Stimulation of Encephalomyocarditis Virus RNA Translation in Wheat Embryo Extracts by Rabbit Reticulocyte Ribosomal Wash

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Encephalomyocarditis virus RNA induced a low level of amino acid incorporation in wheat embryo extracts, but the incorporation did not result in the formation of any viral proteins. However, if wheat embryo extracts were supplemented with a ribosomal wash fraction from rabbit reticulocytes, a large stimulation of amino acid incorporation and the formation of encephalomyocarditis virus-specific proteins were obtained. Among the proteins synthesized were capsid precursor proteins which can be processed by an encephalomyocarditis virus-coded protease to produce the viral capsid proteins.

Encephalomyocarditis virus (EMC) RNA can be translated in several cell-free systems, especially in mRNA-dependent rabbit reticulocyte lysates. Among the proteins synthesized is a protease that catalyzes extensive cleavage of the EMC capsid protein precursors (10, 15). Although cell-free extracts derived from wheat embryos have been shown to catalyze translation of many messengers from both plant and animal sources, EMC RNA and other picornavirus RNAs are translated poorly (see, e.g., reference 5). We report here that under our standard conditions we were unable to translate EMC RNA in wheat embryo extracts. Addition of a ribosomal wash fraction from rabbit reticulocyte ribosomes conferred on these extracts the ability to translate EMC RNA. This suggests that reticulocyte ribosomal wash can provide a factor absent in wheat embryo extracts, a factor essential for EMC translation.

The procedure described by Zagorski (17) was used for preparing wheat embryo S23 extracts from commercial wheat germ. The extracts were pretreated with staphylococcal nuclease to reduce background incorporation (11). A typical nuclease digestion mixture (200 μ l) contained 100 μ l of the S23 extract, 37.5 μ l of "Mix V" (66.7 mM creatine phosphate, 133 μ g of creatine kinase per ml, 0.63 mM each of 19 unlabeled amino acids, 16.6 mM ATP, 2.5 mM GTP), 25 μ l of a buffer-salt solution (200 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], 750 mM potassium acetate, 5 mM magnesium diacetate, 8 mM spermidine), 9 μ l of 0.1 M CaCl₂, and 1 μ l of staphylococcal nuclease (1,500 U/ml). The mixture was incubated at 22°C for 10

min. After incubation, 10 μ l of 200 mM ethylene glycol-bis(β -aminoethyl ether)-*N,N*-tetraacetic acid, 7.5 μ l of [³⁵S]methionine (specific activity, 300 to 400 Ci/mmol), and 10 μ l of calf liver tRNA were added.

Ribosomes were obtained from reticulocyte lysate (14, 15). Lysate was mixed with 0.5 volume of a solution containing 1.5 M sucrose, 0.15 M KCl, and 2 mM dithiothreitol and centrifuged in a Beckman 60 Ti rotor at 50,000 rpm for 2 h. The supernatant was withdrawn, and the ribosome pellet was rinsed briefly with cold distilled water. Ribosomal wash was prepared and concentrated with ammonium sulfate by the procedure of Schreier and Staeheling (13). The ammonium sulfate precipitate was dissolved at a concentration corresponding to 3,000 optical density units at 260 nm of ribosomes per ml of buffer and was stored in small samples at -70°C. Analysis of the preparations of sucrose density gradients showed they were not contaminated with ribosomes.

For protein synthesis reactions, 20 μ l of the treated extract was mixed with EMC RNA (7, 18) usually at 40 μ g/ml, spermidine usually at 0.8 mM, and the concentrated ribosomal wash as noted. The final volume of each reaction mixture was 25 μ l. Incubation was at 30°C for 3 h unless stated otherwise. The conditions for sample preparation, gel electrophoresis, autoradiography, fractionation on preparative gels, and tryptic analysis were as previously described (2, 14, 15).

