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Research Articles

A Small Viral RNA Is Required for in Vitro Packaging of Bacteriophage ϕ29 DNA

PEIXUAN GUO, STEPHEN ERICKSON, DWIGHT ANDERSON

A small RNA of *Bacillus subtilis* bacteriophage ϕ29 is shown to have a novel and essential role in viral DNA packaging in vitro. This requirement for RNA in the encapsidation of viral DNA provides a new dimension of complexity to the attendant protein-DNA interactions. The RNA is a constituent of the viral precursor shell of the DNA-packaging machine but is not a component of the mature virion. Studies of the sequential interactions involving this RNA molecule are likely to provide new insight into the structural and possible catalytic roles of small RNA molecules. The ϕ29 assembly in extracts and ϕ29 DNA packaging in the defined in vitro system were strongly inhibited by treatment with the ribonucleases A or T1. However, phage assembly occurred normally in the presence of ribonuclease A that had been treated with a ribonuclease inhibitor. An RNA of approximately 120 nucleotides co-purified with the ϕ29 precursor protein shell (prohead), and this particle was the target of ribonuclease action. Removal of RNA from the prohead by ribonuclease rendered it inactive for DNA packaging. By RNA-DNA hybridization analysis, the RNA was shown to originate from a viral DNA segment very near the left end of the genome, the end packaged first during in vitro assembly.

PROKARYOTIC RNA SPECIES HAVE BEEN CATEGORIZED INTO transfer RNA (tRNA), ribosomal RNA (rRNA), messenger RNA (mRNA), and small RNA groups, although some RNA species fit into more than one category (*1*). The heterogeneous small RNAs of approximately 200 nucleotides include regulators, enzymes, primers, fragments from processing reactions, and molecules of unknown function. For example, a 4.5*S* RNA is needed to develop or maintain ribosome function in the initiation of protein synthesis in *Escherichia coli* (*2*). We show here that a small RNA transcript of the *Bacillus subtilis* bacteriophage ϕ29 has a novel and essential function in viral DNA packaging. This RNA of approximately 120 nucleotides is not assembled into the mature virion but is a component of the precursor protein shell (prohead) into which the 18–kilobase pair (kbp) ϕ29 DNA–gene product 3 complex (DNA-gp3) is packaged during morphogenesis. This finding may have general significance because common mechanisms are used in packaging double-stranded DNAs of the tailed bacteriophages (*3*). In addition, the observation increases an awareness that molecules other than proteins may be involved in the assembly of DNA viruses. Indeed, this role for RNA as a transient structural component and possible catalyst in viral morphogenesis extends the

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evolvement of novel and multiple biochemical functions for RNA.

An advantage of ϕ29 for studies on the mechanism of DNA packaging is that genome encapsidation occurs efficiently in vitro in extracts of phage-infected cells (*4–8*) or in an adenosine triphosphate (ATP)–dependent, completely defined reaction aided by the purified gene product 16 (gp16) (*9*). Using these systems, we show below that the DNA-gp3 packaging phase of ϕ29 morphogenesis is sensitive to the ribonucleases (RNases) A and T1 but that phage assembly occurs in the presence of RNase that has been treated with an RNase inhibitor (from human placenta, Boehringer Mannheim). Specifically, RNase treatment inactivates proheads by removing an RNA component. The RNA is similar to 5*S* rRNA in electrophoretic mobility and is shown by RNA-DNA hybridization to originate from sequences very near the left end of the viral DNA, the end packaged first during in vitro assembly (*6*).

