Morphogenesis of Bacteriophage $\phi 29$ of *Bacillus subtilis*: Prohead Restoration for DNA-gp3 Packaging and Assembly

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The DNA-protein complex DNA-gp3 of $\phi 29$ is efficiently packaged into purified proheads with the aid of plasmid-derived gp16. The filled heads can be assembled to phage by addition of an extract providing the products for neck-tail assembly (Bjornsti et al., J. Virol. 50:766-772, 1984). However, purified proheads lost their competence to package DNA-gp3 upon storage for 2 months at 4°C. Competence was restored by complementation with extracts of certain mutant-infected cells, and these experiments demonstrated that late proteins were not involved; restoration obtained with 4⁻8⁻14⁻-infected cells was indistinguishable from that obtained with 7⁻8⁻14⁻-infected cells. 2⁻8⁻14⁻ and 3⁻8⁻14⁻ extracts restored about one-third of the capacity to package exogenous DNA-gp3. A 1⁻8⁻14⁻ extract restored activity to package 20.6% of the DNA-gp3 added, but phage were not produced.

In vitro assembly of $\phi 29$ in extracts of mutant-infected cells supplying proheads, gp16, and endogenous or exogenous DNA-gp3 is highly efficient (3-6). In the development of a refined system to study DNA-gp3 encapsidation, we constructed a chimeric plasmid, pUM101, to serve as a source of gp16 (6). Purified DNA-gp3 and proheads were added to a plasmid-containing cell extract, and the gp16 present was sufficient to package more than 20% of the purified DNA-gp3 added in a DNase I-protected form (6).

In this study, we show that purified proheads lose their competence to package DNA-gp3 in vitro upon storage at 4°C. Our operational definition of packaging includes all DNA-gp3 that is DNase I-protected before sucrose gradient centrifugation and is present in fractions 1 through 32 of the gradient as trichloroacetic acid-precipitable radiolabel after centrifugation. These proheads can be restored completely by incubation in certain extracts of mutant-infected cells. Surprisingly, early rather than late viral proteins seem to promote this restoration, because restoration occurs in the absence of the gene 4 product that regulates late transcription (2).

This phenomenon of reversible inactivation of proheads suggests that proheads contain undefined constituents or that defined components undergo conformational change.

MATERIALS AND METHODS

Phage, bacteria, and plasmids. Phage $\phi 29$ (1, 11), reference mutants (8), the nonpermissive host *B. subtilis* *spoA12* (12), and the permissive host *B. subtilis* *sup*⁺⁴⁴ (9) were used. The recombinants *sus16*(300)-*sus14*(1241), *sus7*(614)-*sus8*(769)-*sus14*(1241), and *sus4*(369)-*sus8*(22) have been described (3, 6). The recombinants *sus3*(713)-*sus8*(769)-*sus14*(1241), *sus4*(369)-*sus6*(626)-*sus14*(1241), *sus4*(369)-*sus8*(22)-*sus14*(1241), *sus2*(628)-*sus4*(369)-*sus14*(1241), *sus3*(713)-*sus4*(369)-*sus14*(1241), *sus8*(769)-*sus14*(1241)-*sus17*(741), *sus1*(629)-*sus8*(22)-*sus14*(1241), and *sus8.5*(900)-*sus16*(300)-*sus14*(1241) were constructed for this work. The products of genes 1, 2, 3, and 17 are involved in viral DNA synthesis, the

products of genes 7 and 8 are prohead components, and the mutation *sus14*(1241) provides delayed lysis (10).

In vitro complementation. The procedures for cell growth, infection, radiolabeling, extract preparation, prohead and [³H]DNA-gp3 purification, and evaluation of DNA-gp3 packaging have been described (3-6). Prohead competence was evaluated by packaging of purified [³H]DNA-gp3, assembly of infectious phage, or both. If the infected cell extract to be tested could not make the protein gp16, a protein essential for DNA packaging, then a plasmid pUM101-containing cell extract was added to provide gp16 (6).

RESULTS

Purified $\phi 29$ proheads can efficiently package exogenous DNA-gp3 in a cell extract containing plasmid-specified gp16 (6). The only ¹⁴C-labeled viral proteins detected in these proheads after sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography were gp7 (scaffold), gp8 (major head protein), gp10 (neck-upper collar), and the nonessential protein gp 8.5 (head fibers) (data not shown).

