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Approaches To Determine Stoichiometry of Viral Assembly Components

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Due to the rapidity of biological reactions, it is difficult to isolate intermediates or to determine the stoichiometry of participants in intermediate reactions. Instead of determining the absolute amount of each component, this study involved the use of relative parameters, such as dilution factors, percentages probabilities, and slopes of titration curves, that can be more accurately quantified to determine the stoichiometry of components involved in bacteriophage ϕ29 assembly. This work takes advantage of the sensitive in vitro phage ϕ29 assembly system, in which 10^8 infectious virions per ml without background can be assembled from eight purified components. It provides a convenient assay for quantification of the stoichiometry of packaging components, including the viral procapsid, genomic DNA, DNA-packaging pRNA, and other structural proteins and enzymes. The presence of a procapsid binding domain and another essential functional domain within the pRNA makes it an ideal component for constructing lethal mutants for competitive procapsid binding. Two methods were used for stoichiometry determination. Method 1 was to determine the combination probability of mutant and wild-type pRNAs bound to procapsids. The probability of procapsids that possess a certain amount of mutant and a certain amount of wild-type pRNA, both with an equal binding affinity, was predicted with the binomial equation

$$(p + q)^Z = \binom{Z}{0}p^Z + \binom{Z}{1}p^{Z-1}q + \binom{Z}{2}p^{Z-2}q^2 + \cdots + \binom{Z}{Z-1}pq^{Z-1} + \binom{Z}{z}q^Z = \sum_{M=0}^{Z}\binom{Z}{M}p^{Z-M}q^M,$$

where Z is the total number of pRNAs per procapsid, M is the number of mutant pRNAs bound to one procapsid, and $\binom{Z}{M}$ is equal to $\frac{Z!}{M!(Z-M)!}$. With various ratios of mutant to wild-type pRNA in in vitro viral assembly, the percent mutant pRNA versus the yield of virions was plotted and compared to a series of predicted curves to find a best fit. It was determined that five or six copies of pRNA were required for one DNA-packaging event, while only one mutant pRNA per procapsid was sufficient to block packaging. Method 2 involved the comparison of slopes of curves of dilution factors versus the yield of virions. Components with known stoichiometries served as standard controls. 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. A slope larger than 1 would indicate multiple-copy involvement. By this method, the stoichiometry of gp11 in ϕ29 particles was determined to be approximately 12. These approaches are useful for the determination of the stoichiometry of functional units involved in viral assembly, be they single molecules or oligomers. However, these approaches are not suitable for the determination of exact copy numbers of individual molecules involved if the functional unit is composed of multiple subunits prior to assembly.

Extensive investigation of double-stranded DNA viruses documents common features in DNA packaging, such as the use of a noncapsid enzyme(s) to translocate the viral DNA into a preformed procapsid coupled with the hydrolysis of ATP to provide energy (1a, 3, 4, 7, 9, 13, 15, 22, 27). Bacteriophage ϕ29 is a powerful system for the study of viral assembly because infectious ϕ29 virions can be assembled in vitro (26). With the aid of the DNA-packaging enzyme gp16 and ATP, the genomic DNA of ϕ29 can be packaged in vitro into ϕ29 procapsids with up to 90% efficiency (19). The DNA-filled capsids can subsequently be converted into infectious virions after in vitro addition of the tail protein gp9, the upper collar protein gp11, and the appendage protein gp12, with the aid of the morphogenic factor gp13, all of which are overproduced in *Escherichia coli* from cloned genes (24, 26). The generation of up to 10^8 PFU per ml without a single background plaque (24) provides a sensitive and reliable assay for quantification of packaging components. Additionally, a 120-base virus-encoded RNA (pRNA) is absolutely required for DNA encapsidation (16, 17). This RNA is not present in the mature virion, nor is it required for the assembly of the procapsid into which the viral genomic DNA is inserted during maturation. Mg^{2+} induces a conformational change in the pRNA (10) which leads to its binding to the portal vertex, the location on the procapsid where it is thought that DNA packaging occurs (16, 17, 29, 33, 37).

We have developed a highly efficient method for the inhibition of ϕ29 assembly with the use of mutant forms of the pRNA (31). Phage ϕ29 assembly was severely reduced in vitro in the presence of mutant pRNA and was completely blocked in vivo when the host cell expressed mutant pRNA. The mechanism leading to the high efficiency of inhibition was attributed to two pivotal features. First, the pRNA contains two separate,

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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 only one mutant pRNA molecule into the group (31).

Many biological processes involve multistep reactions. It is relatively easy to determine the concentrations of the initiating substrates and the final products. However, due to the rapidity of the reaction, it is very difficult to isolate and characterize the intermediates or to elucidate intermediate states of the reaction. It would therefore be desirable to have a reliable method to determine the stoichiometry of the substrates or enzymes that actively participate in intermediate reactions. The exact concentrations of the substrates and products of intermediate reactions are difficult to determine, but relative parameters, such as dilution factors, percentages, probabilities, and shapes and slopes of titration curves, can be more accurately determined. A variety of biological systems and molecular processes have been successfully analyzed by mathematical methods (5, 11, 23, 28, 30, 35, 36). The principle of our stoichiometry determination is based on competition binding, inhibition assay, and the theory of permutation and combination. This method takes advantage of a highly sensitive in vitro viral assembly system developed recently. The in vitro ϕ29 assembly system involves eight components, including protein or protein oligomers, enzymes, RNA, genomic DNA, and ATP (24, 26). The copy numbers of these eight components required for the assembly of one virion vary greatly, from 1 (genomic DNA) (26, 31) to more than 9,000 (ATP) (20). Our method for stoichiometry determination is based on the inhibition of in vitro ϕ29 assembly by mutant pRNAs and the comparison of slopes of empirical curves of the yield versus concentration. Using a statistical prediction, we can predict the probability that a given procapsid contains a certain amount of mutant and a certain amount of wild-type pRNA bound. Using this probability, we can predict the yield of infectious virus produced by in vitro assembly under various hypothetical circumstances, such as Z wild-type pRNAs per procapsid being required for DNA packaging and X mutant pRNAs ($Z \geq X$) being needed to inhibit DNA packaging. Titration curves, expressed as the yield of phage assembled in vitro as a function of the concentration of a given ϕ29 assembly component, were also used for stoichiometry determination. The stoichiometries of some of the structural components have been determined (32, 34) and served as standard controls for the determination of titration curves.

