TRANSLATION OF COWPEA MOSAIC VIRUS RNA IN A CELL-FREE EXTRACT FROM WHEAT GERM

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1. Introduction

The genome of Cowpea Mosaic Virus (CPMV) consists of two single-stranded RNAs, which are distributed between two nucleoprotein components [1]. The virions have identical capsids, composed of two different proteins (mol. wt 44 000 and 25 000), in equimolar amounts [2,3]. Both virions or RNAs are necessary for virus multiplication [4]. The two RNA molecules, referred to as M-RNA and B-RNA have mol. wt 1.37×10^6 and 2.02×10^6 [5], respectively. Clearly the coding capacity of these RNAs is more than that necessary for the coat proteins alone. It is not yet known however, where the coat protein cistrons are located on the genome, nor what other cistrons are present. In vitro translation may provide answers to these questions. Furthermore CPMV-RNAs differ from many plant virus RNAs in that they have a poly(A) sequence of about 100–200 residues at their 3′-end [6] and lack the ^{7}mGpppN-cap at the 5′-end [7]. In these respects, CPMV-RNAs are analogous to poliovirus RNA, whose translational strategy is considerably different to that known for plant viruses. The translation of CPMV-RNAs is therefore of particular interest.

CPMV-RNAs have been translated in cell-free extracts from rabbit reticulocytes [8], in which B-RNA directed the synthesis of a large polypeptide of an apparent mol. wt 220 000 in addition to some smaller products, and M-RNA was translated mainly into two large polypeptides with approx. mol. wt 140 000 and 120 000 [9]. The rabbit reticulocyte cell-free system is a heterologous system for translation of a plant virus mRNA and requires tRNA from other sources for efficient translation [8,9].

Since several plant virus RNAs have been translated in wheat-germ cell-free extracts [10–15], we investigated the translation of CPMV-RNAs in such extracts.

This investigation shows that large polypeptides similar to those formed in the reticulocyte system, are formed in wheat-germ extracts, without addition of extra tRNA and the necessity to make the system messenger dependent by ribonuclease treatment [8,9].

Abbreviations: CPMV, cowpea mosaic virus (*Nigeria* strain); M-RNA and B-RNA, the RNA from the middle and bottom components of CPMV after separation of CPMV virions by sucrose-gradient centrifugation; KAc, potassium acetate; $MgAc_2$, magnesium acetate; Tris, Tris(hydroxymethyl)-aminomethane; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulfonate

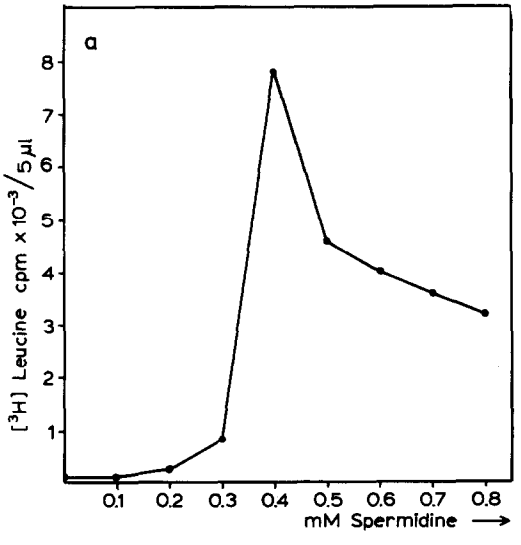
2. Materials and methods

Wheat-germ (General Mills, Vallejo, Cal.) was floated on a mixture of CCl_4 and cyclohexane (500 : 150 ml) and dried on filter paper. Obvious non-embryo material was removed. One gram of dry 'embryo' was ground with crushed glass for a few seconds. After addition of 2 ml homogenization buffer (120 mM KAc, 5 mM $MgAc_2$, 1 mM dithioerythritol (DTE) and either 5 mM HEPES or 6 mM $KHCO_3$) this was ground for 2 min and further extracted in 8 ml homogenization buffer. The extract was centrifuged at 30 000 × *g* at 4°C. The pH of this extract should be about 6.5, to avoid release of endogenous messenger RNA [16]. Pre-incubation is then unnecessary. The pH of the supernatant was adjusted with 1/50 × vol. 500 mM HEPES–KOH,

pH 7.6, and centrifuged again at 30 000 X *g*. The resulting supernatant was dialysed for 16–24 h against 1 litre of buffer containing 120 mM KAc, 5 mM $MgAc_2$, 20 mM Tris–Ac (pH 7.6) and 1 mM DTE, changed after 12 h. Wheat-germ 'S-30' extracts were stored at −70°C in small aliquots after rapidly freezing in liquid nitrogen.

