Virology **281,** 281–293 (2001)
doi:10.1006/viro.2000.0771, available online at http://www.idealibrary.com on IDEAL®

Chemical Modification Patterns of Active and Inactive as Well as Procapsid-Bound and Unbound DNA-Packaging RNA of Bacterial Virus Phi29

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Received August 10, 2000; returned to author for revision September 18, 2000; accepted November 29, 2000

During replication, the lengthy genome of dsDNA viruses is translocated with remarkable velocity into the limited space within the preformed procapsid. We previously found that a viral-encoded RNA (pRNA) played a key role in bacterial virus phi29 DNA translocation. Design of mutant pRNA sets containing two and three inactive mutant pRNAs, respectively, led to the conclusion that the stoichiometry of pRNA in DNA packaging is the common multiple of 2 and 3. Together with studies using binomial distribution of mutant and wild-type pRNA, it has been confirmed that six pRNAs of phi29 form a hexagonal complex to drive the DNA translocating machine. These findings have brought about commonality between viral DNA packaging and other universal DNA/RNA-riding processes including DNA replication and RNA transcription. Chemical modification was used to compare the structures of active and inactive as well as free and procapsid-bound pRNA. Our results explain why certain pRNA mutants are inactive in DNA packaging while remaining competent in procapsid binding, since the mutations were located in a domain involved in DNA translocation that is dispensable for procapsid binding. A mutant pRNA that had reduced procapsid binding was revealed to have a structural alteration within the procapsid-binding region that may account for the binding deficiency. Chemical probing of procapsid-bound pRNA revealed a large area of protection, while a 3-base bulge, $C^{18}C^{19}A^{20}$, was accessible to chemicals. A pRNA with a deletion of this 3-base bulge was fully competent to form dimers, bind procapsids, and inhibit phi29 virion assembly *in vitro;* however, its activity in DNA packaging and virion assembly was completely lost. The results suggest that this bulge is not involved in procapsid binding but may interact with other DNA-packaging components. A computer model showing the location of the CCA bulge was presented.

INTRODUCTION

All linear dsDNA viruses, including herpesviruses, poxviruses, adenoviruses, and linear dsDNA bacterial viruses, package their DNA into a preformed protein shell (procapsid). ATP is consumed to condense the lengthy genomic DNA into the limited space inside procapsids, and one ATP is needed for the packaging of two basepairs of phi29 DNA (Guo *et al.,* 1987b), suggesting that DNA is driven into the capsid by an ATPase motor. Phage phi29 also requires a viral-encoded 120-base RNA (pRNA) to package its genomic DNA. The pRNA secondary structure (Fig. 1) has been partially confirmed by mutagenesis (Zhang *et al.,* 1994; Reid *et al.,* 1994b), crosslinking (Mohammad *et al.,* 1999; Chen and Guo, 1997a), compensatory modification (Zhang *et al.,* 1994), nuclease probing (Reid *et al.,* 1994a; Chen and Guo, 1997a), and chemical modification (Trottier *et al.,* 2000).

To elucidate the role of the pRNA in phi29 DNA packaging motor, it is crucial to know how many copies of the pRNA are involved in each DNA packaging event. We have developed three approaches to determine the stoichiometry of the pRNA. These three approaches led to the conclusion that six pRNAs are involved in each motor.

The first approach for pRNA stoichiometry determination is the use of binomial distribution (Trottier and Guo, 1997; Chen *et al.,* 1997). pRNAs with mutations in the 5′/3′ paired region (the DNA translocation domain) retained procapsid-binding capacity but lost DNA-packaging function. When mutant pRNA and wild-type pRNA were mixed at various ratios in *in vitro* assembly assays, the probability of procapsids that possess a certain amount of mutant and a certain amount of wild-type pRNA was determined by the expansion of a binomial $(p + q)^z$, where Z is the total number of pRNA per procapsid, while p and q represent the percentage of mutant and wild-type pRNA, respectively, used in reaction mixture. For example, if we assume that Z is 3, the probability of all combinations of mutant and wild-type pRNAs on a given procapsid can be predicted by the

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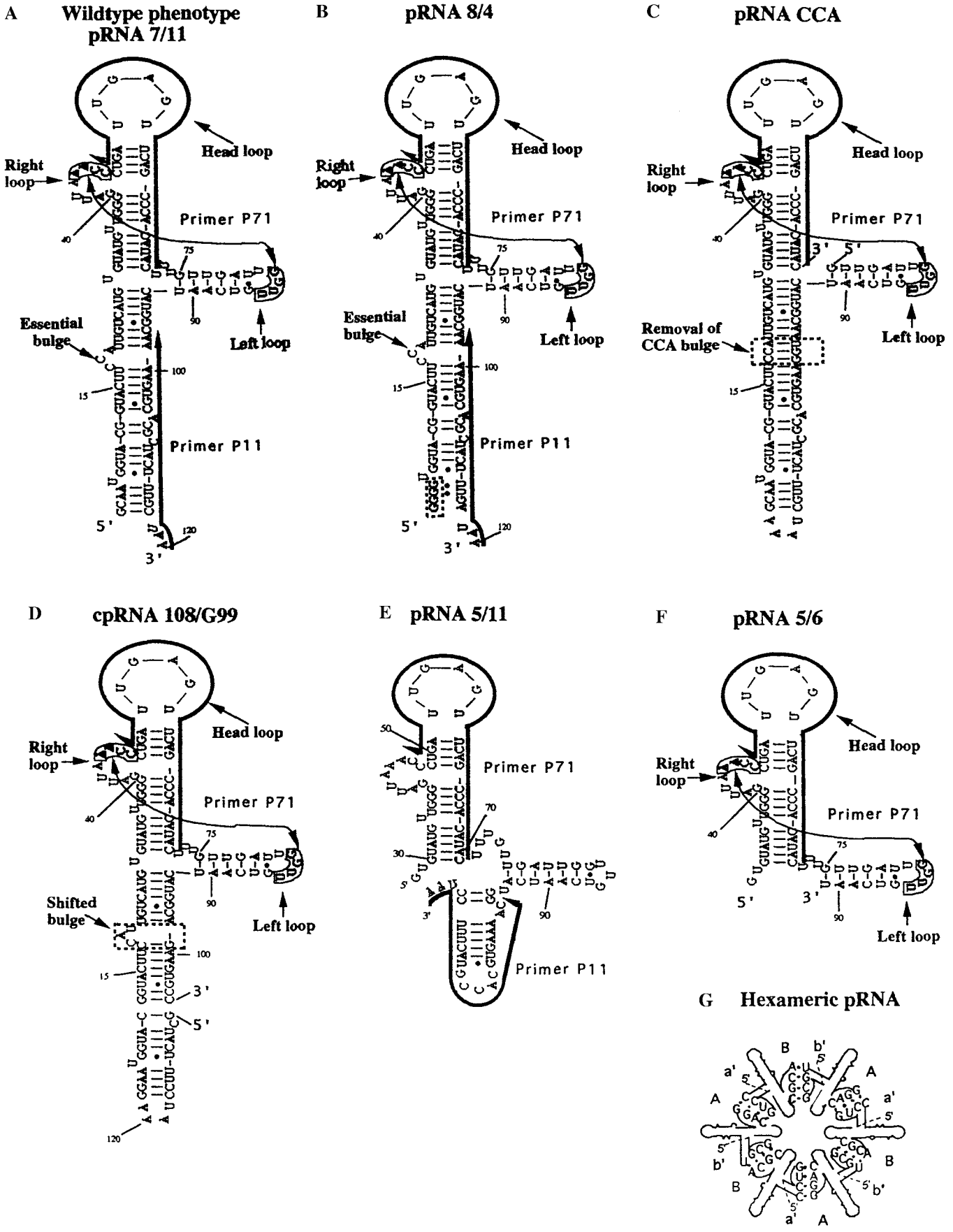
0042-6822/01 $35.00


