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The major coat protein gene of the filamentous *Pseudomonas aeruginosa* phage Pf3: absence of an N-terminal leader signal sequence

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ABSTRACT

From *in vitro* protein synthesis studies and nucleotide sequence analysis it has been deduced that, unlike the major coat proteins of the hitherto studied filamentous bacterial viruses Ff (M13, fd and f1), IKe and Pf1, the major coat protein of the filamentous *Pseudomonas aeruginosa* virus Pf3 is not synthesized as a precursor containing a leader signal polypeptide at its N-terminal end. From the elucidated nucleotide sequence of the Pf3 major coat protein gene it follows that the coat protein is 44 amino acid residues long (mol.wt. 6425). No sequence homology was observed with the major coat protein genes of either the Ff group or IKe but, similar to these phages, 3' ward of the Pf3 coat protein gene a DNA sequence is located which has many characteristics in common with *rho*-independent transcription termination signals.

INTRODUCTION

Filamentous bacterial viruses are simple models for studying processes of molecular assembly. The viruses consist of a closed loop of single-stranded DNA (6000-7500 nucleotides) encapsulated in a sheath of several thousand identical major coat protein subunits. These molecules are of low molecular weight consisting of about 50 amino acid residues (for a review, see ref.1). The ends of the viral filament are distinguished by the presence of a few copies each of one or more minor coat proteins (2-6).

Two structural classes have been defined on the basis of X-ray fiber diffraction patterns of the virions (7-9). Class I includes the F-specific filamentous bacterial viruses Ff, the N-specific virus IKe and the I-specific virus If1. They all have *Escherichia coli* as their host. Class II includes the viruses Pf1 and Pf3, which infect *Pseudomonas aeruginosa* strains K and O, respectively, and Xf, which infects the plant bacterium *Xanthomonas oryzae*. Pf3 only infects cells which harbour the IncP1-specific broad host-range plasmid RP1 (10).

The Ff viruses M13, fd and f1 have been shown to be closely related (11-

13). The DNA of these viruses reveal a mutual homology of about 97%, clearly indicating that they are derived by mutation from a common ancestor. Although the nucleotide sequence of the IKe genome shows a great divergence from that of the Ff group (overall homology 45%)(14), our data nevertheless indicate unambiguously that also IKe and the Ff viruses have evolved from a common ancestor (*cf.*15). Whether the class II viruses, with their different host requirements, DNA compositions and probably also different phage assembly processes, are also descendants from this common ancestor is still very speculative.

With these considerations in mind we have initiated a study towards the structural and genetic organisation of the Pf3 genome. A different genetic organisation might underlie a different process of phage assembly at the host cell membrane. The Ff viruses have a large number of their major coat protein subunits inserted into the membrane. These molecules subsequently displace the DNA-binding proteins from their intracellular complex with DNA, during assembly and transport of the virus out of the cell. The major coat proteins of the Ff virus group are all synthesized in the infected cell in a precursor form (17,18). Concomitant with deposition into the inner cell-membrane a leader peptide of 23 N-terminal amino acid residues is cleaved off (18,19). A similar but not identical precursor-membrane protein relationship has recently been found for the other *E.coli* viruses IKe (14) and If1 (D.Hill & G.B.Petersen, personal communications) and the *P.aeruginosa* virus Pf1 as well (unpublished data), suggesting that all filamentous viruses are similar in this fundamental aspect.

In this study we present the nucleotide sequence of the major coat protein gene of Pf3 and its adjacent signals for transcription and translation. Our results demonstrate that the major coat protein of Pf3, though of low molecular weight (44 amino acid residues), is not synthesized in a precursor form, indicating that deposition of this protein into the host cell membrane is determined by structural parameters different from that of the other filamentous viruses.

MATERIALS AND METHODS

Materials

T4 DNA ligase and the restriction endonucleases used were obtained from Bethesda Research Laboratories. *E.coli* DNA polymerase I (large fragment) was from Boehringer AG. All enzymes were used under the conditions specified by the supplier. 2'-3' dideoxyribonucleoside triphosphates were purchased from

PL-Biochemicals. (α-^{32}P)dATP (410 Ci/mmol), ^{35}S-sulphate (965 Ci/mmol) and the ^{35}S- and ^{3}H-labelled amino acids at the highest specific activity available were obtained from Amersham. PEG-6000 was from BDH. All other chemicals were of analytical grade.

