Morphogenesis of Bacteriophage φ29 of *Bacillus subtilis*: DNA-gp3 Intermediate in In Vivo and In Vitro Assembly

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The assembly of phage φ29 occurs by a single pathway, and DNA-protein (DNA-gp3) has been shown to be an intermediate on the assembly pathway by a highly efficient in vitro complementation. At 30°C, about one-half of the viral DNA synthesized was assembled into mature phage, and the absolute plating efficiency of φ29 approached unity. DNA packaging at 45°C was comparable to that at 30°C, but the burst size was reduced by one-third. When cells infected with mutant *ts3*(132) at 30°C to permit DNA synthesis were shifted to 45°C before phage assembly, DNA synthesis ceased and no phage were produced. However, a variable amount of DNA packaging occurred. Superinfection by wild-type phage reinitiated *ts3*(132) DNA synthesis at 45°C, and if native gp3 was covalently linked to this DNA during superinfection replication, it was effectively packaged and assembled. Treatment of the DNA-gp3 complex with trypsin prevented in vitro maturation of φ29, although substantial DNA packaging occurred. A functional gp3 linked to the 5' termini of φ29 DNA is a requirement for effective phage assembly in vivo and in vitro.

The *Bacillus subtilis* phage φ29 combines a complex morphology with compositional simplicity; five viral proteins are sufficient for the head, neck, and tail (1, 27, 30). The genome is a linear nonpermuted duplex DNA with a mass of about 18 kilobases and intact single strands (1, 2, 20). Single extracted φ29 DNA molecules are sufficient for transfection, and up to 30% of these molecules can be repackaged in vitro to give infectious phage (3a, 31). Protein is bound to φ29 DNA, and φ29 transfection is sensitive to proteolytic enzymes (16, 29). The phenotype of the mutant *ts3*(132) comprises a heat-sensitive virion, thermolabile transfecting DNA and temperature-sensitive DNA synthesis (35, 36, 38). These functions may reflect the bound protein.

The DNA-protein is not degraded by λ-exonuclease or T7 exonuclease, and the 5' termini cannot be phosphorylated by T4 polynucleotide kinase after treatment with proteinase K or alkaline phosphatase. However the 3' termini are substrates for exonuclease III and terminal deoxynucleotidyl transferase (13, 18, 33, 39; M. O. Whittington, Jr., Ph.D. thesis, University of Minnesota, Minneapolis, 1977). Trypsin peptide analysis is consistent with the product of cistron 3, gp3, being linked to the 5' termini by a phosphoester bond from L-serine to 5'-dAMP (15, 33, 41). The protein gp3 is an early protein with a mass of about 30,000 daltons (3, 8, 14).

Mutants of cistron 3 are unable to synthesize viral DNA under nonpermissive conditions (7, 10, 23, 24, 34, 36, 38). 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 (11, 12, 17, 19). The ends of φ29 DNA are flush, and there are inverted terminal repetitions that are 6 base pairs long 5'(-A-A-A-G-T-A-) (9, 40). gp3 is required for continuous DNA synthesis, appears to initiate DNA replication, and perhaps indirectly facilitates rapid dissociation of parent viral DNA from the cell membrane (23, 26, 38).

Salas et al. report that in a mixed infection by *ts2*(98) and *ts3*(132) phage at nonpermissive temperatures most of the progeny are *ts2*(98) (22 of 23), and they reason that *ts3*(132) DNA is not replicated in this complementation (33). In this communication, we demonstrate that the packaging of *ts3*(132) DNA is very ineffective in vivo at nonpermissive temperatures. However *ts3*(132) DNA replication and packaging can be rescued by superinfection that provides native gp3. We have developed a very efficient in vitro DNA packaging and assembly system for φ29 (3a). We use this system to encapsidate DNA-gp3 released from the virion and show that trypsin treatment of the DNA-protein blocks correct DNA packaging, subsequent virus maturation, or both.

