# SYNTHESIS OF PHAGE M13 SPECIFIC PROTEINS IN A DNA-DEPENDENT CELL-FREE SYSTEM*

R.N.H. KONINGS

*Laboratory of Molecular Biology, University of Nijmegen, Nijmegen, The Netherlands*

Received 21 May 1973

## 1. Introduction

M13, a filamentous coliphage, is composed of a single-stranded circular DNA molecule (mol. wt. $1.7 \pm 0.2 \times 10^6$) in a capsid of about 2000 molecules (mol. wt. 5200) coded by gene 8 and a few molecules of protein (mol. wt. 70 000) specified by gene 3 [1, 2, for a review see 3]. The DNA of this phage contains the genetic information for about nine medium sized proteins. Thus far eight genes have been identified [2, 4, 7], the gene order of which has been described recently [5].

After infection of an *Escherichia coli* cell with this phage, not all phage encoded proteins are synthesized in equal amounts [6]. The proteins specified by gene 5 and by gene 8 are synthesized in much larger quantities than the other phage encoded proteins. This suggests that there must be some mechanism(s) to ensure that these proteins are synthesized more frequently than the other phage specific proteins. In order to obtain more information about these regulatory mechanism(s) we have developed a DNA-dependent *in vitro* system.

In this paper it will be demonstrated that phage M13 Replicative Form DNA (RF-DNA) directs the *in vitro* synthesis of at least the proteins specified by gene 5 and by gene 8. Although under the direction of RF-I DNA several other proteins are still synthesized, their gene relationship has not been established yet. As has been observed *in vivo* [6], the proteins encoded by gene 5 and by gene 8 are also synthesized *in vitro* in much larger quantities than the other phage specific proteins. This result will be discussed in relation to the regulatory mechanism(s) which might be responsible for this phenomenon.

* *In vitro* synthesis of the proteins encoded by gene 5 and by gene 8.

## 2. Materials and methods

### 2.1. *Bacteria and phages*

*E. coli* strain AB301 Hfr (λ) met$^-$, RNAase I$^-$ was used both for the preparation of the cell-free extract as well as for the cultivation of wild-type M13 phages. *E. coli* K37, the permissive host for M13 amber mutants, and *E. coli* K38, the non-permissive host, were obtained from Dr. D. Pratt. *E. coli* K12.159F$^+$, UV$^s$, Su$^-$ was used for the cultivation of $^{35}$S-labeled M13 phages.

Phage M13 was obtained from Dr. P.H. Hofschneider, Munich. The M13 nonsense mutant *am*8-H1, the isolation and characteristics of which have been described [2, 4, 6, 7], was a gift from Dr. D. Pratt.

### 2.2. *Materials*

These were obtained from sources given previously [8, 9].

### 2.3. *Preparation of radioactive viral proteins for polyacrylamide gel electrophoresis*

To prepare M13 labeled with radioactive sulfur, *E. coli* K12.159F$^+$ was grown on M9 medium containing $MgCl_2$ in place of $MgSO_4$. When the culture had reached a density of $1 \times 10^8$ cells/ml radioactive sulfate (100 μCi/ml) was added. At a density of $3 \times 10^8$ cells/ml the culture was infected with M13 phages (multiplicity of infection of 10). The infected culture was incubated for an additional 4 hr, after which time the cells were removed by centrifugation. The virus was purified from the supernatant as described by Yamamoto et al. [11].

$^{35}$S-labeled M12 phages were grown essentially as described for the cultivation of radioactive labeled phage M13. The infected cells were incubated for 4 hr, after which time the cells were lysed by the method of Fromageot and Zinder [10]. After centrifugation at low speed, the phages were purified from the supernatant as described by Yamamoto et al. [11].

[$^{14}$C]leucine labeled phage M13 and $^{14}$C-labeled gene 5 protein (mol. wt. 10 000) were generous gifts from Dr. K. Wanner, Max Planck Institut für Biochemie, Munich.

