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Genes *VI, VII,* and *IX* of phage M13 code for minor capsid proteins of the virion

(differential amino acid labeling/amino-terminal sequence copy numbers/chimeric phages)

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ABSTRACT **The minor capsid proteins C and D from phage M13 have been characterized by differential amino acid labeling and amino-terminal sequence analysis. We demonstrate that D protein (M_r 12,260) is the product of gene *VI*, whereas the C component is composed of the products of both gene *VII* (M_r 3580) and gene *IX* (M_r 3650). Our data further show that the proteins of genes *VI, VII,* and *IX* are not subject to proteolytic processing but are packaged into mature virions as their primary translational products. On the basis of incorporation of specific amino acids, the copy numbers of these proteins in M13 virions could be estimated relative to the number of A protein molecules. The M13 phage contains on the average 5 molecules of A protein, 5 molecules of *VI* protein and 3–4 molecules of both *VII* protein and *IX* protein. These copy numbers remained unchanged in M13 recombinant phages of up to two times the length of wild-type phages, a fact that indicates that these minor capsid proteins are located at either one or both ends of the phage filament.**

M13 and its very close relatives fd and f1 are filamentous phages that infect *Escherichia coli* cells carrying the F episome (for a review, see ref. 1). The M13 genome consists of a 6407-nucleotide-long single-stranded DNA molecule (2), which is encapsulated in a tubular protein coat about 895 nm long and 6 nm wide. Over the years there have been reports which suggest that the composition of the virion coat is more complex than originally thought (3–5). The major capsid or B protein (M_r 5200), which is encoded by gene *VIII*, is present in about 2700 copies, which are arranged in a helix around the DNA (6). The gene *III*-encoded minor capsid or A protein (M_r 42,600) is present as only four or five copies (3, 7), which are located at one tip of the filament (4, 5). Besides these two proteins, two additional minor capsid proteins of M_r 3500 and 11,500, designated C protein and D protein, respectively, were recently discovered (8). Their genetic origin and their biological function have not yet been solved.

Assembly of M13 virions does not occur in the cytoplasm: infectious virus has never been detected within the infected cell and the major capsid protein is an integral constituent of the cytoplasmic membrane prior to virus assembly. In the cytoplasm, progeny viral DNA is covered by the DNA-binding protein encoded by gene *V*, forming a filamentous nucleoprotein (9). The assembly process is apparently one of exchange of coat protein for gene *V* protein as the viral DNA leaves the cell. The minor coat or A protein has most probably a very early and late function. It may lead the DNA into the host cell (10, 11) and its presumed site of DNA replication (12) after attachment of the virus to the Fpilus, and there is also evidence which suggests that it functions as a cut-off agent in the final stage of the *V* protein–major coat protein exchange reaction (10). At least four other M13 genes (*I, IV, VI,* and *VII*) are required for virus assembly, but their specific role in this process has yet to be ascertained. Further research in this field is hampered by the fact that the products of some genes (*VI* and *VII*) have not yet been detected either *in vivo* or *in vitro* (13–15), whereas the products of genes *I* and *IV* are present in the infected cell in very low amounts (3, 16).

To gain a better understanding of the assembly functions of these genes and to find out whether the recently discovered minor capsid proteins are host-encoded or M13-specified products, we have studied these proteins in more detail. In this report we demonstrate that D protein is the product of gene *VI* and that the C protein component is composed of two polypeptides, one of which is encoded by gene *VII* and the other by gene *IX*.

MATERIALS AND METHODS

Bacteria and Phages. The *E. coli* strains KA805 (amber suppressor SuI$^+$) and KA807 (amber suppressor SuIII$^+$) were obtained from B. Glickman. M13 and its amber mutant *am*7H2 have been described (17). The recombinant phage fd106Sm2 (18) originated from H. Schaller. Phages M13S100 and S200, containing the 1.8- and 3.0-kilobase-pair Hae II A fragments of pBR322 and pBR325, respectively, were constructed as described in ref. 18.

