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Morphogenesis of Bacteriophage φ29 of *Bacillus subtilis*: Oriented and Quantized In Vitro Packaging of DNA Protein gp3

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The assembly of phage φ29 occurs by a single pathway, and the DNA protein (DNA-gp3) of "packaging intermediates" can be obtained after DNase I interruption of in vitro complementation. A broad spectrum of DNA molecules of variable length was isolated from DNase I-treated proheads. Restriction endonuclease *Eco*RI digestion and electrophoretic analysis of these DNA molecules suggested that DNA-gp3 packaging was oriented with respect to the physical map and was a complex process. Proteinase K-treated exogenous DNA was not packaged. When exogenous DNA-gp3 was predigested with the restriction endonucleases *Bst*EII, *Eco*RI, *Hpa*I, and *Hpa*II, the left-end fragments, ranging in size from 8 to 0.9 megadaltons, were selectively and efficiently packaged. During in vivo and in vitro assembly, DNA-gp3 is packaged into proheads, the "core-scaffolding" protein gp7 exits from the particles, and the DNA-filled heads assume the angular morphology of phage φ29. The packaging of a 4.1-megadalton DNA-gp3 left-end fragment (one third of the genome) resulted in the exit of gp7 and the transition to angularity.

The *Bacillus subtilis* phage φ29 contains seven proteins as structural components of the head, neck, and tail (1, 5, 41, 47). The genome is a linear, nonpermuted duplex DNA of 12 megadaltons (Mdal), with protein (gp3) covalently bound to the 5′ termini (1, 2, 24, 30, 31, 49, 55). Eighteen cistrons have been mapped, and 25 virus-specific proteins, reflecting more than 90% of the coding capacity of the genome, have been identified (5, 13, 25, 38, 39, 45).

Morphogenesis of φ29 occurs by a single pathway, and provisional models of the virion have been published (11, 35, 43, 44). Proheads, the first particles detected, are composed of the major head protein (gp8), the head fiber protein (gp8.5), the neck upper-collar protein (gp10), and the recyclable "core-scaffolding" protein (gp7). There is no evidence that these proteins are cleaved or modified during assembly. Virions lacking the nonessential protein gp8.5 have the absolute plating efficiency (about 1) of wild-type φ29 (1, 46). Pulse-chase labeling experiments demonstrated the in vivo conversion of proheads to DNA-filled heads and φ29 (44). In vivo encapsidation of DNA-gp3 into proheads requires the protein gp16, and the angular DNA-filled heads (11^- heads) no longer contain gp7 (20, 35, 44). The neck lower-collar protein (gp11) and tail protein (gp9) can be added in vitro to DNA-containing heads (10), and the cleaved form of the appendage protein (gp12*) is efficiently added to particles lacking appendages (12^- particles) to produce phage (10, 53). A protein essential for morphogenesis (gp13) can complement 12^-13^- particles (37).

Proheads, DNA-gp3, and gp16 are essential for in vitro DNA encapsidation, and the average yield in complementation extracts is 180 phage per prohead donor cell (average of 9×10^{11} phage per ml) (7, 8). More than 30% of the proheads in the extract are converted to phage, and about 30% of DNA-protein extracted from phage can be repackaged (7, 8). A functional gp3 linked to the 5′ termini of φ29 DNA is a requirement for effective phage assembly in vivo and in vitro, although in vitro packaging of trypsin-treated exogenous [^{3}H]DNA-gp3 into a DNase-protected form can be 60% as efficient as the packaging of untreated control DNA-gp3 (8).

Here we confirm that gp3 is essential for in vitro DNA-gp3 packaging by showing that proteinase K-treated molecules are inactive in the reaction. In addition, we studied this gp3-dependent in vitro packaging process by terminating the repackaging of exogenous [^{3}H]DNA-gp3 by the addition of DNase I after 5 to 12 min of complementation and then characterizing the particles derived from packaging intermediates. We provide evidence for a left to right orientation of DNA packaging (with respect to the

genetic and restriction maps) and demonstrate that left-end restriction endonuclease-generated fragments of DNA-gp3 ranging in mass from about 8 to about 0.9 Mdal are packaged in vitro in a DNase I-protected form. Finally, we provide morphological evidence that in vitro encapsidation of a DNA left-end fragment of 4.1 Mdal can trigger the presumed conformational change(s) of gp8 that results in the conversion of the rounded prohead to the angular morphology of phage φ29.

MATERIALS AND METHODS

Chemicals and isotopes. Spermidine (A grade) and DNase I (bovine pancreatic) were obtained from Calbiochem-Behring Corp., San Diego, Calif. ATP (equine muscle) and egg white lysozyme (3× recrystallized) were purchased from Sigma Chemical Co., St. Louis, Mo. ATP was dissolved in TMS buffer (0.05 M Tris-hydrochloride [pH 7.8], 0.01 M $MgCl_2$, 0.1 M NaCl) and neutralized with 1.5 N ammonium hydroxide to give a stock solution of 20 mM (pH 7.8). Proteinase K (E. Merck, Darmstadt, West Germany) was dissolved in water. High-gelling-temperature agarose was from FMC Corp., Rockland, Maine, and 2-mercaptoethanol was obtained from Eastman Chemical Co., Rochester, N.Y. The restriction endonucleases *Eco*RI, *Bst*EII, *Hae*III, and *Hpa*II were purchased from Bethesda Research Labs, Rockville, Md., and *Hpa*I was from Boehringer-Mannheim Biochemical, Indianapolis, Ind. [^{3}H]thymidine (no. NET 0272; 50 Ci/mmol) and the ^{14}C-labeled amino acid mixture (no. NEC 445E; about 200 mCi/ mmol) were from New England Nuclear Corp., Boston, Mass.

Phage and bacteria. Phage φ29 and previously described mutants were used (39, 47). Recombinants were constructed with mutant *sus14*(1241), a mutation that results in the delayed-lysis phenotype (42). The properties of the permissive host *B. subtilis sup*^{44} (40) and the nonpermissive host *B. subtilis spoA12* (48) have been described previously.

φ29 mutants were grown in the permissive host on tryptose blood agar base plates at 30°C and liberated from the agar overlay by suspension in Difco antibiotic medium no. 3 (Penassay broth [PB]). After centrifugation at 12,000 × *g* for 30 min, the supernatants were filtered through 0.45-μm filters, and the phage were concentrated by centrifugation at 53,000 × *g* at 10°C for 2 h and suspended and stored in TMS buffer.

Radiolabeling. Radiolabeling of φ29 DNA and proteins was as described previously (8), except that ^{14}C-labeled amino acids were added at 20 μCi/ml to label the φ29 proteins.

For labeling of proteins in UV-irradiated cells, bacteria were grown to 4×10^8 cells per ml in PB, concentrated to 2×10^9 cells per ml by centrifugation, UV-irradiated for 10 min (5×10^{-6} joules/mm^2 per s) in PB, infected at a multiplicity of 20, and then diluted to 2×10^8 cells per ml with prewarmed (37°C) PB containing ^{14}C-labeled amino acids (20 μCi/ml) after adsorption at 37°C for 10 min. Proteins were labeled continuously until 60 min after infection, at which time labeling was terminated (25).

In vitro complementation and isolation of DNA "packaging intermediates." Previously described procedures for cell growth, infection, and extract preparation for in vitro complementation were followed, except that PB replaced 416 medium (7). Briefly, the complementation extract was prepared after combining cells infected with the mutants *sus16*(300)-*sus14*(1241) (16^-14^-), supplying proheads, and *sus8*(22)-*sus10*(302)-*sus14*(1241) ($8^-10^-14^-$), supplying gp16 and unable to assemble proheads.

To isolate particles derived from DNA-gp3 packaging intermediates, exogenous wild-type [^{3}H]DNA-gp3 extracted from CsCl-purified phage in TE buffer (0.05 M Tris-hydrochloride [pH 7.8], 0.01 M EDTA) at 70°C for 20 min, was dialyzed against 0.01× TE buffer at 4°C for 2 to 3 h, and 100 μl of this was incubated with 200 μl of the 16^-14^- by $8^-10^-14^-$ in vitro complementation extract on Parafilm at ambient temperature. DNA-gp3 packaging was terminated after 5 to 12 min in 200 μl of the extract by the addition of 40 to 160 μg of DNase I per ml, and the particles derived from the packaging intermediates were isolated by centrifugation of the digest in linear 5 to 20% sucrose gradients (8).

