

Complete Inhibition of Virion Assembly In Vivo with Mutant Procapsid RNA Essential for Phage ϕ 29 DNA Packaging

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A highly efficient method for the inhibition of bacteriophage ϕ 29 assembly was developed with the use of mutant forms of the viral procapsid (or packaging) RNA (pRNA) indispensable for ϕ 29 DNA packaging. Phage ϕ 29 assembly was severely reduced in vitro in the presence of mutant pRNA and completely blocked in vivo when the host cell expressed mutant pRNA. Addition of 45% mutant pRNA resulted in a reduction of infectious virion production by 4 orders of magnitude, indicating that factors involved in viral assembly can be targets for efficient and specific antiviral treatment. The mechanism leading to the high efficiency of inhibition was attributed to two pivotal features. First, the pRNA contains two separate, essential functional domains, one for procapsid binding and the other for a DNA-packaging role other than procapsid binding. Mutation of the DNA-packaging domain resulted in a pRNA with no DNA-packaging activity but intact procapsid binding competence. Second, multiple copies of the pRNA were involved in the packaging of one genome. This higher-order dependence of pRNA in viral replication concomitantly resulted in its higher-order inhibitory effect. This finding suggested that the collective DNA-packaging activity of multiple copies of pRNA could be disrupted by the incorporation of perhaps an individual mutant pRNA into the group. Although this mutant pRNA could not be used for the inhibition of the replication of other viruses directly, the principle of using molecules with two functional domains and multiple-copy involvement as targets for antiviral agents could be applied to certain viral structural proteins, enzymes, and other factors or RNAs involved in the viral life cycle. This principle also implies a strategy for gene therapy, intracellular immunization, or construction of transgenic plants resistant to viral infection.

The assembly process of viruses has been gaining attention recently because of its fundamental importance to research on the development of synthetic viral particle vaccines, the construction of in vivo gene delivery systems, the design for intracellular immunization or antiviral drugs, the assembly of chimeric virus displaying antigenic determinants or ligands, and the study of macromolecular interactions. Viral nucleic acid packaging is a process which may have no analog in the host cell, and thus antiviral agents aimed at this process, including its obligate participants, may be more precisely selective. The assembly processes for all double-stranded DNA (dsDNA) viruses have been shown to be quite similar in that the viral genomic DNA is inserted into a preformed procapsid (2, 4, 5, 9, 14, 20). Bacteriophage ϕ 29 of *Bacillus subtilis* is a linear dsDNA virus for which the assembly process has been studied in great detail (1, 2, 20), and its similarity in assembly to other dsDNA viruses, such as poxvirus, herpesvirus, and adenovirus (5, 11, 20, 28), allows it to be used as a model for dsDNA virus assembly.

An interesting feature of ϕ 29 is that a virus-encoded 120-base RNA molecule is required for DNA packaging (15, 16). This RNA (pRNA, "p" for packaging or procapsid) binds to the portal vertex of the procapsid as an initial step in DNA packaging and is not detected in the mature virion (15, 16). The secondary structure of the pRNA has been proposed and partially confirmed (3, 32, 38–39). Additionally, a pseudoknot

within the molecule has been reported (32). Extensive mutation, truncation, and deletion studies of the pRNA have distinguished essential and nonessential residues of the pRNA molecule and generated tens of mutant pRNA molecules with and without DNA-packaging activities (12, 30, 31, 36–39). With the aid of in vitro-transcribed pRNA, we have been able to assemble infectious virions of ϕ 29 in vitro with 11 proteins from cloned genes (24, 25). It is not clear whether the RNA is used only once for each DNA-packaging event or whether it is reused once DNA packaging is completed. In the absence of pRNA, no DNA packaging occurs in vitro. Currently, the exact role of the pRNA in DNA packaging is unknown. The presence of similar RNA molecules has been identified in *B. subtilis* dsDNA phages SF5, M2, NF, and PZA (3), demonstrated in a chimeric λ - ϕ 29 system (10), and speculated to exist in poxvirus (29), adenovirus (21), *Streptococcus* phage Cp-1 (27), and the transposable Mu-like phage D108 (8). Involvement of a non-protein factor, such as pRNA, in viral DNA packaging provides new insight into RNA-protein and/or RNA-DNA interactions and extends previously demonstrated RNA functions.