Storage of purified competent 16⁻14⁻ ³⁵S-labeled proheads for 2 months at 4°C resulted in loss of the capacity to package exogenous [³H]DNA-gp3 in a DNase I-protected form (Fig. 1). However, when these inactive proheads were added to a 7⁻8⁻-infected cell extract, packaging of [³H]DNA-gp3 was typical of that observed in competent proheads (Fig. 1). Packaging of DNA-gp3 was terminated by the addition of DNase I, and some of the particles were disrupted during sucrose gradient centrifugation. The [³H]DNA-gp3 centering on fraction 5 was at the position of virions and DNA-filled heads and was DNase I protected. The [³H]DNA-gp3 distributed between fractions 10 and 32 was DNase I sensitive after centrifugation (data not shown). Our operational definition of packaging includes all DNA-gp3 that is DNase I protected before centrifugation and is present in fractions 1 through 32 as trichloroacetic acid-precipitable radiolabel after centrifugation (6; Fig. 1). In the experiment described in Fig. 1, ca. 5.2% of the [³H]DNA-gp3 added in complementation sedimented to the position of DNA-filled heads (centering on fraction 5). The phage yield, 6.8 × 10¹⁰ PFU/ml, was relatively low in this restoration experiment, and less than 2% of the DNA-gp3 added to the complementation mixture was assembled to phage. Nevertheless, pro-

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FIG. 1. Inactivity of proheads for in vitro [^3H]DNA-gp3 packaging upon storage. Purified $16^{-}14^{-}$ ^{35}S -labeled proheads ($3\ \mu\text{l}$), stored for about 2 months at 4°C , and $4^{-}8^{-}$ [^3H]DNA-gp3 (ca. 2×10^{11} genome equivalents in $50\ \mu\text{l}$ of $0.01\times$ TE buffer [$0.05\ \text{M}$ Tris hydrochloride, pH 7.8, $0.01\ \text{M}$ EDTA]) were complemented with $100\ \mu\text{l}$ of a *B. subtilis* (pUM101) extract as a source of gp16 (■) or with $100\ \mu\text{l}$ of an extract of $7^{-}8^{-}14^{-}$ -infected cells (□) for 70 min at ambient temperature. After treatment with DNase I ($40\ \mu\text{g}/\text{ml}$) for 10 min, $100\text{-}\mu\text{l}$ portions of the mixtures were centrifuged in 5 to 20% sucrose gradients, and DNase I-protected DNA-gp3 was quantified as trichloroacetic acid-precipitable radioactivity. A 7% spillover of ^{35}S counts into the tritium channel has been subtracted. In the $7^{-}8^{-}14^{-}$ extract, 6.8×10^{10} PFU/ml were assembled.

heads inactivated by storage could regain biological competence for both DNA packaging and neck-tail assembly by incubation in an extract of virus-infected cells.

We quantified the restoration of inactive proheads by measuring virus assembly (Table 1). The average yield obtained with restored proheads, $4.9 \times 10^{11} \pm 3.8 \times 10^{11}$ PFU/ml, was less than the yield obtained with active (fresh) proheads, $8.7 \times 10^{11} \pm 3.6 \times 10^{11}$ PFU/ml. However, the standard deviation of the yields and the range values indicated that restored proheads had comparable activity.

Restoration of DNase I-protected DNA-gp3 packaging by extracts of mutant-infected cells was also determined by sucrose gradient centrifugation (Table 2). An extract of $7^{-}8^{-}14^{-}$ -infected cells restored packaging to $19.6 \pm 11.6\%$ ($n = 6$) of the purified DNA-gp3 added to the complementation mixture. gp3 is essential for viral DNA synthesis (7) and DNA-gp3 packaging (4, 5), and protein synthesis may be reduced in extracts of $3^{-}8^{-}14^{-}$ -infected cells. In 2 of 5

experiments with the $3^{-}8^{-}14^{-}$ recombinant (Table 2), an extract of pUM101-containing cells was added to ensure that gp16 was present in excess. In general, less DNA-gp3 ($7.9 \pm 5.6\%$) was packaged in either the $3^{-}8^{-}14^{-}$ or $2^{-}8^{-}14^{-}$ extract (Table 2; data not shown). Addition of the gp16-containing cell extract did not increase the extent of DNA packaging.

By the use of recombinants that cannot produce gp4, a protein essential for the production of structural and morphogenetic proteins of genes 7 through 16 (2), we demonstrated that late proteins are not essential for the restoration of [^3H]DNA-gp3 packaging by inactive proheads (Table 2; Fig. 2). With this series of mutants, plasmid-specified gp16 is essential for DNA packaging, and the pUM101 cell extract is always present. The DNA-gp3 packaging in the $4^{-}8^{-}14^{-}$ extract ($25.3 \pm 9.9\%$) compares favorably with packaging in the $7^{-}8^{-}14^{-}$ extract (Table 2).

