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Morphogenesis of Bacteriophage ϕ29 of *Bacillus subtilis*: Cleavage and Assembly of the Neck Appendage Protein

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Each of the 12 neck appendages of the *Bacillus subtilis* bacteriophage ϕ29 consists of a single protein molecule with a molecular weight of about 75,000, and on the mature virion the appendages are assembled to the lower of two collars. The appendage protein is cleaved from a precursor protein, P(J), with a molecular weight of about 88,000. This cleavage is independent of neck assembly, occurring during infection by mutants that cannot synthesize the proteins of the upper and lower collars of the neck. The cleaved form of the appendage protein is efficiently complemented in vitro to particles lacking appendages. Thus, cleavage of the appendage precursor protein apparently does not occur in situ on the maturing virus.

Proteolytic cleavage of proteins participating in viral morphogenesis is a widespread phenomenon, occurring in animal viruses (2, 15) and bacteriophages alike (10, 19, 29). Proteolytic modifications in bacterial viruses have been found to play a role in head morphogenesis, and in bacteriophage T4 at least four head proteins (P23, P22, P24, and IPIII) are cleaved during head formation (9, 14, 16, 17). Protein processing reactions may order assembly steps, limit the reversibility of these steps, or relate to DNA packaging (13). They may permit variations of bonding and hence different arrangements of the same protein in a lattice. Cleavage of the phage T4 major head protein (P23) has been found to occur during the conversion between two head precursor particles (18). The conservative mode of the transformation of T4 τ-particles into mature capsids has been interpreted to suggest that the cleavage and rearrangement of P23 occurs in situ on the maturing head (1, 6). Cleavage yielding a minor tail component of bacteriophage T5 has been reported (29).

In comparison with the above, the proteolytic modification of the neck appendage precursor protein of bacteriophage ϕ29 as described in this paper is unusual in that it apparently does not occur in situ on the maturing virus.

Twelve ϕ29 appendages are attached to the lower of two collars, and these structures together comprise the neck of the virus (3, 20). The appendage contains the bulk of the serum-blocking power and is in close contact with the bacterial wall during adsorption (28). Production of the neck appendage protein is under the control of gene J (5, 28), and preliminary genetic studies (unpublished joint data) indicate that this gene is identical with gene B of Moreno et al. (21). Evidence for a precursor-product relationship between the product of gene J, P(J), and the appendage protein has been reported (12) and confirmed by comparison of tryptic digests of these proteins (7). The present data support our previous hypothesis that protein LM2 is the second product of the cleavage reaction (12).

We shall present evidence that each of the 12 neck appendages of ϕ29 is composed of a single protein molecule. The proteolytic processing does not require interaction of the appendage precursor protein with a maturing neck structure, and only the cleaved polypeptide is assembled as a final step of ϕ29 morphogenesis.

MATERIALS AND METHODS

Chemicals and radioactive materials. The serylprotease inhibitor phenylmethylsulfonylfluoride (PMSF; Sigma Chemical Co., St. Louis, Mo.) was dissolved in 95% ethanol to give a stock solution of 6 mg/ml and used at a final concentration of 300 μg/ml. Egg white lysozyme (A grade), DNase I (B grade), and RNase (bovine pancreatic) were from Calbiochem.

The ^{14}C-labeled amino acid mixture (New England Nuclear Corp., NEC 445; 100 μCi/ml, about 200 mCi/mmol) and [^{3}H]amino acid mixture (NET 250; 1 mCi/ml, containing specific activities ranging from about 1 Ci/mmol for valine and serine to about 100 Ci/mmol for isoleucine) were adjusted to neutrality by addition of an equal volume of 0.1 N NaOH immediately before use.

Phage and bacteria. Phage ϕ29 (5) and a clear plaque mutant of spontaneous origin, ϕ29c (5), were employed. Suppressor-sensitive (*sus*) mutants of ϕ29 or ϕ29c were isolated after mutagenesis with hydroxylamine (25) or bromodeoxyuridine (5). The

cistron O mutants were isolated by the bromodeoxyuridine method (24). *sus* mutant M1241 from the collection of Moreno et al. (21) was kindly provided by E. Vinuelà. This mutant has a delayed lysis phenotype, and this mutation has been incorporated into several of our *sus* mutants by recombination.

The properties of the permissive host *Bacillus subtilis* L15 and the nonpermissive host *B. subtilis* SpoA12 have been described (25). The nonpermissive host *B. subtilis* 110NA (8) was obtained from E. Vinuela.

Media. Minimal medium M40 (23) was supplemented with 50 μg of tryptophan per ml (M40t medium). The phage were stored in TMS buffer (0.05 M Tris-hydrochloride, pH 7.8, 0.1 M NaCl, 0.01 M $MgCl_2$) or in antibiotic medium 3 (PB; Difco).

Growth conditions, UV irradiation, and infection. Protocols described previously (5, 12) were generally followed, and variations are detailed in Results. Bacteria were grown to 2×10^8 cells/ml, concentrated to 2×10^9 cells/ml by centrifugation, UV irradiated (50 ergs/mm^2 per s) in M40t when indicated, infected with a multiplicity of infection (MOI) of 40, and then diluted to 2×10^8 cells/ml with prewarmed (37 C) M40t medium (time 0) after a preadsorption period of 5 to 10 min at 22 C. The M40t medium was sometimes supplemented with 100 μg of Casamino Acids (Difco) during growth of the bacteria before infection and with 50 μg/ml after infection (M40ta medium). When mutants with the delayed lysis phenotype were used, host cells were grown in M40t containing 200 μg of Casamino Acids per ml and incubated after infection in M40t containing 100 μg of Casamino Acids per ml. M40ta medium containing 200 μg of Casamino Acids per ml was used in the preparation of extracts of infected cells for in vitro complementation.

Radioactive labeling of proteins. UV-irradiated, infected cells in either M40t or M40ta medium were incubated at 37 C on a gyratory shaker. ^{14}C-labeled amino acids were added at the times and in the amounts indicated for each experiment, usually 2 to 5 μCi/ml for continuous labeling and 15 to 20 μCi/ml for pulse labeling. Viral replication was terminated by diluting the culture into an equal volume of an iced solution containing a chloramphenicol (CAM), sodium azide, and PMSF mixture as described (12). In pulse-chase experiments the radioactivity was chased by addition of Casamino Acids to a final concentration of 1%. Postchase samples were diluted into CAM mixture or frozen in a CO_2-ethanol bath and later thawed at 0 C. The cultures were separated into pellet and supernatant fractions, unless otherwise indicated, and prepared for electrophoresis as described previously (12).

Isolation of viral structures. Cells were grown in M40ta medium and infected at an MOI of 20. The phage were adsorbed for 5 to 10 min at 22 C, and unadsorbed phage were removed by centrifugation. The infected cells were resuspended in prewarmed (37 C) M40ta medium at time 0, labeled with a mixture of ^{14}C-labeled amino acids at 5 min, collected at 35 min on membrane filters (pore size, 0.45 μm; Millipore Corp.), and resuspended in a volume of TMS equivalent to 1/10 of the volume of the original culture. Under conditions of delayed lysis the infected cells (5 ml) were labeled with ^{14}C-labeled amino acids at 35 min, collected by centrifugation at 110 min, and resuspended in 0.1 ml of TMS containing 2 mM sodium azide, 120 μg of PMSF per ml, and 750 μg of lysozyme per ml. After 5 to 10 min of lysozyme treatment at 22 C the cell suspension was placed on ice, and complete conversion to protoplasts was verified by phase-contrast microscopy. The protoplasts were brought to room temperature, and putrescine was added to a final concentration of 300 μg/ml. Nonionic detergent Nonidet P-40 (Shell trademark, Particle Data Laboratories, Elmhurst, Ill.) was then added (18) to a final level of 0.05%, resulting in lysis of the protoplasts. The viscosity was reduced by treatment with DNase I (50 μg/ml) and RNase (25 μg/ml) for 5 min at 22 C. Debris was removed immediately by centrifugation at 12,000 $\times$ *g* for 10 min at 22 C, and the entire supernatant was immediately layered on a sucrose gradient and centrifuged.

