STRUCTURE AND SYNTHESIS OF A LIPID-CONTAINING BACTERIOPHAGE

In vitro protein synthesis directed by bacteriophage PM2

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1. Introduction

Although synthesis of *Alteromonas espejiana** DNA stops soon after infection with bacteriophage PM2, the cellular DNA is not degraded and continues to produce cellular RNA throughout the course of infection [1]. This would imply that cell-specific protein synthesis also occurs to a considerable extent in infected cells. Indeed the overall rate of protein synthesis is the same in control and infected cells, at least until the beginning of cell lysis [2]. This continued synthesis of cellular proteins has made it difficult to investigate PM2-directed protein synthesis in vivo [2]. An in vitro study of PM2 DNA-directed protein synthesis is presented here.

Twelve polypeptides of ~110 000–6000 mol. wt were synthesized in a PM2 DNA-directed cell-free protein synthesizing system from *Escherichia coli*. When *E. coli* ribosomes were used in the cell-free system, the 6000 mol. wt polypeptide became dominant. When *A. espejiana* ribosomes were used, the 6000 mol. wt polypeptide was also synthesized, but comprised a relatively small fraction of the polypeptide spectrum. The polynucleotide-dependent polynucleotide-pyrophosphorylase and endolysine activities, both of which are present in bacteriophage PM2, were also detected as products of the in vitro protein synthesis.

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* The host bacterium for PM2, originally called *Pseudomonas* BAL-31, has recently been reclassified as *Alteromonas espejiana* [14]

2. Experimental

2.1. *General*

Bacteriophage PM2 was grown in *A. espejiana* and was purified by the method in [3]. Superhelical PM2 DNA was extracted from the virus by the method in [4] and purified by sucrose gradient centrifugation [4]. Protein was determined by the methods in [5] or [6].

2.2. *The cell-free protein synthesizing system*

The DNA-dependent protein synthesizing system from RNase-free *E. coli* MRE 600 was prepared by the method in [7]. Standard assays (0.5 ml) contained: 100 μl protein fraction from MRE 600 containing the enzymes for transcription and translation (15 mg protein/ml); 50 μl PM2 DNA (0.5 mg/ml); 25 μl tRNA (20 mg/ml); 50 μl ribosomal fraction (100 mg protein/ml, A_{260} 1500); 10 μg pyruvate kinase in 100 μl, 500 μg phosphoenolpyruvate; and 5 mM each of UTP, CTP, GTP; 50 μl ATP (10 mM); 50 μl 19 L-amino acids (each 2 mM); 50 μl L-[^{14}C]leucine 2.5 or 25 Ci/mol, (2 mM); and 25 μl buffer (0.4 M Tris–acetate, 0.24 M $MgCl_2$, 1.4 M NH_4Cl, 0.02 M 2-mercaptoethanol (pH 7.5) at 25°C).
For measuring in vitro RNA synthesis under the conditions of protein synthesis, [^{3}H]ATP (10 Ci/mol) was used. Incubation was at 37°C for variable time periods. The mixtures were prepared for counting by the following procedure: 3.5 ml icewater and 4 ml 0.06 M $HClO_4$ were added to the mixture. This mixture was heated for 5 min at 80°C to cleave aminoacyl-tRNA. Then the mixtures were either prepared for counting as in [7] or prepared for gel electrophoresis and

liquid scintillation counting by centrifugation of the protein precipitate.

2.3. *Determination of the endolysine activity*

Standard mixtures for in vitro protein synthesis using unlabeled substrates and ribosomes from *E. coli* or *A. espejiana* were incubated at 37°C for 60 min in the presence or absence (control) of PM2 DNA. After the incubation period, purified murein from *E. coli* K12, strain W945T3282, which was labeled with [^{3}H]diaminopimelic acid, was added to the standard assay mixture (18 000 cpm [^{3}H]murein) and incubated further at 25°C for 60 min. Solubilised [^{3}H]diaminopimelic acid was determined in the supernatant of the trichloracetic acid precipitates of the incubation mixtures as in [8].