Marker proteins for polyacrylamide gel electrophoresis were prepared by solubilisation of the respective labeled phages or gene 5 protein in 0.01 M sodium phosphate buffer (pH 7.2) containing 1% sodium dodecyl sulfate, 1% 2-mercaptoethanol, 20% glycerol and 8 M urea. After dissolution, the sample was heated for 3 min at 100°C.

2.4. *In vitro protein synthesis*

The method of preparation of the cell-free extract has been described previously [12]. The reaction mixture contained per ml: 2.5 μCi of either $^{14}$C-labeled amino acid mixture, [$^{14}$C]arginine of [$^{14}$C]tryptophan; 100 μmol of each of the other non-labeled amino acids; 12.5 μmol $Mg^{2+}(CH_3COO^-)_2$; 67.5 μmol Tris-Cl (pH 7.8); 100 μmol $NH_4Cl$; 6 μmol 2-mercaptoethanol; 100 μg tRNA; 0.12 μmol leucovorin; 1.6 μmol ATP; 0.4 μmol of each GTP, CTP and UTP; 16 μmol phosphoenol pyruvate; 20 μg pyruvate kinase and 0.25 ml of the pre-incubated cell-free extract. The amounts of DNA added varied between 50 and 100 μg per ml.

After incubation for 40 min at 37°C, to each 60 μl incubation-mixture, 10 μl 0.2 M EDTA (pH 7.0) and 10 μl pancreatic RNAase (1 mg/ml) was added. After an additional incubation for 15 min at 37°C, the protein

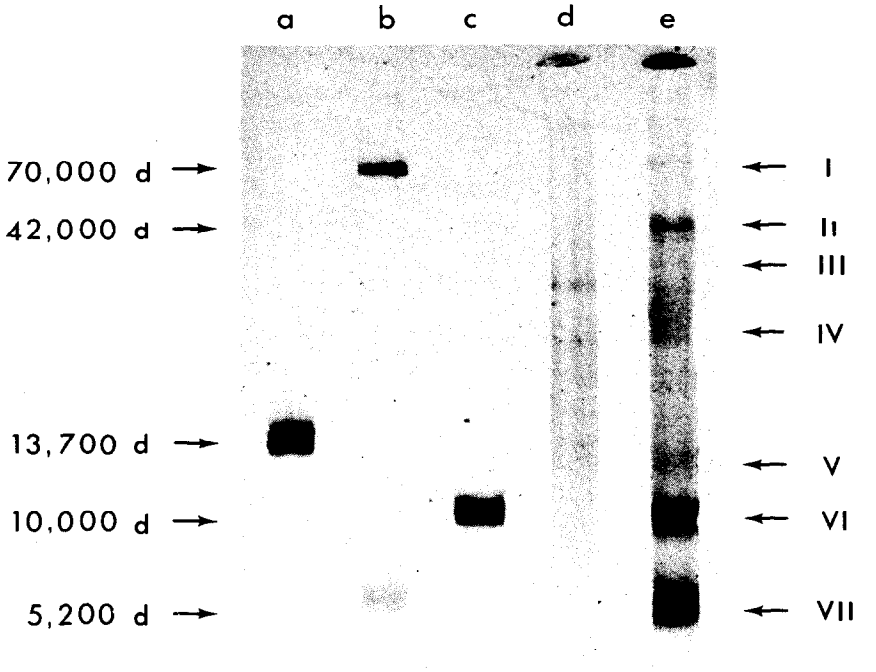


Fig. 1. Autoradiogram of the polypeptides synthesized *in vitro* under the direction of M13 RF-I DNA and separated by SDS-polyacrylamide gel electrophoresis. a) Phage M12 proteins (A-protein, mol. wt. 42 000; coat protein, mol. wt. 13 700) serving as molecular weight markers; b) phage M13 marker proteins labeled with radioactive sulfur; c) gene 5 marker protein; d) products of the endogenous system; e) products synthesized under the direction of M13 RF-I DNA. Incubation was performed in the presence of radioactive labeled amino acid mixture.

was precipitated with $CCl_3COOH$. The precipitate was washed twice with $CCl_3COOH$ and twice with acetone. The proteins were dissolved finally in a small volume of 0.01 M sodium phosphate buffer (pH 7.2) containing 1% sodium dodecyl sulfate, 1% 2-mercaptoethanol, 20% glycerol and 8 M urea. After dissolution the sample was heated for 3 min at 100°C. The proteins were analysed on 10% SDS-polyacrylamide gels [6].

