Journal of Supramolecular Structure 2:337–348 (1974)

PHAGE HEAD SIZE DETERMINATION AND HEAD PROTEIN CLEAVAGE IN VITRO

Gail Pruss, Kathleen Barrett, Judith Lengyel*, Richard Goldsteint, and Richard Calendar

Department of Molecular Biology, University of California, Berkeley, California 94720

Satellite phage P4 causes the head proteins of a helper phage, such as P2, to form a small head. This small head is never found in cells infected by the helper virus alone. This finding, coupled with the dominance of P4 over its helper, indicates that the P4 genome has the potential for specific head size determination. Satellite phage P4 codes for a late protein which is found in the P4 head (45 copies/head). This protein may determine head size. Our finding that the small size of P4 DNA does not determine small head size in an in vitro DNA packaging system lends further support to the idea that a P4 protein determines small head size.

Formation of P2 headlike structures is accompanied by cleavage of P2 head proteins. Cleavage of the major head protein precursor can be observed in vitro after lysis of infected cells with lysozyme. The rate of this in vitro reaction is not affected by deoxyribonuclease; thus there cannot be a tight coupling between DNA packaging and the cleavage of the major capsid protein.

INTRODUCTION

Satellite phage P4 is an unusual virus which requires a helper for successful lytic multiplication (1). When this phage infects *E. coli* lysogenic for a helper such as phage P2, the cells lyse and release 100 P4 satellite phage, but no helper phage (2). Satellite phage P4 can also infect nonlysogenic strains, but no progeny phage are produced (Fig. 1, upper panels). The helper phage genes which are needed by satellite phage P4

*Present address: Biology Division, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139.

†For reprint requests – present address: Department of Microbiology and Molecular Genetics, Harvard Medical School, 25 Shattuck Street, Boston, Massachusetts 02115.

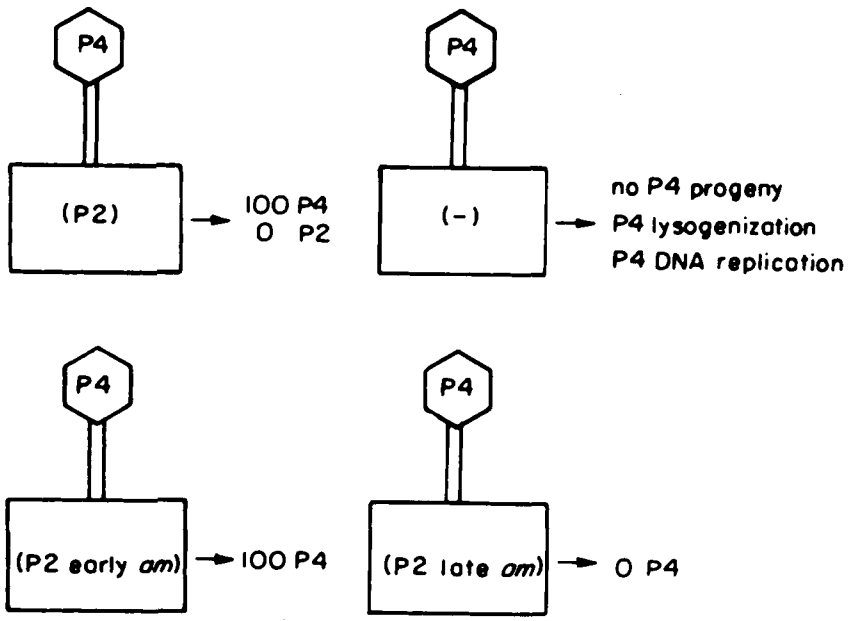

Fig. 1. Results of satellite phage P4 infection of various strains. Cells were grown in broth, and phage were added at a multiplicity of infection between 1 and 10. Details appear in Ref. 2, and E. Six, (in preparation). Lindqvist and Six (3) describe P4 DNA replication in nonlysogenic cells.

