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Isolation and Characterization of Bacteriophage T4 Mutant Preheads

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To determine the function of individual gene products in the assembly and maturation of the T4 prehead, we have isolated and characterized aberrant preheads produced by mutations in three of the T4 head genes. Mutants in gene 21, which codes for the T4 maturation protease, produce rather stable preheads whose morphology and protein composition are consistent with a wild-type prehead blocked in the maturation cleavages. Mutants in gene 24 produce similar structures which are unstable because they have gaps at all of their icosahedral vertices except the membrane attachment site. In addition, greatly elongated "giant preheads" are produced, suggesting that in the absence of P24 at the vertices, the distal cap of the prehead is unstable, allowing abnormal elongation of both the prehead core and its shell. Vertex completion by P24 is required to allow the maturation cleavages to occur, and 24^- preheads can be matured to capsids in vitro by the addition of P24. Preheads produced by a temperature-sensitive mutant in gene 23 are deficient in core proteins. We show that the shell of these preheads has the expanded lattice characteristic of the mature capsid as well as the binding sites for the proteins *hoc* and *soc*, even though none of the maturation cleavage takes place. We also show that 21^- preheads composed of wild-type P23 can be expanded in vitro without cleavage.

The production of the head of bacteriophage T4 proceeds in two stages, prehead assembly and prehead maturation. The prehead which is assembled on the bacterial membrane (21, 24) consists of the precursors to the essential structural proteins of the capsid (12) and also transient core components which will be eliminated during maturation (12, 21). During the conversion from prehead to capsid, most of the core and capsid proteins will be cleaved to shortened forms, the prehead will be released from the membrane, the capsid will expand and become resistant to cold sodium dodecyl sulfate (SDS), DNA will be packaged, and the outer capsid proteins *hoc* and *soc* will be added to the structure (1, 7, 10, 12, 24). The causal relationships among these processes and the sequence in which they occur is not yet clear.

Mutations in two T4 head genes 21 and 24 are known to accumulate normal-appearing prehead-like particles (4, 8) which contain no DNA (13, 16). Unfortunately, these particles are membrane bound and fragile, and until now it has not been possible to isolate them in sufficient quantity and in a sufficiently good state of preservation to determine accurately their protein composition and detailed structure. Gene 21 codes for a precursor to the T4 protease (23), and apparently the *ts*P21 zymogen cannot be activated after a shift to the permissive temperature because none of the maturation cleavages takes place after such a shift (13, 16). Gene 24 codes for the vertex protein of the prehead and capsid (18). Unlike the temperature-sensitive (*ts*) gene 21 mutants, many of the *ts* gene 24 mutants are temperature reversible (3). Gene 24 mutants also differ from 21^- mutants in that they accumulate many polyheads as well as normal-length preheads (14). Production of a third class of preheads is caused by *ts* mutations in gene 23 which codes for the major capsid protein. These structures are irregularly shaped (5) but have been shown to contain most of the precursors to the capsid proteins (19). They differ from 21^- and 24^- preheads in that they are not membrane bound under the conditions of the isolation, and, based on protein composition and structure, they provide an example of an intermediate between the prehead and the capsid.

In this paper we describe the conditions for the isolation of 21^- and 24^- preheads and the related particles produced by a *ts* gene 23 mutant. We describe their origin, composition, morphology, and other characteristics related to the maturation process and show that 23^- preheads are expanded in the absence of protein cleavage. We have partially purified the 24^- preheads but have not been able to sufficiently separate them from contaminating polyheads to allow accurate quantitation of the protein components. How-

ever, we show that a large percentage of the elongated particles made by both amber (*am*) and *ts* mutants in gene 24 under strictly nonpermissive conditions are actually elongated preheads rather than polyheads. Therefore, the 24⁻ defect does not effect initiation as previously thought. The normal length preheads are shown to have gaps at the nonproximal vertex positions, and on complementation of these preheads in vitro with purified P24 or a P24-containing lysate, these gaps are filled and the preheads spontaneously mature to capsids, indicating a role for P24 in the initiation of the maturation cleavages.

MATERIALS AND METHODS

Mutant phage stocks. T4D⁺ was the wild-type strain and the parental from which the mutants were derived. *am* and *ts* strains were obtained from the Cal Tech and Geneva collections, and double mutants were constructed from these. The specific mutants used are listed in Table 1. Stocks were prepared as previously described (9). The designation of multiple mutants follows the nomenclature of Laemmli et al. (14). Proteins are named by genes, e.g., P22 for the protein coded for by gene 22. P22* indicates a processed form of P22.

Isolation of mutant preheads. Mutant lysates were prepared as previously described (19). In all cases the uninfected bacteria were grown at 37°C and transferred to the growth temperature only a few minutes before infection. Infected cultures were collected by centrifugation and then resuspended 100-fold concentrated in the extraction buffer. The extraction buffer was 0.15 M phosphate at pH 6.0, 0.06 M NaCl, 5×10^{-3} M $MgSO_4$, 5×10^{-4} M Ca, 5 to 10 μg of DNase per ml (A grade, SERVA) and 10% glycerol. The low pH is necessary for stabilization of the core structure and for the inactivation of the T4 protease coded for by gene 21. Glycerol is not absolutely necessary for the preservation of the 21⁻ particle, but it is necessary to stabilize the more fragile 24⁻ preheads, and all preheads were extracted under these conditions. Lysis of infected cells in the presence of nonionic detergents such as Nonidet P-40 did not increase the particle yields, and particles produced by these methods were less intact as judged by electron microscopy. The extracted cells were centrifuged for 10 min at 12,000 rpm in the Sorval SS34. The supernatants from two extractions were pooled, and the membrane-bound particles were concentrated by pelleting at 15,000 rpm for 30 min. The pellets were resuspended at 1,000× culture concentration in the extraction buffer, and 0.5 ml was applied to a 15 to 40% glycerol gradient in the same buffer.

In some cases Nonidet P-40 was added to this pellet at a final concentration of 0.5% just before application to the gradient. The gradients were run in the SW41 rotor in a Beckman L2-65 centrifuge at 15°C for 30 min at 41,000 rpm. Fractions (1-ml) were collected, and samples were immediately prepared for gel electrophoresis and electron microscopy. Samples for electron microscopy were fixed in 2% formaldehyde. ^{14}C-labeled cultures were prepared as previously described (19) and then mixed with a fivefold excess of cold, infected cells to maintain concentrations for particle extraction.

In vitro complementation of 24⁻ preheads with P24. The 24⁻*am*- or 24*ts*-infected cells were resuspended and lysed directly into an extract of the appropriate donor lysate. The P24 donor lysate is 21(*N*90)-22(*amE*209)-23(*amH*11), and the isogenic strain 21(*N*90)-22(*amE*209)-23(*amH*11)-24(*amN*65) was used as the control. These lysates were 1-h cultures which were harvested and resuspended in cold extraction buffer at 100 to 200× culture concentration and then sonically disrupted until lysis was complete. The resultant extract was centrifuged at 100,000 × *g* for 1 h, and the supernatant was used as the donor lysate (when purified P24 [22] was substituted for a 24⁺ donor lysate, it was used at a final concentration of 0.1 to 0.5 mg/ml). The lysed cells were incubated with the donor lysate (or P24) for 30 min to 1 h at 30°C, and then the particles were extracted at room temperature to avoid dissociation which takes place in the cold (23). However, after complementation with P24, the low pH is not sufficient to control the cleavage in the 24⁻ mutant preheads, and the complemented preheads were extracted at 10°C to minimize this cleavage in some cases. Further purification of the complemented preheads was carried out as described above.

Electron microscopy. The microscopy was performed as described in Stephen et al. (25), except that 1% uranyl acetate at a pH of 4.5 was used for negative staining.

Electrophoresis. SDS slab gels and radioautograms were prepared as previously described (19). Immune replicas of SDS gels were prepared by the method of Showe et al. (23).

Quantitation from SDS gels. Dye was eluted from stained bands in 25% pyridine, and the adsorbance was measured at 605 nm by the method of Fenner et al. (6). The dye binding capacity of all proteins was assumed to be the same. Radioactive bands were cut from dried SDS slab gels and put into 0.1 to 0.2 ml of concentrated nitric acid and 0.05 ml of 30% hydrogen peroxide. This mixture was heated in a boiling-water bath in a hood until the acrylamide dissolved. Peroxide was added two to three times more during the heating. The dissolved sample was transferred to a scintillation vial in 5 ml of Insta Gel liquid scintillation fluid (Packard). The samples were counted in a Packard liquid scintillation counter (R. van den Broek, personal communication).

