A Soluble Fraction Requirement in the Transfer Reaction of Protein Synthesis by Rice Embryo Ribosomes

Alva A. App and Maria M. Gerosa
Boyce Thompson Institute for Plant Research, Yonkers, New York

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Summary. The requirements for the transfer of 14C-phenylalanine from yeast soluble ribonucleic acid to protein in vitro by rice (Oryza sativa L. var. Bluebonnet) ribosomes have been investigated. An absolute requirement for polyuridylic acid, 2-mercaptoethanol, guanosine triphosphate, magnesium, and potassium or ammonium ions and ribosomes has been demonstrated. Ribosomes washed in 0.5% sodium deoxycholate also required the presence of rice supernatant. The optimum concentration of magnesium ion for the reaction was approximately 7 mm, while 60 mm of either ammonium or potassium ion gave maximum transfer of phenylalanine in this heterologous system. The optimum concentration of guanosine triphosphate required varied with the presence or absence of the phosphoenolpyruvate-pyruvate kinase generating system. Without the system, the optimum concentration was 1.5 mm, but in its presence the optimum was approximately 0.1 mm.

Materials and Methods

Excised rice embryos were prepared from rice seed (Oryza sativa L. var. Bluebonnet) by the method of Johnston and Stern (9). The dry embryos were ground with a cold mortar and pestle for 1.5 minutes in a homogenating buffer containing 0.45 M sucrose, 0.05 M Tris pH 7.5, 5 mM MgCl₂, and 5 mM 2-mercaptoethanol. The homogenate was strained through cheesecloth, centrifuged for 15 minutes at 20,000 g, and the supernatant then centrifuged for 1 hour at 151,000 g. When desired, the middle one-third of the supernatant was collected, and the remainder decanted. The ribosomal pellet was resuspended in 0.05 M Tris pH 7.6, 1 mM MgCl₂, 15 mM KCl, and 5 mM 2-mercaptoethanol (resuspending buffer), centrifuged briefly at 12,000 g, and the supernatant fraction removed and designated unwashed ribosomes. Washed ribosomes were prepared by layering 1.5 ml of the unwashed ribosomes over one-half ml of homogenizing buffer in 2.0 ml Spinco centrifuge tubes and centrifuging for 1 hour at 50,000 rpm. The ribosomal pellet was resuspended as above. Deoxycholate washed ribosomes were first treated with 0.5% (w/v) sodium deoxycholate before layering. Before use, approximately 10.0 ml of the rice supernatant was placed on a 2.0 X 20.0 cm Sephadex G-25 column that had been previously equilibrated with cold resuspending buffer. The early eluting fractions (corresponding to material that is excluded from the G-25 particles) were collected.

It is now commonly accepted that aminoacyl soluble RNA (sRNA) is an intermediate in protein synthesis. The transfer of amino acids from aminoacyl sRNA to protein has been studied in a number of diverse systems, and is commonly found to require GTP, Mg²⁺, a monovalent ion such as NH₄⁺ or K⁺, a sulfhydryl compound, at least 2 transfer factors, and ribosomes programmed with messenger RNA (1,3,4,5,6). In maize, aminoacyl sRNA has been shown to be a probable intermediate in protein synthesis, and transfer of amino acids to protein has been shown to require Mg²⁺ and microsomes, and to be stimulated by K⁺, GTP, a sulfhydryl compound, and a supernatant fraction (12). In addition, indirect evidence for participation of a transfer factor in wheat protein synthesis has been suggested (13).

It was noted during a previous investigation in this laboratory that washed ribosomes were frequently less active than unwashed ribosomes in the polyuridylic acid (poly U) directed amino acid incorporating system (2). In order to separate the formation of phenylalanyl-sRNA and phenylalanine transfer from phenylalanine transfer from phenylalanine-sRNA to protein, the poly U dependent transfer reaction was studied. The data suggest that washing rice ribosomes removes an essential transfer factor, and that this factor can be demonstrated in rice supernatant.

1 This work was supported in part by Grant No. GB-3223 from the National Science Foundation.
pooled, and recentrifuged another hour at 151,000 
\times g$. The upper one-third of the supernatant was
collected and used where indicated in the transfer
reaction assay.