Bacteria and bacteriophages

Bacteriophage Pf3 and its host *P.aeruginosa* PAO1, harbouring the broad host-range plasmid RP1 of the incompatibility group P1, were kindly provided by Dr.D.E.Bradley, St.John's, Newfoundland. The phages M13 and IKe and their respective host bacteria were from our own collection.

Pf3 Phage Growth and isolation of Replicative Form DNA

P.aeruginosa PAO1 cells were grown in R-medium (20) until early log phase and infected with Pf3 bacteriophages at a multiplicity of infection of 10. After incubation for 6 hours at 37°C, cells were harvested by centrifugation and the Pf3 phage particles were concentrated from the supernatant by the addition of PEG-6000 and NaCl to final concentrations of 5% and 0.5 M, respectively. Phages were further purified by CsCl density gradient centrifugation in 5 mM sodium borate, pH 9.0 (21).

Pf3 Replicative Form DNA (RF) was isolated from infected cells essentially as described by Humphreys *et al.*(22) and subsequently purified by two successive CsCl density gradient centrifugations.

In vivo labelling of Pf3 bacteriophages

To obtain ^{35}S-labelled Pf3 coat proteins a fresh plaque was resuspended in 10 ml of M9-medium containing $MgCl_2$ instead of $MgSO_4$ (23). When the culture entered the early log phase 1 mCi of ^{35}S-sulphate was added and the incubation was continued overnight at 37°C. Subsequently, the labelled Pf3 viruses were isolated and purified as described above.

In vitro Protein synthesis and Analysis of the Products

The procedures for the preparation of the DNA-dependent cell-free protein synthesizing system of *E.coli* and the techniques for SDS-polyacrylamide gel electrophoresis and fluorography have been described previously (24).

Molecular cloning and DNA sequencing techniques

Restriction enzyme cleavage maps were constructed as described previously (25). Cloning of isolated restriction fragments in the phage vectors M13mp8 and M13mp9 (26) was by standard methods. Sequencing of the DNA inserts was carried out according to the dideoxy chain-termination method developed by Sanger *et al.*(27) using an 18 bases-long sigle-stranded universal primer. Nucleic acid sequences were analysed using the computer programs written by

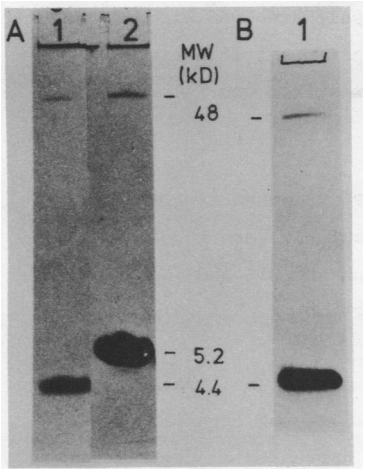

Fig. 1. (A) Electrophoretic analysis of the polypeptides present in Pf3 (lane 1) and M13 (lane 2) virions followed by staining of the gel with Coomassie Brilliant Blue.
(B) Fluorograph of ^{35}S-labelled polypeptides present in Pf3 virions.

Staden (28). Protein hydrophobicity was calculated according to Kyte and Doolittle (29).

RESULTS

To gain information about the protein composition of the Pf3 virion filament, virions were propagated in *P.aeruginosa* cells in the presence or absence of ^{35}S-sulphate. The capsid proteins were isolated from the purified virions and subsequently size-fractionated on SDS-polyacrylamide gels. The proteins were vizualized on the gel by staining with Coomassie Brilliant Blue or with the aid of fluorographic techniques. As shown in Fig. 1, congruent results were obtained. In both cases it was found that the Pf3 virion is composed of two different polypeptide chains. The major component has a molecular weight of about 4500 whereas the minor protein component has an apparent molecular weight of 46,000. In accordance with the nomenclature of other filamentous phages, the major and minor component have further been denoted as gene VIII- and gene III-protein, respectively. In contrast to Ff viruses (2-6), no evidence was obtained so far from our electrophoretic analysis for the presence of other minor coat proteins in Pf3 virions.