Growth and Purification of Radioactive Phages. Amino acid-labeled M13 phages were prepared essentially as described (8) with minor modifications. *E. coli* strain K-38 was grown in 25 ml of M9 minimal medium (19) supplemented with 20 mM glucose but no amino acids were added. At 1×10^8 cells per ml, M13 phages were added (multiplicity of infection 20) and then a total of 1.0 mCi of ^{3}H-labeled amino acid or 0.25 mCi of ^{14}C-labeled amino acid was added in four equal portions at 15, 45, 75, and 105 min after infection (1 Ci = 3.7×10^{10} becquerels). Labeling with [^{35}S] methionine was performed similarly with a total of 0.1 mCi. In all cases amino acids with the highest specific activity currently available were used (Amersham). Amino acid labeling of M13S100, M13S200, and fd106Sm2 was carried out in the presence of ampicillin (25 μg/ml), chloramphenicol (25 μg/ml), or streptomycin (10 μg/ml), respectively. Phages were isolated after a labeling period of 4 hr and further purified by two successive precipitations with 5% polyethylene glycol 6000 in the presence of 0.1% Sarkosyl, followed by centrifugation on CsCl gradients as described (8).

Isolation of Radioactive Phage Proteins. The fractionation of capsid proteins on NaDodSO$_4$/8 M urea polyacrylamide slab gels and their subsequent electrophoretic elution from gel segments have been described in detail (8). After dialysis of the eluate, the radioactive proteins were precipitated, washed, and lyophilized as described by Shaw *et al.* (20).

Automated Edman Degradation. Prior to degradation, the protein samples were subjected to a 3 M HCl treatment as de-

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scribed by Palmiter *et al.* (21). Thereafter the acid was removed by lyophilization and the sample was then washed three times with 1% triethylamine and dried under reduced pressure. Samples were dissolved in 70% (wt/wt) formic acid and 2 mg of parvalbumin was then added as carrier (22). Automated Edman degradation was performed on a Beckman 890C sequenator, using a 0.33 M Quadrol program of Hunkapiller and Hood (23). The recovered phenylthiohydantoin amino acids were dried under nitrogen and dissolved in 200 μl of methanol, and their radioactivity was determined in 5 ml of Lumagel (Baker), using a Packard scintillation spectrometer.

Quantitation of Phage Capsid Proteins. Copy numbers of capsid proteins were determined from tritiated amino acid-labeled phages only. After electrophoretic fractionation of the individual capsid proteins and subsequent fluorography (24), the appropriate gel segments were excised from the dried gel. The gel segments were completely burned with oxygen in a Packard Tri-Carb model 300 sample oxidizer and the radioactivity was determined by adding 10 ml of Lumagel to each [^{3}H]water eluate and scintillation counting. ^{3}H-Labeled standard samples (Packard) were applied for recovery determinations.

RESULTS

Genetic origin of D protein

Electrophoretic analysis of the proteins present in ^{14}C-labeled M13 phages showed that the virion coat is composed of the mature products of genes *III* and *VIII* and two additional small protein components (Fig. 1). The latter, denoted C protein and D protein, have apparent molecular weights of 3500 and 11,500, respectively.

Because conventional ion-exchange and exclusion chromatographic methods for the isolation and subsequent characterization of C and D proteins were unsuccessful, we have attempted to characterize these proteins on the basis of their capability to incorporate several representative ^{3}H-labeled amino acids. In this approach the established nucleotide sequence of M13 DNA (2) was used as a guide for these labeling experiments. From these studies it turned out that D protein can be labeled with all amino acids except histidine (Fig. 1). Because the M13 DNA sequence dictated that gene *VI* codes for a protein of 112 amino acids in which histidine is absent, the genetic relationship between gene *VI* and D protein became apparent. On the other hand, gene *VIII* protein (M_r 5240) also lacks histidine (Fig. 1), and the possibility therefore remained that D protein is not a new capsid protein but merely represents a gene *VIII* protein dimer. This possibility, however, has been ruled out because D protein can be labeled with arginine, an amino acid that is known to be absent from the major coat protein (Fig. 1).