MATERIALS AND METHODS

Chemicals and isotopes. Phenylmethylsulfonyl fluoride, ATP (equine muscles), and trypsin inhibitor (beef
pancreas) were from Sigma Chemical Co., St. Louis, Mo. Phenylmethylsulfonyl fluoride was dissolved in 95% ethanol to give a stock solution of 6 mg/ml and used at a final concentration of 50 μg/ml 6- (p-Hydroxyphenylazo)-uracil (HPUrA), a gift of Dr. W. B. Langley, Imperial Chemical Industries, Ltd., Macclesfield, Cheshire, England, was dissolved in 0.5 M NaOH and used at 50 μg/ml. Spermidine (A grade) and DNase I (bovine pancreatic) were from Calbiochem-Behring Corp., Calif., and 2-mercaptoethanol was obtained from Eastman Chemical Co., Rochester, N.Y. Egg white lysozyme (3× recrystallized) was purchased from Calbiochem-Behring Corp. or Sigma Chemical Co. Trypsin tolylsulfonyl phenylalanyl chloromethyl ketone (12,000 BAEE units per mg [1 BAEE unit = ΔA 253 of 0.001 per min with Na-benzoyl-L-arginine ethyl ester (BAEE) as substrate at pH 7.6 at 25°C]) from Millipore Corp., Bedford, Mass., was dissolved in water or 10 mM CaCl2. Nonidet P-40 was obtained from Particle Data Laboratories, Ltd., Elmhurst, Ill. [3H]Thymidine was from New England Nuclear Corp., Boston, Mass. (NET-027Z; 50 Ci/mmole). The 14C-labeled amino acid mixture (New England Nuclear Corp., NEC-445; about 200 μCi/mmole) was adjusted to neutrality by the addition of 1 N NaOH immediately before use.

Bacteria and phage. The properties of the nonpermissive host B. subtilis Spoa12 and the permissive hosts B. subtilis sust 44 and L15 have been described (26a, 32).

Phage φ29 and previously described mutants have been used to construct recombinants with sus41(1241) (25, 30). The mutation sus41(1241) results in the delayed lysis phenotype. The mutant ts3(132)r2 has been derived from ts3(132) by backcrossing and was obtained by us in 1979 (26). Unfortunately, ts3(132) gave 0.36 ± 0.16% (n = 6) recombination with ts3(132)r2.

To compare ts3(132) with ts3(132)r2, we have used the mutants of both our combined reference collection (25) and those used by Salas et al. (33). All quantitative complementations were internally consistent; for example, both ts3(132) and ts3(132)r2 failed to complement sus3(713) or sus3(91), but complemented sus2(628) and sus2(a23). All recombination values obtained by two-factor crosses were consistent with published values, except that ts3(132) recombined with ts3(132)r2. Both ts3 mutants map between sus2(628) and sus4(369) by three-factor crosses. In summary, we have used quantitative complementation and two- and three-factor crosses and have studied DNA synthesis under nonpermissive conditions with all sus and ts mutants of cistrons 2, 3, 4, and 5 of our combined collection of reference mutants with the exception of ts2(35) and ts5(17). All results are consistent with ts3(132) and ts3(132)r2 being ts3 mutants, and because their phenotypes cannot be distinguished, we have used and will discuss them without distinction.

Infection, radiolabeling, and cell fractionation. B. subtilis Spoa12 was grown to 4 ∙ 10^9 cells per ml in Difco antibiotic medium no. 3 (Penassay broth [PB]), concentrated fivefold by centrifugation, and infected with a multiplicity of infection of 20. To label φ29 DNA or phage for infective lysates, infected cells were incubated with [3H]thymidine (40 μCi/ml) and HPUrA (50 μg/ml) at 5 min and incubated with shaking at 30°C. Radioactive label was chased by the addition of thymidine (200 μg/ml). The same protocol was used to label φ29 proteins with a mixture of 14C-labeled amino acids (10 μCi/ml), except that HPUrA was omitted.

Infected cells were lysed without concentration by lysozyme (100 μg/ml) at 45°C for 30 min. The lysate was vortexed for 15 s after the addition of 0.1 volume of 1 M NaCl and centrifuged 10 min at 12,000 ∙ g. The supernatants (200 μl) were layered on linear gradients of 5 to 20% sucrose in a buffer containing 0.05 M Tris-hydrochloride (pH 7.7), 0.01 M EDTA, 1 M NaCl, and 2 mM Na3 and centrifuged in an SW50.1 rotor at 35,000 rpm for 29 min at 23°C.

To ensure liberation of phage for plating, cells were incubated with lysozyme (500 μg/ml) and DNase I (15 μg/ml) for 30 min at 45°C, and the lysate was treated with Nonidet P-40 for 15 min at 45°C.

In vitro complementation assay. The procedures for infection, extract preparation, and in vitro complementation have been described in detail (3a). Briefly, the complementation extract is prepared from a mixture of cells infected with the mutants sus16(300)-sus14(1241), supplying proheads, and sus8(22)-sus10(302)-sus14(1241), supplying gp16 and unable to make proheads. The infected cells were lysed in TMS buffer (0.05 M Tris-hydrochloride [pH 7.8]–0.01 M MgCl2–0.1 M NaCl) containing 10 mM ATP, 6 mM spermidine, and 3 mM 2-mercaptoethanol. As a source of exogenous DNA for in vitro packaging, 3H-labeled wild-type phage or sus8.5(900)-sus14(1241) phage that lack head fibers were grown in B. subtilis Spoa12 in PB containing 20 μCi of [3H]thymidine per ml. These phage were purified by banding in a CsCl gradient (1), and DNA was released by heating 5 ∙ 10^12 phage per ml in TE buffer (0.05 M Tris-hydrochloride [pH 7.8]–