RESULTS

Isolation and composition of mutant preheads. (i) 21⁻ and 24⁻ mutant preheads. We have identified and quantitated the protein components of the prehead formed by gene 21, 23, and 24 mutants and compared their compositions with that of the wild-type head. Figures 1a and b show the comparison on SDS gels of parallel glycerol gradients of 21⁻ preheads purified with and without Nonidet P-40 treatment. The particles isolated with Nonidet treatment

TABLE 1. *Composition and quantitation of T4 preheads and comparison to wild-type phage*[a]

Protein	No. of molecules/head in mutant:								
	21*ts*(*N*8-*B*90-*N*12)		21*am*(*E*322-*H*29-*N*90)		21(*tsN*8)-23(*tsA*78)		Wild-type		24(*amN*65)
	Dye	^{14}C	Dye	^{14}C	Dye	^{14}C	Dye	^{14}C	Dye
Common to capsid + prehead									
B_1 (B_1*)	42 (3)	40 (3)	44 (3)	39 (3)	ND	30 (3)	43 (3)	45 (3)	+
P20	30 (10)	31 (3)	28 (3)	27 (3)	28 (3)	27 (3)		15 (3)	30
P24 (P24*)	74 (10)	65 (2)	67 (5)	61 (2)	88 (3)	92 (2)	65 (3)	70 (3)	
IPIII (III*)	368 (10)	313 (3)	340 (5)	250 (2)	100 (3)	60 (3)	400 (3)		400
IPI + II (I* + II*)	745 (3)		730 (2)						800
hoc$^+$ 1 band	0	0	0	0	130 (2)	90 (2)	150 (3)	105 (2)	
2 bands					200 2)			230 (2)	
soc	0	0	0	0	500–1,000		1,000 (3)		
Specific for prehead									
P21	106	50				44 (2)			T4PPase and band 4
P22	605 (10)	565 (3)	514 (4)	440 (3)	135 (5)	100			+Cleaved
17K	229 (2)	330 (2)	225 (2)	125 (1)					250
14K (PIPII)	218 (2)	250 (1)	221 (2)	145 (1)	ND	35 (2)			300
17.5K	0	0	0	0		85 (2)			

[a] Compositions of T4 mutant preheads. The values reported are the number of molecules per head based on a value of 1,000 P23 or P23* molecules per head structure. There was no detectable difference in the composition of all of the 21*ts* mutants tested (*N*8-*B*90-*N*12), and repeated determinations were carried out on 21(*tsN*8). The 21*am* mutants *H*29 and *N*90 gave the same results as *E*322, but as these mutants are known to have some low levels of cleavage enzyme, most determinations were done with *amE*322. Quantitation was primarily by extraction of stained bands. Because particle concentrations must be maintained as high as possible (5 to 20 mg/ml) to prevent dissociation during extraction, it was not possible to make labeled particles with a very high specific activity. The numbers in parentheses are the numbers of preparations from which determinations were made. The values for the 24(*amN*65) preheads are from one determination and are only included to demonstrate that there is no enormous difference between these preheads and the 21 preheads. + indicates the presence of a protein, but no quantitation was done. In some isolations not all of the proteins were quantitated because the gels were not suitable.

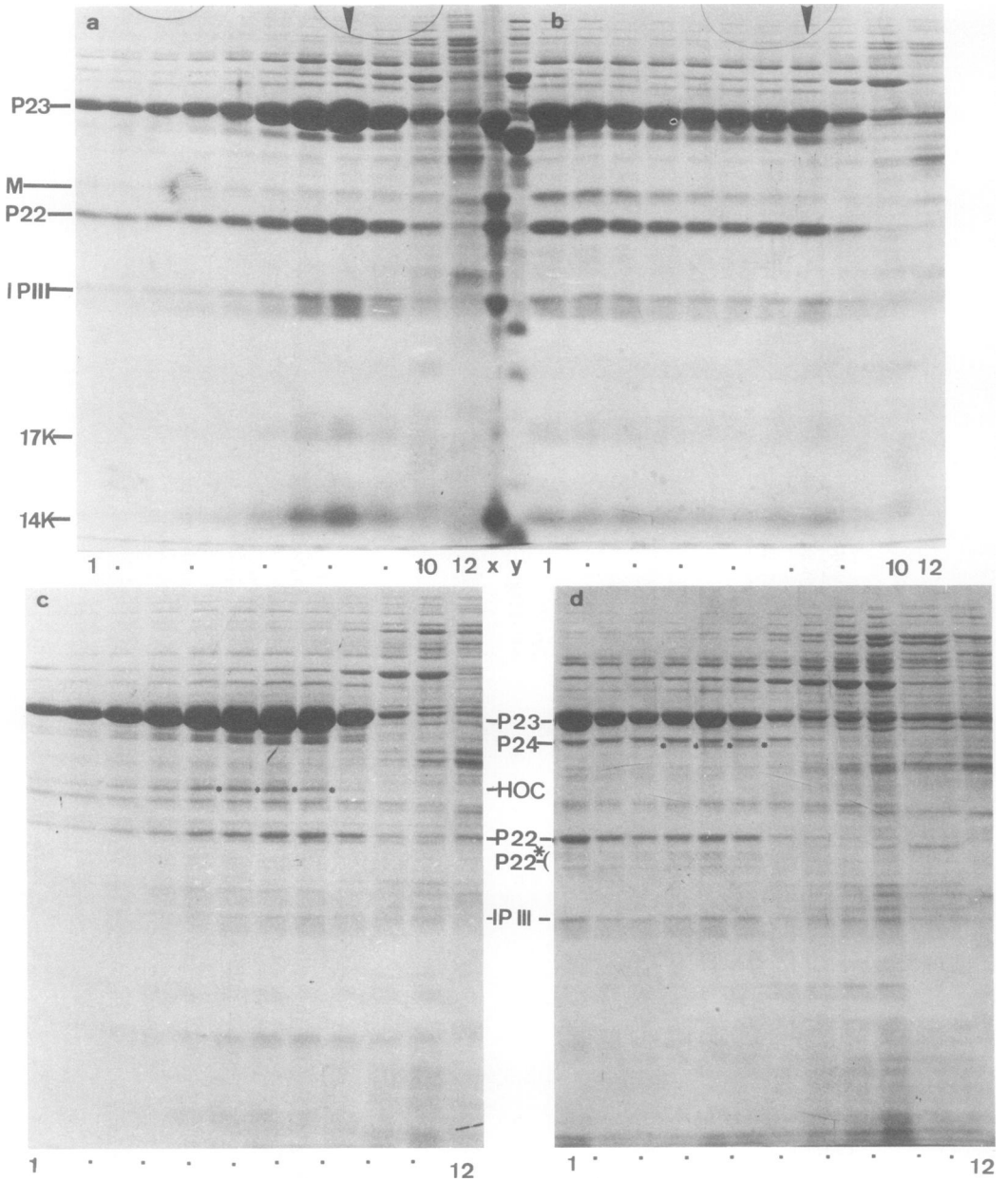

FIG. 1. *Stained 12.5% SDS acrylamide gels (panels a and b) compare the distribution in the glycerol gradients of 21(amE322) preheads prepared with and without Nonidet P-40 treatment, respectively (see the text). Gradient fractions are numbered consecutively from the bottom, and the arrow marks the peak of free preheads in both gradients as determined by electron microscopy. Panels c and d are SDS gels of gradients of 21(tsN8)-23(tsA78) preheads and 24(amN65) preheads, respectively. Both mutants were treated with Nonidet P-40 before application to the gradient, and the 24⁻ preheads were extracted in the presence of a P24 donor lysate. The * in panel c marks the position of the hoc protein, and in panel d it marks the position of the P24 peak, as determined by immune replica in both cases. The positions of the preheads in panels c and d are slightly shifted due to an extended centrifugation. M denotes the position in the gel of the E. coli matrix protein. X is a standard mixture used to identify the position of P22, IPIII, 17K and 14K proteins on the gel; Y is a purified phage standard.*

form a single sharp peak, whereas the particles which have not been treated distribute in two overlapping classes. The Nonidet-treated particles sediment slightly ahead of a lambda phage marker, in agreement with the S value of 420 previously reported (13, 16). The protein classes present in particles produced by the two methods are essentially the same, except that the *Escherichia coli* matrix protein is a major contaminant in the particles prepared without Nonidet treatment. Electron microscopy shows the peak of the preheads prepared with Nonidet to be almost entirely composed of single particles with little or no attached membrane. The upper peak of the gradient in Fig. 1b is composed of a mixture of single particles and small vesicles with two to four particles attached. The lower peak consists primarily of large vesicles with many attached particles and a few contaminating polyheads. Figures 1c and d show similar gradients of 24(*amN*65) particles and 23*ts* particles treated with Nonidet P-40. There is a broad peak of preheads in the expected position on the gradient of the 24-mutant preheads in Fig. 1d. Because these particles were prepared by first complementing with a P24-containing lysate, one can identify the prehead peak against the background of polyhead fragments by determining the peak of the 24 protein which is only incorporated into the preheads. Using the immune replica technique we find that the P24 is associated almost exclusively with this peak. The gradient shown in Fig. 1c shows particles from the double mutant 21(*tsN*8)-23(*tsA*78). The double mutant with 21 was used in this case to prevent cleavage of the precursor proteins by the T4 protease coded for by gene 21. The single mutant 23(*tsA*78) has previously been shown to have a similar composition (19). Treatment with Nonidet had little effect on the sedimentation of 21-23*ts* preheads, because these particles do not seem to be tightly associated with the membrane and isolated particles are rarely seen on vesicles.

Figure 2 compares the protein composition of peak gradient fractions of preheads purified from mutants 21(*tsN*8), 21(*amE*322), 24(*amN*65), 21(*tsN*8)-23(*tsA*78), and 10(*amB*255)-18$^-$-(*amE*18) capsids. The protein compositions of the mutant preheads are discussed in relation to the data from the quantitation which is shown in Table 1. The shell and internal proteins of the mature head were quantitated from purified wild-type phages, because 10$^-$-18$^-$ capsids lose internal proteins during purification. These were used as the standard for comparison with their precursors in the mutant preheads. This comparison provided a measure of the relationship of the mutant preheads to the wild-type prehead. For the 21$^-$ preheads, the calculated value of 350 molecules of IPIII agrees within experimental error with the value of 400 molecules of IPIII* per mature phage. This was also true for the internal protein B_1. There were 40 copies of B_1 in the prehead and 43 to 45 copies of B_1* in the mature phage. We found 65 to 70 copies of the minor capsid protein P24* in the mature phage. This value of P24* is between those previously reported (1, 17), but agrees reasonably well with an expected value of 55 copies based on its position at 11 of the pentameric vertices (18). The previously reported figure of 160 copies of P24* (1) was due to a misidentification of the P24 band. The quantitation of unprocessed P24 in preheads was complicated by the presence of an unidentified contaminating band of very similar molecular weight (Fig. 2) and further complicated in 21$^+$ preheads by some cleavage of P23 to P23* which migrated in almost the same position as P24. We suggest that the low value of 60 copies of P24 as determined on 8% gels is most accurate and that our higher values from higher percentage gels are due to the superposition of bands. Values of P24 as calculated from several percentage gels are listed in Table 1.

