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Biochemistry

Assembly of biologically active proheads of bacteriophage lambda *in vitro*

(phage morphogenesis/DNA packaging/prohead synthesis *in vitro*)

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ABSTRACT Bacteriophage λ DNA can be packaged *in vitro* into preformed proheads to generate plaque-forming units. This complex set of reactions is initiated when λ DNA is mixed with the product of the phage *A* gene, and proheads. Because proheads are an essential early reactant, the system has potential as an assay for the formation of biologically active proheads.

When extracts of cells infected with certain λ head mutants (for example, B^-, C^-, $Nu3^-$, and E^-) are used as the prohead donor, plaque-forming units are not produced. However, when extracts of E^-- and $Nu3^-$-infected cells are first reacted together the combination provides prohead-donor activity to the *in vitro* packaging system. *In vitro* assembled, biologically active proheads have the same sedimentation properties and electron microscopic appearance as "wild-type" proheads isolated from λA^-D^--infected cells. Centrifugation analysis shows that the $Nu3^-$ extract contributes gpE, the major capsid protein, to the reaction in the form of monomers or small polymers.

The morphogenesis of the icosahedral head of bacteriophages comprises an elaborate set of reactions in which both phage and bacterial coded factors are involved (for a review, see ref. 1). Basically, the process can be divided into two stages: the building of the prohead and the encapsidation of the phage DNA into the preformed prohead. The latter stage has been intensively studied in the last few years and, for phages λ, T7, P4, and P2, it has been possible to catalyze, in cell-free extracts, the encapsidation of exogenous DNA by isolated proheads (2–8). Success in reproducing the earlier stage *in vitro*, synthesis of active proheads, has not yet been reported.

In this paper, we describe a system for phage λ in which biologically active proheads are synthesized *in vitro*. A measure of prohead production is possible because the packaging of λ DNA *in vitro* provides a biological assay for proheads. Proheads are an essential early reactant for the series of biochemical steps in which phage DNA, gpA, proheads, phage tails, and other phage-coded gene products interact in sequence to generate λ plaque-forming units (PFU) (2–5, 9). Lambda genes *B*, *C*, *Nu3*, and *E*, and the host gene *groE* are the genes known to be essential in the morphogenesis of biologically active proheads (10, 11, 24). Cells infected with phage mutants in gene *E* produce no heads or head-related particles since gpE is the major capsid protein (12–14). Infection with *C* mutants results in the accumulation of biologically inactive prohead-like structures. Infection with phage with mutations in genes *B* or *Nu3* similarly generates inactive proheads as well as aberrant forms such as "monsters" and tubular structures (11, 14, 15, 24). A phenotype similar to the B^- and $Nu3^-$ cases is observed in *groE* cell mutants infected with wild-type phage (10, 11, 16).

Thus, when one tries to use extracts of cells infected with λ mutants in genes *B*, *C*, *Nu3*, or *E* as prohead-donor, no PFU are detected (5, 11, 24). We have explored the possibility that two extracts prepared from cells infected with two different prohead mutants can complement one another *in vitro*. In this communication, we report on experiments in which biologically active proheads have been synthesized by this type of complementation.

Abbreviations: gpB, gpC, the protein products of gene *B*, gene *C*, etc.; PFU, plaque-forming unit.

MATERIALS AND METHODS

Bacterial Strains. All the bacteria used were derivatives of *Escherichia coli* strain K12. These were: (*i*) QD5003($\lambda i^{21}Sam_7$); (*ii*) 594($\lambda Aam_{32}cI_{857}Sam_7$); (*iii*) 594($\lambda Aam_{11}Bam_{10}cI_{857}Sam_7$); (*iv*) 594($\lambda Aam_{a19}Dam_{15}cI_{857}Sam_7$); (*v*) 594($\lambda Bam_{10}cI_{857}Sam_7$); (*vi*) 594($\lambda Cam_{42}cI_{857}Sam_7$); (*vii*) 594($\lambda Nu3am_{a8}cI_{857}Sam_7$); (*viii*) 594($\lambda Eam_4cI_{857}Sam_7$); (*ix*) W3550($\lambda dg805cI_{857}Sam_7$); and (*x*) W3350.

