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Prohead RNA of bacteriophage φ29: size, stoichiometry and biological activity

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ABSTRACT

We previously demonstrated (Guo *et al.*, 1987. Nucl. Acids Res. **15**, 7081−7090) that purified proheads of bacteriophage φ29 contain an RNA of 120 bases which is essential for DNA packaging. Here we report that this RNA exists primarily as a polymer of *ca.* 174 residues in phage-infected cells and that *ca.* 54 bases are cleaved from its 3′-terminus by adventitious nucleases during the purification of proheads. The long and short forms of the RNA had similar activity in *in vitro* DNA packaging and phage assembly. We report the sequence of the long form of the RNA and show that similar long and short forms can be isolated from the proheads of the φ29 relatives φ21, φ15 and SF5. The concentration dependence in the reconstitution of RNA-free proheads suggests that one copy of the RNA is sufficient to restore DNA packaging activity to RNA-free proheads. However, quantitative measurements indicate that 5 to 6 copies of the RNA are present on proheads isolated from phage-infected cells.

INTRODUCTION

A novel viral RNA molecule was previously found to be a component of purified proheads of the *Bacillus subtilis* phage φ29 and an essential participant in ATP-dependent DNA packaging (1, 2). Sequence analysis showed the RNA to be 120 bases in length and indicated that it was transcribed from the left-most promoter of the phage genome (3). The RNA is a component of the connector or portal protein of the prohead, and it can be detached from the prohead and reattached with the loss and restoration of DNA packaging activity (1, 3).

In the present study we report that φ29 prohead RNA occurs *in vivo* as a 174 residue molecule. The loss of *ca.* 54 bases from the 3′-terminus of the RNA occurred as the result of the action of adventitious nucleases during the purification of proheads. This cleavage did not detectably alter the activity of the RNA in reconstituting RNA-free proheads for *in vitro* DNA packaging and viral assembly. Similar long and short RNA forms were found on proheads of related phages. The long form of the RNA was abundant in φ29-infected cells, and approximately 5 to 6 molecules were present on purified proheads. However, the concentration-dependence of RNA suggested that the binding of only one RNA molecule may be sufficient to restore *in vitro* DNA packaging and viral assembly.

MATERIALS AND METHODS

In Vitro φ29 Assembly in the Defined System

The completely defined *in vitro* DNA packaging system includes purified DNA-gp3, proheads, DNA packaging protein gp16 and ATP (4). To prepare φ29 proheads, *B. subtilis* SpoA12 (*sup*$^-$) was grown to 4×10^8 cells/ml in 416 medium containing 2% (wt/vol)

Difco Bacto-tryptone, 1% Difco yeast extract and 0.17 M NaCl; concentrated to 2×10^9 cells/ml by centrifugation; and infected with the mutant *sus* 16(300)-*sus* 14(1241) at a multiplicity of 15. ϕ21, ϕ15 and SF5 proheads were obtained by infection with wild-type viruses. After 5 min, the infected bacteria were diluted in 416 medium to 2×10^8 cells/ml, incubated with shaking for an additional 70 min at 37° C, collected by centrifugation and concentrated 60-fold by resuspension in SMMP/BSA [2 ×Difco antibiotic medium no. 3 (Penassay broth) containing 0.5 M sucrose, 20 mM maleic acid-NaOH (pH 6.5), 20 mM $MgCl_2$ and 1% BSA] with 100 mg/ml lysozyme. After incubation for 10 to 15 min at room temperature, protoplasts were diluted 4-fold in SMMP/BSA, collected by centrifugation, resuspended in 1/8 volume SMMP/BSA containing 23 units/ml RNase-free DNase I (Boehringer Mannheim) and 25 units/ml RNase inhibitor (human placenta, Calbiochem), and lysed by dialysis against MMS buffer [5 mM maleic acid-NaOH (pH 5.6), 15 mM $MgCl_2$, 0.1 M NaCl and 2 mM sodium azide] for 90 min at 4° C. After three clarification centrifugations, each at 12,000 **g** for 10 min at 4° C, the proheads in the supernatant were isolated by centrifugation in a 10% to 30% linear sucrose density gradient containing MMS buffer (pH 5.6) in the SW28 rotor at 25,000 revs/min (85,000 **g**) for 5.5 hr at 4° C. The purified proheads were pelleted by centrifugation in the 50.2 Ti rotor at 35,000 revs/min (150,000 **g**) for 5 hr at 4° C and resuspended in MMS buffer (pH 6.5) containing 200 to 250 units/ml RNase inhibitor to give about 10^{15} particles/ml. These proheads were also used in the preparation of prohead RNA (see below).