RESULTS

Role of cistron 3 product in assembly of φ29. Cistron 3 mutants do not synthesize φ29 DNA under nonpermissive conditions (7, 10, 24, 34, 36). gp3 is required continuously during DNA synthesis by ts3(132) (23, 26). When cells infected at 30°C by ts3 mutants were shifted to 45°C at 50 min postinfection, DNA synthesis ceased within 2 to 5 min (Fig. 1). DNA replication resumed within minutes if these cells were returned to 30°C at 65 min, but the total synthesis was reduced. DNA synthesis began without
apparent delay when cells infected at 45°C were shifted to 30°C (data not shown).

We observed that when ts3(132)-infected cells had made substantial amounts of DNA at 30°C very few phage were assembled after a shift to 45°C. We decided to investigate this phenomenon in greater detail.

To quantify DNA packaging at the nonpermissive temperature of 45°C, cells were infected with sus14(1241) phage at 30°C and labeled with [3H]thymidine in the presence of HPUra, an inhibitor of host DNA synthesis (4, 21, 34). At 45 min postinfection, when few phage were assembled, a sample of the culture was shifted to 45°C, and at 180 min postinfection, cells of both cultures were lysed and the proportion of DNA packaged into virions was determined by sucrose gradient centrifugation (Fig. 2A). At 30°C, 55% of the [3H]DNA in the gradient sedimented at the position of mature virions (centering on fraction 7), and the burst size was 1,000 phage per cell. At 45°C, 45% of the [3H]DNA was packaged at 180 min, and the total amount of DNA synthesized at 30 and 45°C was roughly equivalent. The burst at 45°C was reduced to 550 phage per cell and, compared with the 30°C culture, packaging at 45°C did not necessarily imply proper assembly.

We infer that DNA with thermolabile gp3 (tsgp3) covalently bound to the 5' termini was produced in cells infected with the mutants ts3(132)r2-sus14(1241) or ts3(132)-sus14(1241). The DNA-protein was labeled with [3H]thymidine at 30°C in the presence of HPUra. At 50 min postinfection, about 25% of the viral DNA had been synthesized and none of the [3H]DNA banded at the viral position in 5 to 20% sucrose gradients (Fig. 2B). Incorporation of [3H]thymidine ceased in the portions of the cultures shifted to 45°C at 50 min, but continued at 30°C even in the presence of excess unlabeled thymidine (data not shown). At 180 min with ts3(132)r2-sus14(1241), 50% of the DNA was packaged at 30°C, whereas less than 12% of the radioactivity was in the viral peak at 45°C (Fig. 2C). With ts3(132)-sus14(1241), more than 47% of the DNA from the 30°C culture was packaged compared with less than 14% of the DNA from the 45°C culture (Fig. 2D). At 30°C, the burst sizes were 315 and 860, respectively, and no phage were assembled after the shift to 45°C. Packaging of ts3(132) DNA at the nonpermissive temperature

FIG. 1. Temperature dependence of DNA synthesis in B. subtilis SpoA12 infected with ts3(132) mutants. Cells at 2 × 10⁹/ml in PB were treated with 50 μg of HPUra per ml for 5 min at 30°C, infected with ts3(132)r2-sus14(1241) (A) or ts3(132)-sus14(1241) (B) phage at zero time at an input multiplicity of 20, diluted 10× with PB containing 40 μCi of [3H]thymidine per ml and 50 μg of HPUra per ml at 5 min, and incubated at 30°C. At 50 min postinfection, a portion of the culture was shifted up to 45°C, and at 65 min postinfection, a portion of this culture was shifted back to 30°C. At the intervals indicated, 20-μl samples were taken for measurement of trichloroacetic acid-insoluble radioactivity. Symbols: ○, 30°C culture; ●, culture shifted to 45°C at 50 min; and △, culture shifted to 45°C at 50 min and back to 30°C at 65 min.
FIG. 2. Ineffective packaging at the nonpermissive temperature of viral DNA with thermolabile gp3 covalently bound to the 5' termini. *B. subtilis* SpoA12 was infected at 30°C with the mutants indicated and labeled with [3H]thymidine under the conditions described in the legend of Fig. 1. At 45 min postinfection (A, B, and C) or at 50 min postinfection (D), when about 25% of the viral DNA had been synthesized, a portion of each culture was shifted to 45°C. At the shift time (B) and at 180 min postinfection (A, C, and D), samples of the infected cells were lysed and the distribution of packaged DNA-gp3 (centering on fraction 7) versus free DNA-gp3 (centering on fraction 33) was determined by centrifugation in 5 to 20% sucrose gradients. Symbols: ○, 30°C culture; ●, culture shifted to 45°C. The ratios of packaged label (30°C/45°C) for the gradients of (A), (C), and (D), respectively, were 1.2, 7.8, and 4.3. The ratio of phage burst sizes (30°C/45°C) in the experiment of (A) was 952/502 or 1.9; the burst sizes at 30°C for the experiments of (C) and (D) were 309 and 827, respectively, and no phage were produced at 45°C.

was much less efficient than wild-type DNA, and the particles assembled at 45°C were noninfectious.