Strains (*i*), (*ii*), (*ix*), and (*x*) have been described in ref. 5. The prophage in strain (*ix*) is missing the genetic material between markers Wam_{812} and Wam_{859}, and *att*. Strain (*iv*) was provided by N. Sternberg and is a pseudorevertant of $\lambda Dam_{15}cI_{857}$ (17). It was provided as $\lambda Aam_{a19}Dam_{15}cI_{857}$; the addition of the Sam_7 mutation and the introduction of this strain as prophage into 594 was done in our laboratory. Strains (*iii*) to (*vi*) and (*viii*) have already been described (14), as has strain (*vii*) (18).

Source of DNA and gpA. The DNA was prepared by phenol extraction of thermally-induced cultures of strain (*ii*) as previously stated (5). gpA was partially purified from thermally-induced 594($\lambda dg805cI_{857}Sam_7$) cells as outlined in ref. 5. The ASI fraction was used as a source of gpA in all the experiments except for the experiment in Fig. 1A, in which the AsII fraction was used.

Preparation of Extracts. The procedures used in preparing cell-free extracts for the assembly reaction *in vitro* have been described elsewhere (5). Extracts to be used for prohead-donor activity were prepared from thermally induced cultures of strains (*iv*) to (*viii*) by ultrasonic irradiation (sonicates) as described by Becker and Gold (5). A sonicate of induced W3350($\lambda dgal_{805}cI_{857}Sam_7$) sometimes served as a source of gpA. Thermally induced 594($Aam_{11}Bam_{10}cI_{857}Sam_7$) cells were always used to catalyze the second stage reaction (5), providing gpD, gpW, gpFII, and tail function to the assay (5, 24). These second-stage extracts were prepared by the lysozyme-freeze-thaw method first described by Kaiser and Masuda (2).

Standard Assay for Prohead Activity. This was carried in two stages as described by Becker and Gold (5). In the first stage, 10 μl of a putative prohead-containing extract (sonicate) was mixed with 5 μl of A^--arrested DNA as the exogenous substrate for packaging and 1 unit of gpA in the presence of Mg^{2+}, spermidine, and ATP (5). After incubation for 15 min at room temperature, the second stage was initiated by the addition of 200 μl of an lysozyme-freeze-thaw extract prepared from $\lambda AamBam$-infected 594 cells (5). After 60 min of incubation

at room temperature, the reaction mixture (containing assembled λ phage) was diluted and plated on QD5003(λ*i*-[21]*Sam*7) as the indicator strain.

Complementation *In Vitro*. We define complementation *in vitro* for prohead function as the ability of two prohead-defective sonicates to react together to yield prohead donor activity in the above assay. Operationally, 100 μl of each of two sonicates prepared as described were mixed together and incubated overnight at 20–22°. Ten microliters of this mixture was subsequently added to the standard assay to test for prohead activity. Prohead assembly *in vitro* is a slow reaction, sometimes characterized by a lag phase of several hours. Overnight incubation is both convenient, and ensures optimum yields of product.

Effect of Extract Concentration on Prohead Yield. The two complementing sonicates were prepared as described above. Samples (0.1 ml) of one extract were mixed with 0.1 ml aliquots taken from a set of dilutions of the other complementing extract. Dilution of the extracts was in buffer A (20 mM Tris·HCl (pH 8.0)/1mM EDTA/3 mM $MgCl_2$/5 mM 2-mercaptoethanol) (5) or in a W3350 sonicate. After overnight incubation, a 20 μl-sample from each mixture was tested as prohead donor in the standard test for prohead activity.