The low amount of P20 in the capsid made it difficult to quantitate accurately, but the value for preheads was consistently double that for phage (30 as opposed to 15) with repeated determinations. Attempts to resolve a possible contaminating band on various percentages of SDS gels have been negative.

The close agreement between the ratios of the related proteins of the capsid with those proteins in the 21$^-$ preheads implies a close relationship of these preheads to the wild-type capsid precursor. The amounts of those core proteins which remain in the phage agree with the amounts of these proteins in the unprocessed core of the 21$^-$ preheads. This suggests that the value for the major core protein P22 (550 to 600 copies) which is completely digested during prehead maturation is also correct. This P22/P23 molar ratio of 0.6 is somewhat higher than the value of 0.48 previously reported (13). However, our isolation procedures have been optimized for preservation of the fragile core which accounts for the higher values. We also identified two new prehead proteins with approximate molecular weights of 17,000 (17K) and 14K. These proteins are present in large amounts (200 to 300 copies) and are probably the two proteins described by Kurtz and Champe (9), which they report are also cleaved during head maturation. The 14K protein is the most acidic of the core proteins with a pI of 4.8 and is cleaved in vitro by purified

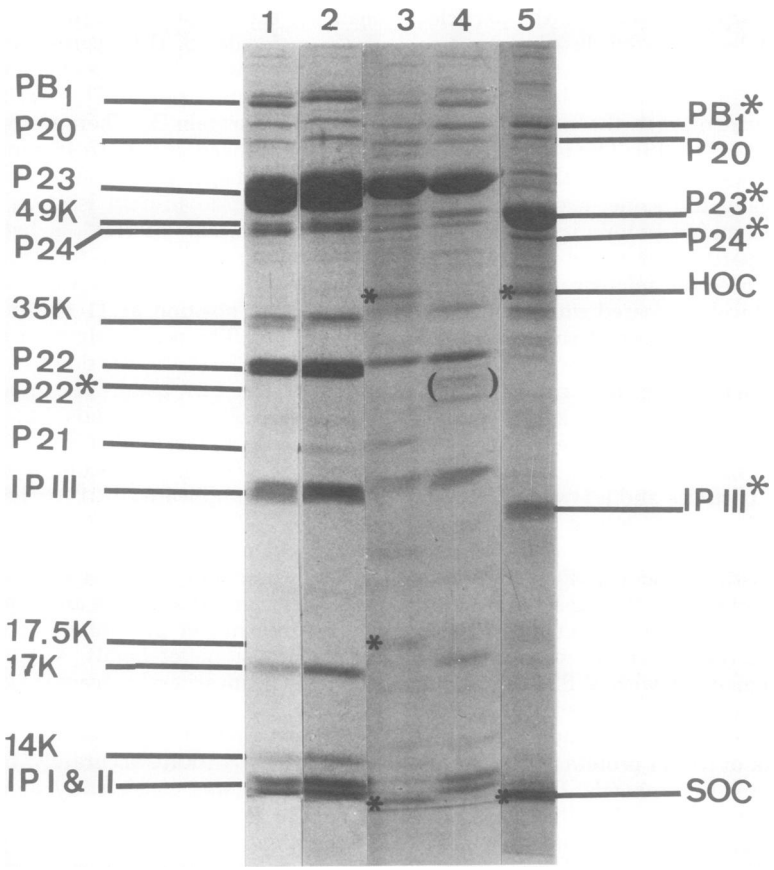

FIG. 2. *Stained SDS acrylamide gels comparing the component proteins in T4 mutant preheads. The samples are peak fractions of gradient-purified preheads which were pretreated with Nonidet P-40 in all cases. Lane 1, 21(amE322); lane 2, 21(tsN8); lane 3, 21(tsN8)-23(tsA78); lane 4, 24(amN65) (complemented with a 21-22-23 lysate); lane 5, T4 capsids. The molecular weights are used to identify band positions for which there is no known gene. The 17.5K protein is present only in lane 3, and the class of bands in parentheses in lane 4 are P22 cleavage products because they react with specific anti-P22 serum on immune replicas of SDS gels.*

T4 prehead proteinase (T4PPase) (Showe, unpublished data). These two proteins were clearly core associated and not capsid associated, for in the 23*ts* mutants which lose their cores they decreased in proportion to the P22 and IpIII. We also found both proteins in 20-21 polyheads whose shell has been shown to consist only of P23(21) but not in 21-22 polyheads which have no cores.

The *ts* gene 21 product (the zymogen of the T4 protease [23]) was associated with the *ts* preheads in an inactive form. We did not classify P21 as a typical core protein because we could not detect it in purified 20^- polyheads. Unlike the 17K and 14K proteins, its quantity was about the same in the core-deficient 23*ts* particle as in the 21*ts* particles. Another 21 mutant, *amH*29, makes a long *am* fragment which can be converted to active T4PPase. At least 4.5 kilodaltons are removed from the C-terminal end of P21 during its activation (23). However, neither the long *am* fragment nor the active T4PPase was associated with the 21(*amH*29) preheads under our conditions of isolation. This suggests that the C-terminal end of the P21 is involved in the association with the prehead, although it is clearly not necessary to maintain the molecule in the proper conformation to allow the conversion cleavages to take place.

The composition of the 24^- preheads is also given in Table 1. Because there is always some unknown amount of contaminating polyhead debris associated with this prehead peak, we have not attempted an accurate quantitation. It is also very difficult to keep these preheads from completing the maturation cleavages once the

P24 has been added back. Extraction of these particles at 10°C was partially successful in controlling the transformation, but there was still considerable P22 cleavage evident. These preheads contained all the proteins associated with the 21⁻ preheads, including the 17K and the 14K proteins. The associated P21 was all in the form of T4PPase or the most immediate precursor. The enzyme remains inactive in vivo in the absence of P24.

(ii) 23*ts* mutant preheads. The 23*ts* preheads differed somewhat in composition from either the 21⁻ or 24⁻ preheads. Although they were composed of unprocessed precursor proteins, the overall composition lay somewhere between the capsid and the prehead. We have shown only the composition of the double mutant 21*ts*-23*ts* to avoid the complication of cleavage during isolation in the single 23*ts* mutant. Table 1 shows that the core content of the 23*ts* (based on both P22 and IPIII) was only 10 to 30% of that of the 21⁻ preheads in a number of isolations. Because these particles are improperly closed, they may lose the core during the purification. Alternatively, the *ts* mutation in gene 23 may prevent formation of a complete core structure. The 17K protein seemed to be missing in the 23*ts* preheads, but a slightly larger protein was present. The minor shell proteins were present in normal amounts compared with the 21⁻ preheads. 23*ts* preheads also contained the outer capsid proteins *hoc* and *soc*. The presence of these two proteins was confirmed by reaction with specific antiserum. *hoc* and *soc* have previously been shown to associate only with cleaved and expanded P23 lattices (7, 25). Their association with the unprocessed 23*ts* preheads in ratios which are approximately the same as those reported for phage (7) suggests that these structures have a P23 shell which is more like that of mature phage than like preheads. This suggestion is supported by several other properties. Table 2 compares the resistance to solubilization by heating in 1% SDS of the *ts*23 preheads with 21⁻ preheads and 10⁻-18⁻ capsids. Preheads composed of wild-type P23 were 100% solubilized under all conditions tested, and, in fact, they are dissociated simply by exposure to low ionic strength (26). The 23*ts* preheads, however, were resistant to solubilization in 1% SDS to over 55°C (as well as to 8 M urea at neutral pH). The stability of the *ts*P23 prehead more closely resembled that of the mature head composed of P23* than that of 21⁻ or 24⁻ preheads. These *ts* particles could be cleaved either by added protease in the case of 21*ts*-23*ts* or by the endogenous protease of the 23*ts* mutant. Therefore, this *ts*P23 still contains the proper conformation for recognition by the T4 protease. This cleavage further increases the stability of the particles so that they are indistinguishable in this respect from normal capsids.

TABLE 2. *Resistance to solubilization in 1% SDS*[a]

Temp (°C)	21(*tsN*8)-23(*tsA*78) for preparation no.:			21(*tsN*8)	Capsids 10⁻(*am*B255) 18⁻(*am*E18)
	1	2	3		
25	14.5	10.3	17.6	60	0
35	15.3	12.0	ND		
45	18.4	14.5	ND		
55	25.0	27.6	33	85	5
65	100	100	100	100	100

[a] Capsids, 21*ts* preheads, and 21*ts*-23*ts* preheads were purified on glycerol gradients. SDS was added to the particles to a final concentration of 1%, and the particles were then incubated at the various temperatures for 1 h. Equivalent samples were applied to the gel. The P23 or P23* bands were cut from the gels after destaining and extracted in pyridine as described. Values are the percent P23 released at various temperatures in comparison to that released after 1 h at 65°C. ND, not done.

Morphology and biological activity of 21⁻ mutant preheads. Preheads produced by gene 21 mutants have previously been described (8, 14, 16), but electron micrographs of negatively stained isolated particles (14, 16) or thin sections (8) revealed very little interpretable structural detail. We have taken advantage of the fact that we can isolate preheads on membrane vesicles to study the intact prehead and its membrane association.