Table 1 shows that in the absence of the ribosomal wash, [¹⁴C]leucine incorporation was only 2-fold over the background, whereas in the presence of the ribosomal wash, a 30-fold stimulation was observed. This stimulatory effect

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TABLE 1. Effect of reticulocyte ribosomal wash on the translation activity of the RNAs from EMC, poliovirus, and brome mosaic virus in the wheat embryo extract^a

Reaction	Incorporation (cpm)		
	EMC ([¹⁴ C]-leucine)	Poliovirus ([³⁵ S]-methionine)	BMV ([¹⁴ C]-leucine)
Without RNA, without ribosomal wash	63	3,622	68
With RNA, without ribosomal wash	123	3,401	10,161
With RNA, with ribosomal wash	1,855	7,348	6,340

^a Conditions for protein synthesis were as described in the text. The RNA concentration was 40 µg/ml for EMC, 20 µg/ml for poliovirus, and 100 µg/ml for brome mosaic virus (BMV). Incubation time was 3 h for EMC and poliovirus RNAs and 90 min for brome mosaic virus RNA. After incubation, duplicate samples (10 µl for [¹⁴C]leucine-labeled samples, 2 µl for [³⁵S]methionine-labeled samples) were spotted on Whatman 3 MM filter paper disks, and the proteins were precipitated, washed, and counted.

increased with the ribosomal wash concentration in the reaction mixture (Fig. 1a). Maximum incorporation of [³⁵S]methionine occurred at 2 µl of the wash per 25 µl of the reaction mixture. (Due to nonspecific adsorption of [³⁵S]methionine to the samples, the actual level of stimulation could not be determined directly.) Figure 1b shows a time course of [¹⁴C]leucine incorporation. It is evident that with the ribosomal wash, incorporation increased rapidly and continued linearly for about 60 min. Maximal incorporation required a polyamine in the reaction mixture. The effect of three levels of spermidine (0, 0.4, and 0.8 mM) on the incorporation activity at different Mg²⁺ concentrations is shown in Fig. 1c. Without spermidine, a broad Mg²⁺ curve with an optimum around 4 mM was obtained, and incorporation was relatively low. With 0.4 mM spermidine, the incorporation was higher and the curve was sharper. With 0.8 mM spermidine, the Mg²⁺ curve was very sharp, and the incorporation at the optimum Mg²⁺ concentration (about 1.6 mM) was about five times higher than the maximum incorporation without spermidine.

The reticulocyte ribosomal wash also stimulated poliovirus RNA translation, but the extent of stimulation was lower than that observed with EMC RNA (Table 1). This lower stimulation may be related to the lower in vitro translation activity of poliovirus RNA (14). Also shown in Table 1 is the effect of the ribosomal wash on the activity of brome mosaic virus RNA, a nat-

ural messenger in wheat. In this case, the ribosomal wash was inhibitory.

Synthesized products were analyzed on sodium dodecyl sulfate-polyacrylamide gels. Figure 2, lane a, shows incorporation in the absence of EMC RNA. Only two faint bands were visible. Lane b indicates the products synthesized in the presence of EMC RNA and in the absence of the ribosomal wash. The pattern was similar to that of lane a and exhibited no identifiable viral protein bands. In contrast, in the presence of ribo-

FIG. 1. Characteristics of the ribosomal wash-stimulated amino acid incorporation. Conditions for protein synthesis were as described in the text. (a) [³⁵S]methionine incorporation as a function of the ribosomal wash concentration. The reaction mixtures contained 0.8 mM spermidine. Duplicate 2-µl samples were counted. (b) Time course of [¹⁴C]leucine incorporation directed by EMC RNA in the presence of the ribosomal wash (2 µl) and spermidine (0.8 mM). Duplicate 5-µl samples were counted. Symbols: Δ, no EMC RNA and no ribosomal wash; ○, EMC RNA but no ribosomal wash; ●, EMC RNA and ribosomal wash. (c) Effect of magnesium ion concentration and spermidine concentration on EMC RNA-directed [³⁵S]methionine incorporation. Ribosomal wash preparation (2 µl) was added to each reaction mixture (25 µl). An amount of [³⁵S]methionine double that used in (a) was used in this experiment. Duplicate 2-µl samples were counted. Symbols: --●--, no spermidine added; --○--, 0.4 mM spermidine; --●--, 0.8 mM spermidine.

