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PHAGE LAMBDA DNA PACKAGING, IN VITRO

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INTRODUCTION

The main problems to be studied in the field of phage morphogenesis are size and shape determination in head and tail, DNA condensation, and various regulative mechanisms to ensure the proper sequential order of morphogenesis steps. Concerning DNA condensation, evidence is accumulating in various systems of complex bacteriophages that DNA is packaged into a preformed precursor particle which sometimes has a smaller size and often a shape different from that of the mature phage head (1–10). The DNA substrate, on the other hand, is often of more than unit size length. Thus, immediately related to the packaging question is the one of size determination of the head and length determination of the DNA. How are pieces of unit length with (as in λ) or without (as in T4) unique ends cut from concatemeric DNA? Is the head size determined by the amount of DNA packaged? What is the relationship between cutting and packaging? Finally, the packaging of the DNA into the phage head has to be explained as a reversible process: condensation during morphogenesis, decondensation during injection into the bacterial host. The protein components, however, have to assemble in a sequential, irreversible manner, each step depending upon the completion of the previous one until a stable capsid is formed to protect its DNA.

In the case of morphogenesis of the head of bacteriophage λ the special relationship between the formation of a normal-sized head and DNA maturation requires that the following observations be explained. Neither in vivo (11, 12) nor in vitro (13) does a substantial amount of phage DNA mature from the concatemeric precursor DNA without simultaneous production of normal-sized phage heads. Conversely, in the absence of precursor DNA, be it as a consequence of overall inhibition of DNA synthesis (14) or as a consequence of a genetic defect that blocks the production of concatemeric DNA from

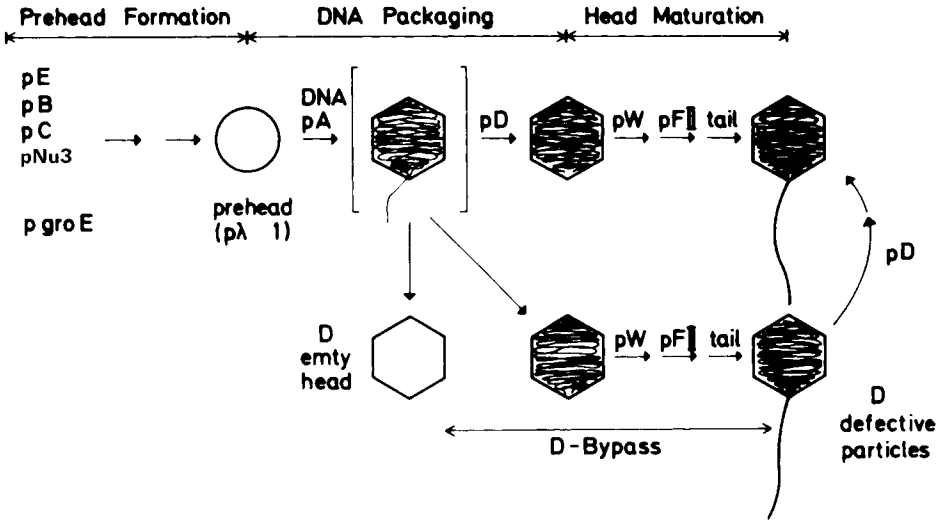

Fig. 1. Pathway of phage λ head morphogenesis. pA, pB . . . symbolize the proteins coded for by the genes A, B . . . .

circular DNA, no normal-sized phage heads are formed (15). These results suggest that both the formation of a mature-sized head and the production of mature DNA are interdependent processes and point toward the packaging process as the event at which both take place.

A number of steps in bacteriophage λ morphogenesis can be studied in vitro: tails are added to mature heads (16, 17) which in turn are built from immature ones by the actions of the W and FII proteins (18,19). The immature heads are formed from preheads which contain no DNA (Fig. 1). This reaction in vitro proceeds as an ATP and spermidine dependent but DNAse and chloroform sensitive step with high efficiency (9, 10). Thereby DNA gets packaged and the head is enlarged. The partners of these reactions, namely preheads, the products of the phage genes A and D and λ DNA, will be introduced in the next section and then their interactions discussed.

PARTNERS OF THE PACKAGING REACTION

Preheads

Using the in vitro complementation test as an assay, preheads can be isolated by centrifugation techniques (10). Their buoyant density in CsCl is that of protein, their sedimentation constant 135S. SDS gel electrophoresis reveals a composition with E protein as major component and the proteins corresponding to the gel bands h3, h6, h7, and h8 as minor components (Table I, Fig. 2). h3 corresponds to the processed form of the B protein (20), h6 and h7 to fused fragments of C and E protein (21) and h8 to B or C protein.