The gene 21 mutants produce primarily preheads and a small number of polyheads mostly with an abnormal cap. Figures 3a and b show the general features of 21⁻ preheads as isolated on membrane vesicles (Fig. 3a) and when released from the vesicles by mild detergent treatment (Fig. 3b) (see above). The vesicle-bound particles have distinct vertices and are more resistant to stain penetration than the free particles (Fig. 3b). On free preheads, the apical vertices can be distinguished from one another by their morphology. One of them is associated with a slight swelling on the inside, and has a distinct extension on the outside of the shell. The extension sometimes carries a membrane fragment as previously noted (16), and may correspond to the fibrous attachment of the prehead to the cell membrane described by Simon (24) from thin sections of wild-type-infected cells. This membrane attachment (or proximal) vertex must be the prospective tail attachment site, for the distal apical vertex has the same morphology as the nonapical vertices, and it is closed by a pentamer of P24 (see below).

The cores shown in Fig. 3b seem to be more highly stain penetrated in the center (as previ-

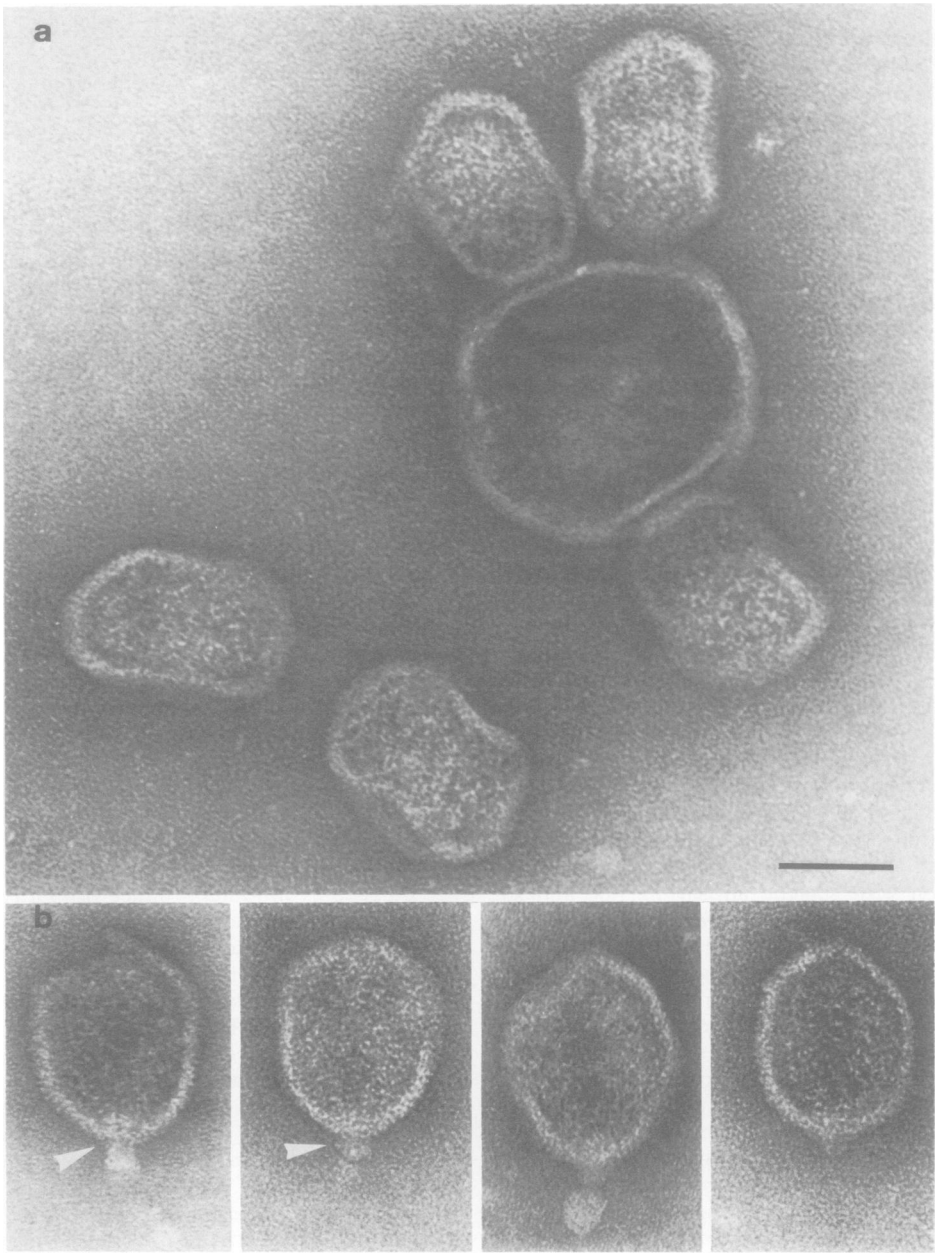

FIG. 3. *(a) Membrane-bound 21ts preheads from the upper peak of a glycerol gradient as shown in Fig. 1b. (b) 21ts preheads isolated on glycerol gradients after treatment with Nonidet P-40 to release them from the vesicles. The arrows indicate the typical "neck" structure which remains on most of the particles. The bar represents 50 nm.*

ously reported for elongated particles [20]), suggesting that it is less dense at the interior. The core itself seems to be intimately associated with the membrane attachment site and seems to merge with the vesicle at that point. The distal "cap" of the core seems less tightly associated with the distal cap of the "shell." These various aspects of the core are better shown in Fig. 4. Preheads which have the outer shell disrupted often still have intact cores. In the case of membrane-bound particles the core is still bound to the membrane attachment site, even when a

considerable amount of the shell is missing (M. K. Showe and L. Onorato, Proc. Natl. Acad. Sci., in press). There was no detectable morphological difference under our conditions between preheads produced by the 21*ts* mutants which contain P21 and those produced by the very N-terminal *am* mutant 21(*amE*322).

Morphology and biological activity of 24⁻ mutant preheads. Preheads produced by mutations in genes 21 or 24 are morphologically indistinguishable in thin sections. However, isolated 24⁻ mutant preheads (*ts* or *am*) are clearly distinguishable from 21 mutant preheads in negative stain. Figure 5a shows the preheads produced by 24(*tsL*90) and 24(*amN*65). These preheads appear more flattened and more highly stain penetrated than those produced in 21⁻ mutants. Close inspection reveals that the 24⁻ preheads have clear gaps at the position of the presumptive vertices except the one at the membrane attachment site. Figure 5b shows the transformation of the 24*am* preheads after complementation with a (21-22-23) donor lysate. These particles are now indistinguishable from the 21⁻ preheads. All of the nonproximal vertices are complete, and the particles are much more resistant to stain penetration. There is no comparable effect when the complementation is carried out with a (21-22-23-24) lysate, whereas complementation with purified P24 gives the

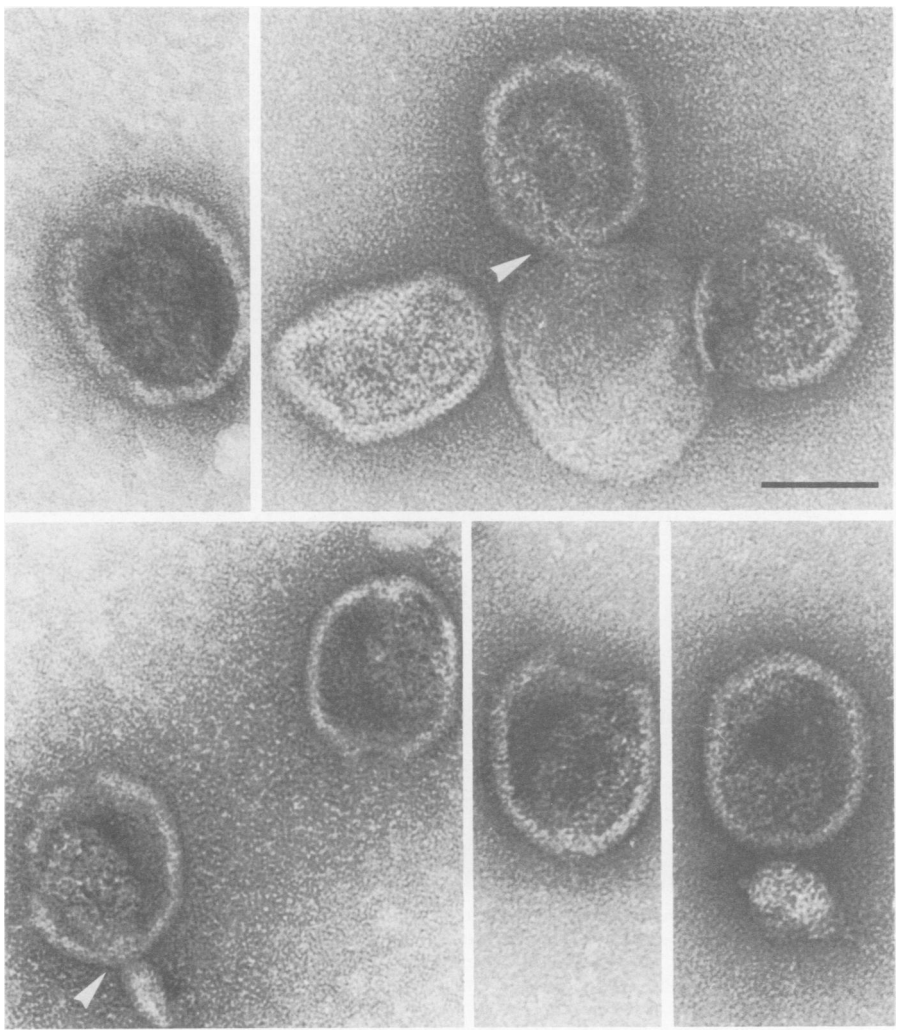

FIG. 4. *Partially disrupted 21ts preheads showing association of the core with the membrane attachment site. The particles were isolated on glycerol gradients, and the examples were selected to show the core attachment. The bar represents 50 nm.*