MATERIALS AND METHODS

Synthesis of pRNA molecules. Wild-type-phenotype pRNA pRT71 was constructed by using *Bgl*II-linearized plasmid pRT71 (29, 38) as a template for in vitro transcription with T7 RNA polymerase. The resulting transcript, pRNA pRT71, was purified by excising the RNA from urea-polyacrylamide gels followed by elution overnight in 0.1% sodium dodecyl sulfate (SDS)–0.5 M sodium acetate–0.1 mM EDTA. The RNA was then ethanol precipitated and resuspended in nuclease-free water. Wild-type-phenotype pRNA P7/P11 was synthesized as described previously (41).

Mutant pRNA P8/P4 was constructed as described previously (38). Briefly, a linear plasmid containing the pRNA-coding sequence was used as a template to generate DNA fragment by PCR with primers containing mutations (38, 41). The purified DNA fragment was used as a template to synthesize mutant pRNA by in vitro transcription with T7 RNA polymerase. The pRNA from the DNA template produced by PCR with primer pairs P8 and P4 was called pRNA P8/P4.

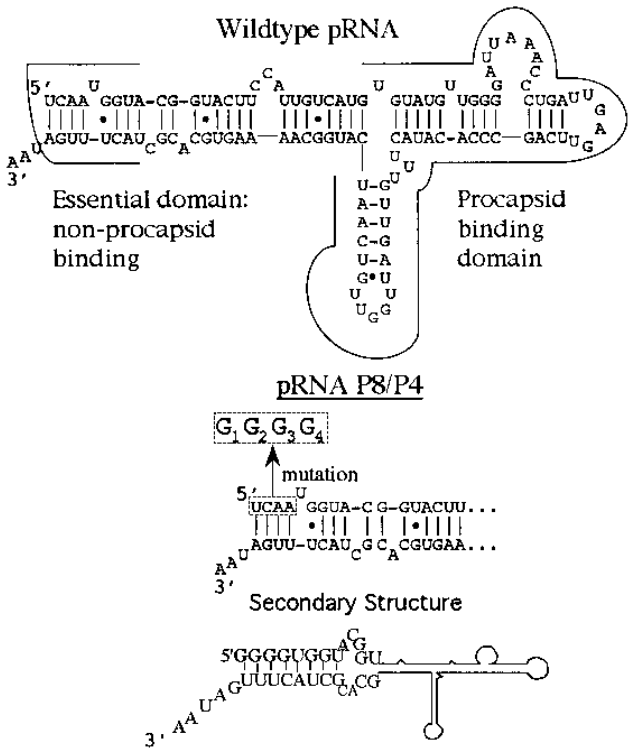

FIG. 1. Predicted secondary structures of wild-type pRNA and mutant pRNA P8/P4. Mutated bases are in boldface. Predictions were made by the method of Zuker (42), and only the lowest-energy structures are shown.

Predictions of the secondary structures of pRNAs (Fig. 1) were made by the method of Zuker (42).

Sucrose gradient sedimentation of procapsid-pRNA mixtures. The purification of ϕ29 procapsids has been described previously (18, 21).

Purified procapsids (18, 21) were dialyzed on a 0.025-μm-pore-size type VS filter membrane (Millipore Corp.) against TBE buffer (89 mM Tris-borate, 2 mM EDTA, pH 8.0) for 10 min at 20°C. ^{3}H-labeled pRNA, synthesized in vitro with T7 RNA polymerase in the presence of [^{3}H]UTP (10 mCi/ml; Amersham), was mixed with procapsids and dialyzed for another 15 min against TBE. The procapsid-pRNA mixtures were then dialyzed against TMS buffer (50 mM Tris-HCl [pH 7.8], 100 mM NaCl, 10 mM $MgCl_2$) for 30 min at 20°C. The mixtures were diluted to 100 μl with TMS and were loaded onto 5 to 20% sucrose (in TMS) gradients and centrifuged in an SW55 rotor for 30 min at 35,000 rpm at 20°C in a Beckman L-80 ultracentrifuge. Fractions were collected from the bottom of each tube and subjected to liquid scintillation counting.

To determine the ability of pRNAs bound to procapsids to exchange with free pRNA, 25 ng of [^{3}H]pRNA pRT71 (or [^{3}H]pRNA P8/P4) was mixed with procapsids and dialyzed as described above. After dialysis of the mixtures against TMS for 30 min, 200 ng of unlabeled pRNA P8/P4 (or unlabeled pRNA pRT71) was added to the procapsid-[^{3}H]pRNA pRT71 (or procapsid-[^{3}H]pRNA P8/P4) mixtures and dialyzed for an additional 30 min at room temperature. The reaction mixtures were prepared for sucrose gradient centrifugation as described above.

In vitro ϕ29 assembly. The purification of gp16 (19) and DNA-gp3 (24) and the preparation of gp9 and gp11-12-13-14 extracts (24–26) have been described previously. *E. coli* extracts containing gp11, gp12, or gp13, separately, were prepared similarly (26, 29a).

In vitro ϕ29 assembly was performed as described previously (24, 26). Briefly, 4 μl of purified procapsid (2 mg/ml) was mixed with 200 ng of pRNA and dialyzed against TBE buffer on a 0.025-mm-pore-size type VS filter membrane (Millipore Corp.) for 15 min and then against TMS buffer for 30 min at room temperature. Next, pRNA-enriched procapsids were mixed with 3 μl 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 that had been dialyzed against gp16 buffer (0.01 M Tris [pH 7.5], 4 mM KCl) for 40 min on ice. The final mixture was incubated for 30 min at 20°C.

