

Formation of bacteriophage MS2 infectious units in a cell-free translation system

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Abstract We show that a simple cell-free translation system from *Escherichia coli*, programmed with phage MS2 RNA, is able to infect F+ *E. coli* cells. The plaques appearing on the *E. coli* host strain are morphologically indistinguishable from those derived from normal phage MS2 infection. This effect is strictly translation-dependent, since an incomplete translation system or the system inhibited by antibiotics leads to no infection. The cell-free based infection is maximal under conditions favouring the highest synthesis of maturation protein (one of the four phage-encoded proteins). The infection is abolished when RNase A or trypsin treatment is included before addition of cells. Similarly, due to RNA and maturation protein degradation, the continued incubation of the translation mixture under protein synthesis conditions significantly decreases infectivity. These findings suggest the formation of 'minimal infectious units', simple complexes of MS2 RNA and maturation protein. Here we describe the first example of bacteriophage infectious unit formation directly performed in a cell-free translation system. A possible application of this phenomenon might be the construction of newly designed RNA vector delivery systems and, moreover, could be an approach for molecular evolution studies.

Key words: Cell-free translation; In vitro phage assembly; RNA-protein interaction; Infectivity; Phage MS2 morphogenesis; RNA vector delivery system; Molecular evolution

1. Introduction

Bacteriophage MS2 is a member of the Leviviridae, the family of single-stranded RNA-containing bacteriophages (for a recent review see [1]). Its RNA codes for four proteins: one is involved in phage genome replication, another acts as a lytic protein, and the two others form the phage particle. An intact phage particle consists of one molecule of RNA, 180 molecules of coat protein, and one molecule of maturation protein, which is responsible for the phage attachment to *E. coli* F-pili and infection [2].

As described, phage particle assembly proceeds through coat protein dimerization and interaction of coat protein dimers with a special stem-loop structure, located at the beginning of the RNA-replicase cistron [3]. This interaction

switches off the expression of the RNA-replicase cistron, being a watershed between the early and late stages of infection [4]. This interaction also triggers the further assembly of coat protein dimers [5], possibly through pentamers to the final icosahedral particle [6].

While MS2 coat protein-RNA interactions and their role in phage assembly have been studied in great detail (for a review see [7]), interactions of phage RNA with maturation protein have not been as thoroughly investigated. It is known that maturation protein protects two regions of phage RNA from RNase digestion, close to the 5' and 3' ends of the RNA [8]. Based on this finding, and bearing in mind the estimate of the molar ratio RNA:protein in RNA-maturation protein complex as 1:1 [9], a model was proposed, according to which phage RNA is bent in half by maturation protein [1]. When the phage particle attaches to the F-pilus, the maturation protein is cleaved into two parts [10] and the RNA straightens and penetrates the cell [1].

Phage RNA-maturation protein complexes were separated from intact phage particles [9] or reconstituted in vitro [11]. In both cases such complexes were shown to be infective for F+ *E. coli* and therefore called 'minimal infectious units' of the RNA phage [11]. Such complexes can also be observed in vivo [10], where newly synthesized maturation protein associates with phage RNA [12]. These data demonstrate the importance of interactions between the maturation protein and RNA for infectivity and possibly for the phage particle assembly.

In the present report, we demonstrate that a simple *E. coli*-based, cell-free translation system is infective for F+ *E. coli* when phage MS2 RNA serves as a translation template. The nature of this infectivity is presumably the formation of RNA/maturation protein minimal infectious units. Therefore, cell-free translation systems can serve as a good tool for elucidating details of RNA phage morphogenesis. The de novo formation of MS2 infectious complexes in a cell-free system is the first such example for bacteriophages and will have a number of applications. In this context, the development of RNA vector delivery systems and, moreover, an approach for molecular evolution studies should be noted and will be discussed.