The known genes of $\phi 29$ have been numbered sequentially from left to right with reference to maps of restriction enzyme sites. Gene 6 is the rightmost early gene (8), and the extract of $4^{-}6^{-}14^{-}$ -infected cells is effective in restoring proheads to competence for [^3H]DNA-gp3 packaging (Table 2). Again, mutation in cistron 2 or 3 is correlated with inefficient restoration of prohead competence to package [^3H]DNA-gp3 (Table 2; Fig. 2).

DNA-gp3 packaging is oriented from left to right with respect to the genetic map, and the DNA-gp3 encapsidation does not necessarily result in effective viral assembly (4, 5). DNA with thermolabile gp3 (*tsgp3*) covalently bound to the 5' termini can be packaged at the nonpermissive temperature, but infectious phage are not constructed (4). Extracts of $1^{-}8^{-}14^{-}$ -infected cells can restore proheads to package DNA-gp3 (Table 3), but the intermediates are not effectively converted to virions. In Table 3, each extract of recombinant-infected cells has the potential to complete neck-tail assembly, and total virus yield is reported. The proportion of the yield shown to have the $4^{-}8^{-}$ genotype of the exogenous DNA by qualitative complementation (more than 1,000 plaques tested) was used to define a kind of efficiency: the total yield of phage having the exogenous DNA genotype was divided by the counts of exogenous [^3H]DNA-gp3 that sedimented to the position of viruses and DNA-filled heads in sucrose gradients. With both active and fully restored proheads, the value for efficiency was 1×10^5 to 3×10^5 (Table 3; data not shown). The restoration of prohead competence by the $7^{-}8^{-}14^{-}$ -infected cell extract is complete when compared to active purified proheads on the basis of [^3H]DNA-gp3 packaging (19.6%), efficiency of assembly to virus of fast-sedimenting exogenous DNA packaged (2.5×10^5), and virus yield (1.8×10^{11} PFU/ml) (Table 3).

TABLE 1. Restoration of inactive proheads for in vitro assembly

Source		Assembly (PFU/ml $\times 10^{11}$)		No. of expts
gp16	Proheads	Yield	Range	
$7^{-}8^{-}14^{-}$ ^a	$16^{-}14^{-}$	8.7 ± 3.6	13–5	6
$7^{-}8^{-}14^{-}$	Inactive proheads ^b	4.9 ± 3.8	14–0.7	9

^a The complete reaction mixture ($100\ \mu\text{l}$), prepared by combining equal volumes of extracts of cells infected with mutants $7^{-}8^{-}14^{-}$ (supplying gp16 and DNA-gp3) or $16^{-}14^{-}$ (supplying proheads and DNA-gp3), was incubated at 23°C for 120 to 180 min (5).

^b Purified $16^{-}14^{-}$ proheads inactivated by storage at 4°C for ca. 2 months cannot package DNA-gp3 in the presence of plasmid-specified gp16 (Fig. 1), and the viral assembly in that case is ca. 10^9 PFU/ml.

Cistron 17 is an early gene on the right end of the viral genome that is involved in DNA synthesis. With the extracts of 8⁻14⁻17⁻-infected cells, exogenous DNA-gp3 packaging is efficient, but the yield, 2.9×10^{10} PFU/ml, is atypically low (Table 3). This low yield may reflect a lower level of endogenous 8⁻14⁻17⁻ DNA-gp3 in the extract, because 34% of the phage in these complementations have the 4⁻8⁻ genotype of exogenous DNA-gp3 compared to the typical proportion of 5 to 10% (Table 3, data not shown). In contrast, the 1⁻8⁻14⁻-infected cell extract can restore inactive proheads for the packaging of purified DNA-gp3 (20.6%), but neither particles containing endogenous 1⁻8⁻14⁻ DNA-gp3 nor particles containing exogenous 4⁻8⁻ DNA-gp3 can be converted to phage by neck-tail assembly (Table 3).

DISCUSSION

In a refined system consisting of purified proheads and a cell extract containing plasmid-specified gp16, about 24% of the purified exogenous [³H]DNA-gp3 added was packaged in a concentration dependence study (6). The equivalent of 25% of the DNA-gp3 that is found at the position of DNA-filled heads in the sucrose gradient can be assembled to phage by subsequent complementation with a 7⁻8⁻14⁻16⁻-infected cell extract (6). Both in vitro prohead utilization and in vitro DNA-gp3 packaging are at least 25% as efficient as comparable processes during in vivo assembly.

