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Gene 24-Controlled Osmotic Shock Resistance in Bacteriophage T4: Probable Multiple Gene Functions

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By use of mixed infections with conditional lethal mutations in the head genes and an osmotic shock-resistant mutant we have demonstrated that osmotic shock resistance is controlled by gene 24. Using acrylamide gel electrophoresis combined with the "immune replicate" technique, we confirmed the positions of gene products 24 and 24* (P24 and P24*). In this paper we have still used the notation "P24," etc., for designating the product of gene 23, etc., although we prefer and use in general the designation "gp23" as introduced by Casjens and King (Annu. Rev. Biochem. 44:585, 1975). The reason for using the old notation is because the illustrations were prepared several years ago.) P24 *ts* showed a significantly slower mobility. Both osmotic shock-resistant and -sensitive mature phages contain 24*. Giants constructed with the Os^r phage showed the same surface lattice as normal phage. Through temperature-shift experiments with 24(*ts*L90) alone and in combinations, we studied the phages which are matured after the shift to permissive temperature in the absence of new protein synthesis. Our results strongly suggest that only a fraction of the total phage complement of gene 24-controlled proteins is involved in determining the phenotype of shock resistance, and the remainder is necessary to mature the head.

T-even bacteriophages are inactivated by loss of their DNA when suspensions in concentrated salt solutions are rapidly diluted. They are not inactivated when diluted slowly, hence, the designation osmotic shock. Anderson et al. (4), who first described this phenomenon, concluded that shock sensitivity is likely to be a property of the protein shell of the phage head. Brenner and his collaborators (8, 34) isolated osmotic shock-resistant mutants and determined that the mutations were not located in gene 23, the structural gene for the major head protein.

Preliminary results of Brenner and his collaborators (S. Brenner, personal communication) suggested gene 24 as the most likely candidate for carrying osmotic shock resistance. They obtained these results by analyzing the progeny of doubly infected cells, in which one infectant was the osmotic shock-resistant (Os^r) mutant and the other was an amber mutant in one of the head genes 20 to 24. All the progenies, except that with 24$^-$, contained a mixed population of Os^r and Os^s phages. This suggested that only the amber mutant in gene 24 was unable to mediate the wild-type phenotype of osmotic shock sensitivity. In the present paper we report similar experiments which confirm the above-mentioned, unpublished results and those reported in preliminary form by Leibo and Mazur (25).

Recent experiments have shown that the cleaved form of the product of gene 24 (P24*) is part of the phage capsid (1, 13, 16, 18, 21, 40). (The notation used refers to the product of a gene as "P" followed by the gene number. The cleaved form of this product is designated by the same notation followed by an asterisk. The reason we have used this system of notation, although we prefer and use in general the designation "gp23" as introduced by Casjens and King (9), is that the illustrations used in this paper were prepared several years ago.) Nonfunctional P24 leads to an accumulation of τ particles which, in the case of *ts* mutants and shift to permissive temperature, can be matured into phage heads (6, 7). This suggests that τ particles (preheads) are the normally occurring precursors of wild-type T4 (36), probably identical with prehead I of Laemmli and Favre (22).

We further confirmed Brenner's observations (S. Brenner, personal communication) that the

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progeny of a cell mixedly infected with the wild type and Os^r mutant consists of a heterogeneous population containing a mixture of fully resistant and fully sensitive phages. Since there appear to be about 60 to 100 copies of P24* per phage capsid, this observation is difficult to explain if all molecules of P24* of the capsid are equivalent in function and if we assume that wild-type P24 (Os^s) and mutant P24 (Os^r) are equally likely to participate in the assembly of an individual phage particle. It appears as if the phenotype "osmotic shock resistance" is related to only a small fraction of the total complement of P24-related proteins needed to fully mature a phage particle. We believe that P24 and its derivatives have multiple functions.

MATERIALS AND METHODS

Bacteria. *Escherichia coli* S/6/5/1 or B^E was used as the host to prepare non-amber phage stocks and as the plating organism. *E. coli* BB or B^E was used as the nonpermissive host for mixed infections with amber phages. *E. coli* CR63 was used as the permissive host to prepare amber phage stocks and for plating these phages. The bacteria were grown in Hershey broth at 37°C on a rotary shaker or with air bubbling. In several experiments M9A, synthetic medium M9 (6) complemented with 1% Casamino Acids (Difco), was used.

Bacteriophages. Because the shock-resistant phages that we and others had studied previously are mutants of T4B, we isolated new *os* mutants of T4D in order to utilize the existing battery of T4D amber mutants for genetic analysis. Shock-resistant (*os*) mutants were isolated from phage T4D in the following way. Mutagen-treated stocks of phage were prepared by inoculating six 10-ml cultures of *E. coli* S/6 in Hershey broth containing 50 μg of 5-bromodeoxyuridine/ml with single-plaque isolates of phage T4D. Selection for shock-resistant mutants was performed by diluting each mutagenized stock into 3.0 M NaCl and diluting it again 100-fold with nutrient broth, both slowly to serve as a control and rapidly to shock the phage. The survivors were plated over a sufficiently wide range of phage concentrations to permit the percent survival to be measured and also to yield plates exhibiting confluent lysis. The top agar layers of these latter plates were scraped into 3.0 M NaCl and shaken vigorously to release the phage. One sample was diluted rapidly and one slowly. Both samples were then replated.

The entire procedure was repeated several times until the rapidly diluted sample exhibited approximately 100% survival as compared to the slowly diluted one. In this way, six separate stocks of shock-resistant mutants were prepared, although the rest of the experiments were limited to one of these, designated T4D *os*10. This stock was purified further by passing it through one growth cycle and picking single plaques of the progeny. Its shock resistance and other properties are similar to those previously described for other *os* mutants (24, 25) derived from T4B.

T4D phage mutants *am*B8 (gene 20), *am*N90 (gene 21), *am*B270 (gene 22), *am*H11 (gene 23), *am*B26 (gene 24), *am*N65 (gene 24), and *ts*N29 *ts*L90 (gene 24) were obtained from R. Epstein and R. Edgar and have been described by Epstein et al. (14). All these mutants, when grown under permissive conditions, are as shock sensitive as the parent T4D, yielding about 5% survival when rapidly diluted 100-fold from 3.0 M NaCl with nutrient broth. These genes, 20 to 24, are all known to be involved in the formation of the normal phage head (14, 23). The preparation and titering of the phage and the osmotic shock procedure have been described previously (24).