Since the discovery of RNA catalytic activities (13, 23), there has been a revolution in the concept about RNA function; that is, RNA can be an enzyme (ribozyme). The use of the hammerhead ribozyme in gene therapy for viral infections introduced catalytic RNA as a new weapon (33). In this report, we document the highly efficient inhibition of bacteriophage ϕ 29 assembly with the use of mutant forms of the viral pRNA. The mechanism leading to the high efficiency of inhibition was elucidated to be due to two functional domains within the pRNA and the multiple copies of pRNA required per procapsid for DNA packaging. Although this mutant pRNA could not

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TABLE 1. Oligonucleotides used

Oligonucleotide	Sequence	Size (nucleotides)	Sense	Location (residues)
P3	5'-TTGCCATGATTGACAAC-3'	17	-	99-83
P8	5'-TAATACGACTCACTAGGGGTGGTAC-3'	25	+	5-10
P15	5'-AAGTACCGTACCATTGA-3'	17	-	17-1
3'Bgl4C	5'-CGCATGTAGATCTTTACCCAGTAGCGT-3'	28	-	120-106

be used for inhibition of the replication of other viruses directly, the principle of using molecules with two functional domains and multiple-copy involvement as inhibitors could be applied to antiviral design for intracellular immunization or transgenic plants and to gene therapy with certain viral structural proteins, enzymes, and other factors or RNA involved in the viral life cycle.

MATERIALS AND METHODS

Synthesis of pRNA molecules. Mutant pRNAs were constructed as described previously (38, 39). Briefly, linear plasmid DNAs were used as templates to generate PCR DNA fragments with primer pairs P7-P4, P8-P4, P7-P94G, and FW-P4 (37, 39), respectively. The purified PCR DNA fragments were used as templates to synthesize pRNAs P7/P4, P8/P4, P7/P94G, and FW/P4, respectively, by *in vitro* transcription with T7 RNA polymerase. The pRNA from the PCR DNA template produced with primer pair P7-P4 was called pRNA P7/P4, with the nomenclature of other pRNAs following this scheme.

³H-labeled pRNA molecules were synthesized by adding 50 μ Ci of [³H]UTP (47 Ci/mmol; Amersham) to the *in vitro* transcription reaction. After *in vitro* transcription, pRNAs were purified by RNaid (Bio 101).

Oligodeoxynucleotides. Oligonucleotides P3, P8, and P15 were used for probing functional domains of the pRNA by a procapsid-pRNA binding assay. Oligonucleotide 3'Bgl4C was used to construct plasmid pRBmutRNA (Table 1).

Procapsid-pRNA binding assays. Sucrose gradient sedimentation was performed to detect the presence of procapsid-pRNA complexes (15). Briefly, [³H]pRNA was mixed with 6.5 μ l of purified procapsid (6 mg of gp8 per ml) and dialyzed on a 0.025-mm-pore-size type VS filter membrane (Millipore Corp.) against TBE buffer (89 mM Tris-borate [pH 8.3], 2.5 mM EDTA) for 15 min at ambient temperature. The mixtures were then dialyzed against TMS buffer (50 mM Tris [pH 7.8], 100 mM NaCl, 10 mM MgCl₂) for 30 min at ambient temperature. After the incubation, the mixtures were diluted to 0.1 ml in TMS, loaded on top of a 5 to 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. After the spin, fractions were collected from the bottom of the tube and prepared for scintillation counting.

***In vitro* ϕ 29 assembly.** The purification of procapsids (17, 19), gp16 (18), and DNA-gp3 (24) and the preparation of neck and tail protein extracts (24-26) have been described previously.

In vitro ϕ 29 assembly was performed as described previously (24, 25). Briefly, approximately 0.7 pmol of purified procapsid (containing 8 μ g of capsid protein gp8) was mixed with 5 pmol (200 ng) of pRNA and dialyzed against TBE buffer on a 0.025-mm-pore-size type VS filter membrane (Millipore) for 15 min and then against TMS buffer for 30 min at room temperature. Next, pRNA-enriched procapsids were mixed with 3 μ l of reaction buffer (10 mM ATP, 6 mM 2-mercaptoethanol, and 3 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 (pH 7.5)-4 mM KCl for 40 min on ice. The final mixture was then incubated for 30 min at ambient temperature.

