Proc. Natl. Acad. Sci. USA
Vol. 78, No. 3, pp. 1717–1721, March 1981
Cell Biology

A mutation downstream from the signal peptidase cleavage site affects cleavage but not membrane insertion of phage coat protein

(filamentous phage/phage assembly)

MARJORIE RUSSEL AND PETER MODEL

The Rockefeller University, New York, New York 10021

Communicated by Norton D. Zinder, November 24, 1980

ABSTRACT **Morphogenesis of filamentous phage includes synthesis of the phage major coat protein in precursor form, its insertion into the host cell plasma membrane, its cleavage to the mature form of the protein, and its assembly there into virions. The M13 mutant am8H1R6 encodes a coat protein in which leucine replaces glutamic acid as residue 2 of the mature protein [Boeke, J. D., Russel, M. & Model, P. (1980) *J. Mol. Biol.* 144, 103–116]. The coat protein precursor produced by this variant is a poor substrate for the *Escherichia coli* signal peptidase both *in vivo* and *in vitro*. This pre-coat protein, which is eventually processed and assembled into viable phage particles, is associated with the membrane fraction of the infected cell. We conclude that the domain recognized by the signal peptidase extends beyond the signal peptide itself. Furthermore, membrane association and signal peptide cleavage can be separated temporally under conditions that permit membrane insertion, cleavage, and phage assembly.**

Under normal conditions the closely related filamentous bacteriophages f1 and M13 grow on their *Escherichia coli* host without causing cell death (1, 2). Phage morphogenesis involves synthesis of large amounts of major coat protein in precursor form (3, 4), insertion of that precursor protein into the cytoplasmic membrane (5), and concomitant cleavage of a 23-amino-acid peptide from the NH_2 terminus of the precursor to form the mature protein (6, 7). These steps appear to mimic the biosynthesis of *E. coli* membrane proteins, and the phage coat protein has many of the characteristics of a typical host membrane protein (8–10). Subsequently phage particles are formed via a poorly understood morphogenetic pathway, which requires the participation of other phage gene products (11). Phage particles are extruded from the host (as they are formed) without causing lysis. The major coat protein of the filamentous phages is, on a molar basis, the predominant protein synthesized in infected cells. This system has been used in order to gain a better understanding of the biosynthesis and route of compartmentalization of prokaryotic membrane proteins.

On the basis of experiments *in vitro*, Chang *et al.* (6, 7) have concluded that formation of the mature filamentous phage coat protein involves cotranslational insertion of the coat protein precursor into membrane vesicles rapidly followed by cleavage of the precursor to the mature form. On the basis of rather similar experiments *in vitro* (12, 13) and subsequent work *in vivo* (14–16), Wickner and coworkers have concluded that pre-coat protein is synthesized on free ribosomes, that the precursor is discharged into the cytoplasmic milieu, and that it subsequently finds its way to the membrane, with proteolytic cleavage again a consequence of membrane insertion.

In consequence of these experimental findings, the two groups have postulated different functions for the precursor portion of the molecule. Chang *et al.* (6, 7) propose that the precursor amino acid sequence functions as a "signal" in the scheme proposed by Blobel and Dobberstein (17), and that the function of the signal is to facilitate the attachment of the nascent chain to the cell membrane (presumably via a receptor) and to differentiate by this means proteins destined for other cell compartments from those that will remain in the cytoplasm. Wickner (18) has proposed that the function of the precursor part of the molecule is to increase the solubility of the protein and to permit protein folding into the membrane without requirement for a receptor-mediated translocation step.

Earlier work (14, 15) together with experiments to be described below shows that newly synthesized coat protein in wild-type-infected cells associates with the cell membrane so rapidly and is cleaved so swiftly that experimental analysis is very difficult. However, bacteria infected with any of several mutant phage process coat protein slowly (14, 15). What these mutants have in common is that phage morphogenesis and extrusion is blocked, while synthesis of the coat protein continues at its normal rate. Cells do not survive infection with such mutant phage, but are rapidly killed, with symptoms that suggest permeabilization of the cell envelope and leakage of cell contents (19). Ultrastructural studies show gross membrane hyperplasia (20) and a number of other changes in cell structure (21). The phospholipid composition of membranes isolated from such mutant-infected cells is abnormal, showing a pronounced elevation of cardiolipin and a depletion of phosphoserine and phosphoethanolamine (22). Spheroplasts formed from such cells are unusually fragile and cannot be isolated under usual conditions. That these changes are due to accumulation of coat protein is shown by infection with gene *VIII* amber mutant phage, which do not make coat protein and do not exhibit these membrane changes (22, 23).

