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In Vitro Packaging of Satellite Phage P4 DNA

(bacteriophage P4/bacteriophage P2/morphogenesis/DNA condensation)

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ABSTRACT **Satellite phage P4 directs the capsid proteins of its helper phage, P2, to form a head which is only one-third the size of the normal P2 head. The P2 head contains a genome of molecular weight 22 × 10^6, while the small P4 head contains a genome with a molecular weight of only 7 × 10^6. We have used *in vitro* DNA packaging to test whether P2 and P4 phage head sizes are determined by DNA size.**

The small DNA of satellite phage P4 added to a P2-infected cell extract was packaged primarily into particles containing three copies of the P4 genome. This process occurred with approximately the same efficiency as P2 DNA packaging in the same cell extract. In contrast, the large DNA of P2 was packaged 300-fold less efficiently than the small DNA of P4 in an extract derived from P4-infected, P2-lysogenic cells. These results suggest that DNA size is not sufficient to determine head size. The results are compatible with DNA packaging via the filling of preformed empty capsids.

In order to understand the morphogenesis of virus heads it will be necessary to construct an *in vitro* system which allows head precursors to assemble into a finished product. Kaiser and Masuda (1) have recently reported such a system, in which genetically marked λ phage DNA was packaged into plaque-forming units (PFU) at an efficieny of 10^{-6} PFU per DNA molecule. Although the efficiency of packaging is low, such a system provides a means to study the role of phage DNA and proteins in the assembly of phage heads.

We report here the results of packaging experiments using satellite phage P4 and its helper, temperate coliphage P2. These phages provide an unusual approach to the study of head morphogenesis: P4 requires the six known head genes of the helper for the synthesis of its own capsid (2) but directs the helper proteins to assemble into a small head (45-nm diameter) which is never found during normal P2 infection (3). (P2 heads are 62-nm in diameter). The double-stranded DNA of P4 is only one-third the size of helper phage DNA, and the volume of the P4 phage head is about one-third that of the P2 head (4). This suggests that genome size might determine capsid size. Alternatively, assembly into a small head may be directed by a P4-induced protein, which is found in P4 heads but not in helper heads (K. Barrett, personal communication).

We suspected that P2-infected-cell extracts might package satellite phage P4 DNA, since the cohesive ends of P2 and P4 DNAs are very similar (5) and provide a logical recognition site for the initiation of DNA packaging. The P2-P4 system, then, might provide a means to study the requirements for formation of the P4-sized head. The results presented here show that P4 DNA alone does not cause formation of small heads *in vitro*, but that in helper-infected cells, P4 DNA is encapsulated into heads of P2 size. We also find that the amount of P4 DNA packaged per head corresponds predominantly to one headful. Thus, a headful of DNA may enjoy some advantage in the assembly or injection process (22). In fact, our results suggest that head size determines genome size, even in the P2-P4 phage system, where the genomes are unique and have fixed ends.

Abbreviation: PFU, plaque-forming units.

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MATERIALS AND METHODS

Chemicals. Twice-crystallized egg white lysozyme was purchased from Worthington Biochemical Corp. Pyruvate kinase (type II: crystalline, from rabbit skeletal muscle) and spermidine trihydrochloride were purchased from Sigma Chemical Co.

Buffers. Lysis buffer contained 10 mM Tris·HCl (pH 8.0), 3 mM EDTA, 10 mM NaCl, and 0.1 mM spermidine (6). P-buffer is described by Lengyel *et al.* (7). DNA for packaging was stored in a sterile solution containing 10 mM Tris·HCl (pH 8.0), 50 mM NaCl, and 0.1 mM EDTA.