2. Materials and methods

2.1. Cell-free translation system

Bacteriophage MS2 RNA (supplied by Boehringer Mannheim) was used as a template in the S-30 cell-free translation system from *E. coli* Q13. Translation mixtures were incubated at 37°C in a 30 µl volume of 50 mM Tris-Ac, pH 8.2, with 130 mM KOAc, 1 mM EDTA, 1 mM ATP, 0.5 mM GTP, 0.5 mM CTP, 0.5 mM UTP, 2 mM dithiothreitol, 0.01% sodium azide, 300 ng leucovorine, 20 amino acids in 50 µM amounts, 5 µM D-[4,5-³H]leucine (72 Ci/mmol, Amersham), 13 µg of *E. coli* tRNA, 4 µl of *E. coli* S-30 ribosomal extract which was pre-

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Abbreviations: PAGE, polyacrylamide gel electrophoresis; PFU, plaque forming unit

pared as described in [13], and 4.4 pmol MS2 RNA unless otherwise specified.

Acetyl phosphate at a concentration of 40 mM and the internal acetate kinase were used as an energy regeneration system [14]. 29 mM $\text{Mg}(\text{OAc})_2$ was found to be optimal for this system. Protein synthesis was monitored by sampling aliquots of 10 μl and counting the radioactivity of the material precipitated in hot 10% trichloroacetic acid, or by 15% PAGE in the presence of SDS and followed by fluorography [15]. The fluorographs were scanned using a CAMAC TLC-Scanner 3 (Camag, Switzerland) and further analyzed by the software package 'CATS', version 4.2. The molar amounts of individual proteins synthesised were calculated afterwards based on their leucine contents [16]. In some experiments, puromycin (Fluka) or chloramphenicol (Fluka) at concentrations 0.5 mM and 77 μM , respectively, were used as inhibitors of protein synthesis.

2.2. Phage assay

F+ *E. coli* DSM 5695 was grown under aerobic conditions in a NZCYM medium until a biomass concentration of 0.3–0.6 g/l (OD_{600} 1–2) was achieved. 100 μl of such cell suspension was mixed with 10–30 μl of translation mixture and 3 ml of melted top agar (47°C) and poured onto plates with hardened bottom agar. The plaques were counted after 8–12 h of incubation at 37°C. In order to test the effect of RNase or protease treatment on cell-free based infectivity, samples were mixed with RNase A (Boehringer Mannheim, final concentration 22 U/ml) or trypsin (Boehringer Mannheim, final concentration 11 U/ml) and further incubated at 37°C for 10 min before addition of cells. As a control normal MS2 phage DSM 5694 (titre: 3.5×10^9 PFUs/ml) was used for infection.

2.3. RNA decay assay

5- μl aliquots were taken at different times after the beginning of the cell-free translation reaction, programmed with 6.6 pmol MS2 RNA, and diluted with 25 μl of 1% SDS. RNA was extracted by phenol/chloroform treatment, analysed by electrophoresis in 1% agarose gels containing formaldehyde, and stained with ethidium bromide [15]. Kinetic data on MS2 RNA degradation were obtained by scanning the resulting agarose gels using a CAMAC TLC-Scanner 3 (Camag, Switzerland) and employing the software package CATS, version 4.2.

3. Results

E. coli S-30 cell-free translation system, supplied with MS2 RNA, produces phage proteins, the most abundant of which is coat protein [17] (see also Fig. 1). Its accumulation is gradual from the beginning of the translation reaction and reaches a plateau after 10–20 min of incubation. Additionally, β -subunit of RNA replicase and maturation protein are synthesised. The amount of β -subunits produced increases from 10 to 20 min of translation and then also stabilises, while maturation protein, being unstable both in vivo and in vitro protein [17], first appears after 10 min and is rapidly degraded later on (Fig. 1). In our experiments the molar ratio of different phage proteins and RNA present after 10 min of cell-free translation (RNA:coat:replicase:maturation) was normally 6:27:1:1.8, as estimated by scanning gels from protein synthesis (Fig. 1) and RNA degradation (see below) assays. The relative amounts of all phage proteins which were synthesised in vitro are close to what is observed in vivo at the end of infection [18]. However, the ratio of 'phage proteins to phage RNA' is much higher in infected cells. This might be one of the reasons why de novo phage formation in a cell-free translation system has never been reported. However, recent data suggest that newly in vitro synthesised coat protein of RNA phage might interact with the operator stem-loop structure at the start of the replicase cistron [19], the process known to be the critical step for phage assembly [5].