The experiments for which results are shown in Table 1 and Fig. 1 to 4 were performed as follows. *E. coli* BB was grown to about 2×10^7 cells/ml, centrifuged, and resuspended in Hershey broth to yield a cell concentration of about 4×10^8 cells/ml. The parental phage mixtures were adsorbed to the bacteria for 7 min in the presence of 0.4 mM KCN at multiplicities of infection within 10 to 15. The unadsorbed phage, amounting to from 1 to 5% of each mixture, was removed by T4 antiserum, and growth was initiated by diluting the infected cells 10^5-fold into Hershey broth. After one growth cycle under nonpermissive conditions, the total progeny of each cross was tested for its phenotypic shock resistance and for its ability to grow under permissive and nonpermissive conditions.

The experiments in Table 2 and Fig. 8 and 9 were performed under the conditions described by Bijlenga et al. (6, 7). *E. coli* B^E was grown to 2×10^8 to 3×10^8 cells/ml in M9A and infected and superinfected with the parental mixtures, each at a multiplicity of about 10. Since, in these experiments, no high dilution was possible, KCN and antiserum were omitted. When necessary, we corrected for unadsorbed phage.

Gel electrophoresis. Discontinuous sodium dodecyl sulfate (SDS)-acrylamide gel electrophoresis was done as described by Laemmli et al. (23), except that slab gels (32, 39) were used. We used autoradiography (15, 29) with ^{14}C-labeled proteins, either pulse labeled as described by Bijlenga et al. (7) or continuously labeled (H. Wunderli, Thesis, University of Basel, Basel, Switzerland, 1976).

For pulse labeling, bacteria were grown in M9 medium to a concentration of 2×10^8/ml and then infected. Lysis inhibition was used throughout. At the beginning of a pulse, 1 μCi of a ^{14}C-labeled amino acid mixture/ml (protein hydrolysate CFB 104, Radiochemical Centre, Amersham, England) was added, and at the end of the pulse it was chased by 1% (final concentration) Casamino Acids (Difco). For preparing samples, cells from 2 ml of a culture of "pregnant" cells were sedimented and resuspended in 0.4 ml of Tris buffer (0.0625 M) saturated with $CHCl_3$ to induce lysis. To this, 0.2 ml of threefold-concentrated SDS sample buffer was added. We learned that protein cleavage reactions can proceed during sedimentation and often cause a loss of some specificity by affecting structures such as polyheads and "crummy" heads which, inside a metabolizing cell, are not cleaved. Hence, in several experiments we used the alternative procedure of alcohol precipitation of infected cells (Wunderli, thesis, 1976). In this case, the infected culture was precipitated by adding two parts of ice-cold ethanol to one part of culture. The flocculate was

then sedimented and resuspended in SDS sample buffer.

For continuous labeling, the cells were grown in M9A (0.1% Casamino Acids) to 4×10^8 cells/ml and infected and superinfected as usual. At 1 min after the first infection, 5 μCi of ^{14}C-labeled amino acids mixture and a second dose of 0.1% unlabeled amino acids were added. The exposure time in autoradiography varied from 2 to 6 days according to the amount of radioactivity present.

The genetic identification of the bands is based on published work (21, 40) and many unpublished experiments by our group (L. Möller-Salamin, R. Bijlenga, H. Wunderli, R. v. d. Broek, M. Showe and V. Mesyanzhinov).

Electron microscopy and image processing. Electron microscopy and image processing of the T4D*os* giants were done by the method described by Bijlenga et al. (5).

RESULTS

Which gene controls resistance to osmotic shock? Mutants of T4 are usually assigned to genes on the basis of complementation tests. Two conditional lethal mutants are said to complement each other if a mixed infection leads to the production of progeny under restrictive conditions. In general, mutants in different genes complement each other whereas mutants in the same gene do not. Since osmotic shock resistance is a property of mature rather than vegetative phage, the concept of a complementation test must be extended somewhat in order to assign an *os* mutant to a gene. The extension is easy and obvious: host bacteria are mixedly infected with *os* and a conditional lethal mutant (e.g., *am*, *ts*) under restrictive conditions, and the progeny population is assayed for osmotic shock resistance. If *all* of the progeny are phenotypically shock resistant, there was no complementation. If the progeny show the same degree of shock resistance as the progeny from a mixed infection of *os* and wild type, there was full complementation.

One can make no a priori prediction with respect to the resistance of the progeny of a mixed infection of *os* and wild type. This must be experimentally determined. *E. coli* B was infected at a constant total multiplicity of infection of 10 but with different proportions of mutant *os*10 and wild type. The progeny population was harvested 30 min after infection and analyzed for osmotic shock resistance. In Fig. 1 the percent survival from the shock is plotted as a function of input ratios of the infecting, parental phages. The shock resistance of the progeny population is approximately proportional to the fraction of *os*10 phage in the input population.

The shock-resistant mutant *os*10 was then tested for complementation with amber mutants

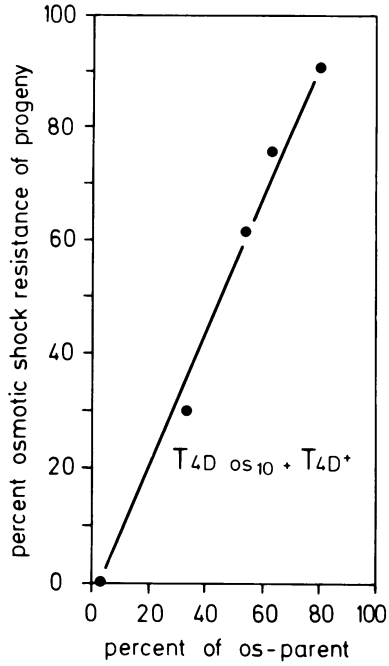

FIG. 1. *Shock resistance of the progeny resulting from mixed infections with various proportions of T4Dos10 and T4D. Five mixtures of the two phages were prepared and used to infect E. coli BB. The shock resistance of the progeny of each of the five infections was tested as described in Materials and Methods.*

TABLE 1. *Genotype and phenotype of shock resistance of parental mixtures and progeny populations of mixed infections with T4D os10 and each of five amber mutants*

