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PROTEIN SYNTHESIZING SYSTEM FROM WHEAT GERM.
EFFICIENT TRANSLATION OF SYNTHETIC AND NATURAL MESSAGES*

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Optimum conditions for translation of eukaryotic, prokaryotic and synthetic templates in wheat germ cell-free extract were determined.

1. Translation of eukaryotic message (BMV RNA and TMV RNA) was at optimum at the same concentrations of K⁺, Mg²⁺, HEPES and spermine. In optimal conditions the efficiency of translation was high, for BMV RNA being equal to 220 pmol and for TMV RNA, to 280 pmol of leucine incorporated per 1 μg of template.

Prokaryotic template (Qβ RNA) was translated under different ionic conditions.

2. Translation of synthetic template [poly(U)] was at optimum at fairly higher concentrations of K⁺ and Mg²⁺ than those optimal for natural template translation.

3. Efficient translation of natural and synthetic templates depends on complete removal of inhibitors found in the wheat germ cell-free extract. Action of these inhibitors could be mimicked by adenine nucleotides.

The wheat embryo cell-free system is widely used in studies on translation in eukaryotes. The reasons for this are as follows: a) The system is versatile and translates efficiently different classes of mRNAs: bacteriophage RNA (Davies & Kaesberg, 1973), plant viral (Marcus et al., 1974a) and animal viral RNA (Davies & Kaesberg, 1974; Davies & Samuel, 1975) as well as cellular eukaryotic mRNA (Roberts & Paterson, 1973). b) Endogenous incorporation in the system is very low. c) The commercially available form of wheat embryo, called wheat germ, is a cheap source of material for preparing the system.

However, the system has some disadvantages. It seems that reproducibility of the system is quite low. For example, in the case of TMV1 RNA translation, as

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1 Abbreviations: BMV, Brome mosaic virus; TMV, tobacco mosaic virus; SAM, S-adenosyl l-methionine; SAH, S-adenosyl-homocysteine; Qβ RNA, phage Qβ RNA; DTT, dithiothreitol (Cleland's reagent); HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid.
different activities as 4 or 250 pmoles of [14C]leucine incorporated per 1 μg of template were reported (cf Roberts & Paterson, 1973; Marcus & Dudock, 1974).

There might be various reasons for such discrepancies. One possibility is that the wheat germ from different sources is of uneven quality (Marcus & Dudock, 1974). On the other hand, the variations in specific activities of wheat germ cell-free systems isolated in different laboratories may be caused by technical differences. For example, the low-molecular-weight substances are removed from the system either by a short dialysis (Shih & Kaesberg, 1973; Marcus et al., 1974a) or by Sephadex filtration (Marcus & Dudock, 1974). In addition, the observed differences may be due to various composition of the incorporation mixtures.

In order to answer these questions we examined the methods of isolation of the cell-free extract from wheat germ and we investigated the optimum conditions for translation of different messages in this system. The system was characterized by studying the translation of BMV RNA. This multicomponent, eukaryotic viral RNA is easy to isolate and is efficiently translated in the wheat system, promoting the synthesis of specific polypeptides (Shih & Kaesberg, 1973, 1976).

The translation of BMV RNA was compared with that of other types of templates: multicomponent RNA from animal virus (reovirus mRNA), large, single-component RNA from plant virus (TMV RNA), prokaryotic mRNA (phage Qβ RNA) as well as a synthetic template, poly(U).

It was observed that three different groups of templates (prokaryotic, eukaryotic and synthetic) were translated under three different sets of conditions. It is of importance that optimum conditions for the translation of different viral eukaryotic messages were quite similar. This finding may facilitate further studies on the translation of other viral RNAs, not always so facile to isolate as BMV or TMV RNAs.

It was shown that the wheat germ extract contained soluble, low-molecular-weight inhibitor(s) of translation, that diminished the aminocacylation of tRNA. Removal of inhibitors by extensive dialysis or Sephadex filtration substantially enhanced the activity of the system and resulted in highly reproducible, efficient translation of the tested messages. Extending previous observations (W. Zagorski, Analytical Biochemistry, in press) it was found that adenine nucleotides, which are present in wheat embryo in rather high concentrations (Cheung & Marcus, 1976), imitated the action of the detected inhibitors.

MATERIAL AND METHODS

Preparation of cell-free extract from wheat embryos and wheat germ. Wheat embryos were isolated according to the method of Johnston & Stern (1957) as described by Shih & Kaesberg (1973) from Kenosha winter wheat. Wheat germ (raw, untoasted) was a kind gift of General Mills, Golden Valley, Minn., U.S.A.