Assembly of ts3(132) during mixed infection. We sought explanations for the ineffective assembly of ts3(132) DNA at 45°C. Renografin
gradient centrifugation (21) was used to demonstrate that membrane-bound \(ts3(132)\) DNA was released normally to the free DNA position. Moreover, proheads were assembled, albeit at a reduced rate, after a shift up to 45°C (data not shown). Ineffective assembly could result in part from a cryptic mutation in another cistron involved in assembly or a structural change in the DNA molecule. Considering the latter possibility, we examined assembly of ts3gp3-sus3 DNA at 45°C. Nonpermissive host cells multiply infected by \(sus3(713)-sus14(1241)\) and \(ts3(132)\) phage at 30°C produce only thermolabile gp3 (ts3p3), and this protein is essential for the replication of both genomes. When the infected cells were collected and lysed after 300 min at 30°C, the mixed burst was about 500 phage per ml, and 45% of the progeny were \(sus3(713)-sus14(1241)\). When the infected cells were shifted up to 45°C at 50 min postinfection, the burst at 300 min was reduced to about 3% of the yield at 30°C. Neither \(sus3\) nor \(ts3\) DNA could be effectively assembled at the nonpermissive temperature with ts3gp3 covalently bound to their 5′ termini.

A series of superinfection experiments was designed to study the packaging of \(ts3\) DNA in the presence of wild-type gp3 at 45°C, and representative results have been presented (Table 1 and Fig. 3). We have used both \(ts3(132)-sus14(1241)\) and \(ts3(132)r2-sus14(1241)\) phage and superinfected with either wild-type \(\phi29\) or \(sus8.5(900)-sus14(1241)\), a mutant unable to make the dispensable head fiber protein gp8.5 (30).

Cells infected at 30°C with \(ts3(132)r2-

### TABLE 1. Superinfection rescue of \(ts3(132)r2-sus14(1241)\) mutant infection

<table>
<thead>
<tr>
<th>Shift to 45°C</th>
<th>Superinfection</th>
<th>Burst size</th>
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<tbody>
<tr>
<td>–</td>
<td>–</td>
<td>270</td>
</tr>
<tr>
<td>+</td>
<td>–</td>
<td>140</td>
</tr>
<tr>
<td>+</td>
<td>–</td>
<td>75 (32% ts)</td>
</tr>
</tbody>
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\(a\) \(B. subtilis\) SpoA12 was infected with \(ts3(132)r2-sus14(1241)\) phage at an input multiplicity of 20, labeled, and incubated at 30°C as indicated in Fig. 1. At 40 min postinfection, one sample of the culture was removed, superinfected with wild-type \(\phi29\) (two experiments) or \(sus8.5(900)-sus14(1241)\) (one experiment) at a multiplicity of 50, and returned to 30°C. At 45 min postinfection, the superinfected cultures and two other samples were shifted to 45°C, and all cultures were incubated an additional 255 min before lysis and being plated on the permissive host \(B. subtilis\) L15.

\(b\) The burst sizes are averages of three experiments.

\(c\) The percentage of the temperature-sensitive genotype was determined by the difference of titers at the permissive and nonpermissive temperatures.

\(d\) The culture was shifted back to 30°C after 15 min at 45°C.