Phages: *os*10 + *am*-mutant		Parents		Progeny	
Mutant	Gene	Genotype non-amber (%)	Phenotype shock resistant (%)	Genotype non-amber (%)	Phenotype shock resistant (%)
*am*B8	20	17.2	14.0	51.5	46.1
*am*N90	21	19.8	13.4	41.5	24.6
*am*B270	22	19.9	14.3	30.1	40.9
*am*H11	23	28.0	12.6	44.6	40.0
*am*N65	24	23.0	12.3	56.2	104.0

from five genes known to be concerned with the T4 head. *E. coli* B was infected (multiplicity of infection = 20) with parental phage mixtures, each containing *os*10 and an *am* mutant. Table 1 gives the osmotic shock-resistant fraction as well as the fraction am^+, both in the parental mixtures and in the progeny populations. In only one case was there a complete failure to complement: the progeny of *os*10 × 24 (*am*N65) were

fully shock resistant. All other pairs showed at least partial complementation. With input mixtures of amber mutants plus about 13% *os* mutants, the progeny exhibited shock resistance ranging from 25 to 46%.

With most amber mutants, the sensitivity to osmotic shock of the progeny of an *os* × *am* mixed infection varied with the proportion of *os* in the parental mixture. Figures 2 and 3 give the results of experiments with 22(*am*B270) and 24(*am*B26), respectively, in which the composition of the parental mixture was systematically varied. When the amber mutant was in gene 22, the curve of shock resistance closely paralleled the curve for am^+ in the progeny. When the amber mutant was in gene 24, however, the curve for shock resistance lay far above the curve for am^+ in the progeny; in fact, except for the "mixture" containing no *os* phage, essentially all of the progeny were resistant to osmotic shock.

The results presented thus far show that, if the *os* mutation lies in one of the five genes

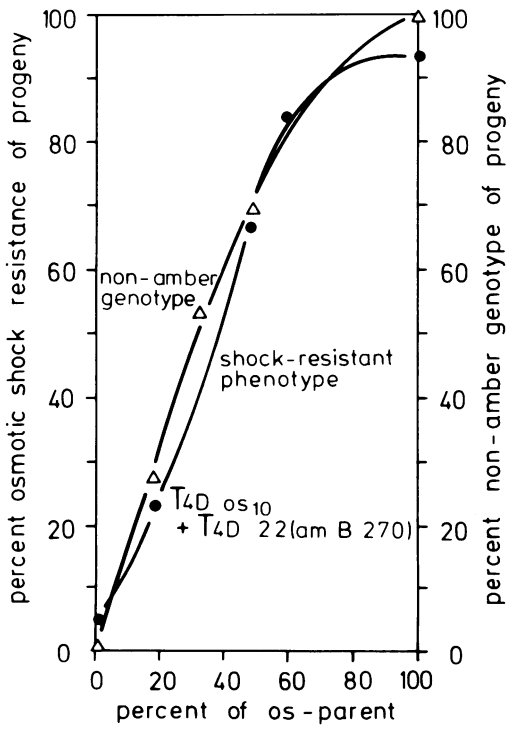

FIG. 2. *Genotype and phenotype shock resistance of the progeny resulting from mixed infections with various proportions of T4Dos10 and T4D22 (amB270). Five mixtures of the two phages were prepared and used to infect the nonpermissive host E. coli BB. Parental mixture and progeny were tested for their shock resistance (Materials and Methods) and for their genotype by plating on E. coli S/6 and CR63.*

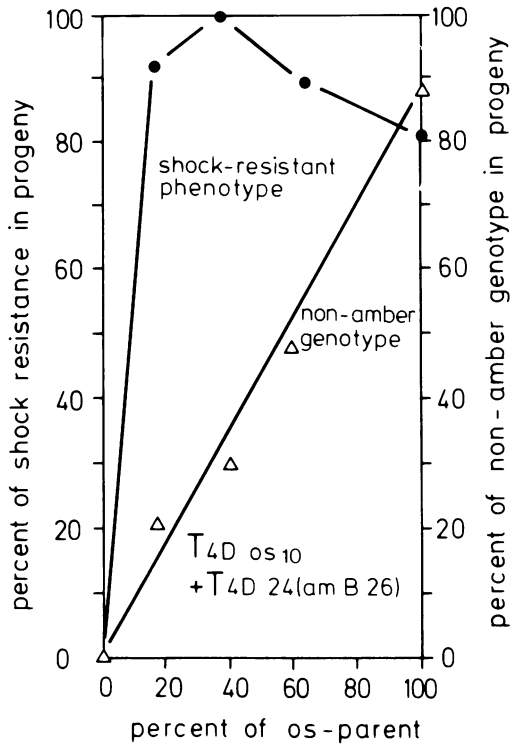

FIG. 3. *Genotype and phenotype shock resistance of the progeny resulting from mixed infections with various proportions of T4Dos10 and T4D24 (amB26). The procedure was the same as that described in Fig. 2.*

tested, then that gene must be gene 24. We considered the possibility, however, that *am*N65 is a polar mutant (38) and that *os* lies in an adjacent, as yet undefined, gene. Temperature-sensitive mutants do not exhibit polar effects in complementation tests. Figure 4 shows the results of mixed infections with *os*10 × 24(*ts*N29). Over a wide range of proportions in the parental mixture, all of the progeny were shock resistant, thus completing the formal demonstration that the *os* mutation is located in gene 24.

In the experiments presented in Table 1 and Fig. 2, 3, and 4, the proportion of am^+ (and hence of *os*) was greater in the progeny population than in the parental mixture. This result was not unexpected. As a result of random adsorption of phage to bacteria, the proportion of am^+ varied from cell to cell. If the product made by the am^+ was limiting, the phage yield would be higher for those cells infected with a higher proportion of am^+. Although we have not systematically studied the phenomenon, the apparent selective advantage of *os*10 in these experiments arose from this statistical effect.

What are the products of gene 24? Dickson

et al. (13) and Laemmli (21) gave evidence for the genetic identification of the product of gene 24, P24, and its cleavage to P24* during phage maturation.

