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In Vitro Assembly of Infectious Virions of Double-Stranded DNA Phage ϕ29 from Cloned Gene Products and Synthetic Nucleic Acids

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Up to 6×10^7 PFU of infectious virions of the double-stranded DNA bacteriophage ϕ29 per ml were assembled in vitro, with 11 proteins derived from cloned genes and nucleic acids synthesized separately. The genomic DNA-gp3 protein conjugate was efficiently packaged into a purified recombinant procapsid with the aid of a small viral RNA (pRNA) transcript, a DNA-packaging ATPase (gp16), and ATP. The DNA-filled capsids were subsequently converted into infectious virions after the addition of four more recombinant proteins for neck and tail assembly. Electron microscopy and genome restriction mapping confirmed the identity of the infectious ϕ29 virions synthesized in this system. A nonstructural protein, gp13, was indispensable for the assembly of infectious virions. The overproduced tail protein gp9 was present in solution in mostly dimeric form and was purified to homogeneity. The purified gp9 was biologically active for in vitro ϕ29 assembly. Higher-order concentration dependence of in vitro ϕ29 assembly on gp9 suggests that a complete tail did not form before attaching to the DNA-filled capsid, a result contrary to earlier findings for phages T4 and λ. The work described here constitutes an extremely sensitive assay system for the analysis of components in ϕ29 assembly and dissection of functional domains of structural components, enzymes, and pRNA (C.-S. Lee and P. Guo, Virology 202:1039–1042, 1995). Efficient packaging of foreign DNA in vitro and synthesis of viral particles from recombinant proteins facilitate the development of ϕ29 as an in vivo gene delivery system. The finding that purified tail protein was able to incorporate into infectious virions might allow the construction of chimeric ϕ29 carrying a tail fused to ligands for specific receptor of human cells.

Infectious single-stranded DNA or RNA viruses have been assembled in vitro by using components entirely or partially purified from virus particles or cells infected with viruses (3, 5, 15, 16, 40) or synthesized de novo with cell extracts (37). Infectious double-stranded DNA (dsDNA) viruses have previously been assembled in vitro with extracts from natural hosts infected with the virus (6, 8, 12, 28–30, 32, 36, 41). Our work shows that an infectious dsDNA virus can also be assembled in vitro with the use of structural proteins and enzymes produced from cloned genes without the involvement of the infectious parental virus.

One striking feature in the assembly of many linear dsDNA viruses, including adenovirus, herpesvirus, poxvirus (perhaps), and bacteriophages T1, T3, T4, T5, T7, P1, P2, P22, Mu, ϕ21, ϕ29, SPO1, SPP1, and λ, is that the viral genome is packaged into a preformed procapsid during maturation (2, 4, 9, 13, 18, 25). Procapsids of dsDNA phages contain a portal vertex which serves as the DNA translocation machinery (2, 4, 14). The procapsid of the *Bacillus subtilis* bacteriophage ϕ29 consists of the major capsid protein gp8 (about 200 copies), head fiber protein gp8.5, scaffolding protein gp7 (180 copies), and portal vertex protein gp10 (12 or 13 copies) (2). The scaffolding protein is necessary for procapsid assembly in vitro (27, 39) but is not necessary for in vitro packaging of the ϕ29 genome (2). Nonessential head fibers (gp8.5, if present, radiate from the apical regions of the head and stabilize the viral structure (2).

The genome of ϕ29 is a linear dsDNA of 19,285 bp. The virus-encoded terminal protein gp3 is covalently linked to each 5′ end of the genome through the formation of a phosphodiester bond between the -OH group of serine residue 232 and 5′ dAMP (43). The terminal protein gp3 functions both as a primer in the initiation of DNA replication (38, 43) and as an enhancer for DNA packaging (7, 17). Full-length ϕ29 genomic DNA has been synthesized in vitro by Takara-LA PCR (44) or by using recombinant gp6, ϕ29 DNA polymerase gp2, deoxynucleoside triphosphate substrates, and terminal protein gp3 as primers (10, 11, 38). The ϕ29 genome is packaged into procapsid with the aid of the DNA-packaging protein gp16 (22). This protein is a DNA-dependent ATPase and binds specifically to the portal vertex of the procapsid (24). The translocation of DNA into the procapsid is energetically unfavorable because of the very compact DNA arrangement within the capsid (9, 28, 29). This unfavorable reaction is driven by ATP hydrolysis (7, 22, 24, 26). Quantification of ATP consumption in DNA packaging has been performed in the ϕ29 system with purified components. One ATP is needed for the translocation of two base pairs of DNA into the procapsid (24).

