RNA (1995), *1*:1041–1050. Cambridge University Press. Printed in the USA.

Confirmation of the helical structure of the 5′/3′ termini of the essential DNA packaging pRNA of phage ϕ29

CHUNLIN ZHANG, TIMOTHY TELLINGHUISEN, and PEIXUAN GUO

Department of Pathobiology and Purdue Biochemistry & Molecular Biology Program, Purdue University, West Lafayette, Indiana 47907, USA

ABSTRACT

Bacteriophage ϕ29 is typical of double-stranded DNA viruses in that its genome is packaged into a preformed procapsid during viral assembly. An intriguing feature of ϕ29 is the presence of a 120-base virus-encoded RNA (pRNA) that is indispensable for DNA packaging. Phylogenetic comparison of similar RNAs in numerous phages has revealed that the secondary structure of the pRNA is well conserved. Computer analysis predicts the presence of an extensive segment of helix with three single-base bulges generated by the pairing of the 5′ and 3′ ends. The desire to understand the role played by the pRNA in DNA packaging has led to a mutational analysis of the 5′-/3′-terminal region, which is believed to be important in DNA translocation. Deletion of 3 bases from the 3′ end of the RNA, shortening the pRNA from 120 to 117 bases, was tolerated without loss of activity, but additional deletion of the base 117 resulted in 100-fold less activity, and a 115-base pRNA was virtually nonfunctional. Additionally, the three unpaired one-base bulges within the helical stretches of the paired proximate ends were nonessential for pRNA activity, as demonstrated by deletion of the bulge individually. An extensive series of helix disruptions by single- and multiple-base substitution almost invariably led to the loss of DNA packaging activity. Additional mutations that restored predicted base pairings rescued pRNA activity. This second site suppression confirmed that the 5′- and 3′-end region was paired and was indeed a helical stretch. The secondary structure was of greater importance than the primary sequence, with the exception of the requirement of an adenine at either the third or fourth position. The specific requirement of an adenine in ϕ29 pRNA at this position, as well as conservation of this position in other phage pRNAs, implicates that this base may play a special role in either the DNA-packaging reaction or the maintenance of the pRNA tertiary structure.

Keywords: compensatory modification; DNA packaging; RNA bulge; RNA helix; RNA secondary structure; viral assembly

INTRODUCTION

The study of viral genome encapsidation is of great interest because it could reveal potential targets for anti-viral therapy. Extensive investigation on DNA packaging of the dsDNA viruses documents common features in this step of the viral life cycle (Earnshaw & Casjens, 1980; Black, 1989; Fujisawa & Hearing, 1994; Guo, 1994). These common features include the use of non-capsid enzyme(s) to translocate the viral DNA into a procapsid (Bazinet & King, 1985; Serwer, 1988; Guo & Trottier, 1994) with the hydrolysis of ATP to provide energy to drive DNA translocation (Feiss et al., 1985; Guo et al., 1987c; Higgins et al., 1988; Morita et al., 1993). DNA packaging is an intriguing problem in virology, with relevance to other biochemical processes, such as molecular motion, bioenergetics, and macromolecular interactions.

The genomic DNA of bacteriophage ϕ29 can be packaged into purified procapsids, produced from overexpressed genes in *Escherichia coli*, in an in vitro system with up to 90% efficiency. The DNA-filled procapsids can be converted to infectious virions after the in vitro addition of neck, tail, and morphogenic proteins produced from cloned genes, thereby providing a convenient assay for the function of packaging components (Guo et al., 1986, 1991b; Lee & Guo, 1994, 1995a, 1995b). One of the most surprising results during the development of the DNA-packaging system was the discovery of a small (120-base) virus-encoded RNA (pRNA) that is absolutely required for DNA encapsidation (Guo et al., 1987b). The presence of similar RNA

Reprint requests to: Peixuan Guo, Hansen Life Sciences Research Building, Cancer Research Center, Purdue University, West Lafayette, Indiana 47907, USA; e-mail: guo@vet.purdue.edu.

molecules has been identified in *Bacillus subtilis* dsDNA phages SF5, M2, NF, GA1, and PZA (Bailey et al., 1990), demonstrated in a chimeric λ/ϕ29 system (Donate & Carrascosa, 1991), and speculated in poxvirus (Parsons & Pickup, 1990), adenovirus (Hatfield & Hearing, 1994), *Streptococcus* phage Cp-1 (Martin et al., 1995) and the transposable Mu-like phage D108 (Burns et al., 1990). Research in our laboratory has demonstrated inhibition of viral encapsidation by targeting the pRNA with antisense oligonucleotides (Zhang et al., 1995a), or mutant pRNA (Trottier et al., 1996). The specificity and efficiency of inhibition in vivo targeting pRNA was unexpectedly high due to the requirement of multiple copies of pRNA to package one DNA genome, and the fact that one inactive pRNA in the group could completely block the packaging (Trottier et al., 1995).

The actual role of the RNA in genome packaging is still largely a mystery. In the presence of Mg^{2+}, the pRNA is known to bind to the portal vertex, the aperture through which DNA passes to enter the capsid. In the absence of other viral components, the pRNA does not bind purified gp16, the viral packaging ATPase (Guo et al., 1987a). Nucleotides 22–84 of the 120-base pRNA are protected from ribonuclease digestion when bound to the portal vertex of the procapsid as demonstrated by RNA footprinting assays (Reid et al., 1994b). The 5′/3′ ends have been proposed to compose the DNA translocation domain, because the DNA-packaging activity is hypersensitive to mutations in this region (Zhang et al., 1994). pRNAs with mutations in the translocation domain are defective in packaging, but often still competent for procapsid binding, indicating a specific function for this 5′/3′ end region (Zhang et al., 1994). Antisense oligos targeting bases 75–91 completely blocked the binding of pRNA to procapsid, suggesting that the location of the procapsid binding domain lies in this region (Zhang et al., 1995a). Phylogenetic analysis of pRNAs from phages M2, NF and SF5, and GA1 show very low sequence homology and few conserved bases, yet, the family of pRNAs appears to have similar secondary structure based on computer prediction (Bailey et al., 1990). Nonetheless, the requirement for pRNA appears to be specific, because pRNAs from related viruses, despite similar secondary structure, failed to function in ϕ29 packaging (Bailey et al., 1990), and mutation of two bases at the 5′ end resulted in a pRNA with 10^5-fold reduction in activity (Zhang et al., 1994). An understanding of the pRNA function and interaction with viral proteins requires a better understanding of the secondary and tertiary interactions within the molecule. Bases 76–78 have been shown to be paired with bases 90–88 (Reid et al., 1994b), and bases 1–2 have been shown to pair with bases 117–116 (Reid et al., 1994a; Zhang et al., 1994). A tertiary interaction, involving a proposed psuedoknot structure in the pRNA, has also been reported (Reid et al., 1994c). Beyond these, the secondary structure presumptions are based on computer predictions and on incomplete digestion with single- and double-strand specific nucleases (Bailey et al., 1990). Confirmation of the secondary structure is needed for further studies on tertiary structure of this pRNA, because S1 and V1 nuclease digestions only reveal whether the region is single- or double-stranded, but do not provide information regarding specific base pairings and interactions. Compensatory modification is one approach to identify base pairings (Couture et al., 1990). The discovery of compensatory mutants that are inactive with some base substitutions, but not others, indicates a specific base requirement in that region. Deletion mutagenesis can be used to define the minimum functional core pRNA structure, and provide the location of nonessential bases. Removal of nonessential base bulges from the helical packaging domain may reduce the size and asymmetry of this domain and allow for NMR or X-ray diffraction analysis. Nonessential base bulges, or their adjacent bases, can also be used as locations for the positioning of new 5′/3′ termini for the construction of active circularly permuted pRNA (cpRNA) molecules (Nolan et al., 1993; Zhang et al., 1995b; Pan et al., 1991) that will facilitate specific labeling of internal bases of pRNA and allow for easier, less expensive, PCR based site-directed mutagenesis of the interior of the pRNA molecule.