In many gels, we and others (e.g., 40) observed for phage and phage heads a band just below P23 which we suspected to have been uncleaved P24. By using different gel concentrations, we show in Fig. 5 that this band is in part due to Pa, a nonidentified protein with abnormal migration behavior. The position of "normally" migrating proteins at a given running time is inversely proportional to the gel concentrations; this behavior can be verified by drawing straight lines connecting the three bands of a given protein obtained in three gels of increasing concentration. These lines all intersect at approximately the same point. Lines connecting bands of abnormally migrating proteins do not join this common point. In Fig. 5, one can see two such abnormally behaving proteins, Pa and Pb. Pa is superimposed with Pwac in 8% gels and is just below P23 in 10% gels, in which it could be mistaken for P24. Lysates show a much more complicated gel pattern and many other abnormally migrating proteins.

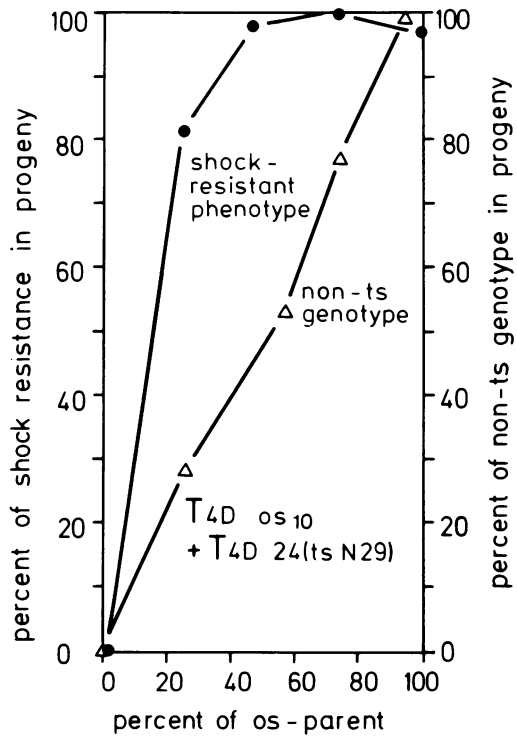

FIG. 4. *Genotype and phenotype shock resistance of the progeny resulting from mixed infections with various proportions of T4Dos10 and T4D24 (tsN29) at 40.5°C. The procedure was the same as that described in Fig. 2, except that the genotypes were determined by plating at 42°C compared to that at 30°C.*

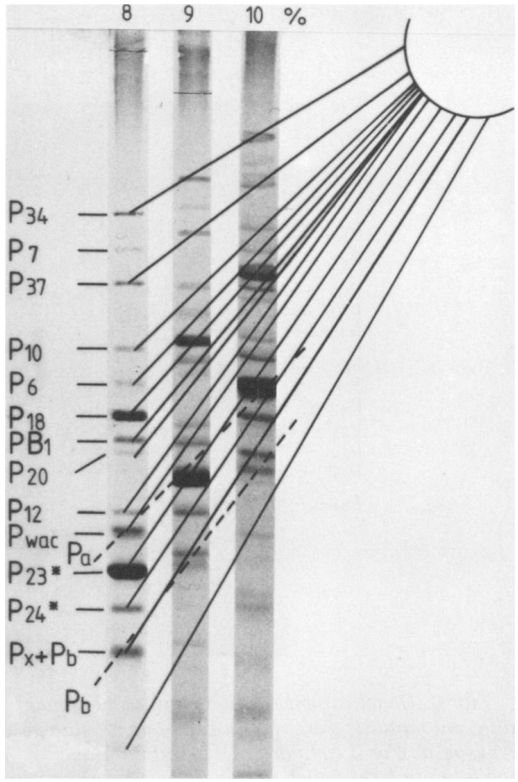

FIG. 5. *Electrophoresis of partly purified phage T4 at different gel concentrations. T4D was produced under conditions of continuous labeling starting 1 min after infection. At 90 min, 0.2 ml of a 10-fold concentrated lysate was layered on a 7 to 35% sucrose gradient in Tris buffer (0.06 M, pH 7.4). It was run on a IEC SW A321 rotor for 25 min at 20,000 rpm. Fractions were collected, and those containing a strong phage peak were dialyzed against Tris and concentrated about 10 times by centrifugation. Samples of 10 μl containing about 25,000 cpm were used for electrophoresis, with 8, 9, and 10% gels.*

In subsequent experiments, we used 8% gels for gene-product identification because, for P24-related proteins, they show the least superposition disturbances by abnormally migrating proteins. Figures 6 and 7 show gels which confirm the identification of P24. In $23^{-} \cdot 24^{-}$ a band (P24) was absent which was present in 23^{-} (Fig. 6, gels i and h, and Fig. 7, gels d and f). We found, however, that a corresponding band of 24*ts* grown at nonpermissive temperature was clearly positioned above P24, just below some P23* (Fig. 7, gels e and f and h to l). By the immune overlay technique (35) we were able to demonstrate serological cross-reaction of this