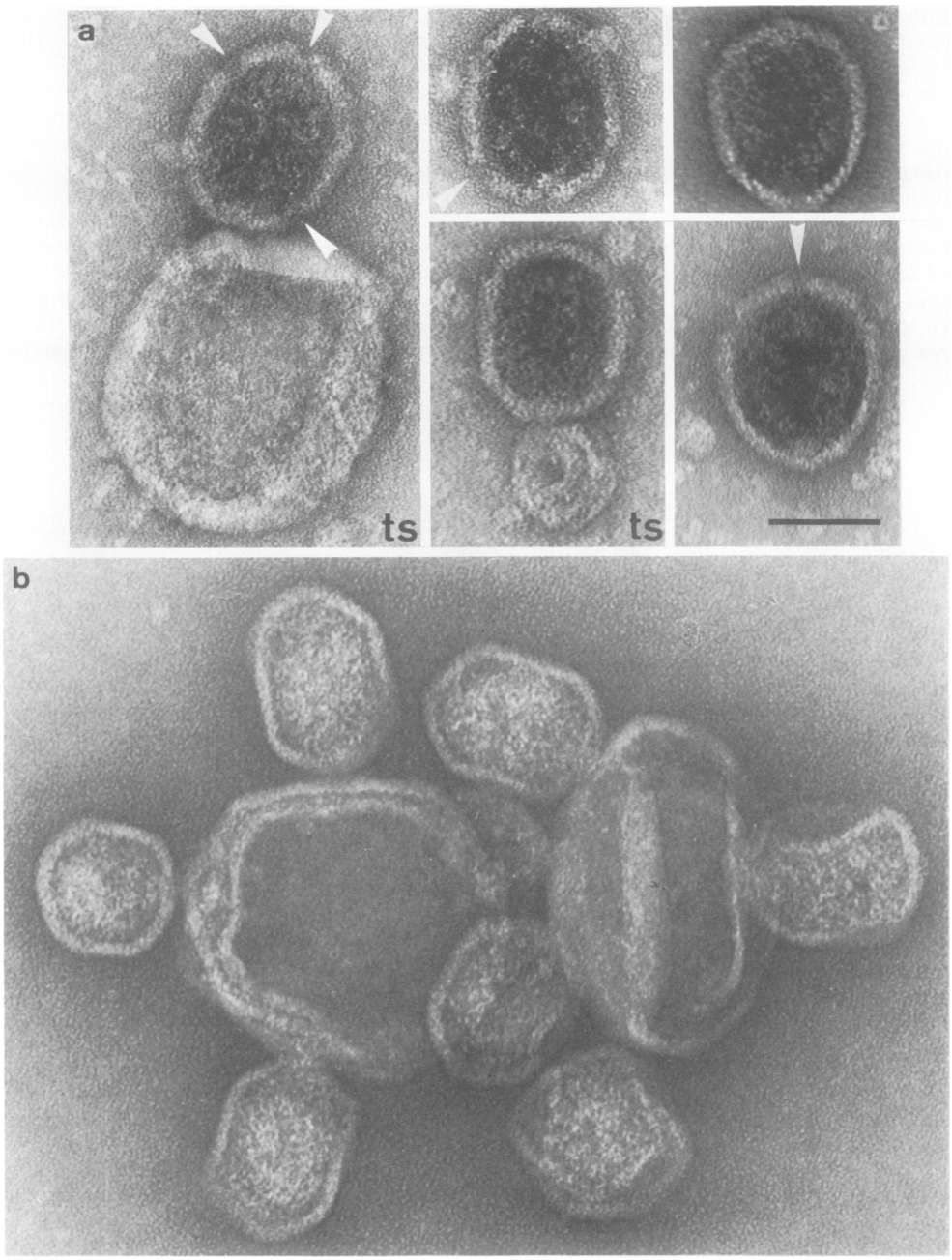

FIG. 5. *24(tsL90) and 24⁻(amN65) preheads (a) and 24⁻(amN65) preheads after complementation with a P24 donor lysate (b). The particles in (a) across the top are from 24⁻ lysate; ts indicates the particles from 24(tsL90); the others are from 24(amN65). The preheads in (b) were produced by complementation of the 24(amN65) particles with a P24 donor lysate. Complementation with purified P24 produces the same transformation. Both (a) and (b) were partially purified by differential centrifugation. The noncomplemented particles were much more fragile than the complemented ones, and there was always much more background debris. The bar represents 50 nm.*

same result as the 24^+ donor lysate. These results are consistent with our earlier finding that anti-P24 serum specifically labels all the vertex positions of 21^- preheads and of capsids except the tail attachment site. From these experiments and the quantitation of P24 in capsids and preheads described above, we conclude that each vertex of the T4 prehead except the membrane attachment site is composed of P24. Because the vertices are positions of fivefold symmetry, the P24 is probably in the form of pentamers. The membrane attachment vertex is morphologically distinct from the other vertices, in both 21^- and 24^- preheads, having a slight swelling where the core, shell, and bacterial membrane seem to join. Because insertion of a pentamer of P24 at this position would close the icosahedral shell completely, it must be the site at which the tail is attached to the mature capsid and it probably contains no P24.

Preheads made by the *ts* gene 24 mutant *tsL*90 also have gaps at their vertices and fail to react with anti-P24 serum. Immune replicas of SDS gels (23) of partially purified 24*ts* preheads show that they contain much less P24 than 24*am* preheads complemented with wild-type P24.

The phenotype of 24 *am* and *ts* mutants has been previously described as production of tau particles (preheads) and polyheads (14, 25). The production of polyheads is difficult to reconcile with our observation that P24 functions at a late stage in prehead assembly, rather than in prehead initiation. However, we found that both types of mutants also produce giant preheads under nonpermissive conditions (Fig. 6a). These are elongated preheads with the 15,5 folding of the P23 shell, which would make them precursors to giant phages. (The folding was verified by optical diffraction of micrographs of particles which were partly emptied of core). Previous failure to observe these structures was probably due to the instability of the capped ends in the absence of complementation with P24. In addition to preheads, giant preheads, and polyheads, 24^- lysates also contain large numbers of a variety of polyhead which has a prehead or giant prehead at one end (Fig. 6b, c, and d). The empty particle shown in Fig. 6d has been diffracted and shown to have the prehead (15,5) folding at the closed end, and a 10,10 folding after the discontinuity. Table 3 lists the distribution of the various types of elongated particles found in 24^- lysates. It shows that about 50% of the elongated structures have a normal or a giant prehead at one end. Some of these maintained the 15,5 folding for their entire length (the giant preheads), whereas others showed a pitch change which resulted in a polyhead with a narrower diameter growing off the prehead. Fifty percent is probably a low estimate for the occurrence of the prehead-polyhead-type structures, because many of the open-ended particles that we counted as polyheads may have broken off preheads in the lysate. This suggests that 24^- polyheads are derived mainly, if not exclusively, from normally initiated structures.

Maturation of 24^- preheads in vitro. If the pH of a 24*am* lysate is raised to 7.6, the precursor proteins are cleaved, but only after the particles begin to dissociate because of the elevated pH. However, after complementation with a 24^+ donor lysate, cleavage occurs even at pH 6.0 and the preheads are rapidly converted to capsids. Figure 7 compares the distribution of P23 and P23* on 15 to 40% glycerol gradients of particles purified after complementation with either a 24^+ or a 24^- donor lysate in parallel experiments.

Once the P24 is added to the particles they cleave spontaneously. It is clear from the gradients that the particles complemented with the 24^+ donor lysate have 50% of the P23 on the gradient in the form of P23* and that the cleaved P23 is found almost entirely at the capsid position. Electron microscopy of this peak confirmed that the P23* was in the form of capsids, and immune replicas of the samples through this peak showed that they contained P24*. There is little or no P23* in the lower part of the gradient, which contains mainly elongated structures. This suggests that T4PPase is associated only with the prehead cap(s) because there is little or no P23 cleavage in the elongated structures. The control gradient of particles prepared with a 24^- donor lysate contains little or no P23*, confirming the requirement for a specific interaction of P24 with the prehead to activate the T4 protease. However, the requirement for P24 only applies to cleavage of intact preheads because active protease can be extracted after particle disruption by sonication or by incubation of the 24^- lysate at pH 7.6. These conditions result in prehead breakdown and, consequently, release of the protease. The association of the protease with the inside of the prehead has been inferred because of the number of its substrates which are core proteins. The outside of the shell of the prehead seems resistant to T4PPase. In attempts to cleave the 21^- preheads with added protease, cleavage occurred only after the particles began to dissociate, and we were never able to recover capsids from these preparations, even when P23* cleavage was 100%.