Sucrose gradient centrifugation. Solutions of 5% (wt/vol) and 20% (wt/vol) sucrose in TMS buffer containing 10^{-3} M sodium azide were used to form 5 to 20% linear gradients in 5-ml cellulose nitrate tubes. The centrifugation parameters are reported in the figure legends. Fractions were collected (40 fractions/tube), and for scintillation counting 10 or 20 μl of each fraction was dissolved in 0.5 or 1 ml of absolute ethanol, respectively, and then in 10 ml of toluene containing 4 g of 2,5-diphenyloxazole and 100 mg of 1,4-bis-[2]-(5-phenyloxazolyl)benzene per liter. PMSF (150 μg/ml) was added to other portions of fractions to be examined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.

Electron microscopy. Grids with carbon-coated Formvar films were floated on drops of the sucrose gradient fractions. After 5 to 10 min, the grids were rinsed by transferring them successively through single drops of 10% sucrose in TMS and 5% sucrose in TMS and water and then were stained with 2% neutral sodium phosphotungstate (28). The specimens were observed in a Philips EM301 electron microscope at 80 kV. For observations of fragile structures, a sample of the fraction from the sucrose gradient was fixed by addition of glutaraldehyde (8%; Polysciences, Warrington, Pa.) to a final concentration of 0.2% immediately after fractionation of the gradient.

Electrophoresis. Proteins were separated by SDS-gel electrophoresis as described previously (12). Standard 12% polyacrylamide slab gels had a thickness of 1.5 mm and a length of 9 cm. For better resolution of proteins in the molecular weight range of the collar proteins, and of the double band of the appendage protein (12), 12 to 20% linear gradient polyacrylamide gels with a length of 23 cm were used. All gels were run with a constant current of 17 mA. Gels were fixed, stained, and destained as described (12).

Protein composition of the virion. *B. subtilis* SpoA12 was grown in either M40t or M40ta medium and infected at an MOI of 10. After a 5-min preadsorption period at 22 C, prewarmed (37 C) M40t medium was added (designated as time 0). Four pro-

cedures were then used to obtain ^{14}C- or ^{3}H-labeled virions. (i) ^{14}C-labeled amino acids (2 μCi/ml) were added at time 0; (ii) the M40t medium was supplemented at time 0 with Casamino Acids (100 μg/ml), and ^{14}C-labeled amino acids (2 μCi/ml) were added at 10 min; (iii) the M40t medium was supplemented at time 0 with Casamino Acids (100 μg/ml), and [^{3}H]amino acids were added in two equal portions at 12 and 17 min to give a final concentration of 4 μCi/ml; (iv) the cells were prelabeled for at least one generation with 5 μCi of D-[U-^{14}C]glucose (ICN, 200 mCi/mmol) by growth in a modified M40t medium in which the concentration of unlabeled glucose had been reduced to 0.1%. After infection and preadsorption, the cells were resuspended in the same radioactive medium, and after 5 min an additional amount of [^{14}C]glucose was added to give a final concentration of 10 μCi/ml. In each case the incorporation of radioactivity into cold trichloroacetic acid-insoluble material was determined at different times after infection by placing culture samples on Whatman 3MM paper disks and treating the disks successively with ice-cold 10% trichloroacetic acid, ice-cold 5% trichloroacetic acid, and ether. After labeling with [^{14}C]glucose, hot trichloroacetic acid-insoluble radioactivity (90 C, 30 min) was also determined. Virions were purified from lysates by banding in CsCl gradients as described previously (3). Samples of the purified phage preparations containing approximately 15,000 ^{3}H or ^{14}C counts/min and about 2×10^{10} PFU were dissociated in SDS and analyzed by electrophoresis on polyacrylamide slab gels as described above. Radioactivity in the resulting protein bands was quantitated either by tracing autoradiographs (12) or by cutting and analyzing fractions of the gels. In the latter, each profile was cut into 1.5-mm fractions with a razor blade, using a supporting block with spacings. Each fraction was placed in a scintillation vial and covered with 20 μl of water. To each vial 0.5 ml of the sample solubilizer Soluene-100 (Packard Instrument Co., Downers Grove, Ill.) was added, and the vials were tightly capped and incubated for at least 12 h at 55 C. This treatment did not dissolve the gel fractions but solubilized the radioactivity quantitatively. Scintillation fluid {toluene-2,5 - diphenyloxazole-1,4 - bis - [2] - (5 - phenyloxazolyl)benzene} was added (10 ml/vial) before counting. Similar polyacrylamide gel fractions were also analyzed by combustion in a Packard Tri-Carb oxidizer, model 306.

In vitro complementation of defective lysates. Recombinants were constructed with representative *sus* mutants of each of the neck cistrons I, O, or J and the delayed lysis mutant M1241 (21). Cells infected at an MOI of 10 were prepared and concentrated 70- to 100-fold in TMS-sodium azide buffer by the method used for the isolation of viral structures. The concentrated cells were converted to protoplasts by treatment with lysozyme (700 μg/ml for 10 min at 22 C). Controls and complementation mixtures containing 20 μl of each protoplast preparation, 2.5 μl of putrescine (6 mg/ml), and 2.5 μl of Nonidet P-40 (1% in water) were incubated for 2 h at 37 C. To assay complementation, 1 ml of PB containing 20 μg of DNase I was added to each sample, the mixtures were incubated for 30 to 60 min at 37 C, and samples were then plated in the standard bacteriophage assay. The genotypes of these viruses were determined by qualitative complementation (25).

RESULTS

Protein composition of the neck appendages. Twelve morphologically distinct appendages are components of the neck of the ϕ29 virion. To determine the protein composition of the appendages, infections of *B. subtilis* with wild-type ϕ29 in the presence of ^{14}C-labeled or [^{3}H]amino acid mixtures or D-[^{14}C]glucose yielded labeled virions that were purified, dissociated, and analyzed quantitatively for constituent proteins after separation by SDS-polyacrylamide gel electrophoresis. During the preparation of each of the four phage samples, the incorporation of radioactivity was linear throughout the period of phage production. Addition of unlabeled amino acids to the growth medium affected total phage production but did not alter the relative distribution of label among the viral proteins (data not shown). The ratio of the appendage protein to the total protein content of the virion is given in Table 1. Assuming a molecular weight of 19 million for the virus (26), a molecular weight of 11 million for the DNA (4), and a molecular weight of 75,300 for the appendage protein (12), the number of appendage protein molecules per virus approximates the number of morphologically distinct appendages observed by electron microscopy.