Wheat embryos were floated in a mixture of CCl₄ and cyclohexane, in the solvents mixed in the ratio 500 : 200 (v/v), and wheat germ in the same solvents mixed in the ratio 500 : 120 (v/v). Flotation was repeated three times, the material was dried on air at room temperature for 20 h and stored at 4°C. The standard
procedure for isolation of the cell-free extract from wheat germ at 0-4°C was as follows. Wheat germ was ground with acid-washed quartz sand in 1:1 (w/v) ratio in the mortar until powdered, then the extraction buffer (10 mM-Tris-acetate, pH 7.6, 50 mM-K-acetate, 3 mM-Mg-acetate, 1 mM-DTT) was added (3-5 ml per 1 g of wheat germ). Grinding was continued for 5 min until a fine paste was obtained. The homogenate was centrifuged at 23,000 g for 10 min, the supernatant was collected and re-centrifuged under the same conditions. Upper three-fourths of the supernatant was collected and submitted to extensive dialysis against the extraction buffer (three changes, 100 volumes each, 24 h, on ice), or filtered through Sephadex G-25 coarse column (21×1 cm) as described by Marcus & Dudock (1974). The column was equilibrated and elution was carried out with the extraction buffer. The cell-free extract from wheat embryos as well as that from wheat germ used in the initial experiments (Figs. 1, 2) were isolated as described by Shih & Kaesberg (1973). Then the preparations were submitted to short-term dialysis (up to 3 h) as suggested in the literature (Shih & Kaesberg, 1973; Marcus et al. 1974a).

After dialysis or Sephadex filtration, the cell-free extract was re-centrifuged at 23,000 g, the heavy precipitate formed was discarded, and the extract was frozen in portions and stored at −40°C.

The postribosomal supernatant (S-105) was obtained by centrifugation of the cell-free extract for 4 h at 105,000 g in the MSE 3×5 ml swing-out rotor. Upper two thirds of S-105 was collected, a portion of it was submitted to extensive dialysis as described for the cell-free extract, whereas the remaining portion was left undialysed. The S-105 preparations were frozen in portions and stored at −40°C.

The concentration of protein in the cell-free extract was usually 32-35 mg/ml and in the S-105 preparations, 23 mg/ml. Before the estimation of protein concentration according to the method of Lowry et al. (1951), proteins were precipitated with 5% trichloroacetic acid, washed twice by centrifugation with trichloroacetic acid and once with a mixture of ethanol and ethyl ether (1:1). This step removed phenolic compounds which affect the protein estimation with Polin reagent.

Preparation of RNAs. Wheat germ tRNA and total, unfractionated BMV RNA were prepared as described elsewhere (W. Zagórski, Analytical Biochemistry, in press). Phage Qβ RNA was a kind gift of Dr. A. Palmenberg. TMV RNA was a gift of Dr. A. Legocki. Single-stranded transcripts of total reovirus RNA obtained in vitro were kindly supplied by Dr. J. Davies.

Translation of poly(U). Standard 100 µl incorporation mixture contained: 50 µl of the cell-free extract; 0.1 µCi of [14C]phenylalanine (spec. act. 60 mCi/mmole); 0.5 mM-DTT; 5 mM-Tris-acetate buffer, pH 7.6; 20 mM-HEPES-KOH buffer, pH 7.5; 8.9 mM-Mg-acetate; 100 mM-K-acetate; 2.5 mM-ATP; 0.375 mM-GTP; 20 mM-creatine phosphate; 6 µg of creatine kinase and 48 µg of poly(U). The incubation was carried out at 31°C for 30 min.

Translation of phage Qβ RNA. Standard 100 µl incorporation mixture contained: 50 µl of the cell-free extract; 1 µCi of [14C]leucine (spec. act. 324 mCi/mmole); 0.5 mM-DTT; 5 mM-Tris-acetate buffer, pH 7.6; 4 nmoles of each of 19 non-labelled amino acids; 4.5 mM-Mg-acetate; 65 mM-K-acetate; 2.5 mM-ATP; 0.375 mM-GTP;
10 mM-creatine phosphate; 6 µg of creatine kinase and 16 µg of Qβ RNA. The incubation was carried out at 31°C for 2 h.

Translation of BMV RNA. Standard 100 µl incorporation mixture contained: 50 µl of the cell-free extract; 1.25 µCi of [14C]leucine (spec. act. 59 mCi/mmol); 0.5 mM-DTT; 5 mM-Tris-acetate buffer, pH 7.6; 20 mM-HEPES-KOH buffer, pH 7.5; 22.5 mMoles of each of 19 non-labelled amino acids; 5.0 mM-Mg-acetate; 95 mM-K-acetate; 2.5 mM-ATP; 0.375 mM-GTP; 20 mM-creatine phosphate; 6 µg of creatine kinase and BMV RNA in concentrations given in Figures.

In some experiments the concentration of ATP in the incorporation mixture was lowered to 1.5 mM, and Mg-acetate concentration to 3.2 mM. The incubation was carried out at 31°C for 250 min.

Translation of reovirus RNA. This was performed in the standard incorporation mixture the same as that used for BMV RNA translation.

Translation of TMV RNA. The incorporation mixture was similar to that used for the translation of BMV RNA except that the concentration of Mg-acetate was 3.6 mM, ATP was 1.5 mM, and 0.8 µCi of [14C]leucine (spec. act. 125 mCi/mmol) was added per 100 µl of the incorporation mixture. The incubation was carried out at 31°C for 2 h.