Fractionation of Extracts by Sedimentation. To determine if the complementing sonicates were contributing relatively large products, or small (monomeric or oligomeric) protein forms, we subjected 1 ml samples of the extracts to centrifugation at 45,000 rpm for 60 min at 4° in the 50Ti rotor of the L2-65B Beckman ultracentrifuge. Relatively large assembly products such as proheads are pelleted under these conditions, while monomers or small polymers remain in the supernatant.

Sedimentation Analysis of the Product of *In Vitro* Complementation. After the standard overnight incubation of two complementing sonicates, 0.1 ml of the mixture was layered on top of a 5–25% sucrose gradient (wt/vol) in buffer A and centrifuged at 45,000 rpm for 45 min at 4° in the SW56 rotor of a Beckman ultracentrifuge. Sixteen-drop fractions were collected from the bottom and 10 μl-aliquots were assayed for prohead activity as described above.

Electron Microscopy. A small drop of extract was layered on top of Formvar, carbon-coated copper grids, and stained by the addition of another small drop of 2% sodium phosphotungstate, pH 7.4, for 30 sec. The preparations were observed in a Philips 300 electron microscope immediately after drying. Nominal magnification factor on the photographic plates was 16,000-fold. Because of the high concentration of the crude extracts, staining was difficult and resolution was poor, but proheads could be seen easily and counted so that a rough estimate of their concentration could be made.

RESULTS

Complementation for Prohead Function *In Vitro*. The first experiments were directed to determining whether complementation *in vitro* for prohead formation could take place. To do this, extracts were prepared from induced lysogens in which the prophages carried mutations in head function-related genes and the ability of these extracts to act as prohead donors, singly or in combination, was measured. The results of such an experiment are shown in Table 1. As expected, an extract prepared from cells induced for an *AamDam* double-mutant prophage provided biologically active proheads, whereas extracts obtained from cells induced for *B, C, Nu3,* and *E* mutant prophages were very poor proheads donors (24). Although each of the six combinations of mutants shown in Table 1 complement in coinfection experiments *in vivo*, only one pair—$Nu3^-$ + E^-—complemented with high efficiency *in vitro*, with a stimulation of approximately 6×10^4-fold over the average background of the individual extracts. Table 1 also shows that the development of competence on the part of the $Nu3^-$-E^- pair is a time-dependent process. When the $Nu3^-$ and E^- extracts were mixed and added within minutes to the assembly assay, as in a standard test for preformed proheads, no PFU were made. A prolonged interaction (overnight) between these extracts is required before prohead-donor function can be detected.

Table 1. Prohead-donor activity of mutant extracts

Extract	Activity, PFU/ml
A^-D^-	1.1×10^7
B^-	80
C^-	3.3×10^2
$Nu3^-$	40
E^-	70
$B^- + C^-$	8.2×10^3
$B^- + Nu3^-$	30
$B^- + E^-$	1.6×10^3
$C^- + Nu3^-$	5.0×10^3
$C^- + E^-$	4.5×10^3
$Nu3^- + E^-$	3.2×10^6
$Nu3^- + E^-$*	4.3×10^3

The sonicates are named by the lambda morphogenetic gene that bears an amber mutation. All extracts prepared after induction of the defective lysogen were incubated overnight at room temperature, then assayed for prohead activity, as described in *Materials and Methods*. + means that the two extracts were mixed prior to overnight incubation.

* The extracts were mixed and, within one hour, a sample was tested for prohead-donor function.

Effect of Relative Concentrations of Complementing Extracts. To assess the optimal concentration ratio of the two complementing extracts for *in vitro* prohead production, we did experiments in which the concentration of one of the two extracts was kept constant while the other was varied by dilution (constant volume). Fig. 1*A* gives the yields of prohead activity generated in response to a 20-fold variation in the concentrations of the $Nu3^-$ and E^- extracts, respectively. The data are summarized in the form of a logarithmic/logarithmic plot. The curve for the $Nu3^-$ extract (gpE donor) as variable shows a threshold and the greater slope. Operationally, the results indicate that a one-to-one ratio of the extracts is close to optimal.