Our in vitro ϕ29 assembly systems have been described (*4–9*). Briefly, extracts were prepared from *B. subtilis* SpoA12 (the *sup*$^-$ host) infected with the suppressor-sensitive (*sus*) 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. Gene product 7 (gp7) of ϕ29 is the prohead scaffold, gene product 8 (gp8) is the major shell protein, and the mutation *sus*14(1241) provides increased yields of proteins by delaying lysis. Extracts derived from infections of the *sup*$^-$ (nonpermissive) bacterial host with the 16$^-$14$^-$ and 7$^-$8$^-$14$^-$ mutants are designated "prohead donor" and "gp16 donor" extracts, respectively. Exogenous [^{3}H]DNA-gp3 can be packaged in a mixture of these extracts on an equal basis with endogenous ϕ29 DNA and appear in filled heads that are separated from unpackaged DNA by sucrose gradient centrifugation. The gp3 of DNA-gp3 is covalently attached to the 5′ ends of the DNA and functions both as a primer for DNA replication (*10*) and as an essential component in packaging (*6*). The completely defined in vitro packaging system includes purified DNA-gp3, proheads, and gp16 in a reaction that requires ATP (*9*). To initiate packaging in the defined system, gp16 first binds to, and is modified by, the prohead. The prohead-gp16 complex then binds DNA-gp3, resulting in a second conformational change in gp16 that permits trapping and hydrolysis of ATP (*11*). Packaging of the 18-kbp genome consumes 9×10^3 ATP molecules, or one ATP per 2 bp of DNA. The gp16 protein is a prohead-dependent and DNA-gp3-dependent adenosinetriphosphatase (ATPase) that contains both A- and B-type ATP-binding consensus sequences and a potential magnesium-binding domain (*12*). The efficiency of ϕ29 DNA-gp3 packaging in vitro, both in extracts and in the defined system, approaches in vivo assembly (*4–9*).

RNases inhibited ϕ29 assembly in extracts and in the defined in vitro system. Assembly of ϕ29 in extracts was sensitive to treatment with the RNases T1 or A (Tables 1 and 2). RNase T1 (Bethesda Research Laboratories or Boehringer Mannheim Biochemicals) and RNase A (Sigma or Boehringer Mannheim) gave similar results. Treatment of extracts or purified proheads with microgram quantities of these RNases reduced phage assembly (measured as plaque-forming units per milliliter) by several orders of magnitude and to background levels. Prior treatment of the RNase A with RNase inhibitor blocked the effect of the enzyme (Table 2), indicating that the inhibition of assembly was due specifically to the action of RNase rather than another contaminating enzyme. The RNases were boiled for 10 minutes before use and had no degradative effect on ϕ29 DNA, as described below.

The extract supplying the prohead was determined to be the target of RNase. Portions of the prohead donor extracts or the gp16 donor extracts were treated with the RNases A or T1 and mixed with complementary untreated extracts. When the RNase-treated prohead donor extracts were complemented with the untreated gp16 donor extracts (Table 3, items c and f), assembly, which was measured in plaque-forming units per milliliter, was similar to that of the complementations in which both extracts were treated with RNase (Table 3, items b and e). Alternatively, when the RNase-treated gp16 donor extracts were complemented with the untreated prohead donor extracts (Table 3, items d and g), the number of plaque-forming units per milliliter was similar to that of the normal control (Table 3, item a). These results indicated that the target of RNase action was the prohead in the prohead donor extract. The low concentrations of RNase used in these extracts, which contain many RNA species, were critical. After RNase incubation with the gp16 donor extracts (Table 3, items d and g), the remaining effective RNase was insufficient to inactivate the proheads in the complementing untreated prohead donor extract before packaging of DNA-gp3. RNA may only be needed for an initiation phase of packaging, and the rate of bacteriophage DNA packaging in vitro can be very rapid (*11, 13*).

RNase also inhibited the DNA-gp3 packaging in the defined in vitro assembly system. When purified proheads, [^{3}H]DNA-gp3, and the DNA packaging protein gp16 are mixed and incubated in the presence of ATP, 30 to 50 percent of the labeled DNA is