A
Wildtype phenotype
pRNA 7/11
Right loop
Head loop
Primer P71
Essential bulge
Left loop
Primer P11
B
pRNA 8/4
C
pRNA CCA
Removal of CCA bulge
D
cpRNA 108/G99
Shifted bulge
E
pRNA 5/11
F
pRNA 5/6
G
Hexameric pRNA

expansion of the binomial: $(p + q)^3 = p^3 + 3p^2q + 3pq^2 + q^3 = 100\%$. The yield of virions from empirical data was plotted and compared to a series of predicted curves to find a best fit. Our results showed that approximately five to six pRNAs were needed for each procapsid to package DNA, explaining the high inhibition efficiency of mutant pRNA (Trottier *et al.,* 1996).

The second approach for stoichiometry determination is by log plots of serial dilution versus the yield of virions assembled *in vitro* (Trottier and Guo, 1997). The larger the stoichiometry of the component, the more dramatic the influence of the dilution factor on the reaction. A slope of 1 indicates that one copy of the component is involved in the assembly of one virion (e.g., DNA). A slope larger than 1 would indicate multiple-copy involvement. Our result also supports the conclusion that the stoichiometry of pRNA in DNA packaging is between 5 and 6.

The third approach for stoichiometry determination was the mixing of inactive mutant pRNAs, each having interactive complementary loops, with one another to determine the common multiples (Guo *et al.,* 1998). Since infectious virions could be produced by mixing two inactive pRNAs with interlocking loops, we showed that the stoichiometry of the pRNA is a multiple of 2. Since infectious virions could also be produced by mixing another set of three inactive pRNAs with interlocking loops, we showed that the stoichiometry of the pRNA is also a multiple of 3. Therefore, we confirmed that the stoichiometry of pRNA to assemble on virions is the common multiple of 2 and 3, that is, 6 or 12. Together with the results from binomial distribution analyses, we confirmed that the stoichiometry of pRNA was 6.

The conclusion of the stoichiometry of 6 is supported by finding that pRNA dimer is the building block to assemble hexamers (Chen *et al.,* 2000). We found that the sequence in the assembly of hexamer is dimer → tetramer → hexamer. The low-resolution 3-D structure of pRNA monomers and dimers has been shown by cryo-atomic force microscopy (Trottier *et al.,* 2000; Chen *et al.,* 2000). The monomer exhibits a "checkmark" shape. The dimer displays an elongated shape, with the size being approximately twice as long as that of the monomer (Trottier *et al.,* 2000). The pRNA binds procapsids in the presence of Mg^{2+}. The hexameric pRNA complex is attached to the connector of procapsids (the unique site on procapsids where DNA enters and exits) (Garver and Guo, 1997). The pRNA also appears to be directly involved in the DNA translocation process and leaves the procapsid after DNA packaging is completed (Chen and Guo, 1997b).

Recently, it was found that several DNA or RNA translocation enzymes, for example, DNA helicases, the *Escherichia coli* transcription termination protein Rho, the yeast DNA polymerase processivity factor PCNA, and the *E. coli* DNA polymerase III holoenzyme, exist as hexamers or in hexameric form (Geiduschek, 1997; West, 1996; Herendeen *et al.,* 1992; Geiselmann *et al.,* 1993). Though the mechanisms of this kind of DNA–protein interaction remain to be elucidated, the finding that six copies of pRNA are attached to a DNA translocation machine might have something in common with these other systems. The processes of both viral DNA packaging and DNA replication (or RNA transcription) involve the relative motion of two components, one of which is nucleic acid. It would be intriguing to show how the pRNA may play a role similar to the role that protein enzymes, such as DNA helicases or the termination factor Rho, play.

To understand the mechanism of pRNA action, it is essential to determine which bases of the pRNA are exposed on the surface when pRNA is associated with the procapsid. The exposed bases may be candidates for interaction with other DNA-packaging components, such as ATP, the DNA-packaging enzyme gp16, or DNA-gp3 substrate.

In this work, structural probing of the pRNA using chemical modification was conducted to assess the effect of mutations and truncations on the conformation of the pRNA. In addition, procapsid-bound pRNA was probed by chemical modification to gain insight into base protection by the procapsid and pRNA conformation when bound to procapsids.

RESULTS

Chemical modification patterns of mutant pRNA 8/4

The change of pRNA bases 1–4 from GCAA to GGGG resulted in a mutant pRNA, 8/4, that is inactive in DNA translocation while being fully competent in procapsid binding. This mutant has been shown to be able to inhibit phi29 assembly *in vitro* and *in vivo* (Trottier *et al.,* 1996) and has been used as a tool for stoichiometry determinations of pRNA (Trottier *et al.,* 1997; Trottier and Guo, 1997; Chen *et al.,* 1997). The pRNA can be presented in solution as a monomer or dimer depending on the sequences of the right- and left-hand loops. Chemical modification of mutant pRNA 8/4 monomers was performed to analyze struc-

FIG. 1. Predicted secondary structures of pRNAs. The locations of hybridization of oligos P11 and P71 used for primer extension are shown as dark lines in the direction 5′ → 3′ (A). Mutated bases 1–4 in pRNA 8/4 are shown in boldface type (B). In pRNA CCA, the bulge $C^{18}C^{19}A^{20}$ was eliminated by inserting the three bases UGG as shown in box (C). In 108/G^{99}, A^{99} was changed to G^{99}, resulted in the shifting of the bulge one base closer to the procapsid-binding domain (D). The numbering of bases in pRNA 5/11 was consistent with that of full-length pRNA, with the 5′ terminal base labeled as base 28 (E). The construction of pRNA 5/6 has been reported (Garver and Guo, 1997) (F). Loop–loop interactions via bases 5′-AACC and 3′-UUGG resulted in the formation of a pRNA hexamer (G).