Bacteriophage Strains. P2 vir_{22} is insensitive to P2 immunity (8). The P2 amber mutant strains used all carry the vir_1 mutation, which prevents the establishment of immunity but does not affect sensitivity to immunity (9). To emphasize the "strong virulent" nature of P2 vir_{22}, the vir_1 (weak virulent) marker of the P2 amber mutants will not be referred to in later sections of this paper. P2 vir_1 am_{12} is defective in cell lysis (10); P2 vir_1 amM_{32} and P2 vir_1 amQ_{34} carry mutations which affect late steps in head synthesis (7, 11). P4 vir_1 is a clear-plaque mutant (12). P4 vir_1 am_1 is an amber mutant unable to synthesize DNA under nonpermissive conditions (3). P2 and P4 stocks were prepared as previously described (7, 3) and were banded two or three times in CsCl.

Bacterial Strains. All the bacterial strains were derivatives of *Escherichia coli* C (13). Strains C-1a (14) and HF4704 (15), which are nonpermissive (sup^-) for amber mutants, were used as hosts for P2 infection. Strain C-1766, which is sup^+ and lysogenic for P2 (3), was the host for P4 vir_1 am_1 infection. The indicator strains used for assaying plaque-forming activity produced in the packaging reaction are all P2-lysogenic derivatives of strain C-1055, a sup^- strain (16): strains C-1749 (3) and C-1197 (made by lysogenization with $P2^+$) were used interchangeably to assay PFU of P2 vir_{22} and P4 vir_1 produced in the packaging reaction. These strains select against both the amber mutation and the sensitivity to immunity of the P2 phage strains used for infection; they select

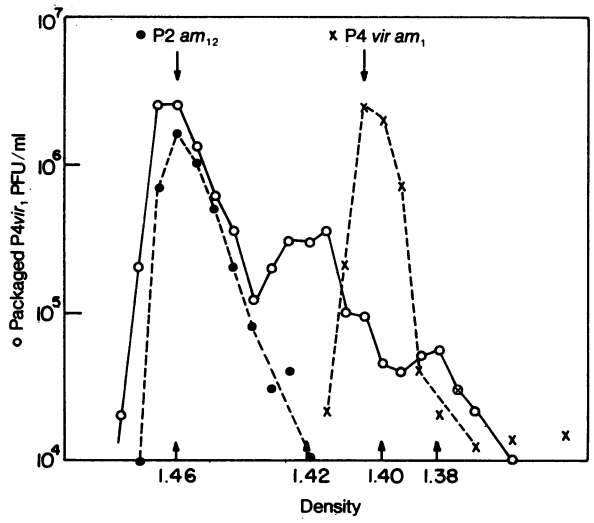

FIG. 1. CsCl equilibrium gradient sedimentation of P4 vir_1 plaque-forming units packaged by 5 ml of unsonicated, whole extract prepared from P2 am_{12}-infected HF4704. (In this experiment, P2 am_{12} infection was allowed to proceed until shortly before lysis would have occurred in P2 lysis$^+$ infected cells.) After the normal period of *in vitro* packaging, the reaction mixture was made up to a density of 1.42 by addition of solid CsCl, and P4 vir_1 am_1 was added as a marker of normal density. The solution was then centrifuged at 32,000 rpm for 48 hr at 4° using an SW50.1 rotor. The 30 fractions collected contained 10 drops each. To determine the approximate density gradient, the refractive index of every fifth fraction was measured. The density values are overestimates, due to the refraction of contaminating cellular material from the packaging mixture. All fractions were titered for P2 am_{12} (●——●), P4 vir_1 am_1 (×- - -×), and P4 vir_1 (O- - -O), as described in *Materials and Methods*. In order to allow the curves to appear on the same scale, the titers of P2 am_{12} are multiplied by 10^{-6}, and the titers of P4 vir_1 am_1 are multiplied by 10^{-4}.

against only the amber mutation of P4 vir_1 am_1. Strain C-1889 is C-1055 lysogenic for P2 amH_{13}, a mutant defective in one of the P2 tail genes (10). This strain was used as an indicator for P2 vir_{22} in the experiment described on line 2, column 1 of Table 3. In that experiment, a selection specific for P2 vir_{22} was necessary since P4 vir_1 is present as a revertant background in the phage stock used for infection. Strain C-1757, which carries the amber suppressor *supD* (11), was used as the indicator for P2 amber phage. Strain C-1758, which is C-1757 lysogenic for P2 (3), was used to assay P4 vir_1 am_1.