In order to address the question of whether or not an MS2 RNA-based cell-free translation system can lead to viable

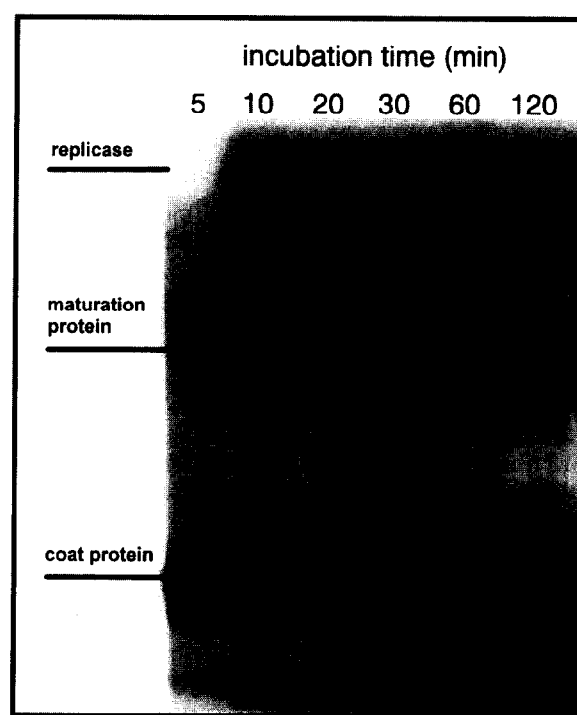


Fig. 1. Time course of protein synthesis in cell-free translation system with MS2 RNA as a template was analysed by 12% SDS-PAGE followed by fluorography. Bands corresponding to replicase β -subunit (60.8 kDa), maturation protein (44 kDa), and coat protein (13.9 kDa) are indicated according to molecular mass standards.

MS2 phages, aliquots at different times after the start of translation reaction were assayed for plaque forming capacity on F+ *E. coli* DSM 5695. Surprisingly, starting with 2 h of *E. coli* growth, plaques were visible on the bacterial lawn, to a maximum of 300–350 plaques per 30 μl of cell-free translation mixture. These plaques were morphologically indistinguishable from those derived from normal phage MS2 infection (Fig. 2). No plaques appeared when aliquots from a blank reaction, lacking MS2 RNA, as well as from an incomplete reaction, omitting S-30 extract or amino acids, were analysed (Table 1). Similarly, after addition of translation inhibitors puromycin or chloramphenicol to the cell-free translation system, no protein synthesis was observed and plaque formation was prevented (Table 1 and data not shown). Incubation of intact MS2 phages with puromycin or chloramphenicol did not affect their infectivity (data not shown). These findings clearly demonstrate that the infectivity of the cell-free translation system for *E. coli* is due to the presence of MS2 RNA and the appearance of its translational products.

The plaque forming capacity of the cell-free translation system was maximal when 4–5 pmol of MS2 RNA were added per 30 μl of translation mixture (Fig. 3). Amounts of MS2 RNA above or below this value strongly decreased the number of PFUs. The dependence of total protein synthesis on MS2 RNA concentration, however, was different: incorporation of radioactive leucine into proteins increased gradually with the increase of mRNA concentration and stabilized at approx. 4 pmol MS2 RNA/30 μl (150 nM) (Fig. 3). When the influence of mRNA concentration on the synthesis of individual phage proteins was checked (Fig. 4), it appeared that