Formally, the $Nu3^-$ extract should contribute gpE, gpB, gpC to the reaction. However, experiments on complementation *in vitro* for prohead assembly involving double-amber mutants (H. Murialdo and A. Becker, in preparation), indicate that this is not the case. In fact, $Nu3^-$ extracts contribute only gpE and gpC in active form, but no active gpB. Accordingly, an extract prepared from cells induced for the doubly-mutant prophage λC^-Nu3^- is a source of gpE alone, for phage-coded prohead functions. Thus, it is possible to generate a dose-response curve on gpE, without creating a variation in the concentration of the other reactants. Fig. 1*B* summarizes the data of an experiment where the concentration of a C^-Nu3^- extract was varied over two orders of magnitude in complementation with an E^- extract and the yield of proheads generated measured. Again the curve is steep and shows a large threshold. Within the region of maximum slope, a doubling of gpE concentration results in a yield differential of at least three orders of magnitude. This

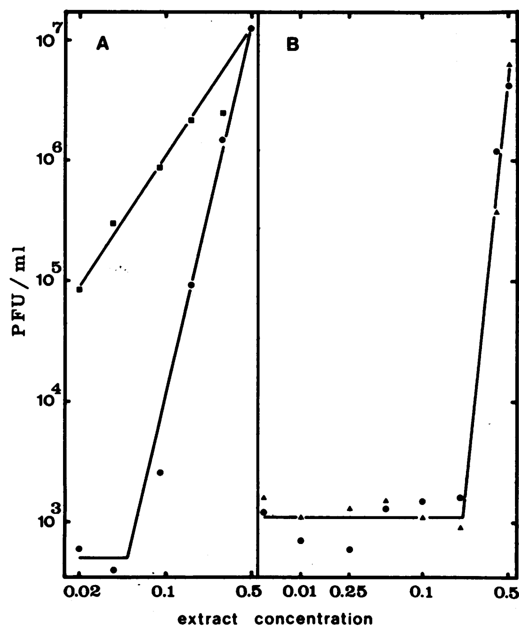

FIG. 1. Effect of extract concentration on prohead yield. The experimental procedure is outlined in *Materials and Methods*. The prohead-donor activities of incubated mixtures are plotted as ordinate. Extract concentration (abscissa) means the volume ratio of the varied extract divided by the total volume of the mixture (0.2 ml). *Panel A: Nu3⁻* extract constant, E^- extract varied (■—■); E^- constant, $Nu3^-$ varied (●—●). *Panel B:* E^- constant, $Nu3^-C^-$ varied. The $Nu3^-C^-$ extract was diluted, prior to mixing, in buffer A (●—●) or in an extract of W3350 (▲—▲) to guard against the possible dilution of required host components.

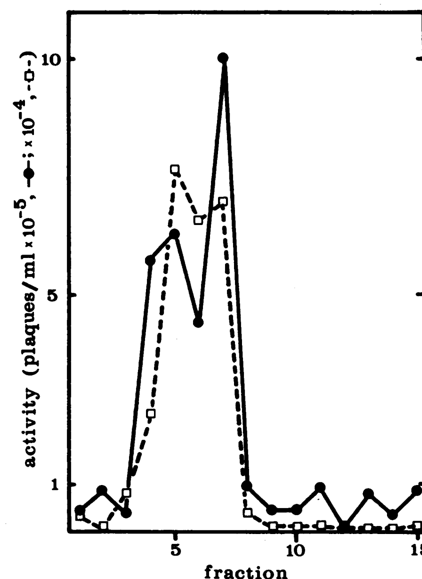

FIG. 2. Preparative sucrose-gradient analysis of prohead-donor activities. For experimental detail see *Materials and Methods*. Mixture of $Nu3^-$ and E^- extract, (□--□); A^-D^- extract (●—●). The activities of the samples prior to sedimentation were: 2×10^6 plaques per ml for the $Nu3^- + E^-$ mixture, and 1.1×10^7 plaques per ml for the A^-D^- extract.

is a minimum estimate, because for the lower gpE concentrations (<0.4, fractional concentration by volume) the data are not significantly different from the background (gpE omitted). Because of this experimental shortcoming, and because the upper asymptote is not determined, more intensive analysis is probably not called for. It is clear, however, from the unmanipulated data, that the transition is extremely steep, somewhat like a phase change.