An exciting aspect in the study of ϕ29 DNA packaging is the discovery that a small viral RNA (pRNA [p for packaging]) encoded by the ϕ29 genome has a novel and essential role in viral DNA packaging (20). This pRNA is not a structural component of the mature virion (20), nor is it required for the assembly of the procapsid (25). The pRNA binds to the portal vertex (connector) of the procapsid and can be detached from purified procapsids in the presence of EDTA and reattached to the pRNA-free procapsids in the presence of Mg^{2+} (19). RNA

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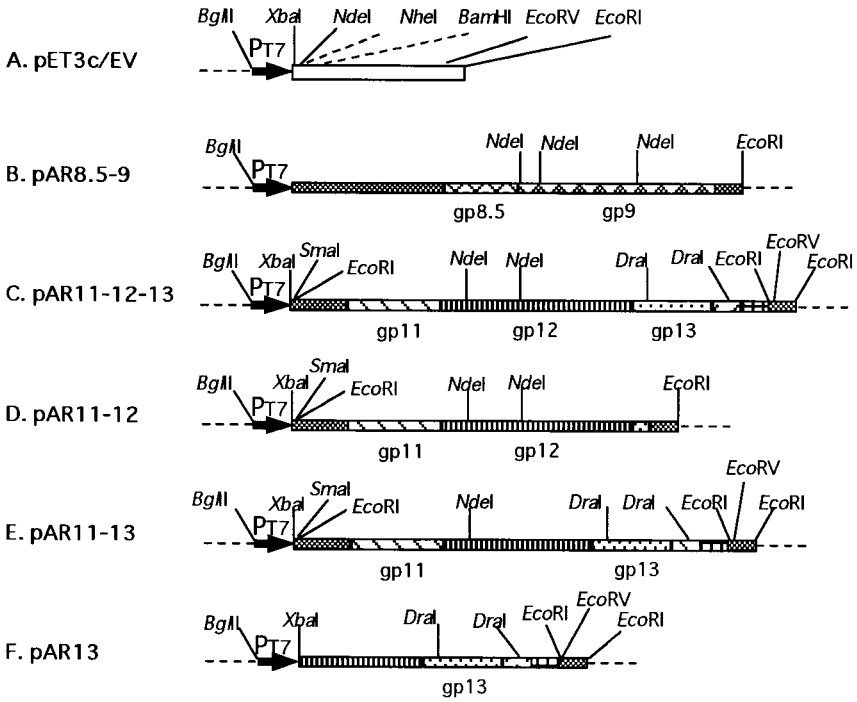

FIG. 1. Outline of plasmid construction showing the polylinker sites, inserts, and certain relevant restriction sites. P_{T7}, T7 promoter.

sequence analysis indicated that the pRNA was transcribed from the left end of the viral DNA, which is the end that enters the procapsid first during DNA packaging (20).

This report describes the in vitro assembly of infectious virions of phage ϕ29 in detail. The purification of tail protein gp9 is also presented. An assay for concentration dependence of phage assembly on this protein revealed that the ϕ29 tail was not preassembled before its attachment to the DNA-filled capsids. Production of infectious virions in vitro with individual components synthesized separately is of paramount importance to studies of the mechanism of viral morphogenesis. The work described here constitutes an extremely sensitive system for the assay of ϕ29 assembly (34). With this system, it has been possible to detect the activity of one mutant pRNA with 10^5-fold reduction in DNA packaging efficiency (34). This activity was too low to be detected by all other assay methods described previously (17, 22). This system has also been used to study the functional domains of the pRNA (49–51) and gp7 (35), as well as to determine the concentration dependence of ϕ29 assembly on its genomic DNA, the DNA-packaging enzyme gp16 (34), and pRNA (48).