Mutations in all but one of the phage genes, growth at high temperature, or growth under a variety of suboptimal conditions leads to the early death of the host cell (ref. 1; unpublished data). Thus experimental results obtained under such circumstances may not be relevant to events that occur in uninfected or wild-type-infected *E. coli*.

We recently described some properties of a mutant phage (am8H1R6), isolated by Pratt (24), whose pre-coat protein is processed much more slowly than is wild-type pre-coat protein (25). Cells infected with this mutant produce phage at nearly normal rates for at least 1 hr after infection. Thus this mutant may be a better analog for studying processing and (export or) membrane insertion of wild-type filamentous phage coat pro-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Vp, f1 gene V protein; Lp, *Escherichia coli* lipoprotein; Cp, coat protein; preCp, pre-coat protein.

tein and, by extension, other transported proteins of the uninfected cell.

MATERIALS AND METHODS

Chemicals. [^{14}C]Phenylalanine (460 mCi/mmol; 1 Ci = 3.7 × 10^{10} becquerels) was purchased from Schwarz/Mann. [^{35}S]Methionine (1–1.2 × 10^3 Ci/mmol) was from New England Nuclear. Crystallized egg white lysozyme was from Reheis Chemical Co. (Chicago, IL), and deoxyribonuclease I (DPFF) was from Worthington. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was from Sigma.

Growth Conditions and Phage Strains. *E. coli* K38 were grown at 37°C in DO salts (26) containing 0.2% glucose, vitamin B_1 at 5 μg/ml, and each of 18 amino acids (no phenylalanine or methionine) at 2 mM in all experiments. am8H1R6 is a non-amber revertant of the gene *VIII* amber mutant of M13, am8H1 (24), with leucine at position 2 of the mature coat protein (25), and was kindly provided by David Pratt. Wild-type f1 is from our collection. Conditions for measuring phage production, for labeling, and for NaDodSO$_4$/urea acrylamide gel electrophoresis have been described (25). *In vitro* translation of f1 RF DNA was by the method of Zubay (27). Subsequent analysis of the products was as described (25).

RESULTS

Processing of Pre-Coat Protein in *E. coli* Infected with f1. In agreement with previous work (14, 15), we find that cells infected with wild-type f1 phage cleave pre-coat protein very rapidly, either while it is still a nascent chain or very shortly after its synthesis is completed. Fig. 1 shows the results of the fastest pulse experiment we have been able to carry out. The time between the addition of [^{35}S]methionine and the addition of the

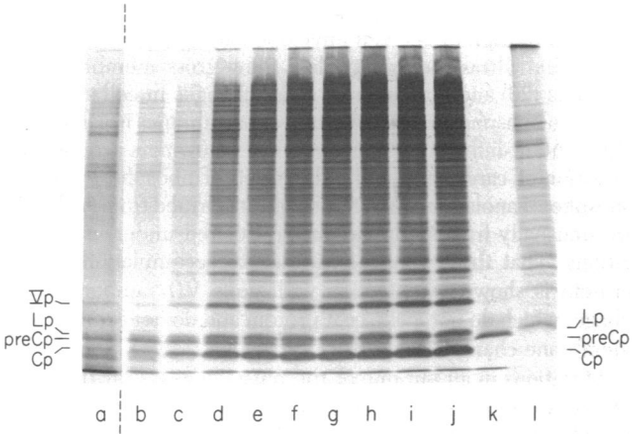

FIG. 1. Rapid processing of wild-type pre-coat protein. Bacteria were infected with wild-type f1 at a multiplicity of 50. At 30 min after infection, 25 μl of culture was added to 20 μCi of [^{35}S]methionine, and 500 μl of 5% trichloroacetic acid was added as rapidly as possible. The elapsed time between addition of isotope and trichloroacetic acid was 3 sec for one pulse and 6 sec for a second pulse. The subsequent pulses were obtained by sampling 25 μl from a 300-μl aliquot of the infected culture to which 100 μCi of [^{35}S]methionine had been added. Incorporation reached a maximum by 20 sec. The samples were precipitated with trichloroacetic acid, dried over NaOH under reduced pressure, resuspended in sample buffer, and analyzed on a NaDodSO$_4$/urea/acrylamide gel. Lanes: a, 3 sec; b, 6 sec; c, 15 sec; d, 35 sec; e, 52 sec; f, 72 sec; g, 100 sec; h, 2 min; i, 4 min; j, 12 min; k, pre-coat protein; l, uninfected control. Lane a is a 6-day exposure. Lanes b–j are a 2.5-day exposure. Vp, f1 gene *V* protein; Lp, *E. coli* lipoprotein; preCp, pre-coat protein; Cp, coat protein.