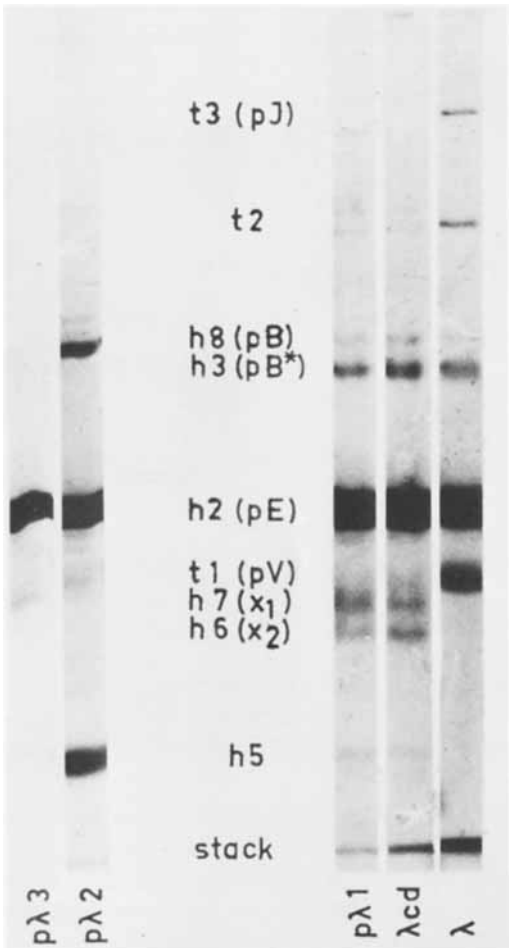

Fig. 2. SDS-polyacryl amide electrophoresis patterns of ^{35}S labeled proteins from petit λ particles and phage λ. Slabgels (51), 12% in acrylamide were used and autoradiographed according to the method of Laemmli (52). Gelbands occurring in phage heads and petit λ particles are given h numbers and those occurring in tails are given t numbers. The assignment to phage gene products and processed phage gene products is given in brackets. Petit λ particles were purified by two steps of sucrose gradient centrifugation (10); phage was purified by CsCl density gradient centrifugation (27). The stack contains proteins of molecular weight lower than 13,000 daltons including the protein h1 (i.e., pD).

pλ3, pλ2, pλ1: petit λ particles obtained from Bam10, Cam42 and Aam32 mutants, respectively. λcd: λ complemented in vitro from radioactively labeled pλ1 (preheads) and unlabeled E^- (Eam4) lysate. λ: λ particles produced in vivo.

In the electron microscope preheads appear as roundish particles of 450 Å diameter (Fig. 8), similar to the petit λ (pλ) particles reported by various authors (see review 22). Since pλ particles appear in lysates of all head mutants except in gene E, these lysates were tested for prehead activity. Lysates of mutants in genes A, D, W, FII, lysates deficient in the production of concatemeric DNA (i.e. fec^-; 15,23) and wild-type lysates contain

TABLE I. Properties of Capsoids of Different Lysates

Particle			Empty Head	pλ1 (Pre-head)	pλ2	pλ3
Occurrence in lysates				A^-, D^-, wildtype fec^-	C^-, $groE^-$	B^-
Activity in in vitro complementation			–	+	–	–
Protein composition						
Band observed	MW $\times 10^{-3}$ daltons	Gene assignment				
h8	62	pB or pC	+	+	+	
h3	56	pB cut	+	+		
h2	38	pE	+	+	+	+
h7	27, 5	(pE+pC) cut,= X_1	+	+		+
h6	25	(pE+pC) cut,= X_2	+	+		+
h5	18	pNu3,			+	
h4	12	pFII	+			
h1	12	pD	+			
Sedimentation constant (S_{20}, w)			145 ± 5	135 ± 5	160 ± 5	135 ± 5

Determination of the protein composition: SDS disk electrophoresis of purified ^{35}S particles. The molecular weights of h1, h2, and h3 as well as of tail proteins according to the measurements of Casjens et al. (27) were used as standards. The assignment of gel bands to proteins was done according to the results of Casjens et al. (27), Murialdo and Siminovitch (25), and Casjens and Hendrix (28). Typical gels are shown in Fig. 2.

The groE strain used was $groE_W$, kindly provided by N. Sternberg. Phage mutants used were Aam32, Bam10, Cam42, Dam15, Fam423. fec^- is thy^- rec A^- (λcIts857 red 113 gam5 Sam7), an appreciated gift from A. Skalka.

Sedimentation constants are mean values of several runs in the Beckmann Analytical Centrifuge with photoelectric scanning at 280 Å wavelength at concentrations between 0.4 and 0.8 mg/ml and at 20°C in TMA buffer (10^{-2} M Tris-HCl, pH 8, 10^{-3} M $MgCl_2$, 10^{-2} M NaN_3).

active preheads (pλ1). Mutants in the phage genes B and C and in the host gene groE (20,24), however, produce particles that are inactive in our assay. They also differ in protein composition from preheads and from each other (Table I, Fig. 2). In pλ3 (B^-) most of the minor bands are missing; in pλ2 (C^- and $groE^-$) a strong additional band (h5) appears that might be a good candidate for a scaffolding protein for prehead formation. Traces of this protein are also found in preheads and in phage. It might be coded for by the gene Nu3 (Murialdo, H., Jara, L., and Ray, P., personal communication).

Thus we conclude that for the production of pλ particles that are active in our assay, the products of the phage genes E, B, and C [and probably others, like Nu3 (25)] and the host gene groE have to be assembled and/or reacted with each

other (Fig. 1). Protein cleavage (protein B ⟶ B*) and protein fusion (proteins E + C ⟶ x_1, x_2) take place prior to packaging of DNA. We do not know yet, at which stage the gene FI product (26) is needed.

D Protein

Four hundred twenty copies each of D protein (12,000 daltons) and E protein (38,000 daltons) are the main components of the mature phage λ head (27, 28). Despite this high amount, D protein does not play a major role in building the phage head, since empty heads lacking D protein are stable (29). D protein which is active in complementation can be isolated either from E defective lysates or from guanidinium hydrochloride denatured phage. It is soluble in distilled water, a property uncommon for structural proteins (30).