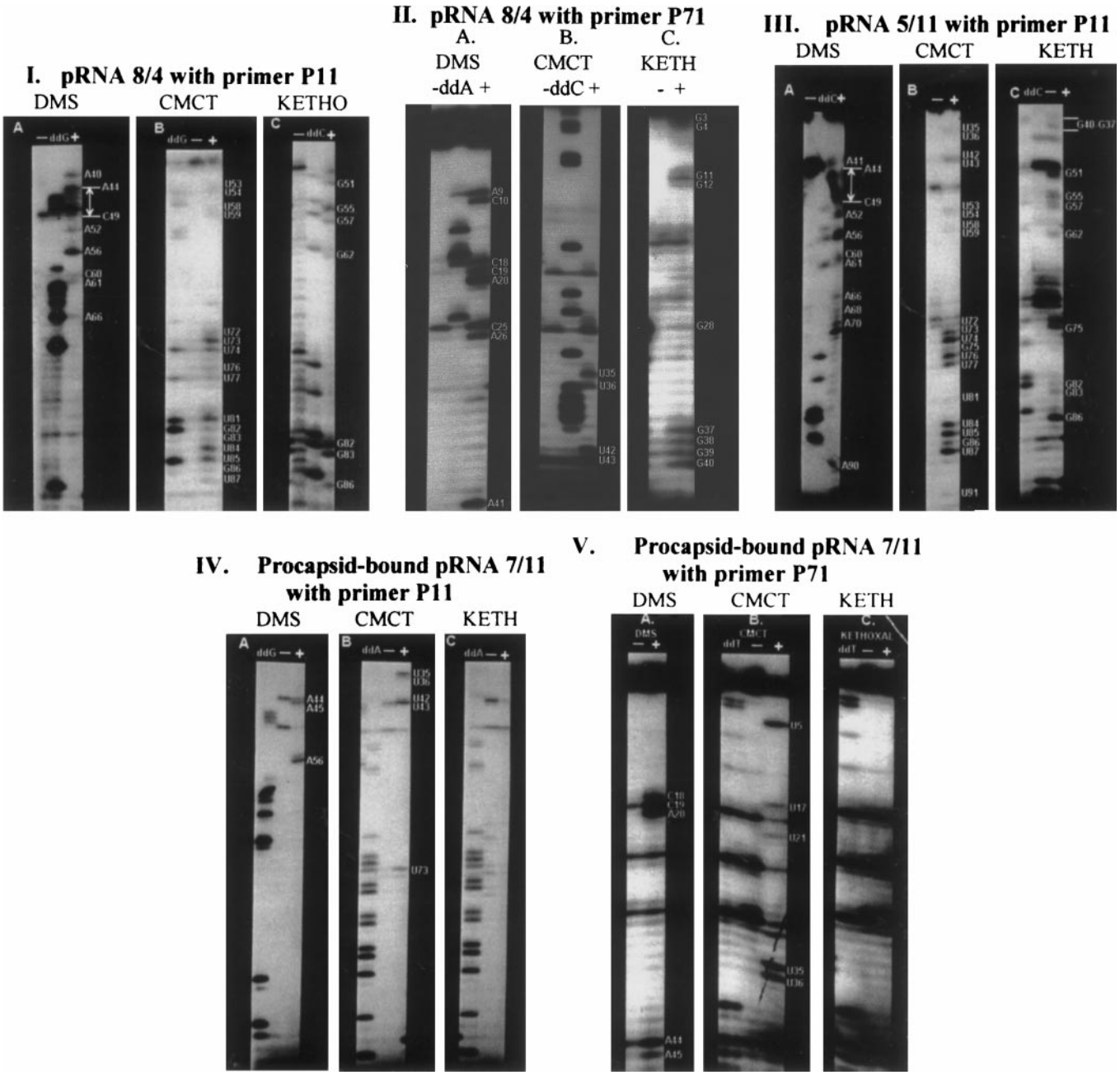


FIG. 2. Primer extension autoradiograms showing chemical modification of pRNA in the presence of Mg^{2+} using pRNA 8/4 as template with primer P11 (I); pRNA 8/4 with primer P71 (II); pRNA 5/11 with primer P11 (III); procapsid-bound pRNA 7/11 with primer P11 (IV); procapsid-bound pRNA 7/11 with primer P71 (V). Autoradiograms of sequencing-type electrophoresis of primer extension were prepared with ^{32}P-end-labeled primer. The lanes marked (−) indicate primer extension reactions performed on unmodified pRNA. The lanes marked (+) indicate primer extension reactions performed on pRNA modified with the chemical indicated. The lanes marked ddA, ddG, ddC, or ddT indicate primer extension reactions performed on unmodified pRNA in the presence of the indicated dideoxy nucleoside triphosphate. The dideoxy lanes were used as molecular weight markers as indicated to the left side of each figure. Bases indicated to the right side of each figure denote bases modified with the chemical indicated.

tural deviations from wild-type pRNA (Figs. 2, parts I and II; Fig. 3B). The results indicate that the right- and left-hand loops are strongly modified, in accordance with the secondary structure. The head loop (bases 62–66) is also modified, albeit less strongly. The CCA bulge is also modified strongly, supporting the presence of a bulge in this area. There are also modifications within predicted helical regions of the pRNA, which are possibly accounted for by the proximity to bulged nucleotides (i.e., base C^{10} is opposite to bulged nucleotide C^{109}) or a helix junction (i.e., bases $C^{25}A^{26}$ are close to a three-helix junction). The mutated bases G^3 and G^4 are modified strongly, suggesting that the mutation disrupted basepairing in this area.