The incubation mixture (100 μl) for translation contained: 50 μl S-30, 20 mM HEPES (pH 7.5, with KOH), 10 mM Tris (pH 7.5, with acetic acid), 3.0 mM Mg^{2+}, 0.4 mM spermidine (HCl), 100 mM K^+ (90 mM as KAc; approx. 10 mM from KOH and ATP), 0.25 mM of each amino acid except for the labelled one, 2.5 mM ATP (K^+-salt), 0.375 mM GTP (tri–Li-salt), 10 mM creatinephosphate (Tris-salt), 10 μg/ml creatine kinase, 0.5 mM DTE and 50 μg CPMV RNA/ml or as described in the results section. The labelled amino acids used were [^{35}S]methionine (diluted to 26 mCi/μmol) or [^{3}H]leucine (diluted to 250 μCi/μmol). The standard incubation time was 1 h at 30°C. [^{35}S]methionine (300 Ci/mmol) and [^{3}H]leucine (45 Ci/mmol) were obtained from the Radiochemical Center, Amersham, England.

Polyacrylamide slab-gel electrophoresis was performed according to Laemmli [17]. For autoradiography the gels were dried on Whatman 3 MM paper and exposed to Kodak Royal-X-omat film. CPMV-RNAs were isolated and separated as described by Klootwijk et al. [7]. Tobacco Mosaic Virus (TMV)-RNA was a gift from Dr L. van Vloten-Doting.