The DNA-filled procapsids were then incubated with 10 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 (24). Extracts of *E. coli* containing

plasmids pARgp11 (20 μl), pARgp12 (20 μl), and pARgp13-14 (10 μl) were used when dilutions of gp11 or gp12 were tested.

Inhibition of ϕ29 assembly with mutant pRNA and prediction of inhibition curves. Inhibition of ϕ29 assembly by mutant pRNAs was performed by first dialyzing procapsids against TBE buffer for 10 min at room temperature, followed by the addition of mixtures of mutant and wild-type pRNAs in various ratios (31). In vitro assembly was then performed as described above.

Inhibition of phage assembly by mutant pRNAs was predicted by determining the theoretical probability of each procapsid containing certain amounts of wild-type and certain amounts of mutant pRNAs, under certain conditions. The following equation was used:

$$(p+q)^Z = \binom{Z}{0}p^Z + \binom{Z}{1}p^{Z-1}q + \binom{Z}{2}p^{Z-2}q^2 + \cdots + \binom{Z}{Z-1}pq^{Z-1} + \binom{Z}{Z}q^Z = \sum_{X=0}^{Z}\binom{Z}{M}p^{Z-X}q^X, \quad (1)$$

where $\binom{Z}{M}$ is equal to $\frac{Z!}{M!(Z-M)!}$, where p and q are the percentages of wild-type and mutant pRNAs, respectively, present in a given reaction, and where $p + q = 1$. Z is the total number of pRNA molecules bound to one procapsid. M and N represent the numbers of mutant and wild-type (or normal) pRNA molecules, respectively, bound to individual procapsids, and $M + N = Z$. X is the minimum number of mutant pRNA molecules bound to individual procapsids to block DNA packaging. Various values for Z, X, p, and q were used to construct theoretical curves.

Determination of stoichiometry by measuring the slopes of log plots of concentration versus products. Concentration dependence was determined with the in vitro ϕ29 assembly system mentioned above in the presence of all components in optimal or excess concentrations except for the component to be tested. DNA-gp3, gp11, and gp12 were serially diluted in TMS buffer, gp16 was serially diluted in 10 mM Tris (pH 7.5)–4 mM KCl–1 mM dithiothreitol after dialysis against the same buffer for 40 min on ice, and pRNA was serially diluted in nuclease-free H_2O.

SDS-polyacrylamide gel analysis of ϕ29 particles. Wild-type ϕ29 particles were obtained by infecting *Bacillus subtilis* 12A with wild-type phage followed by purification of the virus by cesium chloride equilibrium density centrifugation. Purified particles were subjected to SDS–14% polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue. The gel was fixed and destained in 10% methanol–10% acetic acid, washed in 10% glycerol, and left overnight under a glass plate to dry. The semidried gel was scanned with a PDI densitometer, and the percentage of each structural component in ϕ29 particles was measured.

RESULTS

Rationale and background for the design of quantification methods. The basis for the design of the quantification methods described here is that ϕ29 pRNA contains two functional domains, one for procapsid binding and another which is required for DNA packaging but dispensable for procapsid binding. Certain mutant pRNAs with modifications at the 5′-3′ paired helical region are inactive in DNA packaging while retaining procapsid binding activity.

The conclusion that the pRNA contains two functional domains comes from the results of different approaches, including (i) base deletion and mutation (38, 40), (ii) RNase probing (references 29 and 38 and unpublished data), (iii) oligonucleotide targeting (39), (iv) competition inhibition of phage assembly (31), and (v) procapsid binding assays by sucrose gradient sedimentation (31, 39, 40).

5′ and 3′ end deletion and mutagenesis studies with numerous mutant pRNAs revealed that the mutation of more than two bases at the 5′ end of the pRNA to noncomplementary bases in the predicted secondary structure resulted in a complete loss of DNA-packaging activity (38, 40). However, the several inactive mutant pRNAs with mutations or deletions at the 5′-3′ paired region were able to bind procapsids as efficiently as wild-type pRNA (31, 39, 40). RNase probing revealed that the binding patterns of these mutant pRNAs cannot be distinguished from that of wild-type pRNA (reference 38 and unpublished data). These mutants were able to compete with wild-type pRNA for procapsid binding and strongly inhibited phage assembly (31). Antisense oligonucleotides targeting the procapsid binding domain blocked the binding of pRNA to the procapsid and therefore completely inhibited viral assembly in vitro. Antisense oligonucleotides targeting the 5′-3′ DNA-translocating domain completely inhibited viral assembly but did not block procapsid binding (39).

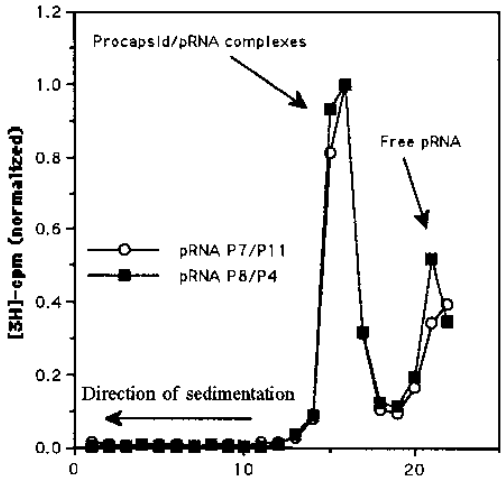

FIG. 2. Comparison of procapsid binding efficiencies of pRNAs. The ^{3}H-labeled mutant pRNA P8/P4 and the wild-type-phenotype pRNA P7/P11 were incubated with procapsids. The [^{3}H]pRNA-procapsid complexes were detected by sucrose gradient sedimentation and are indicated. Unbound RNA remained at the top of the gradient.