The procedures for [^{3}H]DNA-gp3 and gp16 purification have been reported (4). Briefly, gene 16 of ϕ29 was inserted into the plasmid pPLc2833 (5, 6), the recombinant plasmid pPG9 was inserted into *Escherichia coli* M5219, and gp16 was overproduced as lumps under the control of the lambda P_L promoter. Spheroplasts of the cells were lysed at 4°C in the French pressure cell at 69 MPa. The gp16 lumps were isolated from the lysate by differential centrifugation and solubilized on ice in 6 M guanidinium chloride in 10 mM Tris-HCl (pH 7.4) and 50 mM dithiothreitol. The solubilized gp16 was then clarified by centrifugation at 100,000 **g** for 1 hr at 4° C. The supernatant containing gp16 was fractionated by HPLC in a Spherogel TSK 3000 SW column (Beckman) equilibrated with 7.5 M urea, 1 mM dithiothreitol and 10 mM Tris-acetate (pH 5.2). The purity and concentration of gp16 in peaks detected by absorbance at 280 nm were determined by SDS-PAGE, and the fractions were stored at —70° C. gp16 was renatured by dialysis over a membrane filter (0.025 μm, Millipore) against 10 mM Tris-HCl (pH 7.5) and 4 mM KCl for 40 min at 4° C just prior to use. The DNA-gp3, stored at 4° C as fractions from isopycnic CsCl step gradients (4), was dialyzed over a membrane filter against TMS buffer [50 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$ and 0.1 M NaCl] for 30 min at room temperature prior to use. gp16 (200 μg/ml), [^{3}H]DNA-gp3 (1.5×10^{13} molecules/ml), proheads (10^{14} particles/ml) and reaction buffer (10 mM ATP, 6 mM spermidine and 3 mM β-2-mercaptoethanol in TMS buffer) were mixed in the proportions 7:5:3:3 and incubated for 30 min at room temperature. The DNA packaging efficiency was the fraction of the total [^{3}H]DNA-gp3 in the 5% to 20% linear sucrose density gradient that sedimented to the filled head position after centrifugation of the assembly mixture in the SW 50.1 rotor at 35,000 revs/min (120,000 **g**) for 30 min at room temperature.

In order to test the ability of purified RNA to promote DNA packaging activity, purified proheads were made RNA-free by treatment with 0.4 μg/ml RNase A (Sigma) in TBE buffer [89 mM Tris-borate (pH 8.3) and 2.5 mM EDTA] for 15 min at room temperature. After the addition of 400 units/ml RNase inhibitor for 15 min, the RNA-free proheads

(3 μl of about 1.2×10^{14}/ml) were incubated with purified prohead RNA (3μl, 43 to 340 ng) in TBE for 15 min at room temperature. The mixture was then dialyzed over a membrane filter (0.025 μm, Millipore) against TMS buffer for 30 min at room temperature and used in the defined *in vitro* assembly system.

In Vitro φ29 Assembly in Extracts

In vitro assembly of φ29 in extracts has been described (7). Briefly, extracts were prepared from *B. subtilis* SpoA12 infected with the mutants *sus* 16(300)-*sus* 14(1241) or *sus* 7(614)-*sus* 8(769)-*sus* 14(1241), which are defective in this host for production of the DNA packaging protein gp16 or proheads, respectively. gp7 of φ29 is the prohead scaffold, gp8 is the shell protein, and the mutation *sus* 14(1241) provides increased yields of proteins by delaying lysis. Extracts derived from infections with the 16^-14^- and $7^-8^-14^-$ mutants were designated 'prohead donor' and 'gp16 donor' extracts, respectively.