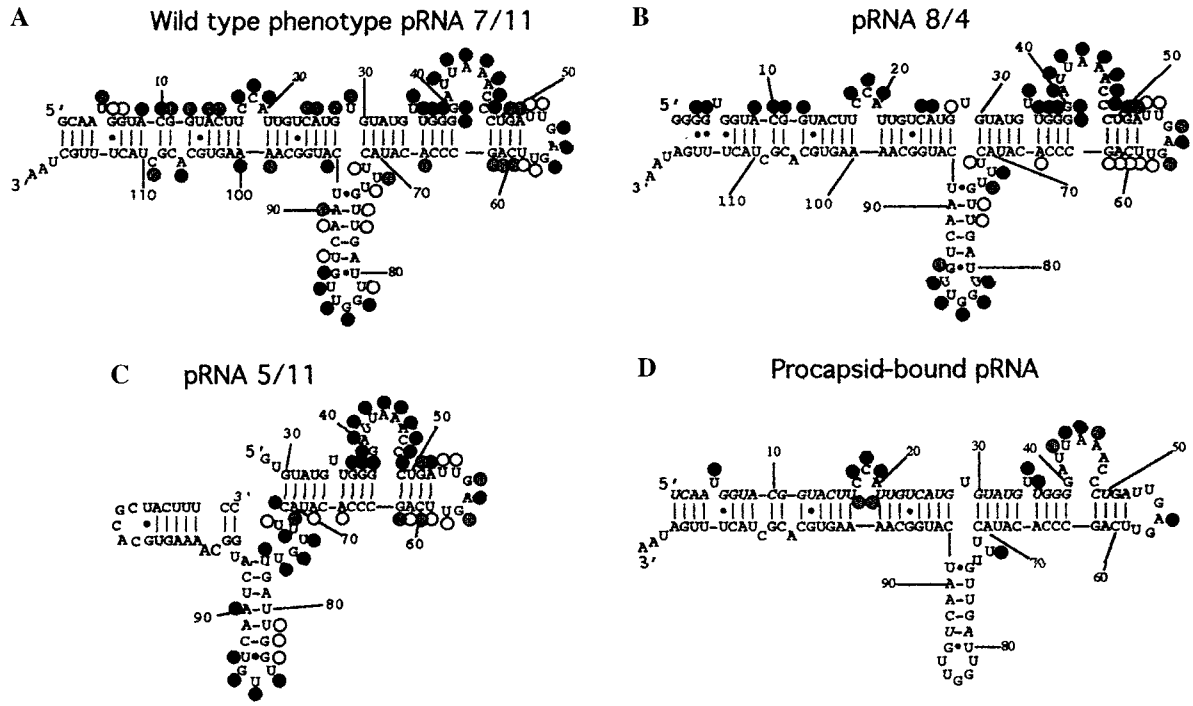


FIG. 3. Summary of chemical modification pattern of wild-type phenotype pRNA 7/11 monomer (A), mutant pRNAs 8/4 (B) and 5/11 (C), and procapsid-bound pRNA (D) showing sites of modification observed at 37°C in the presence of Mg^{2+}. Black circles indicate strongest modification, gray circles indicate moderate modification, and white circles indicate weak modification.

Comparison of modification patterns of wild-type pRNA 7/11 and mutant 8/4

The structure of wild-type pRNA 7/11 was probed previously by chemical modification under identical conditions as RNAs studied in this report (Trottier *et al.,* 2000). There were several similarities between the modification pattern of pRNA 8/4 and that of 7/11 (Trottier *et al.,* 2000) (compare Figs. 3A and 3B). Both the right- and the left-hand loops of each pRNA were modified strongly. The head loop was modified in both RNAs, with differences being mostly in the strength of the modification. The bulged nucleotides U^{35} and base U^{36} were modified strongly in both RNAs and the $U^{72}U^{73}U^{74}$ bulge near the three-helix junction showed similar modification patterns (Figs. 3A and 3B). Significant modification of the CCA bulge occurred in both RNAs as well.

Most of the differences in modification pattern between these two RNAs occurred in the helical regions. The stem of the left stem loop in pRNA 8/4 was modified in a somewhat similar fashion to that of 7/11, with the exception that bases A^{89} and A^{90} were unmodified (Fig. 3B). The stem of the head stem loop of 8/4 showed substantial variation in modification pattern from 7/11. In 7/11, bases C^{60}, A^{61}, and G^{62} were moderately modified, whereas in 8/4, the same bases were modified only weakly (Figs. 3A and 3B). In the helix between the three-helix junction and the right loop, the modification pattern was very similar for both RNAs, with the exception that A^{66} was modified weakly in 8/4 and moderately in 7/11 (Figs. 3A and 3B). Interestingly, bulged nucleotides U^{5} and U^{29} were strongly modified in wild-type pRNA but unmodified in 8/4. Similarly, G^{28} showed moderate accessibility to chemicals in wild-type pRNA (Fig. 3A), while weak modification was observed in mutant 8/4 (Fig. 3B). In the pRNA terminal helix, an increased reactivity of bases in 8/4 was observed with bases C^{25} and A^{25}, which were moderately modified in wild-type pRNA. Bases 3 and 4 were modified strongly in 8/4, but unmodified in wild-type pRNA. Bases G^{11} and G^{12} were moderately modified in wild-type pRNA; however, they were strongly modified in mutant 8/4. Conversely, bases A^{14} and C^{15} were moderately modified in wild-type pRNA but were unmodified in mutant 8/4.

Chemical modification patterns of pRNA 5/11

Extensive investigation of the pRNA, including studies of 8/4, revealed that the pRNA contained two functional domains, a procapsid-binding domain and a 5′/3′ paired helical domain that is essential for DNA packaging (Trottier and Guo, 1997; Garver *et al.,* 1997; Zhang *et al.,* 1994). For structural analysis, it is interesting to isolate a domain of minimal size that is still able to bind procapsids.

Mutant pRNA 5/11 has bases 1–28 deleted (Fig. 1E). This RNA consists mainly of the procapsid-binding domain of the pRNA, i.e., bases 28–91. Bases 92–120 were left in the molecule as a tail to which the primer for reverse transcriptase primer extension is complementary. The predicted secondary structure of this RNA

shows a rearrangement of the left stem loop, which reduces the size of the left loop (Fig. 1E). This pRNA has reduced procapsid-binding affinity (not shown) and was used in chemical modification experiments (Fig. 2) to investigate structural defects in the procapsid-binding region of the pRNA that may be important for procapsid-binding activity.

The right-hand and head loops of the pRNA showed significant modification in pRNA 5/11 (Figs. 2, part III and 3C). The predicted bulged nucleotides between bases 70 and 78 were modified as well. The modification pattern of the left-hand loop indeed confirms the presence of a structural rearrangement in this area, as the predicted loop nucleotides $U^{84}U^{85}G^{86}$ were all modified strongly. The helical nucleotides U^{87} and A^{90} were modified strongly as well (Fig. 3C).

