Characterization of the small RNA of the bacteriophage ϕ 29 DNA packaging machine

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Received June 5, 1987; Accepted July 29, 1987

ABSTRACT

The prohead connector of the bacteriophage ϕ 29 DNA packaging machine was reconstructed with the small RNA that regulates DNA packaging *in vitro*. The complete sequence of the 120 nucleotide RNA proved its origination from the promoter PE1(A1) of the left early region of ϕ 29 DNA, the end packaged first during assembly. The prohead RNA was clearly distinct from eubacterial 5S rRNA in sequence and composition.

INTRODUCTION

Recently we found that a small RNA of bacteriophage ϕ 29 of *Bacillus subtilis* is required for the DNA packaging phase of viral morphogenesis (1). The RNA, about the size of 5S ribosomal RNA (rRNA), hybridizes to a DNA segment near the left end of the ϕ 29 genome, the end that enters the capsid first. The RNA is a constituent of the viral precursor shell (prohead) into which the 18-kilobase pair double-stranded DNA is packaged but is not a component of the mature virion. This role for small RNA as a transient structural component and catalyst in viral assembly adds a new function to their previously demonstrated priming, regulating and enzymatic activities (2). In addition, double-stranded DNA bacteriophages probably utilize a common mechanism for DNA packaging (3, 4), and we imagine that this new role for RNA may be more general. We have now sequenced the ϕ 29 prohead RNA and demonstrated that it is 120 bases in length and transcribed from the promoter PE1(A1) of the left early region of ϕ 29 DNA. The RNA was detached from ϕ 29 proheads and reattached, with concomitant loss and restoration of competence to package DNA-gp3 in the defined *in vitro* system. Moreover, extracts of ϕ 29-infected cells fully restored RNA-free proheads for phage assembly *in vitro*. The attachment site for the RNA was the prohead connector, a collar at the portal vertex of the prohead and an integral part of the DNA packaging machines of several bacteriophages (5).

MATERIALS AND METHODS**Purification of gp16, DNA-gp3 and Proheads**

The purification of gp16 and DNA-gp3 has been described (6). To prepare proheads, *Bacillus subtilis* SpoA12 (the nonpermissive, *sup*⁻ host) 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 $\phi 29$ suppressor-sensitive (*sus*) mutant *sus* 16(300)-*sus* 14(1241) at a multiplicity of 10. 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 resuspended at 2×10^{10} cells/ml in SMMP/BSA [2X Difco antibiotic medium no. 3 (Penassay broth) containing 0.5 M sucrose, 20 mM $MgCl_2$, 20 mM maleic acid-NaOH, pH 6.5, and 1% BSA] containing 100 mg/ml lysozyme. After incubation for 10-15 min at room temperature, protoplasts were diluted 4X in SMMP/BSA, collected by centrifugation, resuspended in 1/4 volume SMMP/BSA containing 5 μ g/ml DNase I, and lysed by dialysis against MMS buffer (5 mM maleic acid-NaOH, pH 6.5, 10 mM $MgCl_2$, 100 mM NaCl and 2 mM sodium azide) for 90 min. After three clarification centrifugations, each at 12,000g for 10 min, the proheads in the supernatant were isolated by centrifugation in a 10-30% linear sucrose gradient containing MMS buffer, pelleted by centrifugation, and resuspended in MMS buffer to give about 4×10^{14} particles/ml.

In vitro Assembly

In vitro $\phi 29$ assembly rivals *in vivo* assembly in efficiency, both in extracts and in a defined system (6-12). Extracts used for complementation were prepared from *B. subtilis* SpoA12 (the nonpermissive, *sup*⁻ host) infected with the mutant *sus* 7(614)-*sus* 8(769)-*sus* 14(1241) that cannot produce the prohead scaffold (gene product 7) and shell (gene product 8) proteins. This extract was a source of the gene product 16 (gp16) required for DNA packaging and other proteins needed to assemble infectious $\phi 29$ particles from DNA-filled heads, and was designated the "gp16 donor extract." The mutation *sus* 14(1241) provides increased yields of proteins by delaying lysis. When purified proheads and exogenous [³H]DNA-gp3 [$\phi 29$ DNA has gene product 3 (gp3) covalently bound at its 5'-termini] are incubated with the gp16 donor extract, the exogenous labeled DNA is packaged on an equal basis with unlabeled endogenous viral DNA and appears in filled heads that are separated from unpackaged DNA by sucrose gradient centrifugation (6, 8). The gp3 of DNA-gp3 functions both as a primer for DNA replication (13, 14) and as an essential component in DNA packaging (10).