Media. HF complete medium (17) was used for growth of strains C-1a and HF4704. Super TPG, which is TPG medium (12) containing 25 μg/ml of each amino acid, 25 μg/ml of each nucleic acid precursor, 0.1 mM spermidine, and 1% (V/V) vitamin mix (Difco supplementary literature, 1968, p. 469), was used for growing C-1766. (TPG and HF media were both buffered with Tris·HCl buffer and contained glucose as the carbon source, plus amino acids and other nutrients, but the burst size of P4 was greater in cells grown in TPG medium than in cells grown in HF medium.) LB medium (18), with NaCl reduced to 0.1 M, was used for growing the indicator strains.

Preparation of Extracts—Whole Extracts. Cells were grown to a density of 6×10^8/ml at 37° with aeration. The culture was then infected with phage at a multiplicity of 10–20 and allowed to continue growing at 37°. At the appropriate time, the culture was cooled quickly by pouring onto frozen basal growth medium. The cells were harvested by centrifugation at 10,000–12,000 × *g* for 10 min and concentrated 100-fold in lysis buffer containing 2 mg/ml of egg-white lysozyme. The cells were lysed by freezing and thawing briefly at 37° (twice). After the second thaw, $MgCl_2$ (12 mM) and dithiothreitol (0.12 mM) were added to the extract which was then sonicated to reduce viscosity (5–10 sec using the "Needle" probe, Bronwill no. BPII-40T, at a setting of 40 on a Biosonic sonicator, from Bronwill Scientific Co.). Sonication results in at least a 20-fold increase in packaging efficiency; this effect is not yet understood.

Unless otherwise noted, P2 am_{12} infection was stopped at the time of maximum packaging competence (calculated using the results in Fig. 3*a*, and assuming a direct proportionality between cell-doubling time and time of maximum packaging competence).

Preparation of Extracts—Supernatant Extract. The whole extract was centrifuged for 15 min at 3,000 × *g* (5,000 rpm in the SS-34 head of a Sorvall RC2-B centrifuge), and the supernatant was removed with a pasteur pipette. Experiments presented which used whole extract have been repeated using the supernatant fraction, yielding results similar to those shown. All extracts were kept on ice and used for the packaging reaction within an hour.

TABLE 1. *Cell fractions required for packaging*

	Immunity-insensitive, am^+ PFU/10^6 DNA molecules	Surviving colony formers/ml in extract
Whole extract	10	7.4×10^6
Supernatant	12	<200
Pellet	4.1	4.8×10^5

Whole extract and supernatant were prepared from *E. coli* C-1a infected with P2 am_{12} as described in *Materials and Methods*. The pellet remaining after preparation of the supernatant was resuspended gently in 0.25 ml of lysis buffer containing 12 mM $MgCl_2$ and 0.12 mM dithiothreitol. Whole extract, supernatant, and pellet were tested for the ability to package P2 vir_{22} DNA as described in *Materials and Methods*.

TABLE 2. *Efficiency of packaging P2 vir_{22} DNA in mixtures of extracts from cells infected with P2 amber mutants*

	Extract 2		
Extract 1	am_{12} (head$^+$)	amM_{32} (head$^-$)	amQ_{34} (head$^-$)
am_{12} (head$^+$)	65	—	—
amM_{32} (head$^-$)	—	20	430
amQ_{34} (head$^-$)	—	—	0.02

P2 amber mutant-infected whole extracts (*E. coli* C-1a host) were prepared as described in *Materials and Methods* with one exception: all infections were allowed to continue for 28 min until just before the normal lysis time of P2 lysis$^+$ infected cells. In the packaging reaction, the head-defective (M_{32}, Q_{34}) extracts were mixed in pairs (25 μl of each). Head$^+$, lysis-defective (am_{12}) extract (50 μl) was used as a control. The efficiency of packaging is expressed in units of immunity-insensitive, am^+ PFU per 10^6 DNA molecules, as in Table 1.