Wild-type and mutant pRNAs bind procapsids irreversibly and with similar affinities in the presence of Mg^{2+}. The procapsid binding affinity of mutant pRNA P8/P4 (Fig. 1), which was completely inactive in ϕ29 DNA packaging, was compared to the procapsid binding affinity of wild-type-phenotype pRNA pRT71 by using a procapsid binding competition assay. A constant amount of ^{3}H-labeled pRNA P8/P4 was incubated with various amounts of unlabeled competitor pRNA (pRT71 or P8/P4) in the presence of procapsids and assayed by filter binding (11a, 16, 29b). The resultant binding curves indicated that mutant pRNA P8/P4 bound ϕ29 procapsids with an affinity similar to that of wild-type-phenotype pRNA pRT71 (data not shown).

The procapsid binding efficiency of mutant pRNA P8/P4 was further investigated by procapsid binding and sucrose gradient sedimentation, as reported previously (31, 39, 40). It was found that this mutant pRNA bound ϕ29 procapsids with an efficiency very similar to that of wild-type-phenotype pRNA P7/P11 (Fig. 2).

To test whether pRNAs bound to procapsids can exchange with free pRNAs in solution, sucrose gradient sedimentation was performed (Fig. 3). First, [^{3}H]pRNA was bound to procapsids. Excess unlabeled competitor pRNA was then added to the procapsid-[^{3}H]pRNA complexes. If unbound pRNA was able to exchange with bound pRNA, a reduction in procapsid binding would be seen when such reactions were compared with reactions in which no unlabeled RNA was added. If the two RNAs cannot exchange, then RNA binding to procapsids should occur at similar levels whether unlabeled competitor RNA was added or not. Previous studies have shown that wild-type pRNA bound to procapsids does not exchange with free wild-type pRNA (29). Figure 3A shows that wild-type-phenotype [^{3}H]pRNA pRT71 bound to procapsids does not exchange with free mutant pRNA P8/P4 over the time period assayed, as evidenced by the presence of two similar radioactive peaks representing the procapsid-[^{3}H]pRNA complexes in

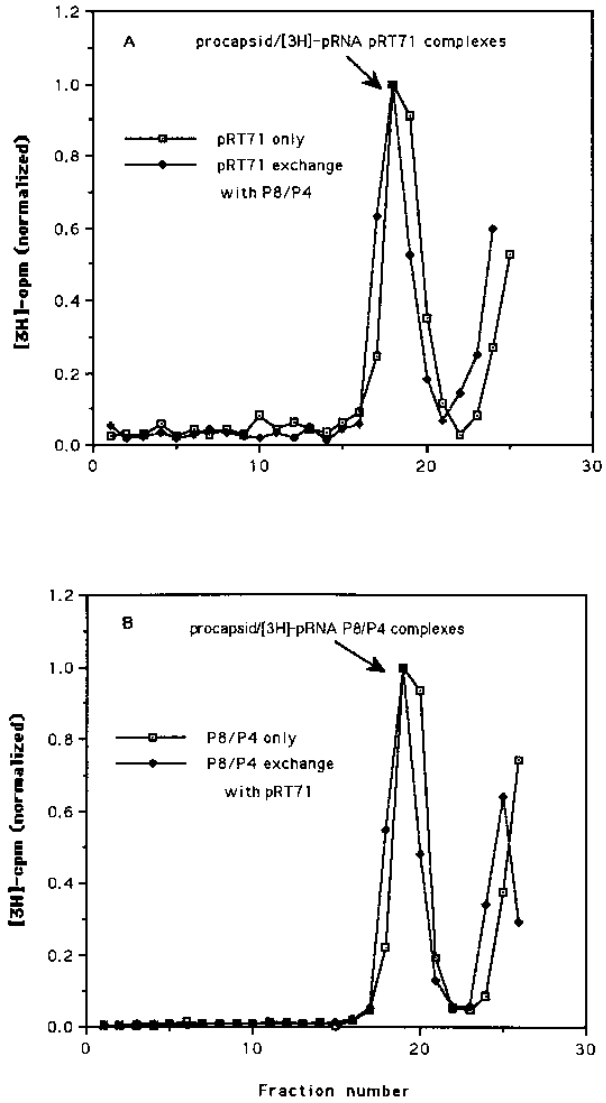

FIG. 3. Assay for ability of free pRNA to exchange with procapsid-bound pRNA. ^{3}H-labeled wild-type-phenotype pRNA pRT71 (A) or ^{3}H-labeled mutant pRNA P8/P4 (B) was mixed with procapsids. After binding, excess unlabeled competitor pRNA P8/P4 or pRNA pRT71 was added to the procapsid-[^{3}H]pRNA pRT71 (A) or procapsid-[^{3}H]pRNA P8/P4 (B) mixture, respectively. The reactions were analyzed by sucrose gradient sedimentation.

the presence and absence of excess unlabeled pRNA P8/P4. Similarly, [^{3}H]pRNA P8/P4 bound to procapsids does not exchange with excess free unlabeled pRNA pRT71 (Fig. 3B).

Generation of predicted-inhibition curves. Equation 1 (see Materials and Methods) was used to predict the levels of inhibition of ϕ29 assembly by mutant pRNAs. For example, if the total number of pRNAs per procapsid required for DNA packaging, Z, is 3, then the probability of all combinations of mutant (M) and wild-type (N) pRNAs on a given procapsid can be determined by the expansion of the binomial $(p + q)^3 = p^3 + 3p^2q + 3pq^2 + q^3 = 100\%$. That is, in the procapsid population, the probability of procapsids possessing either three copies of mutant pRNA, two copies of mutant and one copy of wild-type pRNA, one copy of mutant and two copies of wild-type pRNA, or three copies of wild-type pRNA is p^3, $3p^2q$, $3pq^2$, or q^3, respectively. Suppose that there were 70% ($p \times 100\%$) mutant and 30% ($q \times 100\%$) wild-type pRNAs in the reaction mixture; then, the percentage of procapsids that possessed at least two copies of wild-type pRNA would be the sum of those possessing one copy of mutant and two copies of wild-type pRNA, $3pq^2$, and those possessing three copies of wild-type pRNA, q^3, that is, $3pq^2 + q^3 = 3(0.7)(0.3)^2 + (0.3)^3 = 0.216 = 21.6\%$ (note that this example hypothesizes, for simplicity, that three pRNAs are required for DNA packaging, which is not the case reported in this paper). As another example, if six pRNAs are able to bind each procapsid but only five of the six need to be wild type in order for DNA packaging to occur, the relative yield of phage assembly will be the sum of (i) the probability of each procapsid containing five wild-type pRNAs and (ii) the probability of each procapsid containing six wild-type pRNAs.