TABLE 1. *Copies of the appendage protein in the ϕ29 virion*

Radioactive labeling	% of total virion protein	No. of copies[a]
Amino acid mixture	12.0 ± 1.7[b]	12.7 ± 1.8
Glucose	12.3 ± 0.4[c]	13.1 ± 0.4

[a] Calculated using a molecular weight of 19×10^6 for the virion (26), a molecular weight of 11×10^6 for the DNA (4), and protein molecular weights as previously reported (12).

[b] Mean value was obtained by use of three phage preparations labeled with mixtures of ^{14}C-labeled amino acids or of [^{3}H]amino acids as described in Materials and Methods.

[c] Mean value was obtained by use of four samples of one phage preparation labeled with D-[^{14}C]glucose as described in Materials and Methods. Four profiles from the same slab gel were fractionated, and the radioactivity was determined after combustion in a Packard sample oxidizer, model 306.

Cleavage of the neck appendages from a precursor. The precursor-product relationship of the φ29 protein P(J) and the structural appendage protein Ap was suggested by the observation that synthesis of protein P(J) (molecular weight, about 88,000) begins between 8 and 10 min after infection, whereas the protein Ap (molecular weight, about 75,000) and a smaller protein LM2 (apparent molecular weight, about 18,000) are first detected at 10 to 12 min after infection (12). This precursor role of P(J) was confirmed by use of pulse-chase experiments (Fig. 1). After a radioactive pulse from 12 to 15 min after infection, only small amounts of proteins Ap and LM2 were detected. At intervals after chase of the radioactive pulse, the P(J) band progressively decreases, with concomitant increases of the Ap and LM2 bands. As in previous studies (5, 12) our electrophoretic analysis has been performed separately for the supernatant and the cell pellet fractions, because UV-irradiated, φ29-infected cells are fragile under the conditions employed to terminate viral production and proteolytic processing. In Fig. 2 we show the kinetics of the conversion of the precursor protein P(J) into the Ap and LM2 products as obtained by microdensitometric tracing and quantitation of the same autoradiograph shown in Fig. 1. The values obtained for the corresponding P(J), Ap, and LM2 bands of the supernatant and the cell pellet profiles were added and normalized to the value obtained for the major head protein Hd. The radioactivity in the LM2 band reaches a value of about 20% of the radioactivity in the Ap band or about 16% of the radioactivity in the P(J) band, in reasonable agreement with the ratio of the molecular weights of these proteins. From this and similar pulse-chase experiments we estimate that at about 15 min after infection the half-time of the conversion of protein P(J) into proteins Ap and LM2 is about 3 min. If the radioactive pulse is made from 24 to 27 min after infection when synthesis of structural proteins reaches its maximum rate (7, 12), the conversion is considerably faster (Fig. 3). Immediately after a 2-min pulse more than 50% of the label is already in the Ap band. Five minutes postchase the radioactivity from the P(J) band has almost totally moved into the Ap band. We have performed similar pulse-chase experiments in which either CAM or the serylprotease inhibitor PMSF was added at the time of the chase and have found that the conversion of protein P(J) into proteins Ap and LM2 was not affected (data not shown).

The extent of this proteolytic cleavage was then examined in restrictive infections with *sus* mutants, which are unable to assemble neck or tail structures. Mutants in gene I are defective in synthesis of the upper collar protein C1 and produce very little of the lower collar protein C2 (5, 24). Infection with these mutants leads to the accumulation of isometric head-related structures that are devoid of DNA and lack neck or tail components. Infection with mutants in gene O, defective in lower collar protein synthesis, leads to the production of normal appearing, DNA-containing heads with the upper collar but with no other neck or tail component (manuscript in preparation). With both I and O mutants, the site of appendage attachment, the lower collar, is not assembled. Figure 4 shows that the precursor P(J) does not accumulate in restrictive infections with *sus* mutants defective in neck formation, and that the extent of cleavage is comparable to that observed in the wild-type infection, as judged from the production of proteins Ap and LM2. The same autoradiograph shows that the upper collar protein C1 is missing and the lower collar protein C2 is produced only in traces in the *sus* I302 restrictive infection, whereas C1 is produced but C2 is missing in the *sus* O683 infection (see also reference 24). The bands between proteins C1 and C2 are due to the viral protein P(L) (5) and to a host protein, respectively.

Assembly of neck appendages. The following experiments will show that, in the absence of the appendage protein, all of the other neck and tail structures are assembled and that only the cleaved appendage protein cosediments with nascent and infectious phage particles.

Figure 5A shows the sedimentation in a sucrose gradient of capsids and DNA-containing φ29 particles produced in a restrictive infection with the delayed lysis mutant *sus* M1241 from the collection of Moreno et al. (21). Infectious φ29 particles accumulate in *sus* M1241-infected cells, resulting in a burst of at least 500 PFU/cell at about 2 h after infection in M40ta enriched medium. Electron microscopy of the fractions under peaks I and II demonstrated that peak I contained φ29 particles and peak II contained empty heads (data not shown). The infectivity was found entirely in peak I. Figure 5B shows the sedimentation of structures assembled in a nonpermissive infection with the double mutant *sus* J305-M1241, which is defective for the appendage precursor protein, and also exhibits the delayed lysis phenotype. Empty heads were found at the same position as peak II of the J^+ profile from the infection with phage *sus* M1241, whereas DNA-containing