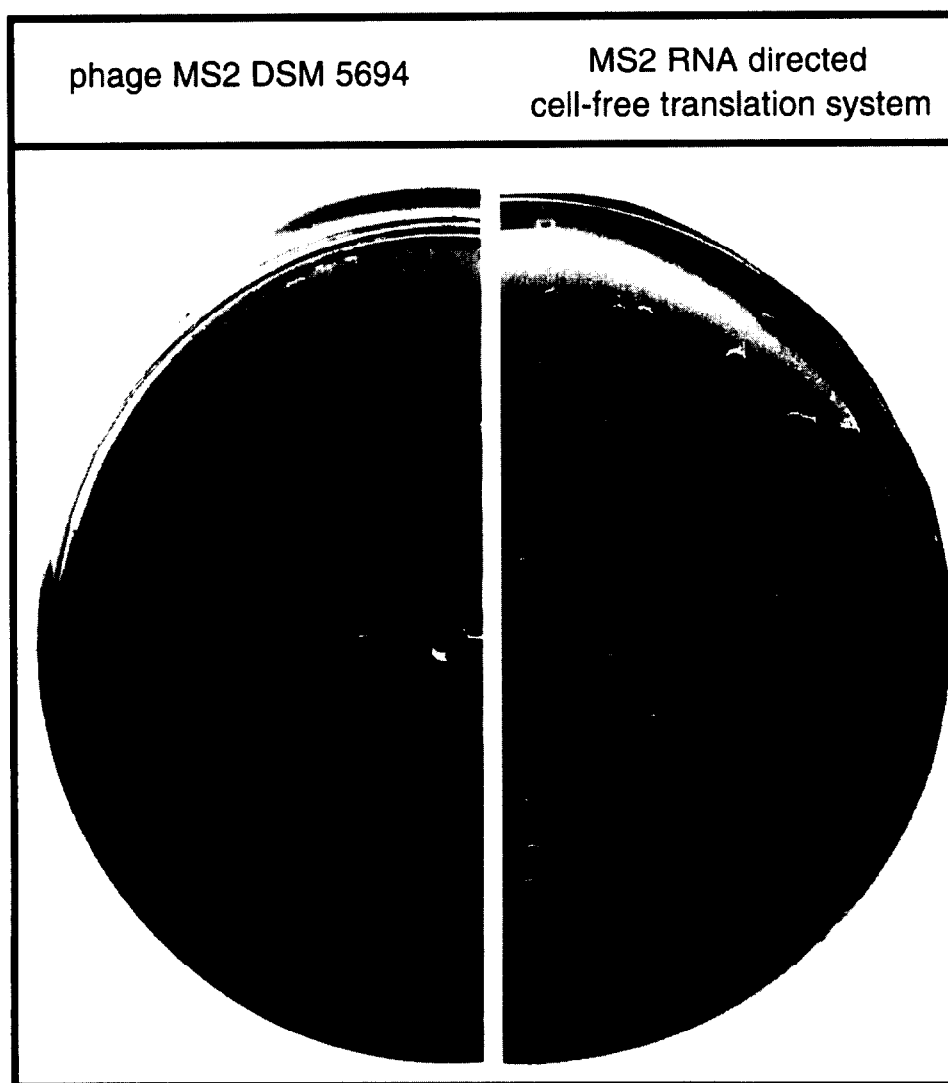


Fig. 2. *E. coli* DSM 5695 was infected with 100 μ l of diluted phage MS2 DSM 5694 (titre: 3.5×10^9 PFU/ml) (left) or 30 μ l of cell-free translation system with phage MS2 RNA as a template (right) as described in Section 2.

the peak of plaque formation coincided with the peaks of coat and maturation proteins. These two proteins constitute the particle of the phage in vivo, which further suggests that the nature of the cell-free translation infectivity is normal phage or something related to it.