Aminoacylation of wheat germ tRNA. The incorporation mixture was similar to that which was used for BMV RNA translation. It was supplemented with the S-105 supernatant instead of the cell-free extract and contained 3.2 mM-Mg-acetate and 1.5 mM-ATP. BMV RNA was replaced with wheat germ tRNA (38 µg per 50 µl of mixture). Non-labelled amino acids were omitted. Constant ratio of the radioactive amino acid to the S-105 supernatant was ensured in order to eliminate the effect of radioisotope dilution. Per 5 µl of S-105 were added: 0.2 µCi of [14C]leucine (spec. act. 125 mCi/mmol), or 0.02 µCi of [14C]phenylalanine (60 mCi/mmol), or 1 µCi of [3H]methionine (6 Ci/mmol). The incubation was carried out at 31°C for 30 min.

Estimation of the adenine nucleotides content in the cell-free extract. This was performed enzymatically as described by Keppler (1974), except that adenine nucleotides were extracted with 9% HClO₄ mixed with the cell-free extract in 1:1 (v/v) ratio. Internal standard (ADP) was added to monitor the efficiency of HClO₄ extraction. NADH oxidation was followed at 340 nm, using Cary 118 spectrometer, equipped with 1 ml cuvettes (1 cm light path).

Counting procedures. After incorporation, the aliquots of the radioactive mixtures were applied onto Whatman 3MM filter paper discs and washed according to Mans & Novelli (1961). For the aminoacylation assay, filter paper discs were washed three times for 10 min with ice-cold 5% trichloroacetic acid, once with ethyl ether-ethanol mixture (1:1, v/v), and finally with ethyl ether. The discs were dried and counted in a Packard scintillator counter, using a toluene scintillator with 60% efficiency for 14C and 7% efficiency for 3H.

Reagents. Radioactive amino acids were from Amersham-Searle (Arlington, Ill., U.S.A.) or from UVVVR (Prague, CSSR). Non-labelled amino acids were from Mann Res. Lab. (New York, N.Y., U.S.A.). ATP (dipotassium salt, A grade),
ADP (potassium salt), AMP monohydrate, creatine phosphokinase, spermine tetrahydrochloride and myokinase (ex rabbit muscle, A grade) were from Calbiochem (La Jolla, Calif., U.S.A.). GTP (trisodium salt), creatine phosphate (monopotassium salt), Tris-(hydroxymethyl)-aminomethane, SAM, SAH, phosphodiesterase from Crotaulus adamantus venom, type III and phosphoenolpyruvate kinase, from Sigma (St. Louis, Mo., U.S.A.). DTT and HEPES were from Aldrich (Milwaukee, Wisc., U.S.A.). Poly(U) was from Miles (Kankakee, III., U.S.A.). Magnesium acetate was from Merck (Darmstadt, F.R.G.), KOH and potassium acetate from Malinckrodt (St. Louis, Mo., U.S.A.) or from Prolabo (Rhône-Poulenc, France). Sephadex G-25 coarse was from Pharmacia (Uppsala, Sweden). Dialysis bags, size 8 were from Union Carbide Corp. (Chicago, Ill., U.S.A.). Lactic dehydrogenase (200 U/mg of protein) was from Biomed (Kraków, Poland).

RESULTS

Isolation of active cell-free extract

The initial experiments were devoted to the comparison of translational activity of the cell-free extract isolated from wheat embryo with that from wheat germ. The extracts examined in these experiments were isolated and their activity was tested according to the standard procedures described in the literature (Shih & Kaesberg, 1973; Marcus et al., 1974a). It was observed that for such extracts primed with BMV RNA, the activity expressed in pmoles of $[^{14}C]$leucine incorporated per milligram of protein varied from preparation to preparation. Nevertheless, the activity of the cell-free extract isolated from wheat embryos was of the same order of magnitude as the activity of the cell-free extract from wheat germ (Fig. 1). Therefore, further experiments were focused on the properties of the system isolated from wheat germ.

To find out the reason for the variations between the cell-free extract preparations obtained from the same batch of wheat germ, we measured their activity in terms of utilization of added mRNA. Incorporation mixtures containing a constant amount of template were titrated with increasing amounts of the cell-free extract proteins (Fig. 2).

Different preparations of the cell-free extract showed very different activities, measured in terms of maximum $[^{14}C]$leucine incorporation. Moreover, the titration curves showed unusual features. The curves had a well defined maximum at about 20 - 30 μl of the cell-free extract per 100 μl incorporation mixture. At higher concentrations of the cell-free extract, incorporation became strongly inhibited. These non-linear titration curves suggested that the cell-free extract contained inhibitor(s) active at the higher concentrations of the cell-free extract. Thus, it could be assumed that in the preparations showing low translational capacity, the inhibitors were present in high concentrations, and that the preparations which were more active were partially depleted of inhibitors.
Fig. 1. Kinetics of [\(^{14}\)C]leucine incorporation into proteins under direction of BMV RNA. The incorporation mixture (100 \(\mu\)l) contained 2.5 mm-ATP, 5.0 mm-Mg-acetate, 63 \(\mu\)g of BMV RNA, cell-free extract (1.75 mg of protein), 700 pmoles of [\(^{14}\)C]leucine (spec. act. 348 mCi/mmole) and other components as described in Methods. At indicated times, 10 \(\mu\)l portions of incorporation mixture were analysed as described in Methods. The cell-free system was isolated from two different preparations (O, ●) of wheat germ or from wheat embryo (Δ) and dialysed for 3 h.