RESULTS

Deletion of bases at the 3′ termini

Our previous data has demonstrated that a 117-base pRNA, with a 3-base deletion at the 3′ end, has an activity similar to that of the full-length 120-base wild-type pRNA (Zhang et al., 1995b). Computer secondary structure predictions indicate that bases 118, 119, and 120 comprise an unpaired tail at the 3′ end of the pRNA. To address the question of what is the minimum length of pRNA required for activity, we synthesized two mutants, pRNA 7/116 and 7/13 (Fig. 1A), with sizes of 116 and 115 bases, respectively. The activity of pRNA 7/116, with 4 bases deleted from the 3′ end, was reduced 100-fold in comparison with wild-type control pRNA. Mutant pRNA 7/13, with a 5-base deletion, showed an almost complete loss of activity. These results indicate that deletions at the 3′ end beyond base 117 dramatically reduce the pRNA activity.

Nonessentiality of the single-base bulges

To investigate the role of the single-base bulges lying within the predicted helical stretches at the 5′ and 3′ ends of the pRNA, deletion mutants were generated and assayed for function. The mutated pRNA 5′dU/11 (Fig. 1A) contained a deletion of the single-base bulge

U_5 located near the 5′ end of the RNA, yet had packaging activity of 3.4×10^7 pfu/ml in comparison with 1.2×10^7 pfu/ml of the wild type, indicating that the bulge at this position is dispensable but augmentative. Another two mutants, pRNA 7/3′dC and pRNA 7/3′dA (Fig. 1A), carried a single-base bulge deletion at the 3′ end of the RNA at bases C_{109} and A_{106}, respectively. Both of these mutants were active in DNA packaging at a level indistinguishable from wild-type control pRNA. These results indicate that the separate deletion of single-base bulges at both the 5′ and 3′ ends of the pRNA molecule does not affect augmentative activity of pRNA.

Loss of DNA packaging activity by base substitutions at the 5′ and 3′ ends of the pRNA.

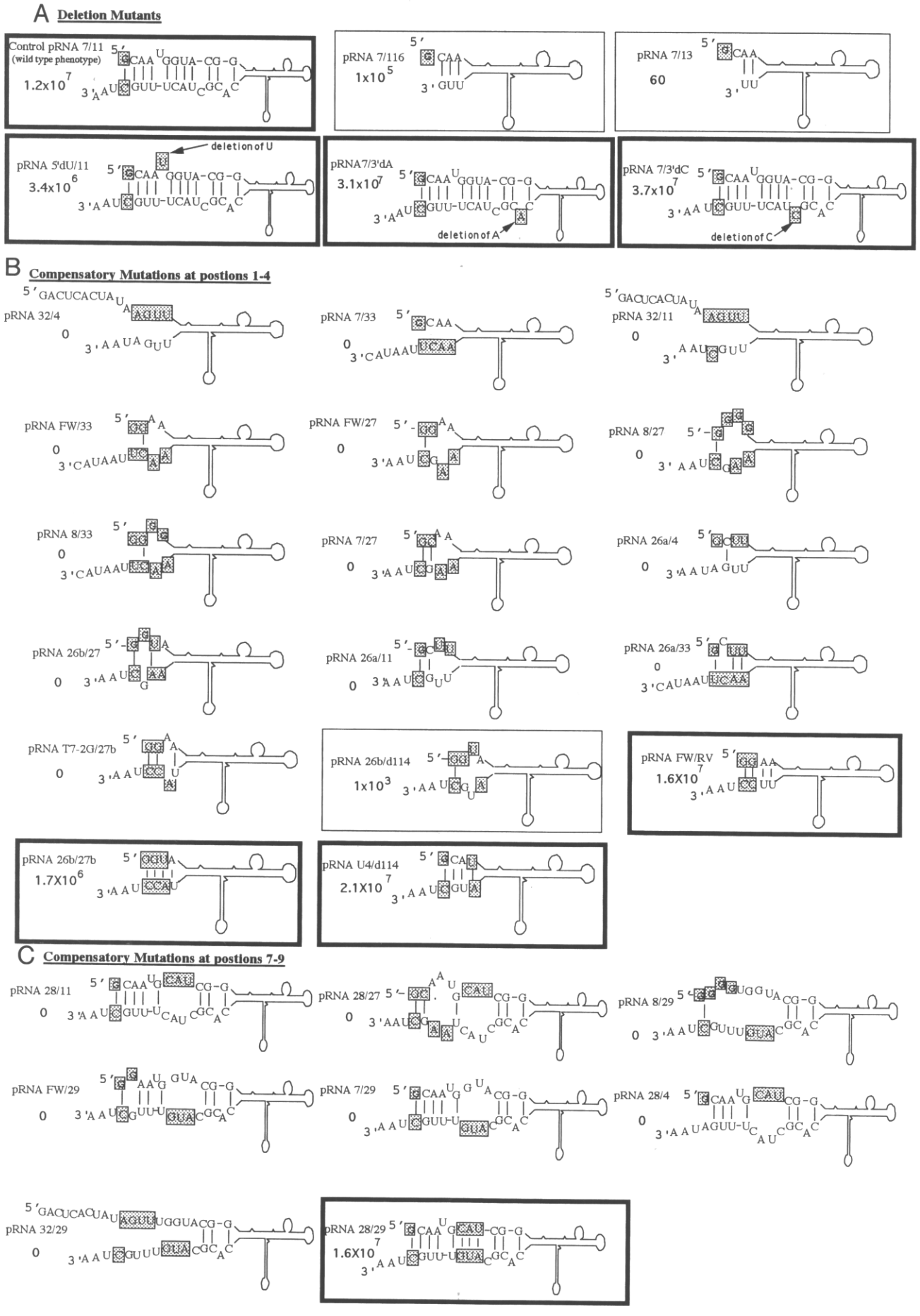
Computer predictions and phylogenetic analysis suggest that the 5′ and 3′ ends of the 117-base pRNA are paired. To confirm this, numerous mutant pRNAs (Fig. 1) were synthesized comprising substitution of the predicted base pairs to mismatched bases, thereby disrupting the helical region. All mutants containing one to four mismatches were nonfunctional in DNA packaging. Interestingly, single-base mismatches appeared to be sufficient to completely destroy the activity of the pRNA, again implicating functionally important secondary structure in the 5′ and 3′ ends of the RNA. An exception to the single mismatch observations was found with the mutant pRNA 26b/124 (Fig. 1D), which had a U_3/U_{115} mismatch pair, yet showed only a 100-fold drop in activity compared to wild-type pRNA. This implies that a mismatch at position three is tolerated in this pRNA. Additionally, a previously constructed mutant pRNA FW/4, which has a mismatch mutation at position one and two, is active with 10^5-fold reduced activity (Zhang et al., 1994).