The completely defined *in vitro* DNA packaging system includes purified DNA-gp3, proheads, and gp16 in a reaction that requires ATP (6, 7, 15). Details are provided in the Table 1 and Fig. 3 legends.

Isolation and Sequencing of Prohead RNA

RNA was released from purified proheads in MMS buffer (containing 10 mM Mg^{+2} , pH 7.8) simply by a 50-fold dilution into TBE buffer (89mM Tris-Borate, pH 8.3, 2 mM EDTA). The RNA-free proheads were pelleted by centrifugation at 125,000g for 3 hr and resuspended in TBE buffer. The RNA in the supernatant was concentrated to the original prohead volume in one step by

Figure 1: Electrophoresis of $\phi 29$ prohead RNA. A) proheads with RNA (about 6×10^{11}); B) the purified RNA used for sequencing and prohead reconstruction (about 1.2×10^{12} prohead equivalents); C) 5S ribosomal RNA; D) isometric $\phi 29$ particles without connectors. The 1% agarose gel was stained with ethidium bromide and photographed over UV light (300 nm).

centrifugation over a membrane with a 30,000 dalton cut-off (Centricon-30, Amicon Corp.) at 4300g for 1 hr at 4°C. [^3H]RNA was isolated from proheads labeled *in vivo* in 416 medium containing 5 $\mu\text{Ci/ml}$ of [^3H]uridine.

The RNA was labeled with [^{32}P] at its 5'- and 3'-ends and then subjected to sequence analysis by enzymatic digestion and urea acrylamide gel electrophoresis (16, 17). For 5'-end-labeling (16), the purified prohead RNA was dephosphorylated by calf intestine alkaline phosphatase (Boehringer Mannheim). After phenol extraction and ethanol precipitation, the RNA was 5'-end-labeled by incubation with [$\gamma\text{-}^{32}\text{P}$] ATP and T4 polynucleotide kinase (U.S. Biochemical). For 3'-end-labeling (17), the purified RNA was incubated directly with [$5'\text{-}^{32}\text{P}$] pCp and RNA ligase, then precipitated by ethanol.

RESULTS AND DISCUSSION

Sequencing of Prohead RNA

RNA was released from proheads simply by dilution into buffer containing EDTA (Fig. 1). The RNA-free proheads were collected by centrifugation, and the RNA in the supernatant was concentrated in one step by centrifugation over a semi-permeable membrane (Fig. 1B). The prohead RNA had a mobility in agarose electrophoresis similar to 5S rRNA (compare Fig. 1B with Fig. 1C).

Figure 2: Sequencing of the prohead RNA. A and B) Autoradiographs of the 20% and 10% denaturing polyacrylamide sequencing gels (16) of the 3'- and 5'-[³²P]-labeled RNA. The labeled RNAs (see Materials and Methods) were purified by electrophoresis on denaturing polyacrylamide gels. Following purification, the 5'- and 3'-end-labeled RNAs were sequenced by partial digestion with RNases (Pharmacia) T1 (G specific), U2 (A specific), phy M (A and U specific), and *B. cereus* (U and C specific). Alkaline hydrolysis (OH⁻) and no enzyme controls (-E) were also run. C) Sequence of the 120-base prohead RNA and its alignment with ø29 DNA positions 320 to 201. Transcription was from the PE1(A1) promoter of ø29 in the right to left direction with respect to the genetic and transcription maps (22, 20). The two arrows at RNA positions 99 and 115 indicate the dyad structure that may relate to the termination of transcription.