Fig. 2. Dependence of [\(^{14}\)C]leucine incorporation under direction of BMV RNA on the concentration of wheat germ cell-free extract. Conditions of incorporation as in Fig. 1, except that incubation mixtures (100 \(\mu\)l) containing 9 \(\mu\)g of total BMV RNA were supplemented with indicated volumes of two different preparations (O, ●) of the cell-free extract dialysed for 3 h.

It was observed that the shape of the titration curve depended on the method of preparation of the system. The inhibition of translation occurring at higher concentrations of the cell-free extract was observed in the extract submitted to the short-term dialysis (up to three hours). When the extract was dialysed longer, the incorporation was substantially enhanced and the titration curve was becoming linear. A lack of inhibition at higher concentrations of the cell-free extract was also observed when the extract immediately after isolation was passed through Sephadex G-25 (Fig. 3). From Figures 2 and 3 it can be seen that after removal of low-molecular-weight substances from the extract the translocation capacity of the system was distinctly raised (up to 1000 pmoles of leucine/100 \(\mu\)l). At this level of incorporation the concentration of radioactive leucine in the incorporation mixture could be a limiting factor. Therefore, beginning with the experiments described in Fig. 3, the concentration of [\(^{14}\)C]leucine was raised from 0.7 to 20 nmoles per 100 \(\mu\)l of incorporation mixture.

The inhibition observed in the extract dialysed for 3 h could not be overcome by the addition of increasing amounts of radioactive amino acids to the incorporation mixture, therefore it was not due to the isotopic dilution. The presence of
Fig. 3. Translation of BMV RNA and poly(U) in the wheat germ cell-free extract deprived of soluble inhibitors.

a. Translation of BMV RNA: the incorporation mixture (25 μl) containing 4 μg of total BMV RNA was supplemented with the indicated amount of the cell-free extract, either dialysed against the extraction buffer for 24 h (○), or filtered through Sephadex G-25 column (△) as described in Methods; 5 nmoles of [14C]leucine (spec. act. 11 mCi/mnmole) was used. Other components of the incorporation mixture as in Fig. 1.

b. Translation of poly(U): the incorporation mixture (50 μl) was supplemented with the indicated amount of the cell-free extract dialysed against the extraction buffer for 24 h (○) or filtered through Sephadex G-25 column (△) as described in Methods.

Inhibitory substances in the cell-free extract was further supported by comparison of BMV RNA-directed polypeptide syntheses in shortly and extensively dialysed extracts. Only one polypeptide migrating on polyacrylamide gel in the region of BMV coat protein was formed in the extract dialysed for 3 h, whereas in the extensively dialysed extract the synthesis of long polypeptides, migrating in the position of BMV proteins 1 and 2, was additionally observed (W. Zagórski, Analytical Biochemistry, in press).

Adenine nucleotides as possible agents responsible for inhibition of translation in the wheat germ cell-free system

It should be noted that in the shortly dialysed cell-free extract the translation of both natural and synthetic template was low. This suggested that in the extract containing inhibitor(s), the reactions which are common for the translation of both templates were affected. This prompted us to investigate the formation of aminoacyl-tRNA in the cell-free extracts prepared in different ways. To correlate the efficiency of translation with that of aminoacylation, charging of tRNA was performed in salt conditions resembling those being optimum for overall BMV
RNA-directed polypeptide synthesis. Indeed, it was observed that aminoacylation was less effective in the presence of undialysed than with the dialysed S-105 supernatant (Table 1).

Table 1

Effect of dialysis on the activity of the wheat germ tRNA charging enzymes

For conditions of tRNA aminoacylation see Methods.

<table>
<thead>
<tr>
<th>S-105</th>
<th>Protein content (µg)</th>
<th>aa-tRNA formed in incorporation mixture (pmoles/50 µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Met-tRNA*</td>
</tr>
<tr>
<td>Non-dialysed</td>
<td>105</td>
<td>10.8</td>
</tr>
<tr>
<td></td>
<td>525</td>
<td>15.1</td>
</tr>
<tr>
<td>Dialysed</td>
<td>105</td>
<td>46.8</td>
</tr>
<tr>
<td></td>
<td>525</td>
<td>61.0</td>
</tr>
</tbody>
</table>


The inhibition of formation of Met-tRNA, Leu-tRNA and Phe-tRNA explained the low level of the BMV RNA and poly(U) translation observed in the undialysed or shortly dialysed cell-free extract.