Restoration of pRNA activity after compensatory changes of corresponding bases 1–4/117–114 and 7–9/112–110

We hypothesized that if the 5′/3′ paired ends were actually helical structures, compensatory mutations should be able to rescue mismatched base mutant pRNAs. To address this issue at position 1–4, 14 mismatch mutations (Fig. 1B, pRNA 32/4, 7/33, 32/11, FW/33, FW/27, 8/27, 8/33, 7/27, 26a/4, 26b/27, 26a/11, 26a/33, T7-2G/27b, and 26b/d114) were used to demonstrate the need for base pairing in the 1–4 region. Three mutant pRNAs, FW/RV, 26b/27b, and U4/d114 (Fig. 1B), were synthesized to serve as compensatory rescue mutations, restoring the base pairings in the region of bases 1–4. All of these rescue pRNAs were found to have wild-type or near wild-type activity. Our attention then shifted to the next "block" of helical structure in the paired end region, bases 7–9. Several mutant pRNAs (Fig. 1C, pRNA 28/11, 28/27, 8/29, FW/29, 7/29, 28/4, and pRNA 32/29) carried mutations that were designed to disrupt pairing at bases 7–9/112–110. These mutants were all inactive in DNA packaging. The compensatory mutant, pRNA 28/29 (Fig. 1C), was constructed to restore base pairing in region 7–9. The packaging activity of mutant pRNA28/29 was similar to that of the wild-type pRNA, confirming that bases 7–9 were paired with bases 112–110, and that the region at bases 7–9 was indeed helical. These results also indicate that no identifiable specific base requirements existed in the 7–9 region other than the satisfaction of Watson–Crick base pairing.

Requirement of one adenine base at position 3 or 4

In testing the compensatory rescue mutants in the region of bases 1–4, it was found that several pRNAs with perfect base pairing had no DNA-packaging activity, indicating the possibility of specific base interactions in this region. Figure 1D shows that pRNA 8/9 was inactive (Zhang et al., 1994), and pRNA 32/33 and 26a/27 (Fig. 1D) have greatly reduced activity, although all predicted base pairs were present after compensatory modification. The loss or reduction of activity of these pRNAs, as well as earlier data suggesting specific base requirements in this area, led to further analysis of this phenomenon. Both pRNA 32/33 and 26a/27 had a change of bases $A_3A_4/U_{115}U_{114}$ to $U_3U_4/A_{115}A_{114}$ with a reduction in activity. This suggests that an adenine at positions three or four may play an important and specific role in DNA packaging or pRNA structural maintenance. The retention of wild-type or near wild-type activity, demonstrated by mutants pRNA 26b/27b and pRNA u4/d114 (Fig. 1D), resulted from compensatory mutations that contained an adenine at either the third or the fourth position, confirming further the apparent need for a specific A-U pair in this region. Mutant pRNAs FW/4, 26b/124, and FW/RV (Fig. 1D) serve to further support the requirement of an adenine in position 3, while demonstrating that positions 1–2 should be paired (pRNA FW/4), and that no bulges should be present in the 3–4 position for maximum activity (pRNA 26b/124). The need for pairing in the 3–4 region is further supported by the A-U pair containing inactive mutants pRNA U4/11, 7/d114 (Fig. 1D), and T7-2G/27b (Fig. 1B). The conclusion that an adenine base at position 3 or 4 is required for activity was supported by our previous finding that mutant pRNA 8/9, with a change of bases $U_1C_2A_3A_4/A_{117}G_{116}U_{115}U_{114}$ to $G_1G_2G_3G_4/\ C_{117}C_{116}C_{115}C_{114}$, was also inactive, though no mismatch was present (Zhang et al., 1994). This data also agreed with the finding that a conserved A-U pair is present at this location in five phage pRNAs in the closely related viruses PZA, M2, NF, SF5, and GA1 (Bailey et al., 1990).