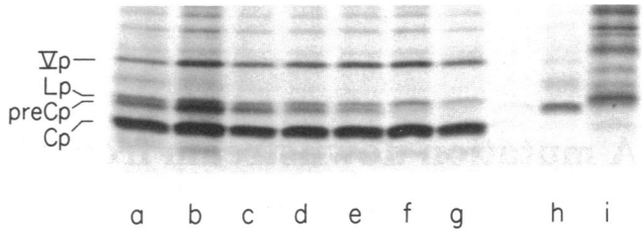

FIG. 2. Kinetics of wild-type pre-coat protein processing. At 30 min after infection with wild-type f1 at a multiplicity of 100, 100 μCi of [^{35}S]methionine was added to 200 μl of culture. Ten seconds later, unlabeled methionine (1.4 mg/ml final concentration) was added, and 25-μl aliquots were removed into 500 μl of 5% trichloroacetic acid after chase periods of: a, 0 sec; b, 9 sec; c, 15 sec; d, 21 sec; e, 28 sec; f, 65 sec; g, 154 sec. The samples were treated as in Fig. 1. Lane h, pre-coat protein; lane i, uninfected control.

5% trichloroacetic acid used to terminate the incorporation was less than 3 sec; the effective pulse length must have been rather less than that, perhaps 1–2 sec. There is somewhat more label in pre-coat protein than in mature coat (see Fig. 1, lane a). As there are three methionine residues in pre-coat but only one in mature coat, about half of the newly synthesized pre-coat has already been processed, suggesting a half-time of less than 2 sec for pre-coat processing. In addition to coat and pre-coat, another band, which migrates faster than coat, may be observed. This presumably represents an incomplete protein, because it disappears rapidly as the pulse time is extended.

Fig. 2 shows a pulse–chase experiment in which the pulse time was 10 sec and the chase was as indicated. Most of the pre-coat protein had already been processed at the time the chase started. The pre-coat that does escape initial processing has a rather long half-life, estimated to be on the order of 60 sec or so from Fig. 2. Thus pre-coat protein has two kinetically distinguishable fates. One is a cleavage to coat protein that occurs either while pre-coat is still nascent or very shortly after it has been completed, whereas the other takes much longer. We cannot determine whether the slow process represents cleavage to coat or degradation; so much coat has accumulated during the 10-sec labeling that an additional small increment during the chase period is not detectable.

Processing of Pre-Coat Protein in *E. coli* Infected with an Amber *V* Mutant Phage. Ito *et al.* (14, 15) have shown that pre-coat protein in amber *I*-, *V*-, and *VII*-infected cells is processed slowly. Gene *V* protein is a single-stranded-DNA-binding protein with, as best as is known, no direct connection to phage morphogenesis (11). Because the delay in the processing may be due to the accumulation of coat protein in the cell membrane rather than to a specific lesion that affects processing, this effect should not obtain early in infection, when not much coat protein has accumulated.

Fig. 3 bears out this supposition. Appreciable amounts of coat protein remain as precursor if labeling is carried out at 30 min after infection or later, but if the labeling occurs at 15 min, no pre-coat protein is detected after a 3-min labeling period.

Processing of Pre-Coat Protein in *E. coli* Infected with am8H1R6. am8H1R6 is a revertant of a coat amber mutant of phage M13; it has a signal peptide identical to that of the wild type (28) but bears a leucine residue instead of a glutamic acid residue at position 2 of the mature coat protein (25). This replacement leads to a decreased rate of processing *in vivo* (25). Detailed kinetics of pre-coat processing shown in Fig. 4 indicate a half-time of about 60 sec.