23*ts* preheads. *ts* gene 23 mutants have been shown to produce preheads which are irregular in size and shape (5, 14). Figure 8a shows preheads isolated from the double mutant 21(*tsN*8)-

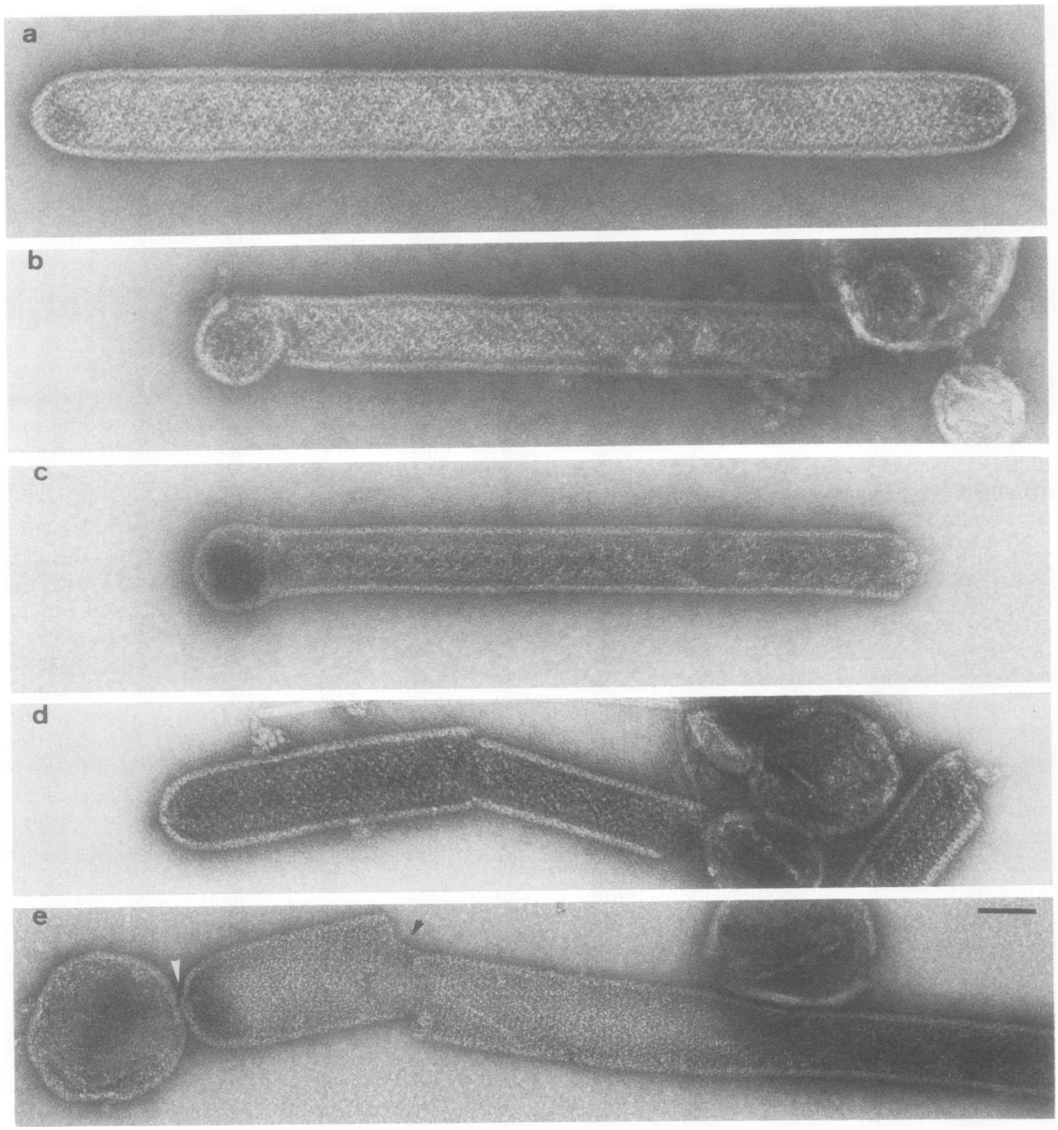

FIG. 6. *Examples of elongated particles produced by 24⁻ (amN65). The particles shown are partially purified by differential centrifugation after complementation with a P24 donor lysate. The distribution of classes of particles in uncomplemented 24⁻ crude lysates is given in Table 2. The core was removed from (e) by addition to the sample before fixation of sodium phosphotungstate at a pH of 7.2 and a final concentration of 0.5% (van Driel, unpublished data). Staining after adsorptions to the grid was with 1% uranyl acetate (pH 4.2) in all cases. The bar represents 100 nm and the magnification is the same in all cases.*

23(*tsA*78) whose protein composition was described above. They are not distinguishable morphologically from the preheads isolated from the single mutant 23(*tsA*78) which we have previously described (19). Many had aberrant or incomplete closures and angular projections which might be vertices at apparently random positions on the surface. Unlike the particles isolated from 21⁻ and 24⁻ mutants, 23*ts* preheads did not appear to be membrane associated in lysates, and the purified particles were not attached to membrane vesicles on isolation. Upon isolation cores showed no regular structure, and the capsid had a smooth appearance in contrast to the rough surface of other mutant preheads. The capsid wall appeared thinner than that of the 21⁻ preheads (Fig. 8b), like that of mature wild-type phage. These characteristics, together with

their resistance to dissociating agents and their association with the outer capsid proteins *hoc* and *soc*, suggested that the *ts*P23 shell is expanded even though the P23 is not cleaved.

To test this hypothesis, double mutants were constructed with 23(*tsA*78) and *am* mutations in head genes which produce polyheads or elongated preheads (genes 20 and 24). Each of these double mutants, when grown under nonpermissive conditions, was found to produce polyheads or elongated preheads which were resistant to cold SDS even though composed of uncleaved *ts*P23. Figure 9a shows a representative 20*am*-23*ts* polyhead. Like the polyheads produced by all of the double mutants, it has a thin wall and a disordered core. To insure that no P23 cleavage

TABLE 3. *Elongated head-related structures in lysates of 24(amN65)*[a]

Time of lysis (min)	Normal closure		Prehead-polyhead	Open polyhead	Not classified	Total
	One end	Both ends				
20	129 (31)[b]		91 (22)	155 (38)	36 (9)	411 (100)
60	146 (33)	14 (3)	55 (12)	192 (43)	41 (9)	448 (100)

[a] Cultures were grown to 4×10^8 and then infected at zero time and 8 min with 24(*amN*65) at a multiplicity of infection of 5. One half of the cultures was centrifuged at 20 min after the first infection, and the other half was centrifuged at 60 minutes. The pellets were resuspended into the donor lysate (21-22-23) 100× concentrated and treated with 5 mg of DNase per ml. The cells were opened by pipetting, and samples for microscopy were fixed with 2% formaldehyde as soon as the lysates could be easily pipetted. This process takes about 5 min from the time the pellet is removed from the centrifuge. Particles with normal closures were selected by eye after testing of 15 selected particles which all proved to have the proper 15,5 folding based on optical diffraction patterns. Particles which were not classified are those which were open at both ends but appeared to be wide enough to be 15,5 polyheads. Total normal initiations, 50%; pitch changes observed, 19%.

[b] Numbers in parentheses are percentages.

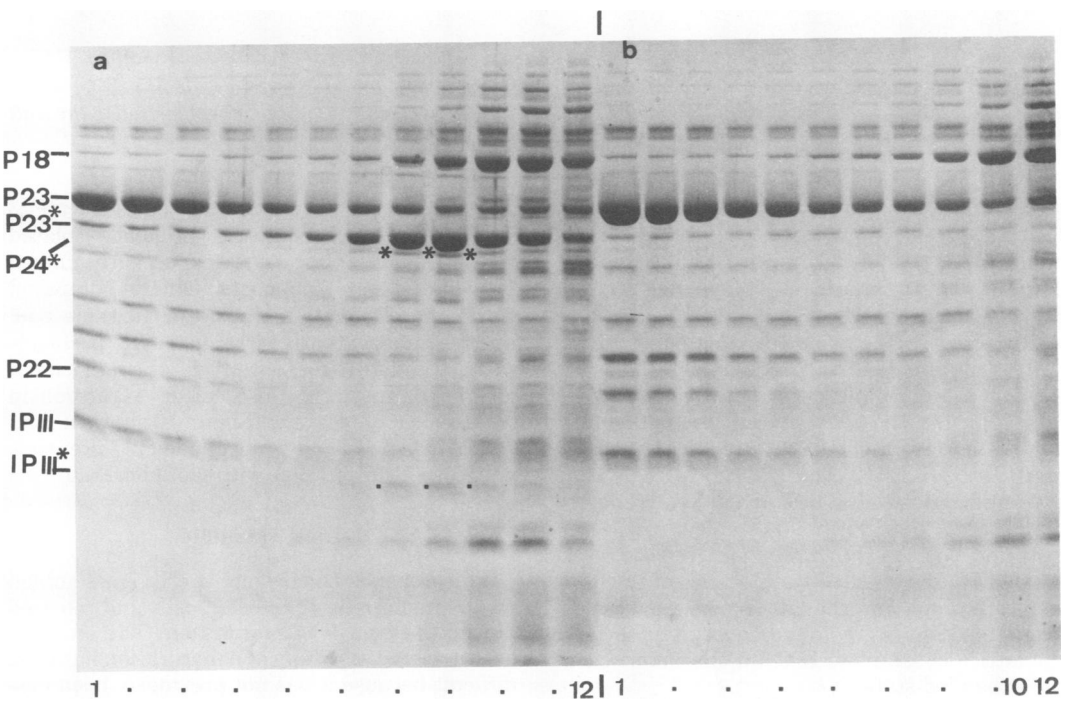

FIG. 7. *12.5% SDS gel of particles purified on glycerol gradients after complementation with a 21⁻-22⁻-23⁻ donor lysate (a) and after complementation with a 21⁻-22⁻-23⁻-24⁻ lysate (b). The gradient fractions are numbered from the bottom, and fraction 11 is missing in (b). The * indicates the presence of P24* in fractions 8, 9, and 10. This was confirmed by immune replicate electrophoresis of a separate gel with specific anti-P24 serum. (■) marks the position of IPIII* on the gradient. The P18 peak indicates the peak of free tails.*