D defective lysates produce three types of particles: preheads (this article), empty heads of normal size (31), and a very small number of defective particles which can only inject their DNA after reaction with D protein (30; Fig. 1). Thus D protein functions both in packaging the DNA (or stabilizing packaged DNA) and releasing the DNA during infection. Its effect on DNA is not due to direct interactions with the DNA; indeed D protein is located at the outside of the capsid (30, 28) and D protein has very little affinity for DNA (30). The effect of D protein on DNA may be through activation of the DNA cutting enzyme in the packaging step (see later) and loosening the DNA-E-protein interactions some time in the head maturation step in order to render the phage head ready to eject its DNA whenever it is needed.

A Protein

A protein (79,000 daltons) is either not present in the phage at all or only in one or two copies (25, 32). It is one of the proteins necessary to produce normal-sized and full phage heads (25). In addition it has termini generating activity, i.e., it produces cohesive ends on λ DNA (13). Its full activity, however, seems only to be expressed in the presence of preheads and D protein (in vivo: 12; in vitro: 10).

DNA as Substrate for Packaging

The natural substrate for packaging in vivo is concatemeric DNA. Monomeric circles, which accumulate in replication inhibited cells (33–35) or in recombination deficient (fec^-) cells (15), are not incorporated into viable phage. Monomeric linear DNA is not found in infected cells other than inside a phage head (12). In vitro the substrate for packaging can be concatemeric or mature linear DNA.

The packaging efficiency for concatemeric DNA endogenous to the in vitro system is about 1% if compared to a phage equivalent of 100 per cell at the time of harvest (10, Table II). Exogenous DNA, however, either deproteinized concatemeric one, or concatemeric DNA originating from an A^- lysate (both still mixed with bacterial DNA) is packaged with a ten-fold lower efficiency (Table II). For monomeric DNA the average efficiency is also about 0.1% [sometimes reaching values of 3% (10)]. An exact com-

TABLE II. Packaging of Exogenous λ DNA

Exogenous imm λ DNA	Incorporation of imm λ DNA as % of Total PFU	Packaging Efficiency of imm λ DNA in % of Input DNA
Concatemeric DNA		
A^- (lysate)	3.5	0.1
E^- (lysate)	40.8	1.0
A^- (deproteinized)	3.3	0.1
E^- (deproteinized)	7.0	0.1
Monomeric DNA		
linear		0.1 – 3
circular		10^{-5}

Each reaction mixture contained equal amounts of λ imm434 D^- F- and λ imm434 E^- concentrated induced lysogens and was prepared, lysed, incubated, and titered as described earlier (10). Lysates containing the concatemeric DNA to be tested were 100 times less concentrated to bring the number of preheads provided by the A defective lysate to a negligible amount. DNA was extracted as described (18). Circular DNA was prepared by phenolextraction of fec^- (15) infected cells and two successive CsCl density gradient centrifugations in presence of ethidium bromide (15). It consisted of 50% covalently closed and 50% nicked circles. Monomeric linear DNA used as reference in this experiment was handled in parallel in all steps and was found to have the same packaging efficiency as without the treatment. Incorporation of λ imm λ DNA as percent of total PFU was calculated for equal concentrations of each lysate or extracted lysate. The total PFUs in the experiment correspond to one particle formed in vitro per induced cell. The packaging efficiency of concatemeric DNA was calculated by comparison with a phage equivalent of 100 per cell at the time of harvest.

parison of efficiencies is difficult, because the added DNA has to compete with the concatemeric DNA already present in the in vitro complementation system for getting packaged and because concatemeric DNA, extracted from a lysate, is fragile and might be broken during handling. Monomeric circular DNA, either in the covalently closed form or with one random nick, is not a substrate for packaging in vitro (or, if so, the particles produced cannot form a plaque; Table II) in keeping with the situation in vivo.

It might be that linear monomeric DNA, which in vivo is not found in unpackaged form, in vitro is used only after annealing and ligation of the cohesive ends, i.e., after production of concatemers. This, however, is unlikely for the following reasons (Hohn, B., et al., to be published):

a) Annealing of DNA, the cohesive ends of which have been melted, does not produce a better substrate than quick cooling of the same DNA.

b) The fraction of the input monomeric DNA which is packaged is independent of its concentration in the complementation mix (Fig. 3).

c) Monomeric DNA is packaged as fast as phenol extracted concatemeric one.

d) Nicotinamide mononucleotide (NMN), which inhibits cellular ligase action (36, 37), does not inhibit the packaging of the linear monomers.