On the basis of the presented results several properties of the detected inhibitory substances could be deduced, i.e., a) they are soluble, low-molecular-weight compounds; b) they are affecting the aminoacylation of tRNA; c) their action is reversible; d) their effect is due to the interaction with some component of incorporation mixture rather than to the direct action on the cell-free extract proteins. Taking into account these properties, it was assumed that a class of substances which could function as such inhibitors would be adenine nucleotides. These water-soluble, low-molecular-weight substances accumulate in wheat embryo in rather high concentrations (Cheung & Marcus, 1976). It is known that high concentrations of ATP inhibit the aminoacylation of tRNA. It is assumed that the inhibition is due to chelating properties of ATP and is caused by the withdrawal of Mg²⁺ ions from the reaction medium by the excess of ATP (Roy & Tener, 1967; Schlimme et al., 1970).

The results presented in Table 2 show that indeed, the three adenine nucleotides, when added to the active cell-free extract, inhibited translation of BMV RNA. Upon addition of adenine nucleotides to the extensively dialysed S-105 supernatant, the aminoacylation of tRNA became diminished (Table 3).

As it can be seen, all three adenine nucleotides inhibited overall protein synthesis and aminoacylation of tRNA. The formation of Met-tRNA was more sensitive to the inhibition induced by adenine nucleotides than the formation of other aminoacyl-tRNAs (Table 3). The same pattern of the inhibition of aminoacyl-tRNA formation was observed in undialysed S-105 supernatant (see Table 1).
At this stage of work it becomes of interest to evaluate the concentration of adenine nucleotides in the non-dialysed extract, and to compare it with concentration of these components in the active extract, depleted of low-molecular-weight components. It was found that in the undialysed extract, adenine nucleotides concentration was high, approaching 1 mM. After 2 - 3 h of dialysis, when inhibition of translation was still persistent, the concentration of adenine nucleotides was still quite high, approaching 0.7 mM. Only after prolonged dialysis or Sephadex filtration, the adenine nucleotides concentration dropped significantly (to 0.09 mM), and simultaneously the incorporating system became fully active.

### Table 2

**Effect of adenine nucleotides on BMV RNA-directed protein synthesis in the extensively dialysed wheat germ cell-free extract**

Values expressed in pmoles of \(^{14}\text{C}\)leucine incorporated in 50 μl assay. Incubation mixture (see Methods) was supplemented with 6 μg of BMV RNA.

<table>
<thead>
<tr>
<th>Concentration of adenine nucleotide added to the system (mM)</th>
<th>Incorporation of (^{14}\text{C})leucine (pmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AMP</td>
</tr>
<tr>
<td>0</td>
<td>830</td>
</tr>
<tr>
<td>2</td>
<td>500</td>
</tr>
<tr>
<td>4</td>
<td>180</td>
</tr>
</tbody>
</table>

### Table 3

**Effect of adenine nucleotides on aminoaacylation of wheat germ tRNA**

50 μl incorporation mixture (see Methods) was supplemented with 105 μg of S-105 proteins. S-105 fraction was extensively dialysed.

<table>
<thead>
<tr>
<th>Incubation mixture</th>
<th>aa-tRNA formed in incorporation mixture (pmoles/50 μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Met-tRNA</td>
</tr>
<tr>
<td>Complete system</td>
<td>36.5</td>
</tr>
<tr>
<td>+2 mM-AMP</td>
<td>35.2</td>
</tr>
<tr>
<td>-4 mM-AMP</td>
<td>30.8</td>
</tr>
<tr>
<td>+2 mM-ADP</td>
<td>34.9</td>
</tr>
<tr>
<td>-4 mM-ADP</td>
<td>32.0</td>
</tr>
<tr>
<td>+2 mM-ATP</td>
<td>21.0</td>
</tr>
<tr>
<td>+4 mM-ATP</td>
<td>9.1</td>
</tr>
</tbody>
</table>

* Values corrected as in Table 1.
Ionic requirement for the translation in the cell-free extract from wheat germ

The optimum conditions for protein synthesis in the wheat germ cell-free system were investigated using the extensively dialysed, fully active cell-free extract. The effect of Mg²⁺ ions is presented in Fig. 4. The optimum Mg²⁺

![Graphs showing the effect of Mg²⁺ ion concentration on translation of different templates in the wheat germ cell-free extract.]

Fig. 4. Effect of Mg²⁺ ion concentration on translation of different templates in the wheat germ cell-free extract.

a, Translation of BMV RNA: the incorporation mixture (50 µl) contained 7 µg of total BMV RNA, Mg-acetate as indicated, and (○), 1 mM-ATP; (△), 1.5 mM-ATP; (□), 2.5 mM-ATP; (●), 3.5 mM-ATP. Other components of the incorporation mixture as described in Methods.

b, Translation of TMV RNA: the incorporation mixture (50 µl) was supplemented with 2.8 µg of TMV RNA, 1.5 mM-ATP and Mg-acetate as indicated. Other components as in Methods.

c, Translation of Qβ RNA: the incorporation mixture (50 µl) was supplemented with 10 µg of Qβ RNA, 2.5 mM-ATP, Mg-acetate as indicated and other components as in Methods.