Table 1. Inhibition of in vitro ϕ29 assembly by RNase T1. RNase T1 [Boehringer Mannheim, 0.3 mg (10^5 unit)/ml] was diluted in TMS. Phage were assembled by mixing a prohead donor extract with a gp16 donor extract or by replacing the prohead donor extract with purified proheads. Prior to complementation, individual extracts or purified proheads (10 μl) were treated with RNase (3 μl) to give the indicated concentrations; after 20 minutes at room temperature, complementation mixtures were made and incubated for 90 minutes at room temperature. Assembly was measured as the number of plaque-forming units (pfu) per milliliter. Experiments represent separate complementations, each with quadruple platings, with the same components. The background was determined by complementing DNase I–treated extracts (10 μg/ml for 20 minutes) or by replacing purified proheads with TMS for complementation. Growth of bacteria, phage infections, and extract preparation have been described (*4, 9*). Briefly, to prepare extracts, infected cells (2×10^8 per milliliter) were centrifuged, and protoplasts were produced by resuspending the cells at 5×10^9 per milliliter in double strength (2×) Difco antibiotic medium No. 3 containing 0.5*M* sucrose and 1 percent bovine serum albumin (BSA). After 5 to 10 minutes at 37°C, the protoplasts were diluted tenfold in this medium without lysozyme, collected by centrifugation, and lysed at 10^{10} cell equivalents per milliliter in reaction buffer containing TMS, 10 m*M* ATP, 6 m*M* spermidine, and 3 m*M* 2-mercaptoethanol. To prepare proheads, protoplasts produced from *sus*16(300)-*sus*14(1241)–infected *B. subtilis* SpoA12 were collected and resuspended at 2×10^{10} cell equivalents per milliliter in the above protective medium containing 0.02*M* $MgCl_2$ and DNase I (10 μg/ml), and lysed by dialysis against TMS for 90 minutes at 4°C. The lysate was clarified by three centrifugations, each at 10,000*g* for 10 minutes at 4°C, and proheads were isolated by successive centrifugations in 10 to 30 percent and 5 to 20 percent linear sucrose gradients.

RNase (μg/ml)	Assembly* (10^9 pfu/ml)	*N*†
	Extracts of prohead donor and gp16 donor	
0	864 ± 87	2
0.012	380 ± 25	4
0.12	42 ± 5	4
1.2	2.7 ± 0.4	4
12	0.3 ± 0.1	4
Background	0.5 ± 0.2	2
	Purified proheads and extract of gp16 donor	
0	192 ± 18	2
0.012	88.2 ± 7.3	4
0.12	7.3 ± 0.5	4
1.2	0.50 ± 0.05	4
12	0.085 ± 0.011	4
Background	0.066 ± 0.010	2

*Mean ± SD †Number of experiments.

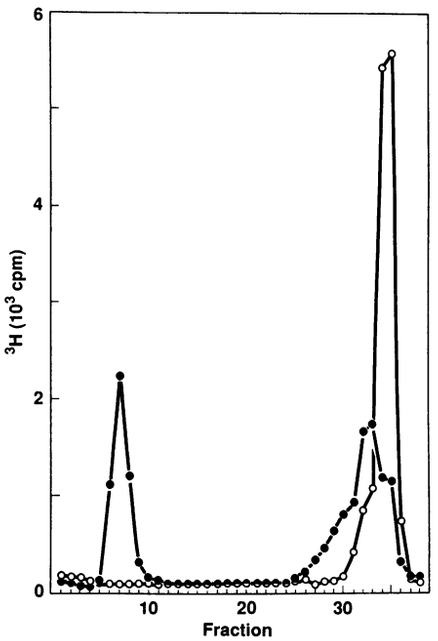

Fig. 1. RNase A destroys prohead competence for DNA-gp3 packaging. Purified proheads were treated with RNase A (10 μg/ml) in TMS or with TMS alone for 30 minutes at room temperature and then centrifuged in sucrose gradients to remove the RNase A. Treated or untreated proheads (3 μl), [^{3}H]DNA-gp3 (10 μl), gp16 (7 μl), and reaction buffer (3 μl) were mixed and incubated for 30 minutes at room temperature. The DNA packaging efficiency was assayed as the fraction of label appearing in filled heads in a linear 5 to 20 percent sucrose gradient after the reaction mixture was centrifuged at 35,000 rev/min (SW50.1 rotor) for 30 minutes at room temperature (*4*, *9*). Sedimentation was from right to left. The closed circles indicate assembly with untreated proheads, with filled heads centering on fraction 7. No filled heads were produced with RNase-treated proheads (open circles), and the unpackaged DNA-gp3, centering on fractions 34 to 35, did not shift to faster sedimenting forms (see text).