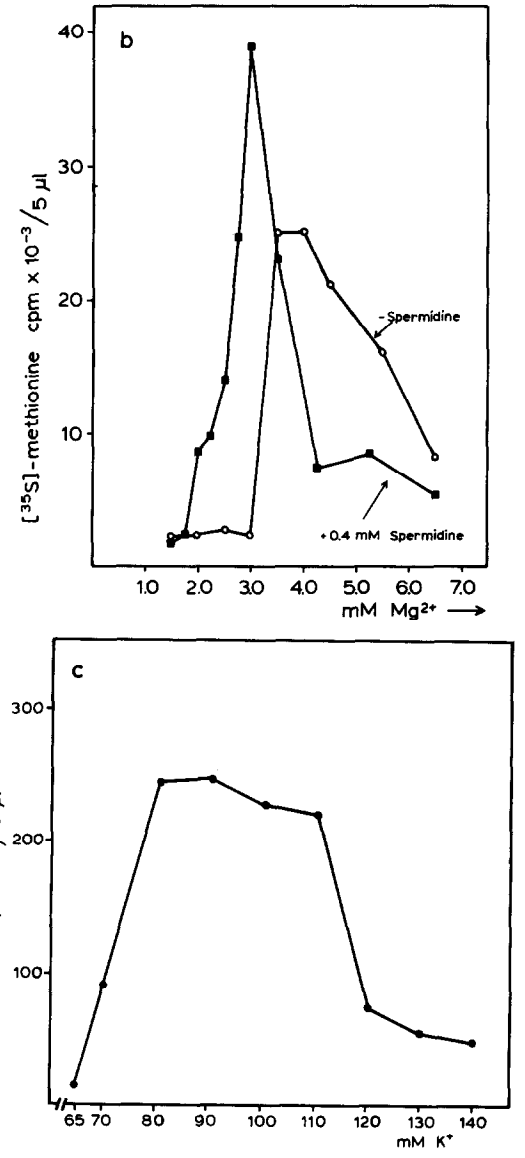


Fig.1. Spermidine, magnesium and potassium requirements for optimum amino acid incorporation directed by CPMV (natural mixture) RNAs. Samples (5 μl) were pipetted on Whatman 3 MM filter discs, which were subsequently washed with hot 5% trichloroacetic acid, then with ethanol and ether. Dried filters were counted in a liquid scintillation counter. (a.) Spermidine concentration curve at 2.9 mM Mg^{2+}, 100 mM K^+ and 50 μg/ml RNA. (b.) Mg^{2+} Concentration curves in the presence (■—■) and absence (○—○) of 0.4 mM spermidine. (c.) Typical K^+-concentration curve, at 2.5 mM Mg^{2+} and 0.2 mM spermine. Similar results were obtained with Mg^{2+}-concentrations from 2.5–3.0 mM, and 0.4 mM spermidine. In this experiment, the [^{35}S]methionine was not diluted with unlabelled methionine.

3. Results

3.1. *Conditions for protein synthesis*

Amino acids incorporation directed by CPMV-RNA was stimulated by addition of spermidine. The optimal concentration as shown in fig.1a is 0.4 mM. In the presence of 0.4 mM spermidine, the Mg^{2+}-concentration optimum is 3.0 mM (fig.1b). The K^+-concentration curve in fig.1c shows that optimal incorporation is reached at concentrations between 80–110 mM. The potassium optimum is similar for the separated B- and M-RNA (not shown). It is stressed that these results are obtained using KAc. When KCl was used, the optimum was 50 mM, and the incorporation was less. Chloride ions may be inhibitory (unpublished observations).

3.2. *Product analysis*

The product of the in vitro protein synthesis under direction of CPMV-RNA were analysed by polyacrylamide–SDS electrophoresis. The products formed from M-RNA at different K^+-concentrations can be seen in fig.2. Large polypeptides are synthesized only at K^+-concentrations of at least 70 mM (tracks B, C, D); concentrations lower than 70 mM give rise to translation into smaller polypeptides (track A).

Spermidine (0.4 mM) or spermine (0.2 mM) not only enhance the incorporation but are required for the translation of CPMV-RNAs into large products (fig.2, track E, F). No coat protein-sized products were observed (cf. track G). B-RNA directed the synthesis of a large polypeptide of approx. mol. wt 220 000, along with several smaller polypeptides (fig.3, track A). M-RNA was mainly translated into two polypeptides of approx. mol. wt 140 000 and 120 000 (fig.3, track B). The natural mixture of both RNAs (i.e., unfractionated) produced a combination of the separated RNA translation products, those from M-RNA being dominant (fig.3, track C).

TMV-RNA was also translated under the same conditions as CPMV-RNAs. As shown also in fig.3 (track D) this RNA directs the synthesis of large polypeptides among which are two polypeptides of approx. mol. wt 165 000 and 140 000, probably corresponding to the large polypeptides found in vivo in TMV-infected tobacco leaves and protoplasts