In the case of the cell-free translation system, the number of PFUs was strongly dependent on the time of incubation of the translation reaction (Fig. 5). Maximal infectivity was obtained

after 10 min of translation but decreased with continuation of the reaction and completely disappeared after 2–5 h. The curve of time-dependent plaque forming capacity strikingly reflects the curve of maturation protein synthesis and degradation (Fig. 4). Maturation protein, which is barely detectable after the first 5 min of translation, but which accumulates in the largest amounts after 10 min and then is rapidly degraded (Figs. 1 and 4), therefore seems to be the limiting point of the

Table 1
Effects of different modifications of the cell-free translation system on translation and PFU formation

Variants	Translation efficiency (%)	Infectivity (%)
Cell-free translation (10 min incubation)	100	100
Cell-free translation (20 min incubation)	110	13
Cell-free translation+puromycin (10 min incubation)	7	1
Cell-free translation—S-30 extract (10 min)	5	0
Cell-free translation—MS2 RNA (10 min incubation)	5	0
Cell-free translation—amino acids (10 min incubation)	5	0
Cell-free translation+trypsin (10 min incubation)	0	0
Cell-free translation (10 min)+trypsin (10 min incubation)	14	0
Cell-free translation (10 min)+RNase A (10 min)	102	0

Translation (measured as total incorporation of [3 H]leucine) and PFU formation in normal cell-free translation system, incubated for 10 min, were equated with 100%.

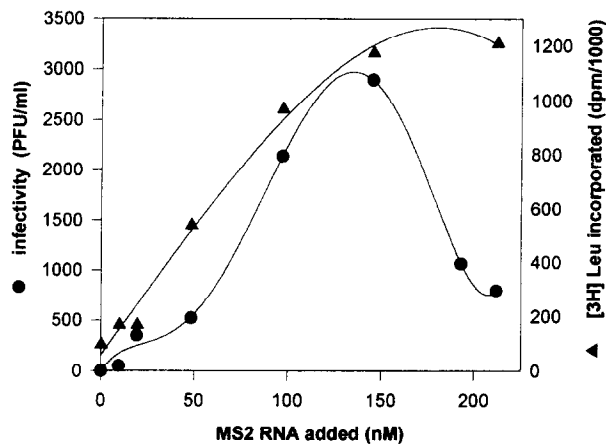


Fig. 3. Dependence of the overall protein synthesis (▲) and the infectivity for F+ *E.coli* (●) on the amount of MS2 RNA added to the system. Translation mixtures were incubated for 10 min and analysed as described in Section 2.

cell-free infectivity. MS2 RNA is also rapidly degraded, providing a second possible barrier to plaque forming capacity: after 5 min of translation only half of the original MS2 RNA retained its full size, and no full-size MS2 RNA was visible on agarose gels stained with ethidium bromide after 40 min of translation (Fig. 4). Thus, the early decrease of plaque forming capacity of the cell-free translation system is most likely to be due to the decay of maturation protein and RNA, molecules that are indispensable for RNA phage infection of intact cells. S-30 – being a crude *E. coli* extract – contains many RNase and protease activities that could account for this decay.

In order to prove that the decrease of infectivity is caused by protease and RNase digestion, we treated samples from the cell-free translation system after 10 min of incubation with trypsin or RNase A for 10 min. As expected, the samples became uninfectious after this procedure (Table 1). The same pretreatment of native MS2 phages with RNase A or trypsin had no obvious effect on the infectivity (data not shown). The strong sensitivity to RNase and protease digestion was first described in the case of minimal infectious units of MS2 phages, consisting of one naked molecule of RNA and maturation protein [9,11]. These complexes, reconstituted in vitro from purified components or separated from phage particles, are rather unstable but infectious for F+ *E. coli* strains protein [9,11]. Such a complex is probably a minimum required for virus infection, since it contains one molecule responsible for infection and one carrier molecule responsible for penetration into a cell. Based on the data presented in this report, we conclude that such minimal infectious units, but not intact MS2 phages, are formed in *E. coli* cell-free translation system with MS2 RNA as a template.