There are three simple explanations that could account sim-

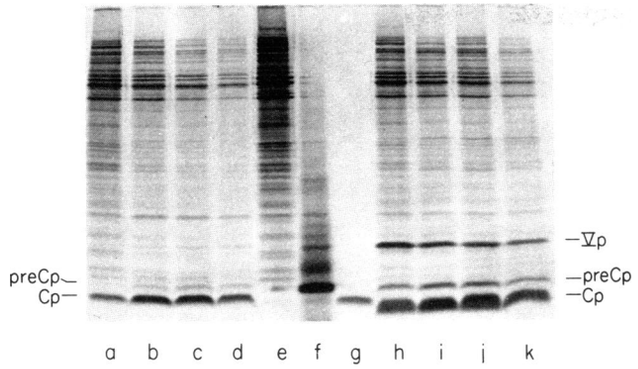

FIG. 3. Rate of pre-coat protein processing at different times after infection. Bacteria were infected with amR13 (gene V^-) or am8H1R6 phage at multiplicities of 60. At 15, 30, 45, and 60 min after infection, 200-μl aliquots of each infected culture were labeled with 1 μCi of [^{14}C]phenylalanine for 3 min. Incorporation was terminated with the addition of 200 μl of 10% trichloroacetic acid. The samples were treated as in Fig. 1. Lanes: a–d, amR13-infected cells; h–k, am8H1R6-infected cells; a and h, 15–18 min; b and i, 30–33 min; c and j, 45–48 min; d and k, 60–63 min; e, uninfected control; f, pre-coat protein; g, wild-type coat protein. We have already noted the anomalous migration of leucine-2-containing coat protein in this gel system (25).

ply for the reduced rate of processing: (*i*) The mutant (leucine-containing) protein may be a poor substrate for the signal peptidase. (*ii*) Assuming that membrane insertion is a prerequisite for cleavage, the mutant protein may not insert into the cell membrane properly, or as fast as wild-type protein. (*iii*) Assembly of phage may be slow, leading to accumulation of coat and pre-coat in the cell membrane, which in turn would lead to a processing block of the sort observed in nonproductive infections described for amber *V*-infected cells (see above). To determine whether slow processing is a consequence of accumulation of coat protein in the membrane, the processing was measured early after infection.

Fig. 3 shows that am8H1R6-infected cells show a delay in the processing of pre-coat protein very early after infection, at a time when the processing of pre-coat in amber *V*-infected cells occurs normally. While the accumulation of coat and pre-coat in the membrane is probably responsible for the processing delay in amber *V* infections, it is not in am8H1R6 infections. am8H1R6 pre-coat synthesized *in vitro* was tested as a substrate for processing *in vitro*. Fig. 5 shows that the extent of processing of this mutant coat *in vitro* is substantially lower than the extent of wild-type coat processing. The Nikkol added to activate the

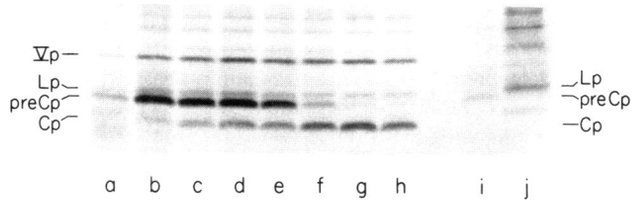

FIG. 4. Kinetics of am8H1R6 pre-coat protein processing. At 30 min after infection with am8H1R6 at a multiplicity of 100, [^{35}S]methionine (100 μCi) was added to a 200-μl culture. One 25-μl aliquot was removed into trichloroacetic acid at 8 sec, and additional 25-μl aliquots were removed into trichloroacetic acid subsequent to addition of unlabeled methionine (1.4 mg/ml final concentration) at 15 sec. The samples were treated as in Fig. 1. Lane a, 8-sec pulse. Lanes b–h, 15-sec pulse followed by chase of: b, 8 sec; c, 26 sec; d, 38 sec; e, 49 sec; f, 105 sec; g, 4 min; h, 6 min. Lane i, precoat protein; lane j, uninfected control.

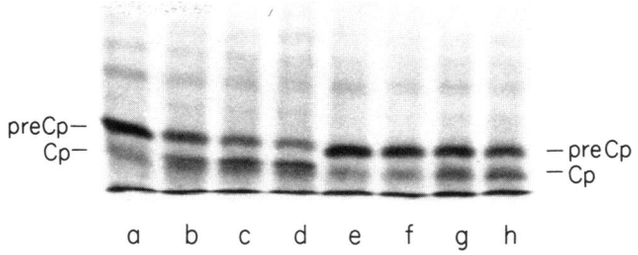

FIG. 5. *In vitro* synthesis and processing of f1 and am8H1R6 coat protein. Synthesis was carried out according to Zubay (27), using DNA at 30 μg/ml. Extracts were preincubated with DNA, 0.33% Nikkol, and *E. coli* cell membranes (6, 7) for 8 min, then [^{35}S]methionine (320 μCi/ml final) was added and synthesis was continued for 10 min. Samples were analyzed as described (25). Lanes: a–d, f1 DNA; e–h, am8H1R6 DNA; a and e, no added membranes; b and f, 1 μl of membranes; c and g, 2 μl of membranes; d and h, 4 μl of membranes.

signal peptidase also disrupts membrane vesicles, and thus the extent of processing reflects the inherent substrate properties of the am8H1R6 pre-coat rather than its transport into the membrane. Evidently some amino acids downstream from a cleavage site can affect processing by signal peptidase.