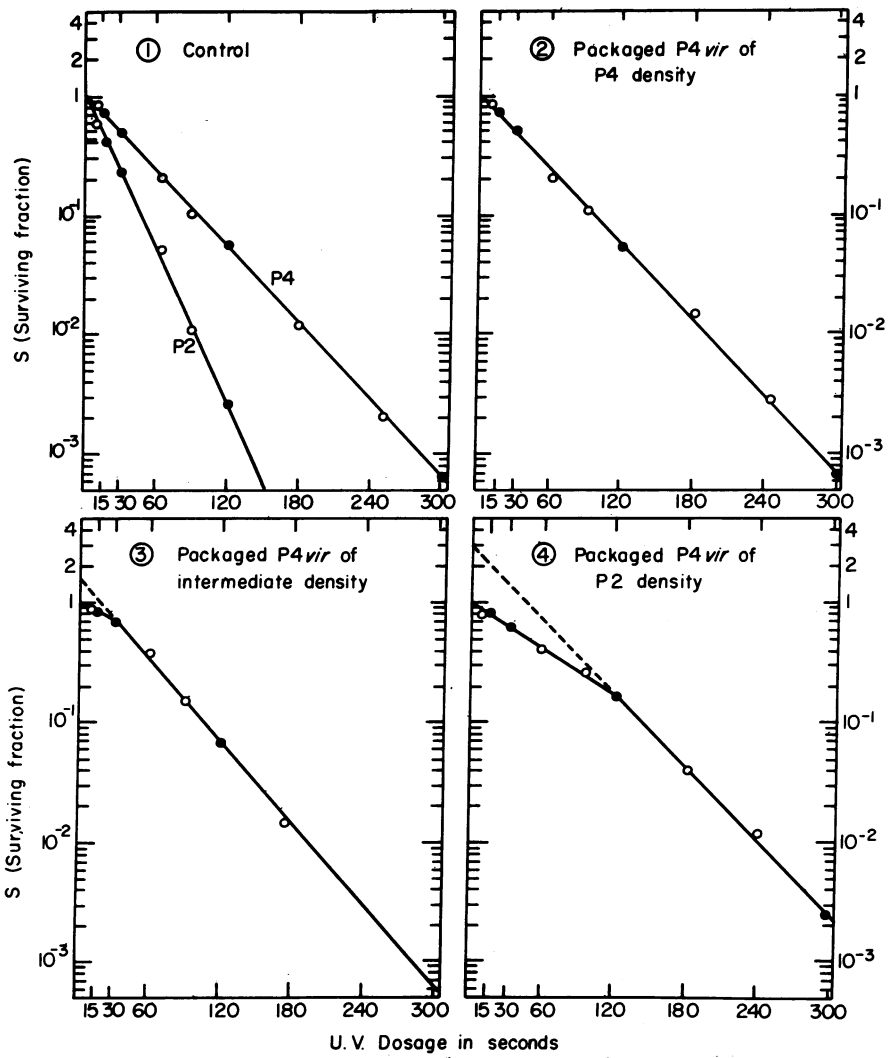

FIG. 2. Ultraviolet light inactivation of the three density classes of P4 vir_1 PFU packaged in a P2 am_{17}-infected cell extract. Fractions from each of the three peaks of P4 vir_1 particles in the CsCl gradient of Fig. 1 were pooled and diluted in a solution containing 80 mM $MgCl_2$, 50 mM Tris·HCl (pH 7.2), and 1% $NH_4(C_2H_3O_2)$. These pooled fractions were then exposed to ultraviolet light at 24 erg per mm^2 per sec. At appropriate time intervals, samples were withdrawn and assayed on *E. coli* C *sup*$^-$ (P2). The data represent a composite of two separate but identical experiments represented by *empty* and *filled circles*.

