pRNA. The arrow indicates packaged  $\phi$ 29 DNA protected from DNase digestion. DNA was packaged in the defined *in vitro* system with the following pRNA: Lanes A–I, DNA packaging with mutant P8/P9, P7/P4, FW/RV, FW/P4, FW/P3, FW/P2, P1/P4, P1/P3, and P1/P2, respectively. Lanes M–O, DNA packaging with mutant P8/P4, P7/P9, and P5/P6, respectively. Lanes K and P, DNA packaging without pRNA as the negative control. Lanes J and Q, DNA packaging with pRT71. Lanes L and R, DNA-gp3 without DNase digestion. DNA packaging activities were only detected with pRNA FW/RV, pRT71, and P7/P4.

3' end of the opposite strand, were changed to  $C_{117}C_{116}$  to restore a complementary configuration. Therefore, the sequence  $G_1 G_2/A_{117}G_{116}$  was changed to  $G_1 G_2/C_{117}C_{116}$  (Fig. 2). This change of the corresponding bases at the opposite strand, which restored the complementary configuration, restored pRNA activity (Table 1). Two additional mutants, FW/RV and pRT71 which were identical to pMbn except containing four additional bases at the 3'-end (Fig. 2), were also active in DNA packaging (Fig. 3, lanes C, J, and Q; Table 1) when the complementary configurations were restored.

#### No restoration of DNA packaging activity by introducing four complementary bases at the 3' end to the inactive mutant P8/P4

The observed reduction of DNA-gp3 packaging activity of Mutant FW/P4 and restoration of DNA-gp3 packaging activity of mutant pMbn, FW/RV, and pRT71 appeared to show that the secondary structure, rather than the specific RNA sequence, is the important factor in DNA packaging function. To investigate this question further, mutant P8/P9 was synthesized and tested. This mutant contained eight mutated bases. Four corresponding bases,  $A_{117}G_{116}U_{115}U_{114}$  at the 3' end of P8/P4 were changed to  $C_{117}C_{116}C_{115}C_{114}$  to restore complementary configuration. Therefore, the  $G_1 G_2 G_3 G_4/A_{117}G_{116}U_{115}U_{114}$  sequence in P8/P4, or  $U_1 C_2 A_3 A_4/A_{117}G_{116}U_{115}U_{114}$  in the wildtype, was changed to  $G_1 G_2 G_3 G_4/C_{117}C_{116}C_{115}C_{114}$ . This change of the corresponding bases at the opposite strand, although restoring the complementary configuration, did not restore pRNA activity in DNA packaging (Fig. 3, lane A; Table 1).

#### Secondary structure of the mutant pRNAs

The secondary structures of all mutant pRNAs were predicted by the methods of Zuker (1989). The secondary structures of the active mutant pRNAs were compared

with those of inactive pRNAs. Mutant P7/P4, which contained a one-base mutation at the 5' end, was active in DNA packaging (Fig. 3, lane B; Table 1); however, its predicted secondary structure was different from the wildtype pRNA (Fig. 2). Mutant P8/P9, which contained an eight-base mutation at the 5' and 3' proximate ends, was inactive in DNA packaging (Fig. 3, lane A; Table 1), although its secondary structure was identical to that of the wildtype (Fig. 2). Mutants that both preserved secondary structure and were active in DNA packaging were mutants pMbn, FW/RV, or pRT71 (Fig. 2). Therefore, secondary structure is not the only determinant of pRNA activity.

## DISCUSSION

The major objective of our research is to understand the role and structure/function relationship of the pRNA in  $\phi$ 29 DNA packaging. The following are possible roles for the pRNA: (1) a morphogenic factor in  $\phi$ 29 procapsid assembly; (2) a structural component; (3) a factor binding to the procapsid or DNA packaging machinery which causes a protein conformational change that is important for biological function; or (4) a catalyst or an enzyme. We have demonstrated that the pRNA is not required for the assembly of functional  $\phi$ 29 procapsid, and it is not present in the mature virion nor is it a structural component of the procapsid (Guo *et al.*, 1991b). It has been well documented that the pRNA binds to the portal vertex (Guo *et al.*, 1987a). One question remaining to be answered is whether the pRNA is only a factor that binds to the portal vertex and brings this DNA packaging machinery to an active conformation, or whether it has addi-

TABLE 1  
ACTIVITY ASSAY OF MUTANT pRNA IN  $\phi$ 29 ASSEMBLY WITH THE SENSITIVE SYSTEM

Mutant pRNA	PFU/ml ( $n = 2$ ) <sup>a</sup>
1. P1/P2	0
2. P1/P3	0
3. P1/P4	0
4. FW/P2	0
5. FW/P3	0
6. FW/P4	$1.6 \times 10^2$
7. P7/P4	$1.4 \times 10^7$
8. P8/P4	0
9. P8/P9	0
10. P7/P9	0
11. FW/RV <sup>b</sup>	$1.3 \times 10^7$
12. pRT71 <sup>b</sup>	$1.05 \times 10^7$
13. pMbn	$1.45 \times 10^7$
14. P5/P6	0

<sup>a</sup>  $n$ , number of experiments.