4. Discussion

Phage MS2 RNA traditionally is a popular mRNA source for studies of prokaryotic translation and its regulation in vitro [20]. However, the de novo formation of the phage in such systems has never been reported. Here we demonstrate that the *E. coli* S-30 cell-free translation system, programmed with MS2 RNA, is able to infect F+ *E. coli* cells. This infec-

tivity, unlike that of normal MS2 phages, is very sensitive to RNase or protease treatment. MS2 RNA and newly synthesised maturation protein are the two entities which are damaged by such treatment. Based on this, we propose that minimal infectious units of MS2 phage, consisting of MS2 RNA and maturation protein [9,11] are formed in the translation system.

Formation of MS2 infectious units in the translation system is de novo in part, since it is based on exogenously added MS2 RNA that conducts the synthesis of protein necessary for infectivity. Although, in experiments presented here, RNA replicase subunit synthesis was observed (see Figs. 1 and 3) and translation mixtures contained all four ribonucleoside triphosphates, RNA replication could not contribute to infectious unit formation, since the omission of CTP and UTP had no effect on infectivity or on translation (data not shown). This agrees with our data (Katanaev and Spirin, unpublished results) and previously reported results [21] failing to demonstrate exogenously synthesised replicase-based RNA replication in cell-free translation systems, which is presumably due to an (over) excess of RNA relative to replicase (see above) [22].

MS2 infectious unit formation in a cell-free translation system is the first such example described for a bacteriophage. De novo poliovirus synthesis was demonstrated in cell-free extracts from HeLa cells when poliovirus RNA was added as a template [23]. Poliovirus polyprotein was synthesised, processed by intrinsic protease, producing poliovirus RNA replicase, and poliovirus RNA was amplified and incorporated into self-assembled virus particles, leading to infectious viruses [23]. The first newly formed polioviruses were detectable between 5 and 10 h of incubation, with the maximum virus production achieved after 15–20 h, while protein synthesis reached a plateau after approx. 9 h [23]. About 10 h

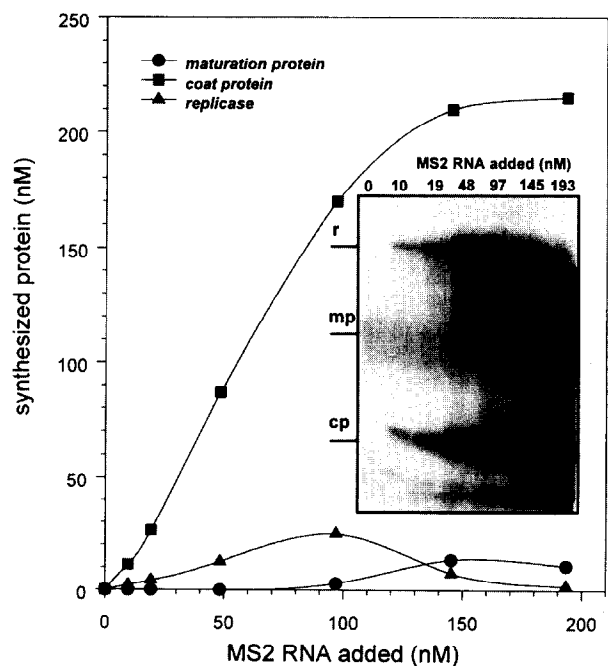


Fig. 4. Synthesis of individual phage proteins directed by different amounts of MS2 RNA added to the cell-free translation system. Translation mixtures were incubated for 10 min and analysed as described in Section 2. Inset: scan from the fluorogram where r is replicase, mp maturation protein and cp coat protein.