Comparison of modification patterns of pRNA 5/11 and wild phenotype pRNA 7/11

Some aspects of the structure of pRNA 5/11 are strikingly similar to that of pRNA 7/11 (compare Figs. 3A and 3C). Bases 30 to 70 of these RNAs, which include the right-hand and head loops, are almost identical in regards to chemical modification. Some important differences include the lack of modification of U^{35} and U^{36} in 5/11 and the weak modification of bases A^{61} and A^{66}, compared to the strong modification of these bases in 7/11. The major differences between the two structures occurs in the left-hand stem and loop regions, most likely as a result of the rearrangement of this loop in 5/11. The modification pattern of 7/11 and 5/11 are consistent with their respective predicted secondary structures in these areas, aside from some modification of bases within predicted helical regions, suggesting a structural alteration in the left-hand loop of 5/11. Interestingly, base A^{90} is strongly modified in both RNAs, yet is within a predicted helix in both cases.

Chemical modification of procapsid-bound pRNA

Chemical modification experiments on procapsid-bound pRNA were performed to analyze bases of the pRNA that were involved in procapsid binding. The chemicals used in this experiment were much smaller than ribonucleases; thus, a finer mapping of procapsid-protected bases can be achieved than by RNase probing.

Figure 2, parts IV and V show representative autoradiograms of chemical modification experiments on procapsid-bound pRNA. A summary of the overall modification pattern of procapsid-bound pRNA is shown in Fig. 3. The most striking feature of the modification of procapsid-bound pRNA was the relative lack of modification of the RNA compared to wild-type pRNA alone (Figs. 3A and 3D). The entire left stem loop was not modified (Fig. 3D). Additionally, only base A^{56} of the head loop was modified. In the right loop, which was modified strongly and completely in unbound pRNA, only bases 42, 43, and 44 were significantly modified and base 45 was only moderately modified (Fig. 3D). The interacting bases 46–48 of the right-hand loop were not modified at all.

TABLE 1

Summary of pRNA Activities

pRNA species	Dimer formation	Procapsid binding	DNA packaging activity (PFU/ml)	Inhibition of *in vitro* virion assembly
7/11	++++	++++	1.0×10^8	−
8/4	++++	++++	0	++++
CCA	++++	++++	0	+++
5/6	−	+	0	+
5/11	±	+	0	+
108/G[99]	ND	+++	0	+++

The helical regions throughout the molecule were resistant to chemical attack, with the exception of base U^{36}, which was strongly modified in both bound and free pRNAs (Figs. 3A and 3D). Also, bases U^{17} and U^{21} were moderately modified in procapsid-bound pRNA (Fig. 2, part V-B), but not in free pRNA (Fig. 3A), suggesting that binding of pRNA to procapsids changed the conformation of pRNA. The CCA bulge was strongly modified in both procapsid-bound and free pRNAs. These data suggest the possibility that bases U^{17} and U^{21}, along with the CCA, are extended from procapsid. The bulged nucleotide U^5 was moderately modified in free pRNA (Fig. 3A) and strongly modified in procapsid bound pRNA (Figs. 2-V-B, and 3A); however, the bulged nucleotides U^{35} and U^{73} were strongly modified in both free and bound pRNA. U^{29} was unmodified in procapsid-bound pRNA and moderately modified in free pRNA (Figs. 3A and 3D).

Functional assay for the CCA bulge

It is generally believed that RNA bulges play critical roles in RNA–protein interactions. The CCA bulge of the pRNA was solvent-exposed and thus did not make contact with the procapsid when the pRNA was bound to it. What is the role of this CCA bulge? To answer this question, a mutant pRNA CCA, with a removal of the CCA bulge, was investigated. This pRNA mutant was inactive in phi29 assembly *in vitro.*

It is known that a prerequisite for DNA packaging is the binding of pRNA to procapsids. It is also believed that the ability of the pRNA to form dimers is an intermediate step in the formation of a pRNA hexamer (Chen *et al.,* 2000). It was found that mutant pRNA CCA was able to form dimers *in vitro* (Table 1, Fig. 4). as well as bind procapsids (Table 1, Fig. 5), indicating that elimination of the CCA bulge did not cause a change in the global pRNA conformation. pRNA CCA was also able to efficiently inhibit *in vitro* virus assembly, a phenomenon

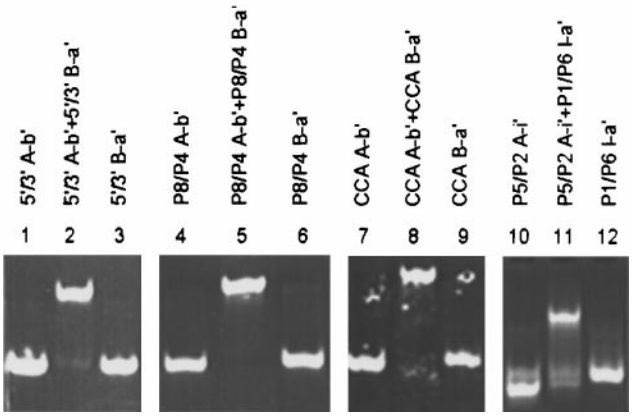


FIG. 4. Native polyacrylamide gel electrophoresis showing the competence of various pRNAs in dimer formation. In each panel, the central lane is the dimer resulting from the mixing of two individual monomeric pRNA. Monomeric pRNAs were electrophoresed alone on either side of each dimer mixture. The two pRNAs in each panel contain alternatively interacting loops, such as A/b′ and B/a′, where A and a′, for example, represent complementary loop sequences with the upper- and lowercase numbers representing the right and left loops, respectively.

indicating that the mutant was able to interlock into hexamers and subsequently block DNA packaging as well as the assembly of infectious virions (Table 1, Fig. 6). These data indicate that elimination of the CCA bulge does not affect procapsid binding and the formation of pRNA dimers and hexamers. However, elimination of this bulge does disrupt DNA-packaging activity, suggesting a potential role for the CCA bulge in interacting with other components of the DNA-packaging machinery. To strengthen our conclusion, another mutant pRNA, 108/G^{99}, was investigated. In 108/G^{99}, A^{99} was changed to G^{99} (Fig. 1D), which was predicted to pair with C^{18}, the first base of the CCA bulge. Therefore, the bulge shifted one base closer to the procapsid-binding domain, and the

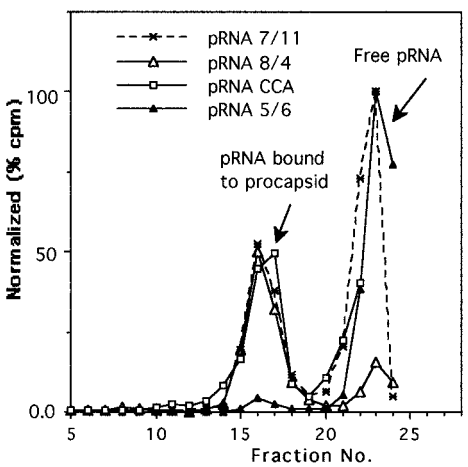


FIG. 5. Sucrose gradient sedimentation analysis for the binding of pRNA to procapsids. Sedimentation is from right to left. The peaks located at fractions 14–18 represent procapsid/pRNA complexes. The peak at the top of each gradient represents free pRNA.