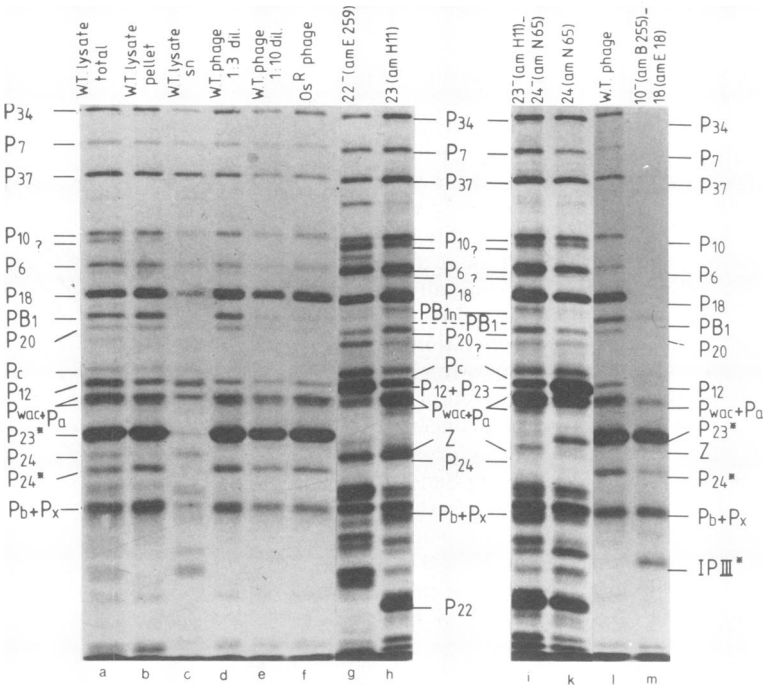

FIG. 6. *Genetic identification of protein bands in gel electrophoresis. Pulse-labeled (15 to 17 min) lysates of different mutants were run on the same 8% slab gel together with continuously labeled purified phages (wild type, os10). For Z compare gels g and i and in Fig. 7 gels b, e, and k. For a wild-type lysate, we show in gel a the total, in b the pellet, and in c the supernatant fraction of a sedimentation in an IEC SW 405 rotor during 90 min at 32,000 rpm. Note that P24 is in the supernatant fluid and while P24* is in the pellet. In purified phage (gels d, e, f, and m) and lysates (gels g and i) a very faint band Z below P23* is visible. It is positioned like P24n of Fig. 7 (gels b, e, and k). For discussion of Z and P24n, see text. The abnormally migrating proteins Pa, Pb, and Pc are those determined in Fig. 5 and 6.*

band with P24 antiserum (Fig. 7, m to p). The gene product of 24*ts* had a lower mobility than what has previously been identified as P24. Ambers $23^- \cdot 24^-$ and 23^- (Fig. 6, gels i and h) showed a faint band Z at the same position as P24n; we failed to demonstrate serological cross-reaction of these bands Z by the immune overlay technique. It is likely that another minor protein moves like P24n.

Figure 6, gels d to f, shows that purified wild-type phage and *os*10 phage are apparently identical, insofar as this sort of gel allows a comparison. P24* was positioned the same in all gels and no P24 was detectable. At the same position as band Z described above, a faint band was present in both Os^r and Os^s phages. We could not decide whether it was a 24-related protein, like P24n, or an unidentified protein (Z) banding at the same position.

We confirmed Laemmli's observation (21) of the movement of label from P24 to P24* in a lysate of wild type grown at 37°C and in the lysates of the mutant 24(*ts*L90) and of wild type after temperature shift-down from 40.5 to 30°C (gels not shown). With the 24 *ts* mutant, about half of P23 was assembled abortively into polyheads; the other half was assembled into τ particles and underwent maturation into capsids (6, 7). We also found that the turnover pattern (gels not shown) obtained in the presence of 25 μg of chloramphenicol/ml added 5 min after pulse labeling was indistinguishable from that without chloramphenicol. This indicates that all precursor proteins made before the shift are functional after the shift and that no newly synthesized proteins are needed for the maturation of the particles.

After centrifugation of a wild-type lysate at 35,000 × *g* for 90 min at room temperature, P24 was found in the supernatant fluid, and P24* was in the pellet (Fig. 6, gels a to c). This is consistent with the interpretation that most of P24 is present as a free, cytoplasmic protein not bound to precursor particles. Similar experiments with lysates of 24*ts* showed the same result: P24 is apparently not appreciably bound to precursors, i.e., τ particles.

Several other observations which are not related directly to the questions we asked should be mentioned here. (i) Proteins P(x) (5, 6) and

hoc (19) have been found to be identical (2). This protein is superimposed with Pb in 8% gels (Fig. 5) but separated into two bands in gels of higher concentrations. (ii) Just below P10 (a major base plate protein [20]), a clearly separated band is present in all lysates studied but not in phage (Fig. 6 and 7). (iii) The native respectively cleaved proteins B1n and B1 (11) were shown to be related to the alteration of the DNA-dependent RNA polymerase (17). Rohrer et al. (33) have isolated from phage the ADP-ribosylation enzyme which is responsible for the alteration by osmotic shock. In collaboration with L. Onorato, B. Dublin, and E. Kellenberger (unpublished data), we have shown by the immune replicate technique (35) that this enzyme is indeed identical to protein B1, which is part of the head (1, 21). Only part of it is released by osmotic shock, while the rest stays associated with the empty head. In a study of five amber mutants in gene 17 (N56, N150, NG332), all showed B1, confirming that this gene is not responsible for these proteins, contrary to other claims (40). We found that cleavage of B1n to B1 does not occur in those mutants where other head proteins are also not cleaved as mutants in the Y genes (governing prehead assembly), D0 mutants (no DNA). Both forms are present in the x genes (governing head maturation).

What determines shock resistance? When cells are mixedly infected with *am* and *wt* at variable proportions, the phage yield can be determined as a function of the input ratio of the parental phages. Such gene dosage experiments have shown an approximately linear response for P23 and for P24 as well (37). We have also found a very similar dosage behavior for these genes.

The results in Fig. 1 show that, in a mixed infection with Os^r and Os^s, the progeny display a shock sensitivity that is proportional to the parental ratio of Os^s/Os^r. The two most straightforward interpretations of these results are as follows: (i) A single copy, or at most very few copies, of the gene product conferring shock-resistance is sufficient to render an individual phage particle completely shock resistant. (ii) Multiple copies of the product are required to confer complete shock resistance on an individual particle. An intermediate number of copies