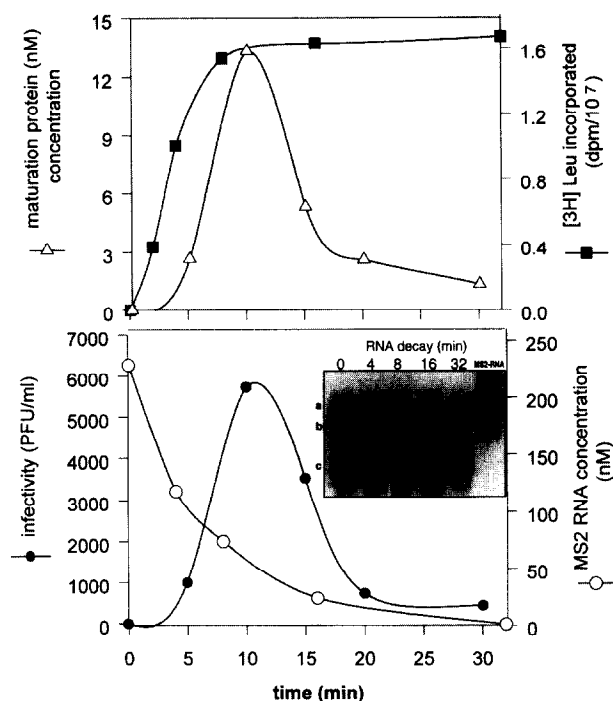


Fig. 5. Time course of the overall protein synthesis (■), maturation protein synthesis and degradation (▲), MS2 RNA degradation (○), and dynamics of PFU formation (●) in the cell-free translation system. Inset: Scan from the ethidium bromide stained agarose gel from RNA decay assay. a, MS2 RNA; b, 23S rRNA; c, 16S rRNA. Pure MS2 RNA served as a standard.

incubation was necessary for effective proteolysis, RNA replication and virus assembly. In our experiments in the S-30 *E. coli* cell-free translation system, termination of the accumulation of phage proteins after 10–20 min and rapid degradation of phage RNA and maturation protein did not favour real phage morphogenesis. However, other *E. coli* cell-free systems, such as the coupled transcription-translation system [24], or the S-100 system [25], or the cell-free continuous-flow systems [26] could be more efficient for this purpose.

Cell-free translation systems seem to be an efficient tool to study details of phage morphogenesis, since its different stages such as RNA translation, replication, contacts with phage proteins, and particle assembly (for reviews on these topics see [7,20,27]) can be independently and easily followed, influenced and changed, which is difficult to do in vivo. These systems also seem to be more productive in understanding mechanisms of phage assembly than in vitro reconstitution experiments, because the latter use pre-synthesised and renatured components, making the process of phage reassembly much more artificial.

Among others, the use of RNA vectors (a research area which has attracted increasing interest during recent years [28–30]), encounters the problem of vector delivery to a cell. For bacteria the only method existing so far is a protoplast-based method, when cells deprived of their cell walls become osmotically sensitive to RNA [31]. However, it is often inconvenient to use such artificially treated cells. Single-stranded RNA bacteriophages, which have recently started to be used as possible alternatives to generally accepted systems of phage display and expression vectors [19,32,33] could find broad applications in future. In this case, the formation of phage

minimal infectious units in a cell-free translation reaction, described in this paper, could serve as a method both to check the correct expression of a new gene in vitro and then to deliver it subsequently to normal bacteria. This new gene could be a sequence for a whole polypeptide (as in [19]) or peptide library (as in [32]) inserted into the coat protein cistron of a phage. Cistrons for maturation protein and replicase, left intact, would serve for vector delivery to bacteria and its propagation therein, respectively.

A lack of proofreading activity during RNA virus amplification leads to the existence of quasispecies inside a viral populations [34]. This provides very high rates of RNA virus evolution and adaptation to changed environmental conditions. The appearance of a new step, (namely cell-free translation of MS2 RNA followed by MS2 infectious unit formation) before *E. coli* is infected by the MS2 phage, can be considered as quite a dramatic change of environmental conditions for the phage. It seems very interesting to try to enrich the MS2 phage population in specimens adapted to new conditions. It could provide new evolutionary and biochemical evidence of how a bacteriophage can change to survive in a changed environment. This work is now in progress.

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