Packaging Reaction. To carry out packaging, 50 μl of extract was incubated for 60 min at 37° in the presence of 5 mM phosphoenolpyruvate, 1 mM ATP, 1.3 μg of pyruvate kinase, and 0.6–0.8 μg of P2 vir_{22} or P4 vir_1 DNA. The final reaction volume was 60 μl; each reaction was performed in duplicate. The DNA was pretreated by heating for 10 min at 70° followed by quick cooling in order to break up end-to-end aggregates (5, 19). P2 DNA heated in this way is packaged in our system with a 10-fold higher efficiency than unheated P2 DNA. In contrast, this treatment had little effect on the efficiency with which P4 DNA was packaged. The reaction was stopped by transferring to 0° and diluting 10-fold with P-buffer containing 50 μl/ml of pancreatic DNase.

RESULTS

Experimental Design. The experiments to be presented are all of one basic type. Cells infected with P2 amber mutant or P4 amber mutant phages were concentrated, lysed, and then sonicated briefly to reduce viscosity. The whole extract thus obtained could be used in the packaging reaction or could first be separated into supernatant and pellet fractions. The packaging reaction consisted of whole or supernatant extract incubated with P2 virulent or P4 DNA plus ATP. After stopping the reaction with deoxyribonuclease, incorporation of the exogenous DNA into viable phage could be determined by plating the reaction mixture on a P2-lysogenic indicator which does not carry an amber suppressor. Such an indicator strain is insensitive to the infecting phage, but is sensitive to phage derived from the exogenously added DNA. In such a packaging reaction, exogenous, genetically marked P2 DNA could be packaged into viable phage with efficiencies between 4 and 400 × 10^{-6} PFU per DNA molecule.

Criteria for In Vitro Packaging. In order to determine whether this process is actually occurring *in vitro*, we have performed two types of experiments. First, we have tested for packaging in the supernatant fraction of our extract. As can be seen in Table 1, full recovery of activity is obtained in the 3,000 × *g* supernatant, while viable cells and presumably spheroplasts are found in the pellet. The amount of packaging activity left in the pellet varies and is probably a