can be defined using conditional-lethal P2 mutants as prophage helpers under non-permissive conditions (E. Six, in preparation). As shown in the lower panels of Fig. 1, early mutants of P2 phage are competent in providing help to satellite phage P4. In contrast, when late mutant P2 helpers are employed, satellite phage P4 progeny are not produced. Therefore, P4 relies on all the head and tail genes of the prophage helper in order to synthesize progeny particles. One of the required helper genes codes for the P2 major head protein; thus the major head proteins of P2 and P4 should be the same. As expected from this genetic data, satellite and helper phages appear similar under the electron microscope (Fig. 2). However, the satellite phage P4 head (d = 450 Å) is smaller than the head of its helper (d = 620 Å) and contains a smaller genome (MW = 7×10^6 daltons in contrast to 22×10^6 daltons for the P2 helper genome (4, 5). The P2 and P4 genomes are not homologous, since they show less than 1% cross-hybridization (B. Lindqvist, Proc. Nat. Acad. Sci., in press). Heads of the small P4 size are not found in cells infected by the helper phage P2 alone (6). Conversely, P4-infected, P2-lysogenic cells contain no heads of the large P2 size (Fig. 3). Furthermore, in cells coinfected with both P2 and P4, P4 is dominant under some conditions (K. Barrett and M. Marsh, unpublished experiments). These facts suggest that P4 possesses the potential for head size determination.

The P2-P4 system provides an unusual opportunity to investigate head size determination, since these two phages make different sized heads from essentially the same

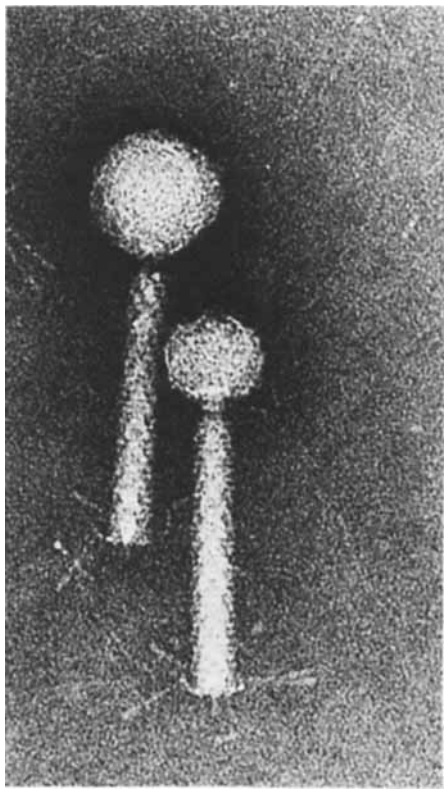

Fig. 2. Temperate phage P2 (left) and satellite phage P4 (right), negatively stained with phosphotungstate. The tail structures are about 135 nm long. The P2 head is 620 Å in diameter, while the P4 head has a diameter of 450 Å. Electron micrograph provided by Robley C. Williams, University of California, Berkeley.

proteins. We have been able to obtain packaging of P4 and P2 DNA in extracts from phage-infected cells, and to observe cleavage of the major head protein precursor in vitro. This allows us to investigate the pathway by which cleavage and packaging occur, as well as the relationship between the two processes. This article is a brief summary of recent studies on P2 and P4 head synthesis. A more extensive review of P2 and P4 morphogenesis will also appear (8).

METHODS

The DNA packaging experiments are all based on the same design (9): Cells infected with P2 amber mutant or P4 amber mutant phages are concentrated, lysed, and then sonicated briefly to reduce viscosity. The whole extract thus obtained may be used

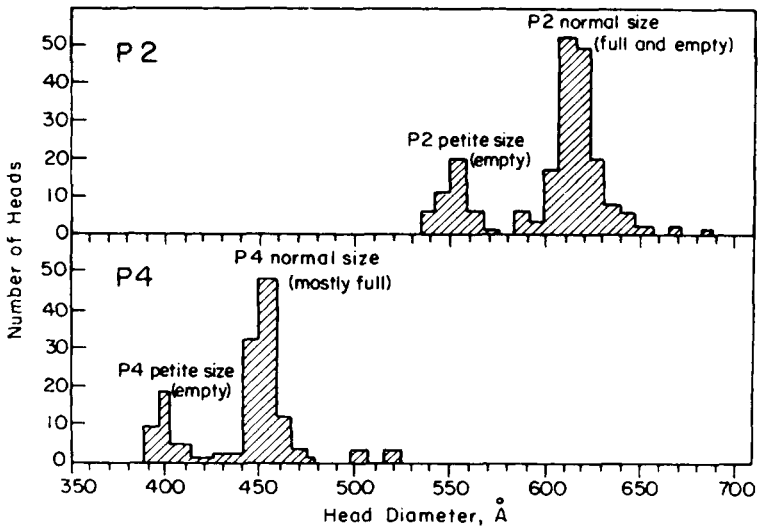

Fig. 3. Histograms showing the size distribution of heads isolated from P2- and P4-infected cells. The number of particles of a specific head diameter are plotted vs. that specific head dimater. After lysis of infected cells, a head- and phage-containing fraction was obtained by differential centrifugation. Particles were observed in negative stain under the electron microscope, and head diameters were measured. Adapted from Ref. 6 with permission of Virology 53:24 © Academic Press.