Table 1 shows the probability that a given procapsid will have M mutant and N wild-type pRNAs bound at increasing percentages of mutant pRNA, under the condition that the total number of pRNAs required for DNA packaging (Z) is six. The formula

$$\left(\frac{Z!}{M!N!}\right)p^Mq^N \qquad (2)$$

was used to calculate each probability value.

Table 2 demonstrates the prediction of the probability of procapsids that are competent for DNA packaging in the presence of various ratios of mutant and wild-type pRNAs. In this case, it was assumed that $X = 1$ and that Z varied from 1 to 6. That is, one mutant pRNA per procapsid is sufficient for inhibition of DNA packaging, while anywhere from one to six

TABLE 1. Probabilities of combinations of procapsids possessing various amounts of mutant and wild-type pRNAs[a]

Mutant RNA (p) (%)	Probability of procapsids with:						
	$N = 6, M = 0$	$N = 5, M = 1$	$N = 4, M = 2$	$N = 3, M = 3$	$N = 2, M = 4$	$N = 1, M = 5$	$N = 0, M = 6$
0	1	0	0	0	0	0	0
10	0.531441	0.354294	0.098415	0.014580	0.001215	0.0000540	0.000001
20	0.262144	0.393216	0.245760	0.081920	0.015360	0.001536	0.000064
30	0.117649	0.302526	0.324135	0.185220	0.059535	0.010206	0.000729
40	0.046656	0.186624	0.311040	0.276480	0.138240	0.036864	0.004096
50	0.015625	0.093750	0.234375	0.312500	0.234375	0.093750	0.015625
60	0.004096	0.036864	0.138240	0.276480	0.311040	0.186624	0.046656
70	0.000729	0.010206	0.059535	0.185220	0.324135	0.302526	0.117649
80	0.000064	0.001536	0.015360	0.081920	0.245760	0.393216	0.262144
90	0.000001	0.000054	0.001215	0.014580	0.098415	0.354294	0.531441
100	0	0	0	0	0	0	1

[a] Predictions were made for various ratios of mutant (p) and wild-type (q) pRNAs. Each procapsid is assumed to contain six copies of pRNA. N and M are the number of copies of wild-type and mutant pRNAs, respectively, attached to one procapsid. $M + N = Z = 6$; $p + q = 1$.

TABLE 2. Predicted probabilities of yields of in vitro ϕ29 assembly in the presence of various ratios of mutant and wild-type pRNAs[a]

Mutant RNA (*p*) (%)	Probability of ϕ29 assembly with:					
	$Z = 6, X = 1$	$Z = 5, X = 1$	$Z = 4, X = 1$	$Z = 3, X = 1$	$Z = 2, X = 1$	$Z = 1, X = 1$
0	1	1	1	1	1	1
10	0.533333	0.600000	0.666667	0.733333	0.800000	0.9
20	0.263333	0.326667	0.400000	0.500000	0.633333	0.8
30	0.116667	0.166667	0.240000	0.333333	0.500000	0.7
40	0.046667	0.076667	0.130000	0.216667	0.366667	0.6
50	0.015667	0.031000	0.063333	0.125000	0.250000	0.5
60	0.004000	0.010000	0.027000	0.063333	0.160000	0.4
70	0.000730	0.002433	0.008000	0.027000	0.090000	0.3
80	0.000063	0.000320	0.001600	0.008000	0.040000	0.2
90	0.000001	0.000010	0.000100	0.001000	0.010000	0.1
100	0	0	0	0	0	0

[a] Predictions were made for conditions where the number of pRNAs per procapsid that were required for packaging (*Z*) was varied from 1 to 6, while the number of mutant pRNAs per procapsid that were sufficient to inhibit packaging (*X*) was predicted to be 1.

wild-type pRNAs per procapsid would be needed for DNA packaging to occur. The simplified formula q^Z (from equation 2) was used to predict the probability, since the only procapsids that would be able to package DNA would have $M = Z$ and $N = 0$. An example of such a probability calculation, when $Z = 6$ and $X = 1$, is as follows. Since it was assumed that six copies of pRNA per procapsid are required for DNA packaging and that one mutant pRNA was sufficient to block DNA packaging, all procapsids possessing one, two, three, four, or five copies of mutant pRNA would be inactive for DNA packaging. Only those procapsids possessing six copies of wild-type pRNA would be competent to package DNA. In this case a procapsid was said to be competent for DNA packaging if it contained *Z* wild-type pRNAs bound. The probabilities of procapsids containing *Z* wild-type pRNAs were equal to the relative yields of in vitro ϕ29 assembly at various percentages of mutant pRNA. The chance that a procapsid will catch six copies of wild-type pRNA in a mixture containing ($q \times 100\%$) wild-type pRNAs and ($p \times 100\%$) mutant pRNAs was q^6.

Determination of the copy number of mutant pRNA sufficient to block DNA packaging of individual procapsids. We have reported that the collective DNA-packaging activity of multiple copies of pRNA may be disrupted by the incorporation of only one mutant pRNA molecule into the group (31). This observation was used to evaluate the quantification method described above.