To test the ability of purified RNA to promote *in vitro* phage assembly, the gp16 donor extract ($7^-8^-14^-$) was rendered RNA-free by the same method as described for proheads above. The RNA-free gp16 donor extract (11 μl), proheads (3 μl of about 1.2×10^{14}/ml) reconstituted with RNA (3μl, 3.2 to 51 ng), and reaction buffer (3 μl) were incubated for 90 min at room temperature. The number of plaque-forming units (pfu) per milliliter were obtained from quadruple platings for each complementation. The phage background was determined by replacing purified RNA with TMS buffer for assembly.

Labeling and Sequencing of Prohead RNA

The long form of φ29 prohead RNA was prepared by phenol extraction of total RNA from phage infected cells or by TBE release of RNA (3) from proheads purified by sucrose density gradient centrifugation. In both cases the RNA was further purified by electrophoresis on polyacrylamide-urea gels prior to labeling. The RNA was dephosphorylated with calf intestine alkaline phosphatase (Boehringer Mannheim; 1 unit/100 pmoles RNA) in 100 mM Tris-HCl (pH 8.0) for 30 min at 55° C. The dephosphorylated RNA was 5′-end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (U. S. Biochemical; 8) or 3′-end labeled with [5′-^{32}P]pCp and RNA ligase (Pharmacia LKB; 9). The labeled RNA was isolated by electrophoresis on 10% polyacrylamide-urea sequencing gels and detected by autoradiography. 3′- and 5′-end labeled RNA was sequenced by enzymatic digestion and separation on polyacrylamide-urea gels (8, 9).

Northern and Dot Blot Hybridization of Prohead RNA

For Northern blot hybridization, total RNA from φ29-infected and uninfected cells (1 μg based on A_{260}) and purified φ29 120 and 174 base RNA from proheads (10 ng) were denatured in 1M deionized glyoxal, 50% DMSO and 10 mM NaH_2PO_4 (pH 7.0) for 60 min at 50° C and chilled on ice. The RNAs were separated on a 1.4% horizontal agarose gel run at 90 volts for 4 hr with recirculating 10 mM NaH_2PO_4 buffer (pH 7.0) (10).

The RNA was transferred by capillarity from the gel to a nylon membrane overnight in 10× SSC (11). The nylon with RNA was baked in a vacuum oven at 80° C for 2 hr and prehybridized for 2 hr in a sealed plastic bag at room temperature in 5× Denhardt's (1× Denhardt's is 0.02% ficoll, 0.02% polyvinylpyrrolidone and 0.02% BSA), 5×SSC, 0.1% SDS, 0.1 mg/ml tRNA and 0.1 mg/ml sheared salmon sperm DNA. The RNA was hybridized with 5 to 10×10^6 cpm of DNA probe in a sealed plastic bag at room temperature in fresh hybridization solution. The synthetic 14 base DNA oligonucleotide probe, 5′ TTATCAAAGTAGCG 3′, was complementary to the 3′ end of the 120 base prohead RNA (3). The probe was 5′-end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase and subsequently purified on a 10% polyacrylamide gel. The membrane was washed

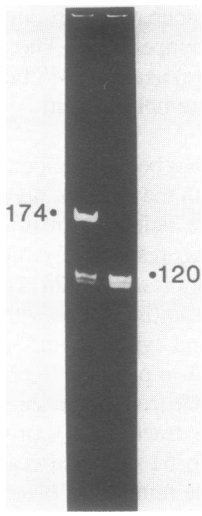

Figure 1: ϕ29 prohead RNA was cleaved from 174 to 120 bases in prohead isolation. Proheads were isolated with (left) and without (right) RNase inhibitor, run on a 6% polyacrylamide-urea gel and stained with ethidium bromide.

with 6× SSC at room temperature, and the RNA was detected by autoradiography.

For the quantitative determination of ϕ29 RNA on proheads by dot blot hybridization (11), proheads, or purified prohead RNA (spectrophotometrically quantified) as standard, were mixed with 1/5 volume of 37% formaldehyde in 6× SSC, incubated for 15 min at 60° C, and chilled on ice. These solutions were spotted onto a nylon membrane prewetted with 6× SSC. The membrane with RNA was baked, prehybridized and hybridized as described above. Spots detected by autoradiography were excised and counted in a scintillation counter. The prohead RNA was quantified by the use of a standard curve obtained with the purified prohead RNA (0.6−20ng).