TABLE 2. Restoration of DNase I-protected DNA-gp3 packaging by extracts of mutant-infected cells^a

Viral genotype ^b		[³ H]DNA-gp3 packaging (%) ^c		No. of expts
Extract	Plasmid	Centering on fraction 5	Fractions 1 through 32	
Series A				
7 ⁻ 8 ⁻ 14 ⁻	± pUM101	9.4 ± 5.5	19.6 ± 11.6	6
3 ⁻ 8 ⁻ 14 ⁻		3.3 ± 4.0	7.9 ± 5.6	5
Series B				
4 ⁻ 6 ⁻ 14 ⁻	+ pUM101 ^d	12.4 ± 5.9	30.6 ± 10.5	3
4 ⁻ 8 ⁻ 14 ⁻	+ pUM101	11.7 ± 6.2	25.3 ± 9.9	6
2 ⁻ 4 ⁻ 14 ⁻	+ pUM101	2.6 ± 0.6	9.1 ± 1.0	3
3 ⁻ 4 ⁻ 14 ⁻	+ pUM101	2.3 ± 0.7	8.0 ± 1.3	3

^a When exogenous DNA-gp3 was packaged by in vitro complementation, the reaction was terminated by addition of DNase I. In the absence of neck-tail assembly, some particles were disrupted during sucrose gradient centrifugation. We define all [³H]DNA-gp3 distributed in fractions 1 through 32 in the sucrose gradient as having been packaged (see Fig. 1). In these experiments, inactive purified proheads were incubated with extracts of mutant-infected cells.

^b The mutant extracts of series A generally reflect the limits of this packaging system. The 7⁻8⁻14⁻ extract is a source of gp16 but does not contain the prohead components gp7 and gp8. The phenotype of a 3⁻ mutant is complicated in that gp3 is essential for DNA synthesis and packaging. In these experiments, 3⁻8⁻14⁻ served primarily to activate proheads and to provide gp16; in 2 of 5 experiments we supplemented this extract with plasmid pUM101-specified gp16 (6).

^c 4⁻8⁻ [³H]DNA-gp3 was purified by CsCl gradient centrifugation (6). Data under the head fraction 5 represent trichloroacetic acid-insoluble radioactivity centering on fraction 5 of the 5 to 20% sucrose gradient, the position of DNA-filled particles (see Fig. 1). Data under the head fractions 1 through 32 represent all acid-insoluble radioactivity in fractions 1 through 32 of the gradient. Data are expressed as a percentage of the [³H]DNA-gp3 added to the complementation reaction.

^d The mutant infections of series B cannot produce gp4, a protein essential for the production of late structural and morphogenetic proteins (genes 7 through 16) (2). The plasmid pUM101 was used as a source of gp16 (6).

FIG. 2. Efficiency of restoration of prohead competence for in vitro [³H]DNA-gp3 packaging: variability and dependence on the complementing extract. Purified 16⁻14⁻ ³⁵S-labeled proheads (3 μ l), inactivated during storage, 4⁻8⁻ [³H]DNA-gp3 (50 μ l), and a *B. subtilis* (pUM101) extract (50 μ l; used as a source of gp16) were complemented for 70 min with extracts (50 μ l) of cells infected with mutant phages 2⁻4⁻ ($\blacktriangle$), 3⁻4⁻ ($\triangle$), 4⁻6⁻ ($\blacksquare$), or 4⁻8⁻ ($\square$). After treatment with DNase I (50 μ g/ml) for 10 min, 100- μ l portions of the mixtures were centrifuged in sucrose gradients as indicated in the legend to Fig. 1.

When proheads purified by sucrose gradient centrifugation were stored for about 2 months at 4°C, the proheads were not able to package DNA-gp3 in the refined system (Fig. 1). However, when these proheads were added to an extract of 7⁻8⁻14⁻-infected cells, [³H]DNA-gp3 was packaged (19.6 ± 11.6%; $n = 6$), and the virus neck-tail was assembled ($4.9 \times 10^{11} \pm 3.8 \times 10^{11}$ PFU/ml; $n = 9$) (Tables 1–3). In a number of experiments, competent proheads and restored proheads were indistinguishable in terms of yield, proportion of exogenous DNA packaged, and efficiency of conversion of packaged DNA-gp3 to virions (Table 3). Inactive proheads did not package enough [³H]DNA-gp3 to be distinguished from background radiation, and the phage yield (10^8 to 10^9 PFU/ml) consisted primarily of phage in the prohead preparation (data not shown).