In Fig. 4A, a series of theoretical curves generated by using equation 1 is presented. For this prediction, *Z* was held constant at 6 while *X* was varied from 1 to 6, as can be seen by the six separate curves. The value of *p* was varied from 0 (0%) to 1 (100%) by increments of 0.1 (10%) for each predicted curve. For these predicted curves it is assumed that mutant and wild-type pRNAs have equal procapsid binding affinities and thus can compete for procapsid binding sites. Additionally, it is assumed that the binding of mutant and wild-type pRNAs to procapsids is irreversible in the presence of Mg^{2+}, a phenomenon which has been observed previously for wild-type pRNA (29) and for mutant pRNA, as described in this paper (Fig. 3). As can be seen, as *X*, the number of mutant pRNAs per procapsid required for inhibition of DNA packaging, increases, the efficiency of inhibition by mutant pRNA decreases. Mutant pRNA P8/P4 (Fig. 1) (38), which was observed to provide the largest amount of inhibition of in vitro assembly when mixed in various ratios with wild-type pRNA (in comparison to other mutant pRNAs previously described [31, 38]), was used in phage assembly inhibition assays to determine the stoichiometry of the pRNA. In vitro assembly was performed in the

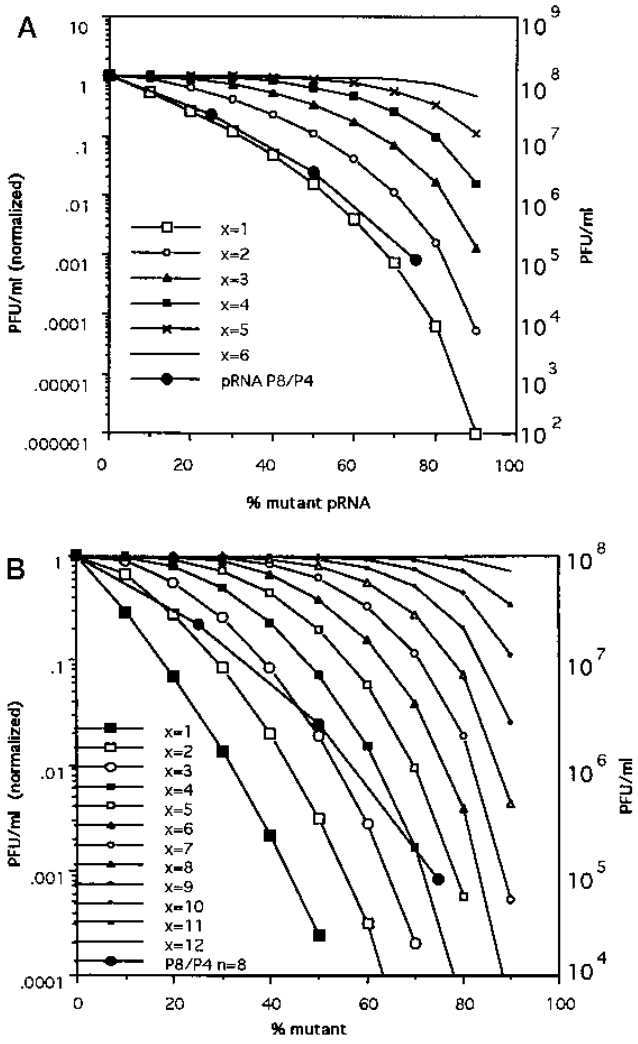

FIG. 4. Theoretical and empirical (mutant pRNA P8/P4) plots of percent mutant RNA versus yield of infectious virions in in vitro phage assembly assays., expressed as PFU per milliliter or normalized. (A) Predictions were made with equation 1, supposing that the total pRNA number, *Z*, per procapsid is 6 and that the minimal number, *X*, of the bound mutant pRNAs required to block DNA packaging is 1, 2, 3, 4, 5, or 6. (B) Predictions were made with equation 1, supposing that the total pRNA number, *Z*, per procapsid is 12 and that the minimal number, *X*, of the bound mutant pRNAs required to block DNA packaging is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12. The typical yield of 10^8 PFU/ml was used for the prediction of the *y* data point for 100% wild-type pRNA. The empirical curve for pRNA P8/P4 was the normalized average from eight experiments.

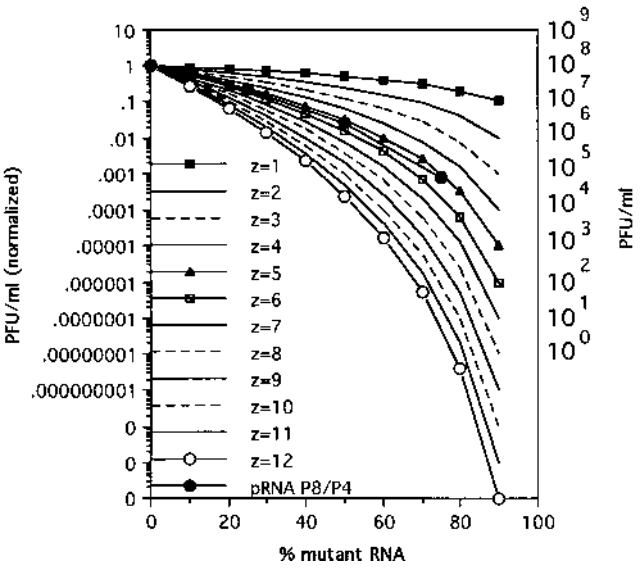

FIG. 5. Theoretical and empirical (pRNA P8/P4) plots of percent mutant RNA versus yield of infectious virions in in vitro phage assembly assays. Predictions were made with equation 1, supposing that the total pRNA number, *Z*, per procapsid is varied from 1 to 12 and that the minimal number, *X*, of the bound mutant pRNA required to block DNA packaging is 1. See Fig. 4 for data explanation.