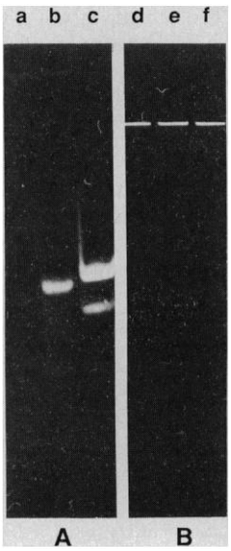

Fig. 2. Effect of RNase on prohead RNA (A) and ϕ29 DNA (B). (**A**) Proheads were incubated with (lane a) and without (lane b) RNase A (10 μg/ml) for 10 minutes before electrophoresis in a 10 percent polyacrylamide gel. The prohead RNA has approximately the same mobility as the 120-nucleotide 5*S* rRNA standard from Boehringer (lane c). (**B**) ϕ29 DNA-gp3 was incubated with RNase A at 10 μg/ml (lane d) or RNase T1 at 10 μg/ml (lane e), or TMS alone (lane f) for 10 minutes at room temperature and then treated with proteinase K (500 μg/ml) prior to electrophoresis in a 1 percent agarose gel.

packaged to give filled heads that are isolated by sucrose gradient centrifugation (*9*). To determine the effect of RNase on this defined in vitro reaction, we mixed 3 μl of proheads (approximately 3×10^{13} particles per milliliter) with 2 μl of either RNase A or RNase T1 to give final enzyme concentrations of 0.4 μg/ml and 3 μg/ml, respectively, or with TMS (0.05*M* tris-HCl, *p*H 7.8, 0.01*M* $MgCl_2$, and 0.1*M* NaCl) as a control. After 10 minutes at room temperature, 10 μl of purified [^{3}H]DNA-gp3 (5×10^{12} molecules per milliliter), 7 μl of gp16 (125 μg/ml), and 3 μl of reaction buffer were added, and the mixtures were incubated for 30 minutes at room temperature. Filled heads containing labeled DNA were not detected when the assembly mixtures containing RNase-treated proheads were assayed on sucrose gradients as described below, whereas 45 percent of the [^{3}H]DNA-gp3 added to the untreated proheads appeared at the filled head position of the gradient. In this experiment, the ratio of gp16 molecules to RNase A molecules in the mixture was 30 to 1.

To confirm that RNase acted on the prohead rather than on some other component of the defined in vitro packaging system, purified proheads were treated with RNase A (10 μg/ml) or mock-treated with TMS for 30 minutes at room temperature and reisolated by sucrose gradient centrifugation. The mock-treated, reisolated proheads packaged more than one-third of the [^{3}H]DNA-gp3 added in assembly to yield filled heads that sedimented to fraction 7 of the sucrose gradient, while the RNase-treated, reisolated proheads were inactive in the [^{3}H]DNA-gp3 packaging reaction (Fig. 1). The data of Fig. 1 also illustrate the shift of the bulk of the unpackaged [^{3}H]DNA-gp3 to faster sedimenting forms in fractions 26 to 33 in the active packaging reaction (closed circles). When RNase-treated proheads were used, this shift did not occur, and the unpackaged [^{3}H]DNA-gp3 was centered at fractions 34 and 35. The faster sedimenting DNA-gp3 represents aggregates, usually dimers and trimers, that are produced in the packaging process in vitro, and formation of these aggregates is correlated with packaging efficiency; moreover, agents that block packaging, such as the nonhydrolyzable ATP analogue [γ-S]ATP, ethidium bromide, and the antibiotics novobiocin, coumermycin A_1, oxolinic acid, and nalidixic acid, block the formation of the aggregates (*11*).