FIG. 3. Replication at the nonpermissive temperature of \(ts3(132)\) DNA in the presence of native gp3. \(B. subtilis\) SpoA12 was infected at 30°C with \(ts3(132)r2-sus14(1241)\) under the conditions described in Fig. 1. At 40 min postinfection, one sample of the culture was removed, superinfected with wild-type \(\phi29\) at a multiplicity of 50, and incubated at 30°C. The primary culture was further subdivided into three samples at 45 min postinfection, and all of the cultures received 200 \(\mu\)g of unlabeled thymidine per ml as a chase. The four cultures were incubated, lysed, and assayed for phage production and for packaged \(\text{[^3]H}DNA-gp3\) (fractions 9 and 10) versus free DNA-gp3 (fractions 33 to 35) by centrifugation in 5 to 20% sucrose gradients as follows: A (○), lysed at 45 min as a control; A (○), maintained at 30°C for 300 min; B (○), shifted to 45°C at 45 min and incubated until 300 min; B (△), superinfected with \(\phi29\), shifted to 45°C at 45 min, and incubated until 300 min.
sus14(1241) phage were superinfected with wild-type phage or sus8.5(900)-sus14(1241) 5 min before a shift to 45°C (Table 1). Without superinfection, the burst size at 45°C was less than 1 phage per cell compared with an average of more than 270 phage per cell at 30°C. The mixed burst produced at 45°C after superinfection averaged 76 phage per cell, and about one-third had the ts3(132)r2 genotype (Table 1). The superinfection result is more meaningful when considering that burst size was reduced by about 50% by a 15-min shift to the nonpermissive temperature (Table 1, line 2). In parallel experiments, cells infected with ts3(132)-sus14(1241) were shifted to 45°C at 50 min when about 28 phage had been assembled per cell. Superinfection with wild-type φ29 and incubation at 45°C restored the burst size to 420 phage per cell, compared with an average burst of 675 phage per cell at 30°C and no phage production at 45°C. Of the progeny of the superinfected cells, 57% were temperature sensitive (data not shown). These results demonstrated that in the presence of normal gp3 substantial DNA synthesis and viral assembly occurred at the nonpermissive temperature in cells that were preprogrammed to produce only ts3(132) phage. In a sense, superinfection rescued the ts3(132) DNA.

These data do not describe the fate of tsgp3-DNA synthesized at 30°C during packaging at 45°C in the presence of free wild-type gp3. The DNA-protein of ts3(132)r2-sus14(1241) was labeled with [3H]thymidine at 30°C in the presence of HPUra. At 45 min, none of the [3H]DNA banded at the viral position in 5 to 20% sucrose gradients (Fig. 3A). In the portion of the culture shifted to 45°C at 45 min, DNA synthesis stopped and almost no [3H]DNA was packaged (Fig. 3B). The labeling after superinfection with wild-type φ29 and incubation at 45°C was not as effective as incorporation at 30°C (cf. Fig. 3A with B), but the burst size was 75 phage per cell, compared with a burst size of 200 at 30°C, and the radioactivity in the viral position was about 15% of that packaged at 30°C. If the only [3H]DNA that was packaged during superinfection was present at shift time, then 13% of the tsgp3-DNA available could have been packaged in the presence of normal gp3. However, the burst after superinfection was 32% ts3, so less than 4% of the preshift DNA could have been packaged. This upper limit is based on the assumption that no ts3 DNA was replicated and packaged at 45°C during superinfection. These results and calculations suggest that free gp3 cannot assist in the packaging of tsgp3-DNA at 45°C in a way that produces a virion; however, free gp3 can rescue the replication of tsgp3-DNA to produce phage that by inference have native gp3 linked to the ts3 genome.

In vitro complementation with DNA-gp3. In vitro DNA packaging and assembly of φ29 can give 180 phage per cell equivalent of proheads (average, 9 × 10^{11} phage per ml). More than 30% of the proheads are converted to virions, and from 5 to 30% of exogenous DNA can be repackaged (3a). Exogenous DNA-gp3, heat released from the virion, and DNA-protein from both a 16'-14" extract and an 8'-10'-14" extract are packaged in the in vitro assay. To provide a valid assay for the biological activity of exogenous DNA-gp3, the complementation system must assemble at least 10^{11} phage per ml, and the two progeny sus phage must be present in the same proportion (Table 2).

<table>
<thead>
<tr>
<th>Trypsin (μg/ml)</th>
<th>Assembly (PFU/ml × 10^{-10})</th>
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<tbody>
<tr>
<td>Control</td>
<td>2.6</td>
</tr>
<tr>
<td>20</td>
<td>0.0001</td>
</tr>
<tr>
<td>2</td>
<td>0.0012</td>
</tr>
<tr>
<td>0.2</td>
<td>2.1</td>
</tr>
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</table>

a The exogenous wild-type DNA-gp3 in 50 μl was treated with trypsin for 45 min at 37°C, and after addition of an excess of trypsin inhibitor (7.5 μg) and incubation for 25 min at 37°C, the mixture was added to 100 μl of the in vitro complementation extract prepared from equal amounts of cells infected with the mutants 16'-14" and 8'-10'-14". For the control, trypsin (20 μg/ml) was pretreated with trypsin inhibitor (7.5 μg) for 25 min at 37°C; after addition to the DNA-gp3, the mixture was incubated for 45 min at 37°C. After 110 min at ambient temperature, the complementation mixtures were plated on the sus+ nonpermissive host to determine exogenous DNA-gp3 packaging and on the su+44 permissive host to measure total assembly. The genotypes of the sus progeny were determined by qualitative complementation.

b In experiment 1, 1.3 ± 0.5 × 10^{11} phage per ml were assembled, and 51% of 1357 sus phage were 16'-14". Exogenous wild-type φ29 DNA (25 μg/ml) gave 2.6 × 10^{10} phage per ml (an efficiency of repackaging of 1.7%) and 20% of all phage assembled.