Figure 3: Reconstruction of proheads and RNA. A) Reattachment of the purified RNA to the proheads. Purified [^3H]RNA in TBE buffer (open circles) and purified [^3H]RNA mixed with unlabeled RNA-free proheads in TBE buffer (closed circles) were dialyzed against TMS buffer for 30 min (see text) and centrifuged in 5-20% linear sucrose gradients in an SW55 rotor at 35,000 rpm for 30 min. Sedimentation was from right to left. The bulk of the [^3H]RNA mixed with proheads cosedimented with the proheads at fractions 20-21, with an unidentified peak at fractions 13-14. The [^3H]RNA alone remained near the top of the gradient, with a small peak at fractions 20-21 representing a small amount of prohead contamination. B) Restoration of RNA-free proheads by the purified RNA for [^3H]DNA-gp3 packaging in the defined *in vitro* system. The RNA-free proheads in TBE (open circles) and RNA-free proheads mixed with purified RNA in TBE (closed circles) were dialyzed against TMS for 30 min and then incubated with [^3H]DNA-gp3, gp16 and ATP (see Table 1 legend). The efficiency of DNA packaging was analyzed by sedimentation of the mixture in a sucrose gradient as described in A. When the RNA-free proheads were mixed with the purified RNA, approximately 30% of the added [^3H]DNA-gp3 was packaged to give filled heads that sedimented to fractions 3-8 in the gradient. The RNA-free prohead control did not show detectable DNA packaging activity.

The purified prohead RNA was labeled with [^{32}P] at its 5'- and the 3'-ends and then subjected to sequence analysis by enzymatic digestion and urea polyacrylamide gel electrophoresis (16, 17). The entire sequence of the viral prohead RNA was read from 20% and 10% sequencing gels such as those shown in Fig. 2, A and B. The substantial sequence overlap achieved between the 20% and 10% gels and the 3'- and 5'-labeled samples in the 10% gel yielded a unique 120-base sequence. This RNA sequence aligned exactly with positions 320-201 near the left end of the 18-kilobase pair ϕ 29 DNA (Fig. 2C). This alignment agrees with our previous RNA-DNA hybridization result that localized the RNA to the Hae II-H fragment subtending positions 176-592 (1). Several sequence ambiguities were encountered. The assignment corresponding to positions 15 and 71 is unclear in the gels shown in Fig. 2. Digestion with the C-specific enzyme CL3 (18) established C's in both

TABLE 1: Purified RNA restored RNA-free proheads for DNA packaging in the defined *in vitro* system.

Comple- mentation*	Proheads	Purified RNA	Pretreatment of RNA with RNase A	Pretreatment of RNase A with RNase inhibitor	Efficiency of DNA packaging (%)**	No. of Expts.
a	Normal	-	-	-	46 ± 4	10
b	RNA-free	-	-	-	~0	10
c	RNA-free	+	-	-	32 ± 5	4
d	RNA-free	+	+	-	~0	4
e	RNA-free	+	+	+	31 ± 6	2
f	None	+	-	-	~0	2

*The complementation mixture contained purified proheads (3 μ l of about 8×10^{13} /ml), RNA (3 μ l of 8×10^{13} /ml prohead equivalents), [3 H]DNA-gp3 (10 μ l of about 5×10^{12} /ml), and gp16 (7 μ l of about 125 μ g/ml). The proheads and RNA (both in TBE buffer, see Materials and Methods) were mixed and dialyzed against TMS buffer for 30 min before complementation. The DNA-gp3 was in TMS supplemented with 10 mM ATP, 6 mM spermidine, and 3 mM mercaptoethanol (8), and the gp16 was in 4 mM KCl, 10 mM Tris-HCl, pH 7.4 (6). RNase A (Sigma Chemical Co.) was dissolved in TMS and used at 0.4 μ g/ml. RNase inhibitor (from human placenta, Boehringer Mannheim) was used at 400 units/ml.