d, Translation of poly(U): the incorporation mixture (100 µl) contained 100 mM-K-acetate, 2.5 mM-ATP and Mg-acetate as indicated. Other components as in Methods.
concentration for the translation of BMV RNA was found to be dependent on the ATP concentration. At the ATP concentrations of 1, 1.5, 2.5 and 3.5 mM, the respective magnesium optima were 3.0, 3.2, 4.6 and 5.6 mM (see Fig. 4a). This showed that the effect of ATP could be compensated by the addition of almost stoichiometrical amounts of Mg^{2+} ions. It seems that the optimum Mg^{2+} concentration for the \textit{in vitro} viral message translation in the studied system should be 2 mM+ATP concn. (mM). Similarly the Mg^{2+} optimum for Qβ RNA translation, estimated in the presence of 2.5 mM ATP, was found to be 4.5 mM. Also in the case of TMV RNA translation, at 1.5 mM-ATP, the optimum Mg^{2+} ion concentration was 3.6 mM.

Interdependence of the Mg^{2+} and ATP optima was reported in studies on aminoacylation of tRNA in other cell-free systems and was attributed to chelation of Mg^{2+} from the incubation mixture by ATP (Roy & Tener, 1967; Schlimm et al., 1970). However, the influence of ATP and Mg^{2+} ions on the overall translation of natural template was more complex than on the aminoacylation alone. When the concentration of ATP exceeded 1 - 1.5 mM, the translation of BMV RNA was diminished even when tested at the appropriate optimum Mg^{2+} concentration. This probably reflects the involvement of ATP in the initiation machinery of eukaryotes (Marcus et al., 1974b). For the optimum translation, more magnesium ions is needed than for balancing the ATP added. This is due to the presence of other components binding Mg^{2+} (e.g. GTP) in the incorporation mixture, as well as to the involvement of Mg^{2+} ions in maintaining the optimum stability of ribosomal 80S couples.

The translation of phage Qβ RNA as well as of TMV RNA depends on Mg^{2+} concentration in the same way as the translation of BMV RNA (Fig. 4b,c). In contrast to that, the translation of poly(U) is at optimum at much higher Mg^{2+} concentration (Fig. 4d). A similar effect is known to occur \textit{in vitro} in prokaryotic protein synthesizing systems, and it is attributed to the requirement of additional amount of Mg^{2+} for the initiation of translation in the presence of synthetic message.

Marcus & Dudock (1974) reported that the translation of TMV RNA is stimulated by the addition of spermine tetrahydrochloride; the effect was thought to be specific for this kind of mRNA—as spermine is a constituent of the TMV virion. However, in our hands, the translation of both tested eukaryotic templates responded in the same way to the presence of spermine (Fig. 5a,b).

Spermine affects in two ways TMV and BMV RNA translation (Fig. 5). It can substitute for Mg^{2+} ions, and, at optimal Mg^{2+} concentration, stimulates the incorporation of [\textsuperscript{14}C]leucine by about 30%. The general effect of spermine can be therefore regarded as a feature of the incorporation system rather than of the specific message only.

The monovalent cation requirements in the translation of different classes of mRNAs were different. Phage RNA was translated at 65 mM-K⁺, the optimum for both TMV RNA and BMV RNA translation was equal to 95 - 100 mM-K⁺, and the optimum for polyphenylalanine synthesis was broad and extended toward higher concentrations of K⁺ ions (see Fig. 6a - d). As it could be expected, the
Fig. 5. Effect of spermine concentration on translation of BMV RNA and TMV RNA in the wheat germ cell free extract.

a. Translation of BMV RNA: the incorporation mixture (50 μl) contained 7 μg of total BMV RNA, 1 mM-ATP, spermine as indicated and (○), 3.2 mM-Mg-acetate or (∆), 2.1 mM-Mg-acetate. For other details, see Methods.

b. Translation of TMV RNA: the incorporation mixture (50 μl) contained 2.8 μg of TMV RNA, 1 mM-ATP, spermine as indicated and (○), 3.7 mM-Mg-acetate or (∆), 3.2 mM-Mg-acetate.

For other details, see Methods.

optimum concentration of K⁺ ions for BMV translation did not depend on ATP concentration in the incorporation mixture (Fig. 6a).

Prokaryotic and eukaryotic message translation was affected in different ways by the presence of HEPES (Fig. 7a-c). In the case of Qβ RNA, translation was inhibited not only in the presence of HEPES buffer but also by higher concentrations of Tris-acetate buffer (Fig. 7d).

After the optimum conditions for protein synthesis were established, the translational capacity of the system fully active after the removal of soluble inhibitors, was measured. It was found (Figs. 8 and 9) that the system incorporated up to 2000 and 3600 pmoles of leucine in 100 μl assay in the presence of saturating amounts of BMV RNA and TMV RNA, respectively. Specific activity of the system, when measured at the mRNA concentrations below the saturation level, was 280 and 220 pmoles of leucine incorporated per 1 μg of BMV RNA or TMV RNA, respectively.