The transfer reaction assay was started by
adding the ribosomal preparation to the reaction
tubes which contained all components except ribo-
somes. The reaction was normally run at 30\degree for
20.0 minutes. The usual reaction mixture in a
total volume of 0.5 ml contained the following
components unless specifically designated otherwise:
7 mm Mg acetate, 50 mm Tris pH 7.6, 28 mm KCl,
0.1 mm GTP, 2.3 mm sodium phosphoenolpyruvate,
2 \mu g pyruvate kinase, 0.1 mm \textsuperscript{14}C-L-phenylalanine,
40 \mu g polyuridylic acid, and 6 mm 2-mercapto-
etanol. The amount of ribosomes and \textsuperscript{14}C-phenyl-
alanyl-sRNA used will be given in each figure
or table.

The reaction was stopped by adding trichloro-
acetic acid to a final concentration of 5\% (w/v),
1.0 mg of bovine serum albumin was added, and
the precipitates washed essentially according to
the method of Sicklevitz (15). Samples were plated
on Millipore filters, dried, and counted in toluene
based scintillator fluid [3 g of 2,5-diphenyloxazole
and 10 mg of 1,4-bis-(5-phenyloxazolyl) benzene
per liter of toluene] on an Ansitron liquid scin-
tillation counter. Since an absolute requirement
for the presence of a sulfhydryl compound (2-
mercaptoethanol) can be demonstrated for the transfer
reaction employing unwashed ribosomes, 2-mercapto-
etanol was always present during the preparation
of ribosomes and all in vitro assays.

The \textsuperscript{14}C-phenylalanyl-sRNA was prepared essen-
tially according to the procedure of Zubay (16)
and Kaji et al. (10). The reaction mixture con-
tained 10 mg of stripped yeast sRNA, 5 mm Mg
acetate, 3.3 mm ATP, 15 mm sodium phosphoenol-
pyruvate, 400 \gamma of pyruvate kinase, 0.1 m Tris pH
7.5, 33 \mu C of \textsuperscript{14}C-phenylalanine (UL, specific
activity 333 \mu C/\mu mole), 18 mg rice supernatant
protein, and 3 mm 2-mercaptoethanol in a total volume
of 10.0 cc. The 151,000 \times g rice supernatant had
been dialyzed overnight and further treated as
described above before being used as a source
of activating enzyme. The mixture was incubated
at 30\degree for 25 minutes, 0.1 volume of 20 \% (w/v)
potassium acetate (pH 5.0) added, then extracted
5 minutes with an equal volume of 88 \% (v/v)
redistilled phenol. The aqueous layer was separated
by brief centrifugation, removed, and the RNA
precipitated at -20\degree with 1 \% NaCl (w/v) and 2
volumes of ethanol. The precipitate was collected
by centrifugation, washed with 90 \% ethanol, re-
dissolved in 0.01 m acetate pH 5.0, reprecipitated
at -20\degree, and washed again. The RNA was re-
dissolved in 0.01 m acetate pH 5.0, and chromatog-
raphed on a Sephadex G-25 column that had been
previously equilibrated with acetate buffer. The
early eluting fraction corresponding to material that
was excluded from the G-25 particles was collected,
pooled, and precipitated at -20\degree with NaCl and
ethanol. The RNA precipitate was collected, dried
under vacuum, and redissolved in acetate buffer and
stored at -20\degree. Assuming no dilution with \textsuperscript{14}C-
phenylalanine during preparation, 600 cpm is equi-
valent to 1 \mu mole of phenylalanine.

Protein concentration was estimated by the
Lowry et al. (11) procedure using bovine serum
albumin as the standard.

The materials used in these experiments were
obtained from the following sources: the \textsuperscript{14}C-
phenylalanine from New England Nuclear Corpora-
tion; ribonuclease from Worthington; polyuridylic
acid with an average sedimentation coefficient of 8
from Miles Laboratories, Elkhart, Indiana; the
GTP, sodium phosphoenolpyruvate, and pyruvic
kinase from Calbiochem; stripped yeast sRNA from
General Biochemicals; and G-25 Sephadex from
Sigma Chemical.