A small viral RNA copurified with the prohead. The ϕ29 proheads, purified from extracts treated with DNase I (10 μg/ml)

Table 2. Inhibition of in vitro ϕ29 assembly by RNase A. RNase A (Sigma) was dissolved in TMS buffer (RNase at 1 mg/ml; 72 Kunitz unit/mg, where 1 unit produces acid-soluble oligonucleotides equivalent to a change in the A_{260} of 1.0 in 30 minutes at *p*H 7.5 in a 1.5-ml reaction volume with yeast RNA as substrate), boiled for 10 minutes, and diluted in TMS. The complementation procedure was given in the legend to Table 1. For studies on RNase inhibitor, RNase A solution was mixed with an equal volume of RNase inhibitor (Boehringer Mannheim, from human placenta; 3×10^4 unit/ml) or TMS (as a control) for 10 minutes at room temperature and dialyzed against TMS; these mixtures (4 μl) were incubated with 3 μl of proheads for 10 minutes before complementation as indicated in Table 1.

RNase (μg/ml)	RNase inhibitor	Assembly* (10^9 pfu/ml)	*N*†
	Extracts of prohead donor and gp16 donor		
0	–	840 ± 87	2
0.03	–	187 ± 30	4
0.3	–	12 ± 3	4
3	–	0.8 ± 0.1	4
Background	–	0.6 ± 0.1	4
	Extract of prohead donor and purified gp16		
0	–	230 ± 43	4
0.03	–	45 ± 6	4
0.3	–	5.6 ± 0.5	4
3	–	0.38 ± 0.05	4
Background	–	0.48 ± 0.09	4
	Purified proheads and extract of gp16 donor		
0	–	180 ± 10	8
0.04	–	65 ± 19	8
0.4	–	4.1 ± 0.4	8
4	–	0.045 ± 0.019	8
Background	–	0.051 ± 0.006	2
	Purified proheads and extract of gp16 donor‡		
0	–	165 ± 12	4
0.4	+	143 ± 11	6
0.4	–	4.2 ± 1.5	6
4	+	112 ± 8	6
4	–	0.04 ± 0.01	6
Background	–	0.06 ± 0.02	4

*Mean ± SD. †Number of experiments. ‡This experiment was like the one above except for the addition of RNase inhibitor where indicated.

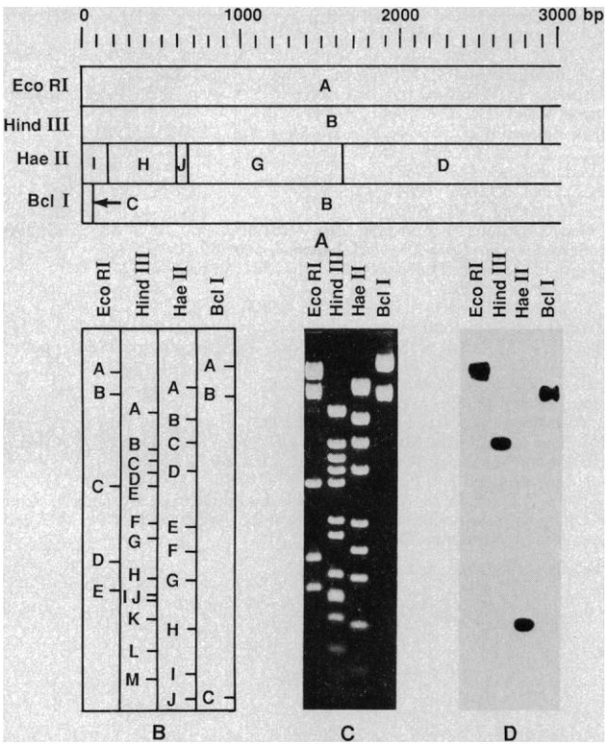

Fig. 3. Hybridization of prohead RNA to restriction fragments of ϕ29 DNA. (**A**) Restriction maps of the left end of ϕ29 DNA for Eco RI, Hind III, Hae II, and Bcl I (*17, 18*); (**B**) restriction fragment designations; (**C**) separation of restriction fragments of ϕ29 DNA in a 1 percent agarose gel; (**D**) autoradiograph of a Southern blot of the fragments of (C) after hybridization with [^{32}P]RNA purified from proheads.

(see legend to Table 1 for prohead purification), were examined directly for RNA content by electrophoresis in a 1 percent agarose gel. Without prior treatment, the proheads released nucleic acid approximately the size of the 5*S* rRNA standard. Release of the nucleic acid from ϕ29 proheads during electrophoresis in tris-borate-EDTA buffer, *p*H 8.3, resembles RNA escape from plant viruses under alkaline conditions or in the absence of Ca^{2+}, Mg^{2+}, or KCl (*14*).