Packaging of proteinase K-treated DNA-gp3 was studied with exogenous wild-type [^{3}H]DNA-gp3 heat-extracted as described above. A portion of the [^{3}H]DNA-gp3 (150 μl) was incubated with 15 μg of proteinase K at 37°C for 30 min. Both DNA samples were extracted with phenol saturated with TE buffer, extracted with ether, purged with nitrogen gas, and dialyzed against 0.01× TE buffer at 4°C. Each of the DNA samples (100 μl) was incubated with 200 μl of the in vitro complementation extract. After 120 min, both extracts were incubated with 60 and 35 U of *Hae*III and *Bst*EII, respectively, at 37°C for 20 min, and 200 μl of each extract was centrifuged in sucrose gradients (8).

For the packaging of exogenous wild-type φ29 DNA-gp3 restriction fragments, a 1:2.6 mixture of CsCl-purified, ^{3}H-labeled (6×10^{11} PFU/ml) and unlabeled (1.3×10^{13} PFU/ml) phage was heated as described above to extract DNA-gp3, and the DNA was diluted to 35 μg/ml in *Eco*RI restriction buffer (0.1 M Tris-hydrochloride [pH 7.8], 0.05 M NaCl, 0.01 M $MgCl_2$, 0.001 M 2-mercaptoethanol). The exogenous [^{3}H]DNA-gp3 (250 μl) was treated with the restriction endonuclease *Bst*EII, *Eco*RI, *Hpa*I, or *Hpa*II (35, 40, 30, or 30 U, respectively) at temperatures suggested by the manufacturers for 3 to 4 h. The digested [^{3}H]DNA-gp3 (100 μl) was added to 200 μl of the in vitro complementation extract. After incubation for 120 min, the mixtures were treated with 8 μg of DNase I, and 200-μl samples were centrifuged in sucrose gradients as described above.

Electrophoresis. For agarose gel electrophoresis, portions of single or pooled fractions from sucrose gradients, as described in the figure legends, were dialyzed against 0.01× TE buffer on 0.025-μm filters (Millipore) and reduced from 200 μl to 20 μl by desiccation at ambient temperature. The packaged DNA-gp3 (50 μl) was heat extracted and treated with 10 μg of proteinase K at 37°C for 30 min; the protease was inactivated at 80°C for 30 min. When indicated, 5 μl of 10× restriction buffer was added, and the DNA was treated with 25 U of *Eco*RI enzyme at 37°C for 3 h. After the addition of 5 to 10 μl of electrophoresis sample buffer (50% [wt/vol] sucrose, 0.1 M EDTA,

0.05% bromophenol blue), the samples were loaded on horizontal 0.7 to 1% (wt/vol) agarose gels (22 by 13.5 by 0.9 cm) prepared in Tris-borate buffer (0.089 M Tris-hydrochloride [pH 8.2], 0.0025 M EDTA, 0.0089 M boric acid) and run for 10 min at 200 V, then for 60 to 120 min at 120 V, and finally for 14 to 18 h at 40 V. Alternatively, the samples were run in vertical 1% (wt/vol) agarose gels (15.6 by 11.3 by 0.3 cm) in Tris-borate buffer for 2 h at 40 V. The gels were stained with 5 μg of ethidium bromide per ml in Tris-borate buffer for 30 min and destained overnight in water. Photographs were made with a Polaroid MP-4 Land camera and Polaroid 4 × 5 type 55 positive/negative film.

The ^{14}C-labeled proteins comprising the particles derived from packaging intermediates were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) on 12 to 19% polyacrylamide gradients as described previously (3).

Electron microscopy. Fresh Parlodion films on 400-mesh copper grids were floated on samples of sucrose gradient fractions that had been fixed with 0.4% (vol/vol) glutaraldehyde, and the preparations were then negatively stained with 1.5% (wt/vol) neutral sodium phosphotungstic acid. The Philips EM301 electron microscope was calibrated from micrographs of a carbon grating replica (E. F. Fullam, Schenectady, N.Y.), and micrographs were taken at magnifications of ×34,000.

RESULTS

A functional gp3 linked to the 5′ termini of φ29 DNA is a requirement for effective phage assembly in vivo and in vitro (8). Trypsin-treated [^{3}H]DNA-gp3 could be packaged in vitro to give particles that resembled φ29 morphologically, but their infectivity was always less than 0.6% of the untreated control.

To further characterize the role of gp3 in in vitro assembly, we compared the packaging of samples of proteinase K-treated and untreated exogenous [^{3}H]DNA-gp3. Heat-extracted wild-type [^{3}H]DNA-gp3 samples with or without proteinase K were phenol extracted, dialyzed, and incubated for 120 min in in vitro complementation mixtures. The mixtures were then treated with *Hae*III and *Bst*EII restriction enzymes to reduce viscosity and centrifuged in sucrose gradients. *Hae*III does not digest φ29 DNA, and *Bst*EII makes a single scission (30, 33). Fast-sedimenting trichloroacetic acid (TCA)-precipitable ^{3}H label in the packaged DNA-gp3 at the position of φ29 on the 60% sucrose shelf at fraction 2 is shown in Fig. 1A. The DNase I-protected label in fractions 1 through 5 represented about 12% of the [^{3}H]DNA-gp3 in the gradient. The 6.2 × 10^8 wild-type phage per ml produced during complementation compared favorably with the predicted yield of about 1.3 × 10^9 phage per ml based on the observed amount of packaging of input [^{3}H]DNA-gp3. The DNase I-sensitive DNA-gp3 in the broad peak centering on fraction 26 was present in part as DNA-

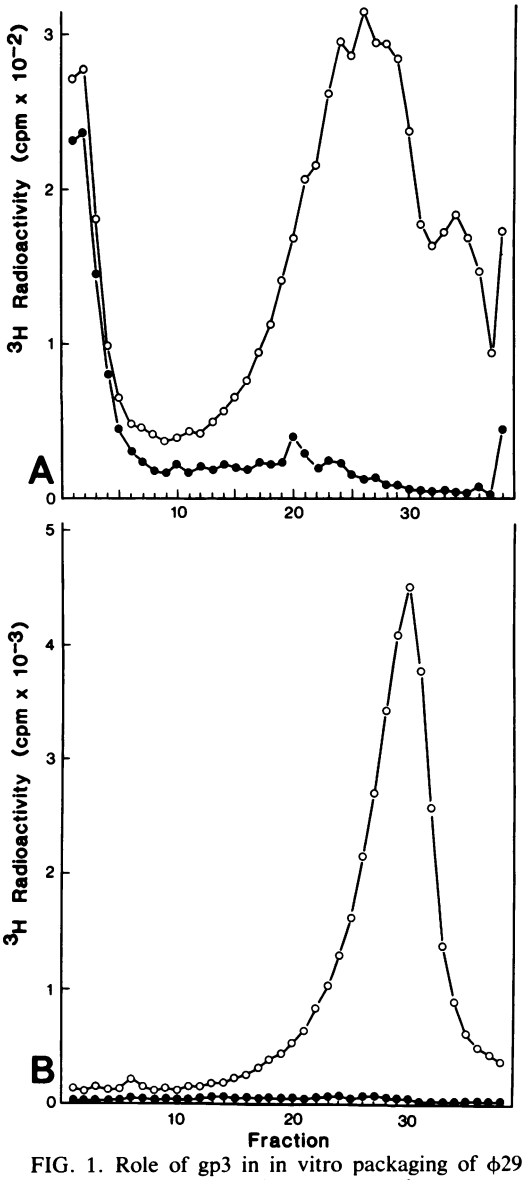


FIG. 1. Role of gp3 in in vitro packaging of φ29 DNA. Untreated or proteinase K-treated exogenous wild-type [^{3}H]DNA-gp3 was packaged in vitro, and the particles were separated in sucrose gradients after treatment of the complementation mixtures with the restriction endonucleases *Hae*III and *Bst*EII. The TCA-insoluble radioactivity in 50-μl samples of sucrose gradient fractions containing untreated (A) and proteinase K-treated (B) [^{3}H]DNA-gp3 is shown before (○) and after (●) treatment of the fractions with 5 μg of DNase I per ml. The wild-type [^{3}H]DNA-gp3 packaged into particles in A (centering on fraction 2) represents 12.4% of the exogenous [^{3}H]DNA-gp3 added to the extract. Wild-type phage in the extract (6.2 × 10^8/ml) were 0.15% of the total phage yield of 4.4 × 10^{11}/ml. Proteinase K-treated exogenous wild-type [^{3}H]DNA-gp3 (B) was not repackaged (<10^6 phage per ml) during complementation that yielded 6.4 × 10^{11} phage per ml.

particle complexes. Although this [^{3}H]DNA-gp3–particle distribution was complicated by *Bst*EII digestion, similar results have been obtained with DNA-gp3 prepared by other methods (7, 8).