A prerequisite for the efficient translation of eukaryotic message is modification of mRNA at its 5' end by introduction of "cap" structure (modified guanosine attached to penultimate base through 5'-ppp-5' link). Both TMV and BMV RNAs are known to contain such structure, when isolated from virions (Zimmern, 1975; Dasgupta et al., 1976). In the case of reovirus mRNA transcribed in vitro, it was observed that the methylation of guanosine at 5' end is mediated by wheat germ enzymes (Muthukrishnan et al., 1975; Both et al., 1975) and SAM, as an obligatory
Fig. 6. Effect of K+ ion concentration on translation of different templates in the wheat germ cell-free extract.

a. Translation of BMV RNA: (○), 2.5 mM-ATP, 5.0 mM-Mg-acetate; (■), 1.5 mM-ATP, 3.2 mM-Mg-acetate. The incorporation mixture (100 μl) was supplemented with 14 μg of total BMV RNA. Other components as in Methods.

b. Translation of TMV RNA: the incorporation mixture was as in Fig. 4b, with 1.5 mM-ATP; Mg-acetate concentration was at optimum.

c. Translation of Qβ RNA: incubation mixture as in Fig. 4c, ATP was 2.5 mM, Mg-acetate concentration was at optimum.

d. Translation of poly(U): incubation mixture as in Fig. 4d, ATP was 2.5 mM, Mg-acetate concentration was at optimum. The incorporation mixture (100 μl) was supplemented with 25 μl of the cell-free extract.

Substrate for this reaction. A cell-free system, depleted of low-molecular-weight substances (and among them, probably SAM) could therefore be inactive in the translation of templates not modified at their 5' ends. To investigate this possibility we tested the influence of SAM and SAH on the translation of BMV RNA and reovirus RNA.
Fig 7. Effect of buffer concentration on translation of different templates in the wheat germ cell-free extract.

a, Translation of BMV RNA: the incorporation mixture (50 μl) was supplemented with 7 μg of BMV RNA, ATP concentration was 1.5 mm, Mg²⁺ and K⁺ concentrations were at optimum. HEPES concentration as indicated; for other components, see Methods.

b, Translation of TMV RNA: conditions of incorporation as in Fig. 4b, HEPES concentration as indicated; for other components, see Methods.

c, Translation of Qβ RNA: conditions of incorporation as in Fig. 4c, HEPES concentration as indicated; for other components, see Methods.

d, Translation of Qβ RNA: conditions as in c, Tris-acetate concentration as indicated.

The addition of SAM or SAH did not affect the translation of BMV RNA (Fig. 10). Translation of reovirus RNA was inhibited by SAH added at a concentration exceeding 300 μm (Fig. 11) but was not stimulated by the addition of SAM (Table 4). This means that the translation of reovirus mRNA is indeed dependent
Fig. 8. Time course of [14C]leucine incorporation directed by BMV RNA. The incorporation mixtures (100 µl) were incubated under optimal conditions for the indicated time. The mixtures contained (●), 25.4 µg or (△), 3.6 µg of total BMV RNA. Stimulation — 100 times over blank not supplemented with BMV RNA. For other details, see Methods.

Fig. 9. Time course of [14C]leucine incorporation directed by TMV RNA. The incorporation mixtures (100 µl) were incubated under optimal conditions (see Methods) for the indicated time in the presence of 16 µg of TMV RNA (△). Control, without TMV RNA (○).

Fig. 10. Effect of SAM and SAH on translation of BMV RNA. The incubation mixture (300 µl) containing 45 µg of total BMV RNA and 75 µl of the cell-free extract was incubated for the indicated time, then 25 µl portions were withdrawn and analysed for hot trichloroacetic acid-precipitable radioactivity. ATP concentration 2.5 mM, Mg-acetate concentration 5.0 mM, other components as in Methods. (○), No additions; (●), with 320 µM-SA; (△), with 4 µM-SAM.

Fig. 11. Effect of SAH on translation of reovirus mRNA. The incubation mixture (20 µl) containing 1 µg of reovirus mRNA was incubated with the indicated concentrations of SAH. For other details, see Methods.
Table 4

Effect of S-adenosylmethionine on translation of reovirus mRNA in wheat germ cell-free extract

Details of incorporation mixture as described in Methods.

<table>
<thead>
<tr>
<th>mRNA (µg/100 µl incorporation mixture)</th>
<th>SAM (8 µM)</th>
<th>Incorporation of leucine (pmoles/100 µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Expt. 1</td>
</tr>
<tr>
<td>5</td>
<td>—</td>
<td>1220</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>1440</td>
</tr>
<tr>
<td>10</td>
<td>—</td>
<td>1940</td>
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<tr>
<td>10</td>
<td>+</td>
<td>1820</td>
</tr>
</tbody>
</table>

on methylation and, furthermore, that SAM is present in the incorporation mixture in concentrations ensuring efficient translation of unmethylated templates. A plausible explanation of this observation is that SAM can be generated from ATP and methionine, present in the incorporation mixture.