2.5. *Preparation of M13 Replicative Form DNA*

The method of preparation of wild-type RF-I DNA and of RF-I DNA, having an amber mutation in gene 8, will be described elsewhere (Konings et al., in preparation).

## 3. Results

3.1. *Characteristics of the cell-free system*

The rate of incorporation of $^{14}C$-labeled amino acids in $CCl_3COOH$ insoluble material was linear for at least 25 min and reached a plateau after about 40 min of incubation. The efficiency of protein synthesis in the coupled system varied greatly with the ionic conditions. The ammonium chloride concentration was optimal in the range of 70 to 120 mM and the magnesium optimum was rather broad centering on 12.5 mM. Compared with the control, the relative stimulation of amino acid incorporation was strongly dependent on the type of $^{14}C$-amino acid added.

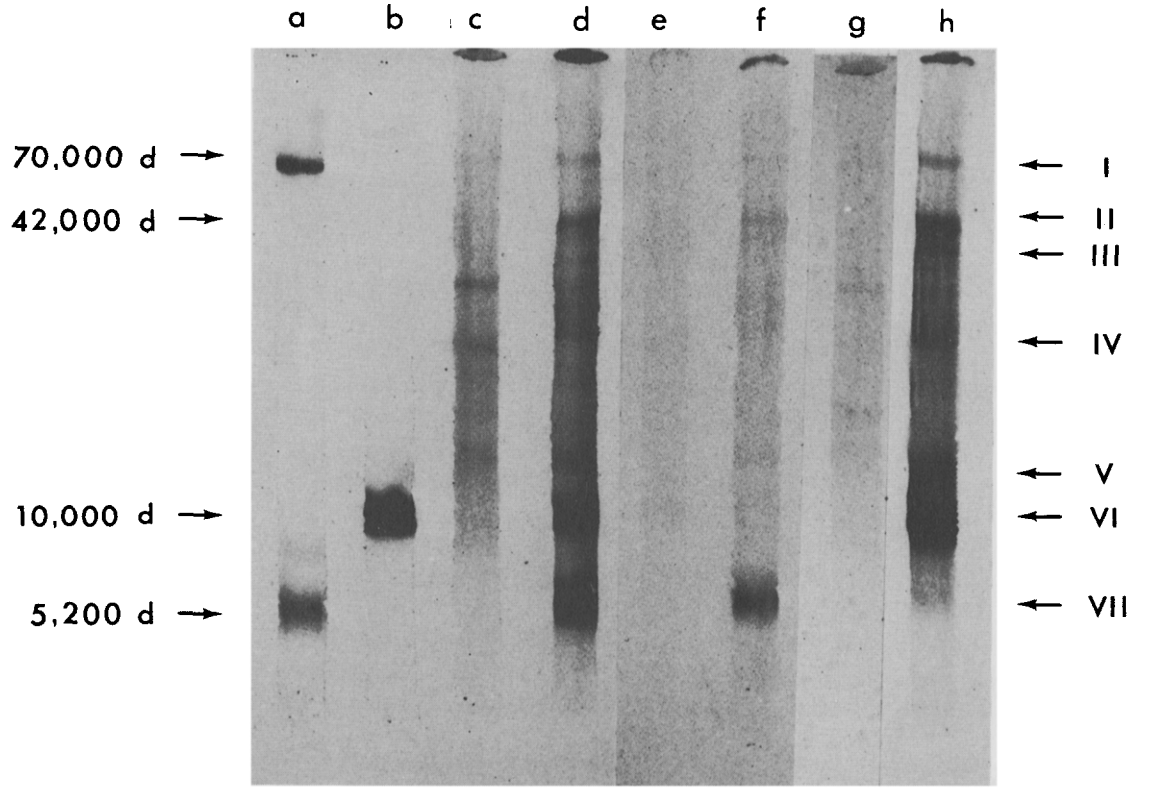


Fig. 2. Autoradiogram of the polypeptides synthesized *in vitro* under the direction of M13 RF-I DNA and separated by SDS-polyacrylamide gel electrophoresis. a) Phage M13 marker proteins; b) gene 5 marker protein; c) products of the endogenous system synthesized in the presence of radioactive labeled amino acid mixture; d) products synthesized under the direction of M13 RF-I DNA in the presence of radioactive labeled amino acid mixture; e) products of the endogenous system synthesized in the presence of radioactive labeled tryptophan; f) products synthesized under the direction of M13 RF-I DNA in the presence of radioactive labeled tryptophan; g) products of the endogenous system synthesized in the presence of radioactive labeled arginine; h) products synthesized under the direction of RF-I DNA in the presence of radioactive labeled arginine.