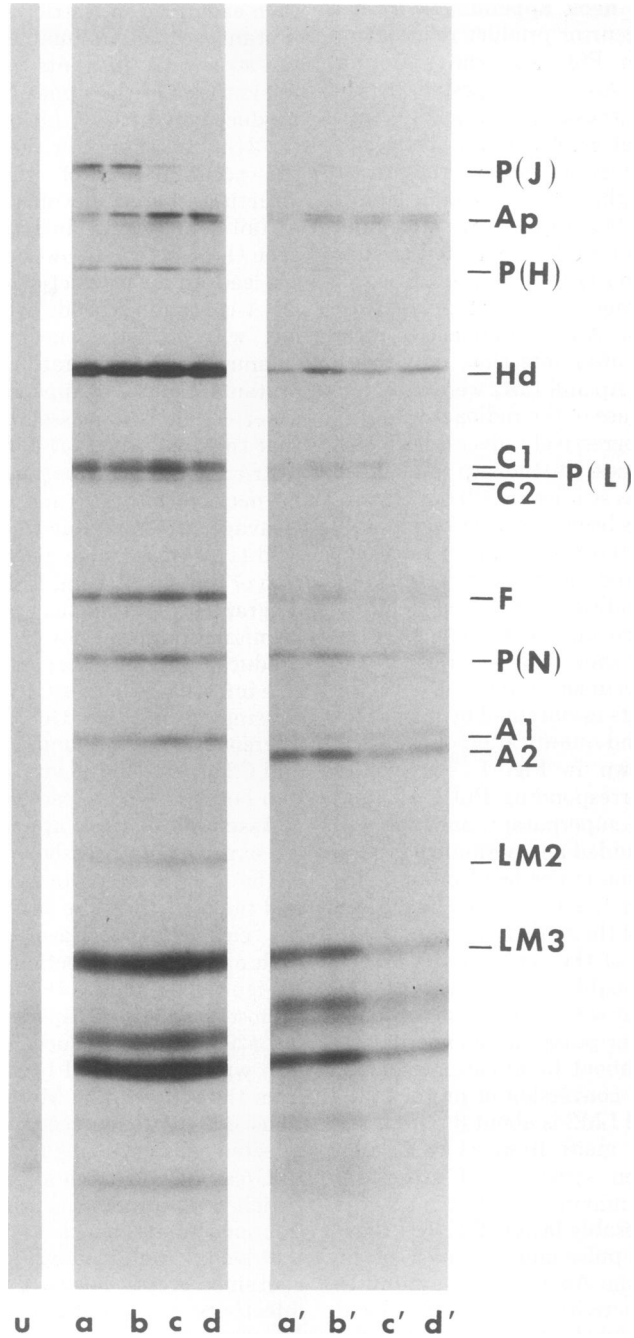

FIG. 1. *Autoradiograph of ^{14}C-labeled ϕ29 proteins demonstrating cleavage of protein P(J) to yield proteins Ap and LM2. B. subtilis SpoA12 was UV irradiated for 10 min, as described in Materials and Methods, and infected with wild-type ϕ29 at an MOI of 50. A ^{14}C-labeled amino acid mixture (20 μCi/ml) was added 12 min after infection and chased 3 min later with a 1,000-fold excess of unlabeled amino acids. An uninfected control culture was pulse labeled, and the radioactivity was chased in the same way. Profiles (a) through (d) are of trichloroacetic acid precipitates of the supernatant fractions of infected cultures, and profiles (a′) through (d′) represent the corresponding cell pellet fractions. (a and a′) Immediately after chase; (b and b′) 2 min after chase; (c and c′) 10 min after chase; (d and d′) 25 min after chase; (u) cell pellet of the uninfected control culture.*

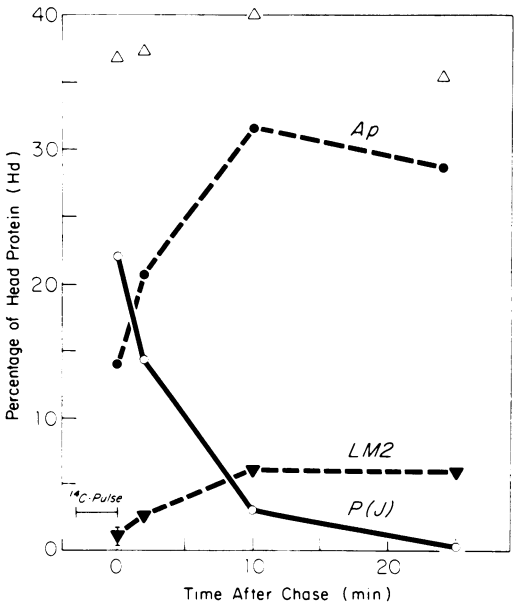

FIG. 2. *Quantitative analysis of the conversion of protein P(J) into proteins Ap and LM2. Microdensitometric tracings of the autoradiograph shown in Fig. 1 were cut and weighed. The values obtained for the corresponding protein bands in the cell pellet and supernatant fractions were added and normalized to the major head protein. Symbols:* (○) *P(J);* (●) *Ap;* (▼) *LM2;* (△) *P(J) + Ap + LM2.*

particles from the J⁻ infection sedimented slightly slower than particles in peak I of the *sus* M1241 profile and apparently lacked only the neck appendages (Fig. 6B). The infectivity in this peak of appendageless particles reached a maximum of 2×10^8 PFU/ml, as compared to 2.4×10^{10} PFU/ml in the corresponding peak obtained from the *sus* M1241 infection (Fig. 5A). A sedimentation profile similar to that shown in Fig. 5B has been obtained with mutant *sus* J716 (data not shown). Figure 6 illustrates the morphology of fast sedimenting viral structures obtained in nonpermissive infection by *sus* mutants in gene J. Figure 6A shows fast sedimenting particles from a *sus* J716 infection in which the cells were concentrated and lysed at 40 min after infection. At this time significant numbers of particles lacking only the appendages had been produced. Heads without tail, but with a small structure centered below the upper collar, were also observed (see arrows). In contrast, when viral assembly was continued for 2 h, as in the *sus* J305-M1241 infection described in Fig. 5B, the fast sedimenting peak consisted exclusively of particles lacking only the neck appendages (Fig. 6B).

To determine the composition of phage particles produced when the appendage precursor protein P(J) is synthesized but the rate of cleavage is reduced, we have used infection with the mutant φ29c, in which the accumulation of protein P(J) has been demonstrated (5, 24). An estimate of the inhibition of cleavage, obtained with a pulse-chase experiment, is shown in Fig. 7. No conversion of protein P(J) into proteins Ap and LM2 was detected, and similar results were obtained with two different doses of UV irradiation of the host cells (3 and 6 min) prior

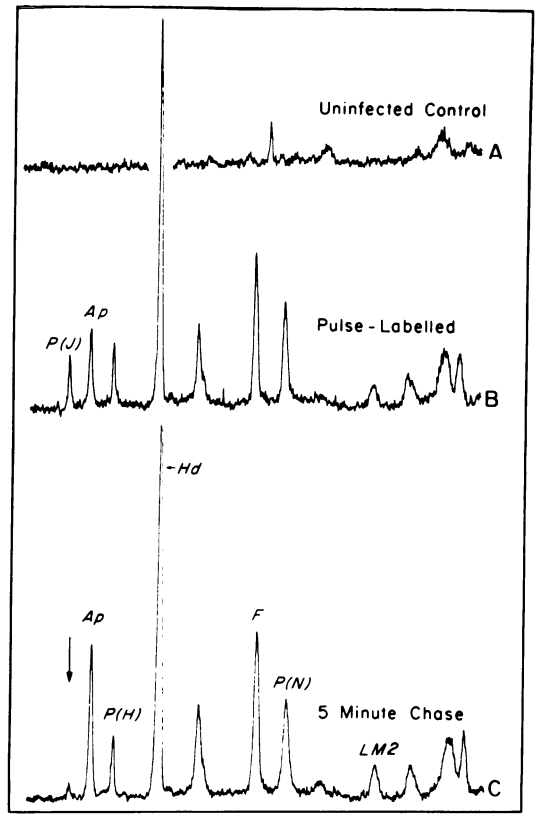

FIG. 3. *Cleavage of protein P(J) late in infection. Cells were UV irradiated for 4 min, infected with wild-type φ29 (MOI of 40) and pulse labeled with* ^{14}C*-labeled amino acids (20 μCi/ml) from 24 to 26 min after infection. A portion of the culture was quickly frozen in* CO_2*-ethanol immediately after the chase (designated pulse labeled). Another portion was frozen 5 min later (designated 5-min chase). A parallel uninfected control culture was labeled in a similar way and frozen immediately after the chase. The frozen cells were thawed at 0 C and treated with lysozyme (300 μg/ml) for 2 h at 0 C. Lysis was completed by feezing and thawing the samples five times. The lysates were treated for 30 min on ice with RNase (10 μg/ml), concentrated by precipitation with 7% trichloroacetic acid, and analyzed by electrophoresis.*