DISCUSSION

Optimization of the preparation of cell-free extract and of conditions for the incorporation allow for high level of total protein synthesis in wheat germ system. In response to TMV RNA, the system incorporates up to 3500 pmoles of leucine in the 100 µl assay. Assuming that the synthesized polypeptides contain on the average about 5% of leucine, it can be calculated that about 7 µg of TMV proteins are formed in one assay. Therefore, the optimized system isolated from commercial wheat germ shows about three times higher translational capacity than the highest reported (Marcu & Dudock, 1974).

Several factors are responsible for the improved capacity of the system. One is the quality of wheat germ. Several batches of General Mills Golden Valley products showed high and comparable activity, whereas the activity of wheat germ "Niblanc" was about ten times lower. Recently, it was demonstrated that manual selection of wheat embryo material from apparently inactive commercial wheat germ allows for isolation of highly active extract (Carlier & Peumans, 1976). It seems also that the purity of salts used for the preparation of the system is essential. Salts from POCh Gliwice were shown to inactivate the system. The high activity of the system is also due to thorough removal of soluble inhibitors of tRNA aminocacylation which strongly diminish the formation of methionyl-tRNA. Decreased formation of initiator-Met-tRNA could unevenly affect the initiation of polypeptide synthesis with different RNA components of total BMV RNA. This would resemble
the effect of initiation inhibitors on the synthesis of haemoglobin chains described by Lodish (1974). Neglecting the existence of soluble inhibitors of translation in wheat germ system could lead to erroneous interpretation of the in vitro experiments.

We are aware of the fact that our assumption that the detected inhibitors are adenine nucleotides is based on circumstantial evidence. However, the addition of adenine nucleotides to the dialysed cell-free extract imitates quite impressively the action of endogenous inhibitors.

It should be mentioned that the concentration of adenine nucleotides required for the inhibition of aminoaecylation (Table 2) is higher than that detected in the cell-free extract. However, the translation of a natural template could be affected by adenine nucleotides at different stages, not only at the level of tRNA aminoaecylation. For example, in the presence of endogenous protein kinase, the initiation factors could be phosphorylated by ATP and inactivated (Sierra et al., 1977). The effects could be additive and therefore the whole process of translation might be more sensitive to inhibition than aminoaecylation of tRNA (compare Tables 2 and 3). In wheat germ homogenate the water-soluble phenolic compounds, reacting with Folin reagent, are present. Such compounds, chelating Mg$^{2+}$ ions, could also diminish the activity of the system.

It seems also that beside the low-molecular-weight soluble inhibitors of aminoaecylation, high-molecular-weight inhibitors of translation of natural templates are present in wheat germ extract. These substances tend to precipitate during dialysis and are removed from the system by low-speed centrifugation. In contrast to soluble inhibitors, these high-molecular-weight substances do not inhibit aminoaecylation of tRNA. Their function and nature are currently under study.

It is of importance that not only plant viral messages are translated in the optimized system with high efficiency. Protein synthesis directed by the transcribed in vitro reovirus RNA was tested under conditions found to be optimal for BMV RNA translation. It was found that in response to 5 µg of reovirus template in 100 µl incorporation mixture, 1300 pmoles of $[^1C]$leucine became incorporated into trichloroacetic acid-insoluble products. One can calculate that in these conditions the system incorporates 260 pmoles of leucine per 1 µg of reovirus RNA. Therefore, in the wheat germ system, at conditions being optimal for the translation of plant viral message, also the animal virus message can be "capped" and translated with comparable efficiency. This finding can facilitate further use of optimized wheat germ cell-free system in the studies on the translation of other eukaryotic viral messages.

Part of the presented results concerning phage Qβ RNA and reovirus RNA translation was obtained by one of us (W. Z.) while working at the Biophysics Laboratory, University of Wisconsin. I am indebted to Dr. Paul Kaesberg for the discussions, laboratory facilities and financial support during these studies.
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CHARAKTERYSTYKA UKŁADU BEZKOMÓRKOWEGO Z ZARODKÓW PSZENICY
AKTYWNE PROWADZĄCE TRANSLACJĘ MATRYC SZTUCZNYCH
I NATURALNYCH

Streszczenie

1. Przebadano warunki translacji wybranych matryc w bezkomórkowym układzie z zarodków pszenicy. Dla optymalnej translacji eukariotycznych matryc (BMV RNA oraz TMV RNA) optymalne stężenia K⁺, Mg²⁺, HEPEs i spermine są podobne. W warunkach optymalnych obie matryce odczytywane są z wysoką wydajnością, wynoszącą dla BMV RNA 220 pmoli, a dla TMV RNA 280 pmoli leucyny na 1 μg RNA.

2. Prokariotyczna matryca (Qφ RNA) odczytywana jest w innych warunkach niż eukariotyczne mRNA. Translacja matrycy syntetycznej [poli(U)] wymaga wyższych stężeń K⁺ i Mg²⁺ niż odczytywanie badanych naturalnych mRNA.

3. Stwierdzono występowanie w układzie rozpuszczalnych inhibitorów procesu translacji i wykazało, że działanie ich podobne jest do działania nukleotydów adenosinowych, których usunięciu poprzez ultrafiltrację lub dąblią towarzyszy aktywacja układu.

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