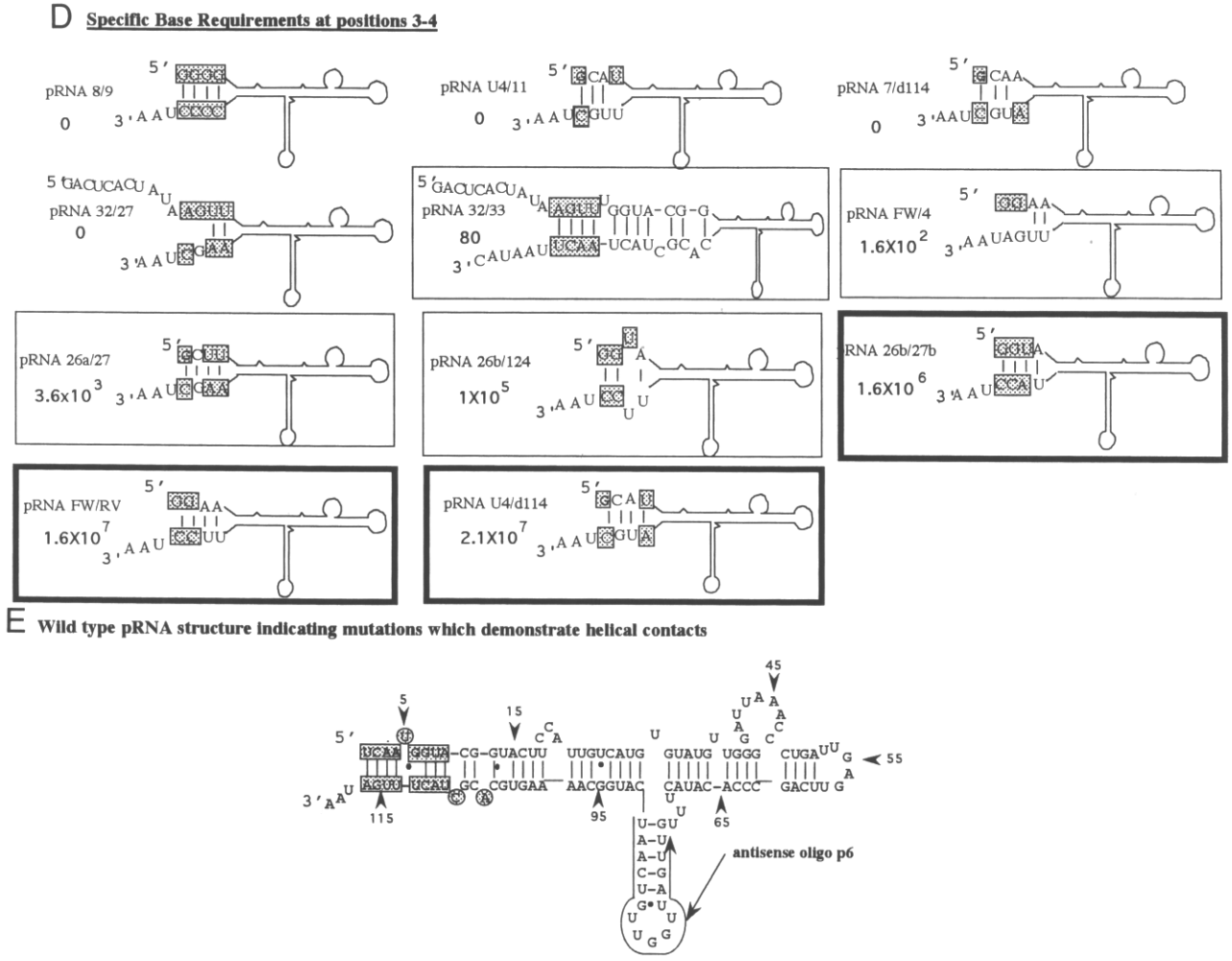


FIGURE 1. Mutation, deletion, and compensatory modification of pRNAs. Activity of each pRNA was determined as its capacity to package ϕ29 DNA in vitro in the defined system, and subsequent conversion of the DNA-filled capsids to infectious virions, expressed in numbers at the left as PFU/mL. Fully active pRNAs are framed in bold lines. Partially active pRNAs are framed in light lines. Inactive pRNAs are not framed. Bases boxed and shaded in gray indicate mutations introduced into the pRNA. Section E shows the predicted secondary structure of ϕ29 wild-type pRNA and the location of hybridization of antisense oligo P6. Base pairings confirmed in this study are boxed. Nonessential single-base bulge deletions are circled.

Inhibition of ϕ29 assembly in vitro with mutant pRNA

It has previously been shown that wild-type pRNA contains a procapsid binding domain located in the middle of the pRNA molecule and a paired 5′/3′ packaging domain (Reid et al., 1994a; Zhang et al., 1994, 1995a; Trottier et al., 1996). To demonstrate that packaging defective mutants generated in this paper were indeed defective for packaging activity and not simply involved in a global change of the pRNA structure, binding assays were conducted to confirm the presence of an intact procapsid binding domain. If these pRNA were distorted in the 5′ and 3′ ends, but were not affected in the procapsid binding domain, they should be able to compete with the wild-type pRNA for the procapsid binding site and therefore inhibit ϕ29 assembly. To examine this speculation, a highly sensitive in vitro ϕ29 assembly assay (Lee & Guo, 1994, 1995b) was used to test the inhibition of phage assembly. Wild-type pRNA was mixed with varied amounts of mutant pRNAs that were not competent in DNA packaging. When 70% of pRNA 8/9 (Fig. 1D) was added to the packaging reaction, plaque formation was reduced to up to four orders of magnitude compared to reactions in which no mutant pRNA was added (Fig. 2). *E. coli* 5s rRNA appeared to produce no inhibition of plaque formation, and mutant pRNAs u4/11 (Fig. 1D), T7-2G/27b, and 26b/d114 (Fig. 1B) all showed strong inhibition to phage assembly (data not shown), indicating that the inhibition was highly specific and the mutant pRNAs contained the intact procapsid binding domains. The log/log plot in Figure 2B showed that the slope of the inhibition curve was larger than 1, indicating that inhibition dependence on concentration of mutant pRNA was higher than first order.

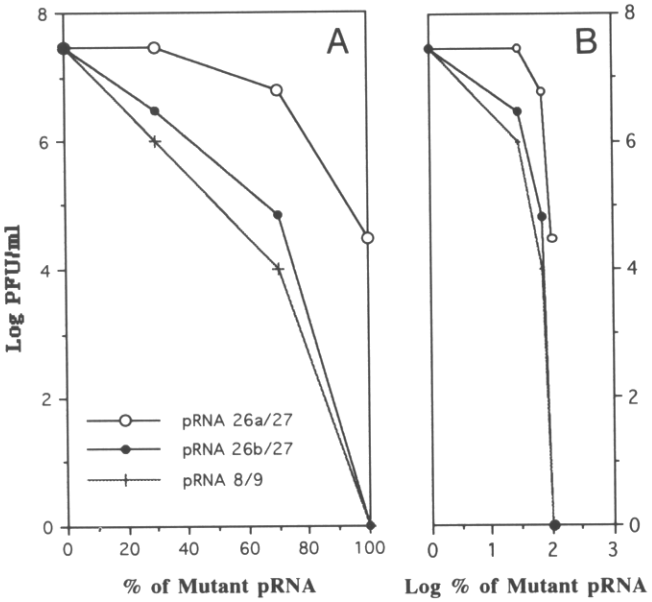


FIGURE 2. Semi-log (**A**) and log/log (**B**) plot of inhibition of ϕ29 assembly in vitro by mutant pRNAs.