2.4. *Determination of the polynucleotide-dependent polynucleotide-pyrophosporylase activity*

Standard mixtures for in vitro synthesis were incubated as above. After the incubation period, 50 μg rifamycin, 50 μg puromycin, $MnCl_2$ to final conc. 2 mM, and 50 μl [^{3}H]ATP (10 Ci/mol, 10 mM) was added to the mixture. Besides these additions, 25 μg PM2 DNA as template for the pyrophosphorylase was added to the control mixture which had been incubated in the absence of DNA. The mixtures were then incubated for a further 30 min and then polynucleotide-dependent polynucleotide-pyrophosphorylase activity was determined as in [9].

3. Results and discussion

The kinetics of PM2 DNA-dependent RNA and protein synthesis under optimal conditions (see section 2) proceeded as described earlier for bacteriophage T4 DNA [7]; the rates are constant within the first 15 min incubation. After this time the rates started to decrease and after 30 min no further RNA or protein synthesis was observed. After 60 min the amount of synthesized protein remained stable.

The labeled protein synthesized in vitro after an 30 min incubation period was analysed by polyacrylamide gel electrophoresis in the presence of dodecyl sulfate. Twelve polypeptides of ~110 000–6000 mol. wt were synthesized in a PM2-DNA directed cell-free protein synthesizing system from *E. coli*. Polypeptides with identical molecular weights were synthesized in vitro using ribosomes from *E. coli* or from the host of bacteriophage PM2, *A. espejiana*. The major difference between the polypeptide spectra of the two products is quantitative. In the presence of ribosomes from *E. coli*, the 6000 mol. wt polypeptide is dominant; in the presence of ribosomes from *A. espejiana* the corresponding polypeptide band is not dominant (fig.1). The background radioactivity

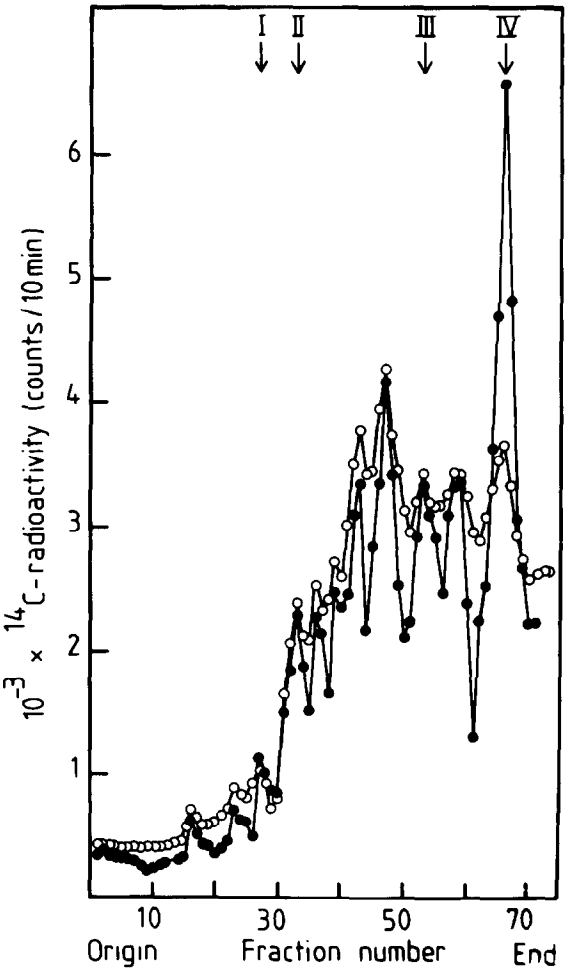

Fig.1. In vitro synthesized PM2-specific proteins. The synthesized proteins were separated by polyacrylamide gel electrophoresis as in section 2. The arrows with the roman numerals I–IV indicate the positions of the structural proteins of bacteriophage PM2. (—•—) Protein synthesis in the presence of *E. coli* ribosomes. (—○—) Protein synthesis in the presence of *A. espejiana* ribosomes.

under the polypeptides synthesized in vitro with < 43 000 mol. wt is increased (fig.1). This is generally observed when labeled polypeptides migrate close together [10]. In the absence of PM2 DNA (control) no labeled polypeptides were synthesized in vitro in the presence of ribosomes from *E. coli* or *A. espejiana*. In this case a background from 10–15 cpm was detected over the entire gel (not shown). The molecular weights of the polypeptides synthesized in vitro were determined and are shown in table 1. Twenty-fold less protein was synthesized in vitro when nicked PM2 DNA (open circles) was used and therefore we were not able to demonstrate the polypeptides which were synthesized under this condition.