Labeling of RNA During Phage Infection

B. subtilis SpoA12 was grown and infected with wild-type ϕ29 or the mutant *sus* 16(300)-*sus* 14(1241) as described above. After adsorption at room temperature for 5 min, the infected cells were diluted 10-fold into prewarmed 416 medium with 2.5 μCi/ml [^{32}P]HPO_4 and shaken at 37° C. At various times, culture aliquots were placed on ice

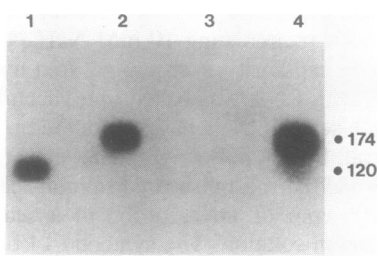

Figure 2: The 174 base prohead RNA predominates in ϕ29-infected cells. Purified 120 and 174 base ϕ29 prohead RNAs (lanes 1 and 2, respectively) and total RNA from ϕ29-infected cells (lane 4) and uninfected cells (lane 3) were analyzed by Northern hybridization and autoradiography employing a prohead RNA-specific probe.

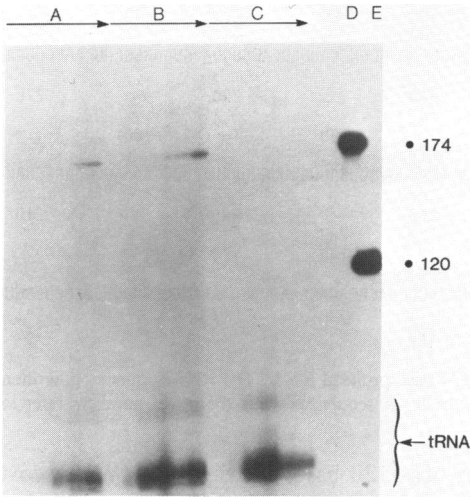

Figure 3: ϕ29 174 base prohead RNA increased with time during infection. The viral RNA was labeled with [^{32}P]HPO_4 in cells infected with wild-type ϕ29 (A) or the mutant *sus* 16(300)-*sus* 14(1241) (B). Uninfected cells were labeled as the control (C). Samples were withdrawn at 5, 10, 20, 30, 40 and 50 minutes after [^{32}P]HPO_4 addition, and the RNAs were extracted and run on a 10% polyacrylamide- urea gel with 5′-[^{32}P]174 base RNA (D) and 5′-[^{32}P]yeast 5S rRNA (120 bases, E) as standards; the RNAs were detected by autoradiography. The lanes in A, B, and C represent samples taken at increasing times, from left to right.

and the cells were collected by centrifugation at 4° C and concentrated 25-fold by resuspension in 10 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$ and 10 mM NaN_3 containing 100 μg/ml RNase-free DNase I and 1 mg/ml lysozyme. After 3 min at 37° C, SDS was added to a 1% final concentration and the RNA was phenol extracted prior to electrophoresis and autoradiography.

RESULTS

ϕ29 Prohead RNA was Cleaved from 174 to 120 bases During Prohead Isolation

The RNA previously found on purified ϕ29 proheads, active in DNA packaging in the

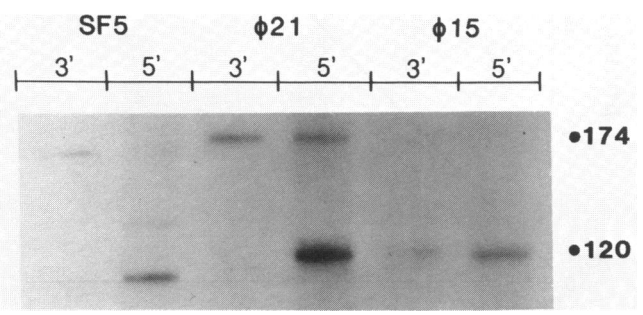

Figure 4: Proheads of the ϕ29 relatives SF5, ϕ21 and ϕ15 have RNAs of about 174 and 120 bases (ϕ29 174 and 120 RNAs, not shown, were run as standards). The RNAs were isolated from purified proheads by phenol extraction, labeled with ^{32}P at the 3′- or 5′-ends, run on a 10% polyacrylamide-urea gel, and detected by autoradiography.