In contrast, proteinase K-treated DNA (Fig. 1B) presented a more uniform profile in the sucrose gradient, and the position of the peak (centering on fraction 30) suggested that this DNA was not associated with the prohead. In the absence of gp16, DNA-gp3 is not packaged (7), and the proheads would center on fraction 20 under these centrifugation parameters (data not shown). The proteinase K-treated DNA had the φ29 unit length when examined by agarose gel electrophoresis (data not shown). After proteinase K treatment and in vitro complementation, less than 0.4% of the [^{3}H]DNA-gp3 sedimenting in fractions 1 through 5 was DNase I protected, and less than 10^6 wild-type phage per ml were produced. This is close to the reversion level of the 16^- parent when 6.4×10^{11} phage per ml are assembled from endogenous DNA during complementation (Fig. 1, legend). Therefore, untreated DNA-gp3 was at least 30-fold more effective than proteinase K-treated DNA in the in vitro DNA packaging mixture, and there was no positive evidence that the proteinase K-treated wild-type DNA was packaged (less than 10^6 PFU/ml).

Isolation and characterization of DNA-gp3 packaging intermediates. When exogenous [^{3}H]DNA-gp3 is incubated for 80 min in the in vitro complementation mixture, treated with DNase I, and then centrifuged in a sucrose gradient, most of the DNase I-protected TCA-precipitable [^{3}H]DNA-gp3 and assembled φ29 cosediment and center on fraction 4 of the gradient (8). However, when exogenous [^{3}H]DNA-gp3 encapsidation is terminated by addition of DNase I at 3 min, up to 50% of the [^{3}H]DNA-gp3 is found in fractions 10 to 20, between the positions of φ29 and proheads (8). Partially filled prohead-like structures are observed in these fractions by negative staining and electron microscopy, and we assume that these particles are derived from packaging intermediates by DNase treatment.

Figure 2 shows the sedimentation analysis after the interruption of [^{3}H]DNA-gp3 packaging with DNase I after 5 or 10 min. After 5 min of complementation, more than 14% of the exogenous [^{3}H]DNA-gp3 added was present in the packaging intermediates position (centering on fraction 16), and this TCA-insoluble radioactivity represented about 39% of the exogenous DNA-gp3 packaged into φ29 by this reaction mixture in 160 min (about 2.2% [9.9×10^9 phage per ml] of the assembled phage had the wild-type genome). After 10 min of complementation, [^{3}H]DNA-gp3 also appeared at the position of DNA-filled particles (centering on fraction 6). More than 64% of the exogenous DNA-gp3 that was ultimately packaged was positioned in the gradient in the peaks centering on fractions 6 and 17, although very few phage (4×10^7 per ml) had been assembled. This is consistent with our demonstration that about 0.1% of virus assembly is completed during the first 20 min of in vitro complementation (7). If DNase I (5 μg/ml) was first mixed throughout the sucrose gradient and the corresponding gradient fractions from a 10-min complementation were then incubated at

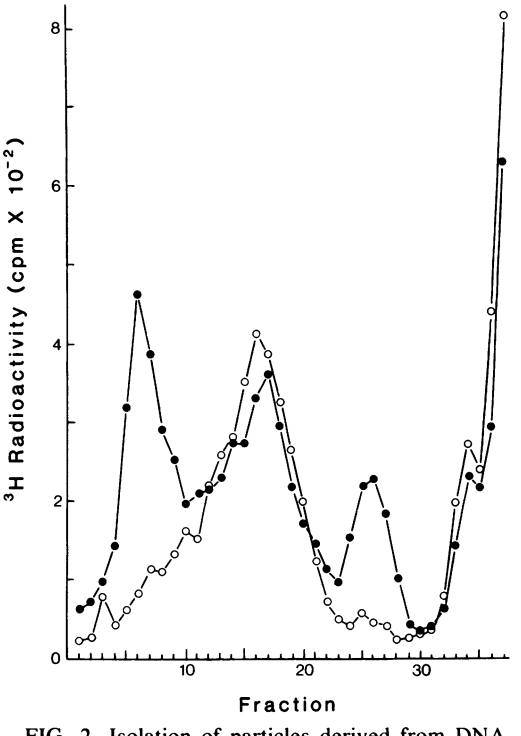


FIG. 2. Isolation of particles derived from DNA-gp3 packaging intermediates. [^{3}H]DNA-gp3 was heat-extracted from CsCl-purified wild-type phage and dialyzed against 0.01× TE buffer at 4°C. Two 100-μl samples of the [^{3}H]DNA-gp3 were mixed with separate 200-μl volumes of the in vitro complementation extract. After 5 (○) or 10 (●) min, 200-μl volumes of the mixtures were treated with 20 μg of DNase I for 5 min, and the particles were separated by sucrose gradient centrifugation (8). Sedimentation is from right to left. TCA-insoluble radioactivity in the 5-min intermediate peak (centering on fraction 16) and the 10-min intermediate peaks (centering on fractions 6 and 17) represents 14.2%, and 10.2 and 12.9%, respectively, of the exogenous [^{3}H]DNA-gp3 added to the extracts. The number of PFU produced in the in vitro complementation mixture after 120 min was 4.2×10^{11} per ml on the permissive host, and 9.6×10^9 per ml had the exogenous wild-type genome.

37°C for 30 min, only the [^{3}H]DNA-gp3 of the uncharacterized peak (centering on fraction 26) was rendered TCA soluble (data not shown).

As the prohead is converted to the DNA-filled head both in vivo and in vitro, the core-scaffolding protein (gp7) exits, the structure develops the angularity characteristic of the virion, and the abortive structure, the so-called "empty head," appears (7, 20, 35, 44). The composition of the "prohead" component of the packaging intermediates was determined by SDS-PAGE and autoradiography (Fig. 3). The prohead structural proteins gp8, gp8.5, and gp10 were present in each fraction and are a useful reference. We used nonpermissive infection by the *sus* mutants $7^-10^-14^-$ and 16^-14^- (Fig. 3, legend) to demonstrate the positions of gp7, gp10, and gp16 in our SDS-PAGE system. With 12 copies of the upper-collar protein gp10 per prohead (12) and molecular weights of 35,000 and 14,000 for gp10 and gp7, respectively (25), we estimated the number of gp7 molecules in the packaging intermediates. From fractions 22 to 2 (Fig. 3), the average number of gp7 molecules per particle, based on densitometry of the autoradiograph, decreased from about 75 to less than 5. This trend is consistent with the reports that DNA-filled heads do not contain gp7 (35, 44). The apparent number of copies of gp7 per intermediate particle is probably overestimated, particularly for fractions 20 through 10, because of the high probability of a decreasing but significant presence of proheads in these fractions. gp16, a protein required for in vitro DNA packaging (7), was not detected in the packaging intermediates.

To determine whether the intermediates shown in Fig. 2 were separated on the basis of DNA content, DNA was heat extracted from the particles, digested with proteinase K, and examined by agarose gel electrophoresis (Fig. 4). Particles in fractions of the usual prohead position (fractions 20 and 21) contained DNA smaller than the 0.44-Mdal *Eco*RI fragment E, whereas fast-sedimenting particles (fraction 3) contained genome-length DNA-gp3. DNA of particles in fractions 20 through 18 formed a series of about 10 faintly discernable bands in the agarose gels with masses of 1 to 3 Mdal. In particles in fractions 18 through 15, DNA molecules with a mass of about 3.2 Mdal accumulated. As more DNA-gp3 of greater length was packaged and particles sedimented further into the gradient (fractions 16 through 10), the DNA content seemed to be increasingly heterogeneous. Distinct bands appeared in a given channel, suggesting that packaging is quantized.