MATERIALS AND METHODS

Construction of plasmids. A 4,202-bp *Nhe*I-*Eco*RI fragment containing the genes coding for the fiber protein gp8.5 and tail protein gp9 was isolated from plasmid pBlue6K (21), which contains all of the structural genes of the ϕ29 procapsid. This fragment was ligated into expression vector pET3c/EV, which was derived from pET3c (42) by the deletion of an *Eco*RV fragment (from bases 172 to 368) at the *Xba*I-*Eco*RI sites to produce plasmid pAR8.5-9 (Fig. 1).

An *Eco*RI fragment (bases 9862 to 15806 of the ϕ29 genome) containing the gene coding for upper collar protein gp11, appendage protein gp12, and nonstructural protein gp13 of phage ϕ29 was isolated from the ϕ29 genome and inserted into the *Eco*RI site of pBluescript SK(+) (Stratagene) to generate plasmid pBlue11-12-13. A 6.0-kb *Xba*I-*Eco*RV fragment was isolated from this plasmid and inserted into the *Xba*I-*Eco*RV sites of plasmid pET3c/EV, resulting in plasmid pAR11-12-13 (Fig. 1). In this plasmid, the active gp14 gene could not be expressed because of the presence of an amber mutation.

To construct plasmid pAR13, the *Sma*I-*Nde*I fragment from plasmid pAR11-12-13 was deleted by digestion with both *Sma*I and *Nde*I, treatment with mung bean nuclease, and ligation of the resulting blunt ends (Fig. 1). To construct plasmid pAR11-13, the *Nde*I fragment in plasmid pAR11-12-13 was deleted by *Nde*I digestion and self-ligation (Fig. 1). To construct plasmid pAR11-12, the *Xba*I-*Dra*I fragment of plasmid pAR11-12-13 was isolated and inserted into the *Xba*I-*Eco*RV sites of plasmid pET3c/EV (Fig. 1).

The resulting plasmids were transformed into the expression host *Escherichia coli* HMS174(DE3)/pLysS, which contained the compatible lysozyme-producing plasmid pLysS (42, 46).

Purification of components necessary for ϕ29 DNA packaging. The synthesis and purification of pRNA (50), procapsid (21, 34), DNA-gp3 (20, 34), and gp16 (22, 23) have been reported previously.

Purification of tail protein gp9. An overnight culture (10 ml) of *E. coli* HMS174(DE3) containing plasmid pAR8.5-9 was inoculated into 1 liter of LB broth with 50 μg ampicillin per ml and incubated at 37°C for 3 h. After addition of isopropylthiogalactopyranoside (IPTG) to a final concentration of 0.5 mM, the *E. coli* culture was incubated for an additional 3 h at 37°C, and cells were collected by centrifugation. The cell pellet was resuspended in 25 ml of buffer A (50 mM Tris-Cl [pH 8.0], 50 mM NaCl, 1 mM EDTA, 0.01% Nonidet P-40, 300 μg of phenylmethylsulfonyl fluoride per ml, 2 mM dithiothreitol) and lysed by passage through a French press cell. The cell debris was removed by centrifugation at 10,000 rpm in a JA20 rotor (Beckman) for 30 min, and the supernatant was passed through a DEAE-Sephadex column (25 by 20 mm) equilibrated with buffer A. The flowthrough fractions were applied to a phosphocellulose P11 column (25 by 20 mm) and eluted with a 100-ml 0.05 to 1 M NaCl gradient in buffer A. The fractions containing tail protein gp9 were identified with a UV detector (280 nm) and by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and the protein concentration was determined by a Bio-Rad assay with bovine serum albumin as a standard.

In vitro assembly of infectious ϕ29. A quantity of 5 μl of purified procapsids was mixed with 1 μl of pRNA (1.6 μg/μl) and dialyzed on a 0.025-μm-pore-size membrane (type VS filter; Millipore Corp.) against TBE (89 mM Tris-borate [pH 8.3], 2.5 mM EDTA) for 15 min at ambient temperature and then against TMS (35) for 30 min at ambient temperature. The presence of Mg^{2+} in TMS buffer promotes the binding of pRNA to the procapsid. These pRNA-enriched procapsids were mixed with 3 μl of reaction buffer (10 mM ATP, 6 mM spermidine, 3 mM β-mercaptoethanol, 50 mM Tris-Cl [pH 7.8], 10 mM $MgCl_2$, 100 mM NaCl), 10 μl of DNA-gp3 that had been dialyzed against TMS for 40 min at ambient temperature, and 3 μl of DNA-gp3-packaging protein gp16 that had been dialyzed against 0.01 M Tris-Cl (pH 7.5)–0.04 M KCl for 40 min on ice. These mixtures were then incubated for 30 min at ambient temperature.