**Efficiency of DNA packaging was the percentage (mean $\pm$ SD) of [3 H]DNA-gp3 added in the defined system that was packaged into proheads to yield filled heads that sedimented to fractions 3-8 of 5-20% sucrose gradients (Fig. 3).

positions (data not shown), in agreement with the DNA sequence. Ambiguities in the RNA sequence at positions 112 and 113 presumably were caused by 3'-terminal heterogeneity. Analysis of other RNA digests (not shown) demonstrated that the base at position 113 is a U, also in agreement with the DNA sequence. The RNA sequence suggested a G at position 112, whereas the DNA sequence assigns a C to this position. We have designated position 112 in the RNA to be cytosine in agreement with the DNA sequence for two reasons. First, cleavage at C residues yielded the least distinct signals. Second, the G band at position 112 did not align exactly with the corresponding alkaline hydrolysis band and was of lighter intensity than other adjacent G cleavages, and thus was presumed to be a satellite band. Several other shadow bands were seen with the 3'-labeled RNA, especially in the 20% gel (Fig. 2A). We believe that these bands reflect length heterogeneity of the viral RNA at its 3'-terminus. The presence of a minor and slightly longer component is faintly visible in Fig. 2B. The 5'-labeled RNA gave no evidence of heterogeneity. By comparison to the DNA sequence (19), the RNA was transcribed, most likely, from the early promoter PE1(A1) (20, 21) near the left end of ϕ 29 DNA, the end packaged first during *in vitro* assembly (10). The RNA sequence is shown in Fig. 2C in a left to right orientation, although the direction of transcription is from right to left

TABLE 2: RNA in a gp16 donor extract restored RNA-free proheads for phage assembly.

Complementation*	gp16 donor extracts	Proheads	Phage assembly 10 ⁸ pfu/ml (mean $\pm$ SD)		No. of Expts.
a	No treatment	None	0.27 $\pm$	0.04	6
b	No treatment	Normal	911 $\pm$	180	7
c	Treated with 0.4 μ g/ml RNase A, then RNase inhibitor	RNA-free	0.62 $\pm$	0.14	4
d	No treatment	RNA-free	739 $\pm$	267	5

*Proheads (3 μ l of about 8×10^{13} /ml) were mixed with gp16 donor extracts (10 μ l) and incubated for 90 min at room temperature. Experiments represent separate complementations using different samples of extracts and proheads. Use of the RNase inhibitor was described in the Table 1 legend.

with respect to the ϕ 29 genetic and transcription maps (22,20). The sequence agrees with the promoter PE1(A1) start sequence determined previously (23).

Though ϕ 29 prohead RNA and eubacterial 5S rRNA share the same size, the prohead RNA was clearly distinct from 5S rRNA in sequence and composition. Prohead RNA contained 30A, 41U, 27G and 22C residues, while 5S rRNA (*Escherichia coli*) contains 23A, 20U, 41G and 36C residues (24).

TABLE 3: Attachment of prohead [³H]RNA to connector protein (gp10) assayed by filter binding*

Ligand	³ H cpm (mean $\pm$ SD, n=4)
gp10	1579 $\pm$ 111
gp16	77 $\pm$ 5
BSA	79 $\pm$ 5
TBE	85 $\pm$ 4

* The basic method has been described (30, 31). Purified [³H]RNA (2.5 μ l in TBE, 3442 $\pm$ 79 cpm) was mixed on parafilm with the purified connector protein gp10 (10 μ l, 500 μ g/ml in TBE), purified gp16 [10 μ l, 125 μ g/ml, as prepared for the defined *in vitro* DNA packaging system (6)], bovine serum albumin (BSA) (10 μ l, 500 μ g/ml in TBE), or TBE buffer (10 μ l) as a background control. TMS buffer (100 μ l) was then added without mixing. After 20 min at room temperature, 11 μ l of 2 M NaCl solution was added, and individual mixtures were transferred onto 2.4 cm GF/C glass fiber filters (Whatman) prewetted with high salt TMS (TMS with 0.325 M NaCl) over a stack of absorbent paper. After 8 rinses with 1 ml of high salt TMS, the filters were soaked in an excess of high salt TMS for 10 min before drying and counting.