Orientation of DNA packaging. The restriction endonuclease *Eco*RI cleaves the φ29 genome into five fragments (28, 32, 51). The protein gp3

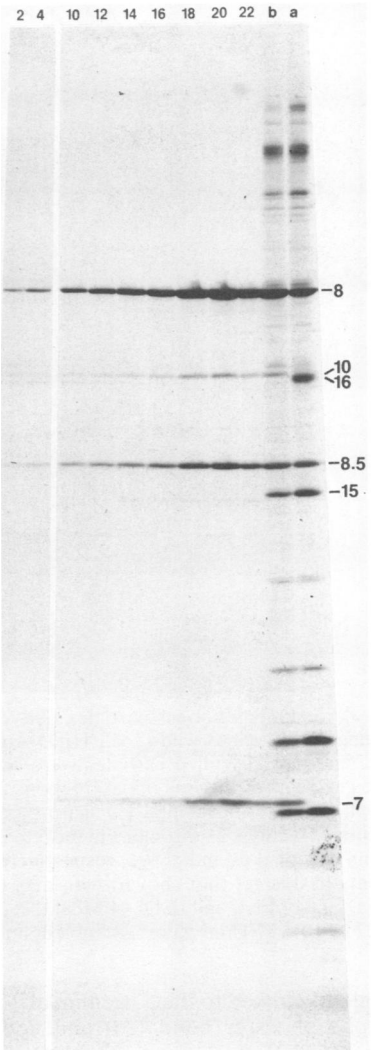


FIG. 3. Autoradiograph of the ^{14}C-labeled proteins of the particles derived from in vitro DNA-gp3 packaging intermediates. Heat-extracted exogenous wild-type [^{3}H]DNA-gp3 (100 μl) was incubated with 200 μl of complementation extract prepared from 16^-14^-- and $8^-10^-14^-$-infected cells grown in PB containing ^{14}C-labeled amino acids (20 μCi/ml). After 10 min, DNA-gp3 packaging in a 200-μl portion was terminated by the addition of 8 μg of DNase I, and the particles were separated by sucrose gradient centrifugation. As a source of φ29-specific marker proteins, the nonpermissive host *B. subtilis spoA12* was UV irradiated, infected with the mutant *sus7*(614)-*sus10*(302)-*sus14*(1241) or 16^-14^-, and labeled with ^{14}C-amino acids (20 μCi/ml) in PB for 60 min as described in the text. Profiles (right to left) are of samples of the infected cell pellets of (a) $7^-10^-14^-$ and (b) 16^-14^-, and samples of fractions 22, 20, 18, 16, 14, 12, 10, 4, and 2 of the sucrose gradient after SDS-PAGE and autoradiography as described previously (3). Numbers on the right are gene designations that mark the positions of φ29 gene products.

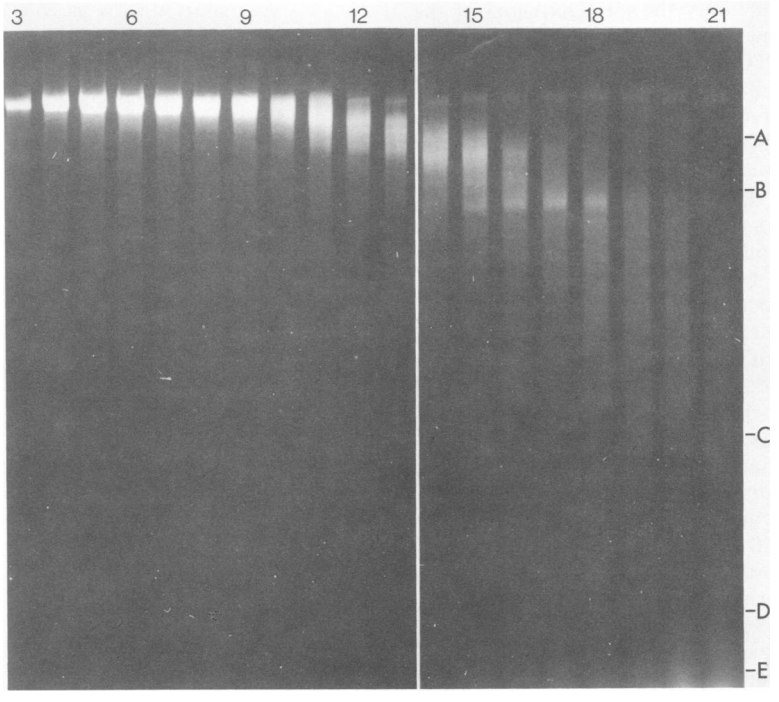


FIG. 4. Sedimentation of particles derived from packaging intermediates is a function of DNA content. Packaging of exogenous wild-type [^{3}H]DNA-gp3 and endogenous DNA was interrupted by DNase I treatment at 10 min, and partially filled particles were separated in a sucrose gradient. DNA was heat-extracted from the particles in 50 μl of the individual fractions 3 through 21, treated with proteinase K (10 μg), and separated by electrophoresis in a horizontal 1% agarose gel as described in the text. The profiles (right to left) correspond to consecutive fractions 21 through 3 in the 5 to 20% sucrose gradient, with fractions 20 and 3 representing the usual positions of proheads and phage, respectively. The letters at the right reflect the relative positions of ϕ29 *Eco*RI fragments in this gel (not shown) with masses previously determined as (A) 6.1 Mdal, (B) 3.5 Mdal, (C) 1.25 Mdal, (D) 0.64 Mdal, and (E) 0.44 Mdal (28, 32, 51). When the complementation mixture was incubated for 120 min, 5.7×10^{11} PFU/ml were assembled, and 3.7% of the phage had the exogenous wild-type genome.

is covalently linked to the 5′ termini of ϕ29 DNA by a phosphoester bond (26) and defines the terminal *Eco*RI fragments A and C (32). The fragment order has been shown to be A, B, E, D, C, with fragment A being assigned to the left end of the physical and genetic maps (28). The cleavage of the partially packaged DNA-gp3 of packaging intermediates by *Eco*RI was used to define the orientation of packaging.

The repackaging of exogenous [^{3}H]DNA-gp3 was interrupted at 10 min by the addition of DNase I, and the DNA-containing particles were fractionated on a sucrose gradient. (The exogenous DNA packaged when this complementation was allowed to proceed to completion accounted for less than 1% of the 4.5×10^{11} phage assembled per ml.) The DNA-gp3 was heat-extracted from particles in pooled fraction pairs, treated with proteinase K and the restriction endonuclease *Eco*RI (when indicated), and examined by electrophoresis in an agarose gel (Fig. 5).

As successive sucrose gradient fractions were examined, we demonstrated that the particles contained, on the average, DNA-gp3 of progressively greater length (see also Fig. 4). If DNA encapsidation is oriented from left to right, then the first *Eco*RI fragment to be packaged in a DNase-protected form would be fragment A. The *Eco*RI-treated DNA molecules are displayed in alternate profiles in Fig. 5. Substantial amounts of fragment A were packaged in the particles of fractions 12-13, and the amount increased in the particles of fractions 8-9 and 5-6. In addition, there was a progressive increase in the amount of fragment B in particles that contained DNA of greater mass. Similarly, in the profiles of fractions not treated with *Eco*RI, the mass of the packaged DNA molecules progressively increased to that of the ϕ29 marker DNA of profile 1. However, if the orientation of packaging is from right to left, then the smaller *Eco*RI fragments C, D, and E should be predominant in the profiles exhibiting the DNA first packaged (16-17 and 12-13); they were not. (This portion of the gel is not shown in Fig. 5.)