in the packaging reaction directly or may first be separated into supernatant and pellet fractions. The packaging reaction consists of whole or supernatant extract incubated with ATP and P2 virulent DNA or P4 DNA. After stopping the reaction with deoxyribonuclease, incorporation of the exogenous DNA into viable phage can be determined by plating the reaction mixture on a P2-lysogenic indicator which does not carry an amber suppressor. Such an indicator strain is not sensitive to the infecting phage, but is sensitive to phage derived from the exogenously added DNA. In the packaging reaction, exogenously added DNA can be packaged into viable phage with efficiencies between 4 and 400 $\times$ 10^{-6} PFU/DNA molecule (10).* Further details of the DNA packaging procedure appear in Ref. 10.

Other methods are described in figure and table legends.

RESULTS AND DISCUSSION

In Vitro DNA Packaging

Head size might be determined by the genome size, by interactions between proteins, or by a combination of the two. In order to distinguish between these possibilities, we have employed an in vitro DNA packaging system similar to that described by Kaiser and Masuda (9).

We suspected that P2-infected cell extracts might package satellite phage P4 DNA,

*PFU = plaque-forming units.

TABLE I. In Vitro Packaging of P2 and P4 DNA*

Infected extract	PFU/DNA molecule $\times 10^6$ P2 DNA	P4 DNA
E. coli + P2 	3.6	0.9
E. coli (P2) + P4 	0.02	5.9

*Packaging of P2 virulent DNA and P4 DNA in extracts of P2-infected cells or P4-infected, P2-lysogenic cells. The procedure is outlined in Methods section; a detailed procedure appears in Ref. 10.

since the cohesive ends of P2 and P4 DNA are very similar (11) and provide a logical recognition site for the initiation of DNA packaging. The P2-P4 system, then, might provide a means to study the requirements for formation of the P4-sized head. Our first goal was to test whether P4 DNA could determine small head size in extracts of P2-infected cells which contain only large heads and free protein (6). A second goal was to test whether P2 DNA can determine large head size in extracts of P4-infected P2-lysogenic cells which contain only small heads and free protein (6). The data in Table I show that extracts of P4-infected P2-lysogenic cells package the large DNA of P2 very poorly, as though small head size had been predetermined by P4 gene products. Extracts of P2-infected cells, which contain large heads and free head proteins, can package both the large DNA of P2 and the small DNA of P4 equally well. It might seem that the small P4 genome had caused small heads to form in an extract of P2-infected cells, but this is not the case. The small DNA of P4 is packaged in P2-infected cell extracts into phage particles whose density is much greater than that of P4. Figure 4 shows that these P4 phage band in three peaks in a CsCl equilibrium density gradient, with 90% of the material at the density of P2, 10% at a density intermediate between P2 and P4, and 1% somewhat lighter than P4. We believe that these phage have P2-sized heads and contain 3, 2, and 1 copies of the P4 genome, respectively. Although not enough pure product is available to do meaningful electron microscopy, this contention is supported by the kinetics of phage inactivation by ultraviolet light (Fig. 5). The heavy phage containing P4 DNA show an initial shoulder, followed by a first order inactivation which can be extrapolated back to three hits per PFU. The intermediate density phage exhibit a two-hit inactivation curve, while the light phage exhibit an ordinary one-hit inactivation curve. Thus, the small DNA of P4 was not sufficient to determine a small head size in extracts of P2-infected cells. In fact, the simplest conclusion is that head size determines genome length, even in the P2-P4 system, where the phage genomes have unique (nonpermuted) sequences and fixed ends (4,5).