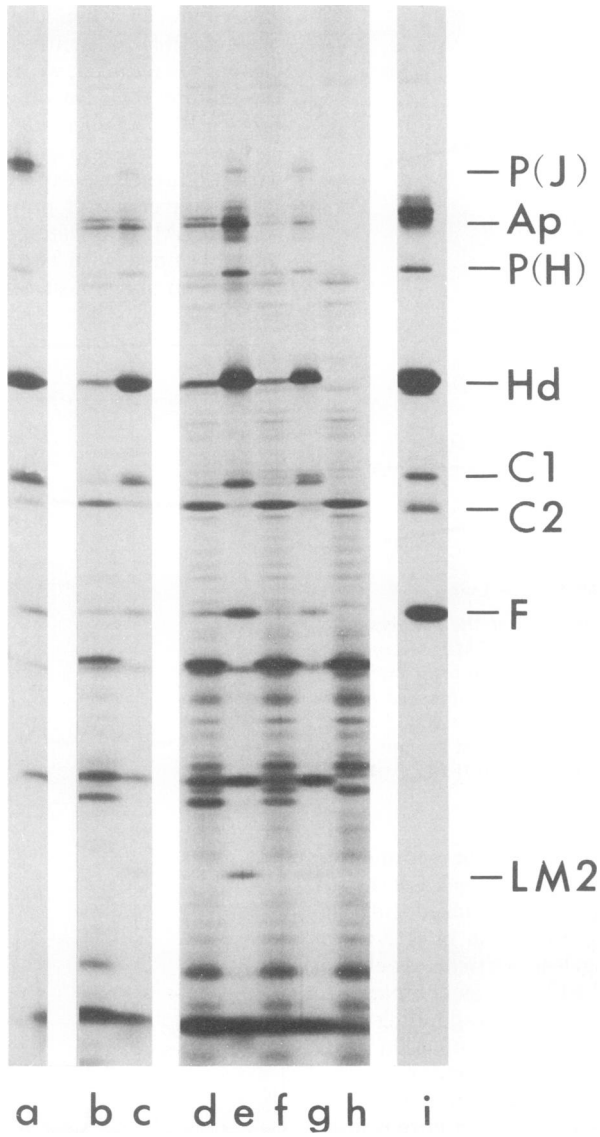

FIG. 4. *Autoradiograph of ^{14}C-labeled ϕ29 proteins produced in restrictive infections with representative sus mutants in genes I and O. B. subtilis SpoA12 was UV irradiated for 7 min, infected and labeled throughout infection with a mixture of ^{14}C-labeled amino acids. Cell pellet fractions and trichloroacetic acid precipitates of the infected culture supernatants were analyzed by SDS-gel electrophoresis on a linear gradient of 12 to 19% polyacrylamide. Profiles (b), (d), (f), and (h) represent cell pellet fractions. Profiles (a), (c), (e), and (g) represent trichloroacetic acid precipitates of supernatants of the infected cultures. (a) sus O771c; (b and c) sus O683; (d and e) sus I302; (f and g) wild-type ϕ29; (h) uninfected control; (i) purified ϕ29 virions.*

to infection. The precursor protein P(J) slowly disappears without apparent conversion into the normal products Ap and LM2 at late postchase times that are close to the time of lysis. The burst size of ϕ29c in unirradiated cells varied between about 10 to 50% of that of the wild type, depending on the composition of the medium (M40t or PB). We therefore asked whether the correlation between low burst size and reduction of cleavage would reflect a different role of the P(J) and Ap proteins in the assembly process. Phage from a lysate of ϕ29c-infected, unirradiated cells were concentrated by high-speed centrifugation and sedimented in a sucrose gradient, yielding the results shown in Fig. 8. Fractions were assayed for infectivity

and analyzed for viral proteins and for viral structures by gel electrophoresis and electron microscopy, respectively. The infectivity cosedimented with the peak of radioactivity, which is centered around fraction 14. No fractions of this peak contained the appendage precursor protein P(J). The shoulder at fractions 17–20 was mainly due to bacterial flagella, as determined by electron microscopy and by the presence of a dense host band in the electrophoretic profiles. Only traces of the viral head proteins Hd and F (5, 12) were found in this shoulder. Fractions 13 through 16 were also examined by electron microscopy, and typical particle morphology is illustrated in Fig. 9. Phage particles completely lacking appendages but having an otherwise normal appearance were a significant component, and these particles seem to be identical with those from the *sus* J716 and *sus* J305-M1241 infections (Fig. 6). Quantitative analysis, performed by tracing autoradiographs of the electrophoretic profiles of these fractions, has shown that the amount of the cleaved protein Ap varied between 65 and 90% of the normal stoichiometric amount (Fig. 8, φ29 profile). This quantitative analysis has been performed on fractions from two similar sucrose gradients, using as a reference CsCl-purified wild-type virus (3) labeled under the same conditions. Loss of appendages during purification of the wild-type virus has never been observed under comparable conditions. On the contrary, mature particles with a full complement of appendages are very stable. Appendages can be removed only by use of rather severe treatments such as dimethyl sulfoxide at a concentration higher than 70%, which also removes the head fibers and the tail (20, 27).

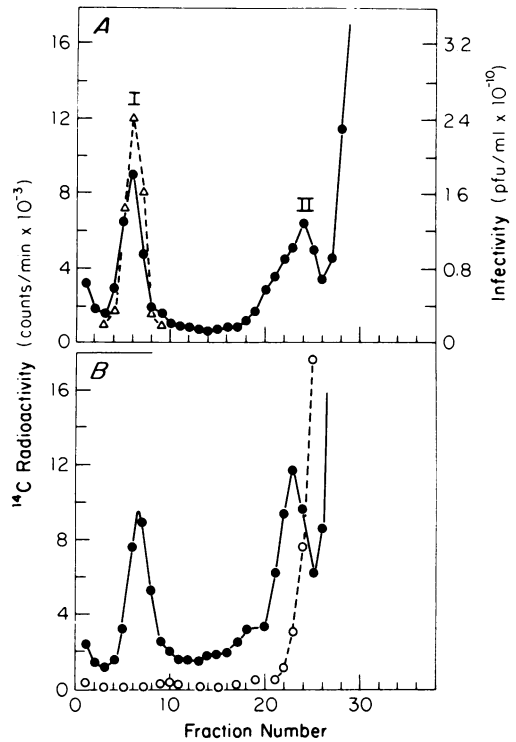

FIG. 5. *Sucrose gradient profiles of lysates from infection of B. subtilis SpoA12 by the delayed lysis mutant sus M1241 (A) and by the double mutant sus J305-M1241 (B), respectively. The infected cultures and the uninfected control culture were lysed under controlled conditions as described in Materials and Methods. Centrifugation of the 5 to 20% sucrose gradients was at 22 C in a Spinco SW50.1 rotor at 35,000 rpm for 30 min. The ^{14}C radioactivity was determined in 20-μl samples from each fraction. Symbols: (●) sus M1241 (A) and sus J305-M1241 (B); (△) infectivity; (○) uninfected control.*

In vitro complementation between extracts defective in neck proteins. We have shown that the protein P(J) is cleaved in nonpermissive infections by *sus* mutants in genes I and O, which are defective in the synthesis of both collar proteins or the lower collar protein, respectively (Fig. 4). To determine whether the cleaved appendage protein produced in these infections was biologically active, we developed an in vitro complementation assay. Table 2 shows that particles of J$^-$ lysates [defective for the appendage precursor protein P(J)] were efficient acceptors in vitro of appendage protein donated by I$^-$ or O$^-$ lysates. In each case, the newly formed infectious particles had the J$^-$ genotype. CAM (300 μg/ml) had no significant effect on the level of complementation. The generally lower PFU values obtained from comparable numbers of infected cells when *B. subtilis* SpoA12 was the host (Table 2, column B) probably reflect a lack of expression of the delayed lysis phenotype of the *sus* M1241 mutation in this strain. Complementation resulted in a 100- to 600-fold increase in titer over the background, defined as one-half the value indicated in Table 2 for the J$^-$ extracts alone. The phage titers obtained in the mixed extracts I$^-$ + J$^-$ and O$^-$ + J$^-$ compare well with the PFU obtained in vivo from mixed infection with the mutants *sus* I302 and *sus* J305. Among these in vivo progeny we have found similar proportions of the I$^-$ and J$^-$ parental genotypes, as well as a low but significant level of recombination. The predominance of the J$^-$ genotype in progeny obtained in our in vitro assay is additional evidence that in vitro complementation occurred.