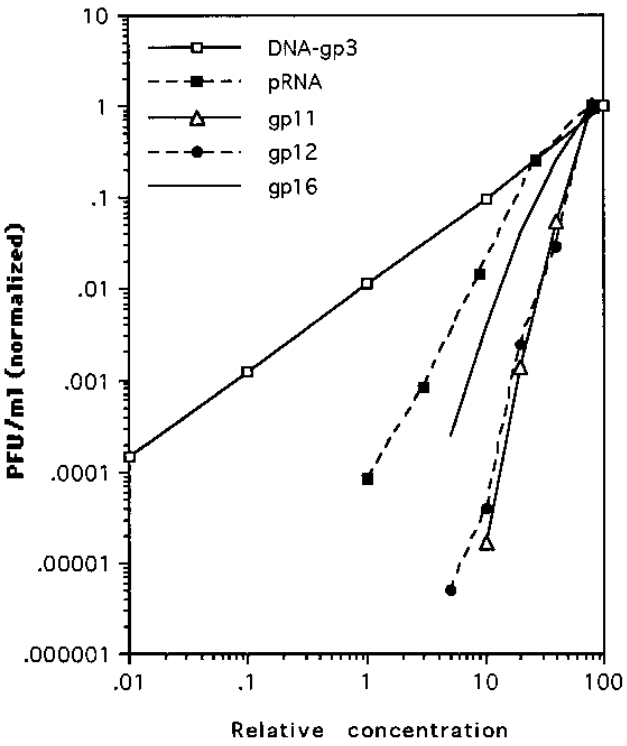

FIG. 6. Dose-response curves with log plots showing DNA-gp3, pRNA, gp16, gp11, and gp12 concentration dependences of phage ϕ29 assembly in vitro. The curve increases linearly as a function of procapsid and DNA-gp3 concentrations, indicating a first-order response. The slope of the curve for DNA-gp3 was 1; the curves for pRNA, gp16, gp11, and gp12 showed slopes larger than 1.

presence of various ratios of mutant pRNA P8/P4 to wild-type pRNA. Mutant pRNA competes with wild-type pRNA for procapsid binding and inhibits in vitro assembly. The yield of assembled virus as a function of the percentage of mutant pRNA was plotted along with the predicted curves of Fig. 4A. As can be seen, the empirical curve of inhibition most closely matches the predicted curve generated when $Z = 6$ and $X = 1$.

Figure 4B shows another set of theoretical curves generated by using equation 1, but under different conditions. In this case, *Z* was held constant at 12, while *X* was varied from 1 to 12. The *p* and *q* values were varied as before. These predicted curves indicate that inhibition of ϕ29 as a function of the percentage of mutant pRNA becomes greater as the number of pRNAs required to inhibit DNA packaging (*X*) decreases. When the empirical data for inhibition of phage assembly by mutant pRNA P8/P4 shown in Fig. 4A were superimposed onto the predicted curves of Fig. 4B, the slope and magnitude of inhibition observed did not match any of the predicted curves.

Determination of the stoichiometry of pRNA in DNA packaging. Figure 5 shows experimental inhibition of ϕ29 assembly obtained with mutant pRNA P8/P4 plotted along with predicted inhibition curves under the conditions that $X = 1$ and *Z* varied from 1 to 12. The experimental curve lies between the curves for $Z = 5$ and $Z = 6$, suggesting that five or six pRNAs are required for DNA packaging, while only one mutant pRNA per procapsid (*X*) would be sufficient to inhibit the process.

Determination of stoichiometry by measuring the slopes of log plots of concentration versus products. Approximation of the stoichiometry of factors involved in viral morphogenesis by observation of log plots of assembly product versus amount of assembly component added was utilized in early phage studies (5, 23). Similar techniques have been used more recently by others (6, 24, 26, 28). When limiting amounts of a certain component are used in assembly assays, the slope of the titer reduction as a function of component concentration reflects the stoichiometry of the component being tested (23). The defined in vitro ϕ29 assembly system was utilized in such a manner for a quantitative assay of assembly components. The entire system currently consists of (i) recombinant procapsids purified from *E. coli*; (ii) purified ϕ29 genomic DNA-gp3; (iii) purified gp16, a DNA-packaging ATPase; (iv) pRNA; (v) purified tail protein gp9; (vi) an *E. coli* extract containing the lower collar protein gp11; (vii) an extract containing appendage protein gp12; and (viii) an extract containing morphogenic factor gp13 (24, 26). In vitro assembly was performed with decreasing concentrations of DNA-gp3, pRNA, gp16, gp11, and gp12. Plots of log PFU per milliliter versus log concentration of each component were established. The slope of the curve for each component is the intrinsic parameter reflecting the stoichiometry of the component, since the higher the copy number, the stronger the influence of its concentration on the products.

The curve reflecting DNA-gp3 concentration dependence was linear with a slope of 1 (Fig. 6) (26, 31), indicating that, as expected, only one copy of DNA-gp3 was needed for the assembly of one virion. DNA-gp3 can be considered one component, since the DNA and the 5′-end terminal protein gp3 are covalently linked.

It has been reported that there are 24 copies of gp12 in each ϕ29 virion (8) but that the protein is present as a homodimer before assembly (34). It has been clearly demonstrated by electron microscopy that ϕ29 contains 12 appendages (2). The 12 appendages are commonly referred to as morphological units. Thus, the gp12 concentration dependence of ϕ29 assembly may reflect a stoichiometry of 12 despite the fact that more than 12 gp12 molecules may exist in the completed virion.

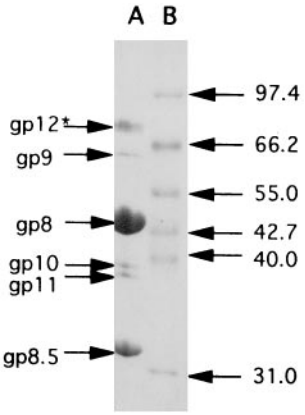

FIG. 7. SDS-polyacrylamide gel of purified wild-type ϕ29 particles stained with Coomassie brilliant blue. Lane A, purified ϕ29 with each component indicated; lane B, molecular mass standard (Promega) with sizes in kilodaltons.