We followed the flow of label from pre-coat into mature phage for wild-type- and am8H1R6-infected cells (Fig. 6). After a short pulse in wild-type-infected cells all of the coat protein initially associated with the cellular fraction chased into extracellular phage with a half-time of about 2 min. Label introduced into coat protein in am8H1R6-infected cells flowed into extracellular phage more slowly (half-time about 4 min) and not as completely. Some of this lag is attributable to the slower processing time of the am8H1R6 pre-coat, because the half-time for its processing *in vivo* is on the order of 1 min (as compared with less than 2 sec for wild-type coat), but we cannot exclude

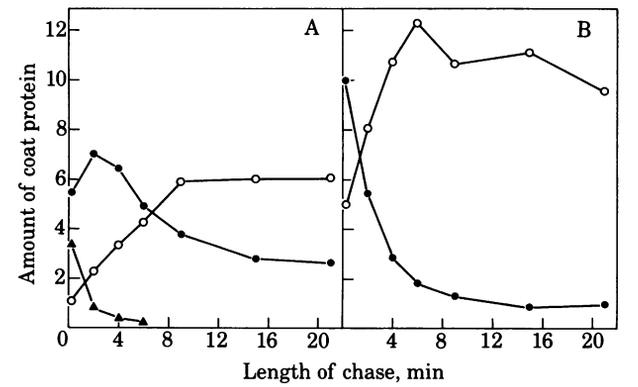

FIG. 6. Kinetics of phage release. Cells were infected with am8H1R6 (*A*) or wild-type (*B*) phage at multiplicities of 100. At 30 min after infection, 20 μCi of [^{35}S]methionine was added to 1.6 ml of infected cells. Unlabeled methionine (2 mg/ml final concentration) was added 60 sec later, and 200-μl aliquots were removed onto ice at various times. The aliquots were centrifuged at 4°C, and the cell pellets and supernatants were separately precipitated with trichloroacetic acid and treated as described for Fig. 1. Appropriately exposed autoradiograms were scanned by a Joyce–Loebl densitometer, and areas of peaks of interest were measured. Mature coat protein contains a single methionine residue; pre-coat protein synthesized *in vitro* contains three, one of which is NH_2-terminal. It is not known whether pre-coat protein synthesized *in vivo* retains the NH_2-terminal methionine. The amount of pre-coat protein present in *A* has been somewhat arbitrarily calculated by assuming its removal. ●, Coat protein in cell pellets (intracellular); ○, coat protein in supernatants (extracellular); ▲, pre-coat protein.

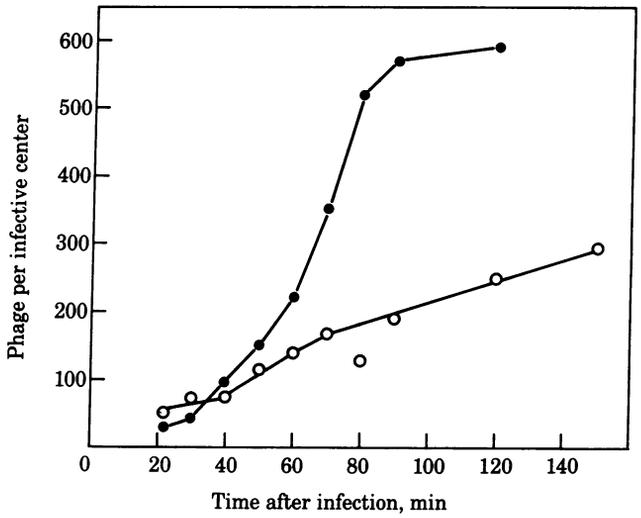

FIG. 7. Kinetics of phage production. Bacteria were infected with am8H1R6 or with wild-type phage at multiplicities of 10. After a 10-min adsorption period, the cultures were diluted 1:10^4 (growth tube) to prevent multiple cycles of infection. At 20 min after infection, after treatment with anti-f1 serum to remove unadsorbed phage, infective centers were assayed. At various times, aliquots of the growth tube were sampled into $CHCl_3$-saturated tryptone broth and assayed. ○, am8H1R6; ●, wild-type f1.