The DNA-filled procapsids were then incubated with 18 μ l of gp8.5-9 extract from *Escherichia coli* containing plasmid pARgp8.5-9 and 20 μ l 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.

Construction of plasmid pRBmutRNA. To construct plasmid pRBwtRNA, a 6.8-kb *EcoRI-HpaI* fragment from plasmid pRB381-L550 (7) (modified and kindly provided by M. Wang and H. Zalkin) was ligated with a 375-bp *PvuII-EcoRI* fragment, containing the pRNA coding sequence under the control of T7 promoter, from plasmid pRT71 (kindly provided by R. Reid and D. Anderson).

Plasmid pBlue102 (36) contains the pRNA coding sequence under the control of its natural promoter. A *HindIII-BglII* DNA fragment containing the coding sequence of the mutant pRNA 3'-4G with the pRNA natural promoter (PE1) (15, 22, 34) was generated by PCR with pBlue102 as a DNA template, M13 reverse primer, and primer 3'Bgl4C, containing a *BglII* site near the 3' end as well as a four-base mutation at the 3'-end of the pRNA gene (Fig. 1). After *HindIII-BglII* digestion, the PCR fragment was ligated with a 6.0-kb fragment from plasmid pRBwtRNA that was digested with *HindIII* and partially digested with *BglII*, resulting in plasmid pRBmutRNA.

Transformation of *B. subtilis*. Transformation of *B. subtilis* DE1 was accomplished by using the Paris method (6) as modified by Wang (34a). Briefly, *B. subtilis* DE1 was grown for 4 h at 37°C with shaking (200 rpm) in BG medium [15 mM (NH₄)₂SO₄, 80 mM K₂HPO₄, 43 mM KH₂PO₄, 2.5 mM sodium citrate · 7H₂O, 1 mM MgSO₄, 0.5% glucose, 0.025% casein hydrolysate, 0.1% yeast extract]. A 2.5-ml culture was mixed with 2.5 ml of MG2 [15 mM (NH₄)₂SO₄, 80 mM K₂HPO₄, 43 mM KH₂PO₄, 2.5 mM sodium citrate · 7H₂O, 6 mM MgSO₄, 0.5% glucose, 0.01% casein hydrolysate] and grown for 90 min at 37°C at 300 rpm. After centrifugation, the cell pellet was kept on ice, and the supernatant was mixed with 0.425 ml of glycerol and 50 μ l of 50% glucose. The cell pellet was then resuspended gently in 1.0 ml of the supernatant mixture mentioned above. Cell aliquots (100 μ l) were quick-frozen in a dry ice-ethanol bath and stored at -70°C. After thawing at 37°C, 45 μ l of competent cells was mixed with 75 μ l of transformation buffer [1.5 mM (NH₄)₂SO₄, 8 mM K₂HPO₄, 4 mM KH₂PO₄, 0.5 mM sodium citrate, 20 mM MgCl₂, 0.5% glucose, 1 mM EGTA]. A volume of 100 μ l of cell-transformation buffer mixture was mixed with 1 μ l of plasmid DNA in a 1.5-ml Eppendorf tube and incubated at 37°C for 20 min. After the addition of 0.4 ml of prewarmed LB, the mixture was incubated at 37°C for 90 min and then plated (0.25 ml) to select for neomycin resistance.

Infection of *B. subtilis* expressing mutant pRNA. *B. subtilis* DE1 harboring plasmid pRB381-L550, pRBwtRNA, pUM102, or pRBmutRNA was grown in 416 medium containing 10 μ g of neomycin per ml for 3 h at 37°C. Serial dilutions of purified wild-type ϕ 29 were plated with DE1 cells on LB-neomycin (10 μ g/ml) plates and assayed for plaque formation.

Secondary-structure predictions for the pRNA. Secondary structures for the various RNAs tested in this study were predicted by the method of Zuker (40) with the Genetics Computer Group (University of Wisconsin) program, and only the lowest-energy structures were selected.