SDS-polyacrylamide gel electrophoresis was performed to determine the relative concentrations of gp10 and gp11 in purified ϕ29 virions (from infected cells) (Fig. 7). Densitometer scanning of the gel provided relative intensities of each Coomassie blue-stained band. The relative intensity of the portal vertex protein, gp10, and the lower collar protein, gp11, were 2.3 and 2.4%, respectively. The reported molecular masses of gp10 and gp11 are 35.8 and 33.8 kDa, respectively. The similar intensities and molecular masses of these two proteins indicate that gp10 and gp11 are present in approximately equal copy numbers in ϕ29 particles. Previous work has indicated that there are 12 or 13 (12, 32) copies of gp10 per virion. Thus, densitometer scanning indicates that the copy number of gp11 per virion is roughly 12 or 13, which agrees with the copy number determined by an alternate approach in Dwight Anderson's lab recently (1). These results are consistent with our concentration dependence curves (Fig. 6), which showed that the curves of gp11 and gp12 concentration dependence were very similar.

The dose-response curves for gp16 and pRNA were located between those for gp11 and gp12 and for DNA-gp3, indicating that the stoichiometry for gp16 and pRNA was more than one but less than the stoichiometry for gp11 or 12. It has been reported that multiple copies of gp16 are involved in the assembly of one virion (6, 19). As described earlier, the stoichiometry of pRNA was determined to be between five and six. It is interesting to find that the curves for gp16 and pRNA do not overlap, since it is hypothesized that one of the DNA-packaging components that the pRNA may interact with is gp16. The significance of the difference is unknown. Whether the difference is due to possible reusability of the pRNA remains to be elucidated. The concentration dependence curve for the component with stronger reusability would exhibit a smaller slope, since fewer copies than the actual stoichiometry are needed if recycling occurs.

DISCUSSION

Reid et al. (29) reported that on the average each procapsid contains 5.8 ± 2.7 or 6.0 ± 3.5 pRNAs. Our previous log plots suggest that multiple copies of pRNA are needed for the packaging of one ϕ29 genome (31). We used in vitro ϕ29 assembly to determine how many pRNAs are required for DNA packaging. Concentration dependence curves with various structural components as standards and inhibition of assembly with mutant pRNA were used for the stoichiometry determination. The principle for the design of this method is that in multistep reactions, it is difficult to calculate the absolute concentrations of the substrates, enzymes, and products involved in the reaction. However, the relative parameters, such as dilution factors, percentages, probabilities, and slopes of titration curves, can be more accurately and easily quantified. That is, instead of determining the exact amount of each component to be used in quantification assays, various dilutions of components were used. A statistical analysis to predict results under various conditions allows observed and predicted results to be compared. Because our method of calculation is based on relative amounts, such as those detected by the shapes and slopes of data curves, rather than on absolute concentrations, predicted and empirical curves can be more readily compared in order to find matches and thus determine the stoichiometry. Such an approach may reduce experimental error.

The observed inhibition of ϕ29 assembly by mutant pRNA P8/P4 indicated that multiple pRNAs were required for DNA packaging while only one mutant pRNA per procapsid was necessary to inhibit packaging. One problem with determining the stoichiometry of the pRNA with the competitive inhibition approach is the large number of predicted curves with relatively small differences between them. However, the large difference between the slopes of the observed inhibition plot and many of the predicted curves allows most of the proposed scenarios to be discounted, leaving only a few to choose from. In fact, the observed inhibition of ϕ29 assembly by mutant pRNA P8/P4 supports the conclusion that either five or six pRNAs are required per procapsid for DNA packaging to occur, while only one mutant per procapsid is sufficient to inhibit packaging.

The significance of five or six pRNAs per procapsid being required for DNA packaging remains to be investigated. The pRNA may play a role in the energetics of DNA packaging. It has been shown that, in extract complementation studies, gp16 shields the pRNA from RNase A digestion when the pRNA is already bound to procapsids (14). Additionally, the pRNA causes a fourfold increase in the ATPase activity of the packaging ATPase gp16, suggesting a pRNA-gp16 interaction (14). The fact that multiple copies of gp16 are required for DNA packaging thus may have relevance to the requirement of five or six pRNAs for each DNA packaging event.

The ability to extend this stoichiometry determination method to the quantification of stoichiometry of other components required for ϕ29 assembly is yet to be tested. In this report, we have shown that the gp11 and gp12 concentration dependence of ϕ29 assembly is higher than that for gp16, which in turn is higher than that for the pRNA. Each of these concentration dependence curves reflects a requirement for multiple copies of each component in ϕ29 assembly, in contrast to genomic DNA, of which only one is required per virion. It should be noted that the concentration dependence studies in this work reflected the minimum copy number requirement for each component to assemble an infectious virion and did not necessarily reflect the optimal amount of component used for assembly if such a component is reusable. Also, the curves may not reflect the actual copy number of a component present in the completed phage if the component is present as an oligomer prior to assembly. Additionally, concentration dependence curves could not address the question of whether all of a given component present in a completed phage is required for infectivity.

A quantification of the amounts of gp11, gp12, and gp16 required for the assembly of one virion was not performed by phage assembly inhibition, as it was for the pRNA, due to the

fact that mutant forms of these proteins that can inhibit phage assembly have not yet been constructed. Indeed, the fact that these proteins are almost completely uncharacterized, in regard to domain structure, has hampered the construction of inhibitory mutants of these proteins. It is interesting that the stoichiometry of the pRNA in ϕ29 assembly was consistent with previous quantifications of the number of pRNAs bound to each procapsid (29). It appears that the conditions for such a quantification were ideal in that both mutant and wild-type pRNAs had similar procapsid binding affinities, both mutant and wild-type pRNAs bound to procapsids irreversibly in the presence of Mg^{2+}, and each of the five or six pRNAs bound to each procapsid appeared to be essential for DNA packaging. Such ideal conditions may not exist for other components involved in ϕ29 assembly. Additionally, it may not be possible to generate mutant forms of certain assembly components that can compete with wild-type components and inhibit viral assembly or infectivity. Currently attempts are being made to construct gp11, gp12, gp16, and tail protein gp9 mutants that can inhibit ϕ29 assembly and thus can be used to assess our stoichiometry quantification system.

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