Extracts were prepared as described previously (34) from *E. coli* harboring a single plasmid containing one or multiple genes. The DNA-filled capsids were incubated with 18 μl of purified tail protein gp9 (see below) and 20 μl of extracts

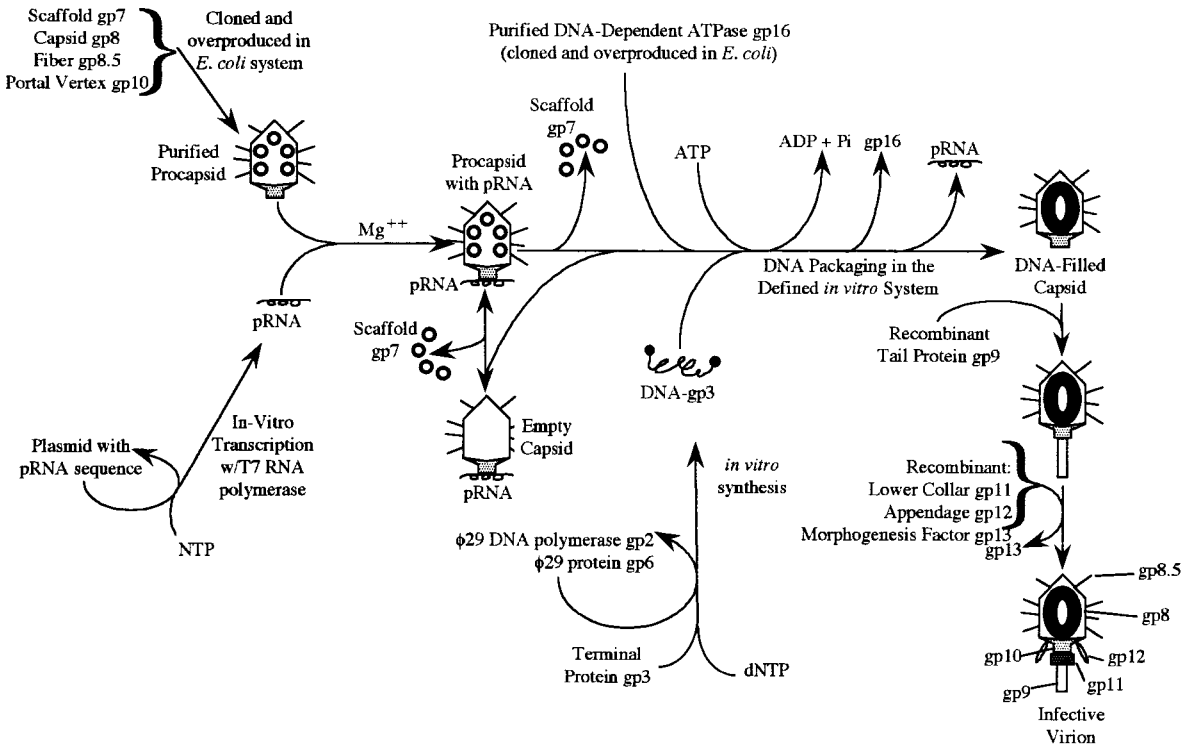

FIG. 2. Steps in the synthesis of infectious virion of ϕ29 in vitro. NTP, nucleoside triphosphate.

containing plasmids pAR11-12-13, pAR11-12, pAR11-13, and/or pAR13 for 2 h at ambient temperature. The mixtures were plated on *B. subtilis* $su^{+44}(sup^{+})$.

Cryoelectron microscopy. Electron micrographs were prepared by Tim Baker and Norman Olson as reported previously (21). Briefly, the samples were applied to carbon-covered electron microscope grids. After the grids were blotted with filter paper, they were quickly plunged into liquid ethane and transferred into liquid nitrogen and then into specimen holder that was maintained at about −165°C. The samples were observed with a Philips EM420 transmission electron microscope.