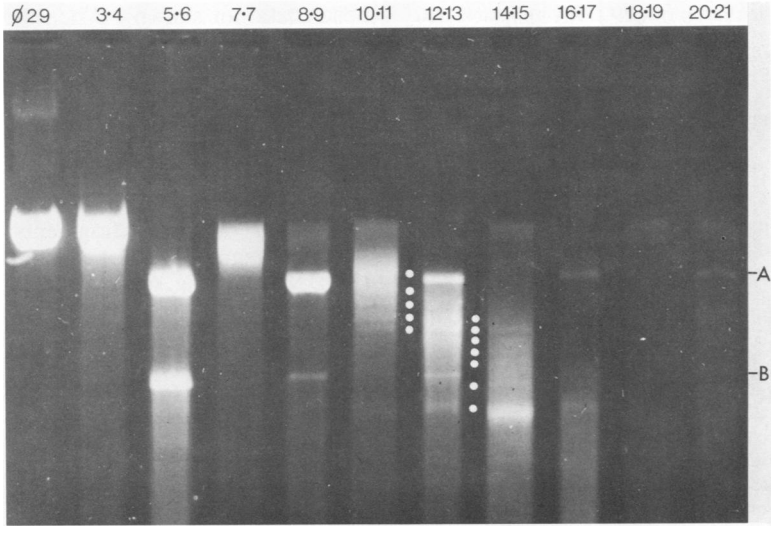


FIG. 5. In vitro packaging of φ29 DNA-gp3 is quantized and complex. After DNase I interruption of in vitro packaging of exogenous wild-type [^{3}H]DNA-gp3 at 10 min, partially filled particles were separated in a sucrose gradient. Sequential fraction pairs were pooled and treated as described in the text. Samples of alternate fraction pairs were incubated after the addition of restriction buffer and *Eco*RI enzyme; DNA molecules were then separated by electrophoresis in a horizontal 1% agarose gel as described in the text. Profile 1 is of intact φ29 DNA. Profiles 5-6, 8-9, 12-13, 16-17, and 20-21 are of corresponding *Eco*RI-digested fraction pairs. Alternate profiles 3-4, 7-7, 10-11, 14-15, and 18-19 are of untreated fraction pairs. A and B mark the positions of *Eco*RI fragments A and B (6.1 and 3.5 Mdal, respectively). A portion of the complementation extract incubated for 160 min produced 4.5×10^{11} phage per ml; 3.7×10^9 phage per ml had the exogenous wild-type genome.

In a similar experiment, in vitro DNA encapsidation was terminated at 12 min with DNase I; the particles were fractionated in a sucrose gradient, and the DNA was prepared and treated with *Eco*RI as in the experiment shown in Fig. 5. The *Eco*RI fragments were separated by electrophoresis in a vertical 1% agarose gel, and the relative amounts of the fragments were determined from densitometer tracings of Polaroid negatives of the stained gel (Table 1). In particles that sedimented in sucrose gradients with or near proheads (fractions 19 and 20), there was very little DNA (Fig. 4 and 5; Table 1). Only *Eco*RI fragment A could be detected in fractions 19 and 13 of the gradient (Table 1). In fractions 10 and 7 we could also detect *Eco*RI fragments B and E. As the DNA content of the intermediate particles in the gradient increased (Fig. 4 and 5), fragments to the right of *Eco*RI-A appeared and increased in proportion until they approximated the ratio of the fragments found in *Eco*RI-digested φ29 DNA (Table 1). Fragment C, the right-hand terminus of the genome, was not detected in these densitometer tracings (Table 1). The absence of *Eco*RI-C was not an artifact of heat extraction or of our methodology, because fragment C was present when DNA-gp3 was heat-extracted from intermediate particles isolated in the absence of DNase I (data not shown).

Some elements of the complexity of the packaging process are also shown in Fig. 4 and 5. Although some rather small DNA fragments were protected from DNase I (Fig. 4, fractions 18 through 21), DNA with a mass of 3.2 Mdal (about 27% of the genome) accumulated in particles in fractions 14-15 (Fig. 5). Particles of this and successive fractions contained molecules of greater mass that appeared in the agarose gel as a series of at least 10 bands (compare fractions 14-15, 12-13, and 10-11, Fig. 5). These DNA bands were reproducibly present in all of these agarose gels and were in good alignment from profile to profile. We estimated that the bands contained DNAs ranging in mass from 3.2 to 7.2 Mdal; the bands between the *Eco*RI-A and *Eco*RI-B fragments contained DNA with estimated masses of 3.6, 3.8, 4.0, 4.2, 4.4, 5.3, and 5.8 Mdal, respectively. The band with 7.2-Mdal DNA was slightly above *Eco*RI fragment A in the gel and constituted about 60% of the genome (fractions 7-7 and 10-11, Fig. 5).

In vitro packaging of φ29 genome fragments. To determine whether DNA fragments could be repackaged, we digested DNA-gp3 with restriction endonucleases and added both right and left-end fragments having gp3 in the native conformation to the complementation extract. The restriction endonuclease *Bst*EII cleaves φ29 DNA once and yields an 8-Mdal left end (A fragment [30]) that might be preferentially pack-

TABLE 1. Orientation of ϕ29 DNA-gp3 packaging

*Eco*RI restriction fragments[a]	Ratio in ϕ29 DNA-gp3 (control)	Ratio in gradient fraction[b] no.:				
		4	7	10	13	19
A/A		0.6	1.0	0.6	0.9	0.08
A/B	1.7	1.4	2.1	6.2	—	—
A/E	13.9	11	34	—	—	—
A/D	9.5	17	—	—	—	—
A/C	4.8	—	—	—	—	—

[a] The DNA-gp3 control values are the ratios of the molecular weights of the respective fragments. The A/A ratio is the amount of fragment A in the indicated fraction relative to that in fraction 7.

[b] DNA packaging in a 200-μl in vitro complementation extract incubated with 100 μl of heat-extracted exogenous wild-type [^{3}H]DNA-gp3 was terminated at 12 min by the addition of 20 μg of DNase I to 200 μl of extract, and the particles derived from packaging intermediates were separated by sucrose gradient centrifugation. Portions (150 μl) of fractions 4 through 20 were dialyzed against 0.01× TE buffer and desiccated to 35 μl, and the packaged DNA-gp3 was heat extracted. The samples were treated with 10μg of proteinase K at 37°C for 30 min, and the protease was heat inactivated. After incubation with 5 μl of 10× restriction buffer and 25 U of *Eco*RI enzyme, the DNA molecules were separated by electrophoresis in vertical agarose gels and photographed as described in the text. Densitometer tracings of the negatives were made with a Joyce-Loebl densitometer, and the areas under the peaks were measured with a Zeiss MOP digitizer. The ratios were calculated from the areas of the peaks. Dashes indicate the absence of fragment B, E, D, or C from the fraction.

aged. Moreover, DNA-filled heads assembled in vivo differ from proheads in their angularity and lack of the core-scaffolding protein, gp7 (20, 35, 44). The successful packaging of a series of partial genome fragments would generate particles that may also help define the mechanism of the transition from prohead to DNA-filled head. For example, [^{3}H]DNA-gp3 was partially digested with the restriction endonuclease *Bst*EII and added to the complementation extract; after 120 min, the mixture was treated with DNase I and then sedimented in a sucrose gradient (Fig. 6A). When DNA-gp3 was heat-extracted from the ^{3}H-labeled particles and examined by electrophoresis in 0.7% agarose gels, two molecular species were present, one of 12 Mdal (the ϕ29 genome) and one of 8 Mdal (Fig. 7A). About 6.5% of the exogenous DNA-gp3 added was packaged as fragment A (the equivalent of 10% unit-length DNA packaged) (Fig. 6A). When particles containing the *Bst*EII fragment A were examined by negative staining and electron microscopy, more than 95% of the 2,000 particles examined had the angularity of the DNA-filled head (data not shown). When the protein composition of the particles (fraction 9) was examined by SDS-PAGE and fluorography (3, 9), the core-scaffolding protein gp7 was not detected. gp7 was present in the proheads of fraction 20 of Fig. 6A (data not shown).

These experiments were repeated with the restriction endonucleases *Eco*RI (five fragments), *Hpa*I (eight fragments), and *Hpa*II (eleven fragments) (30) to cleave the exogenous [^{3}H]DNA-gp3 (Fig. 6 and 7 and data not shown). In each instance only the left-end restriction fragment was packaged in a DNase I-protected form. When the sedimentation distance of the particle into the 5 to 20% linear sucrose gradient was plotted versus the molecular weight of the respective left-end fragments, a linear relationship was obtained over the molecular weight range of 8 to 0.9 Mdal (Fig. 8).