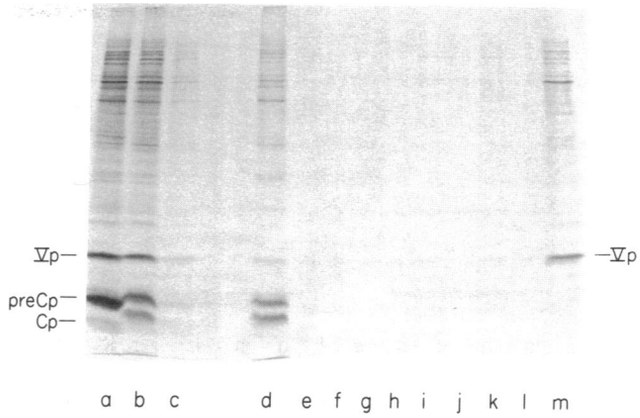

FIG. 8. Location of pre-coat protein in am8H1R6-infected cells. Cells were infected with am8H1R6 phage at a multiplicity of 40. At 30 min after infection, 50 μCi of [^{35}S]methionine was added to 500 μl of culture. After 30 sec, dinitrophenol and CCCP were added to final concentrations of 2 and 0.1 mM, respectively, and 15 sec later the culture was chilled on ice. An aliquot was removed into trichloroacetic acid at this time. All manipulations were performed at 0°C or 4°C. The culture was centrifuged, and the pellet was resuspended in 500 μl of 0.1 M Tris·HCl, pH 8.1/18% sucrose/10 mM EDTA containing 2 mM dinitrophenol, 0.1 mM CCCP, and 4 × 10^9 unlabeled carrier cells. The cells were incubated with lysozyme (100 μg/ml final concentration) on ice for 10 min and pelleted, and the supernatant was removed. The pellets were resuspended in 100 μl of Tris/sucrose/dinitrophenol/CCCP and made 10 mM in $MgCl_2$. DNase I was added to a final concentration of 50 μg/ml, and the spheroplasts were lysed by adding 400 μl of 2 mM dinitrophenol/0.1 mM CCCP and sonicated (Heat Systems; micro tip; setting 1–2) for 5 sec. An aliquot was taken into trichloroacetic acid. After addition of EDTA (10 mM final) 100 μl of the lysate was layered onto 12 ml of 15% sucrose/3 mM EDTA/2 mM dinitrophenol/0.1 mM CCCP with a 0.5-ml 70% sucrose cushion and centrifuged in a Beckman SW 40.1 rotor for 2 hr at 40,000 rpm and 0°C. Fractions (1.2 ml) were collected, precipitated with trichloroacetic acid, and treated as in Fig. 1. Lanes: a, lysate sampled at end of pulse period; b, lysate sampled after sonication of spheroplasts; c, aliquot of pooled gradient fractions; d–m, gradient fractions from bottom to top.

the possibility that the am8H1R6 mutation is somewhat pleiotropic and independently affects the rate of assembly. What we *can* say, however, is that, as shown in Fig. 7, cells infected with am8H1R6 continue to produce phage at a nearly normal rate for at least 60 min.

Cellular Location of am8H1R6 Pre-Coat. Processing of pre-coat in am8H1R6-infected cells is sufficiently slow for much of the label incorporated to be recovered as pre-coat if the pulse time is short. Fig. 8 shows the distribution of pre-coat, coat, and gene *V* protein in gradient fractions from disrupted cells. We note that the bulk of the pre-coat and coat protein is located in a fast-sedimenting fraction—e.g., associated with the cell membrane—whereas most of the gene *V* protein remains in the slowly sedimenting, or cytoplasmic, fraction. These experiments were carried out by pulse-labeling for 30 sec, then adding CCCP [which inhibits processing *in vivo* (29)] and dinitrophenol [which inhibits processing *in vitro* (29)] and subsequently carring out the lysis procedure at 0°C. Notwithstanding these precautions, and notwithstanding our observation that am8H1R6 pre-coat protein is processed inefficiently by the signal peptidase, Fig. 8, lanes a and b, shows that considerable processing occurred between the time the pulse ended and the time at which the spheroplasts were lysed.

DISCUSSION

Hitherto signal sequence mutants have been described in which the mutant protein is neither transported nor cleaved (30–32). In addition, Lin *et al.* (33) have described a mutant prolipoprotein which is transported but not cleaved. am8H1R6 differs from these in that membrane insertion occurs but cleavage is slow and in that the mutation is in the protein itself, rather than in the signal peptide of the precursor. This mutant, therefore, serves to further differentiate between membrane recognition and proteolysis, two processes that normally occur in concert. It also shows that the domain affecting signal peptidase activity extends beyond the signal peptide itself.