Previously we have demonstrated that the major coat protein of M13 is synthesized both *in vitro* as well as *in vivo* as a precursor molecule with an extra leader- or signal sequence of 23 amino acid residues at its amino-terminal end (11,18). Similar observations have been made for fd and f1 (12, 13,18,19) and quite recently also for phage IKe (14) and Pf1 (R.Konings, unpublished data). To find out whether this is also true for Pf3, or more

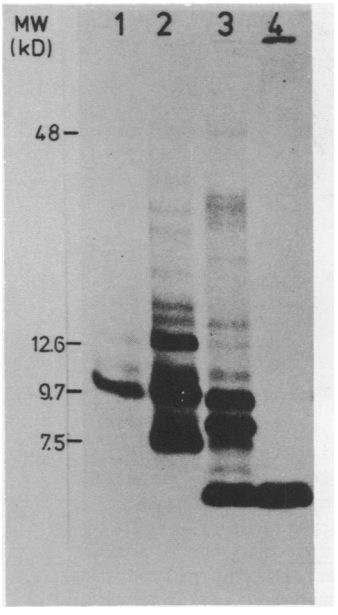

Fig. 2. Fluorograph of ^{35}S-methionine labelled polypeptides synthesized in a DNA-dependent *in vitro* protein synthesizing system of *E.coli*, in the absence of exogenous DNA (lane 1) or the presence of the replicative form DNAs of the phages M13 (lane 2) and Pf3 (lane 3) respectively. In lane 4 ^{35}S-labelled Pf3 major coat protein was run in parallel.

generally whether this is a general property of filamentous viruses, we have investigated the major coat protein synthesis in more detail. For this purpose, protein synthesis studies were carried out in a coupled transcription-translation system of *E.coli* which was programmed by the replicative form I DNA of Pf3. As shown in Fig. 2, the genome of the *P.aeruginosa* phage Pf3 is expressed efficiently in the *E.coli* cell-free system. At least eleven polypeptides ranging in size from 4500 to about 50,000 are formed of which the smaller polypeptides are the most predominant. A similar pattern, in which particularly the products of genes V and VIII are most abundantly present, has also been obtained during our studies on the *in vitro* synthesis of the proteins encoded by the replicative form DNAs of phage M13 an IKe (14,17,30) (*cf.* Fig. 2). The 9kD protein encoded by Pf3 RF co-migrates on the SDS-poly acrylamide gel with one of the major Pf3 encoded polypeptides synthesized in the infected cell that preferentially binds to single-stranded but not to double-stranded DNA (data not shown). The 4.5 kD protein co-migrates exactly with the major coat protein present in Pf3 virions.

To obtain evidence whether the latter two polypeptides are identical, and thus whether they are products of the same gene, we have studied Pf3 RF-directed *in vitro* protein synthesis in the presence of the ^{3}H-labelled amino acids tyrosine, proline and cysteine, which we knew from our amino acid

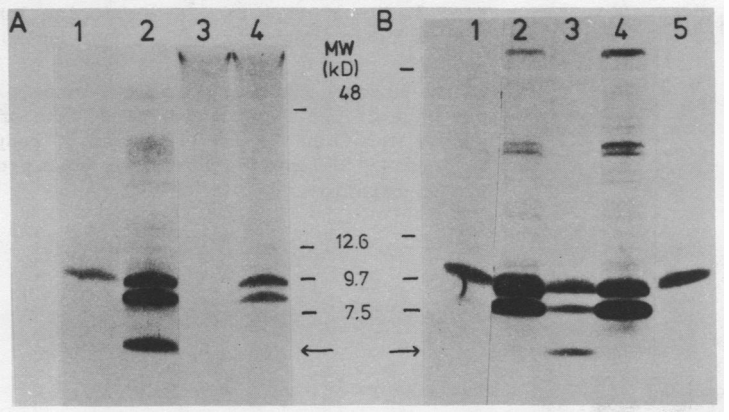

Fig. 3. (A) Fluorograph of the polypeptides synthesized *in vitro* in the absence (lanes 1 and 3) or presence of replicative form DNA of phage Pf3 (lanes 2 and 4). The polypeptides were labelled with ^{35}S-methionine (lanes 1 and 2) and ^{35}S-cysteine (lanes 3 and 4) respectively.
(B) Fluorograph of the polypeptides synthesized *in vitro* in the absence (lanes 1 and 5) and presence of Pf3 RF (lanes 2, 3 and 4). The polypeptides were labelled with ^{3}H-proline (lanes 1 and 2), ^{35}S-methionine (lane 3) and ^{3}H-tyrosine (lanes 5 and 6) respectively. The arrows refer to the position of migration of Pf3 major coat protein (*cf.* Fig. 2).