RESULTS

Cloning and expression of the genes coding for the tail (gp9), lower collar (gp11), appendage (gp12), and nonstructural (gp13) proteins. The tail protein (gp9) of ϕ29 was expressed from plasmid pAR8.5-9 (Fig. 1), and the other proteins were expressed from plasmid pAR11-12-13, pAR11-12, pAR11-13, or pAR13 (Fig. 1) in *E. coli* HMS174(DE3)/pLysS. The bacteria also carried a plasmid pLysS containing the lysozyme gene. The expression of lysozyme made the cells fragile and facilitated the preparation of *E. coli* extracts. The proteins gp9 and gp12 could be detected easily in SDS-PAGE, but the proteins gp11 and gp13 were difficult to detect because of the low level of expression and overlapping with *E. coli* proteins in the gel (data not shown). In these plasmids, the transcription termination signal for T7 RNA polymerase was removed, but the ϕ29 proteins were still expressed well.

In vitro assembly of infectious ϕ29 virions. The entire in vitro ϕ29 assembly system (Fig. 2) consists of (i) recombinant procapsids purified from *E. coli*, (ii) DNA-gp3 synthesized in vitro or purified from mutant phage infected bacteria, (iii) purified gp16, the DNA-packaging ATPase, which was expressed from a cloned gene, (iv) pRNA transcribed in vitro with T7 RNA polymerase, (v) purified tail protein gp9 or *E. coli* extract containing tail protein, (vi) *E. coli* extracts containing the coexpressed lower collar protein gp11, appendage protein gp12, and nonstructural protein gp13, and (vii) reaction buffer containing ATP.

The ϕ29 DNA-gp3 was packaged in vitro into the procapsid with the aid of ϕ29 pRNA, gp16, and ATP. The DNA-filled capsids were incubated with purified gp9 and/or *E. coli* lysates containing the tail protein gp9, lower collar protein gp11, appendage protein gp12, and nonstructural protein gp13. After incubation, the mixtures were plated on *B. subtilis* $su^{+44}(sup^{+})$ to determine the titer of infectious virions assembled. Approximately 5.8×10^7 PFU/ml was produced when all components, i.e., DNA-gp3, procapsid, pRNA, gp16, gp9, gp11, gp12, and gp13, were present (Table 1; Fig. 3B). No plaques were produced when any one of the components was absent (Table 1). These results indicate that the infectious virions were produced in vitro in the absence of both parental virus and infected host extracts.

To confirm that the plaques were produced by phage ϕ29, viruses from a lawn of *B. subtilis* infected with in vitro-synthesized virions were purified by CsCl density gradient and prepared for cryoelectron microscopy. Electron micrographs show

TABLE 1. In vitro assembly of infectious ϕ29 virions

Expt	DNA-gp3	Procapsids	pRNA	gp16	gp9	gp11	gp12	gp13	PFU/ml (10^7)
1	+	+	+	+	+	+	+	+	5.8
2	−	+	+	+	+	+	+	+	0
3	+	−	+	+	+	+	+	+	0
4	+	+	−	+	+	+	+	+	0
5	+	+	+	−	+	+	+	+	0
6	+	+	+	+	−	+	+	+	0
7	+	+	+	+	+	+	−	+	0
8	+	+	+	+	+	+	+	−	0