We believe that a special P4 protein determines small head size, since we have found a protein in the P4 head which is not in the P2 head (Fig. 6; Barrett and Calendar, in preparation). This protein can be detected in cells infected by P4 alone; thus it must be

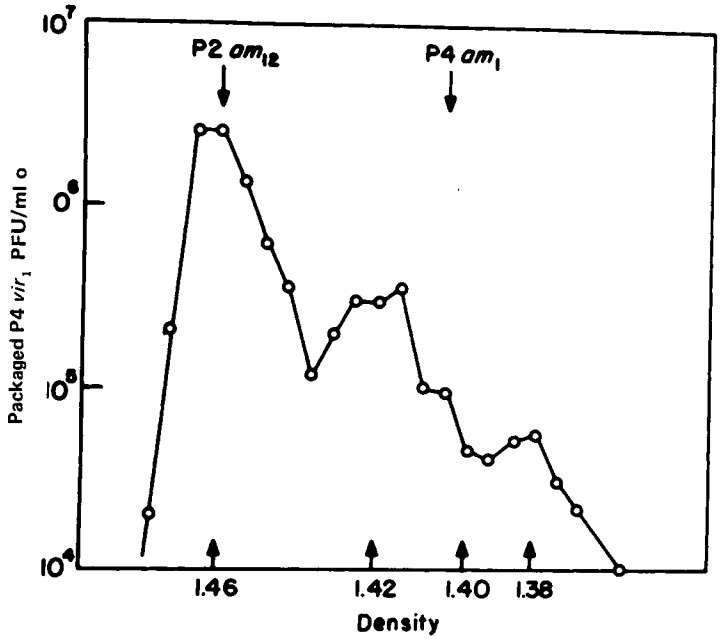

Fig. 4. Density analysis of P4 plaque-forming units packaged in a whole extract prepared from P2-infected *E. coli*. The procedures for packaging and equilibrium sedimentation in a CsCl density gradient are described in Ref. 10.

a P4 gene product. About forty-five copies of this protein are found in each P4 head. There are only a few ways to arrange this number of copies in T = 3 or 4 structure which is thought to represent the P4 head (8).

Figure 7 shows one model for the placement of the putative P4 size-determining protein. The human heads represent the major capsid protein of P2 and P4, while the hippopotamus heads represent the special satellite phage protein, which lies at the center of each hexamer and pentamer. This protein might determine small head size by changing bond angles between the protein subunits of the hexamers, so that the bond angles between the hexamers become more acute. We contend that protein determines the small head size of satellite phage P4, although more genetic and biochemical experiments are needed to prove this. One useful approach would be to study the synthesis of P2 and P4 heads and the cleavage of the capsid proteins to see at what level head size determination occurs.

Capsid Protein Cleavage In Vitro

The major capsid protein (N*) and two minor proteins (h_1 and h_2) of the P2 head, which are also present in the P4 head, are derived from the P2 N gene product by cleavage