Binding of the mutant pRNAs to procapsid was further investigated (Fig. 3) by sedimentation assays in the presence or absence of antisense oligo P6 (Fig. 1E), which has been shown to specifically inhibit the binding of pRNA to procapsid by binding to the pRNA lower hairpin loop at positions 75–91 (Fig. 1E) (Zhang et al., 1995a). The competence of mutant pRNAs to bind procapsid was demonstrated by sucrose gradient sedimentation of procapsids in the presence of [^{3}H]-pRNA molecules. [^{3}H]-pRNA/procapsid complexes migrate toward the bottom of the gradient, producing the observed peak between fractions 16–20 on the gradient, whereas unbound pRNA remains at the top of the gradient. The data for pRNA 26a/27 (Fig. 1D) was chosen to demonstrate the results of this assay. When no oligo was added to the reaction, binding of pRNA 26a/27 to procapsid occurred, as demonstrated by the peak between fractions 16–22. When antisense oligo P6 was added to the reaction, little binding of prohead by pRNA was observed (Fig. 3), indicating that the binding observed was specific. pRNA 7/11 (Fig. 1A), which was shown to have the procapsid binding and DNA-packaging activity of wild-type pRNA (Zhang et al., 1995b), was used as positive pRNA control. The peak observed at fractions 23–27 in all samples (Fig 3) was due to unbound RNA that remained in the top portion of the gradient. In the absence of procapsids, [^{3}H]-pRNA remained on top of the gradient (data not shown). Other pRNAs with mutations or deletions at either the 5′ or 3′ ends, such as 26b/27 (Fig. 1B) and 8/9 (Fig. 1D), were tested and found to also have bound procapsid specifically, whereas *E. coli* 5s rRNA did not bind procapsids (data not shown). The results suggested that these mutant pRNAs retained their procapsid binding ability, confirming that mutants affected only the DNA-packaging activity due to the modification of the domain at 5′/3′ paired ends.

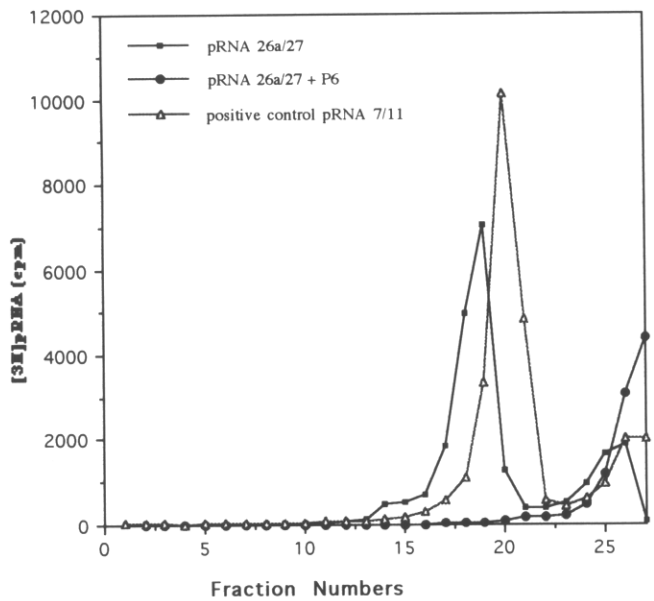


FIGURE 3. Demonstration of procapsid binding competency and specificity of the mutant pRNAs. Purified ϕ29 procapsids were mixed with [^{3}H]-pRNAs in the presence or absence of antisense oligo P6, and assayed by sucrose gradient sedimentation indicated as from right to left. Peaks between fractions 17 and 22 were [^{3}H]-pRNA/procapsid complexes. In the presence of antisense oligo P6, the peak representing the pRNA/procapsid complexes was not observed. Difference between the intensity and location of control and experimental peaks was due to variation during the collection of fractions from the multiple individual gradients.

DISCUSSION

It is logical to hypothesize that the bulged bases, not restricted into the plane of the helix by base pairing, may be more free to interact with proteins, DNA, or other regions of the RNA, and therefore may play an important role in RNA structure and function (Schroeder et al., 1991; Zacharias & Hagerman, 1995). Deletion mutagenesis has implicitly demonstrated that the bulged bases, U_5, A_{106}, and C_{109}, have little function in the structure or activity of the pRNA individually. It has been demonstrated previously that the three-base bulge, $C_{18}C_{19}A_{20}$ (Fig. 1E), is essential, whereas all other single-base bulges, U_{29} and U_{35}, of the pRNA can be deleted without diminishing pRNA activity in DNA packaging (Reid et al., 1994a, 1994c). It is now clear that all five single-base bulges of the pRNA molecule are nonessential for activity when deleted individually. This information will aid in the future determination of the minimum "core" functional unit of the active pRNA. The definition of a minimum core pRNA may, in the future, aid in the solution of the structure of the packaging domain of the pRNA by X-ray diffraction or NMR. Current work is underway in generating circularly permuted pRNAs with open-

ings at these nonessential locations to determine if openings at these locations are better tolerated than those previously attempted. The demonstration of these nonessential bases opens new doors to specific labeling and specific incorporation of radioactive labeling or photo crosslinking agents in the cpRNA to determine the interaction of the pRNA with the DNA packaging machinery.

Of 27 mutant pRNAs in which base pairings were destroyed, none were active in DNA packaging at wild-type control levels. In the entire sequence of the pRNA, there is not a single mismatch, with the exception of G-U pairs, as is the case for pRNAs of phage GA1, SF5, M2, NF, and PZA (Bailey et al., 1990). Our purposeful disruption of the proposed helix in the DNA-packaging domain provided the evidence that the region was indeed helical. Most single-base mismatches could completely destroy the activity of the pRNA, supporting the observation that a paired DNA-packaging domain is critical for activity. One of the pRNAs, pRNA 26b/124 (Fig. 1D), was unusual in that this RNA had a single mismatch, U-U, at bases 3/115, yet packaged DNA with only a 100-fold loss of activity. This is possibly due to the fact that the two terminal end bases, bases 1 and 2, were interacting with bases 117–116 as G-C pairs, and an A-U pair was present in position 4, hence generating less distortion of the helix by the thermodynamic stability of the G-C pairings. Despite this one unusual result, the data generated by the induced mismatches greatly support the presence of a helical DNA packaging domain and the importance of paired ends.

Mutation of the pRNA to nonfunctional form through introduction of mismatches, followed by compensatory rescue mutations has provided significant information about the secondary structure and specific requirements of the DNA-packaging domain of the pRNA. Previous research (Reid et al., 1994a; Zhang et al., 1994) as well as the work contained in this communication, has shown that positions 1 and 2 can be altered to any base without loss of activity as long as a compensatory change of bases 2/116 is also made. Bases 1 and 117 do not have to be paired for a fully active pRNA molecule, but lack of pairing in both positions 1 and 2 shows a significant loss of activity. Base pairing in positions 3 and 4, as well as 7–9, is absolutely required for fully active pRNA. The compensatory mutation data presented clearly indicate base pairing at positions 1–4 and 7–9, thereby furthering our knowledge of the secondary structure of the DNA-packaging domain of the pRNA.