<sup>b</sup> The RNA sequences of FW/RV and pRT71 were identical but from different templates, a PCR DNA template, and a plasmid DNA template, respectively.

tional enzymatic activity in the DNA packaging reaction. This question can be answered in part by determining whether the DNA packaging domain is separated from the procapsid binding domain within the  $\phi$ 29 pRNA.

When the pRNA was bound to the procapsid, RNA footprinting analysis has revealed a protected region, spanning residues 22 to 84 (Reid *et al.*, 1993). This result agrees with our finding that the procapsid binding domain was localized between residues 11 and 99 (Zhang and Guo, unpublished data). Mutations in the procapsid binding region that did not affect the predicted pRNA secondary structure had less of an effect on the pRNA activity than mutations that altered the RNA secondary structure (Wichitwechkarn *et al.*, 1992). Deletions within the aforementioned procapsid-protected region resulted in significant decreases in procapsid binding (Wichitwechkarn *et al.*, 1992), while deletions or mutations outside of this region had little or no effect on procapsid binding, as RNA molecules with mutations at the proximate 5' and 3' ends were tested (Zhang and Guo, unpublished data). Our results in this paper indicate that a mutation of more than one base at the 5' and/or the 3' proximate end of the pRNA resulted in dramatic reduction or loss of DNA-gp3 packaging activity. The fact that the pRNA still can bind to the procapsid, yet be deficient in allowing packaging to occur, indicates that the pRNA is not only a factor that binds to the portal vertex, but also that the pRNA itself contains at least one domain with DNA packaging function, that is independent of the portal vertex binding activity. It further implies that the proximate 5' and 3' ends of the pRNA were involved in interactions with other components during DNA packaging. Possible candidate components for the interaction might be the DNA, gp16, gp3, ATP, or Mg<sup>2+</sup>.

Mutation of both bases 1 and 2 resulted in a dramatic loss of packaging activity. As can be seen in Fig. 2, bases 1 and 2 are predicted to be base paired with bases 117 and 116, respectively, in wild-type pRNA. Mutation of bases 1 and 2 in FW/P4 caused an alteration in the secondary structure of the molecule (Fig. 2), resulting in 10<sup>5</sup>-fold reduction of DNA packaging activity (Table 1). This hypothesis was tested when bases 117 and 116 were mutated, so that they were complementary to the already mutated bases 1 and 2, respectively. This pRNA, pMbn, was found to be active in the packaging of DNA. It has also been shown that mutations that altered the pRNA secondary structure had a substantial effect on pRNA activity (Wichitwechkarn *et al.*, 1992). This observation led to the speculation that sequence specificity is not as important as secondary structure regarding the function of the pRNA, as proposed previously (Wichitwechkarn *et al.*, 1992). However, results of the study of mutant P8/P9 did not justify this speculation. Mutant P8/P9 contained an eight-base mutation, four at the 5' end and the other four complementary bases at the 3' end in such a way that mutation did not alter the pRNA sec-

ondary structure as analyzed by the computer program of Zuker (1989) (Fig. 2), which was the same program used by both Bailey *et al.* (1990) and Wichitwechkarn *et al.* (1992). DNA-gp3 packaging assay showed that mutant pRNA P8/P9 was inactive (Fig. 3; Table 1), even though its secondary structure was identical to the wild-type pRNA (Fig. 2), suggesting that the secondary structure was not the only factor contributing to pRNA packaging activity. The relationship between function and tertiary structure has to be further investigated.

Previously, pRNA had been purified from two sources: Separation from procapsids purified from infected cells (Guo *et al.*, 1987a,b) and purification from *B. subtilis* harboring a plasmid containing the pRNA gene and its native promoter (Wichitwechkarn *et al.*, 1992). For this work, we have been able to synthesize active pRNA *in vitro* with T7 RNA polymerase and DNA templates from PCR directly. This provides a very simple procedure to generate large quantities of wild-type or a variety of mutant pRNAs, which facilitates the identification of pRNA domains for protein binding or DNA packaging. Moreover, large quantities of pRNA will enable the crystallization of pRNA for X-ray diffraction studies.

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