c In experiment 2, 3.8 ± 0.9 × 10^{11} phage per ml were assembled, and 46% of 2744 sus phage were 16'-14". Exogenous wild-type φ29 DNA (13.3 μg/ml) gave 4.1 × 10^{10} phage per ml (an efficiency of repackaging of 5%) and 11% of all phage assembled.
Repackaging of exogenous DNA-gp3 by in vitro complementation. Two 50-μl samples of exogenous 8.5-14°C [3H]DNA-gp3, prepared as described in the text, were added to separate 100-μl volumes of the in vitro 16-14°C x 8×10^{-14} complementation extract on Parafilm at 23°C. After 3 min or 80 min, the mixtures were treated with 13 μg of DNase I per ml for 15 min. The trichloracetic acid-insoluble radioactivity in phage (centering on fraction 4), intermediates (centering on fraction 16), and an uncharacterized peak (centering on fraction 29) was determined after centrifugation of 100-μl samples in 5 to 20% sucrose gradients. Symbols: O, 80-min mixture; and ●, 3-min mixture.

FIG. 4. Repackaging of exogenous DNA-gp3 by in vitro complementation. Two 50-μl samples of exogenous 8.5-14°C [3H]DNA-gp3, prepared as described in the text, were added to separate 100-μl volumes of the in vitro 16-14°C x 8×10^{-14} complementation extract on Parafilm at 23°C. After 3 min or 80 min, the mixtures were treated with 13 μg of DNase I per ml for 15 min. The trichloracetic acid-insoluble radioactivity in phage (centering on fraction 4), intermediates (centering on fraction 16), and an uncharacterized peak (centering on fraction 29) was determined after centrifugation of 100-μl samples in 5 to 20% sucrose gradients. Symbols: O, 80-min mixture; and ●, 3-min mixture.

Packaging of trypsin-treated DNA-gp3 during in vitro assembly. Wild-type [3H]DNA-gp3, prepared as described in the text, was divided into two 50-μl portions. One was incubated with 20 μg of trypsin per ml for 45 min at 37°C, followed by the addition of 7.5 μg of trypsin inhibitor and incubation for 20 min at 23°C. The second was mixed with trypsin (20 μg/ml) that had been pretreated with 7.5 μg of trypsin inhibitor for 20 min at 23°C, and the mixture was incubated for 45 min at 37°C. Each sample was mixed with 100 μl of the in vitro 16-14°C x 8×10^{-14} complementation extract on Parafilm, incubated at 23°C for 80 min, treated with 13 μg of DNase I per ml for 15 min, and diluted fivefold with water. Trichloracetic acid-insoluble counts in fast-sedimenting particles (centering on fraction 4) and the uncharacterized peak (centering on fraction 28 or 31) were determined after centrifugation of 200-μl samples in 5 to 20% sucrose gradients. Symbols: O, [3H]DNA-gp3 incubated with trypsin that had been pretreated with trypsin inhibitor; the number of PFU produced in vitro was 9.5 × 10^{11}/ml on the permissive host su^44 and 1.4 × 10^{11}/ml on the nonpermissive host SpoA12; ●, trypsin-treated [3H]DNA-gp3; the number of PFU produced in vitro was 9.3 × 10^{11}/ml on the permissive host and 3.7 × 10^{9}/ml on the nonpermissive host. The [3H]DNA packaged into fast-sedimenting particles represented 19 and 10%, respectively, of the exogenous DNA-gp3 added to the complementation mixture.

FIG. 5. Packaging of trypsin-treated DNA-gp3 during in vitro assembly. Wild-type [3H]DNA-gp3, prepared as described in the text, was divided into two 50-μl portions. One was incubated with 20 μg of trypsin per ml for 45 min at 37°C, followed by the addition of 7.5 μg of trypsin inhibitor and incubation for 20 min at 23°C. The second was mixed with trypsin (20 μg/ml) that had been pretreated with 7.5 μg of trypsin inhibitor for 20 min at 23°C, and the mixture was incubated for 45 min at 37°C. Each sample was mixed with 100 μl of the in vitro 16-14°C x 8×10^{-14} complementation extract on Parafilm, incubated at 23°C for 80 min, treated with 13 μg of DNase I per ml for 15 min, and diluted fivefold with water. Trichloracetic acid-insoluble counts in fast-sedimenting particles (centering on fraction 4) and the uncharacterized peak (centering on fraction 28 or 31) were determined after centrifugation of 200-μl samples in 5 to 20% sucrose gradients. Symbols: O, [3H]DNA-gp3 incubated with trypsin that had been pretreated with trypsin inhibitor; the number of PFU produced in vitro was 9.5 × 10^{11}/ml on the permissive host su^44 and 1.4 × 10^{11}/ml on the nonpermissive host SpoA12; ●, trypsin-treated [3H]DNA-gp3; the number of PFU produced in vitro was 9.3 × 10^{11}/ml on the permissive host and 3.7 × 10^{9}/ml on the nonpermissive host. The [3H]DNA packaged into fast-sedimenting particles represented 19 and 10%, respectively, of the exogenous DNA-gp3 added to the complementation mixture.
wild-type DNA was quantified by sucrose gradient centrifugation after in vitro complementation (Fig. 5). Of the untreated exogenous [3H]DNA-gp3 added, 19% was repackaged (fractions 3 to 7), and 15% of the infectious phage produced in vitro was wild-type. After trypsin treatment (20 µg/ml), 10% of the [3H]DNA was packaged, but effective assembly was less than 0.04% (3.7 × 10^6 phage per ml) of the untreated control (Fig. 5). Packaging of trypsin-treated DNA varied from 30 to 66% of the untreated control, whereas effective assembly never exceeded 0.6% (data not shown).