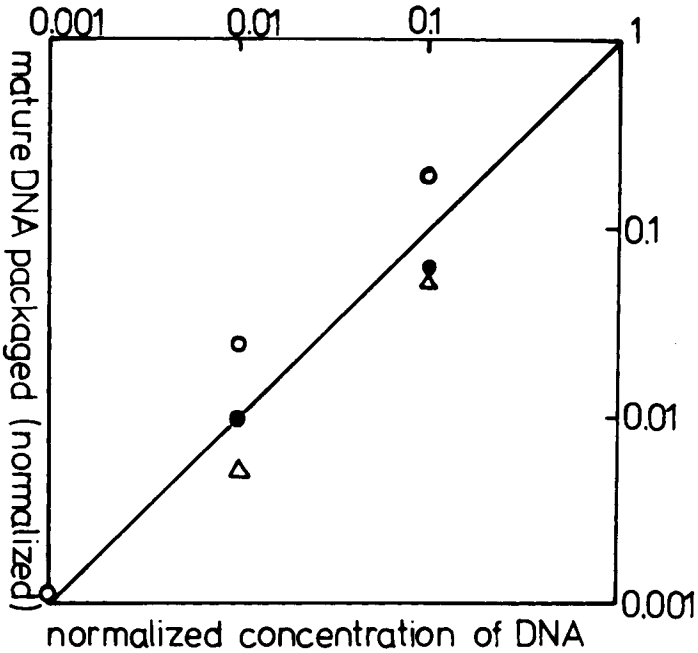

Fig. 3. Packaging of monomeric DNA, concentration dependence. DNA, phenol extracted from phage λcIts857 Sam 7 was heated to 75° C for 5 min and then chilled in ice in different dilutions. Packaging of this DNA was assayed in a complementation mix containing a λimm434 D^-F^- lysate as prehead source and a λimm434 E^- lysate as protein source, according to the procedure described earlier (10). Theoretical values are represented by the full line, whereas the results of three independent experiments are represented by circles, filled circles, and triangles, respectively.

e) Results mentioned in a), b), and c) could be explained by assuming annealing and ligation of the exogenous DNA with endogenous DNA that had received a "ter" cut already. This seems to be unlikely, however, in view of the fact that "ter" mediated recombination could not be detected so far (38–41); neither could the rapid in vivo circularization of infecting DNA be achieved in vitro (42). Thus cohesive end alignment for ligation seems to be an enzyme (annealase) mediated process, preferentially performed on *infecting* DNA (42).

The cutting of DNA needs A protein and ATP (13). Both factors are also needed for the packaging of monomeric DNA (Hohn, B., et al., to be published), pointing to a dual function of A protein (see below) and a need for ATP not only for DNA cutting but also for the packaging itself.

A MODEL FOR THE PACKAGING–CUTTING REACTION

There exists a strong interdependence of A protein and D protein action: A protein, as termini generating enzyme, has to be activated by D protein [in the absence of D pro-

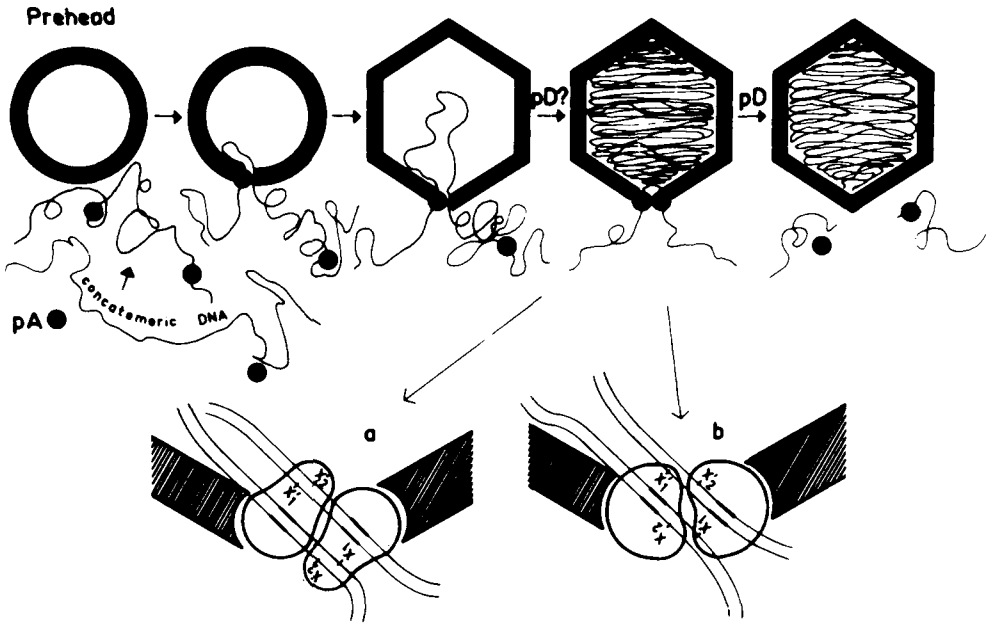

Fig. 4. Model for DNA packaging and cutting. The thick stretches on the DNA in a) and b) symbolize the sites to which the A protein molecules bind. For details of the model see text.

tein only few cohesive ends of the DNA are produced (12)]. D protein binding to preheads, on the other hand, requires A protein (A^- pλ do not contain D protein; Table I). To resolve this paradox we assume, as has been done in a related context by Kaiser and Masuda (43), that A protein binds to DNA in one of the initial steps of the packaging reaction, but does not cleave the DNA until the head acquires its final form. A DNA–A protein complex would easily explain how a prehead and the DNA to be packaged find each other: the A protein, having affinities to both the DNA and the prehead, enables the contact. Consistent with this proposed A protein interaction is the following result (Table II): In complementation of crude lysates DNA originating from an A^+ lysate (which contains A protein) is packaged preferentially over DNA originating from an A^- lysate (which contains no A protein). This, together with the facts about prehead, D and A protein listed above leads us to propose the following model of the packaging–cutting mechanism (Fig. 4): The DNA–A protein complex reacts with the prehead and triggers the head enlargement. As a consequence, DNA packaging starts, probably beginning with the left end (38, 39; Sternberg and Gottesmann, personal communication). The packaging proceeds, possibly only after reaction of the D protein with the capsid, until all the DNA located between two bound A protein molecules is encapsulated. Thus the two molecules dimerize and thereby, as well as by direct or indirect interaction with the D protein, they become enzymatically active and cut the DNA. Thus, a DNA containing particle is produced

which serves as substrate for the final maturation steps.