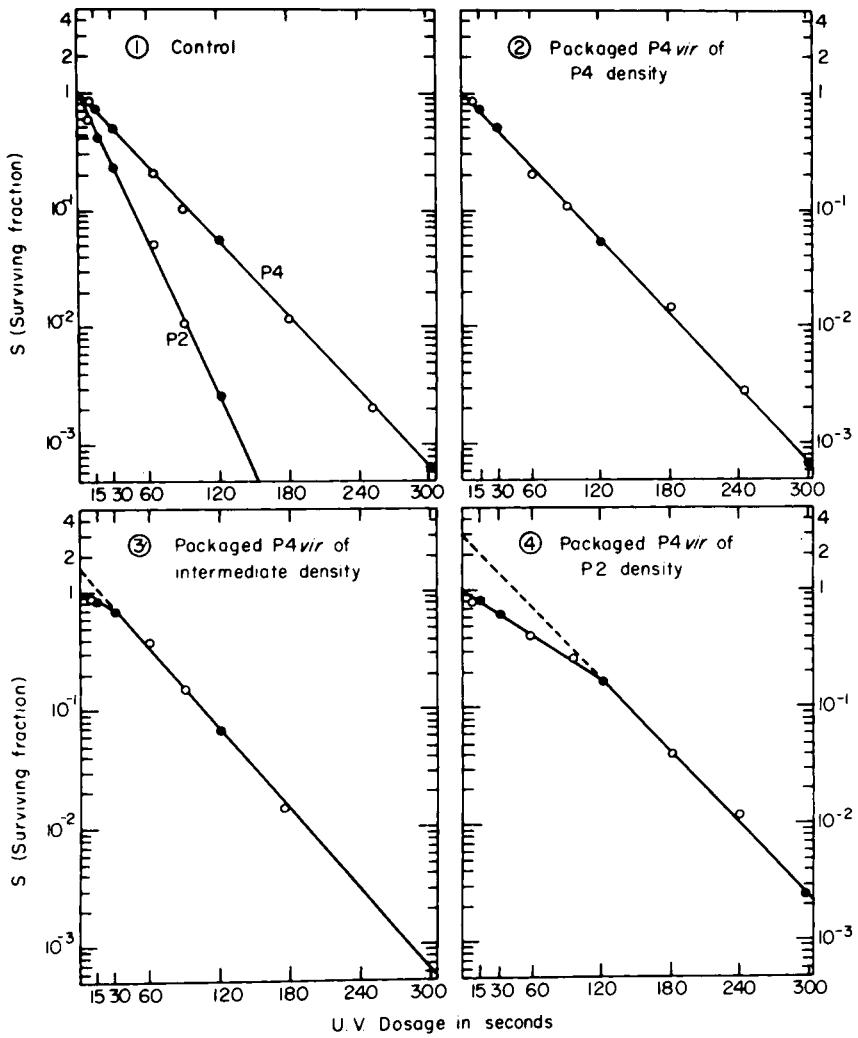

Fig. 5. Ultraviolet light inactivation of the three density classes of P4 plaque-forming units packaged in an extract of P2-infected cells. Fractions from each of the three peaks of P4 in the CsCl gradient of Fig. 4 were pooled, diluted, exposed to ultraviolet light for varying periods of time, and assayed for surviving plaque-forming units (10).

(13). The evidence for this cleavage comes from two sources: analysis of the proteins produced by phage amber mutants and the kinetics of appearance of the proteins N, N*, h_1 and h_2. The amber mutant P2 amN_{209} does not synthesize any of the four proteins N, h_1, h_2, or N* under nonpermissive conditions. Of these four proteins, only N is labeled during a pulse of radioactive amino acids. After a "chase" with nonradioactive amino