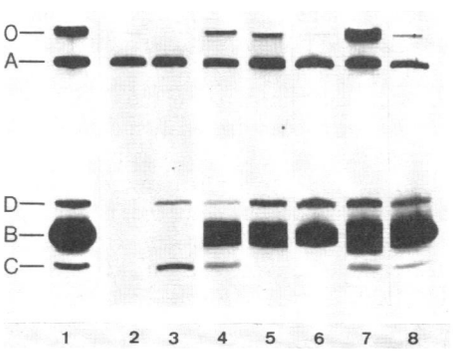

FIG. 1. NaDodSO$_4$/urea/polyacrylamide gel electrophoresis of M13 phage capsid proteins that were labeled with a ^{14}C-labeled amino acid mix (lane 1), histidine (lane 2), arginine (lane 3), methionine (lane 4), proline (lane 5), lysine (lane 6), tryptophan (lane 7), or aspartic acid (lane 8). Individual amino acids were all ^{3}H-labeled. A and B refer to the gene *III*-encoded minor capsid protein (M_r 42,600) and the gene *VIII*-encoded major capsid protein (M_r 5240). Sometimes a protein aggregate of unknown composition that does not enter the separating gel was noted (lanes 1 and 7; O, origin), particularly when B protein-labeled phages were disrupted at high concentrations. A similar phenomenon was noted by Grant *et al.* (25).

Our initial attempts to characterize D protein by amino acid analysis were only partly successful. The overall amino acid composition of electrophoretically purified, uniformly ^{14}C-labeled D-protein, as determined with ^{14}C-labeled gene *V*, *VIII*, and *III* proteins as internal radioactive standards, did show the absence of histidine, low percentages of arginine and methionine, and relatively high amounts of leucine, as expected for gene *VI* protein, but also suggested that the protein was still contaminated, most probably with major capsid protein (data not shown).

To demonstrate unambiguously that D protein originates from gene *VI*, M13 phages labeled with a single radioactive amino acid were prepared and the electrophoretically isolated D protein was then subjected to amino-terminal sequence analysis by automated Edman degradation. The results obtained are presented in Fig. 2. The DNA sequence predicts that gene *VI* protein contains a single methionine residue, which is located at the amino terminus of this protein. Edman degradation of D protein from [^{35}S]methionine-labeled phages, however, did not liberate any radioactivity after the first degradation step (Fig. 2), despite the fact that D protein can be labeled with methionine (Fig. 1). To find out whether D protein molecules are blocked by a formyl group at their amino terminus, prior to sequential degradation, the protein was subjected to a HCl treatment essentially as described by Palmiter *et al.* (21). It appeared that only after such treatment could the amino-terminal sequence of this protein be determined, strongly suggesting that this protein is present in the virion in its *N*-formylated form. The yield of Edman degradation after this 3 M HCl treatment was low and ranged between 20% and 40%. Accordingly, D protein from phages that were labeled with proline, glycine, or leucine were treated with HCl and subjected to automated sequence analysis. With the exception of two degradation steps, the radioactivities obtained after the various sequence runs were found in full accordance with the amino-terminal sequence of gene *VI* protein as deduced from the DNA sequence (Fig. 3). The exceptions are the positions 3 and 6, where in both cases either a glycine or a proline residue could be proposed. On the basis of repetitive yield of Edman sequence analysis, which was usually about 92%, and the pronounced intensity of proline at position 6 as compared to proline residues 2 and 8, it is obvious that we are elucidating the amino-terminal sequence of two different proteins. Proline residues 2 and 8 agree with the *VI* protein sequence, whereas a proline at position 6 fits with the amino-terminal sequence of mature *VIII* protein. A similar conclusion emerged from the sequence run of [^{14}C]glycine-labeled D protein. The glycine residue at position 6 belongs to *VI* protein, and glycine at position 3 fits again with the sequence of mature *VIII* protein. From these degradation results we infer that D protein consists of *VI* protein but also of *VIII* protein dimers. We calculated that a residual dimerization of the major capsid protein of only 0.2% is sufficient to cause the anomalous degradation results. Such low values cannot be excluded in the NaDodSO$_4$/8 M urea/polyacrylamide system applied to fractionate these phage proteins.