The experiments described above have confirmed earlier findings (14, 15, 34) that, in cells infected with wild-type filamentous phage, most pre-coat protein is processed very rapidly to mature coat, perhaps before synthesis is complete. In order to study the details of membrane insertion and processing, previous workers have used cells infected with mutant phage that produce coat protein but do not assemble phage (14, 15) [or cells infected with wild-type phage under conditions that preclude efficient phage assembly (16)]. Under such conditions membrane insertion and processing of pre-coat protein is greatly delayed, and free pre-coat protein has been found in the cell cytoplasm (refs. 14 and 16; unpublished results). We believe that the delay in membrane insertion and processing observed under these conditions is attributable to the "loading" of the cell membrane with coat protein. Such accumulated coat protein either directly blocks the normal receptor sites in the membrane or renders the membrane incompetent for transport in some more indirect fashion. This leads to the cytoplasmic discharge of free pre-coat protein. The presence of substantial amounts of pre-coat protein in the cell cytoplasm would then reflect the pathological state of the membrane and not be related to the events that occur during normal infection. In support of this hypothesis we offer the following observations: (*i*) In amber *V*-infected cells, taken as prototypic of cells infected under "nonproductive" conditions, processing is delayed late after infection but seems to be normal early after infection. (*ii*)

Processing of the pre-coat protein of am8H1R6 is delayed both early and late after infection. The proximal cause of this processing delay appears to be a defective signal processing site, because *in vitro* cleavage of the mutant pre-coat protein is slow, and because assembly and export of viable phage in cells infected with this mutant seem to be normal. (*iii*) Pre-coat protein produced in cells infected with am8H1R6 is membrane associated; it is not found free in the cytoplasm. (*iv*) Pre-coat protein in cells infected with wild-type M13 that are treated with CCCP to inhibit processing is membrane associated, not free in the cytoplasm (13, 35).

Even in wild-type infections a small fraction of pre-coat protein may fail to associate properly with the membrane. This would account for the minor, kinetically distinguishable form of pre-coat seen in Figs. 1 and 2, which has a long half-life.

We reported earlier (6, 7) that efficient sequestration and proteolytic cleavage of f1 pre-coat protein into membrane vesicles occurs only when the vesicles are present during synthesis of the pre-coat protein, and not when they are added after translation is finished. This result was also obtained by Mandel and Wickner (12). On this basis, and reasoning by analogy with eukaryotic systems, we postulated that the "signal hypothesis" (17, 36) is applicable to the f1 coat protein system.

Mandel and Wickner (12) made the additional finding that proteolysis by a detergent extract of membranes can also occur posttranslationally, but only if a detergent is present during translation. They suggested that the detergent prevents the folding of the completed protein and leaves the signal peptide exposed. This latter result supports our hypothesis that *in vivo* the interaction between pre-coat protein and the membrane occurs while the pre-coat is still a nascent chain.

Our current outline of the process of membrane insertion/proteolysis of filamentous phage coat protein follows: Synthesis of f1 pre-coat occurs with concomitant binding of the NH_2-terminal amino acids (signal peptide) to the plasma membrane of the infected cell, which accounts for the rapidity with which wild-type filamentous coat and pre-coat become membrane associated. It also accounts for the rapid membrane association of the pre-coat protein of mutants such as am8H1R6 and for the appearance in wild-type-infected cells of structures that, in the electron microscope, closely resemble eukaryotic rough endoplasmic reticulum (20). Under "nonproductive" conditions, the membrane is overloaded and becomes incompetent to support this association. The resulting pre-coat protein will tend to end up in the cytoplasm. Proteolytic cleavage of pre-coat is contingent upon and follows successful insertion of the coat protein into the membrane but is not required for the membrane association *per se*. From our failure to find pre-coat protein in mature am8H1R6 phage, and on *a priori* grounds, proteolytic cleavage is probably a prerequisite for phage assembly.

There are probably at least three discrete steps required for the successful integration of coat protein: the initial recognition between the signal peptide and the membrane (or membrane receptor), actual insertion, and cleavage. Date *et al.* (35) report that, in cells treated with CCCP, pre-coat is membrane associated but not accessible to the signal peptidase. Furthermore, while an intact signal peptide is necessary for transport (30–32), it is not always sufficient (37), which again suggests a postrecognition step.

We have no direct evidence bearing on the orientation of am8H1R6 pre-coat protein in the membrane of the infected cell. However, we observe cleavage to mature coat protein in am8H1R6-infected cells during the preparation of membranes even when the preparations are held in a mixture of CCCP and dinitrophenol. In contradistinction to pre-coat protein formed in the presence of CCCP, therefore, am8H1R6 pre-coat can become accessible to the signal peptidase and must therefore be in a different state.