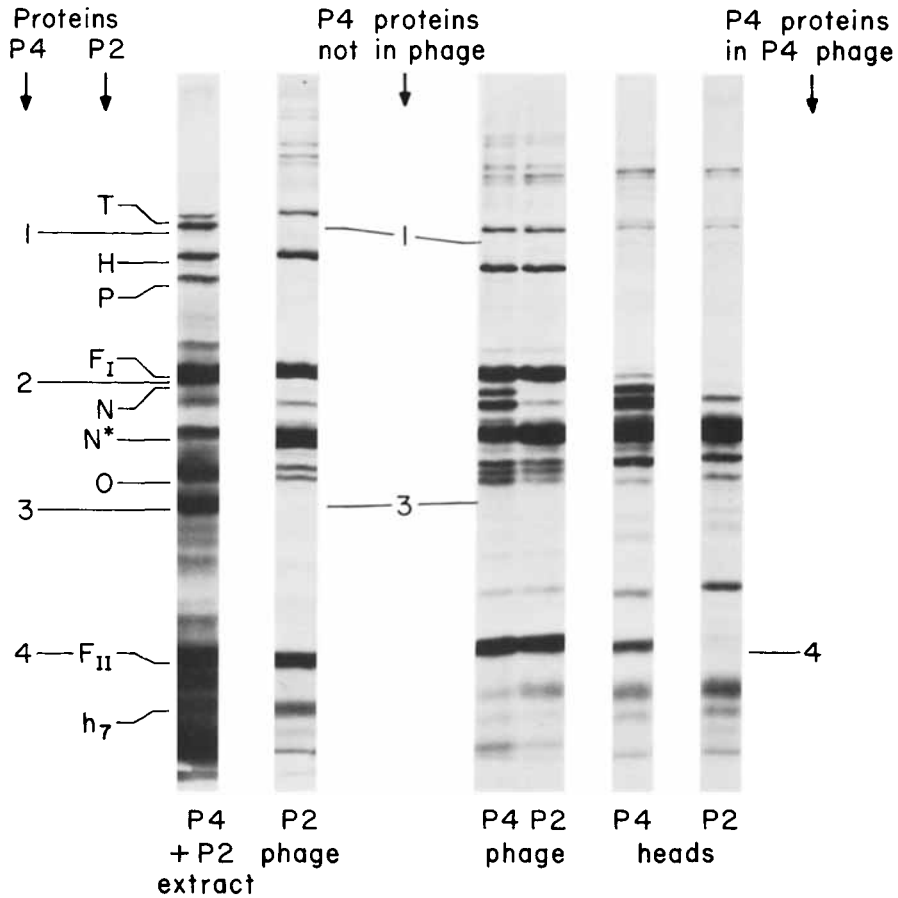

Fig. 6. Analysis of proteins from the P2 and P4 phage particles. *E. coli* cells treated with ultraviolet light to reduce host protein synthesis were infected by P2 and P4, labeled with radioactive amino acids, and lysed by boiling in 2% sodium dodecyl sulfate. The labeled extract was electrophoresed on 10% acrylamide gels (12) alongside labeled phage and phage heads purified by CsCl gradient centrifugation (Barrett and Calendar, in preparation). The letters represent P2 phage proteins identified by Lengyel et al. (13, 14), while the numbers refer to satellite phage P4 proteins defined by Barrett and Calendar. The autoradiogram shown is overexposed to emphasize minor proteins.

acids, N disappears, while h_1, h_2, and N* appear. A tentative pathway for the cleavages is presented in Fig. 8. We do not yet know whether proteins h_1 and h_2 are intermediates in the N to N* conversion, end products of N cleavage, or fusion products similar to those described for phage λ by R. Hendrix (this issue). The final elucidation of these relationships depends on peptide mapping analysis.

The product of the P2 O gene is also cleaved, but to low molecular weight material which has not been detected on gel electrophoresis. The O and N protein cleavages are

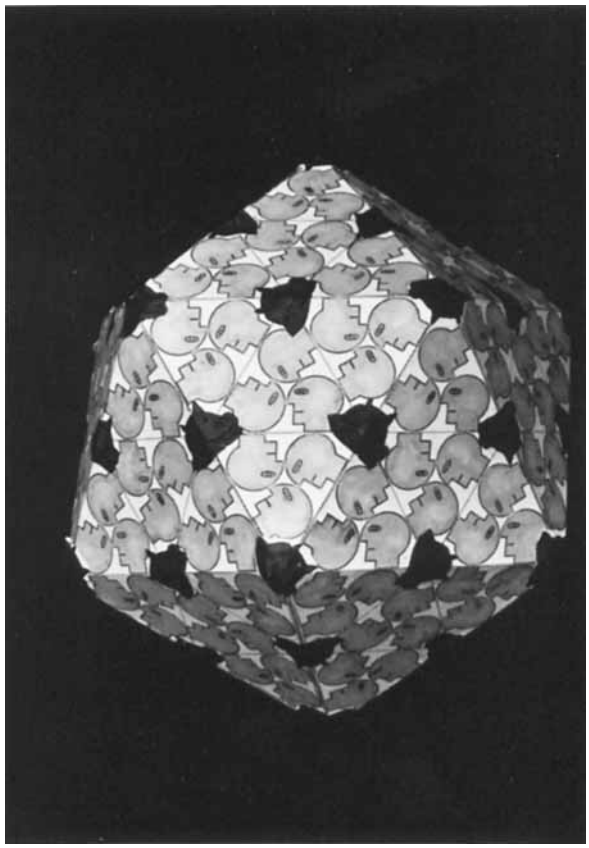

Fig. 7. A model for placement of the putative P4 size-determining protein. The human heads represent the major capsid protein, while the hippopotamus heads represent P4 protein 4.

	$N \longrightarrow$	$h_1 \longrightarrow$	$h_2 \longrightarrow$	N^*
MW in thousands	44.3	42.2	40.7	36.1

Fig. 8. Hypothetical cleavage pathway.

interdependent, as shown by the failure of either protein to be cleaved if the other protein is absent due to an amber mutation, or defective due to a ts mutation. This fact, coupled with the approximately equimolar synthesis of the O and N proteins, suggests that these proteins associate into a 1:1 complex before cleavage occurs. Whether this complex is a headlike structure or consists merely of a few molecules each of the O and N proteins is a question which remains to be answered.

We do not yet know what causes cleavage. Mutations in P2 head genes other than O and N do not affect cleavage. Cells infected with mutants in these other genes accumulate empty headlike structures which must contain cleaved N protein. The cleavage activity could be due to: a) a phage gene not yet defined by mutation, b) a host enzyme, or c) autocatalytic activity of the associated O and N proteins.