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                    20                  40                  60
                    :                   :                   :
5'-UCAAUGGUACGGUACUUCCAUUGUCAUGUGUAUGUUGGGGAUUAAACCCUGAUUGAGUUC
                  :                   :                   :
                310                 290                 270

                    80                  100                 120
                    :                   :                   :
AGCCCACAUACUUUGUUGAUUGGUUGUCAAUCAUGGCAAAAGUGCACGCUACUUUGAUAA
                  :                   :                   :
                250                 230                 210

                  140                 160
                  :                   :
CCUUUUACAUGCGACACAGACGAAGCGCUAAAACGUGGGAUUCUGUGUCGUUUU-3'
                  :                   :                   :
                190                 170                 150
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Figure 5: Sequence of the 174 base prohead RNA. The RNA sequence is written from left (5') to right (3'). The upper numbers refer to the RNA sequence, while the lower numbers refer to the genomic sequence (12) reading from right to left.

defined *in vitro* system, was 120 bases in length (3). We discovered subsequently that the size of the RNA depended on the method employed in prohead purification. An example of this result is shown in Fig. 1. When prepared in the usual way, no RNA larger than 120 bases was seen, but occasionally one (Fig. 1, right lane) or more (data not shown) slightly smaller fragments were also observed. At least one of these smaller fragments was able to restore DNA packaging activity to RNA-free ϕ29 proheads (data not shown), but we have not yet further investigated their structures. A strikingly different result was obtained when human placenta ribonuclease inhibitor was added to protoplast suspensions

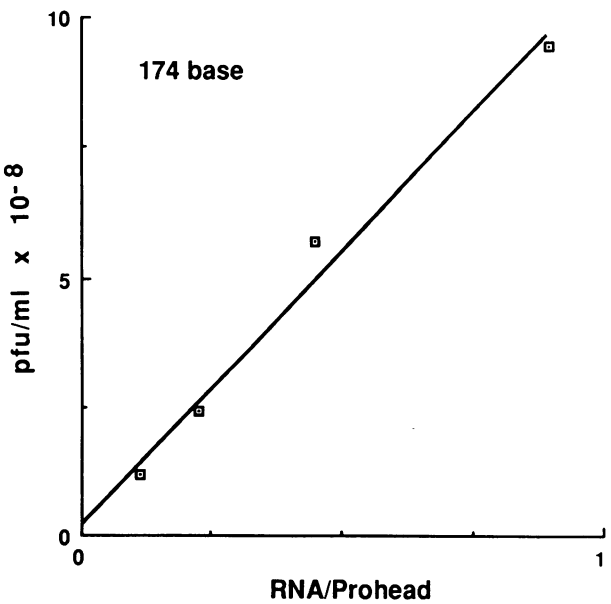

Figure 6: The RNA concentration dependence in prohead reconstitution was first order when ϕ29 was assembled in extracts. RNA-free ϕ29 proheads were reconstituted with purified 174 base prohead RNA and complemented with RNA-free gp16 donor extracts to assemble ϕ29.

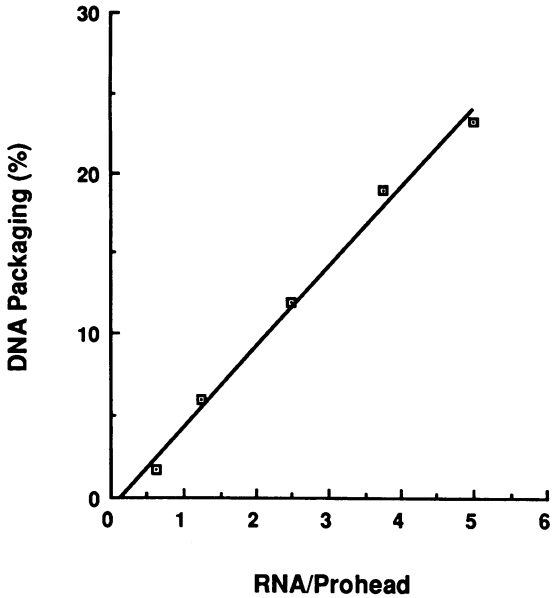

Figure 7: The RNA concentration dependence in prohead reconstitution was first order for ϕ29 DNA-gp3 packaging in the defined *in vitro* system. RNA-free ϕ29 proheads were reconstituted with purified 174 base prohead RNA and complemented with [^{3}H]DNA-gp3, gp16 and ATP to produce filled heads that were isolated in sucrose density gradients. DNA packaging efficiency was the percent of the total label in the gradient found in filled heads.

prior to lysis for the preparation of proheads. Under these circumstances the majority of the RNA subsequently isolated from proheads contained *ca.* 174 residues (Fig. 1, left lane).