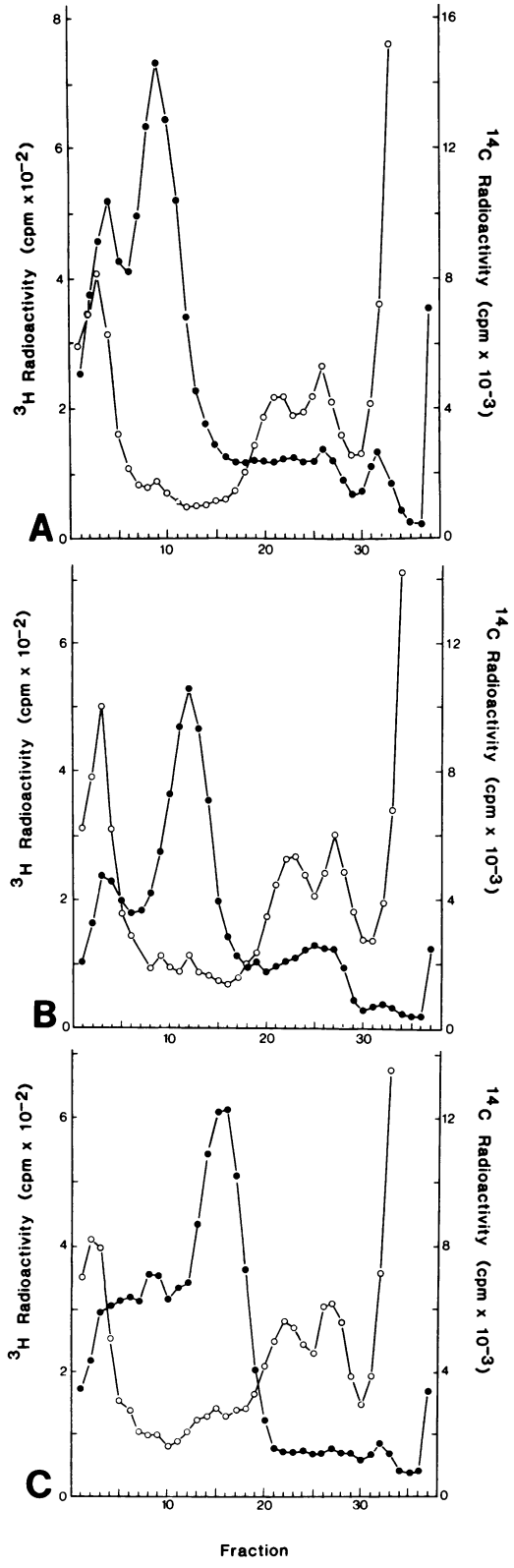
With *Eco*RI digestion, about 11% of the exogenous [^{3}H]DNA-gp3 added was packaged; 8.8% was packaged as fragment A (6.1 Mdal) in a discrete peak centering on fraction 12 (Fig. 6B and 7B). These particles lacked gp7 and had the angularity of DNA-filled heads (data not shown).

In the more complex *Hpa*I digest (eight fragments), the 4.1-Mdal fragment A was preferentially packaged, and this encapsidation of about one third of the genome resulted in the loss of gp7 and the transition to particle angularity (Fig. 6C, 7C, 8, and 9; data not shown).

DISCUSSION

DNA-gp3, 16$^-$ proheads, and the DNA-filled 11$^-$ and 12$^-$ particles have been shown to be intermediates of the ϕ29 assembly pathway both in vivo and by in vitro complementation (7, 8, 10, 35, 44, 53). Morphogenesis of ϕ29 is an imperfect process both in vivo and in vitro. Less than 50% of the DNA-gp3 is packaged, between 20 and 50% of the proheads are not converted to phage, and more than 20% of the particles that sediment with proheads are empty heads that are artifacts of interrupted assembly. During in vitro encapsidation more than 30% of the proheads are assembled into virions, and 5 to 30% of exogenous DNA-gp3 is repackaged. The in vitro complementation can yield 500 phage per cell equivalent of proheads (however, the average yield is 180 phage [9×10^{11} phage per ml]), and exogenous DNA-gp3 competes effectively with endogenous DNA-gp3 from both extracts, the genomes occurring in equal proportions in assembled phage (7, 8).

A single phenol-extracted ϕ29 DNA-gp3 molecule is sufficient for transfection, and this transfection is sensitive to proteolytic enzymes (27, 47). Furthermore, transfection by DNA-gp3 of



the *ts3*(132) mutant is thermolabile (54). Proteinase K digestion can reduce protoplast transformation yield by more than 99.99% (unpublished data). Functional gp3 is also required for effective assembly. DNA-gp3 of the mutant *ts3*(132) made at the permissive temperature is not effectively packaged at 45°C; about 12 to 14% of the DNA-gp3 is in particles that are similar to φ29 in morphology but are not infectious (8). Trypsin digestion can reduce the in vitro packaging of exogenous DNA-gp3 from 19 to 10%, but phage production is reduced to less than 0.04% (3.7×10^8 phage per ml) (8). Capsid proteins are typically added with heat-extracted exogenous DNA-gp3 during complementation. When more than 99% of [^{35}S]methionine-labeled virus proteins are removed (substantially all of the remaining protein is gp3), about 1% of the exogenous DNA-gp3 is repackaged (7). About 12% of the phenol-extracted exogenous DNA-gp3 used in the present study was repackaged (Fig. 1A), and about 50% of the repackaged [^{3}H]DNA-gp3 that sedimented to the virus position in the sucrose gradient was in infectious phage. After proteinase K treatment, less than 0.4% of the exogenous wild-type [^{3}H]DNA-gp3 was repackaged, and although 6.4×10^{11} phage per ml were assembled from endogenous DNA-gp3, less than 10^6 wild-type phage per ml were present. These results suggest that functional gp3 is essential for DNA packaging as well as for the production of infectious phage (Fig. 1B).

When exogenous DNA-gp3 encapsidation is terminated with DNase I at 3 min, up to 50% of the repackaged DNA is found in fractions 10 to 20 of a sucrose gradient, positioned between

FIG. 6. Discrete terminal fragments of *Bst*EII-, *Eco*RI-, and *Hpa*I-treated exogenous wild-type [^{3}H]DNA-gp3 are selectively packaged in vitro. Exogenous [^{3}H]DNA-gp3 was digested with *Bst*EIII (A), *Eco*RI (B), or *Hpa*I (C) and packaged in vitro, and the particles were separated in sucrose gradients. Sedimentation is from right to left. ●, TCA-insoluble radioactivity. DNA in particles from the peaks centered on fractions 9 (A), 12 (B), and 16 (C) corresponds in mass to the φ29 DNA left-end A fragments of 8, 6.1, and 4.1 Mdal produced by the enzymes *Bst*EII, *Eco*RI, and *Hpa*I, respectively (30, 33) (Fig. 7). Infected bacteria labeled with ^{14}C-amino acids were used in parallel complementation experiments to identify the positions of particles. Plots of TCA-insoluble ^{14}C radioactivity (○) in panels A, B, and C mark the positions of φ29 (centering on fraction 3), proheads (centering on fraction 22), and an uncharacterized DNase I-sensitive particle-DNA complex (centering on fractions 26-27). The number of PFU produced from endogenous DNA in the complementation reaction mixtures containing the *Bst*EII-, *Eco*RI-, and *Hpa*I-treated exogenous DNA-gp3 at 120 min was 8.1×10^{11}, 2.0×10^{12}, and 8.6×10^{11} per ml, respectively.