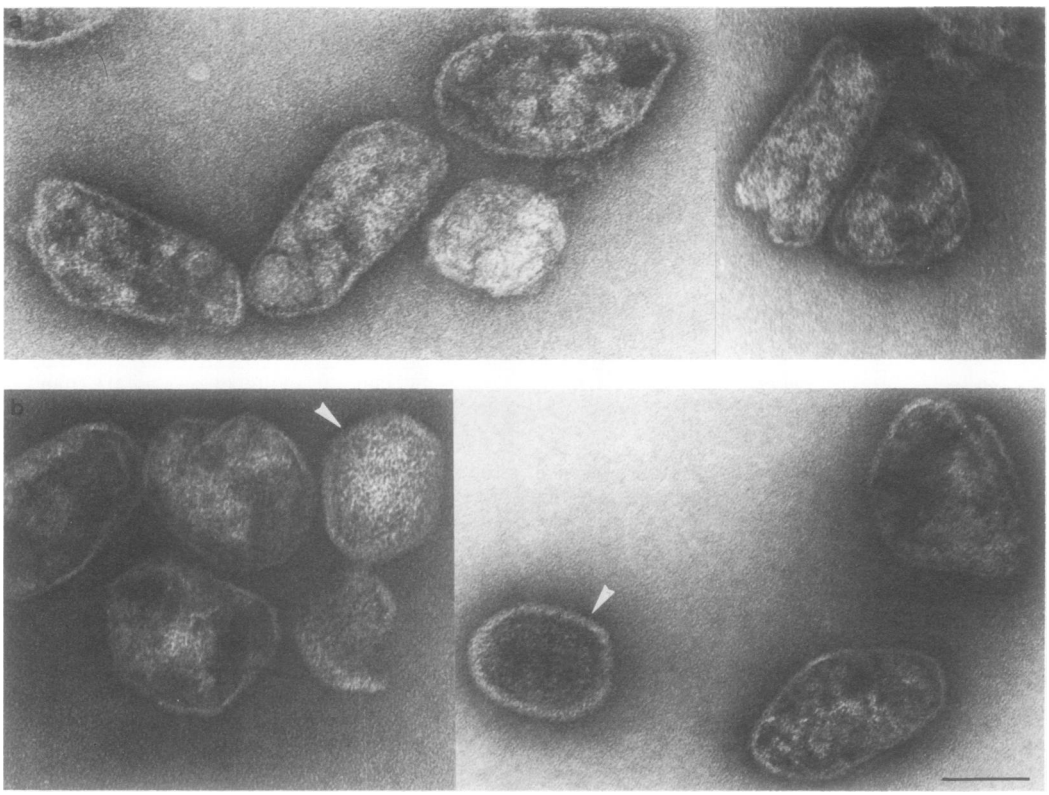

FIG. 8. *The 21(tsN8)-23(tsA78) preheads shown in (a) were purified on a glycerol gradient (see legend to Fig. 1c). (b) shows a mixture of gradient-purified 21(tsN8⁻), 23(tsA78), and 21(tsN8) for comparison of the wall thicknesses. The bar represents 50 nm, and the arrows indicate the 21(tsN8) unexpanded preheads.*

might take place after cell lysis, we compared the few polyheads produced by gene 21 mutants with those produced by the double mutant 21-*ts*23*ts* (Fig. 9b and c). The single-mutant polyheads always had regular cores and thick walls, whereas the particles isolated from the double mutant had an irregular core and thin walls. To see whether the putative expansion takes place in the cell or after lysis, a 21*ts*-23*ts* double-mutant-infected culture was lysed directly into 1% SDS. Figure 9d shows a polyhead prepared in this way. The core has been removed by the SDS, but the shell is stable to the detergent and has the thin-wall characteristic of expanded P23 lattices. When particles of this type were diffracted and the lattice constant was determined, they were found invariably to have the lattice constant of 12.9 nm characteristic of the expanded state.

We conclude that the *ts* shell protein produced by 23(*tsA*78) assembles into a lattice which can expand in the cell in the absence of the cleavage which is thought to accompany expansion of wild-type P23 lattices. The ability to expand without P23 cleavage is not unique to the *ts*P23 lattice. About 50% of the 24⁻ preheads isolated on the gradient in Fig. 1d are expanded as judged by wall thickness or particle diameter compared with normal-looking preheads on the same grid. Furthermore, extensive washing of 21⁻ preheads with Nonidet P-40 to remove the associated membrane fragments also causes expansion in the absence of cleavage. Figure 10 shows a mixture of expanded and nonexpanded 21⁻ preheads produced by treatment with Nonidet P-40.

DISCUSSION

The prehead of bacteriophage T4 is assembled on the membrane of infected cells and released during the course of maturation. The study of both prehead assembly and maturation has been difficult because it has not previously been possible to isolate and purify either intermediates on the pathway to formation of the wild-type head or preheads blocked in maturation by mutations in the head genes. We have now found conditions for the isolation and purification of

head-related structures produced by mutations in three head genes, 21, 23, and 24. The composition, morphology, and other properties of these particles have clarified several aspects of T4 prehead assembly and head maturation.

Prehead composition and assembly. The finding that gene 24 mutants make preheads with gaps at their non-proximal vertices confirms our previous (18) localization of P24 on preheads and capsids. In the absence of P24, preheads are unstable to isolation, possibly because the shell dissociates at the gaps at the vertex positions. Only by adding P24 to the preheads in vitro can 24^- particles be stabilized sufficiently for isolation on our glycerol gradients. McNicol et al. (17) have described a *ts* mutation in gene 23 which allows the production of viable phage by gene 24 *am* mutants. They suggested that the major shell protein, P23, might substitute for the missing P24 in this

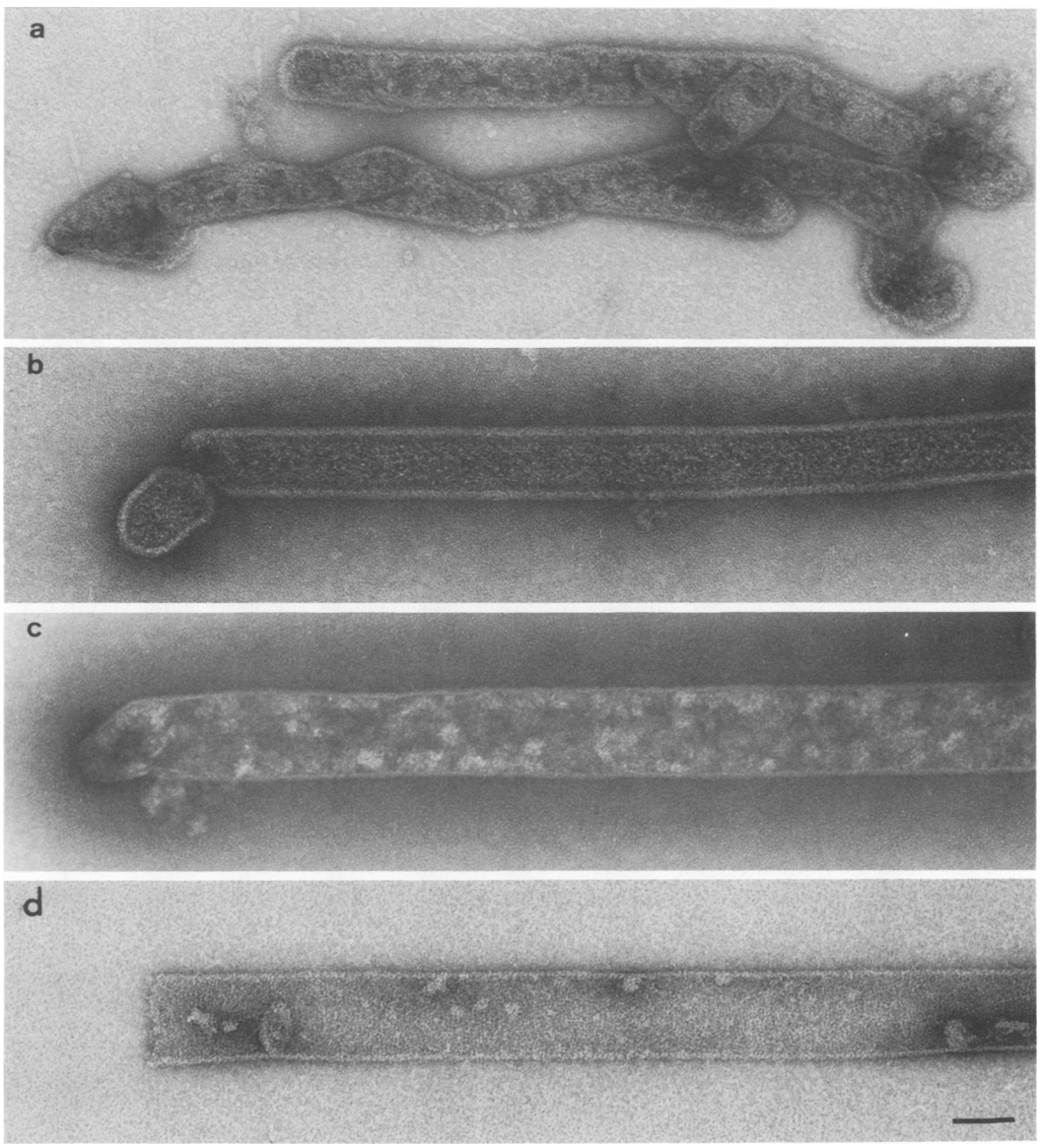

FIG. 9. *(a) 20(amN50)-23(tsA78) polyheads; (b) 21(tsN8) polyhead; (c) 21(tsN8)-23(tsA78) polyhead; (d) 21(tsN8)-23(tsA78) polyhead extracted into 1% SDS at room temperature and washed several times with water to remove the SDS. The bar represents 50 nm.*