RESULTS

Inhibition of ϕ 29 assembly *in vitro* with mutant pRNA. The predicted secondary structures of pRNAs used in this report are shown in Fig. 1. Other mutants have been previously described. Mutant pRNA P8/P4 has been shown to be inactive, mutant pRNA FW/P4 has been shown to have severely reduced activity, and mutant pRNA P7/P4 has been shown to be fully active in ϕ 29 DNA packaging (38, 39). Mutant pRNA P7/P94G was shown to have no DNA-packaging activity (unpublished data), and this result served as the basis for the design of the mutant pRNA for *in vivo* inhibition. All mutations were localized at the 5' and 3' ends. The two functional domains of the molecule, one for procapsid binding and one that is indispensable in DNA packaging but not involved in procapsid binding (30-32, 37-38a), and the proposed locations of the hybridization of antisense oligonucleotides P3, P8, and P15 (37) are indicated in Fig. 1. Oligonucleotide P8 contains the T7 promoter and a four-G mutation corresponding to the 5' end of the pRNA and does not inhibit ϕ 29 assembly (37).

A highly sensitive *in vitro* ϕ 29 assembly assay (24) was used to test the ability of each of the aforementioned mutant pRNAs to inhibit phage assembly. In each assay, 5 pmol of pRNA with wild-type phenotype was mixed with various amounts of competing mutant pRNA (Fig. 2). When 0.5 pmol of mutant pRNA was added to the packaging reaction, up to a 10-fold reduction in plaque formation was observed. When the amount of mutant RNA added was increased to 4.5 pmol, plaque formation was further reduced up to 4 orders of magnitude compared with when no mutant was added. The amount of inhibition varied depending on which mutant RNA was used, and *E. coli* 5S rRNA appeared to produce little or no

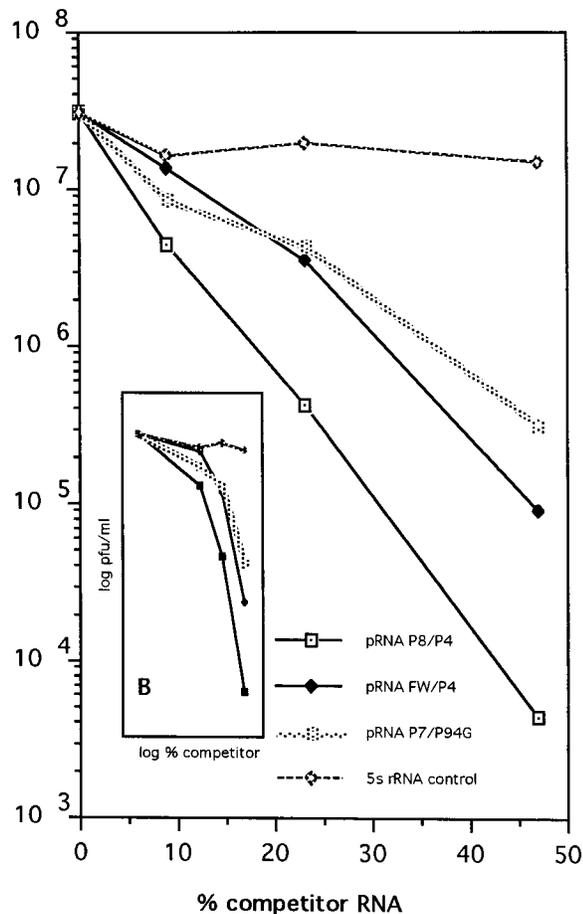


FIG. 2. Inhibition of $\phi 29$ assembly in vitro by mutant pRNAs. (A) The log titer of infectious virions assembled in vitro was plotted against the percentage of competitor RNA in the reaction. Five picomoles of $\phi 29$ pRNA (wild-type phenotype) was mixed with increasing concentrations of mutant pRNA, or *E. coli* 5S rRNA as a control, and dialyzed against TBE buffer before addition of procapsid. In vitro phage assembly was performed, and the reactions were subsequently plated on *B. subtilis* su⁺⁴⁴. Competitor RNAs were added in increasing concentrations of 0, 0.5, 1.5, and 4.5 pmol. (B) Log titer plotted against the log percentage of competitor RNA. A hyperbolic curve was observed with increasing competitor RNA concentrations, showing the higher-order inhibitory effect of mutant pRNAs.