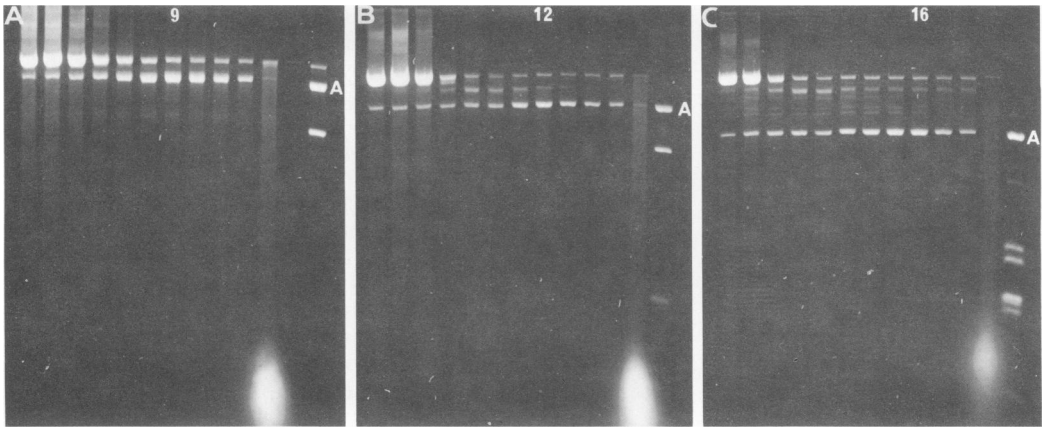


FIG. 7. Left-end fragments of *Bst*EII-, *Eco*RI-, and *Hpa*I-treated exogenous [^{3}H]DNA-gp3 are selectively packaged in vitro. DNA-gp3 was heat-extracted from particles in 50 μl of the sucrose gradient fractions shown in Fig. 6 after the addition of 5 μl of 10× TE buffer and then treated with 10 μg of proteinase K. As markers, approximately 0.05 μg in 20 μl of *Bst*EII-, *Eco*RI-, or *Hpa*I-digested [^{3}H]DNA-gp3 was also incubated with 10 μg of proteinase K. Electrophoresis of these samples was done in horizontal 0.7% agarose gels as described in the text. (A) From right to left: *Bst*EII-digested ϕ29 DNA and the sucrose gradient fractions 36, 26, 12, 11, 10, 9, 8, 7, 6, 5, 4, and 3 (shown in Fig. 6A). (B) From right to left: *Eco*RI-digested ϕ29 DNA and the sucrose gradient fractions 27, 15, 14, 13, 12, 11, 10, 9, 7, 5, 4, and 3 (shown in Fig. 6B). (C) From right to left: *Hpa*I-digested ϕ29 DNA and the sucrose gradient fractions 27, 18, 17, 16, 15, 14, 13, 11, 9, 7, 5, and 3 (shown in Fig. 6C). The A fragments produced by cleavage of ϕ29 DNA with the restriction enzymes *Bst*EII, *Eco*RI, and *Hpa*I have masses of 8, 6.1, and 4.1 Mdal, respectively (30, 33). The right-end *Bst*EII fragment B is 4 Mdal. The *Eco*RI fragments B through E range from 3.5 to 0.44 Mdal, and the *Hpa*I fragments B through H range from 1.17 to 0.49 Mdal (30, 33).

DNA-filled particles and proheads (8). We believe that these particles are derived from DNA-packaging intermediates, and their composition and properties are consistent with this belief. Our basic method was to add exogenous [^{3}H]DNA-gp3 to the complementation extract, interrupt packaging with DNase I, and fractionate the intermediate particle population by sucrose gradient centrifugation. Most of the exogenous DNA-gp3 that would ultimately be packaged (64%; Fig. 2) appeared in the intermediate fractions 10 through 20, and at early times accumulated in a noninfectious form at the position of DNA-filled heads (at 10 min, less than 4 $\times 10^7$ phage per ml were assembled from endogenous DNA). The particles that centered on fraction 27 were an anomaly. We believe that they reflect the instability of intermediate particles that have packaged a large fraction of the genome because they appear late in time, have become DNase I sensitive, and have the morphology of the empty head (8, 44) (Fig. 2; data not shown). The intermediate particles of all fractions had the protein composition of proheads with the exception of the core-scaffolding protein gp7 (Fig. 3). However, DNA-filled heads do not contain gp7, and the progressive loss of gp7 across the gradient is consistent with these observations (20, 35, 44). It is interesting that protein gp16, essential for DNA packaging, was not detected in these particles. As the particles sedimented further into the gradient, they had a greater DNA content, and the DNA-gp3 of the particles of a given fraction were greater in length and more heterogenous until they approached genome length (Fig. 4 and 5). The organization inherent in the left to right orientation of packaging that was observed by means of cleavage with the restriction endonuclease *Eco*RI is consistent with these particles being intermediates (Fig. 5 and Table 1). Morphogenesis occurs in cells as a series of discrete events that are not in phase, so that at early times there would be a broad array of intermediate particles in the complementation extract (36). Our results suggest that by fractionating these particles in sucrose gradients we partitioned the DNA encapsidation process itself. It appears that DNA-gp3 is packaged into typical proheads in a progressive, oriented fashion, such that longer and longer molecules become DNase I protected, and that this process is efficient in assembling a high percentage of the [^{3}H]DNA-gp3 of the intermediate particles into phage (8) (Fig. 2, 4, and 5 and Table 1).

Our study of orientation also suggests quantized packaging of DNA-gp3. We believe that this complexity is not an artifact of DNase treatment or DNA preparation but a real phenomenon. We employed high concentrations of

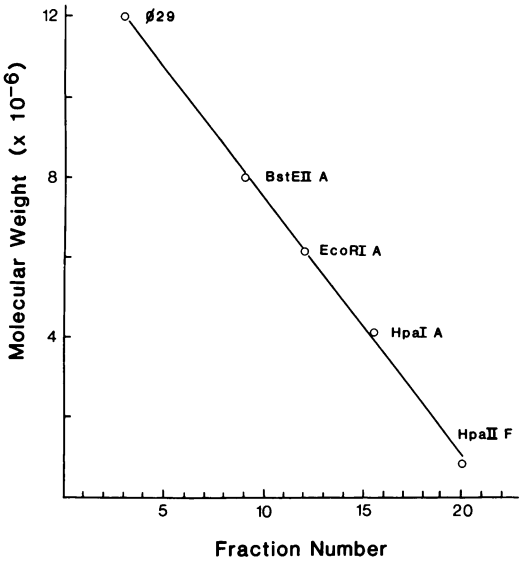


FIG. 8. Sucrose gradient sedimentation of particles containing φ29 DNA left-end restriction fragments is a linear function of DNA content. Samples of heat-extracted exogenous wild-type [^{3}H]DNA-gp3 were digested with the restriction endonuclease *Bst*EII, *Eco*RI, *Hpa*I, or *Hpa*II. These DNAs (100 μl) were each incubated with 200 μl of the in vitro complementation extract for 120 min. DNase I was added to 40 μg/ml, and particles with packaged left-end fragments (Fig. 6 and 7) were separated in sucrose gradients. Sedimentation distances (expressed as peak fractions) were plotted versus the molecular weights of the respective fragments. Between 8.1×10^{11} and 2.0×10^{12} phage per ml were assembled from endogenous DNA during these in vitro complementations.

DNase I in interrupting packaging, levels sufficient to render exogenous [^{3}H]DNA-gp3 completely acid soluble in the complementation extract in the absence of gp16 or ATP (data not shown). In addition, we used four restriction endonucleases to generate left-end fragments from DNA-gp3 that ranged in mass from 8 to 0.9 Mdal. With these digests used as exogenous DNA, complementations were terminated by DNase I at 120 min, and the packaged DNA molecules were examined by agarose gel electrophoresis (Fig. 6 and 7; data not shown). In each instance only a single band was present.

We showed that particle sedimentation was a function of the molecular weight of the left-end DNA restriction fragment packaged (Fig. 8). With *Bst*EII, 6.5% of the exogenous DNA-gp3 added was packaged as a left-end fragment. With *Eco*RI-A, 8.8% (the phage genome equivalent of 17.1%) of the exogenous DNA added was packaged. It was apparent (Fig. 6 and 7) that substantial amounts of the gp3-bound left-end fragments were packaged, but no other DNA fragments (other than partial digests) were packaged. Virtually no *Bst*EII-B–gp3 was packaged in a DNase I-protected form (Fig. 7). With each fragment, the fragment length was conserved in the particles (Fig. 6 and 7), the prohead had assumed the angularity of the DNA-filled head (Fig. 9), and gp7, the core-scaffolding protein, was not detectable in SDS-PAGE gels by fluorography (unpublished data). We cannot discuss particle angularity or gp7 content after packaging of the 0.9-Mdal fragment of HpaII because the particles could not be resolved from proheads in this sucrose gradient.