**DISCUSSION**

Ortín et al. (29) observed protein bound to φ29 DNA extracted from virions, and the results of Yanofsky et al. (38) that the ts3(132) virion and its transfecting DNA were thermolabile gave biological meaning to the observation. Salas et al. (33) used tryptic peptide analysis to identify the product of cistron 3, gp3, linked to the 5′ termini of the phage DNA. In their study, with mixed infection by ts3(132) and ts2(98) phage at nonpermissive temperatures, the ts3(132) genotype was substantially underrepresented among the progeny. Salas et al. (33) reasoned that ts3(132) DNA was not replicated in this in vivo complementation. Unfortunately, we cannot reproduce these results with the mutants ts3(132) and ts2(98) obtained both before or after publication by Salas et al. (33). With Bacillus amyloliquefaciens strain HM, B. subtilis 110NA or our strain B. subtilis SpoA12 at 42, 44, and 46°C, both parents were present in the burst of the in vivo complementation in equal numbers. The genotype of 40 ts3(132) clones was determined by genetic recombination, and the phage were ts3 mutants. With a ts3(132)r2 by ts2(98) complementation, 20% of the progeny phage were ts3 (data not shown).

We are considering the hypothesis that the proper assembly of the virion, particularly the neck-tail complex, requires functional gp3. In the virion, this gp3 must be positioned to make contact with the membrane and to initiate the entry of the genome into the host cell. Our results show that the requirements for DNA packaging are less stringent than those for proper assembly or maturation of the virion. Operationally, DNA packaging merely means stability to DNase I and sedimentation in sucrose gradients to the position of the virion.

We have shown that the absolute plating efficiency of both φ29 and sus8.5(900)-sus14(1241) is near one when the phage are assembled at 37°C (1, 30). Morphogenesis at 37°C is an imperfect process, for "empty heads" accumulate during any infection that results in DNA packaging. These entities are present in vivo and after in vitro complementation (3a, 5, 22, 28). In sucrose gradients, more than 20% of the particles sedimenting with proheads can be empty heads (10, 28).

With φ29 infection at 30°C, more than 50% of the [3H]DNA-gp3 synthesized was packaged when examined by sucrose gradient centrifugation, and the burst size could exceed 1,000 phage per cell. Compared with 30°C, roughly an equivalent amount of DNA was synthesized and packaged at 45°C, although the burst size was reduced by one-third. This calculation does not include the abortive assembly that produces empty heads.

DNA synthesis and packaging occur simultaneously during much of the latent period, and because functional gp3 is integral to both processes, our timing might seem arbitrary and unusual. We made temperature shifts when viral DNA, but not phage, was present and at times when packaging had begun. The results were internally consistent, although the use of ts3(132), 45°C, and different shift times led to great variability in the burst size. Packaging of ts3(132) DNA at nonpermissive temperatures was studied by shifting a culture sample to 45°C when about 25% of the DNA had been made and no [3H]DNA-gp3 was at the position of the phage in sucrose gradients. Again, about 50% of the viral DNA was packaged at 30°C, and the burst size was more than 300 phage per cell. Infectious phage were not assembled at 45°C, but about 12 to 14% of the ts3gp3-DNA was packaged (Fig. 2C and D).

The phenotype of ts3(132) is complicated, and although ts3(132)r2 has been backcrossed, we are still concerned that packaging inefficiency at 45°C may reflect other chromosomal damage in this mutant. The hypothesis that ts3gp3 directly affects assembly is consistent with and supported by the following observations. ts3gp3-DNA synthesized at 30°C was not effectively packaged after a shift to 45°C (Fig. 2 and 3), and sus3(713) DNA linked to ts3gp3 was not assembled to phage at 45°C (see above), although DNA has been demonstrated to pass from the membrane to a "free" form by Renografin gradient centrifugation and proheads were continuously assembled at 45°C (data not shown). At the nonpermissive temperature, ts3(132) DNA synthesis ceased abruptly (Fig. 1), and native gp3 is required continuously for DNA replication (23, 26, 38). However, during superinfection at 45°C, the superinfecting genome produced native gp3 and replicated, and the DNA was assembled to phage in the presence of excess ts3gp3 and all ts3(132) gene products (Table 1).