Results

The time course for the transfer of \textsuperscript{14}C-phenyl-
alanine from \textsuperscript{14}C-phenylalanyl-sRNA to protein in
the presence of rice ribosomes is shown in figure 1.
The initial lag in the rate of the reaction has been
noted previously and probably represents for-
mation of a \textsuperscript{14}C-phenylalanyl-sRNA, poly U, ribo-
some complex (2). The reaction is essentially
complete by 20 minutes. Addition of 1 \mu g of
ribonuclease to the reaction mixture completely
abolishes all incorporation, and there is a linear
relationship between ribosome concentration and the
final quantity of \textsuperscript{14}C-phenylalanine transferred
within the ribosome concentrations employed here.

There is a strict requirement of a monovalent
ion for the transfer reaction (fig 2). K\textsuperscript{+} and
NH\textsubscript{4}\textsuperscript{+} seem equally effective in meeting this
requirement. A GTP concentration of approximately

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|}
\hline
Time in Minutes & 0 & 10 & 20 \\
\hline
CPM & 0 & 600 & 1200 \\
\hline
\end{tabular}
\caption{Time course of transfer of \textsuperscript{14}C-phenylalanine from yeast sRNA to protein in presence of rice ribosomes. In addition to the components listed under Methods, the reaction mixture contained 195 \mu g of ribosomal protein and 1.2 mg of \textsuperscript{14}C-phenylalanyl-sRNA containing a total of 3900 cpm.}
\end{table}

Fig. 1. Time course of transfer of \textsuperscript{14}C-phenylalanine from yeast sRNA to protein in presence of rice ribosomes. In addition to the components listed under
Methods, the reaction mixture contained 195 \mu g of ribosomal protein and 1.2 mg of \textsuperscript{14}C-phenylalanyl-sRNA containing a total of 3900 cpm.
transphosphorylation reactions, experiments designed to assess the specificity of transfer reaction for GTP were not performed.

The data in figure 4A indicate the transfer reaction is completely dependent upon the presence of poly U. The reaction is also dependent upon Mg$^+$, and a concentration of 7 mM appears optimum (fig 4B). Finally, the amount of $^{14}$C-phenylalanine transferred is obviously dependent upon the concentration of $^{14}$C-phenylalanyl-sRNA preparation in the reaction mixture (fig 5). Although used routinely, addition or omission of $^{12}$C-phenylalanine at a concentration of 0.1 mM did not affect the amount of $^{14}$C-phenylalanine transferred, indicating that activation of any free $^{14}$C-phenylalanine in the mixture is not making a significant contribution to the transfer reaction.

The results in table I indicate that washing of rice ribosomes reduces their ability to carry out the transfer reaction. Washing with 0.5% (w/v) sodium deoxycholate practically abolishes the reaction. However, the ability to carry out the transfer reaction can be almost completely restored by the addition of the rice supernatant fraction. The rice supernatant by itself does not exhibit the

**Fig. 2.** Effect of KCl and NH$_4$Cl on the transfer reaction. The reaction mixture contained all the components listed under Methods with the exception of KCl. As indicated, various amounts of NH$_4$Cl or KCl were employed. In addition, 200 μg of ribosomal protein and 1.4 mg of $^{14}$C-phenylalanyl-sRNA containing 7200 cpm were present.

1.5 mM appears optimum for the reaction, but in the presence of a regeneration system the optimum concentration drops to approximately 0.1 mM (fig 3). In parallel experiments employing GTP at its optimum concentration in each case, the use of the regeneration system resulted in approximately double the rate of $^{14}$C-phenylalanine transferred (unpublished data). Because of the possibility of

**Fig. 3.** Effect of GTP concentration on the transfer reaction. A) All the components of the reaction mixture listed under Methods were present with the exception that various concentrations of GTP were employed. In addition, 270 μg of ribosomal protein and 1.7 mg of $^{14}$C-phenylalanyl-sRNA containing 15,000 cpm were used. B) Conditions were similar to above except the phosphoenolpyruvate and pyruvic kinase regeneration system were not present, and 1.1 mg of $^{14}$C-phenylalanyl-sRNA containing 3900 cpm were used.