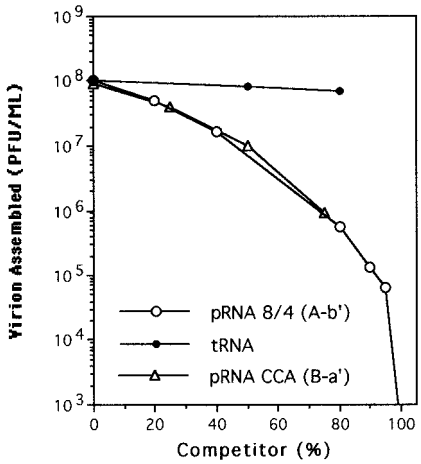


FIG. 6. Competitive binding of pRNA to procapsid and incorporation of pRNA 8/4 or pRNA CCA into hexameric complexes as demonstrated by inhibition assays. pRNA B-a′ plus pRNA A-b′ was mixed with a varying ratio of competitor pRNA 8/4(A-b′) or pRNA CCA(B-a′), respectively, in phi29 *in vitro* virion assembly assays. The yield of virion production (PFU/ml) vs percentage of competitor RNA is plotted to determine inhibition efficiency. Inhibition of virion assembly by competitor RNA indicates an ability to incorporate into pRNA hexamers. Yeast tRNA was used as a negative control.

bulge was changed from CCA to CAU. Mutant 108/G^{99} was found to be inactive in DNA packaging and phi29 assembly; however, it was fully competent in procapsid binding (Table 1) and was able to inhibit phi29 virion assembly *in vitro,* supporting the conclusion that the CCA bulge plays a role in DNA packaging.

Phylogenetic analysis of the CCA bulge

Phylogenetic analysis of similar pRNAs from bacterial viruses SF5, B103, Phi29/PZA, M2/NF, and GA1 (Chen *et al.,* 1999) shows very low sequence identity and few conserved bases, yet, the family of all pRNAs appears to have similar predicted secondary structures with a bulge located at a similar location as the CCA bulge of phi29 (Fig. 7).

Computer modeling of pRNA to assess the CCA bulge

A computer model of the pRNA hexamer has been proposed (Zhang *et al.,* 1998). This model was based on previous work concerning basepairing in the pRNA secondary structure and on the finding that the 5′-AACC of the right loop interacts intermolecularly with the 3′-UUGG of the left loop. No distance constraint data on the three-dimensional structure of the pRNA were available at that time for use in the modeling. Recently, we reported that the dimer is the building block in hexamer formation (Chen *et al.,* 2000; Garver and Guo, 2000). We have also probed the structure of the pRNA dimer by chemical modification and cryo-AFM (Trottier *et al.,* 2000), revealing that the dimer is formed via hand-in-

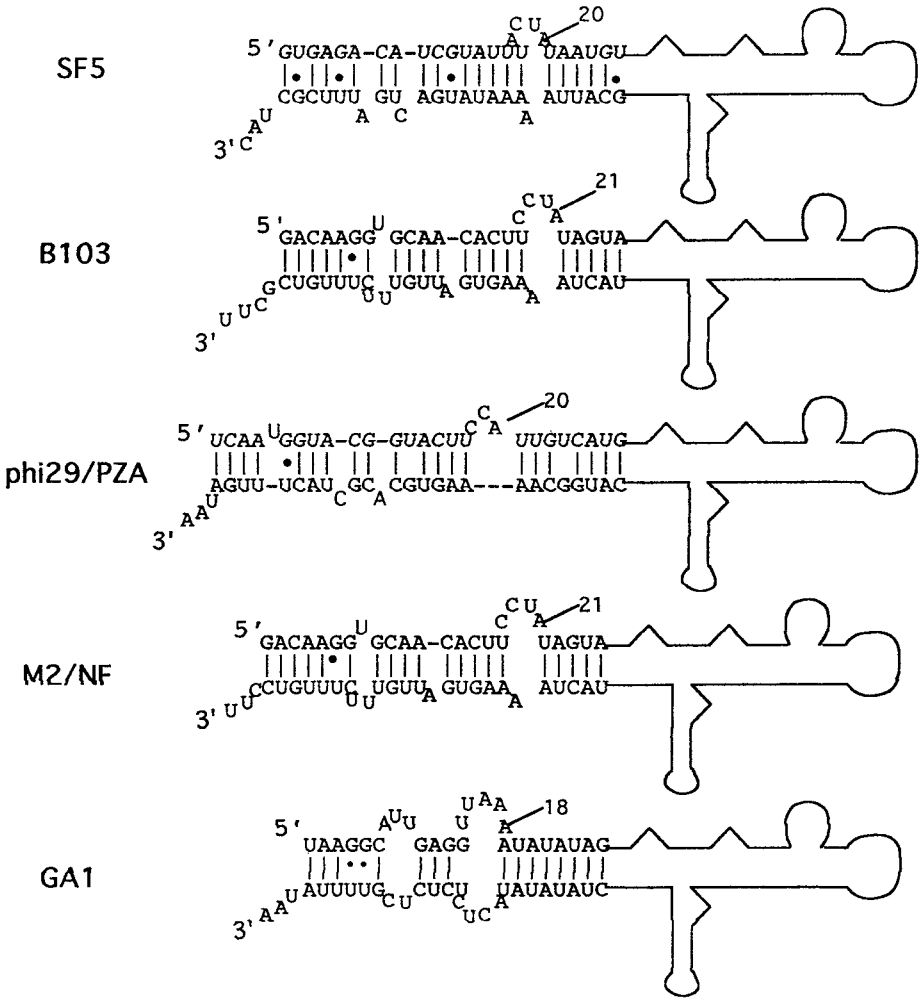


FIG. 7. Phylogenetic analysis of similar pRNAs from bacterial viruses SF5, B103, phi29/PZA, M2/NF, and GA1 showing a very low sequence identity and the conserved bulge located at a location similar to the CCA bulge of phi29.

hand interaction. Data from chemical modification of procapsid-bound pRNA and free pRNA revealed that the CCA bulge is solvent-exposed in both cases (this work). To accommodate the new findings concerning the CCA bulge, a 3-D structure of the pRNA was reconstructed by computer modeling (Fig. 8) with the program MC-SYM (Major *et al.,* 1991). This monomer model is extracted from the dimer model (F. Major and N. Bourassa, personal communication) to reflect the following new information: (1) the CCA budge sticks out; (2) the pRNA is reoriented so as to generate a conformation able to interact hand-in-hand with another pRNA with two hands instead of one hand as demonstrated in a previous model.