Cleavage of the N protein may be involved in head size determination, since the two minor cleavage products h_1 and h_2 are present in different amounts in P2 and P4 heads (8, 15). There are about 4 and 10 copies of h_1 and h_2, respectively, in the P2 head, while the P4 head contains about 20 molecules of h_1 and 50 molecules of h_2.

In order to assess the nature of the presumed O:N precleavage complex and the source of the cleavage activity, we have studied cleavage of the N and O proteins in vitro in a cell extract similar to that used for in vitro DNA packaging. P2-infected cells are pulse-labeled with ^{35}S-methionine, concentrated 100-fold, and lysed by freezing and thawing in the presence of lysozyme-EDTA. Lysis is 98–99% complete as determined by plating for colony-formers and by observation under a phase contrast microscope. The cleavage of N to N* observed in this extract (Fig. 9) occurs at the same rate and to half the extent found in vivo. The reaction is complete by 25 min at 37°, at which time about 40% of the N protein is cleaved to N*.

Some requirements for cleavage in vitro are shown in Table II. Magnesium ions stimulate the reaction slightly, but ATP is not required. The cleavage is not inhibited by DNase; thus, cleavage is not coupled obligatorily to DNA packaging.

Sonication of the extract releases about 65% of the N protein and half of the cleavage activity into a low speed supernatant fraction (5000 rpm for 15 min in a Sorvall SS-34

TABLE II. Requirements for In Vitro Cleavage

Components Added	% Cleavage
Complete	43
Complete minus $MgCl_2$	37
Complete plus ATP (5 mM)	41
Complete plus DNase (40 μg/ml)	49

Infected cell extracts were prepared as described in Fig. 9 except that sonication was omitted. An aliquot of extract was removed before the addition of $MgCl_2$ and DTT in order to test the effect of Mg^{2+} on cleavage. The complete reaction consists of extract of 2.2×10^9 bacteria containing 10 mM $MgCl_2$ and 0.1 mM DTT in a final volume of 50μl. The reaction mixtures were incubated and analyzed as described in Fig. 9. Percent cleavage was calculated as $(N^*/N + N^*) \times 100$. The amount of N cleavage in an unincubated extract (6%) was subtracted from the values for the incubated extracts to obtain a value for the net cleavage of the N protein which occurred during incubation.