The 174 Base Prohead RNA was Present in ϕ29-infected Cells

Three different methods were utilized to determine the size of the prohead RNA present in ϕ29-infected cells. First, a [^{32}P]-labeled synthetic oligodeoxynucleotide complementary to the 3'-end of the 120 base RNA was used to detect prohead RNA by Northern hybridization in total RNA extracts of phage-infected cells. The predominant form of hybridizing RNA was indistinguishable from the 174 residue RNA observed on proheads isolated in the presence of RNase inhibitor, while a negligible quantity of the shorter form was seen (Fig. 2). Prohead RNA in ϕ29-infected cells was sufficiently abundant to be detected by *in vivo* labeling with [^{32}P]-phosphate. Fig. 3 shows an autoradiogram of a polyacrylamide gel of low molecular weight RNAs extracted from [^{32}P]-labeled *B. subtilis* cells either uninfected (lane C) or after varying times of infection with wild-type ϕ29 (lane A) or with the mutant *sus* 16(300)-*sus* 14(1241) (lane B). A labeled RNA species of *ca.* 174 residues was readily observed after about 30 min of labeling in cells infected with either phage, but this RNA was not seen in uninfected cells. No labeled RNA was seen in phage-infected cells which corresponded to the 120 base form found on purified proheads. Finally, the 174 base RNA was isolated from purified proheads and from total RNA of infected cells and sequenced (see below). All of these results suggested that the RNA present on ϕ29 proheads during DNA packaging *in vivo* was *ca.* 174 residues in length and that this RNA was cleaved to 120 residues during the purification of proheads.

Proheads of the ϕ29 Relatives ϕ21, ϕ15 and SF5 Contained RNA

RNAs were extracted from purified proheads of the ϕ29 relatives ϕ21, ϕ15 and SF5. These

total RNAs were then subjected to both 5′- and 3′-end labeling, electrophoresis and autoradiography (Fig. 4). RNAs approximately corresponding in size to the 120 and 174 base RNAs seen with ϕ29 were observed with all three related phages. Both of the two forms of SF5 RNA appeared slightly smaller than their ϕ29 counterparts but in no case were larger RNAs observed. Greater relative amounts of the short form RNA were seen after 5′-end labeling, presumably reflecting degradation of the long form during alkaline phosphatase treatment. This result suggested that degradation of the long form occurred not only on the prohead but also with the isolated RNA.

The Sequence of the 174 Base ϕ29 Prohead RNA

The long form of ϕ29 RNA isolated both from purified proheads and from total RNA extracts of whole cells was labeled with [^{32}P] at its 5′- and 3′-termini and subjected to sequence analysis by enzymatic digestion and polyacrylamide-urea gel electrophoresis as done previously for the short form of the RNA (3). The two forms of RNA had identical 5′-sequences, indicating that they differed in length at their 3′-termini. Moreover, all preparations of RNA exhibited approximately comparable 3′-terminal heterogeneity, each containing about equal amounts of 172-, 173- and 174- residue molecules (data not shown). This result suggested but did not prove that this heterogeneity was present *in vivo*. The sequence of the longest form of the RNA is shown in Fig. 5. The previously observed sequence of the 120 base RNA agreed with the ϕ29 gene sequence except for an ambiguity at residue 112 which we attributed to 3′-terminal heterogeneity (3). The sequence observed for the 174 base RNA agreed with the genome sequence at position 112 and was identical to residues 320 through 147 of the DNA, from right to left with respect to the transcription and genetic maps (12, 13).