Maximal stimulation was obtained when the DNA concentration was between 50 and 100 μg/ml. Due to the high endogenous incorporation, the relative stimulation was not as high as has been described for more purified systems [13]. However, the number of amino acid molecules incorporated per ribosome equalled that of the more purified systems. Furthermore, as will be shown, the background incorporation did not have a disturbing effect on the evaluation of the results. The synthetic activity of the coupled system was destroyed by pancreatic DNAase (2.5 μg/ml). Extra addition of purified *E. coli* RNA-polymerase had no effect on the relative stimulation.

3.2. *Analysis of the in vitro product*

Autoradiographic analysis of the *in vitro* product was performed to determine the size and number of the polypeptides synthesized *in vitro* under the direction of M13 RF-I DNA (fig. 1d and 1e). For comparison, the gel patterns of the hitherto characterised phage specific proteins, specified by gene 3, gene 5 and gene 8, respectively, are also shown (fig. 1b and 1c). It can be concluded from these results that addition of RF-I DNA to the cell-free system resulted in the synthesis of at least seven proteins (I–VII), with molecular weights ranging from 5000 to 70 000 daltons. No equivalent proteins were synthesized in the endogenous system. Three of these *in vitro* proteins (I, VI and VII) have electrophoretic mobilities which are identical with those of the M13 specific marker proteins (fig. 1b and 1c). Since the M13 proteins encoded *in vivo* by the genes no. 1, 2, 4, 6 and 7 have not been identified yet (cf. [3, 6, 7], it is impossible at present to predict a gene relationship for the other M13 DNA directed *in vitro* proteins.