Genetic origin of C protein

As is shown in Fig. 1, the smallest minor capsid protein C cannot be labeled with histidine, proline, or lysine. The only M13 gene products the sizes of which are ≈3500 daltons and that lack

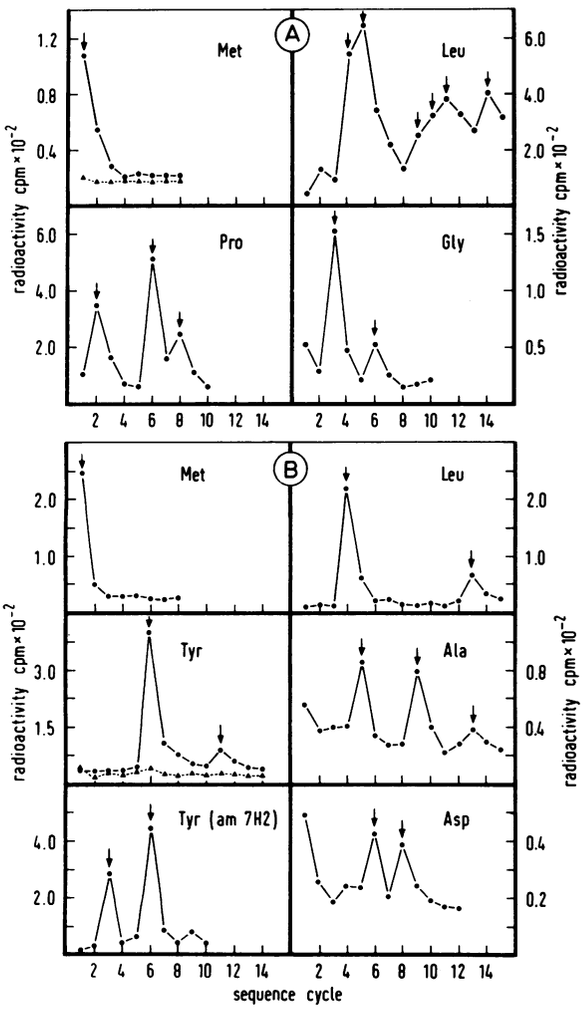

FIG. 2. Amino-terminal sequence anaylsis of: (*A*) capsid protein D, and (*B*) capsid protein C, both isolated from M13 phages that were labeled with a single ^{3}H- or ^{14}C-labeled amino acid as indicated. The broken lines indicate the sequence runs of the proteins that were not pretreated with 3 M HCl; the solid lines represent the runs of treated protein samples. *am*7H2 represents the degradation of [^{3}H]tyrosine-labeled C protein from am-7 phages propagated in SuIII^{+} *E. coli*.

these amino acids are those of gene *VII* and the recently discovered gene *IX* (17, 26). From the nucleotide sequence of both genes it is also evident that gene *VII* contains aspartic acid, which is absent in gene *IX*, and that tryptophan is present in

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     1                                  10
A    Met-Pro-Val-Leu-Leu-Gly-Ile-Pro-Leu-Leu-Leu-Arg-Phe-Leu

     1                                  10
B    Met-Glu-Gln-Val-Ala-Asp-Phe-Asp-Thr-Ile-Tyr-Gln-Ala

     1                                  10
C    Met-Ser-Val-Leu-Val-Tyr-Ser-Phe-Ala-Ser-Phe-Val-Leu
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FIG. 3. Sequences A, B, and C represent the amino-termini of *VI*, *VII*, and *IX* proteins, respectively, as deduced from the M13 DNA sequence (2). The amino acids given in boldface are those found after Edman degradation of C and D proteins that were labeled with single radioactive amino acids.

gene *IX* but not in gene *VII*. C protein can, however, be labeled with both amino acids (Fig. 1), suggesting that this protein is a mixture of both gene products.