was demonstrated by infectious phage assembly in the highly sensitive phage assembly assay (24) and by sucrose gradient sedimentation in the presence of antisense oligonucleotides (37). Phage assembly in vitro was completely blocked by antisense oligonucleotides P15, which is complementary to the 3'

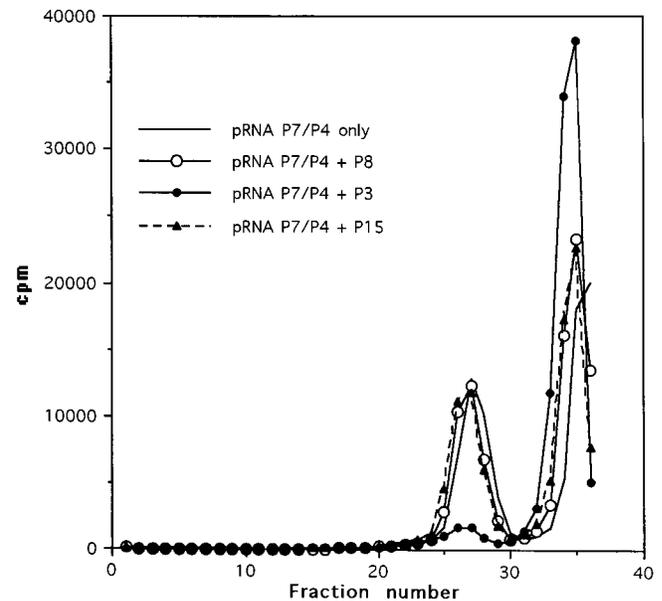


FIG. 3. Demonstration of two functional domains of pRNA by oligonucleotide targeting, assayed with sucrose gradient sedimentation. Sedimentation was from right to left. Purified $\phi 29$ procapsids were mixed with 0.5 pmol of [³H]pRNA P7/P4 (wild-type phenotype) in the presence or absence of antisense oligonucleotides (300 pmol). [³H]pRNA P7/P4 was used alone or mixed with oligonucleotide P8, P15, or P3 before procapsid binding. The peak between fractions 25 and 29 was [³H]pRNA-procaspis complexes.

end of the pRNA, and P3, which is complementary to an internal region of the pRNA. However, control oligonucleotide P8 did not block phage assembly (Table 1) (37).

To define the mechanism of oligonucleotide action, sucrose gradient sedimentation was performed to assay the binding of pRNA to procapsids (Fig. 3). Binding of pRNA to procapsids occurred when no oligonucleotide was added to the binding reaction, as shown in Fig. 3 by the peak between fractions 25 and 29. When oligonucleotide P3 was added to the reaction, a large decrease in procapsid binding by pRNA was observed (Fig. 3). When control oligonucleotide P8 (contained mostly T7 promoter sequence) was added to the binding reaction, there was no alteration of pRNA-procaspis binding. Interestingly, oligonucleotide P15 did not block the binding of pRNA to procapsids (Fig. 3), although it strongly inhibited infectious phage assembly in the in vitro system (Table 1). These results indicate that the oligonucleotides acted to inhibit phage assembly in different ways, one by preventing pRNA binding to the procapsid, which is the case for P3, and the other by

TABLE 2. Resistance of *B. subtilis* to $\phi 29$ infection conferred by mutant pRNA^a

<i>B. subtilis</i> strain	Plasmid vector	Promoter	pRNA gene	pRNA expression	Output virus (PFU/plate)		
					2.5×10^{4b}	2.5×10^2	2.5×10^0
12A	None	None	None	—	Total lysis	248	47
DE1(pRB381-L550)	pRB381	None	None	—	Total lysis	174	35
DE1(pRBwtRNA)	pRB381	T7	Wild type	—	Total lysis	157	16
DE1(pUM102)	pUB110	PE1(A1)	Wild type	+	Total lysis	92	1
DE1(pRBmutRNA)	pRB381	PE1(A1)	Mutant	+	0	0	0

^a Various dilutions of wild-type $\phi 29$ were plated onto *B. subtilis* DE1 cells harboring plasmid pRB381-L550, pRBwtRNA, pUM102, or pRBmutRNA in the presence of 10 μ g of neomycin per ml. The input titer of phage was determined by plating on *B. subtilis* 12A cells in the absence of neomycin. It should be noted that *B. subtilis* cells harboring plasmid pRBmutRNA were unable to produce plaques even when the input virus level was 10^6 PFU per plate.