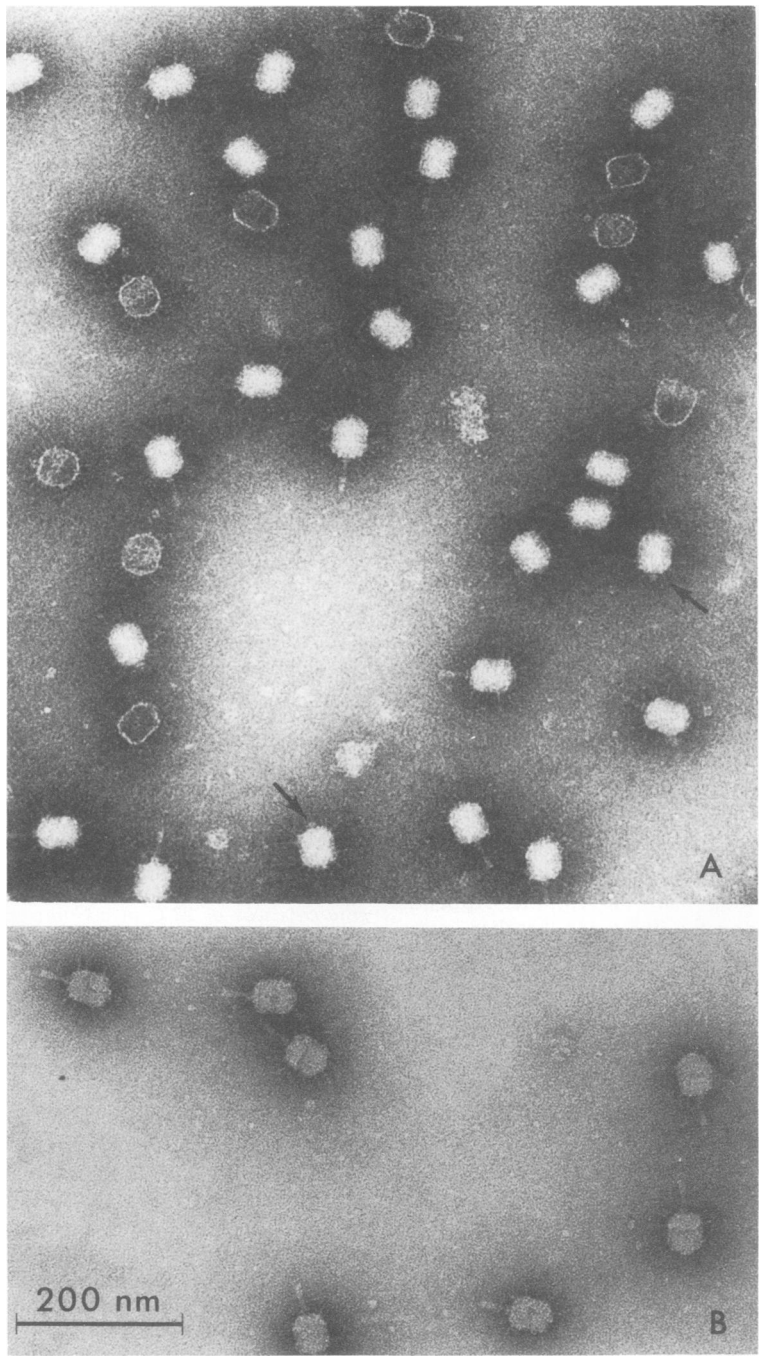

FIG. 6. *Electron micrographs of intermediates in assembly produced in nonpermissive infection with the appendage mutants (A) sus J716 and (B) sus J305-M1241. These particles were purified as described in Fig. 5 and stained without fixation. The magnification bar represents 200 nm.*