DISCUSSION

Chemical modification has been used previously to analyze the structure of phi29 pRNA monomers and dimers (Trottier *et al.,* 2000). In this paper, chemical modification of active and inactive as well as procapsid-bound and unbound pRNAs was performed to provide insight into structural alterations. When bound to procapsids, wild-type pRNA undergoes drastically reduced chemical modification (Fig. 2, parts IV and V). This is somewhat to be expected, since the procapsid-binding domain of the pRNA is quite large. Only a few bases within this domain are modified, suggesting a large area of protection by the phi29 procapsid. This observation is in agreement with nuclease probing, which shows large areas of protection of the pRNA by procapsids (Reid *et al.,* 1994a). The phylogenetically conserved base A^{56}, in the head loop, is strongly modified in the presence of procapsids. Ironically, mutagenesis studies have shown that changing the base at position 56 from A to C does not affect the DNA-packaging activity of the pRNA but does alter its procapsid-binding competence (Wichitwechkarn *et al.,* 1992).

One interesting feature about the modification pattern of procapsid-bound pRNA is the lack of reactivity of

FIG. 8. Computer modeling of pRNA with the program MC-SYM (Major *et al.,* 1991), presented as a stereo image with the CCA bulge shown in red. The right and left loops for intermolecular interaction are shown in white and yellow, respectively.

helical regions. This lack of reactivity compared to unbound pRNA extends to bases not located in the procapsid-binding domain, suggesting that procapsid binding stabilizes overall pRNA folding. A similar stabilization of RNA structure by protein binding was shown to occur when certain ribosomal RNAs bound to ribosomal proteins (Moazed *et al.,* 1986). In the case of ribosomal RNAs, enhanced reactivity of some bases was observed

along with the overall "tightening" of RNA secondary structure, suggesting a conformational change of the RNA upon protein binding. Only bases U^{17} and U^{21}, which flank the CCA bulge, show enhanced reactivity toward chemical modification when the phi29 pRNA was bound to procapsids. Similarly, enhanced accessibility of bases 37–40 to nuclease digestion was observed when the pRNA was bound to procapsids (Reid *et al.,* 1994a); however, these bases were accessible to double-strand specific nucleases and thus such regions of the pRNA would likely not be sensitive to chemical modification. Both lines of evidence suggest a conformational change in the pRNA upon procapsid binding.

One model for pRNA function in DNA packaging proposes that the pRNA sits on the procapsid, leaving the 5′/3′ terminal helix free for interactions with other DNA-packaging components, such as genomic DNA-gp3, ATP, or the packaging enzyme gp16 (Chen and Guo, 1997b; Garver and Guo, 1997; Guo *et al.,* 1998). It is logical to assume that any such intermolecular interactions would involve solvent-exposed bases within the terminal helix. When the pRNA is bound to procapsids, only a few bases remain accessible to chemical modification. As shown by mutagenesis, bases U^5, A^{106}, and C^{109} are all nonessential for pRNA activity, as is base U^{29}, which is closer to the boundary of the procapsid-binding domain, indicating that these bases may not be involved in such interactions (Reid *et al.,* 1994b,c; Wichitwechkarn *et al.,* 1992; Zhang *et al.,* 1995a). The CCA bulge, conversely, is required for pRNA activity and remains modification sensitive. Thus, the CCA bulge is extended beyond the procapsid and may be a prime candidate for the binding site of other DNA-packaging components.

The capsid of dsDNA bacterial viruses contains a 12-fold symmetrical connector embedded in a protein shell with 5-fold rotational symmetry (Tao *et al.,* 1998; Ibarra *et al.,* 2000; Badasso *et al.,* 2000). A model was previously proposed that the relative motion of the two symmetry-mismatched rings could provide a driving force for DNA translocation. The finding that six copies of pRNA were bound to the connector and worked sequentially (Chen and Guo, 1997b; Chen *et al.,* 1997; Trottier and Guo, 1997; Guo *et al.,* 1998; Zhang *et al.,* 1998; Hendrix, 1998) is potentially relevant to this model. To make two rings rotate relatively, at least one additional component is needed to provide a propelling force. The DNA-packaging enzyme gp16 and/or the pRNA could be the candidates for this third component. The finding that the CCA bulge was essential for pRNA activity in addition to being accessible to chemicals while bound to procapsids makes this bulge an attractive focal point in the search for pRNA interactions with other phi29 DNA packaging components.

We reported here that the CCA bulge was critical for DNA translocation while being dispensable for procapsid binding. We propose that the pRNA binds to the connector, leaving the 5′/3′ ends free for interaction with an additional component, such as ATP, gp16, or the genomic substrate DNA-gp3. Interaction of the pRNA or a pRNA/gp16 complex, which is tethered to the connector, with the capsid or DNA would provide the propelling force for connector rotation. RNA bulges have been shown to play critical roles in RNA–protein interactions (Haasnoot *et al.,* 1986; Peattie *et al.,* 1981; Schroeder *et al.,* 1991; Zacharias and Hagerman, 1995). The CCA bulge might be involved in interacting with ATP, gp16, or DNA-gp3. We previously proposed that the pRNA itself is part of an ATPase, which possesses at least two conformations, a relaxed form and a contracted form. Alternating between contraction and relaxation of the pRNA or the pRNA/gp16 complex, driven by ATP hydrolysis, allows sequential binding and release of the complex, which in turn drives the rotation of the connector to allow DNA insertion into the capsid.

MATERIALS AND METHODS

Synthesis and purification of pRNAs

Wild-type and mutant pRNAs were synthesized by *in vitro* transcription using T7 RNA polymerase as described (Zhang *et al.,* 1995b). Secondary structure predictions for pRNAs were made using the method of Zuker (1989).

Mutant pRNA CCA was a circularly permuted RNA with G^{75} and C^{71} serving as new 5′ and 3′ ends, respectively (Zhang *et al.,* 1995b). The 5′-$C^{18}C^{19}A^{20}$ bulge was removed by inserting three complementary bases, 3′-GGU, between bases 99 and 100 (Fig. 1C). The DNA templates for making pRNA CCA were prepared by two-step PCR (Guo *et al.,* 1998), cloned into pGEM-T vector (Promega), and sequenced.