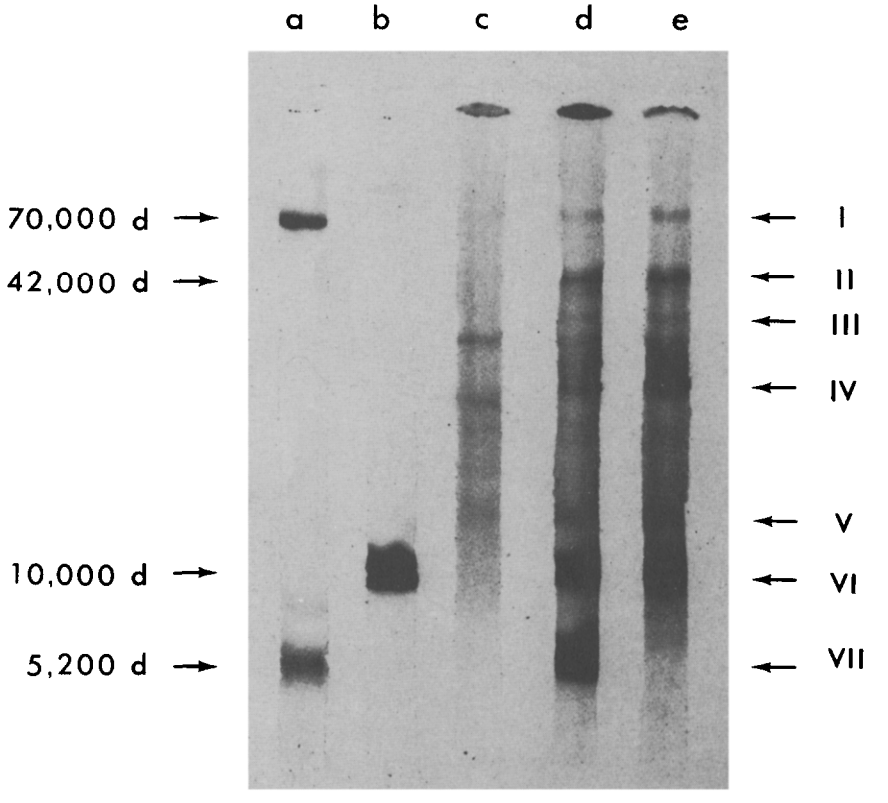


Fig. 3. Autoradiogram of the polypeptides synthesized *in vitro* under the direction of either wild-type RF-I DNA or RF-I DNA having an amber mutation in gene 8 (M13 *am*8-H1). a) Phage M13 marker proteins; b) gene 5 marker protein; c) products of the endogenous system; d) products synthesized under the direction of wild-type RF-I DNA; e) products synthesized under the direction of M13 RF-I DNA having an amber mutation in gene 8 (M13 *am*8-H1). Incubation was performed in the presence of radioactive labeled amino acid mixture.

In order to provide evidence that the *in vitro* synthesized proteins (VI and VII) are identical with the *in vivo* proteins specified by gene 5 and gene 8, respectively, we studied the M13 DNA directed protein synthesis either in the presence of [$^{14}$C]tryptophan (not present in gene 5 protein; 14), or in the presence of [$^{14}$C]arginine and [$^{14}$C]histidine (neither of which are present in gene 8 protein; 15, 16). As shown in fig. 2, no synthesis of a low molecular weight protein co-migrating with gene 5 marker protein could be detected when protein synthesis was studied in the presence of [$^{14}$C]tryptophan (fig. 2e and 2f). Similar results were obtained for the protein co-migrating with the gene 8 marker protein, when protein synthesis was studied in the presence of either [$^{14}$C]arginine (fig. 2g and 2h) or [$^{14}$C]histidine (unpublished results). These results support strongly the conclusion that both low molecular weight *in vitro* synthesized proteins (VI and VII) are identical with the native viral proteins encoded by gene 5 and gene 8, respectively.

Definite proof for the identity of the *in vitro* synthesized proteins can be derived when *in vitro* protein synthesis is studied under the direction of a DNA preparation having an amber nonsense mutation in the gene coding for the protein under investigation. In the latter case, under non-permissive conditions, no protein should be synthesized which co-migrates with the native marker protein. Substitution of RF-I DNA, having an amber mutation in gene 8 (M13 *am*8-H1), for wild-type RF-I DNA in the cell free system resulted in a complete disappearance of the protein which originally co-migrated with the native gene 8 protein (fig. 3d and 3e). This result was independent of the type of $^{14}$C-amino acid used to label the *in vitro* proteins (unpublished results).

It is interesting to note that, instead of the synthesis of gene 8 protein, we were unable to detect the appearance of a new faster migrating polypeptide produced by premature termination of peptide synthesis at the site of the amber mutation. Therefore, it seems more likely that the amber mutation is located proximal to the 5′- rather than to the 3′-end of this mutated gene.

Striking is the fact that M13 DNA still codes for another low molecular weight protein (V, mol. wt. 12 000). This protein can be excluded as an aggregation product of either gene 5 or gene 8 protein, since the synthesis of this protein can also be detected when protein synthesis is studied in the presence of either [$^{14}$C]tryptophan (fig. 2f) or [$^{14}$C]arginine (fig. 2h, cp. fig. 3e). In contrast to a study published previously [6], both from our studies on the proteins present within the virion as well as from our *in vitro* studies, we did not find any indication for the existence of a M13 DNA encoded mini-protein (mol. wt. 2000).