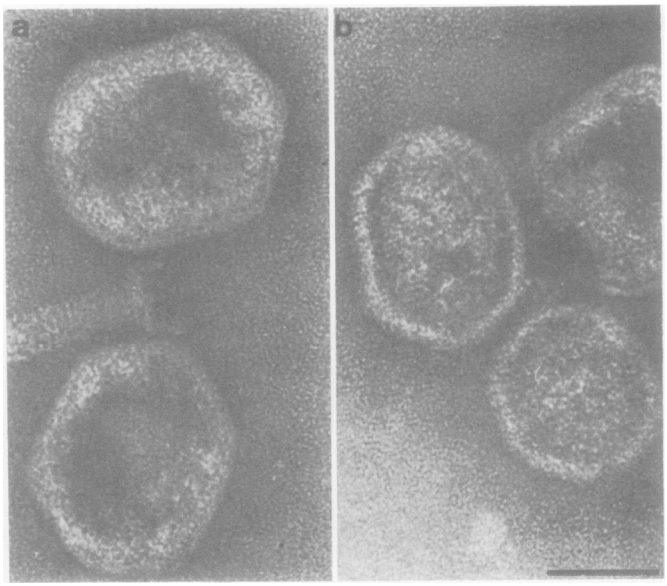

FIG. 10. *21⁻ preheads expanded in vitro. The particles shown are derived from 21(tsN8). They were extracted by the methods described in the text and then subjected to two successive treatments with 0.5% Nonidet P-40 followed by centrifugation. Approximately 50% of the particles are of the expanded type shown in (a); (b) is a sample of unexpanded particles taken from the same grid space for comparison of size. The bar represents 50 nm.*

mutant. Our localization of P24 at the prehead vertices suggests that at high temperature, the *ts*P23 produced by this mutant might form the prehead vertices as well as the rest of the shell, permitting maturation of the preheads to capsids. The function of P24 as a specialized vertex protein for the P23 shell further supports the suggestion of McNicol et al. (17) of an evolutionary relationship between P23 and P24.

We have found that both *ts* and *am* gene 24 mutants produce, in addition to normal-length preheads, large numbers of elongated structures attached to bacterial membrane by the same structure which attaches the preheads. The shell attached to the membrane, as judged by either its diameter or folding (as determined by optical diffraction), has the normal prehead lattice. We propose that these elongated particles represent preheads which are normally initiated but which continue to elongate because the gaps at the vertices make the caps unstable and allow the continued addition of P23 at the vertex positions. If the pitch of the lattice changes, a prehead-polyhead or giant prehead-polyhead (Fig. 6b through e) results. If the folding remains 15,5 until a cap is added, a giant prehead results (Fig. 6a). Laemmli and Eiserling (11) showed that the initiation of polyhead production by 20⁻*am* mutants requires a higher pool of precursors than normal prehead production, being delayed by about 10 min relative to the appearance of infective phage. However, the elongated structures made by 24⁻ mutants are produced simultaneously with the 24⁻ preheads (14). This would be expected if they arise as aberrations in elongation of normally initiated preheads. We conclude that P24 functions in T4 head assembly to stabilize the prehead by inserting subunits, probably pentamers, at the icosahedral vertex positions except for the membrane attachment vertex. It is not required to specify the form of the prehead, because the majority of the structures formed have the morphology of wild-type preheads (14), and *ts*24 preheads have been shown to be capable of conversion to infectious phages by a temperature shift in vivo (3). The production of small numbers of giant phages by growth of 24*ts* mutants at intermediate temperature or mixed infections with 24*am* and 24⁺ phage (2) probably represents the maturation of some of the giant preheads of the type produced under strictly nonpermissive conditions. Our results show P24 to have two separable functions, one in prehead assembly and the second in maturation. In the first, it stabilizes the vertices, and in the second, it triggers the maturation cleavages (see below). Thus, the addition of P24 to the prehead may serve to bridge these two stages in T4 head formation.

If P24 is required only at a late stage in prehead assembly, what T4 head gene products are required earlier to specify the form? Mutations in genes 20, 22, and 40 can cause P23 to assemble into polyheads, but do not make struc-

tures with the 15,5 folding of preheads. We have presented evidence (18) that all of the P20 of the capsid is present at the proximal vertex, because it can be removed together with a structure at one of the apical vertices under conditions which leave the other vertices intact. Our micrographs of purified preheads show that they have a similar structure at the vertex which is attached to the bacterial membrane. The prehead core, shell, and the bacterial membrane join at this vertex. Either the core or the shell of the prehead is sufficient to attach the particle to the membrane at this point, in agreement with thin sections which show continuity of the prehead core and bacterial membrane (24). We have argued that P20 functions to initiate normal prehead assembly by forming an initiation complex with fivefold symmetry on the bacterial membrane. Our micrographs (Showe and Onorato, Proc. Natl. Acad. Sci., in press and Fig. 6e) suggest that this complex binds both the core and the shell of the prehead and may serve to determine the subsequent assembly and structure of the core as well as the head shell. This is in agreement with the observation of Paulson and Laemmli (20) that the cores of 20^- polyheads have a different repeat from those of giant preheads. Because P22 is the principal essential component of the T4 prehead core, it may further explain why 22^- mutants are unable to initiate prehead formation.

We find that all of the classes of preheads contain about twice the amount of P20 found in mature capsids. Although other explanations are possible, it may be that the initiation complex on the bacterial membrane contains some P20 which is left behind during normal prehead maturation.

We have found that core-containing preheads contain substantial amounts of two proteins not found in mature phage. These must be core components, because they are not present in the core-deficient 23*ts* preheads and can be purified from core-containing polyheads (our unpublished data). Their molecular weights, 14K and 17K, suggest that they are identical to two proteins described by Kurtz and Champe (9) as being cleaved in vitro by lysates of T4 infected cells. The smaller has been identified as the precursor to one of the two trichloroacetic acid-soluble peptides found in the mature capsid (9).

Prehead maturation. Our experiments demonstrate three steps at which prehead maturation can be blocked, all independent of DNA packaging. They are: conversion of P21 to T4PPase, cleavage of the precursor proteins by T4PPase, and lattice expansion.

The first step is blocked in the *ts* gene 21 mutants. These make morphologically normal preheads which contain the *ts*P21. We have previously shown that *ts*P21 can be converted to T4PPase antigen by the addition of wild-type T4PPase in vitro (23). However, the 21*ts* preheads cannot be matured to capsids by the addition of exogenous T4PPase. This suggests that all of the peptide bonds which are susceptible to the enzyme are inside the prehead, including those of the P21. The location of P21 within the prehead is not known. We find that it is not present in highly purified 20^- polyheads which contain the prehead core proteins (unpublished data). This suggests that it is not a typical core protein, but is associated either with all of the vertices or with the initiation complex at the proximal vertex.

A second step in the control of prehead maturation is illustrated by the 24^- preheads. In 24^--infected cells, P21 is converted to T4PPase, less rapidly than in the wild type but more rapidly than in other head-gene mutants which accumulate more P21 and intermediate P21 cleavage products (23). However, the T4PPase in 24^--infected cells is inactive. It can be activated by raising the pH of a lysate to 7.6, the pH optimum of T4PPase, or by completing the preheads by the addition of P24 even at pH 6.0. We do not know the mechanism by which T4PPase is kept inactive in head mutant-infected cells. It may be caused by association with an inhibitor of T4PPase activity that we have isolated from lysates (22) whose function is to prevent protein cleavage before prehead assembly is complete. However, we do not find this polypeptide associated with 21^- or *ts*23 preheads. The activation of prehead cleavage by P24 addition could result either from incorporation of T4PPase with the P24, replacement of inhibitor at the vertices by P24, or some change in the structure of the prehead brought about by vertex completion. We cannot distinguish among these possibilities for the moment because we have not yet succeeded in isolating a mutation in the gene which codes for the protease inhibitor.

A third step in prehead maturation, lattice expansion, has previously been shown to occur only on structures in which P23 cleavage has taken place (7, 25). However, we have found that preheads and polyheads made from *tsA*78 protein are expanded, even though the P23 is not cleaved. This expansion appears to occur in the cell because the particles can be extracted from cells which are lysed directly into SDS and these particles already contain the *hoc* and *soc* proteins. Although the significance of this finding for wild-type prehead maturation is not clear, it does show that P23 cleavage per se is not required for either lattice expansion or the formation of the *hoc* or *soc* binding sites.

Because 21^- and 24^- preheads can also expand

without cleavage, this is not a special property of the *ts*P23. How is this expansion of the 23*ts* particle in vivo related to the expansion of the 21^- and 24^- preheads in vitro? We have already stated that in addition to being expanded, the isolated 23*ts* preheads differ from the 21^- and 24^- preheads in that they are not membrane bound and that they contain no ordered core. The conditions or treatment which trigger expansion in the 21^- and 24^- preheads cause both core disruption and dissociation from the membrane. A role for the core in the stabilization of the unexpanded P23 shell is further indicated by the observation that 22*am*-23*ts* polyheads made at permissive temperatures on the su^- host are expanded while the core containing 20^-*am*-23*ts* or 24^-*am*-23*ts* polyheads made under the same conditions are not (unpublished data). This suggests that in the absence of P22 the metastable *ts*P23 shell cannot maintain the unexpanded state. Our micrographs of elongated 24^- preheads clearly show that both the core (Showe and Onorato, Proc. Natl. Acad. Sci., in press) and the shell (Fig. 6e) are tightly associated with the membrane via the proximal vertex. It is not unreasonable to suggest that the disruption of these attachments might trigger the expansion of the shell. In vivo, the activation of T4PPase associated with this proximal vertex could have the same effect.

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