FIG. 2. Translation products synthesized in the wheat embryo extract. Conditions for protein synthesis gel electrophoresis were as described in the text. Lane a, No EMC RNA and no ribosomal wash; lane b, EMC RNA but no ribosomal wash; lanes c to i, samples withdrawn at 10, 20, 30, 40, 50, 60, and 120 min, respectively, from a reaction mixture containing EMC RNA and ribosomal wash; lane j, EMC proteins synthesized in rabbit reticulocyte lysate.

somal wash, several distinctive protein bands appeared (lane i). Three of these bands, designated proteins WAI, WA, and WB, migrated like the known EMC capsid protein precursors A1, A, and B, and a fourth, designated WG, migrated like EMC protein G. Figure 2, lanes c to i, shows the EMC RNA-directed products as a function of time. Some products of relatively low molecular weights were formed first, followed by the appearance of protein WB in 30 min (lane e) and proteins WA and WAI in 40 min (lane f). Protein WG did not appear until after 1 h of incubation. It is known that the amino termini of proteins A1, A, and B all map at a common initiation site (3, 4, 10) and that protein G maps immediately to the right of protein A (4). The mobility and time of appearance of WAI, WA, WB, and WG suggest that they are the wheat embryo-synthesized counterparts of proteins A1, A, B, and G. This in turn suggests that proper translation and processing of these proteins has occurred.

However, Fig. 2 shows that most EMC proteins were absent, e.g., proteins F and C, the two major primary products translated from the 3' half of the viral RNA. This partial translation is

undoubtedly a result of premature peptide chain termination and a well-documented deficiency of wheat embryo extracts. Also missing were viral capsid proteins ϵ , α , and γ . Formation of the capsid proteins from the capsid precursors requires the activity of an EMC protease (10, 15), and probably it was not synthesized because the location of the protease gene is in the 3' half of EMC RNA (9, 10, 15, 16).

Two other products are apparent in Fig. 2, protein WD (discussed below) and protein WEP. We believe that WEP is the counterpart of protein EP found in EMC RNA-programmed reticulocyte lysates. Protein EP is produced early in translation and is distinct from protein A1 (Ann Palmenberg, personal communication). It may result from initiation of translation at a second site as has been reported for polio RNA (8).

To further establish the identity of the proteins synthesized in the wheat embryo extracts, tryptic peptide maps were made and compared with those from the corresponding proteins made in reticulocyte lysates. Thus, [^{35}S]methionine-labeled proteins were isolated from preparative gels and were digested with trypsin. The resulting peptides were mixed with the tryp-

tic peptides from [^3H]leucine-labeled brome mosaic virus coat protein (used as internal markers) and chromatographed on a cation exchange column. The tryptic profiles of proteins A1 and WA1 are shown in Fig. 3a and b, respectively. The two patterns were nearly identical except near the exclusion fractions, where the A1 pattern contained two additional peaks. This suggests that WA1 and A1 are similar but are not identical. Coelectrophoresis of differentially labeled WA1 and A1 showed that WA1 migrated about 2% faster than A1 so that, in fact, it may be slightly smaller than A1. The tryptic peptides from WA and WB were subsets of the peptides from WA1 and were similar to the peptides from proteins A and B (data not shown). Figure 3c represents the pattern from a protein designated WD (the protein band in Fig. 2, lane i, that migrated slightly more slowly than protein D). It is clear from the pattern that the tryptic peptides from WD were a subset of those from WA1 (Fig. 3b) and were considerably different from those from protein D (Fig. 3d). Protein WD, therefore, is a shorter chain of WA1 and is not related to protein D.