It has been demonstrated that mutant pRNA 8/9 (Fig. 1D), containing altered 1–4/117–114 positions from $U_1C_2A_3A_4/A_{117}G_{116}U_{115}U_{114}$ to $G_1G_2G_3G_4/C_{117}C_{116}C_{115}C_{114}$, was inactive (Zhang et al., 1994). We again showed that mutant pRNA 26a/27 (Fig. 1 D), containing altered 1–4/117–114 positions from $U_1C_2A_3A_4/A_{117}G_{116}U_{115}U_{114}$ to $G_1C_2U_3U_4/C_{117}G_{116}A_{115}A_{114}$, lost activity by 10^4-fold. It had already been shown that any 1–2 compensatory mutation mutant is active, implying that the loss of function in pRNA 8/9 and 26a/27 was due to the alteration of position 3–4. Additional data implying a sequence-specific requirement at position 3–4 were collected from mutant pRNA 32/33 (Fig. 1D) in which the sequence $A_1G_2U_3U_4/U_{117}C_{116}A_{115}A_{114}$ was generated. It should be noted that this RNA was made with additional bases extending from the end that have been found to not affect the activity of other pRNAs (Zhang et al., 1995b). This mutant showed no packaging activity, again implying a sequence specific requirement at position 3–4. It was apparent, from several mutant pRNAs, that G-C and U-A pairs were unable to replace all A-U base pairs at position 3–4, respectively, again suggesting an adenine at position 3 or 4 was required for pRNA function. To continue investigating this issue, control pRNA sequences of $G_1C_2A_3A_4$ were altered to $G_1C_2A_3U_4$ and to $G_1C_2U_3A_4$ to test whether the A at position 3 or 4 had to be paired with a U at position 115 or 114. The presence of base pairing in position 3–4 also appears to be a requirement for function, as shown by mutant pRNA U4/11 and 26b/124 with U-U mismatches in positions 3 and 4 (Fig. 1D). The demonstration of the requirement for an A-U pair at 3–4/115–114 position is supported by the phylogenetic studies showing that an A-U pair is conserved at this position in all pRNAs of phages SF5, M2, NF, and PZA (Bailey et al., 1990). From the success of these mutations, it is clear that an A-U pair is involved in either the DNA packaging mechanism or simply in maintaining the structure of the pRNA through intramolecular contacts. Prohead binding assays demonstrate that mutants without this adenine base are competent for procapsid binding, precluding its interaction with portal vertex. The adenine base in this position is present as part of a base pair, precluding interaction between the purine ring and the other bases in the pRNA. It has been shown that the 2′-hydroxyl group of certain bases are critical for interaction or base–base contact (Pyle & Cech, 1991; Musier-Forsyth & Schimmel, 1992; Yap & Musier-Forsyth, 1995). It is unclear why adenine is required in position 3–4, and why the purine base guanine could not serve as substitute if the requirement of base pairing is fulfilled. It remains to be demonstrated whether the A-U pair is required for interaction with genomic DNA, the ATPase gp16, or other portions of the pRNA. It is clear that a single A-U pair is not of sufficient length or complexity to serve as a specific protein-binding recognition site, unless serving as a small part of a specific tertiary binding domain on the RNA. The specific adenine base may play a critical role in the currently undefined ATP-driven DNA translocation reaction. The ability of the required adenine to reside in either the third or fourth position indicates that the role of this adenine has some

degree of flexibility in the overall spatial arrangement of the base, which may make modeling this region of the pRNA into part of an active site for packaging problematic. The work described herein serves to direct our research into new areas. The demonstration of a key conserved base in the helical DNA-packaging domain will undoubtedly lead to new insight into the structure and function of this pRNA.

MATERIALS AND METHODS

Synthesis and nomenclature of mutant pRNAs

Twenty-two oligonucleotides were used as PCR primers (Table 1). Two plasmid DNAs, pRT71 (Reid et al., 1994b) and cpDNA3A (Zhang et al., 1995b), were used as PCR templates for the amplification of DNA fragments for use in the synthesis of mutant pRNAs as described previously (Zhang et al., 1994, 1995b). Briefly, linear plasmid pRT71 or cpDNA3A were used as templates to generate PCR DNA fragments with primer pairs containing either T7 or SP6 promoter and mutations to pRNA. PCR fragments were then used as templates to synthesize mutant pRNAs by in vitro transcription with either T7 or SP6 RNA polymerase (Milligan et al., 1987). The mutant pRNA produced from the PCR template using primer pairs P26a and P4 was called mutant pRNA 26a/4, other pRNAs derived from different primer pairs were named in a similar fashion. The subscript numbers following the bases indicate the base location. For example, G1 represents the first guanine base at the 5′ end of the pRNA. [^{3}H]-labeled pRNA molecules were synthesized by adding 10 μCi of [^{3}H]-UTP (47 Ci/mmol, Amersham) to the in vitro transcription reaction.

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

Purification of the DNA packaging enzyme gp16 (Guo et al., 1986), DNA-gp3 (Lee & Guo, 1994), procapsid (Guo et al., 1991a, 1991b), tail protein gp9 (Lee & Guo, 1995b), and neck and tail proteins (Lee & Guo, 1994) have been described previously.

Mutant pRNA activity assay with the ϕ29 assembly system

Construction of the highly sensitive ϕ29 assembly system for the assay of pRNA activity has been reported previously (Lee & Guo, 1994, 1995a, 1995b). The assay is based on the assembly of infectious ϕ29 virions in vitro with pRNA transcribed in vitro and proteins produced and purified from cloned genes. Briefly, approximately 0.7 pmol (8 μg of gp8 capsid protein equivalent) of procapsid was mixed with 1.5 pmol (60 ng) of pRNA and dialyzed against TBE buffer (89 mM Tris-borate, pH 8.3, 2.5 mM EDTA) on a 0.025-μm type VS filter membrane (Millipore Corp.) for 15 min at ambient temperature. The mixtures were then dialyzed against TMS buffer (50 mM Tris-HCl, pH 7.8, 100 mM NaCl, 10 mM $MgCl_2$) for 30 min. Next, pRNA-enriched procapsids were mixed with

TABLE 1. Oligonucleotides used in synthesis of mutant pRNAs.