## 4. Discussion

It can be concluded from these studies that under the direction of M13 RF-I DNA at least two proteins are synthesized (fig. 1, VI and VII), which are identical with the native viral proteins encoded by gene 5 and gene 8, respectively. It can be concluded also that these proteins are synthesized in much larger quantities than the other phage encoded proteins (fig. 1e). This result reflects the *in vivo* situation in which the same phenomenon has been observed [6]. To explain these noticeable differences in the level of gene expression, several mechanisms can be proposed:

i) The genes coding for gene 5 and gene 8 protein are transcribed more efficiently than all other M13 genes, resulting in a higher mRNA concentration coding for these proteins (transcriptional control).

ii) All mRNA's coding for the distinct phage specific proteins are synthesized in equal amounts, but their rate of translation is different (translational control).

iii) RF-I DNA is transcribed into several distinct polycistronic mRNA's which have overlapping polynucleotide sequences. Due to this phenomenon, some genes are transcribed more frequently than the other genes, resulting in a higher rate of the synthesis of their encoded proteins.

Our preliminary results on the isolation and characterisation of M13 specific mRNA from infected cells indicated that several mRNA's with different length exist, which have in common the coding information for at least one protein. For this reason we favour the latter model, proposed originally by Okamoto et al. [17]. This model is furthermore supported by several studies on the *in vivo* and *in vitro* synthesis of phage specific mRNA [17–21].

**Acknowledgements**

The author would like to thank Mrs. Josephine Jansen for her excellent technical assitance. The generous gifts from Drs. K. Wanner and K. v/d Hondel of labeled gene 5 protein and wild-type RF-I DNA, respectively, are gratefully acknowledged. Thanks are also due to Drs. J. Schoenmakers and A. Berns for their stimulating discussions.

**References**

[1] Salivar, W.O., Tzagoloff, H. and Pratt, D. (1964) Virology 24, 359.
[2] Pratt, D., Tzagoloff, H. and Beaudoin, J. (1969) Virology 39, 42.
[3] Marvin, D.A. and Hohn, B. (1969) Bacteriol. Rev. 33, 172.
[4] Pratt, D., Tzagoloff, H. and Erdahl, W.S. (1966) Virology 30, 397.
[5] Lyons, L.B. and Zinder, N.D. (1972) Virology 49, 45.
[6] Henry, T.J. and Pratt, D. (1969) Proc. Natl. Acad. Sci. U.S. 62, 800.
[7] Pratt, D., Tzagoloff, H., Erdahl, W.F. and Henry, T.J. (1967) in: Molecular Biology of Viruses (Colter, J.S. and Parachych, W., eds.), p. 219, Acad. Press Inc., New York.
[8] Ward, R., Konings, R.N.H. and Hofschneider, P.H. (1970) European J. Biochem. 17, 106.
[9] Konings, R.N.H. and Bloemendal, H. (1969) European J. Biochem. 7, 165.
[10] Fromageot, H.P.M. and Zinder, N.D. (1968) Proc. Natl. Acad. Sci. U.S. 61, 184.
[11] Yamamoto, K.R., Alberts, B.M., Benzinger, R., Lawhorne, L. and Treiber, G. (1970) Virology 40, 734.
[12] Konings, R.N.H., Ward, R., Francke, B. and Hofschneider, P.H. (1970) Nature 226, 604.
[13] Gesteland, R.F. and Kahn, C. (1972) Nature New Biol. 240, 3.
[14] Oey, J. and Knippers, R. (1972) J. Mol. Biol. 68, 125.
[15] Braunitzer, G., Asbeck, F., Beyreuther, K., Höller, H. and Wettstein, v. G. (1967) Hoppe-Seyler's Z. Physiol. Chemie 348, 1689.
[16] Beyreuther, K. (1968) Thesis, University of Munich.
[17] Okamoto, T., Sugiura, M. and Takanami, M. (1969) J. Mol. Biol. 45, 101.
[18] Sugiura, M., Okamoto, T. and Takanami, M. (1969) J. Mol. Biol. 43, 299.
[19] Takanami, M., Okamoto, T. and Sugiura, M. (1971) J. Mol. Biol. 62, 81.
[20] Jacob, E., Jaenisch, R. and Hofschneider, P.H. (1970) Nature 227, 59.
[21] Takanami, M. and Okamoto, T. (1973) Bose Institute Symposium.