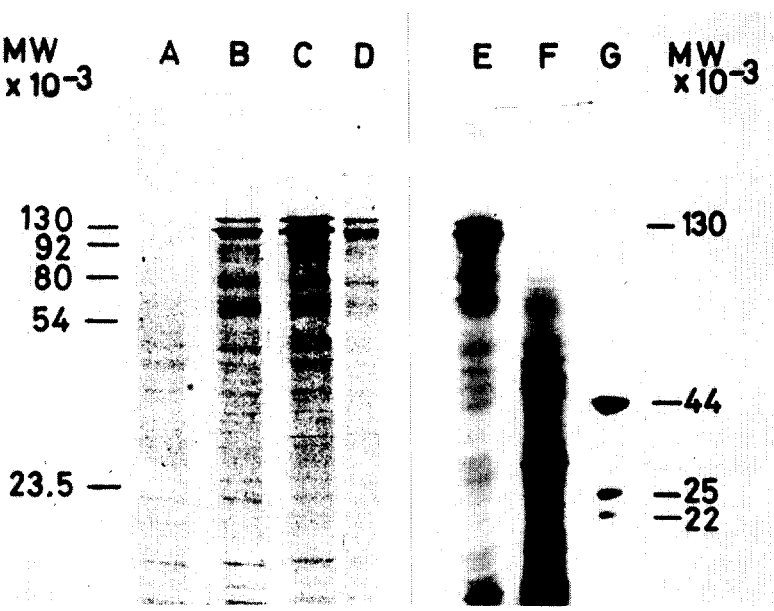


Fig.2. Analysis of the CPMV-RNA translation products, labelled with [^{35}S]methionine under different conditions. Experiment 1: M-RNA at 50 μg/ml; 2.5 mM Mg^{2+} and 0.2 mM spermine. K^+ was mostly as the acetate salt (see Materials and methods). Track A: at 65 mM K^+. Track B: at 70 mM K^+. Track C: at 110 mM K^+. Track D: at 130 mM K^+. Experiment 2: Track E: at 4.0 mM Mg^{2+}, no spermidine. Track F: at 3.0 mM Mg^{2+} and 0.4 mM spermidine. Electrophoresis was performed as described [17] using 7.5–15% gradient gels. Track G shows the positions of coat proteins.

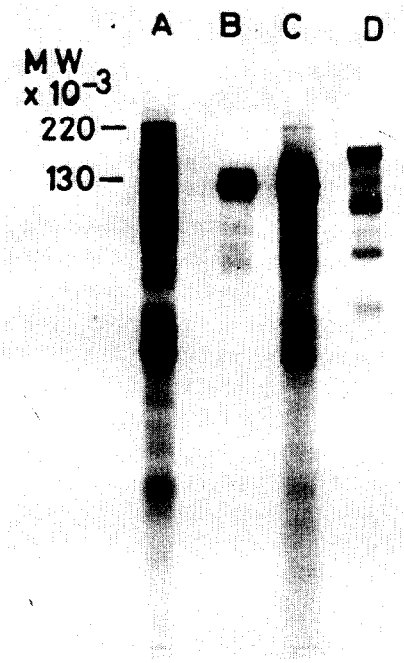


Fig.3. Translation of CPMV-RNAs under optimal conditions: analysis of the molecular weights of the products and comparison with TMV-RNA translation products. Track A: Natural mixture CPMV-RNA products. Track B: B-RNA products. Track C: M-RNA products. Track D: TMV-RNA products. Electrophoresis was performed as described [17] using a 12% gel.

(Zaitlin and Hariharasubramanian [18], Patterson and Knight [19]) and in *Xenopus* oöcytes and reticulocyte lysates (Hunter et al. [20]).

4. Discussion

Our results show that CPMV-RNAs can be translated into large polypeptides in wheat-germ extracts. Concentrations of K^+ higher than 70 mM are necessary for this translation; lower potassium concentrations give rise to lower molecular weight products, probably as a result of premature termination. Addition of spermidine (or spermine) is required for the translation into large products. The molecular weight estimations of the largest polypeptides, 220 000 for B-RNA (2.02×10^6) and 140 000 for M-RNA (1.37×10^6) suggest that almost the full-length (about 90%) of these RNAs can be translated. The origin of the smaller products may be due to processes such as premature termination, degradation of the RNA, hidden breaks in the isolated RNA or some proteolytic activity. The proportion of these products is somewhat variable.

It was observed, however, that the production of the two major products (120 000 and 140 000) from M-RNA is consistent. These 'doublet' polypeptides are also synthesized in reticulocyte extracts. The possibility of a double initiation site (as is the case with TMV-RNA) or heterogeneity of the RNA is being investigated.

The translation of CPMV-RNA into large polypeptides in wheat-germ extracts, as previously obtained in reticulocyte lysates [8,9], supports the suggestion that these RNAs can function as monocistronic messengers. The fact that full size translation products can be formed and no coat proteins have been identified among the small products, raises the interesting possibility that in vivo CPMV-RNAs are translated into large precursor molecules from which the functional virus specific proteins such as coat proteins and replicase are formed by a process of post-translational cleavage. The fact that translation into the large polypeptides reported here requires about 90% of the genome coding capacity, implies that the regions of RNA coding for the coat proteins must have been translated, as part of these large polypeptides. There is insufficient RNA remaining to code separately for coat proteins, as is the case with TMV [20].

The results reported here suggest further similarity between CPMV-RNAs and polio-like virus RNA in addition to the 5′- and 3′-terminal structures. Investigation of the post-translational cleavage phenomenon is in progress.

Acknowledgements

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