The ϕ29 Prohead Contained 5 to 6 Copies of RNA

To determine the number of copies of RNA per prohead, purified proheads were counted by electron microscopy, and the RNA was quantified by dot blot hybridization with a ^{32}P-labeled oligonucleotide probe. Electron microscopic counts for three preparations of proheads were $1.4 \pm 0.2 \times 10^{15}$, $7.0 \pm 1.7 \times 10^{14}$, and $1.1 \pm 0.2 \times 10^{15}$ per milliliter. The corresponding RNA copies were $7.0 \pm 0.9 \times 10^{15}$, $3.4 \pm 0.8 \times 10^{15}$, and $7.2 \pm 2.2 \times 10^{15}$ per milliliter, giving RNA/prohead ratios of 5.0, 4.9, and 6.5, an average of 5.5 copies of RNA per prohead. The number of latex beads and proheads counted in 22, 30 and 11 electron micrographs in these three determinations were 2943, 7242; 3634, 4954; and 2461, 2134; respectively. Similar measurements with mature ϕ29 phage showed less than 0.2 RNA molecules per particle.

The Concentration Dependence of RNA in Prohead Reconstitution was First Order

The concentration dependence of RNA in prohead reconstitution was studied by the addition of RNA to RNA-free proheads and complementation with an RNA-free gp16 donor extract. The concentration dependence was first order with the 174 base RNA when 0.1 to 0.9 RNA per prohead was used (Fig. 6). When the RNA/prohead ratio was increased to two, the phage assembly was 1.4×10^9 pfu/ml, and the response reached a plateau. Further increases in the RNA/prohead ratio resulted in very little increase in phage production. The activity of the 120 base RNA in this assay was identical (data not shown). In the defined *in vitro* system in which the efficiency of DNA-gp3 packaging was measured, the relationship was similarly first order, however RNA to prohead ratios of 0.6 to 5 were used (Fig. 7). When the RNA/prohead ratio was increased to 10 and 20, the DNA packaging percentages remained constant at 28.6 and 25.9%, respectively. The difference in the total RNA requirement between the extract (Fig. 6) and the defined (Fig. 7) *in vitro* assembly

systems is inexplicable at this time. The results suggested that a single copy of RNA on each prohead was sufficient to package ϕ29 DNA-gp3 but did not clarify the functional significance of the 5 to 6 copies of RNA found on the prohead.

For unknown reasons, phage production with reconstituted proheads in RNA-free extracts was relatively inefficient (Fig. 6). The extracts contained 10^{10} infected cell equivalents per milliliter, and only about 0.1 phage per infected cell equivalent was produced. Constituents of the extract needed for phage assembly may have been unstable or limiting.

DISCUSSION

The experiments reported here demonstrate that the 120 base prohead RNA, previously shown (3) to play an essential role in the *in vitro* packaging of ϕ29 DNA, exists as a polymer of *ca.* 174 residues in phage-infected cells. The truncated molecule present on the purified proheads employed in the completely defined *in vitro* packaging system (4) arose by nuclease cleavage of the 174 residue polymer during purification. The *in vitro* activities of the two forms of RNA, both in DNA packaging and subsequent phage assembly, were indistinguishable.

The structure of ϕ29 prohead RNA is under investigation. The present observation that approximately 54 residues can be cleaved from the 3'-terminus of this RNA, either while it is bound to the prohead or free in solution, suggests that it may be organized into two structural domains. This view is strengthened by the finding of similar sized RNAs which can also be cleaved into shorter forms on the proheads of related *B. subtilis* phages. The putative 3'-domain is not required for *in vitro* DNA packaging and presumably performs some additional role in phage morphogenesis. The sequence determination of prohead RNAs from ϕ29 relatives is in progress. Thus far, several quite different sequences have been found, and we anticipate that these will be useful in developing a model of secondary structure by phylogenetic sequence comparison.

The abundance of prohead RNA in ϕ29-infected cells underscores its functional importance in phage morphogenesis. The prohead contains 5 to 6 copies of prohead RNA, but the RNA is absent from the mature phage. It was shown previously that prohead RNA binds specifically to purified gp10, the connector or portal protein in ϕ29, and is not found on isometeric particles that lack the connector (1). The connector contains 12 copies of gp10 (14) and the concentration dependence for gp16 in DNA packaging was higher order (15; unpublished data), suggesting the involvement of multiple copies of this protein as well. On the other hand, the RNA concentration dependence in the reconstitution of RNA-free proheads for DNA packaging was first order, suggesting that only a single copy of RNA is required. Taken together, these observations suggest that prohead RNA may have multiple functions in ϕ29 morphogenesis.

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