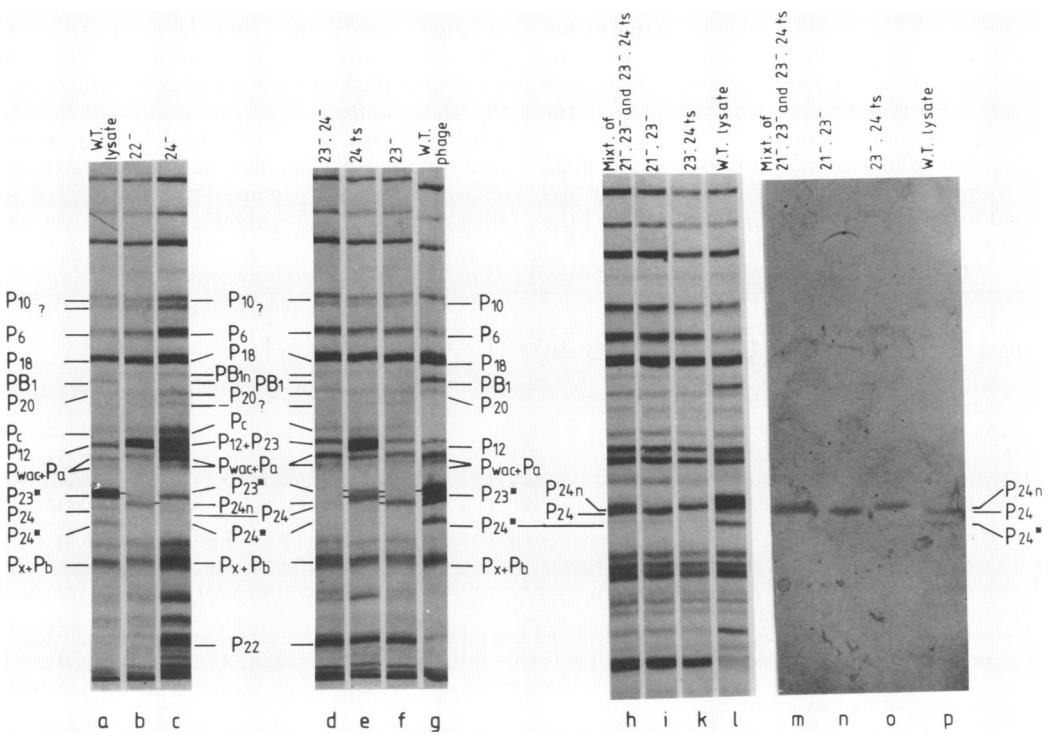

FIG. 7. *Genetic and serological identification of protein bands in gel electrophoresis. The procedures were the same as in Fig. 6 (gels a to l). On gels h and l an "immune replicate" was made and is shown in gels m to p. The temperature-sensitive mutant in 24 (gels e, h, and k) is 24 (tsL90) grown to 40.5°C. Gels a to g and h to l are each from the same slab. Note the occurrence of P24n in gels b, e, h, and k and their cross-reaction with anti P24* antiserum (replicates m and o).*

confers intermediate resistance.

Because osmotic shock resistance is a phenotypic property of phage, one test of these propositions is to subject the progeny of a mixed infection to two successive osmotic shocks. The prediction of interpretation (i) is that the survivors of the first shock will be as shock resistant as the Os^r parents. We attempted to distinguish between these propositions by subjecting the progeny of two different Os^r + Os^s mixtures to two successive osmotic shocks. Furthermore, we compared the observed percentage survival with the calculated one, assuming that interpretation (i) is correct, i.e., that one *os* product per particle confers complete shock resistance. The results of this test are shown in Table 2. The upper half of the table shows the observed percentages of survival of the parental and progeny populations of pure Os^s (wild type) and pure Os^r (shock-resistant) phages subjected to successive shocks. Approximately 5 to 10% of Os^s phage survived each shock, and approximately 75 to 100% of Os^r phage survived each shock. We then prepared two parental mixtures of Os^s + Os^r, and, using the observed responses of the pure populations, calculated the allele ratios in the parental mixes. The fact that the allele ratios calculated for each mixture after two successive shocks agree rather closely, and that the observed survivals after the second shock of the parental mixes also agree with the calculated ratios, is an indication of the accuracy of the procedure.

The test of interpretation (i) was to calculate the frequency of Os^r phages in the progeny of a mixed infection, assuming that a survivor of the first osmotic shock to the progeny is as shock resistant as an Os^r parental phage. The results in the lower half of Table 2 show that the observed response can be approximately predicted by assuming that a given phage particle in the progeny is phenotypically either completely shock sensitive or shock resistant.

Kinetics of Os^r phage assembly. Using several *ts* mutants in gene 24, Bijlenga et al. (7) showed that the P24*ts* produced at nonpermissive temperatures (40°C) becomes active after shift-down to permissive temperature (30°C). Infective phage is produced by these mutants after shift-down and even in the presence of chloramphenicol. The τ particles accumulated at nonpermissive temperatures are converted quantitatively into phage heads (6). We have utilized this phenomenon to obtain evidence concerning the possible functional differentiation of the products of gene 24 with respect to osmotic shock.

Figure 8a shows the maturation of phage after temperature shift-down and the addition of 25 μg of chloramphenicol/ml for wild type, *os*10, and 24(*ts*L90). With wild type and *os*10, about 30 phages matured within some 20 min; with 24*ts*, maturation was delayed by about 25 min, although nearly the same level of 20 to 25 phages/cell was eventually produced. This and other unpublished results lead us to believe that this delay reflects the time necessary for the inactive $P24ts^i$ to be converted into an active conformation, $P24ts^a$. The osmotic shock resistance of the phages produced was as expected: at all times the phages produced by 24*ts* were as

TABLE 2. *Resistance of parental and progeny populations to two successive osmotic shocks*[a]

Phage	Parents				Progeny (% survival)			
	1° Shock		2° Shock		1° Shock		2° Shock	
	% Survival	% Os^r	% Survival	% Os^r	Observed	Expected	Observed	Expected
Os^s	9.1	0.0	0.7	0.0	5.1	—	0.5	—
Os^r	97.4	100.0	88.3	100.0	83.8	—	72.9	—
Mix-A	45.3	(41.0)[b]	37.2	(41.7)[b]	35.1	(37.7)[c]	23.1	(30.5)[c]
Mix-B	27.4	(18.9)[b]	19.8	(21.8)[b]	18.2	(21.2)[c]	13.1	(15.3)[c]

[a] T4D wild type (Os^s) and T4D *os*10 (Os^r) alone or mixed were used to infect *E. coli* B^E at multiplicities of 5 or 10 (there were no significant differences; results were averaged), and after 8 min the complexes were superinfected at a multiplicity of 5. The progeny were havested after 90 min and assayed for shock resistance. Values in parentheses are calculated.

[b] Allele ratios in the parental mixes were calculated from their shock resistances, using the survival data on the pure parents: e.g., in Mix-A, the % Os^r is $100 \times (45.3 - 9.1)/(97.4 - 9.1)$ using the 1° shock data and $100 \times (37.2 - 0.7)/(88.3 - 0.7)$ using the 2° shock data. Comparison of these two values (41.0 and 41.7) is a measure of the accuracy of the procedure.

[c] The expected survival values of the mixed progeny population were calculated on the assumption that a "single copy" determines shock sensitivity or resistance (interpretation i, see text). The allele ratio in the parents (average of 1° and 2° values) and the survival data of the pure progeny were used in the calculation e.g., in Mix-B % survival expected after the 1° shock is $(83.8)(20.4/100) + (5.1)(1 - 20.4/100)$ and after the 2° shock is $(72.9)(20.4/100) + (0.5)(1 - 20.4/100)$. These values (21.2 and 15.3) are to be compared to their respective observed values (18.2 and 13.1), not to each other.