We thank N. D. Zinder for advice and for critically reading the manuscript, J. D. Boeke for helpful suggestions, and D. Pratt for providing am8H1R6. This work was supported in part by grants from the National Institutes of Health and the National Science Foundation.

1. Marvin, D. A. & Hohn, B. (1969) *Bacteriol. Rev.* **33**, 172–209.
2. Hoffman-Berling, H., Durwald, H. & Beulke, I. (1963) *Z. Naturforsch.* **18b** 893–898.
3. Pieczenik G., Model P. & Robertson, H. D. (1974) *J. Mol. Biol.* **90**, 191–214.
4. Sugimoto, K., Sugisaki, H., Okamoto, T. & Takanami, M. (1977) *J. Mol. Biol.* **111**, 487–507.
5. Webster, R. E. & Cashman, J. S. (1978) in *The Single-Stranded Phages* eds. Denhardt, D. T., Dressler, D. & Ray, D. S. (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp. 557–569.
6. Chang, C. N., Blobel, G. & Model, P. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 361–365.
7. Chang, C. N., Model, P. & Blobel, G. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 1251–1255.
8. Makino, S., Woolford, J. L., Tanford, C. & Webster, R. E. (1976) *J. Biol. Chem.* **250**, 4327–4332.
9. Wickner, W. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 4749–4753.
10. Wickner, W. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 1159–1163.
11. Horiuchi, K., Vovis, G. F. & Model, P. (1978) in *The Single-Stranded DNA Phages*, eds. Denhardt, D. T., Dressler, D. & Ray, D. (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York), pp. 113–137.
12. Mandel, G. & Wickner, W. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 236–240.
13. Wickner, W., Mandel, G., Zwizinski, C., Bates, M. & Killick, T. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 1754–1758.
14. Ito, K., Mandel, G. & Wickner, W. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 1199–1203.
15. Ito, K., Date, T. & Wickner, W. (1980) *J. Biol. Chem.* **255**, 2123–2130.
16. Date, T. & Wickner, W. T. (1980) *J. Virol.*, in press.
17. Blobel, G. & Dobberstein, B. (1975) *J. Cell Biol.* **67**, 835–851.
18. Wickner, W. (1979) *Annu. Rev. Biochem.* **48**, 23–45.
19. Hohn, B., von Schutz, H. & Marvin, D. A. (1971) *J. Mol. Biol.* **56**, 155–165.
20. Schwartz, F. M. & Zinder, N. D. (1967) *Virology* **34**, 352–355.
21. Onishi, Y. & Kuwano, M. (1971) *J. Virol* **7**, 673–678.
22. Woolford, J. L., Jr., Cashman, J. S. & Webster, R. E. (1974) *Virology* **58**, 544–560.
23. Webster, R. E. & Rementer, M. (1980) *J. Mol. Biol.* **139**, 393–405.
24. Pratt, D., Tzagoloff, H. & Beaudoin, J. (1969) *Virology* **39**, 42–53.
25. Boeke, J. D., Russel, M. & Model, P. (1980) *J. Mol. Biol.* **144**, 103–116.
26. Vogel, H. J. & Bonner, D. M. (1956) *J. Biol. Chem.* **218**, 97–106.
27. Zubay, G. (1973) *Annu. Rev. Genet.* **7**, 267–287.
28. Boeke, J. D. & Model, P. (1979) *Virology* **96**, 299–301.
29. Date, T., Zwizinski, C., Ludmerer, S. & Wickner, W. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 827–831.
30. Emr, S. D., Schwartz, M. & Silhavy, T. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 5802–5806.
31. Bedouelle, H., Bassford, P. J., Jr., Fowler, A. V., Zabin, I., Beckwith, J. & Hofnung, M. (1980) *Nature (London)* **285**, 78–85.
32. Bassford, P. & Beckwith, J. (1979) *Nature (London)* **277**, 538–541.
33. Lin, J. J. C., Kanazawa, H., Ozols, J. & Wu, H. C. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 4891–4895.
34. Smilowitz, H., Carson, J. & Robbins, P. W. (1972) *J. Supramol. Struct.* **1**, 8–18.
35. Date, T., Goodman, J. M. & Wickner, W. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 4669–4673.
36. Emr, S. D., Hall, M. N. & Silhavy, T. J. (1980) *J. Cell Biology* **86**, 701–711.
37. Moreno, F., Fowler, A., Hall, M., Silhavy, T. J., Zabin, I. & Schwartz, M. (1980) *Nature (London)* **286**, 356–359.