To confirm this, automated Edman degradation was also carried out with C protein that was labeled with single amino acids. The results obtained are shown in Fig. 2. Also with this protein an amino-terminal sequence could be determined only after a 3 M HCl treatment. Untreated [^{3}H]tyrosine-labeled C protein showed no radioactivity peaks even after 15 degradation steps, whereas treated C protein revealed a peak of great intensity at position 6 and one of low intensity at position 11 (Fig. 2). The intensity of the latter did not fit the value expected on the basis of the repetitive yield of the Beckman sequencer, an observation that, in turn, strongly suggests that the two tyrosine residues belong to two different proteins. This was also evident from our observations that at position 6 a tyrosine was found but also an aspartic acid residue, and at position 13 both a leucine and an alanine residue were noted. By comparing the positions of the various radioactive residues with the amino-terminal sequence of *VII* and *IX* proteins as deduced from the DNA sequence (Fig. 3), our interpretation of these data is that tyrosine-6 belongs to gene *IX* and tyrosine-11 to gene *VII*. Leucine-4 and leucine-13 are both derived from *IX* protein. Consistent with the repetitive yield, alanine-5 and alanine-13 belong to gene *VII*, whereas the higher intensity of alanine-9 exactly fits with the gene *IX* sequence. In a similar way we infer that the aspartic acid residues at position 6 and 8 belong to gene *VII*. We do not yet have a plausible explanation for the occurrence of radioactivity at position 1 of the aspartic acid label, because at that position the occurrence of methionine was also demonstrated.

That C protein indeed is composed of the proteins encoded by gene *VII* (33 amino acids) and gene *IX* (32 amino acids) is further supported by the results of our labeling studies with the M13 phage mutant *am*7H2. We previously demonstrated (17) that this mutant bears two mutated sites. One is a C → T change in gene *VII* at position 1114 of the DNA sequence, which alters that CAG codon into a TAG codon. The other is a C → T change outside gene *VII* but in the gene *IX* sequence (position 1141), where a CGT (Arg) codon is converted into a TGT (Cys) codon. Consequently, gene *IX* protein encoded by this mutant contains only one instead of two arginine residues. When *am*7H2 phages were propagated in SuI^{+} *E. coli* cells in the presence of [^{14}C]arginine and the proteins of the purified phages were analyzed on polyacrylamide gels, the radioactivity contained in the band representing C protein was markedly reduced as compared to wild-type phages (Fig. 4).

That *VII* protein also forms part of the C protein component is confirmed by our Edman degradation data of *am*7H2 phage proteins. When this mutant phage was propagated in SuIII^{+} *E. coli* cells in the presence of [^{3}H]tyrosine, a tyrosine residue was noted in C protein not only after the sixth but also after the third degradation step (Fig. 2). Residue 6 reflects gene *IX* protein, whereas residue 3 corresponds with a tyrosine suppression at the amber codon in gene *VII*, namely at position 1114 of the DNA sequence.

Copy numbers of C and D proteins

The great abundance of major coat protein as compared to C and D protein and the well-known tendency of the major coat protein to aggregate even under strongly denaturing conditions make an estimation of their copy numbers in phage particles very difficult *a priori*. Unless certain precautions have been made, cross-contamination is expected to occur during the electrophoretic separation of these phage proteins. We have circumvented these problems by analyzing the radioactivity distribution of D protein in [^{3}H] arginine-labeled M13 phages in

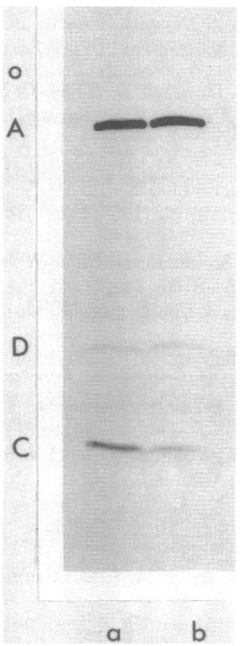

FIG. 4. NaDodSO$_4$/8 M urea/polyacrylamide gel electrophoresis of the capsid proteins of M13 wild-type phage (lane a) and *am*7H2 phage (lane b) that were propagated in SuI$^+$ *E. coli* cells in the presence of [^{3}H]arginine. The radioactivities applied were 12,503 and 11,718 dpm, respectivley.