Preliminary Characterization of the *In Vitro* Complementation Product—Sedimentation Properties. The results shown in Table 1 indicate that $Nu3^-$ and E^- extracts can complement one another to form active proheads as defined by their ability to enter the assembly of infectious phage particles *in vitro*. Although considered unlikely, it was possible that the proheads formed *in vitro* were grossly different than those formed *in vivo*. To test this, and as a first step in characterizing the product of the complementation reaction, we compared the sedimentation and electron microscopic appearance (see below) of this *in vitro* product with authentic proheads isolated from A^-D^--infected cells.

For sedimentation analysis, $Nu3^-$ and E^- extracts were mixed, incubated overnight at room temperature, and 0.1 ml of the mixture was then layered on top of a sucrose gradient, centrifuged, fractionated, and the fractions analyzed for prohead activity as described in *Materials and Methods*. For comparison, an A^-D^- extract was also incubated overnight at room temperature and subjected to identical treatment and analysis. The results are presented in Fig. 2. It is clear that within the resolution provided by this analysis there seems to be no difference in the sedimentation velocity of the proheads made *in vitro* and the "wild-type" proheads obtained from A^-D^- extracts by the criterion of biological activity.

Molecular Nature of the gpE Involved in the Complementation Reaction *In Vitro*. Induction of lysogens carrying a *Nu3* defective prophage results in the formation of aberrant head-related structures called "monsters" and a relatively small proportion of petit λ (14, 18). These petit λ, as opposed to wild type or A^-D^- petit λ, are not head precursors for *in vitro* DNA packaging reactions (11, 24, and Table 1). To determine whether the petit λ produced upon induction of a *Nu3*-defective lysogen were activated to biologically active proheads by some components present in the E^- extracts, we centrifuged a 1 ml sample of a $Nu3^-$ extract at 45,000 rpm for 60 min. These conditions are known to pellet petit λ and monsters. A noncentrifuged sample of the $Nu3^-$ extract, as well as the supernatant of the centrifuged sample were each mixed with an E^- extract, incubated overnight at room temperature, and assayed for prohead activity by standard conditions. For comparison, an A^-D^- extract was treated identically. The results of this experiment are presented in Table 2.

Table 2. Differential centrifugation analysis of complementing extracts

Extract	PFU/ml
A^-D^-	3.7×10^7
A^-D^- hs sup	$<5.0 \times 10^2$
$Nu3^- + E^-$	2.4×10^7
$Nu3^-$ hs sup + E^-	3.4×10^7
$Nu3^-$	$<5.0 \times 10^2$
$Nu3^-$ hs sup	$<5.0 \times 10^2$
E^-	$<5.0 \times 10^2$

For nomenclature and procedures see legend to Table 1. hs sup refers to the high-speed supernatant of a 1 ml sample after 60 min centrifugation at 45,000 rpm in a 60Ti rotor.

First, these results show that ultracentrifugation indeed removed proheads very effectively from the suspension, because the prohead activity of the A^-D^- extract dropped by a factor of more than 7×10^4 after centrifugation. The complementing activity of the $Nu3^-$ extract however remained unaltered by