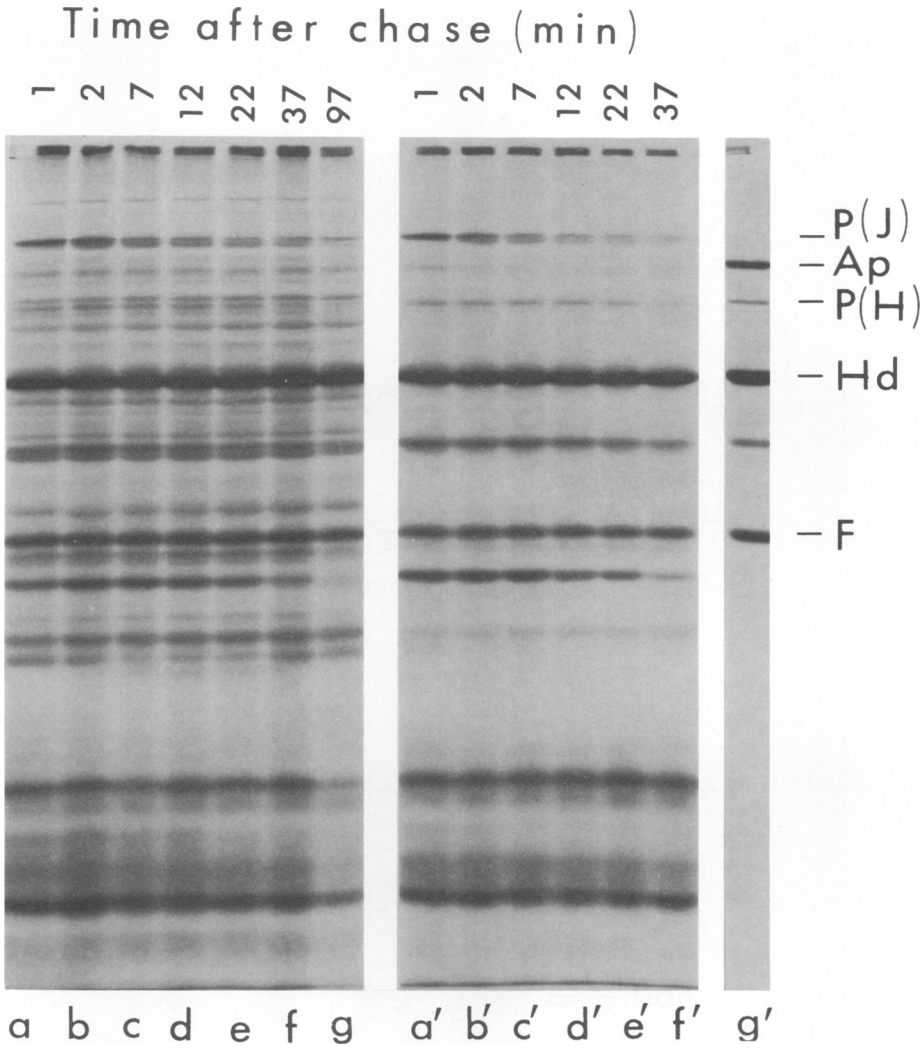

FIG. 7. *Autoradiograph of ^{14}C-labeled φ29 proteins showing the fate of pulse-labeled P(J) produced in φ29c infection. B. subtilis SpoA12, UV irradiated for 3 or 6 min as described in Materials and Methods, was infected with φ29c at an MOI of 50. Adsorption was at 22 C for 10 min. The infected cultures were then incubated with agitation at 37 C and pulse labeled 20 min later with a mixture of ^{14}C-labeled amino acids (15 μCi/ml). The radioactivity was chased with a 1,000-fold excess of Casamino Acids 3 min later. Culture samples were transferred into an iced CAM-sodium azide-PMSF solution at the time indicated below and processed for SDS gel electrophoresis as described (12). The profiles represent trichloroacetic acid precipitates of whole lysates. Total trichloroacetic acid-precipitable radioactivity was determined for all postchase samples, and in the 3-min UV samples it was constant until 37 min postchase; in the 6-min UV sample it was constant until 12 min postchase. Profiles (a) through (g) and profiles (a') through (f') represent lysates of cells infected after 3 or 6 min of UV irradiation, respectively. (a and a') One minute after chase; (b and b') 2 min after chase; (c and c') 7 min after chase; (d and d') 12 min after chase; (e and e') 22 min after chase; (f and f') 37 min after chase; (g) 97 min after chase; (g') purified φ29 virions.*

DISCUSSION

When Mendez et al. (20) examined the protein composition of substructures obtained by partial dissociation of φ29 virions, the neck appendages were identified with one protein band in the SDS-polyacrylamide gel electrophoretic profile. In determining the ratio of the appendage protein Ap to the total protein content of the virion, the doublet band of Ap shown in Fig. 1 was considered one protein

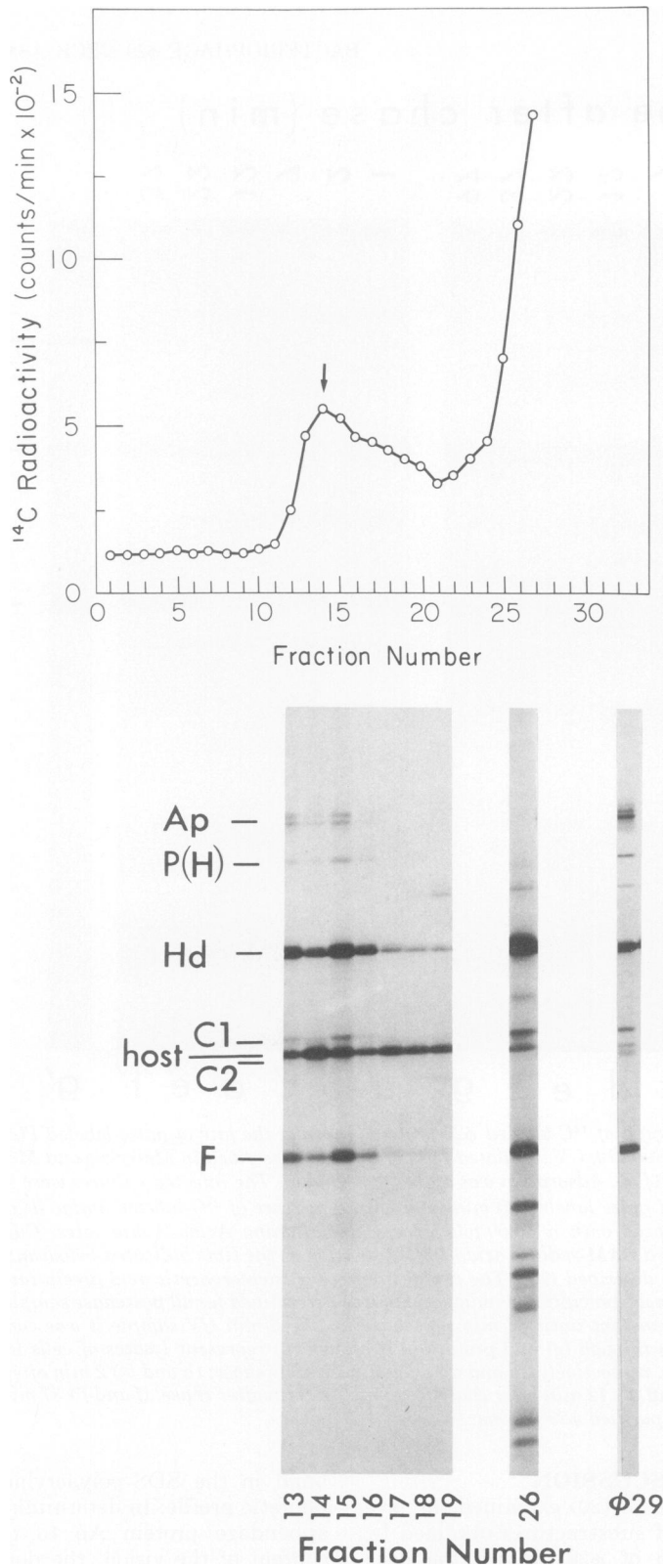

FIG. 8. *Protein composition of purified virions produced in infection with mutant φ29c. B. subtilis SpoA12 was grown in M40t medium, infected with φ29c, and labeled with* ^{14}*C-labeled amino acids as described in Materials and Methods for the radioactive labeling of φ29 virions. The same procedure was followed as for the*

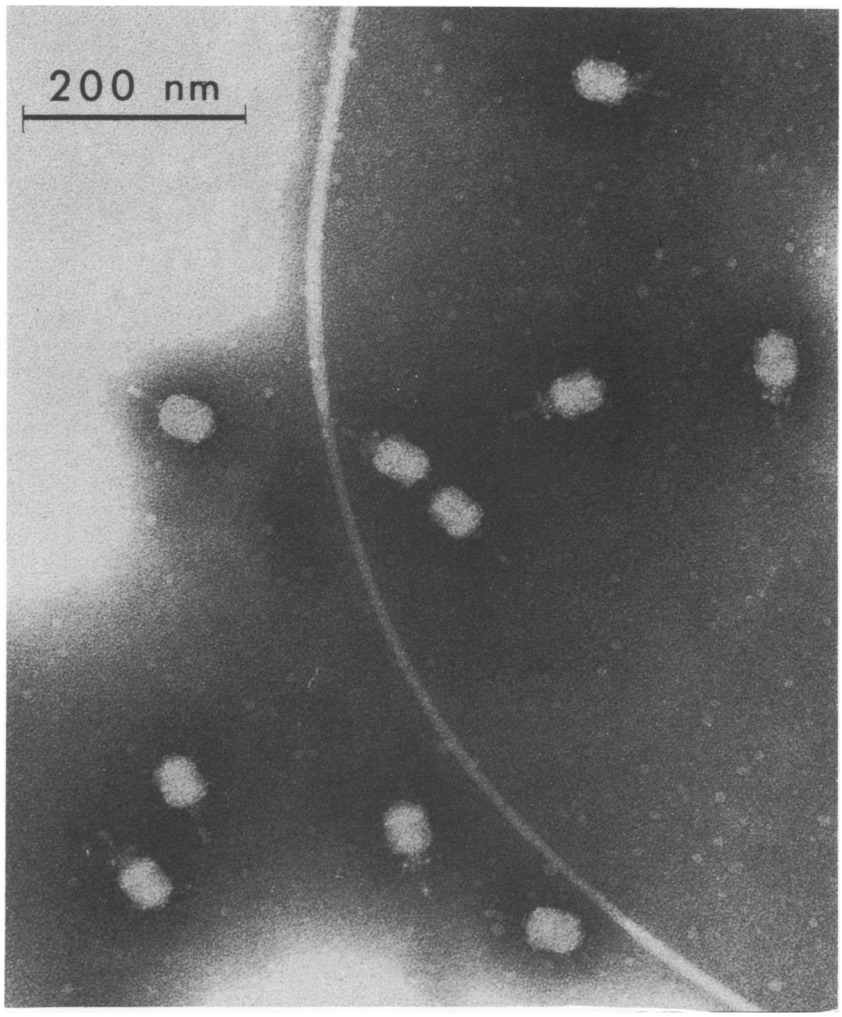

FIG. 9. *Electron micrograph of φ29c particles from fraction number 14 of the sucrose gradient described in Fig. 8. The sample was negatively stained without fixation. The magnification bar represents 200 nm.*