In reticulocyte lysates, the EMC capsid protein precursors A1, A, and B can be processed by the *in vitro*-synthesized EMC protease (10, 15). To determine whether proteins WA1, WA, and WB can be cleaved by the EMC protease,

[^{35}S]methionine-labeled proteins WA1, WA, and WB were mixed with an unlabeled reticulocyte lysate mixture which contained EMC RNA translation products, including the EMC protease. Sparsomycin was added to the combined mixture to inhibit further protein synthesis. After incubation, samples were analyzed on gels. Lane a of Fig. 4 was a control experiment in which a mock wheat embryo extract (containing no EMC RNA) was mixed with the EMC products containing reticulocyte lysate mixture. The purpose of this control was to make certain that no residual protein synthesis occurred after the two extracts were mixed. As expected, the pattern shows that only the wheat embryo endogenous proteins existed. Lane b represents the [^{35}S]methionine-labeled precursors mixed with a mock reticulocyte lysate containing no EMC RNA. The pattern shows the normal band pattern of WA1, WA, and WB. Lanes c and d show the radioactive precursor sample incubated for 3 and 16 h, respectively, with the unlabeled reticulocyte mixture containing EMC translation products. It is clear from the pattern that some products with the mobility of capsid proteins ϵ , α , and γ , along with a considerable amount of the intermediate protein D1, were formed.

Although WA1, WA, and WB could be cleaved by the EMC protease, Fig. 4 indicates that the

FIG. 3. Tryptic patterns of proteins A1, WA1, D and WD. Solid line represents peptides from EMC RNA-directed products. Dashed line represents peptides from brome mosaic virus coat protein. (a) Protein A1; (b) protein WA1; (c) protein D; (d) protein WD.

FIG. 4. Cleavage of the precursor-like proteins by the EMC protease. Lanes c and d: A normal reaction mixture of the wheat embryo extract was prepared, and sparsomycin (0.1 mM) was added at the end of incubation. To this mixture, 5 volumes of a nonradioactive reticulocyte reaction mixture, which contained EMC RNA and had been incubated for 1 h, was added. The combined mixture was further incubated for 3 h (lane c) and 16 h (lane d). Lane a: A mock wheat embryo reaction mixture containing no EMC RNA was mixed with 5 volumes of the reticulocyte lysate mixture and incubated for 16 h. Lane b: The precursor-containing wheat germ reaction mixture was mixed with 5 volumes of a mock reticulocyte lysate mixture containing no EMC RNA and incubated for 16 h. Lane e: Capsid protein precursors synthesized in the reticulocyte lysate were mixed with the nonradioactive reticulocyte reaction mixture and incubated for 3 h.

efficiency of the cleavage was less than that found with A1, A, and B (15). A simple explanation for this low cleavage efficiency is that the ionic conditions of the wheat embryo extract are not favorable for proper folding of the precursor chains into the correct conformation recognizable by the EMC protease. Another possibility is that the wheat extracts lack a proper primary cleavage enzyme. WA1, WA, and WB may be in peptidyl-tRNA forms so that they are not able to fold into the correct conformation. A third possibility is the presence of a protease inhibitor in the wheat embryo extract.

The reason for the partial translation of EMC

RNA in the wheat embryo extract is not clear. We tried to correct the partial translation by changing reaction conditions, such as varying the incubation temperature and the ionic conditions, lowering the RNA concentration, shifting salt concentrations, or adding reticulocyte pH 5 enzymes. None of these was useful. This partial translation, or preferential termination after the capsid precursor coding region, is a phenomenon encountered in a number of cell-free systems (e.g., references 1, 6, 12). Perhaps this reflects some deficiency of the *in vitro* systems, but it may reflect a mechanism involved in regulation of picornaviral protein synthesis. We have observed that in the reticulocyte lysates the rate of ribosome movement decreases after the ribosomes traverse the region coding for the capsid precursors (15). This slowdown results in a temporary accumulation of the capsid precursors. Also, we have observed that for certain reticulocyte lysate preparations, the addition of exogenous tRNA is important for complete translation of EMC RNA. For these preparations, if tRNA is not added, primarily the capsid precursors will be synthesized. It is possible that all these phenomena are related and that they may reflect the existence of a mechanism important for the expression of the 3' half of the picornavirus genomes.

Our results demonstrate clearly that wheat embryo extracts lack a factor (or factors) essential for the translation of EMC RNA. Obviously, this factor is not required for the translation of brome mosaic virus RNA (an homologous RNA) or for the large number of heterologous mRNAs that are translated efficiently in these extracts. This factor exists in reticulocyte ribosomal wash and should be purifiable from it.

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