The results shown in Table 1 and Fig. 3 were consistent with ts3(132) DNA being synthesized,
packaged, and assembled to phage in the presence of wild-type gp3 at 45°C. The question remains, can tsgp3-DNA be packaged in the presence of excess native gp3? If we assume that no ts3(132) DNA is replicated after superinfection and a shift to 45°C, we can suggest an upper limit for packaging of tsgp3-DNA at 45°C in this experiment. About 13% of the DNA present at shift time could account for all packaging at 45°C after superinfection (Fig. 3B). Since 32% of the phage produced were temperature sensitive, only about 4% of the tsgp3-DNA present at shift time could have been assembled. This unrealistic upper limit would represent inefficient packaging, and assembly in the absence of superinfection never exceeded one phage per cell (Table 1, Fig. 2 and 3). On occasion, a shift to 45°C after wild-type superinfection could restore the burst size to more than 60% of the yield at 30°C (420/675), and more than half of the progeny were ts3(132) (see above).

In vitro assembly permits a more direct study of the requirement for gp3 in ϕ29 maturation. Repackaging of exogenous DNA-protein, released from the virion, was studied in a complementation extract that assembles endogenous DNA to phage (3a). The 16'14" extract supplies proheads and DNA-protein, and the 8'10'14" extract provides the DNA-protein and gp16, a protein essential for in vivo packaging. About 10^{12} phage per ml are assembled, and both genomes are present in equal proportions among the assembled phage. DNA-gp3 is an intermediate in ϕ29 assembly, since only DNA-protein can be effectively assembled (up to 30% of exogenous DNA added), and this exogenous DNA-protein can appear in 33% of all phage assembled in a mixed extract (3a; Table 2, Fig. 4; data not shown). To date, DNA-gp3, proheads, and the 11' and 12' particles have been shown to be assembly intermediates by in vitro complementation (3a, 6, 37).

Trypsin (20 μg/ml) digestion of either exogenous wild-type or sus8.5(900)-sus14(1241) DNA-gp3 reduced the biological activity to the background level of the complementation assay (Table 2; data not shown). The gp3 is linked to the 5' termini of ϕ29 DNA by a phosphoester bond (15), but we do not know how accessible the gp3 is to proteolytic enzymes and we have not measured the extent of proteolysis during trypsin treatment. Extensive purification of the DNA-gp3 until more than 99% of the protein was gp3 reduced the biological activity of the DNA-protein (3a), and protease degrades components of the complementation assay. Trypsin and trypsin inhibitor were used because the DNA-gp3 enzyme digest could be tested without reisolation of the DNA. There is slight inhibition of the in vitro complementation by the addition of these proteins, but the data seem reasonable (Table 2).

The sucrose gradient analysis of in vitro packaging of trypsin-treated DNA (Fig. 5) gave results similar to those for ts3(132) DNA at 45°C of Fig. 2. The trypsin digestion reduced DNA packaging from 19 to 10% of the [3H]DNA added, but reduced effective in vitro assembly to less than 0.04% (3.7 x 10^6 phage per ml) (Fig. 5). Packaging was reduced by 30 to 66% by trypsin digestion, but effective assembly to phage was always less than 0.6% of the untreated control.

Phage assembly was more sensitive to the structural integrity of gp3 than DNA packaging, both in vivo with ts3(132) infection and during in vitro complementation. Particles obtained by packaging wild-type or trypsin-treated exogenous [3H]DNA-gp3 were examined after sedimentation in sucrose gradients by negative staining and electron microscopy. Wild-type DNA-gp3 gave 6.1 x 10^8 phage per ml, about 26% of the particles assembled in vitro. With trypsin treatment, about 18% of the DNA-gp3 was re-packaged, but only 7.0 x 10^8 phage per ml were produced. Of the 2,000 particles of each preparation, 98 and 95%, respectively, had the morphology of ϕ29 assembled in vivo (data not shown).

Our results demonstrated that functional gp3 linked to 5' termini was a requirement for effective phage assembly in vivo and in vitro. The presence of excess free gp3 during in vivo complementation rescued replication but not the effective packaging of DNA with covalently bound tsgp3. An accurate model for ϕ29 should incorporate gp3, and the mechanism of assembly should define the role of gp3. Caution should be used in the interpretation of complementation experiments involving mutants with a complex phenotype, and DNA packaging should not be confused with viral assembly in an operational sense.

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