Wang and Brezinsky (44) have proposed a model for the termini generating function according to which two cohesive sites of DNA are aligned in an antiparallel and symmetrical fashion and cut by two A protein molecules in concert. This alignment, of course, could be facilitated through completion of packaging of a unit of DNA. A hypothetical alignment protein thus becomes superfluous. The A protein molecules might bind to the cohesive sites (44) or, more likely, to A protein binding sites close to the right of them (to the right, because packaging proceeds from left to right; see above), explaining the requirement for A protein for the packaging of monomeric DNA that only has cohesive ends but no intact cohesive site.

According to our model a cohesive site is not necessarily cut by the A protein bound close to it; it also could be cleaved by the A protein bound to the distant binding site (Fig. 4a). Alternatively, the A protein could create one cohesive end out of each of the two aligned cohesive sites (Fig. 4b). After its action the A protein bound to the left A protein binding site would either be incorporated into the phage head or released as such or in an inactivated form. The A protein bound to the right A protein binding site would ensure continuous packaging of another DNA unit into the next head.

The model is consistent with or explains the following findings:

a) A phage head is necessary for the production of mature DNA (12). We now know that actually a prehead is needed for the reaction which consequently is converted to a head.

b) The DNA cutting reaction is necessary if a full head is to be built from prehead and concatemeric DNA. In the absence of A protein not only can DNA not bind to prehead, but also head enlargement cannot be triggered. In the absence of D protein, however, heads can be enlarged but they cannot retain the DNA. Directly or indirectly the presence of D protein activates the ter enzyme.

c) The first-packaging-then-cutting sequence would ensure the vicinity of the DNA ends to the head opening. This has been demonstrated at least for the right end of the DNA (45, 46, 53, 54). Such a close proximity of the end to be injected first, is of course necessary for a successful infection.

d) The postulate that two cohesive sites have to come into close proximity after a DNA unit is packaged and that only then the bound A protein molecules become activated explains nicely the fact that at least two cohesive sites are needed on concatemeric or circular DNA to function as substrate for stable packaging (44).

e) The theory that the A protein binding site is located to the right of the cohesive site is compatible with a finding of Syvanen and Kaiser (personal communication) according to which DNA with a left cohesive *end* and a right cohesive *site* can be incorporated into viable phage, while one with a right cohesive *end* and a left cohesive *site* cannot: In order for λDNA to serve as substrate for cutting it has to have two A protein binding sites. An active cutting enzyme is an A protein dimer. For the *recognition* of DNA, however, only one binding site is necessary as could have been predicted from the observed polarity of packaging and cutting. Linear mature DNA is recognized and packaged, but does not need to be cut anymore.

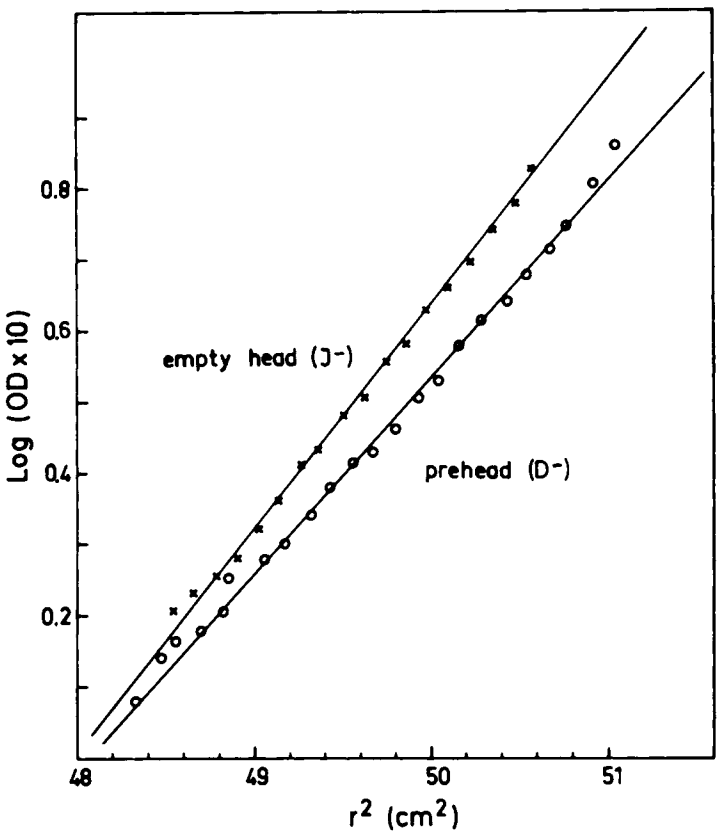

Fig. 5. Petit λ 1 particles and empty heads at sedimentation equilibrium. The logarithm of optical density is plotted against the square of radial distance in the rotor. Speed 800 rpm. For details see legend of Table III.

HOW IS THE HEAD ENLARGED?

We consider two extreme models for the head enlargement: increase of the amount of protein or a conformational change of the tertiary structure of the subunits coupled with a rearrangement of the quarternary structure.