^b Input virus level (PFU per plate).

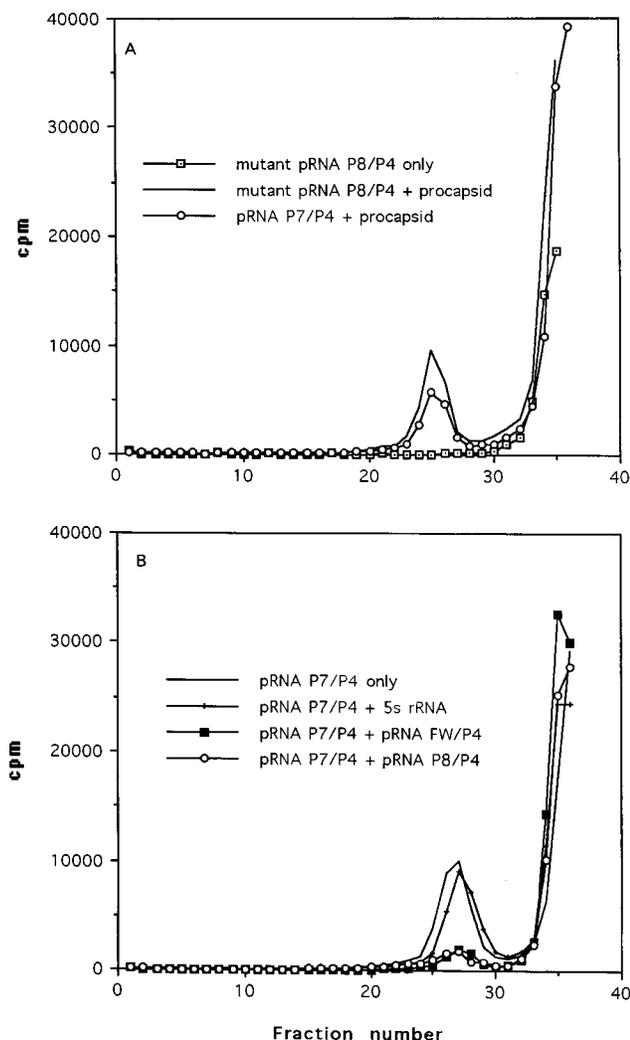


FIG. 4. Demonstration of procapsid binding competency of mutant pRNA P8/P4 by procapsid-pRNA complex formation (A) and competition binding (B) assays. Purified ϕ 29 procapsids were mixed with [3 H]pRNA (2.5 pmol of each) and assayed by sucrose gradient sedimentation. Sedimentation was from right to left. The peaks between fractions 23 and 27 in panel A and fractions 25 and 29 in panel B were [3 H]pRNA-procapsid complexes. Mutant P8/P4 bound to procapsid as efficiently as P7/P4 (A). In panel B, binding of pRNA P7/P4 to procapsids was strongly inhibited by pRNA P8/P4 (13.8 pmol) but not by 5S rRNA (20 pmol).

blocking the RNAs involvement in another DNA translocation function, which is the case for P15. These results also support the conclusion that the pRNA contained two domains, one for procapsid binding and the other involved in another DNA translocation function yet to be defined.

The mutant pRNA P8/P4 contained a four-base mutation at the 5' end, which resides in one of the essential domains, but is intact as far as procapsid binding domain is concerned. The competence of pRNA P8/P4 to bind procapsid was demonstrated by sucrose gradient sedimentation of procapsids in the presence of [3 H]RNA molecules (Fig. 4A) as well as by a competition binding assay (Fig. 4B). [3 H]pRNA-procapsid complexes migrate toward the bottom of the gradient, producing the observed peak, whereas unbound pRNA stays at the top (Fig. 4A). Binding of pRNA P8/P4 to procapsid was revealed by the formation of pRNA P8/P4-procapsid complexes with a

sedimentation rate similar to that of pRNA P7/P4-procapsid complexes, as evidenced by a peak between fractions 24 and 27. The result suggested that mutant pRNA P8/P4 retains its procapsid binding ability despite having no DNA-packaging activity. A competition assay was performed by adding excess unlabeled mutant pRNA P8/P4 or mutant pRNA FW/P4 to [3 H]pRNA P7/P4 and then mixing the pRNA with procapsids. A severe reduction in the formation of [3 H]pRNA P7/P4-procapsid complexes indicated that unlabeled pRNA P8/P4 and pRNA FW/P4 were able to compete with [3 H]pRNA P7/P4 for procapsid binding. The presence of excess *E. coli* 5S rRNA had no effect on the binding of pRNA P7/P4 to procapsids. These results indicate that mutant pRNA molecules bind specifically to procapsids and can compete with wild-type pRNA for procapsid binding sites.