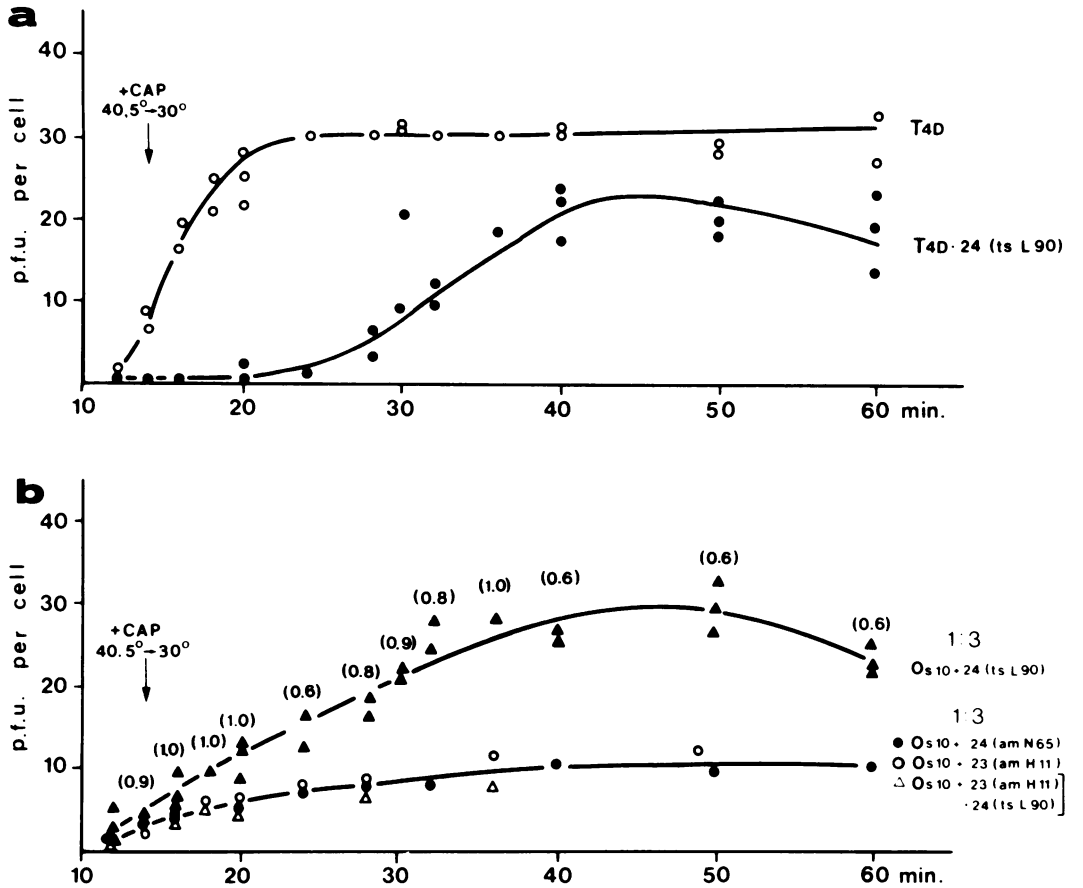

FIG. 8. *Maturable phage after temperature shift-down and in the presence of chloramphenicol. E. coli B^E grown at 40°C was infected and superinfected, each with a total multiplicity of 10. At 12 min after infection, the temperature was shifted to 30°C and 25 μg of chloramphenicol/ml was added to the cultures. Samples were taken at the times indicated and lysed by chloroform. PFU were always determined under permissive conditions. (a) kinetics of active phage maturation for wild type and 24 (tsL90) obtained from three experiments. The experimental values for T4Dos10 were very similar to those for T4D, but have been left out so as not to complicate the graph. (b) Results for mixed infections of os10 with: ▲, 24 (tsL90); ●, 24(amN65); ○, 23(amH11); or △, 23(amH11).24(tsL90). The ratio is always one os10 to three of the others. The numbers in brackets are the shock resistance (expressed as fractions) averaged from three experiments, from which the points of the curves were also calculated.*

shock sensitive as the wild type. As a control we checked these phages by shocking them at 50, 40, and 30°C; there was no difference. R. K. L. Bijlenga (personal communication) tested phages made under permissive conditions using different *ts* mutants in gene 24 and found that they are also shock sensitive at both 30 and 40°C.

Figure 8b shows the results of the same type of experiment as in Fig. 8a, but performed with mixed infections of *os*10 with 24(*ts*L90), 24-(*am*N65), 23(*am*H11), or 23(*am*H11)·24(*ts*L90), always in the ratio of 1:3. In the three combinations with ambers the yield of maturable phages was reduced to about 10/cell, a value which roughly corresponds to the expected gene dosage of 1:3. The available amounts of P23 and P24 are apparently limiting with respect to active phage production.

With *os*10 + 24*ts* (Fig. 8b) the final number of maturable particles was 25 to 30 per cell. The above results on the limitation of mature phage production by the availability of P23 and P24 suggest that the largest part of P24ts^i produced at high temperature is converted into an active form (P24ts^a) after shift-down; complete activation is achieved after some 30 min.

The time course of maturation with a mixed infection of *os*10 + 24*ts* was between those of the two parents. The behavior towards osmotic shock is interesting; the matured phages were all shock resistant (Fig. 8b, values in parentheses),

at least those harvested up to 35 to 40 min after the temperature shift. After this time a small proportion of sensitive phages appeared approximately at the same time at which an unexplained slight decrease of plaque-forming ability started. According to the experiments with *os*10 + 24*am* discussed above, the amount of P24·*os* (Os^r) available should only be sufficient to completely mature 10 phages. The maturation of the remaining 15 phages appeared to be dependent on the presence of P24*ts*. Nevertheless, these phages have the phenotype of osmotic shock resistance. These results will be discussed below.