species. The calculated numbers of appendage protein molecules per virion reported in Table 1 probably represent an upper limit because of our choice of values for total protein weight of the virion and for the molecular weight of the appendage protein (12, 26). The data indicate that each morphologically distinct appendage unit is composed of one polypeptide of about 75,000 to 80,000 molecular weight (12, 20, 22). If the appendage unit as observed in the electron microscope (3) is considered ellipsoid, with a length of 10 nm and a width of 5 nm, and it is assumed to consist of protein with a partial specific volume of 0.73 cm^3/g (26), then the calculated weight of each appendage unit falls in the same range.

The production of the appendage protein is a function of cistron J (5), and we have reported a precursor-product relationship between the product of cistron J, P(J), and both the append-

purification of wild-type virions, but after differential centrifugation the high-speed pellet was resuspended in TMS buffer and then sedimented at 4 C in a 5 to 20% sucrose gradient for 30 min at 38,000 rpm in a Spinco SW65 rotor. Proteins in fractions from the sucrose gradient were concentrated by precipitation with trichloroacetic acid and analyzed by SDS-gel electrophoresis on a 12 to 20% linear gradient of polyacrylamide. Gel profiles of fractions 13 through 19 and of fraction 26 are shown, together with the profile of virions obtained from wild-type infection under the same conditions of growth and labeling.

TABLE 2. *In vitro complementations*

Extracts[a] mixed	PFU[b] × 10^{-7}		Genotype[c] (%)
	A	B	
I^-	2.0	0.35	I^- (100)
O^-	22.5	2.0	O^- (100)
J^-	5.0	0.3	J^- (100)
$I^- + O^-$	19	2.0	
$I^- + J^-$	260	75	J^- (99)
$O^- + J^-$	330	91	J^- (98)
Mixed infection[d]			
I^-, J^-	460		I^- (47), J^- (50)
M1241	4,300		

[a] The double *sus* mutants were: I302-M1241, O683-M1241, and J305-M1241. M1241 has a delayed lysis phenotype.

[b] The results of two experiments are presented, in which the extracts for complementation were produced in (A) *B. subtilis* NA110 and (B) *B. subtilis* SpoA12, respectively. In experiment A chloramphenicol (300 μg/ml) was present in the complementation mixtures. For plating, the suppressor strain *B. subtilis* L15 was used in each case. Production of viable phage is expressed as PFU/6 × 10^8 to 8 × 10^8 cell equivalents in the complementation mixtures.

[c] Determined by qualitative complementation (25). We have tested at least 130 clones, transferred at random from each in vitro assay.

[d] The MOI was 10 for each mutant.

age protein Ap and a low-molecular-weight product, LM2 (12). Infection with a mutant having a clear plaque (c) phenotype, φ29c, permits the uncleaved P(J) to accumulate and causes a parallel reduction in burst size (5). Carrascosa et al. (7) have confirmed and extended these observations. They have demonstrated the relationship of P(J) to Ap (their proteins P-NP1 and NP1) both in pulse-chase experiments and by comparison of peptides obtained from tryptic digests. Our results shown in Fig. 1, 2, and 3 are in agreement with theirs, although Carrascosa et al. (7) did not report evidence of the fragment we have named LM2 (12). An accumulation of the precursor protein P-NP1, similar to the accumulation of P(J) reported in Fig. 7, occurs in nonpermissive infection with temperature-sensitive mutants of cistron B of the map of Moreno et al. (7, 21). This cistron corresponds to cistron J of our genetic map (25; joint unpublished data).

We have developed evidence supporting the model that proteolytic cleavage of the appendage precursor protein P(J) occurs prior to assembly and does not occur in situ on the maturing particle. In summary, (i) proteolytic activity is expressed soon after the onset of phage structural protein synthesis at 10 to 12 min after infection (Fig. 5 and 6 of reference 12), and viral structures can be detected in thin sections only at about 20 min after infection (unpublished data); (ii) this proteolytic activity is expressed in the absence of production of neck proteins (Fig. 4); (iii) there is efficient in vitro assembly of appendage protein cleaved in vivo in the absence of neck assembly (Table 2); and (iv) affinity of the uncleaved protein for maturing viral particles is not detected, at least in infection with φ29c (Fig. 3). Furthermore, P(J) is cleaved in infection by *sus* mutants in the cistrons A, C, and E (5) that are DNA negative and are apparently blocked at early stages of viral assembly (unpublished data).

Restrictive infection with cistron J *sus* mutants in which protein P(J) is not produced yields particles that appear to lack only the neck appendages. The in vitro complementation data of Table 2 support the conclusion that these particles are true intermediates in φ29 assembly. The efficiency of in vitro complementation of these particles by proteins cleaved in the absence of the neck structure is evidence that cleavage and assembly can occur efficiently as uncoupled reactions.

Limited cleavage of P(J), as in infection with φ29c, results in the production of a limited number of particles containing cleaved appendage protein but no precursor protein P(J); however, both SDS-polyacrylamide gel electrophoresis and electron microscopy have shown that the average number of appendages on these particles is less than 12. The great majority of particles had either a full complement of appendages or no appendages after isolation on sucrose gradients and staining for electron microscopy, and thus assembly of appendages may be a highly cooperative process, with the limited quantity of cleaved protein only used to produce particles with a full complement of appendages. However, intrinsic instability of the particles with less than 12 appendages and the experimental manipulations could also explain these observations.

We have observed an increase in the rate of protein P(J) cleavage at about 25 min after infection, as compared to the early times of structural protein synthesis (Fig. 2 and 3). If this is an autocatalytic reaction, the rate may reflect substrate concentration (8, 12). A phage-induced protein produced late in infection may stimulate the reaction, and more detailed studies on the nature of φ29c and on the possible role of host proteolytic enzymes are in progress. Unfortunately, investigation of cleavage has been limited to infection of UV-irradiated cells because of the extensive host protein synthesis

in unirradiated cells. Therefore, caution should be exercised in the interpretation of cleavage rates. Nevertheless, it has been demonstrated (12) that production of the proteins P(J), Ap, and LM2 begins in unirradiated φ29-infected *B. subtilis* at the same time and in the same order as in UV-irradiated cells. Production of the above proteins has also been studied in *B. subtilis* irradiated with different doses of UV light and infected with either wild-type φ29 or φ29c. No major effect of the UV dose on the pattern of appearance of these proteins has been detected (unpublished data). Therefore, the infection of UV-irradiated cells provides a reasonable model for analyzing precursor-product relationships between φ29 proteins.

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