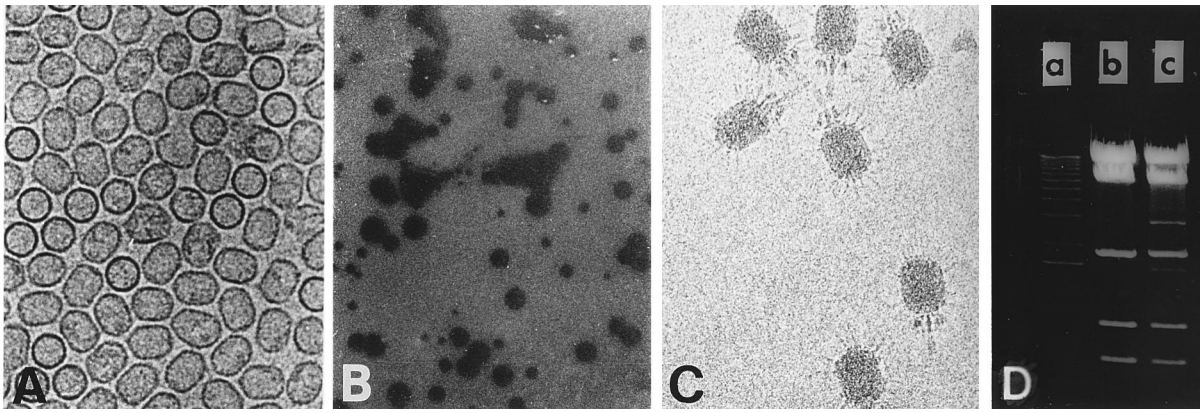

FIG. 3. Products in the assembly of infectious virions of ϕ29 in vitro. (A) Cryoelectron microscopy (magnification, ×90,000) of the procapsids purified from *E. coli*. (B) Plaques formed on a *B. subtilis* lawn plated with the ϕ29 particles assembled in vitro. (C) Cryoelectron microscopy (magnification, ×90,000) of the virions purified from plaques. (D) *Eco*RI restriction mapping of wild-type ϕ29 genomic DNA (lane b) and DNA isolated from the purified virions synthesized in vitro (lane c), assayed by agarose gel electrophoresis with a 1-kb ladder (lane a).

virions with morphology and size typical for phage ϕ29 (Fig. 3C). Note that the virions in Fig. 3C are full of fibers. These virions are the progeny of the synthetic virions rather than the actual synthetic virions themselves. The in vitro-synthesized virions were assembled with genomes and structural proteins originating from different sources. The procapsids were produced in an *E. coli* recombinant system, and as can be seen in Fig. 3A, only a small number of head fiber proteins are evident on these procapsids. The synthetic virions were constructed with these recombinant procapsids and thus contained fewer head fiber proteins than the subsequent progeny. Therefore, it can be said that the synthetic ϕ29 particles were chimeras with their protein coats derived from exogenous genes.

To further confirm the identity of the particle, DNA-gp3 was extracted from the purified virions by heat treatment of the virions at 75°C for 15 min. DNA-gp3 exited the capsid during the heat treatment. The free DNA-gp3 in solution was digested with the restriction enzyme *Eco*RI. Electrophoresis on a 1% agarose gel showed that the restriction map of the genome of virions assembled in vitro was comparable to that of the wild-type ϕ29 virions (Fig. 3D).

Requirement of the nonstructural protein gp13 for ϕ29 assembly. The function of gp13, which is not a structural component of the mature virion, is not completely understood. Hagen et al. (27) reported that mutant 13^- phage lacked DNA and frequently had either a complete neck and tail or a neck structure, and thus protein gp13 might interact with lower collar gp11 and tail gp9 proteins to stabilize the completely assembled phage ϕ29. To study the role of gp13, the gp13 gene was deleted from plasmid pAR11-12-13 to generate plasmid pAR11-12 (Fig. 1). With an *E. coli* extract containing this plasmid, no infectious phages were detected (Table 2, experiment 4). If an extract containing plasmid pAR13 or pAR11-13 was added together with the extract containing plasmid pAR11-12, the number of plaques produced was similar to the number produced when the extract containing pAR11-12-13 was used (Table 2, experiments 6 and 8), indicating that gp13 was indispensable for the assembly of infectious ϕ29 virions.

When an *E. coli* extract containing plasmid pAR11-13 instead of pAR11-12-13 was used for in vitro ϕ29 assembly, no plaques were produced, confirming that gp12 was required for the assembly of infectious ϕ29 (Table 2, experiment 5).

Purification of tail protein gp9. Protein gp9 was purified from an induced culture of *E. coli* HMS174(DE3) containing plasmid pAR8.5-9. The crude extract (Fig. 4, lane 2) prepared from these cells was passed through a DEAE-Sephadex anion-exchange column to remove nucleic acids. Protein gp9 did not bind to DEAE-Sephadex (Fig. 4, lane 3). The flowthrough solution, which contained protein gp9, was applied to a cation-exchange phosphocellulose column. Protein gp9 bound to this column and eluted at 0.2 M NaCl (Fig. 4, lane 5). Approximately 2.4 mg of protein gp9 was obtained from 1 liter of induced bacterial cultures, as determined by a Bio-Rad protein assay.

