Requirement of chain initiation factor 3 and ribosomal protein S1 in translation of synthetic and natural messenger RNA

J.E. Sobura, M.R. Chowdhury, D.A. Hawley and A.J. Wahba

Laboratory of Molecular Biology, University of Sherbrooke School of Medicine, Sherbrooke, Quebec, Canada

Received 5 August 1976

ABSTRACT

Amino acid incorporation directed by poly(A), poly(U) or R17 RNA has been examined in S1-depleted protein synthesizing systems. We observe that the translation of either synthetic or natural messenger RNA is strictly dependent on the presence of chain initiation factor 3 and ribosomal protein S1. With poly(A) or poly(U) both IF-3 and S1 stimulate amino acid incorporation at least 25-fold, and with R17 RNA the stimulation is approximately 15-fold. More than one copy of S1 per ribosome decreases amino acid incorporation directed by poly(U) or R17 RNA. Initiation complex formation with R17 RNA is also stimulated optimally by the addition of one copy of S1 per ribosome. The function of IF-3 and S1 in protein synthesis is considered.

INTRODUCTION

The 30S ribosomal protein S1 of Escherichia coli was previously shown to be identical with the protein synthesis interference factor "i" as well as with subunit I of Q8 replicase1−3. In this laboratory, S1 was found to inhibit amino acid incorporation directed by pyrimidine-rich polynucleotides. Initiation complex formation and translation of R17 RNA were also reduced in the presence of excess S1. The extent of inhibition of translation by this protein is a sensitive function of the messenger concentration in the assay. No effect by S1 was observed on the translation of endogenous mRNA, poly(A) or polynucleotides rich in adenylate residues4. S1 binds to single-stranded RNAs5−12 and because of its strong affinity for polypyrimidines, it can be easily purified by chromatography of the E. coli cell extract on poly(C) cellulose13.

Previous studies on the function of S1 in translation were performed with a cell-free system that contained considerable
amounts of this protein. In that system, chain initiation factor 3 is essential for the translation of R17 RNA and will stimulate amino acid incorporation 6 to 10-fold with synthetic polynucleotides. For the work presented here, we prepared 30S ribosomal subunits free of S1 and we removed S1 as well from the E. coli supernatant by chromatography of the S150 fraction on poly(C)-cellulose. We find that IF-3 and S1 are required for the translation of R17 RNA, poly(A) and poly(U), and that intact phage RNA is bound only by S1-containing ribosomes. The presence of S1 in excess of one copy per ribosome causes a decrease in amino acid incorporation directed by poly(U) and R17 RNA.

MATERIALS AND METHODS
Preparation of 30S ribosomal subunits free of S1. Ribosomes were prepared from freshly grown E. coli MRE600 cells and were washed twice with 1.0 M NH₄Cl. Subunits were prepared according to Traub et al. with the following modifications. Twenty six ml 10-30% linear sucrose gradients in buffer A (10 mM Tris-HCl, pH 7.8, 100 mM NH₄Cl, 0.5 mM magnesium acetate and 6 mM 2-mercaptoethanol) over 6 ml cushions of 30% sucrose in the same buffer, were layered with 4 ml samples containing 1200 A₂₆₀ units of purified ribosomes in buffer A, and centrifuged for 10 hrs at 0° in a Spinco No. 30 rotor. 30S ribosomal subunits were recovered from the gradients, pelleted and washed three times with buffer B (20 mM Tris-HCl, pH 7.8, 1.0 M NH₄Cl, 10 mM magnesium acetate and 1 mM dithiothreitol). After the final wash they were resuspended in buffer C (20 mM Tris-HCl, pH 7.8, 0.5 M NH₄Cl, 10 mM magnesium acetate and 1 mM dithiothreitol), heat activated at 37° for 50 min and cooled slowly. 50S subunits were recovered as pellets from the first preparative gradients. They were further purified by an additional sucrose density gradient centrifugation and resuspended in buffer C. All ribosomal particles were stored at 0°. The 30S subunits were essentially free of 50S particles whereas preparations of the latter contained 8% of the smaller subunits. SDS-gel electrophoresis of the washed 30S particles showed the absence of S1 (Fig. 1). When equimolar amounts of 30S and 50S subunits were analyzed at 0° on sucrose density
Figure 1. Densitometer tracings of SDS-polyacrylamide gels containing 30S ribosomal proteins.
(A) 1.0 A$_{260}$ unit of 2x washed 30S particles;
(B) 1.0 A$_{260}$ unit of 5x washed 30S particles.
The direction of migration is from top to bottom.

gradients at 5 mM magnesium acetate, 58-60% of the particles reassociated to give 70S ribosomes. This was observed with four different preparations of subunits. The purified 30S particles were found to bind 0.9 copy of $^{14}$C-labeled SI (Fig. 2).

Preparation of SI. SI was isolated from the 45-55% (NH$_4$)$_2$SO$_4$ fraction of the 1.0 M NH$_4$Cl ribosomal wash and purified as previously described$^5$. This protein was also provided by G. Carmichael who prepared it from E. coli MRE600 cell extracts by poly(C)-cellulose chromatography$^{13}$. The preparation of $^{14}$C-labeled SI was carried out by reductive methylation with [$^{14}$C]formaldehyde (59.2 mCi/mmol, NEN). The purity of the labeled protein was estimated to be greater than 95% as judged by SDS-polyacrylamide gel electrophoresis, and its specific radioactivity was 2440 cpm per ug. Its activity in poly(U) translation remained unchanged after modification.