The restoration of prohead competence by incubation of inactive purified proheads in cell extracts did not seem to require late proteins. Extracts of cells infected with recombinants were able to restore proheads even though they were unable to produce prohead constituent proteins, i. e., recombinants 7⁻8⁻10⁻14⁻, 7⁻8⁻14⁻16⁻, 8⁻9⁻14⁻, etc. (data not shown). Finally, suppressor-sensitive mutants in cistron 4

TABLE 3. Effect of viral genotype on prohead restoration for DNA-gp3 packaging and assembly^a

Genotype ^b	Prohead status	[³ H]DNA-gp3 packaging (%) ^c		Efficiency ($\times 10^3$) ^d	Yield (PFU/ml $\times 10^{11}$)
		Centering on fraction 5	Fractions 1 through 32		
7 ⁻ 8 ⁻ 14 ⁻	Active	9.8	18.2 (2)	135 (2)	2.2 (2)
7 ⁻ 8 ⁻ 14 ⁻	Inactive	9.4	19.6 (6)	250 (1)	1.8 (5)
8 ⁻ 14 ⁻ 17 ⁻	Inactive	11.1	29.4 (3)	110 (2)	0.29 (3) ^e
1 ⁻ 8 ⁻ 14 ⁻	Inactive	8.6	20.6 (2)	1.7 (1)	0.01 (2)

^a Inactive purified proheads were incubated with extracts of mutant-infected cells to test for restoration of exogenous DNA-gp3 packaging and assembly to phage.

^b The early genes 17 and 1 are located on the right and left ends of the chromosome, respectively, and their products are purported to affect DNA synthesis.

See footnote c of Table 2. The number of experiments used to compute the average values are in parentheses.

^d Efficiency is defined as the ratio of phage with the exogenous genotype 4⁻8⁻ produced in complementation to the counts of exogenous [³H]DNA-gp3 that sediment in the sucrose gradient to the position of viruses and DNA-filled heads (centering on fraction 5). The efficiency obtained in typical complementation is about 1×10^5 to 3×10^5 .

^e The reduction in viral yield may reflect a low level of endogenous DNA-gp3 in the extract. More than 34% of the assembled phage have the 4⁻8⁻ genotype of exogenous [³H]DNA-gp3.

are unable to synthesize any known structural or morphogenetic proteins with the exception of the early protein gp3. The 4⁻8⁻14⁻-infected cell extract could package $25.3 \pm 9.9\%$ ($n = 6$) of the purified exogenous DNA-gp3 added (Fig. 2; Table 2).

Incubation of inactive proheads with an extract containing early proteins seemed sufficient to restore competence for DNA-gp3 packaging. The cistrons of $\phi 29$ have been numbered sequentially from left to right on the $\phi 29$ chromosome. The known cistrons that code for early proteins are 1 through 6 and 17. gp2 and gp3 are essential for DNA-gp3 synthesis (see reference 7 for review). In both 3⁻4⁻14⁻-infected and 2⁻4⁻14⁻-infected cell extracts, prohead competence did not seem to be fully restored. With these recombinants and 3⁻8⁻14⁻, about one-third of the capacity to package DNA-gp3 was restored (Fig. 2; Table 2). Cistrons 4, 6, and 17 did not seem to be required for the restoration of inactive proheads to package DNA-gp3, and with 8⁻14⁻17⁻ the efficiency of functional neck and tail assembly seemed comparable to that of 7⁻8⁻14⁻ extracts (Fig. 2; Tables 2 and 3). The results with the 1⁻8⁻14⁻ extract were unusual in that more than 20% of the exogenous DNA-gp3 was packaged, but virions were not assembled from either endogenous 1⁻8⁻14⁻ or exogenous 4⁻8⁻ DNA-gp3 (Table 3).

We have demonstrated that DNA-gp3 packaging is oriented from left to right, requires a functional gp3 and gp16, and is quantized (4-6). If we apply our operational definition of DNA-gp3 packaging, purified proheads lose most if not all of their competence to package DNA-gp3 upon storage at 4°C (Fig. 1). A typical prohead can regain competence to package DNA-gp3 in a 4⁻ extract, and competence in a 7⁻8⁻14⁻ extract is similar to that of active (fresh) proheads

both in terms of DNA-gp3 packaging and virus yield (Tables 1 and 2). However, DNA-gp3 packaging in restored proheads may not always be correct. For example, a random orientation of packaging might result in the reduced packaging observed in 2⁻4⁻14⁻ and 3⁻4⁻14⁻ extracts and an inability to assemble a functional neck and tail in a 1⁻8⁻14⁻ extract (Tables 2 and 3).

Reversible inactivation of proheads is a mysterious and interesting phenomenon. Since we did not detect any minor prohead proteins (unpublished data), inactivation may reflect a change in conformation of the connector or shell. An understanding of prohead stability or competence may lead to identification of proteins and conformational changes that function in substrate recognition or the initiation of DNA-gp3 packaging.

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