D protein is added to the particle during or after DNA packaging. This addition, however, has no influence on the head enlargement since D defective mutants produce normal-sized heads (25). It is unlikely that additional molecules of E protein cause the size increase: 1) preheads, empty heads, D defective heads (Table III, Fig. 5) as well as full heads (47) have the same triangulation number (48) T = 7, corresponding to 420 E protein molecules. 2) Isolated preheads are complemented in vitro by E defective lysates (10). Consequently any E protein molecule added to a prehead must come from the breakdown of another prehead. This can be tested. A mixture of isolated heavy and light preheads is complemented with an E^- lysate and the resulting phage checked for its density (Fig. 6).

TABLE III. Molecular Weight of Prehead and Empty Head

	MW $\times 10^{-6}$ daltons	Triangulation Number (T) Fitting
Measured values		
Prehead (A^- or D^-)	16 ± 1	7
Empty head	20 ± 2	7 (pE + pD)
Empty head lacking D protein	18 ± 1	7
Calculated values		
pE T = 4	10	
pE T = 7	17	
pE T = 9	22	
pE + pD T = 7	22	

Molecular weights of preheads from A defective lysates and from D defective lysates and of empty heads from J (tail) defective lysates were determined with the equilibrium method in an AN-J rotor of the analytical Beckmann Ultracentrifuge. Mean values of several runs for 60 hr at 800 and 1200 rpm and 8°C are given. Particles were dissolved at concentrations of 0.15–0.30 mg/ml in TMA buffer (see Table I). A partial specific volume of 0.74 cm^3/g was assumed. The molecular weight of empty heads lacking D protein was determined by Buchwald et al. (29). Calculated values were obtained by assuming a molecular weight of the E protein (pE) of 38,000 and the D protein (pD) of 12,000 (27) and by taking a 7.5% increase in protein mass into account due to the minor protein bands observed in SDS gels. Typical plots of sedimentation equilibrium are given in Fig. 5.

Both types of preheads are transformed conservatively, since no phage particles of intermediate density are observed. The possibility that additional molecules of E protein cause the size increase is thus excluded.

Is the prehead–head transition accompanied by protein processing? In phage λ morphogenesis processing of minor protein components does occur (21); preheads, however, already contain the processed forms (Table I). A comparison of gelbands of purified radioactively labeled preheads with those of purified phage particles resulting from complementation of these preheads with unlabeled E defective lysate reveals no difference in the pattern, neither in position nor in intensity of the bands (Fig. 2). Minor forms of protein processing as a cause for the structural shift, like cutting of a small number of amino acids, phosphorylation, amidation, and glycosylation are, however, not excluded and have to be checked. As for the E protein, the amino acid composition of this protein derived from prehead and head is the same within a limit of four amino acids (49).

The fact that the major protein processing reactions observed occur during prehead formation and not during packaging of the DNA does not necessarily mean that processing has no relation to packaging. It might for instance create binding sites for the DNA that later become necessary for the DNA packaging.

If a hollow sphere is increased in size without the addition of new material the thickness of the wall has to decrease. In the case of the prehead–head transition one can assume that the subunits are quenched and thus the capsid thickness is decreased. Lickfeld

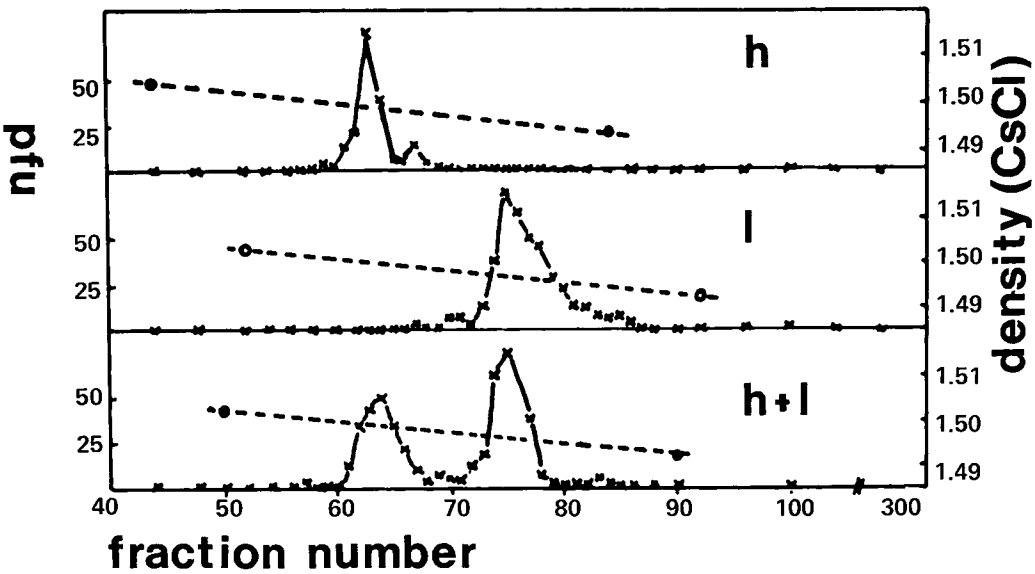

Fig. 6. Density of λ complemented from heavy and from light preheads. Preheads purified from A^- cultures grown in $D_2O-{}^{15}NH_4Cl$ medium and $H_2O-{}^{14}NH_4Cl$ medium, respectively, were complemented separately with crude E defective, unlabeled lysate. Complemented heavy (h) and light (l) phage were banded in CsCl density gradients which were run in the A321 fixed angle rotor for 60 hr at 30,000 rpm at 4°C in the International Centrifuge. A mixture of heavy and light preheads (h + l) was complemented and banded similarly. The gradients were collected in 300 fractions, each of which was tested for plaque-forming units.

et al. (50) in fact observed in thin sections of λ infected bacteria preheads with a wall thickness of about 6 nm and empty heads with a wall thickness of about 5 nm (Fig. 7).