order to eliminate any radioactivity contribution of *VIII* protein dimers to D protein. The ratio of ^{3}H label in *III* protein to the ^{3}H label in the D protein band, as estimated by oxidizing the corresponding gel segments in a Packard sample oxidizer and scintillation counting, was found to be 8.1–9.5 to 1. This ratio, coupled with the known arginine content of *III* and *VI* protein, allowed us to calculate the copy numbers of *VI* protein. Assuming 5 copies of *III* protein (4, 5), a value of 4.7 ± 0.5 was found for *VI* protein in wild-type and *am*7H2 mutant phages (Table 1).

In a similar way we estimated the copy numbers of *VII* protein from [^{3}H]aspartic acid-labeled phages and of *IX* protein from [^{3}H]tryptophan-labeled phages. As shown in Table 1, the estimated values were 3.3 and 3.5 copies for *VII* and *IX* protein, respectively, which suggests that both proteins are present in phage particles in equimolar amounts. With [^{3}H]valine labeled phages a value of 7.6 and with [^{3}H] arginine-labeled *am*7H2 phages a value of 6.2 was found. Because genes *VII* and *IX* in these phages have identical numbers of valine and arginine residues, the latter values represent the sum of *VII* and *IX* protein copies in mature phage particles. They are in fair agreement with the copy numbers found for these small capsid proteins.

Location of C and D protein in M13 virions

Woolford *et al.* (4) and also Goldsmith and Konigsberg (5) clearly demonstrated that the minor capsid protein encoded by gene *III* is located at only one tip of the phage filament. To find out whether the other minor capsid proteins are also located at or clustered near one tip of the filament, we have estimated the copy numbers of these proteins in M13 recombinant phages of various lengths. For this purpose we used the helper-independent phages M13S100, M13S200, and fd106Sm2, the lengths of which were 1.2, 1.5, and 2.0 times the length of wild-type phage particles, respectively. Our results obtained with these phages are presented in Table 1. They show that with increasing lengths of the phage particles the copy numbers do not increase but remain of the same order of magnitude as that in wild-type

Table 1. Copy numbers of *VI*, *VII*, and *IX* proteins in M13 wild-type and chimeric phages

Phage	^{3}H label used	Copy numbers of proteins		
		*VI**	*VII*†	*IX*†
Wild type	Tryptophan	ND	—	3.5 (0.5)
Wild type	Aspartic acid	4.3 (0.7)	3.3 (0.4)	—
Wild type	Valine	ND	3.8	3.8
AM7H2	Arginine	5.4 (0.1)	3.1 (0.1)	3.1 (0.1)
Wild type	Arginine	4.3 (0.3)	3.8 (0.4)	3.8 (0.4)
M13S100	Arginine	4.1	3.3	3.3
M13S200	Arginine	3.9 (0.3)	3.2 (0.2)	3.2 (0.2)
fd106Sm2	Arginine	3.2 (0.5)	2.9 (0.3)	2.9 (0.3)

* The values given are calculated from the determined amounts of ^{3}H label in the D and *III* protein bands. Radioactivities were determined from several independent phage preparations and the molar ratio between D and *III* protein was calculated on the basis of the known number of amino acid residues as deduced from the DNA sequence (2). The copy number of *VI* protein was then derived by multiplying the molar ratio by the copy number of *III* protein. The latter value is assumed to be 5 on the basis of data of Woolford *et al.* (4) and Goldsmith and Konigsberg (5).