The size of the prohead nuleic acid was confirmed by electrophoresis in a 10 percent polyacrylamide gel in the presence of the RNase inhibitor diethyl pyrocarbonate (Fig. 2A). Again, the bulk of the

Table 3. Effect of RNase on the extract containing the ϕ29 prohead. Individual prohead donor or gp16 donor extracts were treated with RNases A or T1 and complemented with untreated extracts to determine the sensitive extract. The methods used are described in the legends to Tables 1 and 2, and the in text.

Mixtures of prohead donor and gp16 donor extracts	RNase treatment of extract or extracts	Assembly* (10^9 pfu/ml)	*N*†
a	None	860 ± 170	4
	Ribonuclease A (0.3 μg/ml)		
b	Both	9.7 ± 0.1	5
c	Prohead donor	14.2 ± 3.1	5
d	gp16 donor	740 ± 130	5
	Ribonuclease T1 (1.2 μg/ml)		
e	Both	1.3 ± 0.1	3
f	Prohead donor	3.2 ± 0.6	3
g	gp16 donor	665 ± 70	3

*Mean ± SD. †Number of experiments.

Table 4. Reconstruction of RNA-free proheads with purified prohead RNA. The gp16 donor extract was treated with RNase A (0.4 μg/ml, 10 minutes, room temperature) and then with RNase inhibitor (from human placenta; 400 units/ml, 10 minutes, room temperature). The complementation procedures are described in the legends of Tables 1 and 2.

Complementation in RNA-free gp16 donor extract	RNA-free proheads	Purified prohead RNA	Phage assembly* (10^7 pfu/ml)	*N*†
a	−	−	0.7 ± 0.1	8
b	+	−	0.8 ± 0.1	4
c	−	+	0.7 ± 0.1	2
d	+	+	140 ± 20	4

*Mean ± SD. †Number of experiments.

prohead nucleic acid had a mobility similar to the 120-nucleotide 5*S* rRNA (compare lanes b and c). The faster band in the 5*S* rRNA standard was due to secondary structure, since glyoxal-treated 5*S* rRNA ran at the position of the prohead RNA as a single band. Proheads treated with RNase A at 10 μg/ml for 30 minutes at room temperature showed no bands (lane a), confirming that the prohead nucleic acid was RNA. The boiled RNase A used in this experiment had no effect on ϕ29 DNA, nor did RNase T1 (Fig. 2B).

The RNA in purified proheads was quantified by analyzing prohead concentration by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and prohead RNA concentration by PAGE. The head-to-neck connector of the ϕ29 particle is a dodecamer of gene product 10 (gp10) (*15*), and bands of this protein stained with Coomassie blue were compared by densitometry to stained bands of a bovine serum albumin standard. Similarly, bands of prohead RNA stained with ethidium bromide were compared to stained bands of the 5*S* rRNA standard. If the bulk of the proheads contained RNA, each particle had approximately two copies.

The prohead RNA was a transcript from the left end of ϕ29 DNA. Purified proheads were treated with SDS (1 percent) and proteinase K (500 μg/ml) for 30 minutes at 55°C, and the RNA was extracted with phenol and chloroform. With [α-^{32}P]ATP as substrate, a poly(A) tail was added to the RNA in the presence of ATP:RNA adenyltransferase [poly(A) polymerase] from *Escherichia coli* (New England Nuclear) (*16*) and used as a hybridization probe with ϕ29 restriction enzyme fragments that were separated by agarose gel electrophoresis, denatured, and transferred to Zeta-Probe (Bio-Rad Laboratories) by electroblotting. The [^{32}P]RNA hybridized specifically to denatured restriction fragments from the left end of ϕ29 DNA (*17, 18*), including Eco RI-A (~9000 bp), Hind III-B (~2900 bp), Hae II-H (416 bp), and Bcl I-B (~5700 bp), but not to the HAE II-I (176 bp), Hae II-J (61 bp), or Bcl I-C (73 bp) fragments (Fig. 3). Thus the prohead RNA is a viral transcript from a ϕ29 DNA segment near, but not extending to, the left end of the genome. The transcript may originate from the *B. subtilis* RNA polymerase binding site A1 (*19*) and promoter P_E1 with its "Pribnow-box" at positions 328 to 333 (*18*) from the left end of the genome. The A1 site may be a weak initiation site in vivo because early transcription initiating at this site was not confirmed by S1 mapping experiments with 5′ end-labeled RNA (*20*). Since the prohead RNA was about 120 nucleotides long and was homologous to sequences of the Hae II-H fragment, it might include open reading frame 2 (*18*). RNA of 120 nucleotides would have a molecular size of about 40,000 daltons. This corresponds to the size of an early RNA produced in ϕ29-infected cells in the presence of chloramphenicol (*21*).