Structural elements on the surface of intact phage heads and petit λ particles are difficult to see and to interpret. On damaged phage heads and damaged petit λ particles, however, clustering of subunits becomes visible (Fig. 8). Capsomers in phage heads are seen as small rings 7 nm apart, each surrounded by six others. Capsomers in petit λ are larger, 9 nm apart, and again each is surrounded by six others. On the basis of similar micrographs of damaged λ particles and of exceptionally well-preserved (D protein containing?) polyheads Williams and Richards (47) have interpreted the λ surface structure as a clustering in trimers of the large (E) subunits and a clustering in hexamers and pentamers of the small (D) subunits (Fig. 9d and e). Because of the observed size, distance, and arrangement of capsomers on the pλ surface we assume that in this case the E protein molecules are clustered in hexamers and pentamers. Fig. 9a shows a model of this arrangement. In Fig. 9b and c possible ways of how the subunits could rearrange during head enlargement are depicted. We can imagine that the initiation of this rearrangement process causes heavy distortions of bondings in the neighborhood ensuring a fast progression of the rearrangement over the entire capsid surface. The head enlargement which causes the inner volume of the capsid to increase to more than double might occur so fast that a

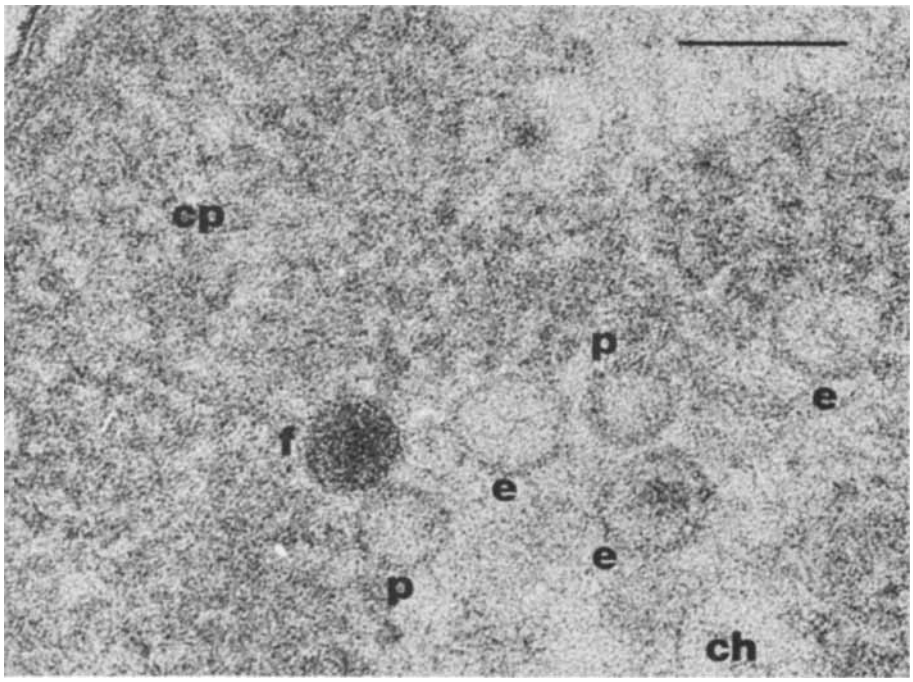

Fig. 7. Thin section of B10 (λimm434 cItsSam7) 40 min after induction. pλ (p), empty headlike particles (e), and full head (f). The particles are located in the chromosomal area (c), very close to or on the interior surface of the cytoplasm (cp). Bar represents 100 nm.

negative pressure can build up inside that could "suck in" at least part of the DNA. This idea has also been put forward independently by P. Serwer for T7 (8).

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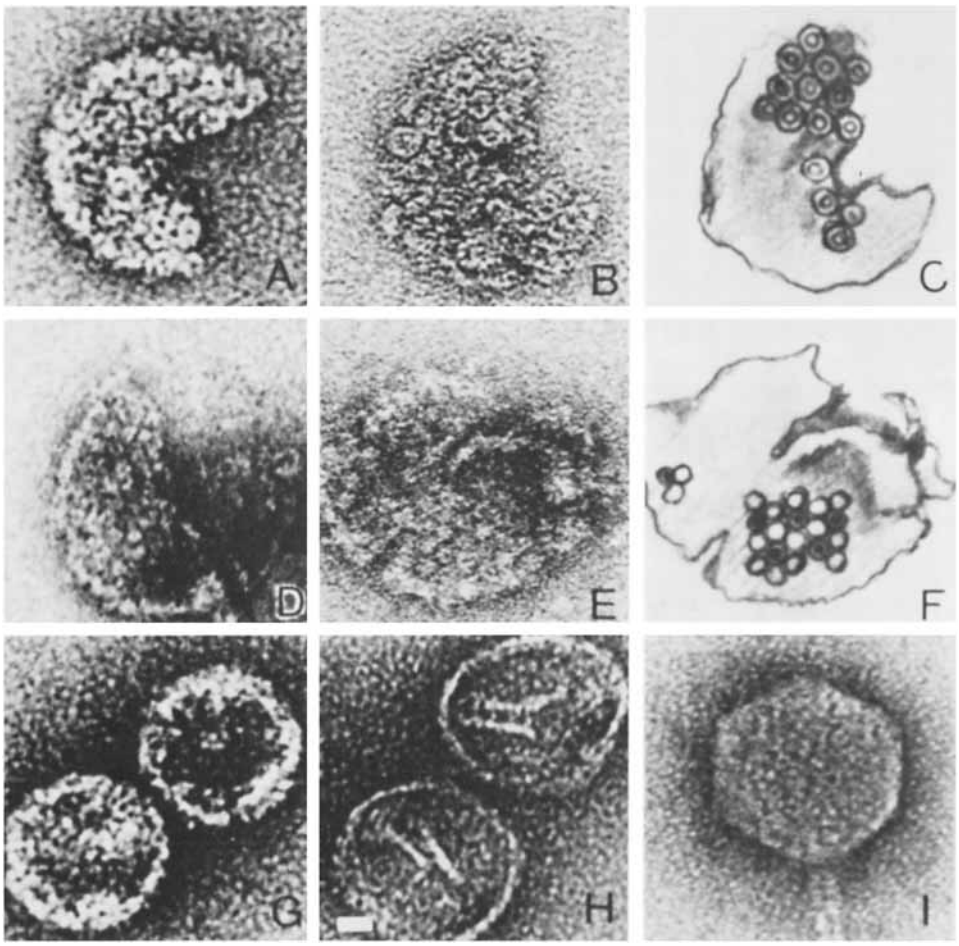