† Copy numbers of *VII* and *IX* proteins were estimated as described above. The values obtained with arginine- and valine-labeled phages are based on the assumption that these proteins are present in virions in equimolar amounts as evidenced by the experiments with tryptophan and aspartic acid. Parentheses indicate SEM. ND, not determined.

particles. Our interpretation of these data is that an insertion of C and D protein molecules in a periodical array along the longitudinal axis of the filamentous protein tube is very unlikely. On the basis of the constant ratios of *III* protein to C and D protein in longer filaments, we infer that the minor capsid proteins encoded by genes *VI*, *VII*, and *IX*, although not located *per se* on different ends, are clustered on the tips of the filamentous phage particles.

DISCUSSION

Despite our detailed knowledge of the molecular biology of the single-stranded filamentous DNA phages, the process of virion assembly on the host cell membrane is still a largely unsolved problem. Although there exists indirect evidence that genes *VI* and *VII* are involved in this process, further research in this field was greatly hampered by the fact that the products of these genes were still completely unknown (3, 13–16). Recently, their identification has been greatly facilitated as the expected sizes of these proteins and their primary sequence have been deduced from nucleotide sequence data (2, 17, 26). Similarly, from the DNA sequence the existence of a new gene, gene *IX*, has been postulated (17, 26). Our data presented here demonstrate that the products of genes *VI*, *VII*, and *IX* are minor constituents of the phage coat, where they are present in only a few copies per virion. These data are based on direct sequence analysis of wild-type proteins and were confirmed, where possible, by specific amino acid substitutions to be expected from conditionally lethal phage mutations. An identical conclusion has recently been reached by Lin *et al.* (27). Their data on f1 are in fair agreement with our data on M13, which strengthens the argument for the presence of these minor capsid proteins in all filamentous phages of this type.

Our Edman degradation studies further demonstrate that the proteins encoded by genes *VI*, *VII*, and *IX* are not subject to proteolytic cleavage at their amino terminus as is the case with *III* and *VIII* protein but are packaged into mature virions as their primary translational products. An intriguing aspect is that these minor capsid proteins can be degraded only after treat-

ment with 3 M HCl, a procedure that characteristically has been applied for deformylating the amino termini of proteins. Until now we could not exclude the possibility that some obscure circumstances have shielded a free amino terminus and, consequently, simulated the presence of an *N*-formyl group in these proteins. Because such a blocking or shielding was not apparent during degradation of similarly isolated B protein, our observation argues for an *N*-formylated status of these three proteins in M13 virions. With respect to the amino terminus of *VII* and *IX* protein, our data are less clear. They suggest but do not prove that these proteins bear a formylmethionine residue. In accordance with this is our finding of a phenylthiohydantoin derivative of [^{35}S]methionine after the first degradation step and the failure to degrade [^{35}S]methionine or [^{3}H]tyrosine-labeled C protein prior to 3 M HCl treatment. Not in accordance is the radioactivity at position 1 in [^{3}H]aspartic acid-labeled protein. A definite conclusion on the amino-terminal status of these proteins awaits further investigations.

Data are now also available on the location and possible function of these three minor capsid proteins. In this study evidence is presented that these proteins, like the *III* protein, are also located at either one or both ends of the phage filament. By shearing normal length fd and f1 phages and treating the mixture with anti *III* protein antibodies, Grant *et al.* (25) very recently provided evidence that D protein is located at the *III* protein end of the phage and, consequently, that C protein is at the opposite end. Such a clustering of *VI* and *III* proteins and of *VII* and *IX* proteins is of great biological importance because their synthesis is under strict control. Genes *III* and *VI* belong to one operon (10), whereas genes *V* and *VII* form a second operon (28). Moreover, recent protein synthesis studies with several M13 amber mutants have shown that gene *IX* protein synthesis also is controlled by gene *V* expression (unpublished data). An overall picture emerges that the four minor capsid proteins of the F-specific filamentous phages can be divided in two functional groups. One consists of the products of one operon (*III* and *VI*), which trigger the entry process of phage DNA into the host cell but also its reversed process—i.e., the final stages of packaging the DNA when it leaves the cell—whereas the other group, composed of products of the other operon (*VII* and *IX*), most probably directs the very early events of chanelling the *V* protein–DNA complex through the host cell membrane. Our primary goal is to study the regulation of synthesis of these minor capsid proteins.

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