analysis data to be absent in the Pf3 coat protein. As shown in Fig. 3, under these labelling conditions the *in vitro* synthesis of the 4.5 kD polypeptide can no longer be demonstrated. From these data and the observation that both proteins have identical electrophoretic mobilities we have concluded that the major coat protein and the 4.5 kD protein are products of the same gene and that, in contrast to the major coat proteins of the other filamentous phages, the major coat protein of the Pf3 virion is not synthesized as a precursor molecule. The latter conclusion is strengthened by our following observation. It is well known that the *in vitro* protein synthesizing system is not capable of processing primary translation products unless signal peptidase is liberated from residual membrane fragments by the addition of non-ionic detergent (17,19). Pre-addition of such compounds to the system, however, did not alter the electrophoretic mobility of the *in vitro* synthesized 4.5 kD protein (data not shown).

To obtain information in which respect the structural and biochemical characteristics of the Pf3 major coat protein differ from those of the major coat proteins of the class I filamentous viruses, we have elucidated the amino acid sequence of the Pf3 coat protein by nucleotide sequence analysis of its corresponding gene. To this end restriction enzyme cleavage maps of

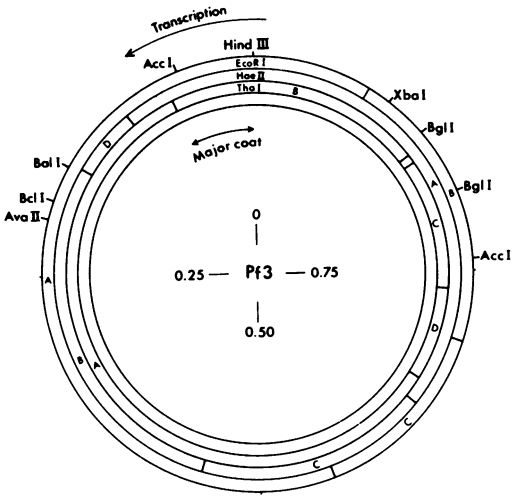

Fig. 4. Restriction enzyme cleavage maps of Pf3 replicative form DNA. The region coding for the major coat protein and the direction of transcription are indicated. One map unit corresponds to 5833 basepairs.

Pf3 RF were established (Fig. 4). Subsequently, the gene coding for the major coat protein was localized with the aid of restriction fragment directed *in vitro* protein synthesis studies (17, 24). The major coat protein gene was found to be located on the smallest fragment obtained after cleavage of fragment *Tha*I-B with *Hin*dIII (Fig.4). This fragment, which is about 410 basepairs long, was then subcloned in the phage vectors M13mp8 and M13mp9 which had been digested with both *Hin*dIII and *Sma*I. The nucleotide sequence was established by the dideoxy chain-termination sequencing technique (27) using an 18 bases long universal primer. The deduced nucleotide sequence of the Pf3 major coat protein gene is presented in Fig. 5.

Inspection of this sequence revealed that there is only one open reading frame present. This sequence starts with the ATG codon at position 120 and is 132 nucleotides long (termination codon TGA at position 252). It has the potential to to code for a polypeptide which is 44 amino acids long and in which the amino acid residues tyrosine, proline, cysteine, glutamic acid and asparagine are absent. The size and deduced amino acid sequence of this polypeptide are in excellent agreement with the molecular weight and the amino acid composition established for the Pf3 major coat protein and with the data from our *in vitro* protein synthesis studies. We therefore conclude that this open reading frame represents the nucleotide sequence of the major coat protein gene. Reading in phase from the ATG codon at position 120 in the 5' direction the first nonsense (TAA) codon is already found at position 96 which, in turn, excludes the possibility that the major coat protein is

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                aagcttgccggaaggttcaggcttgcaaattggcgggatgttatt
                1

gctactttccgccaccgcttggggtattcaacaaatagcccgtttacttttaaatcgtt
   50                                                  100
    SD          Met Gln Ser Val Ile Thr Asp Val Thr Gly Gln
gatgaggtgtctttt ATG CAA TCC GTG ATT ACT GAT GTG ACA GGC CAA
                                                       150
Leu Thr Ala Val Gln Ala Asp Ile Thr Thr Ile Gly Gly Ala Ile
CTG ACA GCG GTG CAA GCT GAT ATC ACT ACC ATT GGT GGT GCT ATT