Oligo names	Orientation	Sequences[a]	Size (bases)	Location on pRNA (base #)
P4	Reverse	5′ TTATCAAAGTAGCGTGCAC 3′	19	120–102
P6	Reverse	5′ ATTGACAACCAATCAAC 3′	17	91–75
P7	Forward	5′ TAATACGACTCACTATAGCAATGGT 3′[b]	25	1–8
P8	Forward	5′ TAATACGACTCACTATAGGGGTGGTAC 3′[b]	27	1–10
P9	Reverse	5′ TTAGGGGAGTAGCGTGCAC 3′	19	120–102
P10	Reverse	5′ GCAAAGTAGCGTGCACTTTTG 3′	21	117–97
P11	Reverse	5′ TTAGCAAAGTAGCGTGCACTTTTG 3′	24	120–95
P13	Reverse	5′ AAAGTAGCGTGCACTTT 3′	17	115–99
P26a	Forward	5′ GTAATACGACTCACTATAGCTTTGGTAC3′[b]	28	1–10
P26b	Forward	5′ TAATACGACTCACTATAGGTATGGTACGGTACT 3′[b]	33	1–16
P27	Reverse	5′ GCTTAGTAGCGTGCACTTTTG 3′	21	120–100
P27b	Reverse	5′ TTAGGTAAGTAGCGTGC 3′	17	120–104
P28	Forward	5′ TAATACGACTCACTATAGCAATGCATCGGTACT 3′[b]	33	1–16
P29	Reverse	5′ GCAAACATGCGTGCTCTTTTG 3′	21	120–100
P32	Forward	5′ ATTTAGGTGACACTATAGACTCACTATAAGTTTGGTACGGTAC 3′[c]	43	1–15
P33	Reverse	5′ GTATTATAGTTAGTAGCGTGC 3′	21	120–100
P116	Reverse	5′ CAAAGTAGCGTGCACTTTTG 3′	20	116–97
5′PT7dU	Forward	5′ TAATACGACTCACTATAGCAAGGTAC 3′[b]	26	1–9
3′PdC	Reverse	5′ TTAGCAAAGTACGTGCAC 3′	18	120–103
3′PdA	Reverse	5′ GCAAAGTAGCGGCACTTTTG 3′	20	117–98
5′PT7u4	Forward	5′ TAATACGACTCACTATAGCATTGGTACG 3′[b]	28	1–11
3′Pd114	Reverse	5′ TTAGCATAGTAGCGTGCA 3′	18	120–102
5′T7W2G	Forward	5′ GTAATACGACTCACTATAG 3′[b]	19	0–1

[a] Promter sequences are indicated by underlining.
[b] T7 promoter sequence.
[c] SP6 promoter sequence.

3 μL of reaction buffer (10 mM ATP, 3 mM 2-mercaptoethanol, and 6 mM spermidine in TMS), DNA-gp3 that had been dialyzed against TMS for 40 min, and the packaging ATPase gp16, which had been dialyzed against 0.01 M Tris-HCl, pH 7.5, 4 mM KCl for 40 min on ice. The final mixture was incubated for 30 min at ambient temperature.

The DNA-filled procapsids were then incubated with 18 μL of gp8.5-9 extract from *E. coli* containing plasmid pARgp8.5-9 and 20 μL of gp11-12-13-14 extract from *E. coli* containing plasmid pARgp11-12-13-14 for 2 h at room temperature to complete the assembly of infectious phage. The activity of each mutant pRNA reported in this paper was measured by the number of plaque-forming units per milliliter produced when the mutant pRNAs were used in the aforementioned assay. The activity is based only on the number of plaques generated, in comparison to a control pRNA. Mutant pRNA molecules, which produced fewer PFU/mL than the control pRNA, when used in the plaque-forming assay, were said to have reduced activity.

Inhibition assays for mutant pRNAs were performed with the same assembly system mentioned above. Varied amounts of mutant pRNA were mixed with pRNA 7/11, which has been shown to have the wild-type phenotype (Zhang et al., 1995b), before incubating with purified procapsids.

Procapsid-pRNA binding assays

Sucrose gradient sedimentation of procapsid-[^{3}H]-pRNA mixtures were performed to detect the presence of procapsid–pRNA complexes (Guo et al., 1987a). Briefly, [^{3}H]-pRNA (60 ng) was mixed with 10 μL of purified procapsid (4 mg/mL) and dialyzed on a 0.025-μm type VS filter membrane against TBE for 15 min at ambient temperature. The mixtures were then dialyzed against TMS buffer for 30 min at ambient temperature. After the incubation, the mixtures were diluted to 0.1 mL in TMS and loaded onto the top of a 5–20% sucrose gradient in TMS and spun in a Beckman L-80 ultracentrifuge at 35,000 rpm for 30 min at 20 °C in an SW55 rotor. Following centrifugation, fractions were collected from the bottom of the tube and prepared for scintillation counting.

Computer prediction of mutant pRNA secondary structure

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

ACKNOWLEDGMENTS

We thank Drs. R. Reid and D. Anderson for plasmid pRT71, and Dr. Elke Scholz for procapsid purification. This work was supported by NIH grant GM 48159 to P.G.

Received September 28, 1995; returned for revision November 6, 1995; revised manuscript received November 16, 1995

REFERENCES

Bailey S, Wichitwechkarn J, Johnson D, Reilly B, Anderson D, Bodley JW. 1990. Phylogenetic analysis and secondary structure of the *Bacillus subtilis* bacteriophage RNA required for DNA packaging. *J Biol Chem 265*:22365–22370.

Bazinet C, King J. 1985. The DNA translocation vertex of dsDNA bacteriophages. *Annu Rev Microbiol 39*:109–129.

Black LW. 1989. DNA packaging in dsDNA bacteriophages. *Annu Rev Microbiol 43*:267–292.

Burns CM, Chan HLB, DuBow MS. 1990. In vitro maturation and encapsidation of the DNA of transposable μ-like phage D108. *Proc Natl Acad Sci USA 87*:6092–6096.

Couture S, Ellington AD, Gerber AS, Cherry JM, Doudna JA, Green R, Hanna M, Pace U, Rajagopal J, Szostak JW. 1990. Mutational analysis of conserved nucleotides in a self-splicing group I intron. *J Mol Biol 215*:345–358.

Donate LE, Carrascosa JL. 1991. Characterization of a versatile in vitro DNA packaging system based on hybrid λ/ϕ29 proheads. *Virology 182*:534–544.

Earnshaw WC, Casjens SR. 1980. DNA packaging by the double-stranded DNA bacteriophages. *Cell 21*:319–331.

Feiss M, Sippy J, Miller G. 1985. Processive action of terminase during sequential packaging of bacteriophage λ chromosomes. *J Mol Biol 186*:759–771.