Fig. 8. Electron micrographs of phage λ capsoids. Preheads: A) and B), squashed particles; C), graphical interpretation of B) showing the size and array of capsomers; G), intact particle. Empty heads: D) and E), squashed particles; F), graphical interpretation of E); H), intact empty head. Intact phage: I).

Negative staining with uranylacetate. Bar represents 10 nm.

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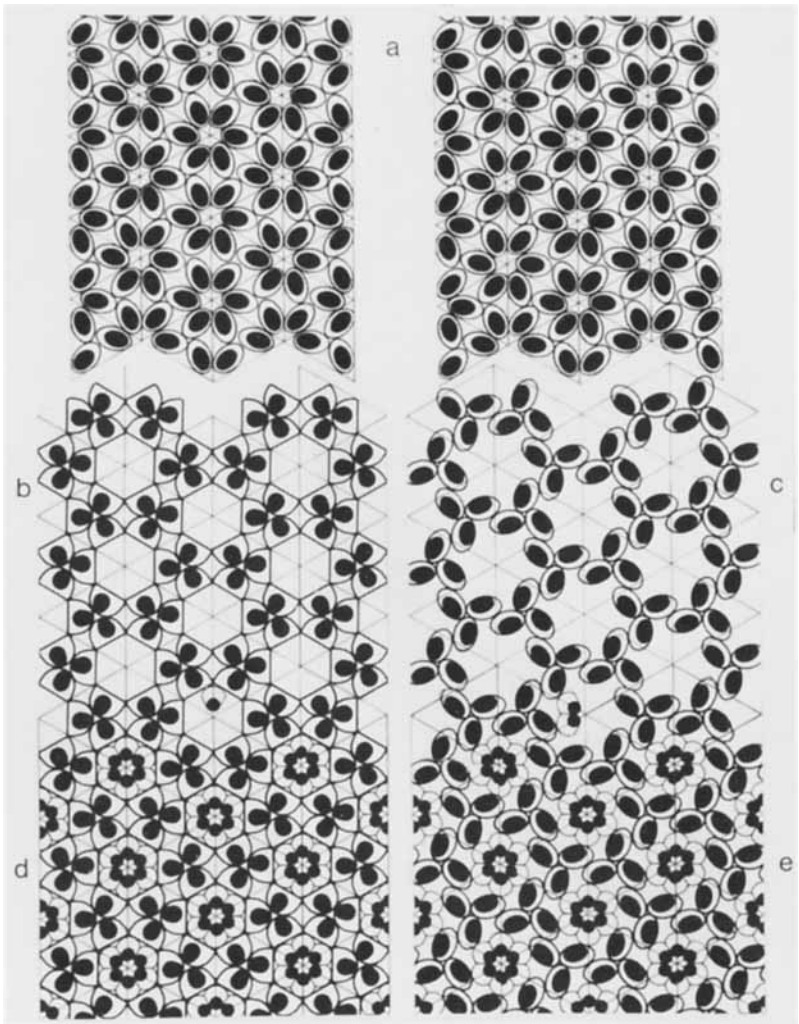

Fig. 9. Hypothetical lattices. a) E protein subunits as present on the surface of preheads. Each subunit can be seen as part of a hexamer or as part of a trimer; protruding parts of the molecules (black areas) are drawn, however, to pronounce the hexameric clustering as it becomes visible by negative staining (Fig. 8).

b, c) Two extreme possibilities are considered as explanation for the enlargement of the lattice: the shape of the subunit is extensively altered to favor the trimer clustering and to provide space in between six trimers [b)], or the trimers are rotated around their centers allowing new protein interactions to occur (dimer bonding) and others (i.e., hexamer bondings) to be disrupted [c)]. In this case the black areas have been slightly shifted to pronounce trimer clustering.

d, e) Smaller protein subunits (D protein) are intercalated as hexamers in each of the centers between 6 E-protein trimers.

Operations performed to enlarge the lattice could analogously be performed on icosahedral surfaces obtained by folding the lattice according to the rules of Caspar and Klug (48). The smaller subunits in this case will be added as hexamers on the faces and as pentamers on the corners of the icosahedron.

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