Ile Val Leu Ala Ala Val Val Leu Gly Ile Arg Trp Ile Lys Ala
ATT GTT CTG GCC GCT GTT GTG CTG GGT ATT CGC TGG ATC AAA GCG
200
Gln Phe Phe ***
CAA TTC TTT TGA tccgtccttgggcttttggcctcaatcgttataaggggcttc
      250

ggctcccttattcgtttagcggctaaaatttttcaattcacggggctttatggagatt
300                                                  350

atggaatgggtctacattatttatttgggttttgtcttgccctttttctttttccgcg
                                              400
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Fig. 5. Nucleotide sequence of the 410 basepairs long *Tha*I-B/*Hin*dIII fragment encoding the Pf3 major coat protein. Numbering starts at the first nucleotide of the *Hin*dIII cleavage site (*cf.* Fig. 4).

synthesized with an amino-terminal signal sequence of about 15-30 residues as found for the major coat proteins of other filamentous phages. Another, though very unlikely candidate for translational initiation would be the GTG codon two triplets upstream the ATG codon. If so, the major coat protein synthesized *in vitro* would clearly have been discriminated from that present in intact Pf3 virions on the SDS-polyacrylamide gel systems used. Moreover, applying the "perceptron algorithm" derived matrix as published by Stormo *et al.* (31) to distinguish translational initiation sites, unambiguously indicated that the ATG codon is the only candidate for translational initiation.

DISCUSSION

The coat of the filamentous *Pseudomonas aeruginosa* phage Pf3 appears to be composed of at least two polypeptides of which the major one has a molecular weight of 4600 while that of the minor component is 46,000. In this respect Pf3 resembles the other known filamentous phages which have their DNA encapsulated in a sheath of several thousand identical coat proteins (ranging in size from 44 to 53 amino acids) and which ends in a few copies

of a high molecular weight protein. In Ff viruses this minor coat protein (gene III-protein, mol.wt. 44,750) mediates productive infection of the virus to its bacterial host (32-35) by adsorption to the F-pilus. We assume that the 46 kD protein present in Pf3 virions exerts a similar function by mediating adsorption of the virus to the Inc-P1 encoded pili of *P.aeruginosa* PAO1 cells.

For the major coat proteins of the filamentous phages studied so far it has been demonstrated that they are transmembrane proteins and that they are proteolytic cleavage products of precursor molecules, so called pre-coat, containing a hydrophobic amino-terminal leader peptide. This signal peptide is cleaved off by the signal peptidase concomitant with the deposition of the mature coat protein in the inner cell membrane (19,32). The data presented in this study demonstrate, however, that such a deposition mechanism is not valid for the major coat protein of Pf3. In contrast to the F- and N-specific filamentous *E.coli* phages, the primary translation product of the major coat protein gene of the *P.aeruginosa* phage Pf3 is not proteolytically cleaved but assembled as such in the mature virion.

To find out in how far the basic protein structure of the Pf3 major coat protein differs from that of the class I viruses, the nucleotide sequence of the coat protein gene was established. The amino acid sequence predicted from the DNA sequence confirmed that the major Pf3 coat protein is only 44 amino acid residues long and that its real molecular weight is 4625. Moreover the amino acid sequence found was completely consistent with the data incorporated in a recent report (16) describing the differences and similarities among filamentous phages. When the nucleotide sequence of the and its border regions is compared with the corresponding gene VIII of the other filamentous phages, *i.e.* the Ff phages and IKe, it is evident that they are completely different. Within the coding sequence of the M13 and IKe genes several long stretches of mutual sequence homology were noted which is consistent with our conclusion that these DNA genomes, though diverged considerably, are derived from a common ancestor (14,15). No regions of homology were found, however, between either one of these *E.coli* phages and Pf3 suggesting that Pf3 virus is derived from an independent ancestral virus.