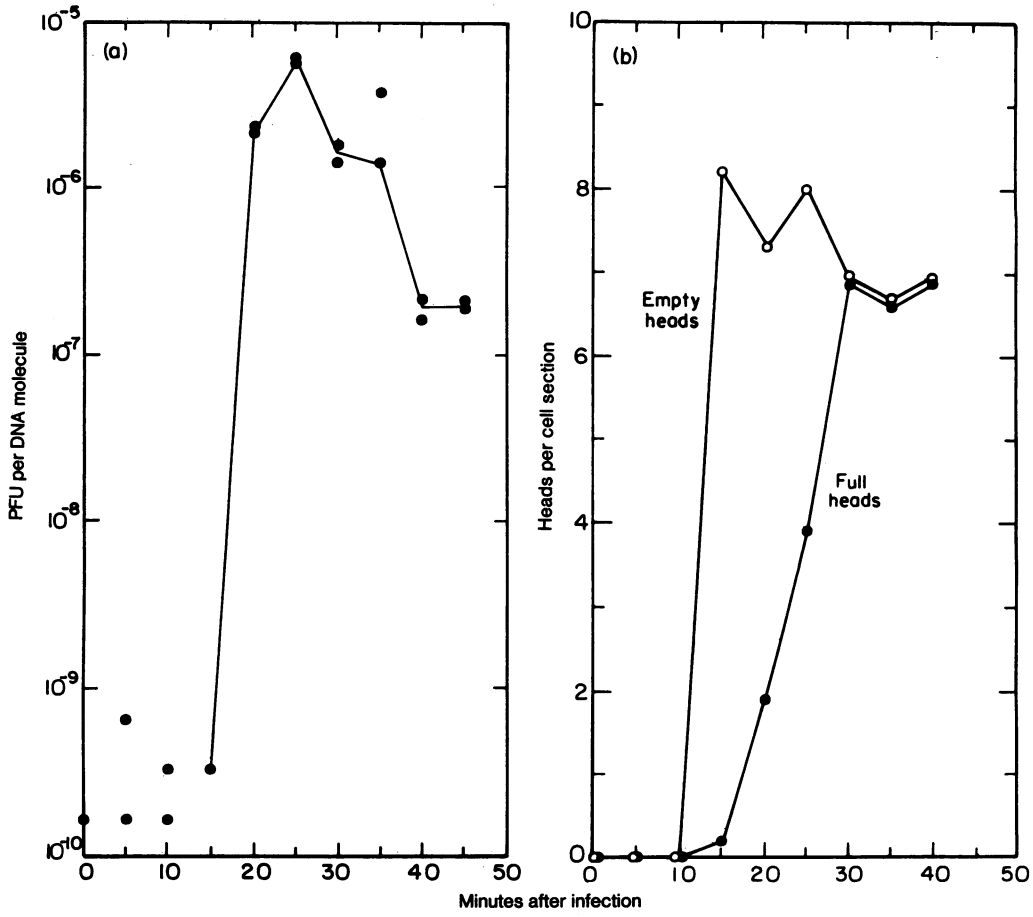

FIG. 3 (*a*). *Efficiency of DNA packaging as a function of time after infection.* Aliquots (25-ml) of a culture of HF4704 were removed at various times after infection with P2 am_{12}, quick-cooled, and stored at 0° until the last sample was taken. Whole extracts were then prepared and tested for packaging as described in *Materials and Methods*. Only one data point is shown on the graph where duplicate packaging reactions gave identical results. FIG. 3 (*b*). *Concentration of intracellular headlike particles as a function of time after infection.* Aliquots (10-ml) of cells taken as described in Fig. 3 (*a*) were fixed, pelleted, embedded, sectioned, and stained with uranyl acetate and lead citrate as described previously (15). The thin sections were observed in a Siemens Elmiskop I electron microscope at 20,000–30,000 magnification. The number of empty (O——O) and filled (●——●) heads per cell section were tabulated.

function of the amount of supernatant trapped in the pellet. Secondly, we have carried out the packaging assay under conditions which require complementation between two head-defective cell extracts for the product of viable phage. As can be seen in Table 2, a mixture of head-defective extracts derived from P2 M$^-$-infected cells and P2 Q$^-$-infected cells can package P2 DNA at a level greater than that seen in an extract of cells infected with a lysis defective mutant, P2 am_{12}. The high packaging background observed in the case of P2 amM_{32}-infected cell extracts is 100-fold greater than expected from the leakiness of the amM_{32} mutation *in vivo* (11). The reason for this finding is not clear, but we believe that head assembly may be slowed down in P2 M$^-$-infected cells, resulting in the accumulation of precursors which package DNA *in vitro* during our 60-min incubation period.

Head Size Determination In Vitro. The above experiments do not examine the mechanism of packaging. Since this is the question of interest, we have taken advantage of the unique properties of the P2-P4 system to investigate whether DNA forms the core around which free capsid condenses. If DNA alone forms a size-determining core, P2 PFU should be obtained when P2 DNA is added to a P4-infected-cell extract. Moreover, P4 PFU of normal density should be obtained when P4 DNA is added to a P2-infected-cell-extract. Neither of these expectations was fulfilled. In Table 3 it can be seen that packaging of P2 DNA in a P4-infected-cell extract is reduced 300-fold compared to packaging of P4 DNA in the same extract. In contrast, packaging of P4 DNA in a P2-infected-cell extract occurs at a level comparable to that seen with P2 DNA. Moreover, these P4 PFU are grossly abnormal: when banded in CsCl they show a trimodal distribution (Fig. 1) About 90% of the activity bands with the heavy density characteristic of P2 phage, while about 10% has a density between that of P2 and P4; 1% of the activity has a density slightly lower than that of P4. These results strongly suggest the packaging of trimers, dimers, and monomers of P4 DNA into P2-sized heads. To confirm this interpretation, we tested each class of P4 PFU for inactivation by ultraviolet light. The results of these experiments are shown in Fig. 2. The P4 of lowest density (Fig. 2.2) is inactivated with single-hit kinetics, identical to those seen with the control P4 (Fig. 2.1). The P4 phage of intermediate density (Fig. 2.3) and heavy density (Fig. 2.4)