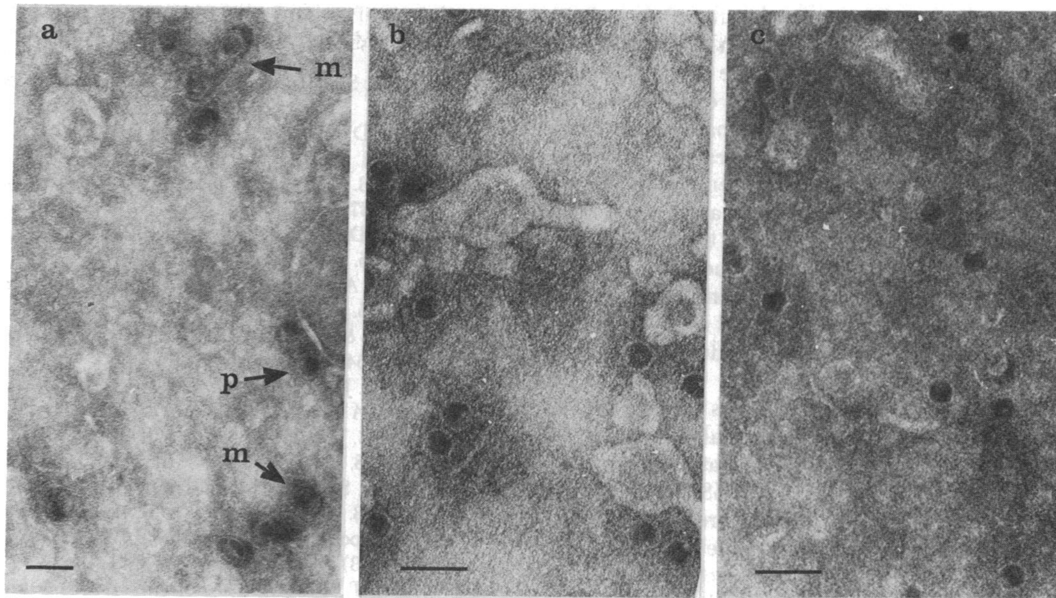

FIG. 3. Electronmicrographs of defective extracts. For details see *Materials and Methods*. The extracts are: (*a*) *Nu3*$^-$; (*b*) *A*$^-$*D*$^-$; and (*c*) a mixture of a *Nu3*$^-$ high-speed supernatant and an E^- extract. All observations were made after 24 hr of incubation of the extracts at room temperature. The bars represent 100 nm. The biological activities of the extracts were: (*a*) $<5 \times 10^2$; (*b*) 3.7×10^7; and (*c*) 3.4×10^7 plaques per ml.

the same treatment. These results show that the complementing activity of the *Nu3*$^-$ extract is independent of the presence of the inactive petit λ particles and monsters found in these extracts, and that the gpE used for complementation is in a monomeric or small oligomeric form, so that it is not removed by this type of sedimentation.*

Electron Microscopic Assessment of the Elements of the Reaction and the Complementation Product. In conjunction with the above measurements of biological activity, electron microscopic observations revealed the absence of head-related particles in the E^- extract. *Nu3*$^-$ extracts contained moderate amounts of petit λ particles and monsters (Fig. 3*a*) which were removed by high-speed centrifugation (45,000 rpm in a 60Ti rotor for 60 min) to below the concentration at which they can be found with ease with the electron microscope under the conditions used (about 1 to 2 particles per grid square in a 400 mesh grid, which is approximately equivalent to about 10^6 particles per ml). Fig. 3*b* shows an electron micrograph of an A^-D^- extract. There were about 2000 particles per photographic plate as an average, which is roughly equivalent to a concentration of 5×10^{10} particles per ml. The high-speed centrifugation removed these particles, again below the concentration at which they could be found in the electron microscope (picture not shown). Finally, Fig. 3*c* is an electron micrograph of a mixture of the E^- extract and the high-speed supernatant of the *Nu3*$^-$ extract (compare Table 2), after incubation overnight at room temperature. About 130 proheads could be seen per photographic plate as an average, which is roughly equivalent to a concentration of 1.3×10^9 particles per ml. These observations show that the complementation reaction involves an extensive polymerization of relatively small components, nonobservable in the electron microscope under the reported conditions, into observable particles that resemble, at this level of resolution, normal λ proheads. A more detailed analysis of their ultrastructure is deferred pending their purification. Purification of these particles seems feasible because of the relatively high yields obtained.