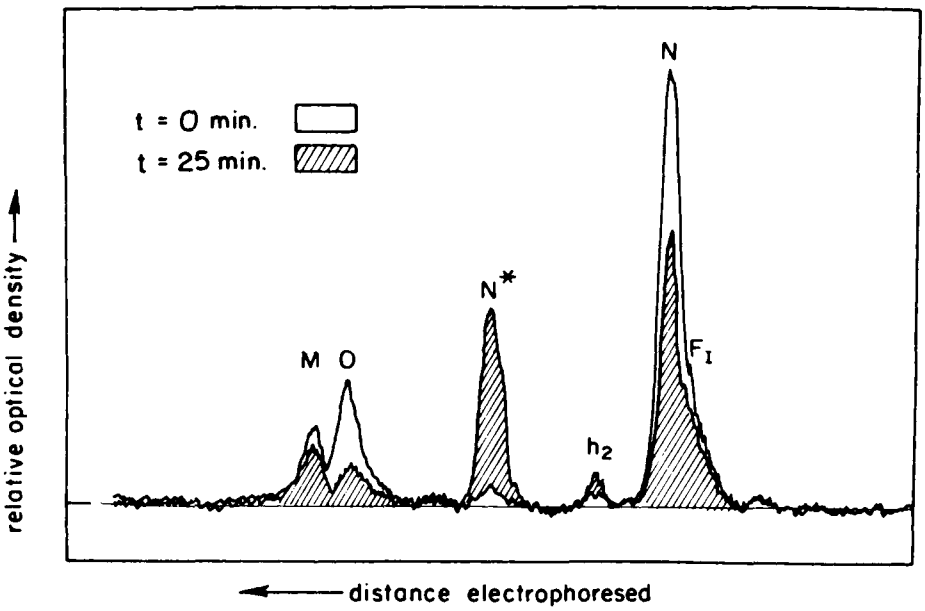

Fig. 9. In vitro capsid protein cleavage. Strains, media, and buffers are described in Pruss et al. (10). *E. coli* C-la was grown at 37°, with aeration, to a density of 5 X 10^8 per ml in Super TPG medium containing 2.5 μg/ml methionine. The cells were then concentrated 10-fold in KAB and mixed with the lysis-defective phage, P2vir_1 am_{12}, at a multiplicity of infection of about 10. (KAB is 100 mM NaCl, 10 mM Tris pH 7.4, 5 mM $CaCl_2$). Cells plus phage were incubated for 10 min at 0° to allow adsorption, followed by 5 min at 37° (without aeration) to allow injection. At the end of this time (defined t = 0), the infected cells were diluted 10-fold in Super TPG medium lacking methionine and allowed to continue growing. At 20 min the cells were pulse labeled by the addition of ^{35}S-met (0.5 μg/ml; 50 Ci/M); the pulse was terminated at 22 min by pouring the culture onto a 500-fold excess of unlabeled methionine layered on top of frozen basal growth medium. The cells were then harvested by centrifugation and resuspended in 1/100th volume of lysis buffer containing 2.0 mg/ml lysozyme. The cells were lysed by freezing and thawing at 37° two times. 13 mM $MgCl_2$ and 0.13 mM DTT were added to this extract which was then sonicated briefly to reduce viscosity. The in vitro cleavage reaction consisted of 50 μl of extract incubated at 37° for 25 min. The reactions were stopped by the addition of 150 μl of electrophoresis sample buffer. All samples were boiled for 2 min and centrifuged at 17,500 X g for 15 min to remove insoluble material. They were then electrophoresed on an 8% slab gel, autoradiographed, and scanned with a double beam recording microdensitometer (Joyce, Loebl and Company, Ltd., model MK III C). The profile obtained after 25 min incubation is cross-hatched in order to distinguish it from the profile obtained at zero time.

rotor). We are presently attempting to isolate the precleavage complex from this supernatant in order to study cleavage in a more defined system.*

Using the in vitro packaging and cleavage system, it should be possible to analyze the action of specific gene products in P2 and P4 head assembly. We hope that one of the results of this study will be an increased understanding of the mechanism of head size determination.

ACKNOWLEDGMENTS

This research was supported by grants AI 08722, CA 02245 and GM 01389 from the U.S. National Institutes of Health. R. Goldstein was the recipient of an American Cancer Society Postdoctoral Fellowship (PF-663).

REFERENCES

1. Six, E. W., Bacteriol. Proc. 138 (1963).
2. Six, E. W., and Klug, C., Virology 51:327 (1973).
3. Lindqvist, B. H., and Six, E. W., Virology 43:1 (1971).
4. Inman, R. B., Schnös, M., Simon, L. D., Six, E. W., and Walker, D. H., Virology 44:67 (1971).
5. Inman, R. B., and Bertani, G., J. Mol. Biol. 44:533 (1969).
6. Gibbs, W., Goldstein, R. N., Wiener, R. W., Lindqvist, B., and Calendar, R., Virology 53:24 (1973).
7. Six, E. W., and Lindqvist, B. H., Virology 43:8 (1971).
8. Goldstein, R. N., Lengyel, J. A., Pruss, G., Barrett, K., Calendar, R., and Six, E., "Current Topics in Microbiology and Immunology," in press, Vol. 68
9. Kaiser, A. D., and Masuda, T., Proc. Nat. Acad. Sci. U.S. 70:260 (1973).
10. Pruss, G., Goldstein, R. N., and Calendar, R., Proc. Nat. Acad. Sci. U.S. 71:2367 (1974).
11. Wang, J. C., Martin, K. V., and Calendar, R., Biochemistry 12:2119 (1973).
12. Laemmli, U. K., Nature 227:680 (1970).
13. Lengyel, J. A., Goldstein, R. N., Marsh, M., Sunshine, M. G., and Calendar, R., Virology 53:1 (1973).
14. Lengyel, J. A., Goldstein, R. N., Marsh, M., and Calendar, R., submitted to Virology (1974).
15. Lengyel, J. A., Ph.D Thesis, University of California, Berkeley (1972).
16. Studier, F. W., J. Mol. Biol. 79:237 (1973).

*Both M. Showe and U. Laemmli (this issue) have proposed that in phage T4, cleavage in vitro occurs spontaneously in a headlike structure. Recent in vitro experiments of Murray Woolf, working with P2 phage are consistent with this model.