Involvement of multiple copies of pRNA for the assembly of one virion. Besides the feature of possession of two functional domains, the mechanism leading to the high efficiency of inhibition was attributed to another pivotal feature: multiple copies of pRNA were involved in the assembly of one virion. The concentration dependence of phage assembly on pRNA was determined to investigate the molecule's stoichiometry in phage assembly, using increasing amounts of pRNA in the highly sensitive *in vitro* ϕ 29 assembly system. When the concentration dependence of phage assembly on DNA was tested, the log/log response curve for DNA concentration did not reach a plateau even though up to 1 μ g of DNA was used (24) (Fig. 5A). The linear plot with a slope of 1 indicates that DNA concentration dependence was first order, and thus only one DNA genome was required for the assembly of one infectious virion. There is a deviation from linearity observed at low virus titers (PFU per milliliter) (Fig. 5A). This deviation can be explained by the fact that in the *in vitro* assembly assay, the presence of a very small number of plaques (<10 per plate) results in the observed low titers. A low concentration of DNA-gp3 might be more sensitive to digestion by nonspecific DNase present in the packaging mixture. Such a small number of plaques cannot be used for accurate quantitative analysis, and thus only the upper portion of the curve was taken into account. The dose-response curve of phage assembly on pRNA,

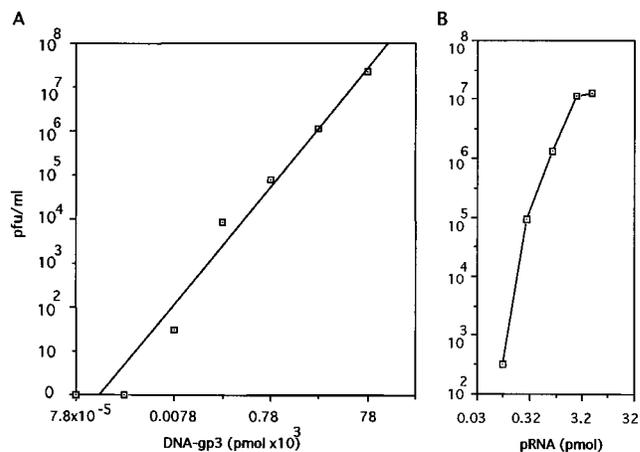


FIG. 5. Dose-response curves with a log/log plot showing pRNA (B) and DNA (A; as a control) concentration dependence in phage ϕ 29 assembly *in vitro*. (A) The best fit curve increases linearly as a function of DNA-gp3 concentration, indicating a first-order response. (B) pRNA with wild-type phenotype was used in increasing concentrations, i.e., 0.09, 0.28, 0.83, 2.5, and 5 pmol. The log of picomoles pRNA was plotted against the log of the titer. The hyperbolic curve indicated that the concentration dependence of pRNA is higher than first order.

however, was higher than first order. There was a dramatic increase in the number of phages assembled until the concentration of pRNA reached 2 $\mu\text{g}/\text{ml}$. However, the number reached a plateau at concentrations higher than 2 $\mu\text{g}/\text{ml}$ (Fig. 5B). The hyperbolic curve with an initial slope greater than 1 indicated that multiple copies of pRNA were needed for the assembly of one infectious virion. This finding is in accord with previous studies which showed that six pRNA molecules bind each procapsid (3, 30, 35). This higher-order dependence of pRNA in viral replication concomitantly resulted in its higher-order inhibitory effect. This finding suggested that the collective DNA-packaging activity of multiple copies of pRNA perhaps could be disrupted by as few as one mutant pRNA.