Efficient Reconstruction of Proheads and RNA

The purified prohead RNA sedimented slowly in a sucrose gradient (Fig. 3A, open circles), and when mixed with the RNA-free proheads under appropriate conditions as described below, reattached to the proheads almost quantitatively (Fig. 3A, closed circles). Proheads sediment typically to fractions 20-21 in these gradients (7, 8). The RNA-free and reconstructed proheads were tested for competence to package [³H]DNA-gp3 in the defined *in vitro* system, measuring the percentage of label that sedimented as filled heads to fractions 3-8 in sucrose gradients (6). The RNA-free proheads were inactive in DNA-gp3 packaging in the defined system (Fig. 3B, open circles; Table 1b), but their activity was restored following reconstruction with the purified prohead RNA (Fig. 3B, closed circles; Table 1c). Use of RNase A with and without RNase inhibitor (1) indicated that the restoration activity was due specifically to the RNA (Table 1, e and d). RNA itself had negligible DNA packaging activity (Table 1f), demonstrating that the RNA preparation did not contain a significant number of native proheads. Similarly, the RNA-free proheads were restored by RNA in a gp16 donor extract, in this case measuring phage production (Table 2). If the extract was treated with 0.4 µg/ml RNase A and then incubated with RNase inhibitor, phage assembly in plaque-forming units (pfu/ml) with the RNA-free proheads (Table 2c) was close to the background level (Table 2a). If the extract was not treated with RNase A, phage production with normal and RNA-free proheads was similar (Table 2, b and d), demonstrating that the RNA-free prohead was reconstructed efficiently by the RNA in the extract.

The Prohead RNA was Attached to the ø29 Connector

Interestingly, the RNA-free proheads and RNA had to be resuspended and mixed in TBE buffer (containing 2 mM EDTA, pH 8.3) and then dialyzed against TMS buffer (containing 10 mM Mg⁺², pH 7.8) for reconstruction prior to DNA packaging. If the components were resuspended and mixed in TMS, no DNA packaging activity was detected. Isometric ø29 particles produced in gene 10 mutant infections lack the connector (25, 26), a gp10 dodecamer in the base of the prohead (27) through which the DNA passes in packaging. These particles lacked RNA (Fig. 1D), suggesting that RNA was attached specifically to the connector structure in the normal prohead. Moreover, connector protein produced by the cloning and high level expression of gene 10 in *Escherichia coli* (28) and purified to homogeneity by phosphocellulose chromatography (29) was able to bind the purified [³H]-labeled prohead RNA in a filter binding assay (30, 31) (Table 3). The binding assay with 5'-[³²P]-labeled RNA gave similar results. Cosedimentation of the RNA and purified connector protein in sucrose gradients confirmed the results of the filter binding assay. Taken together, the results indicated that conformation of the connector and/or the RNA was Mg⁺² dependent; once the proheads and RNA were separated, addition of Mg⁺² might have changed the conformation of the connector and/or RNA to block the interaction(s) needed for reconstruction and restoration of biological activity. Conformational changes in the connector might trigger both the

initiation and termination of ϕ 29 DNA-gp3 packaging, act as a signal for proper neck/tail assembly, and ultimately regulate DNA-gp3 ejection from the particle after adsorption to the host; RNA is involved at least in the packaging initiation events as a structural component and possibly as a catalyst. It is now feasible to determine the structure of the RNA, to conduct footprinting studies, and to localize the RNA in the connector by Fourier filtering of electron micrographs of two-dimensional crystals of connectors (27) and by use of three-dimensional reconstructions (32).

ACKNOWLEDGMENTS

We thank Charlene Peterson, Mary Ann Gomes, Stephen Erickson and Lini Lu for technical assistance. The work was supported by grants DE-03606 to D.A. and GM-26832 to J.W.B. from the National Institutes of Health.

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