Properties of tail protein gp9. The ϕ29 tail is composed of multiple subunits of gp9 proteins. A question remaining to be answered was whether the entire tail is preassembled before attaching to the DNA-filled capsid, which is the case in the assembly of the tails of phages T4 and λ. The size of the purified tail protein gp9 or its oligomeric complex was determined by velocity sedimentation. Figure 5 shows that in the gradient, gp9 migrated faster than bovine serum albumin, which had a larger molecular mass (66 kDa) than gp9 (62 kDa), and migrated to the same location as alcohol dehydrogenase, which is a 141-kDa tetramer composed of four 35-kDa monomers. This finding suggested that the gp9 tail proteins did not exist as monomers and formed oligomers, mostly dimers, in solution. The concentration dependence of phage assembly on gp9 was determined to investigate the stoichiometry. The log/log plot (Fig. 6) of gp9 concentration versus phage assembly shows that the response curve was hyperbolic. With a decrease in the gp9 concentration, there was little decrease in the num-

TABLE 2. In vitro ϕ29 assembly with different combinations of neck and tail extracts

Expt	Neck and tail extracts[a]	PFU/ml (10^5)
1	gp9	0
2	gp11-12-13	0
3	gp9 + gp11-12-13	1.9
4	gp9 + gp11-12	0
5	gp9 + gp11-13	0
6	gp9 + gp11-12 + gp13	2.0
7	gp9 + gp13	0
8	gp9 + gp11-12 + gp11-13	3.9

[a] Extracts were incubated with DNA-filled capsids prepared by the defined in vitro DNA packaging system, using DNA-gp3.

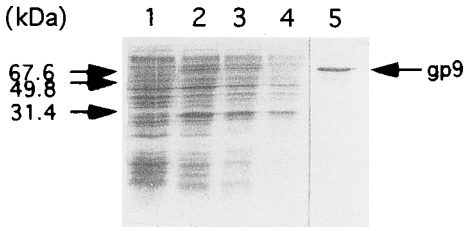

FIG. 4. SDS–15% polyacrylamide gel showing the purification of tail protein gp9. Lanes: 1, *E. coli* lysate containing vector plasmid pET3c/EV only; 2, *E. coli* lysate containing plasmid pAR8.5-9; 3, flowthrough solution of lysate in lane 2 from a DEAE-Sephadex column; 4, flowthrough solution of lysate in lane 3 from a phosphocellulose column; 5, purified gp9 eluted in the fraction with 0.2 M NaCl.

ber of phages assembled until the concentration of gp9 reached 246 ng/ml. However, the number decreased dramatically at gp9 concentrations lower than 246 ng/ml. A slope greater than 1 indicated that multiple copies of gp9 were needed for the assembly of one infectious virion. The higher order of gp9 concentration dependence indicated that the entire tail was not preassembled before attaching. Dimeric gp9 was added to the DNA-filled capsid one by one during tail maturation.

DISCUSSION

An in vitro dsDNA phage ϕ29 assembly system was developed by using recombinant proteins (Fig. 2). The nucleic acids were also synthesized in vitro. Since even in the presence of the genome, the omission of any one of the components resulted in the production of no infectious virions (Table 1), the infectious virus described here was, in this sense, truly synthetic rather than a result of native replication.

In the in vitro ϕ29 assembly system, the appendage protein gp12 was required for the assembly of an infectious virion (Table 2) even though it was known that this protein was not needed for neck and tail assembly (27). Protein gp12 was reported to be essential for phage adsorption to host cells through interaction with its receptor (47). Therefore, the phage could not adsorb and infect the bacteria without this protein. ϕ29 tail assembly was preceded by neck assembly with the lower collar protein gp11. The tail was not assembled on the phage capsid in cells infected with mutant 11^- phage (27). The function of the nonstructural protein gp13 is still obscure.