Bacteriophage PM2 contains two known enzymatic activities: a polynucleotide-dependent polynucleotide-pyrophosphorylase [9] and an endolysine [8]. Both activities were detected as products of PM2-DNA directed protein synthesis using ribosomes from *E. coli* or *A. espejiana* (table 2).

The presence of nucleolytic and proteolytic enzyme activity in *A. espejiana* (data not shown) prevented us from preparing a homologous cell-free protein synthesizing system. We therefore used the cell-free protein synthesizing system from *E. coli* [7] with ribosomes from *E. coli* or *A. espejiana*, the host of bacteriophage PM2. In vitro protein synthesis with double-stranded linear bacteriophage DNA species is somewhat more difficult to manipulate. Optimal in vitro transcription occurs at much higher ionic strength and requires higher Mg^{2+} concentrations than

Table 1
The molecular weights of the proteins synthesized in vitro

Polypeptide	Mol. wt ($\times 10^{-3}$)
1	(110)
2	87
3	68
4 (I)	42
5	38
6	35
7 (II)	27
8 (III)	12.5
9	10.5
10	(7.8)
11 (IV)	(6.5)

In the gel system used, the calibration curve is linear only up to 100 000 mol. wt and therefore the molecular weight of polypeptide 1 is only approximate. The molecular weights of polypeptides 10 and 11 are also approximate since the gel system gives uncertain values in this range. The roman numerals in parentheses refer to the polypeptides having the same molecular weights as the structural proteins of bacteriophage PM2

Table 2
In vitro synthesis of PM2 specific enzymes

A. In vitro protein synthesis which occurred in the presence of:	Poly A synthesis by the polynucleotide-pyrophosphorylase which was synthesized in vitro (trichloroacetic acid precipitable [^{3}H]AMP)	
	+ PM2 DNA	– PM2 DNA (control)
Ribosomes from *E. coli* MRE 600	571 cpm	63 cpm
Ribosomes from *A. espejiana*	840 cpm	41 cpm
B. In vitro protein synthesis which occurred in the presence of:	Solubilisation of [^{3}H]diaminopimelic acid from labeled murein by the endolysine activity which was synthesized in vitro	
	+ PM2 DNA	– PM2 DNA (control)
Ribosomes from *E. coli* MRE 600	7005 cpm	53 cpm
Ribosomes from *A. espejiana*	6253 cpm	49 cpm

(A) Polynucleotide-dependent polynucleotide-pyrophosphorylase activity
(B) Endolysine activity. The assay conditions are in section 2

in vitro translation and therefore a compromise between the two optimal salt requirements in such in vitro DNA-dependent protein synthesis system must be made [7,11]. Fortunately the salt requirements (Mg^{2+}, NH_4^+) for optimal RNA synthesis of the superhelical PM2 DNA [12] are close to the requirement for optimal protein synthesis [7]. Twelve polypeptides were synthesized in vitro when supercoiled PM2 DNA was used. Twenty-fold less protein was synthesized in the presence of nicked PM2 DNA. This may be related to the reported 6-fold lower amount of in vitro RNA synthesis when nicked PM2 DNA (open circles) was used in the assay [12]. In the presence of *E. coli* ribosomes, the protein having the same molecular weights as protein IV was the major product. Ribosomes from *B. stearothermophilus* and *E. coli* have been shown to recognize different initiation signals of f2 RNA [13]. Thus all of the differences described above could be due to the employment of a protein synthesizing system of *E. coli* which is not the homologous one for expression of the PM2 genome. On the other hand, protein IV has a polynucleotide-dependent polydeoxyribo- and polyribonucleotide-pyrophosphorylase activity [9] and protein III has an endolysine activity [8]. Since both activities were synthesized in vitro, we conclude that in vitro protein synthesis with the *E. coli* heterologous system is able to produce PM2-specific proteins.

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