Yoshikawa and Ito (57) compared the two-dimensional peptide maps of the gp3 molecules linked to the 5′ termini of the DNA and concluded that they are identical. However, we consistently resolved two forms of gp3, trivially named AF and BF for their position in SDS-PAGE (4, 5; unpublished data). We showed that gp3 is necessary for packaging (8) (Fig. 1), that packaging is oriented from left to right (Fig. 5 and Table 1), and that only the left-end DNA-gp3 restriction fragments are packaged in a DNase I-protected form (Fig. 6, 7, and 8). If gp3 is both necessary and sufficient to initiate packaging, then these data suggest that the proteins linked to the 5′ termini are not identical. Proper recognition of the left end by the appropriate protein could occur as a part of DNA replication or as a result of a protein-binding nucleotide recognition sequence near this terminus of the genome. The first *pac* sequence that had a role in DNA maturation and encapsidation was found in P22, and one has been located for the *B. subtilis* phage SPP1 (17, 34). With φ29 DNA, replication begins at either end of the molecule, proceeds by strand displacement, and generates unit-length molecules with gp3 covalently linked to the 5′ termini (22, 23, 29, 31). No obvious cleavage reaction is required to initiate packaging, and if one function of a specific gp3 in DNA replication is to recognize the gp3 of the left terminus, then proteins might be necessary and sufficient to initiate packaging without a *pac* site, and we must consider that the terminal proteins might not be identical. The ends of φ29 DNA are flush, and there are inverted terminal repetitions that are 6 base pairs long 5′(AAAGTA); to this extent the ends are similar. Yoshikawa et al. (56) have shown that 48% of the nucleotide sequence of the first 60 base pairs is identical between the two ends of the DNA. These results are in fair agreement with those of Escarmis and Salas (19), and the differences in sequence near the ends could provide a site(s) that could account in part for the orientation of packaging that we observed (Fig. 5, 6, and 7). These results suggest that the identity of the gp3 protein bound to the termini and the effects of base sequence on packaging, if any, are open questions.

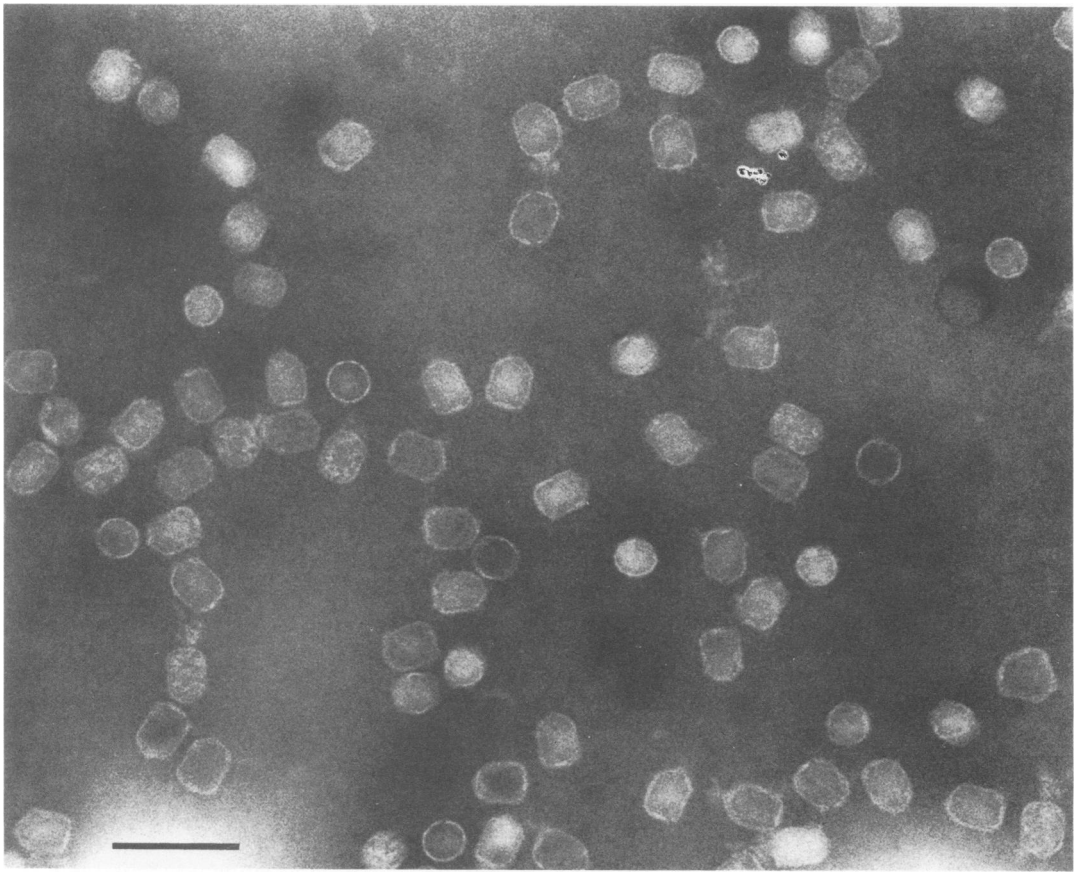


FIG. 9. Particles that have packaged the 4.1-Mdal *Hpa*I-A fragment have angularity. This electron micrograph is of fraction 15 of the sucrose gradient shown in Fig. 6C which contains ^{14}C label. Fixation, staining, and microscopy are described in the text. Bar, 100 nm.

In the absence of gp7, the core-scaffolding protein, the major head protein gp8 does not organize into any stable structure, and proheads are not formed (20). Pulse-chase experiments have shown that gp7 recycles and is not present in DNA-filled heads, but the relationship of gp7 exit to encapsidation is not known (35, 44). The number of gp7 molecules per particle decreased across the gradient from 75 to less than 5, but the rate of exit of gp7 did not seem to be commensurate with the rate of DNA-gp3 packaging (Fig. 3 and 4; data not shown). On the average, most of the gp7 had exited from particles of fractions 12 through 14, which contained about one third of the genome. When the *Hpa*I left-end fragment (4.1 Mdal) was packaged (Fig. 8), the gp7 had exited (data not shown), and the particles had the angularity of the DNA-filled head (Fig. 9). These results led to the hypothesis that in the presence of gp16 an early interaction of DNA-gp3 with the prohead triggers a conformational change(s) in the major head protein gp8. This change results in an abrupt transition to angularity and a rapid exit of gp7, events that are not necessarily correlated with the amount of DNA packaged. This model is consistent with the large number of empty heads observed in vivo and in vitro that are the result of abortive DNA-gp3 packaging. We are testing this model with smaller left-end DNA fragments and different methodology.

DNA packaging in λ, T7, T1, and Mu phages is believed to be oriented in the opposite direction of DNA ejection (18). Krawiec et al. (37) observed the spontaneous release of φ29 DNA-gp3 from $12^{-}13^{-}$ particles and reported that the *Eco*RI-A fragment tends to remain associated with the capsid protein. If this phenomenon reflects DNA injection during infection, then the orientation of packaging and ejection would be opposite for φ29 as well.

In adenoviruses, a terminal protein molecule is covalently bound to each 5′ end of the genome which contains 100-base-pair inverted

terminal repeats (6, 50). As with φ29, the protein is required for DNA replication and is postulated to prime new strand synthesis (14, 15). Selective capsid association and packaging of subgenomic DNA sequences in adenovirus types 3-, 7-, and 16-infected HeLa cells has been demonstrated (16, 21, 52). Restriction endonuclease digestion of DNA fragments extracted from CsCl-purified incomplete particles indicates that the DNA-protein complex is packaged from left to right on the genetic map (16, 21, 52). A sequence of DNA, approximately 290 to 390 base pairs from the left end of the genome, may be involved in the recognition of a packageable substrate (21). A role for the terminal protein in adenovirus DNA-protein packaging or assembly has not been demonstrated.

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