Is osmotic shock resistance reflected in a lattice difference? Bijlenga et al. (5) investigated the giant T4 phages produced as a consequence of P24 dosage. Therefore, we examined the giant phages produced by a multiple infection by T4*os* and T4 24(*am*N65) at about equal inputs. The giants were isolated, and electron micrographs of them were processed as described by Bijlenga et al. (5). It is obvious (Fig. 9) that the lattice and the unit cell morphology are indistinct from those of "wild-type" giants.

DISCUSSION

Our first conclusion is that resistance to osmotic shock in bacteriophage T4D is determined by gene 24, confirming previously reported preliminary results (25; S. Brenner, personal communication). This conclusion is consistent with all the experiments presented. A phage carrying a conditional lethal mutation in gene 24 is not able to contribute the Os^s phenotype to the progeny obtained from mixed infection with *os*10 and ambers in different genes under nonpermissive conditions. Hence, a P24-related protein determines this phenotype either directly or indirectly.

Second, we found no detectable differences between the general chemical composition and the basic lattice structures of osmotic shock-resistant and -sensitive phages. The cleaved product of gene 24 was present in both. We found, however, that a P24*ts* has a lower electrophoretic mobility. Among the many possible reasons for different electrophoretic mobilities of SDS-denatured proteins (molecular weight, chemical modifications, and conformational differences), the most commonly encountered one reflects different lengths of the peptide chains. Perhaps the native gene product is larger than P24 and the final P24* of the virion is reached after two steps of cleavage.

Finally, our observations suggest that one or very few molecules of gene product 24 itself or one derived from it determine osmotic shock resistance.

We prefer the following simple working hypothesis to explain the production of shock-resistant phages. In a first step, a few copies of P24 interact with the phage prehead, performing some functions in the maturation pathway and/or conferring shock resistance directly to the phage. Additional P24-related proteins would be responsible for later maturation steps.

We can only speculate about the molecular mechanism of osmotic shock resistance. One possibility is that it reflects a difference in the "permeability" of the protein coat to solvent and

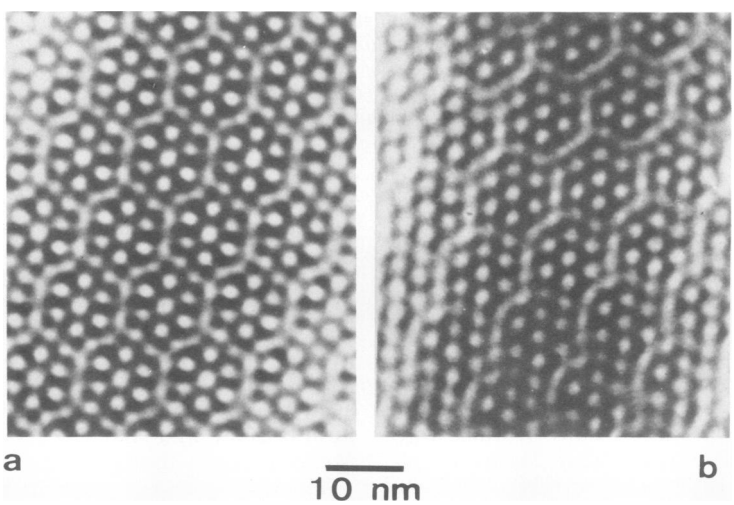

FIG. 9. *Processed electron micrographs of negatively stained (uranyl acetate) surface lattices. Normal, "wt" giants (a) and Os 10 giants (b) were produced by gene dosage with 24am. After optical diffraction and filtering, the average was further enhanced by lateral displacement and a symmetrization according to a two-fold axis. (The wild-type giant is a courtesy of U. Aebi and R. Bijlenga [5].)*

solute flux. This latter suggestion follows by analogy with the osmotic response of cells. That is, both T4 phages and human erythrocytes withstand a rapid dilution from 1.5 M NaCl into isotonic saline, but more than 90% of both phage and cells are destroyed by rapid dilution from 3.0 M NaCl (26, 27). In the case of cells, the explanation seems clear. When suspended in solutions above some critical concentration, the cells take up solute. Upon rapid dilution, solvent enters the cells more rapidly than the solute can leave, the cells swell in an attempt to achieve osmotic equilibrium, and the cells lyse at some critical volume. Phages may behave similarly. The osmotic responses of cells reflect the well-known semipermeability properties of the membrane. Whether the protein shell of the phage head functions in a similar fashion is unknown. However, observations that are consistent with this interpretation include the behavior of *os* phages during CsCl gradient centrifugation (24, 30), the replacement of internal proteins of *os* phages by inorganic cations (3), and the different "permeability" of *os* and *wt* phages to methylene blue (12).

The question relevant to phage structure is: are the "pores" which determine the permeability distributed regularly over the surface lattice of the capsid, or are they confined to particular points, such as the vertices or the attachment site of the tail? If all the pores are affected, then one must assume a long-range, cooperative effect of P24* because, as we have seen, only a few molecules seem to determine the Os phenotype. We have demonstrated in this paper that the surface lattice of both Os^r and Os^s giants show hoc and soc, proteins previously identified by Ishii and Yanagida (19) and Aebi et al. (2). Thus, it is unlikely that these minor proteins play a role as "pore-fillers," particularly since we also know that osmotic shock-resistant phages can be isolated from T2 phage, in which these minor proteins are absent. The second possibility (that the pore size at the vertices is determinant) is supported by the finding that P24* is indeed located there (31).

Our hypothesis that a few P24-related protein molecules in the maturing particle confer osmotic shock resistance underlines the rather exceptional multifunctional role of this gene:

(i) An additional amount of P24-related proteins is needed in order to proceed in the maturation pathway.

(ii) Except for mutants in gene 21, responsible for the T4ppase which also cleaves P24 (35), only the mutants in gene 24 lead to an accumulation of membrane-attached preheads. All other head-related particles are free in the cytoplasm (41, 42). It thus appears likely that a P24-related protein is responsible for the detachment of the prehead from the membrane.

(iii) By gene dosage experiments with P24, one obtains an increased amount of giant heads (5), suggesting also a form-giving (morphopoietic) role of gene 24. On the other hand, McNicol et al. (28) have reported the isolation of a mutant in which the requirement for P24 for capsid morphogenesis is bypassed.

Since a particle which is morphologically indistinct from the prehead can now be assembled in vitro (R. van Driel and E. Couture, submitted for publication), we hope that further such studies will enable us to understand the mechanisms of some of the above-enumerated gene 24-related functions.

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