The purified prohead RNA can restore inactive RNA-free proheads for phage assembly in extracts. RNA can be isolated from the purified proheads in the presence of 2 m*M* EDTA. The RNA-free proheads were inactive in a prohead donor extract treated

with RNase A and then with RNase inhibitor (Table 4, item b). Purified RNA alone did not increase phage production (Table 4, item c). However, the inactive RNA-free proheads and the purified prohead RNA can be reconstructed, resulting in phage production (Table 4, item d), albeit at a relatively low efficiency compared to complementations of normal extracts and proheads (Tables 1 and 2). It is not clear whether this lower efficiency of assembly is due to alteration of the extracts with RNase and RNase inhibitor treatment or the quality of the purified RNA or RNA-free proheads.

In conclusion, we have demonstrated that a small RNA transcript of about 120 nucleotides from the far left end of the ϕ29 genome was a component of the ϕ29 prohead and that it had an essential role in ϕ29 DNA packaging in vitro. Some data have been obtained for systematic investigations of the possible role or roles of the small RNA in the initiation events of DNA-gp3 packaging (*11*) and the formation of the prohead-dependent and DNA-gp3–dependent ATPase (*12*) (as mentioned earlier). Treated proheads do not produce the aggregated forms of DNA-gp3 (Fig. 1), which are correlated with packaging efficiency (*11*), suggesting that an initiation step in DNA-gp3 packaging is blocked.

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Bacterial Resistance to β-Lactam Antibiotics: Crystal Structure of β-Lactamase from *Staphylococcus aureus* PC1 at 2.5 Å Resolution

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β-lactamases are enzymes that protect bacteria from the lethal effects of β-lactam antibiotics, and are therefore of considerable clinical importance. The crystal structure of β-lactamase from the Gram-positive bacterium *Staphylococcus aureus* PC1 has been determined at 2.5 angstrom resolution. It reveals a molecule of novel topology, made up of two closely associated domains. The active site is located at the interface between the domains, with the key catalytic residue Ser^{70} at the amino terminus of a buried helix. Examination of the disposition of the functionally important residues within the active site depression leads to a model for the binding of a substrate and a functional analogy to the serine proteases. The unusual topology of the secondary structure units is relevant to questions concerning the evolutionary relation to the β-lactam target enzymes of the bacterial cell wall.

MANY TYPES OF DEFENSE MECHANISMS EXIST IN THE biological world, and relatively lowly life forms often exhibit a surprising degree of sophistication in these matters. One such system that has been used to advantage by man is the defense that some fungi mount, through the production of β-lactam antibiotics, against bacterial attack. These compounds kill bacterial cells by irreversibly inhibiting a number of enzymes concerned with cell wall synthesis and repair (*1*). A degree of complexity is added by the ability of some bacteria to defeat this fungal defense mechanism, primarily through the production of membrane-bound and excreted enzymes which hydrolyze β-lactam rings. These enzymes are known as β-lactamases or penicillinases (E.C. 3.5.2.6) (*2*). A variety of β-lactams are produced by fungi, and in turn, there are at least three different classes of β-lactamases produced by bacteria (*3*). A further layer of complexity is provided by the production of inhibitors of β-lactamases by some fungi (*4*).

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