* Sedimentation velocity analyses of the complementing activity in *Nu3*$^-$ extracts in preparative sucrose gradients have not been successful. These failures could be due to the dilution on gradients of gpE below the concentration threshold for polymerization (Fig. 1), or due to separation of gpE from another essential component. Further experiments are needed to clarify this point.

DISCUSSION

Reconstitution of viruses from elemental protein components and nucleic acids *in vitro* has until now only been achieved for viruses of helical symmetry, such as tobacco mosaic virus (19) and some small RNA-containing plant viruses (20). For the more complex DNA-containing phages such as λ, the fact that it was possible to package DNA into preformed proheads and convert the intermediate to PFU *in vitro* (2–5) provided an opportunity to attempt to construct biologically competent proheads from prohead-precursor proteins in cell-free extracts. Indeed, as outlined in this report assembly of λ proheads *in vitro* proceeds when two suitably complementing extracts are mixed.

The efficiency of the reaction *in vitro* is difficult to assess because one cannot readily estimate the concentration, for example, of active gpE added to the crude system. As a potential model for the physicochemical study of mechanism, however, it appears significant that proheads are assembled *in vitro* to produce a relatively large yield. Thus, by the criterion of counting prohead-like particles in the electron microscope, the ratio of such particles made *in vitro* to those made *in vivo* and extracted from an equivalent mass of λA^-D^--infected cells is 2–3%. Assessed by the criterion of biological activity, this ratio can be of the order of 30 percent as shown in Table 1. It is possible that proheads made *in vivo* are damaged by the extraction procedure, leading to the apparent higher specific activity of the *in vitro* made product.

Experiments reported here show that formation of proheads proceeds when *Nu3*$^-$ + E^- extracts are used. Combinations of other extracts do give some complementation, but the yields are low (Table 1). The reasons for this latter finding are not known at present. It is possible that *Nu3*$^-$ and E^- extracts contain preformed sub-assembly complexes that enter directly into the reaction of prohead formation, increasing its overall

efficiency. If so, such complexes must be absent or poorly represented in the other combinations shown in Table 1. It is also possible that E^- extracts contain an active preformed core that includes gpNu3 (18). Conversely, $Nu3^-$ extracts may be contributing an appropriate form of gpE, the major coat subunit of proheads (11, 13). As shown by the results described in Table 2, active gpE derived from $Nu3^-$ extracts seems to be present in a slowly sedimenting form—a monomer, or an oligomer such as a hexamer or pentamer. It remains possible that, for some of the pairs of extracts that fail to enter a productive complementation *in vitro*, one or more essential gene products are present in an aberrant, irreversibly modified form—a consequence of abnormal morphogenesis *in vivo* due to the mutation(s) present.

The morphogenesis of λ proheads proceeds through a complex set of reactions in which certain structural components of the finished prohead arise as the result of alterations of primary gene products. Thus, gpB, a polypeptide of 58,000 molecular weight, is cleaved to the form gpB* (53,000) present in finished proheads (21, 22). The two other minor proteins, X1 and X2, are formed by the fusion of gpC to a minor fraction of gpE, and cleavage (23). X1 and X2 differ from each other in the size of the gpC-derived moiety present (23). gpNu3 is present only transiently during prohead assembly; is eventually lost from the nascent proheads, and seems to be extensively degraded (18). All these protein alterations are blocked in the absence of the phage-specific gpE or gpNu3, and the host-derived gpgroE (10, 11, 18, 21, 22). Therefore, if the product of the complementation *in vitro* between E^- and $Nu3^-$ extracts is a biologically active prohead, as seems to be the case, these alterations are operative *in vitro*. The results reported here suggest that most of the events in the morphogenesis of this complex DNA-containing virus may be subject to *in vitro* analysis, and that the action of certain form-generating functions may be describable in biochemical terms.

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