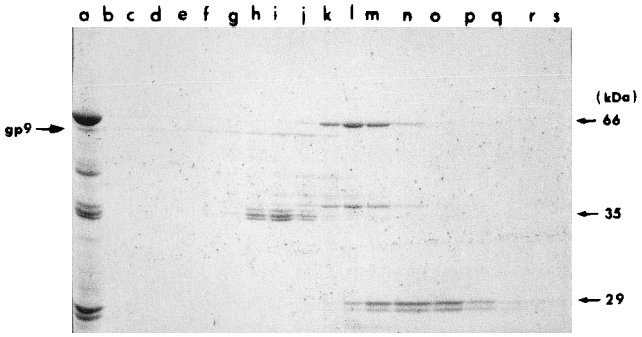

FIG. 5. SDS-PAGE (10% polyacrylamide gel) for size determination of purified gp9 by sucrose gradient velocity sedimentation. One hundred microliter of purified gp9 (62 kDa) was sedimented, in a 15 to 30% (wt/vol in TMS) linear sucrose gradient in a Beckman SW55 rotor for 24 h at 40,000 rpm at 20°C, with the molecular weight markers alcohol dehydrogenase (141-kDa tetramer composed of four 35-kDa monomers), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa). Sedimentation was from right to left.

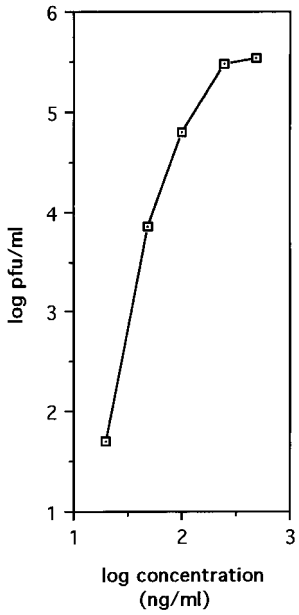

FIG. 6. Concentration dependence of in vitro ϕ29 assembly on the tail protein gp9. The purified gp9 (0.25 mg/ml) was serially diluted, and 10 μl per dilution was used for in vitro ϕ29 assembly. The dose-response curve is hyperbolic, indicating that the concentration dependence on gp9 is higher than first order.

Hagen et al. (27) reported that this protein stabilized the completely assembled virion; however, the protein was absolutely required for in vitro infectious ϕ29 assembly, as demonstrated in this report. Obviously, protein gp13 must have a more important role than just stabilizing the assembled virion.

In phages T4 and λ, the entire tail is preassembled independently before attaching to the DNA-filled capsid (13, 33). Moreover, in the assembly of the tails of phages T4 and λ, the tape measure (1) and ruler (31) proteins, respectively, determine the length of the tail. The tail of ϕ29 is composed of only one kind of protein, but the mechanism for determination of the length of the ϕ29 tail is unknown. The purified protein gp9 was biologically active in in vitro phage assembly and existed in solution as oligomers (mainly dimers; Fig. 5). The concentration dependence of ϕ29 assembly on the purified protein gp9 was higher than first order (Fig. 6), indicating that more than one gp9 oligomer was necessary for tail assembly. Therefore, the complete tail was not preassembled before attaching to the DNA-filled capsid. Otherwise, the concentration dependence would have been first order.

Production of infectious virions in vitro with proteins from cloned genes is an important step in the study of the mechanism of viral assembly. The work described here constitutes a highly sensitive assay system for the studies of ϕ29 assembly. This system detected in vitro-assembled infectious phages with a titer range of 0 to 6×10^7 PFU/ml. The in vitro viral assembly makes the manipulation of viral components and the study of their function more manageable. This system can be used for the analysis of components in ϕ29 assembly and dissection of functional domains of structural components, enzymes, and pRNA, utilizing truncation, deletion, and in vitro mutagenesis (34, 35, 48–51). The demonstration that foreign DNA is efficiently packaged into the purified procapsids (17, 22, 25) suggests that ϕ29 has the potential to be used as a gene delivery system. The notion that purified tail protein gp9 was able to incorporate into complete virions may facilitate the construc-

tion of chimeric proteins or fusion proteins of gp9. Ligands for specific receptors of certain human cells could be fused with gp9 and incorporated into the in vitro-assembled viral particles. The chimeric phage could then be used as a vector to carry the DNA into a specific cell. As a nonpathogen gene delivery vector, ϕ29 may have many significant advantages over other proposed animal viral vectors.

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