**Fig. 4.** Effect of poly U and Mg acetate concentration on the transfer reaction. A) All the components of the reaction mixture listed under Methods were present except various indicated concentrations of poly U were employed. In addition, 1.1 mg of $^{14}$C-phenylalanyl-sRNA containing 3900 cpm and 280 μg of ribosomal protein were used. B) Conditions were identical to above with the exception that 40 μg of poly U was present in all tubes and Mg$^+$ concentration was varied as indicated.
It should be noted that only a 30 to 40% transfer of 14C-phenylalanine into protein was normally obtained with this system. The failure to obtain nearly 100% transfer may be due in part to the fact that phenylalanyl-sRNA spontaneously hydrolyzes at pH 7.6 in Tris (7, 14). In addition, since this is a heterologous system, there is always the possibility that more than 1 species of yeast sRNA may be charged with 14C-phenylalanine by the rice activating enzyme preparation. All of these species may not contain the correct anticodon to recognize the synthetic messenger attached to the ribosome.

There is apparently considerable variation in the binding of transfer factors to ribosomes (1, 3, 7, 12). Ribosomes from Saccharomyces fragilis may be washed as often as 5 times without exhibiting a supernatant requirement for the transfer reaction (5). On the other hand, washing rice ribosomes 1 time with deoxycholate can entirely abolish the transfer reaction unless the ribosomes are supplemented with rice supernatant. Some of the variation in tenacity of binding of transfer factor to ribosomes may be related to the presence or absence of messenger. With Bacillus cereus, for example, binding of transfer factor to ribosomes in vitro is apparently dependent upon the prior attachment of messenger RNA to the ribosomes (8). Since both dry wheat (13) and rice (unpublished data) embryo ribosomes apparently have little messenger associated with them, their transfer factor(s) may be loosely bound.

The nature of the transfer factor(s) in rice supernatant is not known and is currently under investigation. Variation in content of this factor(s) in rice supernatant may account in part for some of the variation in rates of incorporation previously noted in the poly U system with washed ribosomes.

Table I. Effect of Rice Supernatant on the Transfer Reaction of Washed and Unwashed Ribosomes

<table>
<thead>
<tr>
<th>Ribosomal preparation</th>
<th>No supernatant</th>
<th>With supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm</td>
<td>cpm</td>
</tr>
<tr>
<td>Unwashed</td>
<td>1975</td>
<td>2891</td>
</tr>
<tr>
<td>Unwashed</td>
<td>2084</td>
<td>2764</td>
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</tr>
<tr>
<td>Washed</td>
<td>727</td>
<td>2636</td>
</tr>
<tr>
<td>Washed with Na deoxycholate</td>
<td>102</td>
<td>2519</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>42</td>
</tr>
</tbody>
</table>

Table I indicates that the transfer reaction of unwashed ribosomes is enhanced 50% by the addition of supernatant fraction.

Discussion

The results reported here indicate that the rice ribosomal system can transfer 14C-phenylalanine from yeast 14C-phenylalanyl-sRNA to protein (probably polyphenylalanine) in the presence of poly U, Mg++, K+ or NH4+, GTP, 2-mercaptoethanol and a factor(s) found in rice supernatant. An absolute requirement for poly U, Mg++, K+ or NH4+, GTP, and the supernatant factor (with washed ribosomes) has been demonstrated. Similar requirements for the transfer reaction have been demonstrated in systems derived from bacteria, mammalian cells, yeast, and maize (1, 3, 4, 5, 6, 12).

It should be noted that only a 30 to 40% transfer of 14C-phenylalanine into protein was normally obtained with this system. The failure to obtain nearly 100% transfer may be due in part to the fact that phenylalanyl-sRNA spontaneously hydrolyzes at pH 7.6 in Tris (7, 14). In addition, since this is a heterologous system, there is always the possibility that more than 1 species of yeast sRNA may be charged with 14C-phenylalanine by the rice activating enzyme preparation. All of these species may not contain the correct anticodon to recognize the synthetic messenger attached to the ribosome.

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Literature Cited