TABLE 3. *Packaging of P2 vir_{22} DNA and P4 vir_1 DNA in extracts of P2-infected cells and P4-infected, P2-lysogenic cells*

Infected cell extract	PFU/10^6 DNA molecules: With P2 vir_{22} DNA	With P4 vir_1 DNA
E. coli sup^- + P2 am_{12}	3.6	0.91
E. coli sup^+ (P2) + P4 vir_1 am_1	0.02	5.9

Supernatant extracts were prepared and tested for packaging as described in *Materials and Methods*, using *E. coli* C-1a as host for P2 infection and *E. coli* C-1766 as host for P4 infection. The packaging of P4 vir_1 DNA is detected at levels more than 1000-fold above the background of am^+ revertants in the P4 vir_1 am_1-infected cell extracts.

show the multi-hit kinetics expected for phage containing two copies and three copies, respectively, of the phage genome (20). We believe, therefore, that multimers of P4 DNA are being packaged into heads of P2 size.

Are Empty Heads Packed with DNA? The above results are compatible with the idea that phage DNA is packaged into preformed heads. To further investigate this possibility, we used electron microscopy to correlate the onset of packaging competence in the extract with the time of appearance of heads in infected cells. Fig. 3 shows that the ability to package is not detected until 10 min after headlike structures have appeared, supporting the idea that preformed heads are a precursor to heads packed with DNA *in vitro*.

DISCUSSION

We have demonstrated that the small DNA of satellite phage P4 is packaged in a P2-infected-cell extract primarily as particles containing three copies of the phage genome. In contrast, the large DNA of P2 phage is packaged poorly in a P4-infected-cell extract. Thus, the size of P2 and P4 DNAs could not be determining head size in our *in vitro* system. It seems more likely that the small size of the P4 head was determined by a P4-induced late protein which is found in the P4 head at about 45 copies per head, and which is absent from P2 heads (K. Barrett, personal communication). Mutants affecting this protein will have to be isolated in order to test this hypothesis critically. The putative "size-determining protein" might not be essential for plaque formation, since P4 DNA can be incorporated into P2-sized heads *in vitro* (Table 3, Figs. 1 and 2).

The discovery that three classes of P4 PFU are produced when P4 DNA is added to a P2-infected extract is also consistent with the idea that DNA is packaged into preformed capsids (21). The observation that packaging ability in the extract appears significantly later than the appearance of phage heads in the infected cells (Fig. 3*b*) is compatible with this interpretation. We cannot, however, rule out co-condensation of DNA and protein as the pathway of head morphogenesis, since empty heads seen in our thin sections may have been derived from unstable preheads which originally carried DNA.

Preliminary experiments indicate that the packaging of P4 DNA into P2-sized heads is extremly rare *in vivo*, when *E. coli* is coinfected with P2 and P4 (amounting to <1% of the P4 produced) (Souza, Goldstein, and Pruss, unpublished results). This observation is difficult to reconcile with our *in vitro* results, namely, that P4 DNA is packaged as efficiently as P2 DNA in a P2-infected-cell extract. One possible explanation is that P4 DNA exists in the cell only as circular molecules of single genome length (12). These molecules may not be good substrates for packaging into P2-size heads. The observed dominance of P4 in a coinfection with P2 may also account for the *in vivo* results (K. Barrett, unpublished results). Alternatively, P4 may induce a protein which associates specifically with P4 DNA and prevents it from being incorporated into a P2 head.

It is interesting to note that *in vitro* packaging has only been observed using phages with cohesive-ended DNA. One interpretation of this result is that circular or concatenated DNA molecules, which are easily formed from cohesive-ended molecules, are required substrates for packaging. Since P4 DNA trimers are packaged into P2 heads, we are probably, observing packaging of concatenated P4 DNA. In contrast, P2 DNA may not be packaged efficiently from very long concatenated precursors, since packaging activity is stimulated when end-to-end aggregates of P2 DNA are dissociated by heating. Clearly more research on the form of the DNA packaged will be needed to interpret these observations.

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