Fujisawa H, Hearing P. 1994. Structure, function and specificity of the DNA packaging signals in double-stranded DNA viruses. *Virology 5*:5–13.

Guo P. 1994. Introduction: Principles, perspectives, and potential applications in viral assembly. *Sem Virol 5*(1):1–3.

Guo P, Bailey S, Bodley JW, Anderson D. 1987a. Characterization of the small RNA of the bacteriophage ϕ29 DNA packaging machine. *Nucleic Acids Res 15*:7081–7090.

Guo P, Erickson S, Anderson D. 1987b. A small viral RNA is required for in vitro packaging of bacteriophage ϕ29 DNA. *Science 236*:690–694.

Guo P, Erickson S, Xu W, Olson N, Baker TS, Anderson D. 1991a. Regulation of the phage ϕ29 prohead shape and size by the portal vertex. *Virology 183*:366–373.

Guo P, Grimes S, Anderson D. 1986. A defined system for in vitro packaging of DNA-gp3 of the *Bacillus subtilis* bacteriophage ϕ29. *Proc Natl Acad Sci USA 83*:3505–3509.

Guo P, Peterson C, Anderson D. 1987c. Prohead and DNA-gp3-dependent ATPase activity of the DNA packaging protein gp16 of bacteriophage ϕ29. *J Mol Biol 197*:229–236.

Guo P, Rajogopal B, Anderson D, Erickson S, Lee CS. 1991b. sRNA of bacteriophage ϕ29 of *Bacillus subtilis* mediates DNA packaging of ϕ29 proheads assembled in *E. coli*. *Virology 185*:395–400.

Guo P, Trottier M. 1994. Biological and biochemical properties of the small viral RNA (pRNA) essential for the packaging of the double-stranded DNA of phage ϕ29. *Sem Virol 5*:27–37.

Hatfield L, Hearing P. 1994. The NFIII/OCT-1 binding site stimulates adenovirus DNA replication in vivo and is functionally redundant with adjacent sequences. *J Virol 67*:3931–3939.

Higgins RR, Lucko HJ, Becker A. 1988. Mechanism of *cos* DNA cleavage by bacteriophage lambda terminase—Multiple roles of ATP. *Cell 54*:765–775.

Lee CS, Guo P. 1994. A highly sensitive system for the assay of in vitro viral assembly of bacteriophage ϕ29 of *Bacillus subtilis*. *Virology 202*:1039–1042.

Lee CS, Guo P. 1995a. Sequential interactions of structural proteins in phage ϕ29 procapsid assembly. *J Virol 69*:5024–5032.

Lee CS, Guo P. 1995b. In vitro assembly of infectious virions of dsDNA phage ϕ29 from cloned gene products and synthetic nucleic acids. *J Virol 69*:5018–5023.

Martin AC, Lopez R, Garcia P. 1995. Nucleotide sequence and transcription of the left early region of *Streptococcus pneumoniae* bacteriophage Cp-1 coding for the terminal protein and the DNA polymerase. *Virology 211*:21–32.

Milligan JF, Groebe DR, Witherell GW, Uhlenbeck OC. 1987. Oligoribonucleotide synthesis using T7 RNA polymerase and synthetic DNA templates. *Nucleic Acids Res 15*:8783–8798.

Morita M, Tasaka M, Fujisawa H. 1993. DNA packaging ATPase of bacteriophage T3. *Virology 193*:748–752.

Musier-Forsyth K, Schimmel P. 1992. Functional contacts of a trans-

fer RNA synthetase with 2′-hydroxyl groups in the RNA minor groove. *Nature 357*:513–515.
Nolan JM, Burke DH, Pace NR. 1993. Circularly permuted tRNAs as specific photoaffinity probes of ribonuclease P RNA structure. *Science 261*:762–765.
Pan T, Gutell RR, Uhlenbeck OC. 1991. Folding of circularly permuted transfer RNAs. *Science 254*:1361–1364.
Parsons BL, Pickup DJ. 1990. Transcription of orthopoxvirus telomeres at late times during infection. *Virology 175*:69–80.
Pyle AM, Cech TR. 1991. Ribozyme recognition of RNA by tertiary interactions with specific ribose 2′-OH groups. *Nature 350*:628–631.
Reid RJD, Bodley JW, Anderson D. 1994a. Characterization of the prohead–pRNA interaction of bacteriophage ϕ29. *J Biol Chem 269*:5157–5162.
Reid RJD, Bodley JW, Anderson D. 1994b. Identification of bacteriophage ϕ29 prohead RNA (pRNA) domains necessary for in vitro DNA-gp3 packaging. *J Biol Chem 269*:9084–9089.
Reid RJD, Zhang F, Benson S, Anderson D. 1994c. Probing the structure of bacteriophage ϕ29 prohead RNA with specific mutations. *J Biol Chem 269*:18656–18661.
Schroeder R, Ahsen UV, Belfort M. 1991. Effects of mutations of the bulged nucleotide in the conserved P7 pairing element of the phage T4 td intron on ribozyme function. *Biochemistry 30*:3295–3303.
Serwer P. 1988. The source of energy for bacteriophage DNA packaging: An osmotic pump explains the data. *Biopolymers 27*:165–169.
Trottier M, Zhang CL, Guo P. 1996. Complete inhibition of virion assembly in vivo with mutant pRNA essential for phage ϕ29 DNA packaging. *J Virol*. Forthcoming.
Yap LP, Musier-Forsyth K. 1995. Transfer RNA aminoacylation: Identification of a critical ribose 2′-hydroxyl-base interaction. *RNA 1*:418–424.
Zacharias M, Hagerman PJ. 1995. Bulge-induced bends in RNA: Quantification by transient electric birefringence. *J Mol Biol 247*:486–500.
Zhang CL, Garver K, Guo P. 1995a. Inhibition of phage ϕ29 assembly by antisense oligonucleotides targeting viral pRNA essential for DNA packaging. *Virology 211*:568–576.
Zhang CL, Lee CS, Guo P. 1994. The proximate 5′ and 3′ ends of the 120-base viral RNA (pRNA) are crucial for the packaging of bacteriophage ϕ29 DNA. *Virology 201*:77–85.
Zhang CL, Trottier M, Guo P. 1995b. Circularly permuted viral pRNA active and specific in the packaging of bacteriophage ϕ29 DNA. *Virology 207*:442–451.

Confirmation of the helical structure of the 5'/3' termini of the essential DNA packaging pRNA of phage phi 29.

C Zhang, T Tellinghuisen and P Guo

RNA 1995 1: 1041-1050