DISCUSSION

The $\phi 29$ pRNA is a 120-base molecule which contains two separate, essential domains. The internal domain encompassing residues 22 to 84 is involved in procapsid binding (30–32, 37–38a). A second domain located at the 5' and 3' termini is not involved in procapsid binding but plays an essential, albeit unknown, role (38, 38a). The ability of pRNAs with mutations in the 5' and 3' termini to inhibit phage assembly *in vivo*, an RNA with a mutation at the 3' terminus was cloned into a plasmid of *B. subtilis*. *B. subtilis* DE1 cells expressing the mutant pRNA were completely resistant to plaque formation, while cells harboring control plasmid pRB381-L550, pRBwtRNA, or pUM102 were not (Table 2). The mutant pRNA was under the control of the pRNA's natural promoter (an early promoter) (15, 22, 34); thus, the mutant molecule is expressed constitutively. Mutant pRNAs present in the cell presumably bind to procapsids and inhibit DNA packaging. In accordance, *B. subtilis* cells expressing a mutant pRNA were completely resistant to plaque formation when infected with high titers of wild-type $\phi 29$. The inability of DE1 cells containing plasmid pRB381-L550, pRBwtRNA, or pUM102 to resist infection with $\phi 29$ indicates that resistance to infection is conferred by the presence of mutant pRNA and not the presence of the vector plasmid or products, neomycin, the pRNA coding sequence, or wild-type pRNA.

The ability of the mutant pRNAs to inhibit $\phi 29$ assembly so effectively is ascribed to several factors. One is that the pRNA itself has two separate domains, one to bind procapsid and one that is essential for DNA translocation. Therefore, mutant pRNAs which have approximately normal procapsid binding affinity and thus can compete well with wild-type pRNA for procapsid binding can be constructed. As a result, binding of the inactive pRNA to procapsid interfered with the packaging of viral DNA. Another reason for the high efficiency of inhibition could be the number of pRNA molecules per procapsid that are required for DNA packaging. It has been reported that six pRNA molecules bind each procapsid (30, 35). Consequently, any one of these sites can be occupied by a mutant pRNA, increasing the chances of a mutant pRNA binding to the procapsid. Also, Fig. 5B shows that the concentration dependence of *in vitro* $\phi 29$ assembly on pRNA is higher than first order, indicating that more than one pRNA per procapsid is needed for phage assembly. It is unclear how many mutant pRNAs are required to bind procapsid to inhibit DNA packaging. However, the observed high efficiency of inhibition of $\phi 29$ assembly produced by these mutant pRNAs suggests that fewer than six mutants molecules per procapsid can cause blockage of DNA packaging, and perhaps only one mutant molecule is sufficient. In addition, the high efficiency of inhibition of DNA packaging by mutant pRNAs is possibly en-

hanced by the observation that pRNAs bind to procapsid irreversibly in the presence of Mg^{2+} ion (30). Since mutant pRNAs can inhibit DNA packaging (38), it is probable that once mutant pRNAs bind a procapsid, the procapsid is rendered incompetent in DNA packaging, regardless of the presence of excess wild-type pRNA.

The apparent success of targeting multicopy, multidomain factors for inhibition studies suggests the use of other such biomolecules as targets for gene therapy or drug design. Chemicals binding to such domains can inhibit certain biological functions while leaving the other unaffected. The unaffected domain is then free to occupy binding sites that would otherwise be taken by untreated molecules. If the molecule is required in multiple copies, then there is a possibility that not all of the molecules need be treated in order exert an inhibitory effect, thus producing the desirable attribute of a lower drug dosage requirement. The principle of using molecules with two functional domains and multiple-copy involvement could be applied to certain viral structural proteins, enzymes, and other factors involved in the viral life cycle. This principle also implies the use of such similar factors for gene therapy, intracellular immunization, and transgenic plants. Viral products or components with multiple functional domains and multiple-copy involvement in viral replication could be placed within the cell to confer resistance of the cell to viral infection. A high efficiency of resistance would be predicted, as evidenced by the mutant $\phi 29$ pRNA inhibition observed in this study.

ACKNOWLEDGMENTS

We thank R. Reid and D. Anderson for plasmid pRT71, M. Wang and H. Zalkin for *B. subtilis* DE1 cells and plasmid pRB381-L550, and K. Garver for technical assistance.

This work was supported by NIH grant R01GM48159 to P.G.

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