Chemical modification of RNAs

RNAs were modified with the chemicals dimethyl sulfate (DMS), 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluene sulfonate (CMCT), and β-etoxy-α-ketobutyraldehyde (kethoxal) (Ehresmann *et al.,* 1987; Moazed *et al.,* 1986). pRNAs were modified as follows, with ideal amounts of chemicals in each reaction determined empirically.

DMS

Purified pRNA (15 pmol) was incubated in buffer D (50 mM sodium cacodylate, pH 7.0, 10 mM $MgCl_2$, and 100 mM NaCl) at a final volume of 50 μl. One microliter of DMS (diluted 1:3 in 100% ethanol) was added to the reaction. Unmodified control RNA was prepared by including 1 μl of 100% ethanol to the reaction instead of DMS. The reactions were incubated for 3 min at 37°C.

Reactions were terminated by the addition of 6.5 μl DMS stop buffer (1.0 M Tris–acetate, pH 7.5, 1.0 M 2-mercaptoethanol, 1.5 M sodium acetate, 0.1 mM EDTA) and incubated on ice for 10 min.

Reaction volumes were brought up to 200 μl and extracted once with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and once with an equal volume of chloroform:isoamyl alcohol (24:1), followed by ethanol precipitation at −20°C for several hours. Alternatively, the reactions were ethanol precipitated directly after termination of the reaction. Pelleted RNA was resuspended in 8 μl DEPC-treated H_2O.

CMCT

Purified pRNA (15 pmol) was incubated in buffer C (50 mM sodium borate, pH 8.0, 20 mM magnesium acetate, 100 mM NaCl) at a final volume of 25 μl. To the RNA was added 25 μl of CMCT (12 mg/ml in buffer C). For unmodified control RNAs, 25 μl of buffer C without CMCT was added. Reactions were incubated at 37°C for 30 min and then phenol extracted and ethanol precipitated as for DMS modification.

Kethoxal

Reactions were similar to the modification of pRNA by DMS, except that 5 μl of kethoxal (42 mg/ml in 20% ethanol) was added to each reaction. For unmodified control RNAs, 5 μl of 20% ethanol was added instead of kethoxal. Reactions were incubated for 1–2 h at 37°C and phenol extracted and/or ethanol precipitated as described for DMS modification.

Chemical modification of procapsid-bound pRNA

Procapsids from phi29-infected *Bacillus subtilis* cells or from *E. coli* were purified as described (Guo *et al.,* 1987a). To attach 7/11 to procapsids, approximately 1 μg of pRNA was mixed with 90 μl of procapsid (approximately 10^{11} procapsids per microliter) and dialyzed against TBE buffer (89 mM Tris–borate, pH 8.3, 2 mM EDTA) for 15 min on a Millipore type VS 0.025-μm filter and then against buffer D (or buffer C when CMCT was used) for 30 min at room temperature. Procapsid-bound pRNA was separated from unbound pRNA by ultracentrifugation of the mixture through a 5–20% sucrose gradient for 4 h at 4°C (Beckman L-80 ultracentrifuge, SW55 rotor, 35,000 rpm). Procapsid/RNA complexes in the pellet were resuspended in 40 μl of buffer D (or buffer C when CMCT was used).

Procapsid/RNA complexes were treated with the chemicals DMS, CMCT, and kethoxal essentially as described above, with a few exceptions. The amount of procapsid/pRNA complex used in each reaction was determined empirically to provide adequate amounts of RNA for primer extension and was generally 5 μl, although the amount used varied for each procapsid preparation. For DMS modification, DMS diluted 1:2 in 100% ethanol was used instead of the 1:3 dilution for pRNA alone. For CMCT modification, 25 μl of 18 mg/ml CMCT in solution was used instead of the 12 mg/ml CMCT in solution used for pRNA alone. Various amounts of kethoxal were used for modification experiments with this chemical.

Other treatments of the procapsid/pRNA complex were as described for free pRNA above except that all samples were extracted once with phenol:chloroform:isopropyl alcohol (25:24:1) and once with chloroform:isopropyl alcohol (24:1) followed by ethanol precipitation.

Reverse transcriptase primer extension

Primer extension of RNA requires additional bases for primer annealing in order to read the succeeding bases. Thus, chemical modification of certain regions of the pRNA (i.e., primer annealing sites) could not be mapped. The 3′ tail of p5/11 served as a primer annealing site. This pRNA was also used in procapsid binding and DNA-packaging assays (Figs. 1 and 8 and Table 1).

RNA (1.5 pmol) was mixed with 0.1 pmol of ^{32}P-end-labeled primer in H_2O or TE buffer, pH 7.6, and heated to 90°C for 2 min. The mixtures were slowly cooled to 30°C in a water bath (approximately 1.5 h). RNA/primer mixtures were mixed with 0.5–1 unit of avian myeloblastosis virus reverse transcriptase (Promega), 1 μl of 10 mM dNTPs (2.5 mM each), and 2 μl of 5× RT buffer (250 mM Tris–HCl, pH 7.9, 30 mM $MgCl_2$, 10 mM spermidine, 50 mM NaCl) in a final volume of 10 μl. Reactions were incubated at 55°C for 30 min and terminated by the addition of an equal volume of 2× loading buffer (98% formamide, 10 mM EDTA, 0.01% bromophenol blue, 0.01% xylene cyanol). Samples were heated to 90°C for 2 min and immediately transferred to ice for 2–5 min. Samples were subjected to sequencing-type polyacrylamide gel electrophoresis and dideoxy sequencing lanes were run adjacent to experimental chemical modification reactions to facilitate mapping of individual bases. Modified bases were mapped by identifying stops in the primer extension reactions that did not occur in unmodified RNA control reactions. Such stops occur one nucleotide prior to the modified base.

Assay for pRNA dimer formation, procapsid binding, DNA packaging, virion assembly, and inhibition using mutant pRNAs

Native polyacrylamide gel electrophoresis to detect pRNA dimer formation and sucrose gradient sedimentation to analyze binding of pRNA to procapsids have been

reported previously (Trottier and Guo, 1997; Chen and Guo, 1997a,b). The test for the DNA-packaging activity of pRNAs (Guo *et al.,* 1986, 1987b), the procedure for *in vitro* virion assembly (Lee and Guo, 1995; Guo *et al.,* 1991), and the *in vitro* assembly inhibition assay (Chen *et al.,* 1997, 1999; Guo *et al.,* 1998) to test whether mutant pRNA could be incorporated into the hexameric pRNA complex have also been reported previously.

ACKNOWLEDGMENT

This work was supported by NIH grant GM59944 to Guo. We thank Nancy Bourassa and Francois Major for personal communication, for providing the preliminary data of the pRNA dimer model, for their critical reading of this manuscript, and for their comments and suggestions concerning the presentation of the monomer model in this paper.

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