Interesting structural homology is noted 3' ward of the major coat protein gene. Immediately distal to gene VIII of the filamentous phages a transcription termination signal is located, consisting of a G-C rich region which has the potential to form a stable stem-loop structure followed by a string of T-residues (14,36,37). A nucleotide sequence with

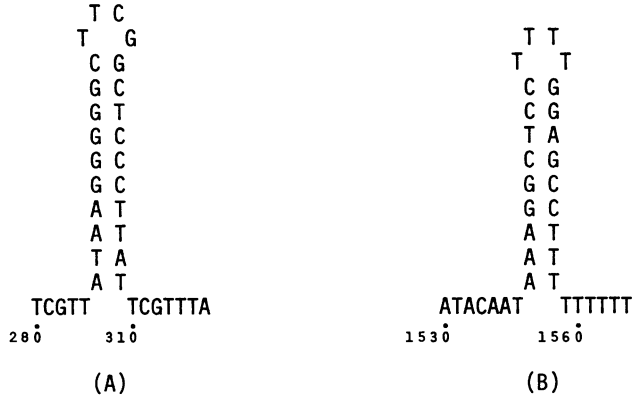

Fig. 6. Structural similarity of the DNA region located 3' ward of the Pf3 major coat protein gene (A) and the *rho*-independent transcription termination signal of M13 (B).

similar secondary structure characteristics (Fig. 6) is found at position 285 through 314 of the Pf3 DNA sequence. Since in Pf3 infected cells several phage RNA transcripts are formed which have terminated at this site (unpublished data) we infer that this DNA sequence represents a *P.aeruginosa*- specific transcription termination signal.

For the F-specific filamentous viruses it has been demonstrated that within the major coat protein a "stop-transfer" sequence is present which, after partial vectorial discharge of the nascent polypeptide through the inner cell membrane, is responsible for anchoring of this coat protein molecule in the membrane. This stop-transfer signal consists of a region of approximately 18 hydrophobic amino acids followed at its carboxy-terminal end by several charged residues (38,39). Inspection of the Pf3 coat protein sequence indicates that also in this molecule a region with similar characteristics and probably identical function is present (Fig 7). Which region of the Pf3 coat protein is responsible for its vectorial discharge through the cell membrane is by no means clear. It might well be that the signals for insertion and stop-transfer overlap each other. If true, the amino-termini of the mature coat proteins of the viruses Ff and Pf3 almost certainly have a different orientation with respect to the cytoplasmic side of the inner cell membrane. This in turn will have significant consequences for the morphogenetic reactions occurring during the transition of the Pf3 major coat protein from the membrane-bound to the DNA-bound state. With respect to the mechanism of insertion of the major coat protein of Pf3 into

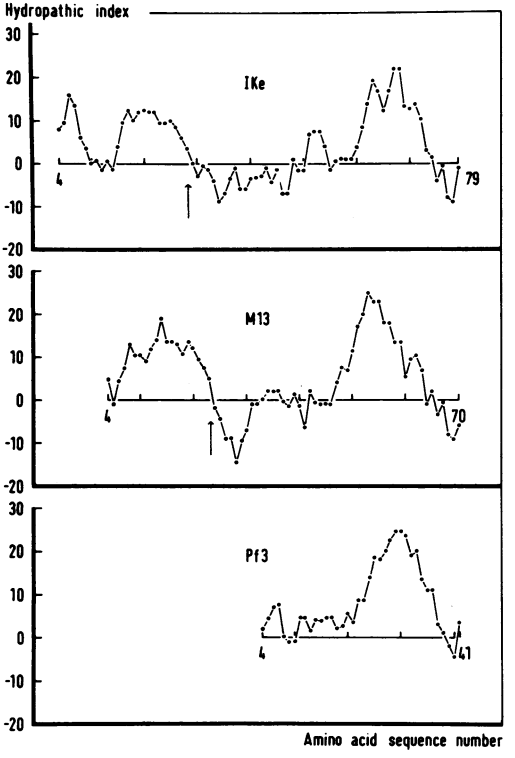

Fig. 7. Hydropathic index profiles of the major coat proteins of IKe, M13 and Pf3. The proteolytic cleavage sites in the primary translation products of gene VIII of IKe and M13, leading to the mature coat proteins, are indicated (arrow). The sequences have been aligned by their C-termini to emphasize the similar distribution of hydrophobicity in the proteins.

the cellular membrane a final remark should be made. Protein synthesis studies have indicated that approximately 40 amino acids of the carboxy terminal end of the nascent polypeptide chain are contained within the ribosome. This, together with our observations that the primary translation product of the major coat protein gene of Pf3 is only 44 amino acids long and that it does not contain a signal peptide, suggests that, in contrast to the major coat protein of the Ff-phage, the major coat protein of Pf3 is not inserted into the membrane *co*-translationally but instead *post*- translationally.

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