Removal of SI from the E. coli supernatant. Poly(C) was coupled by ultraviolet irradiation to Whatman CF11 cellulose as described elsewhere$^{13,17}$. Five ml of an S150 fraction
Figure 2. Stoichiometry of binding $^{14}$C-labeled S1 to 30S particles. Subunits were incubated at 37° with varying quantities of $^{14}$C-labeled S1 in 0.1 ml containing 0.01 M TrisCl, pH 7.5, 0.1 M NH$_4$Cl and 5 mM magnesium acetate (buffer D). After 10 min, the reaction mixtures were applied to the top of 17 ml 5-25% sucrose density gradients in buffer D and centrifuged at 4° in a Spinco SW 27 rotor for 20.5 hr at 19,000 rpm. Gradients were analyzed by using a Buchler Autodensiflow and a Gilford flow cell. The fractions were counted in Aquasol (NEN) in a Packard Tri-Carb scintillation counter.

(85 mg of protein) dialyzed against buffer E (20 mM Tris-HCl, pH 7.6, 10 mM magnesium acetate and 10 mM 2-mercaptoethanol) were passed through a column (0.5 x 12 cm) of poly(C)-cellulose, previously equilibrated with the same buffer. The fractions (0.5 ml each) containing A$_{260}$ absorbancy were combined and the protein concentration was determined by the method of Lowry et al.$^{16}$. Although S1 in the E. coli supernatant reacted with antibodies prepared against the purified ribosomal protein, no reaction was obtained with the eluted fractions (Fig. 3). S1 that was bound to poly(C)-cellulose was removed with 8.0 M urea and dialyzed against buffer E.

Assay systems. Translation of synthetic polynucleotides and natural mRNA were performed as previously described.$^{14}$ Ribosomal binding of f$[^{14}$C]Met-tRNA in the presence of AUG or R17 RNA was determined as in previous work.$^{19}$ IF-1, IF-2 and IF-3 were purified from the 1.0 M NH$_4$Cl ribosomal wash.$^{20}$
Crude IF-2 was prepared by passing the 35-80% (NH₄)₂SO₄ fraction of the 1.0 M NH₄Cl ribosomal wash over a DEAE-cellulose column²⁰•²¹.

RESULTS

Translation of poly(A) and poly(U). A protein synthesizing system containing both IF-3 and S1 shows a 25 to 40-fold increase in amino acid incorporation over a system without these two components. Addition of S1 alone has a negligible effect on translation (Fig. 4, Curve I), whereas addition of IF-3 alone will stimulate the system approximately 10-fold (from 40 to 450 pmol). Only when IF-3 and 0.3-1 copy of S1 per ribosome are added is maximum stimulation of poly(U) translation (from 40-1000 pmol) achieved (Fig. 4, Curve II). Likewise in the poly(A) translation system, a 40-fold stimulation of lysine
Figure 4. The effect of SI on the translation of synthetic polynucleotides. [\textsuperscript{14}C]Phenylalanine incorporation in the poly(U) assay was determined as previously described\textsuperscript{14}. Each reaction mixture contained 2.9 \mu g of IF-3 when added, 180 \mu g of SI-free S150 fraction, 65 pmol each of 30S and 50S subunits, 2 \mu g of poly(U), 84 mM NH\textsubscript{4}Cl, 14 mM magnesium acetate, and SI as indicated. A blank of 19 pmol (minus poly(U) or minus IF-3, minus poly(U)) was subtracted from each value. Conditions for the translation of poly(A) were the same as above except for the use of [\textsuperscript{14}C]lysine and 2 \mu g of poly(A) in each incubation mixture. A blank of 133 pmol (minus poly(A) or minus IF-3, minus poly(A)) was subtracted from each value in the poly(A) assay. Incubation was for 20 min at 37\degree. I, poly(U) and poly(A) translation without IF-3; II, poly(U) translation with IF-3; III, poly(A) translation with IF-3.

incorporation (from 40-1660 pmol) is attained with IF-3 and one copy of SI per ribosome (Fig. 4, Curve III). When SI is added in excess of the ribosomes, only poly(U) translation is inhibited (Fig. 4, Curve II).

Translation of R17 RNA. The effect of SI on the translation of R17 RNA is negligible in the absence of the initiation factors (Fig. 5, Curve I). However, when one copy of SI per ribosome is added together with the initiation factors, amino acid incorporation is stimulated (from 5 to 87 pmol) more than 15-fold (Fig. 5, Curve II). Translation is inhibited with
Figure 5. The effect of SI on R17 RNA translation. The assay system for the translation of R17 RNA was previously described. Each reaction mixture contained 372 µg of S1-free S150 fraction, 65 pmol each of 30S and 50S subunits, 40 µg of R17 RNA, 76 mM NH₄Cl, 12 mM magnesium acetate and SI as indicated. Factor additions were: IF-1, 0.3 µg; IF-2-α, 0.2 µg; IF-3, 2.8 µg. A blank of 8 pmol (minus R17 RNA or minus initiation factors, minus R17 RNA) was subtracted from each value. Incubation was for 20 min at 37°. I, translation without initiation factors; II, translation with initiation factors.

levels of SI in excess of the ribosomes.

By using purified initiation factors and a high speed supernatant free of SI we were able to determine which component of the ribosomal wash is required for the translation of R17 RNA. As illustrated in Table I, Expt. I, optimum amino acid incorporation is obtained with IF-1, IF-2-α, IF-3 and one copy of SI per ribosome. When IF-2-α is omitted from the reaction mixtures (Table I, Expt. II), optimum translation is still achieved with IF-1, IF-3 and one copy of SI per ribosome. In the absence of added SI, a crude fraction of IF-2 together with IF-1 and IF-3 will stimulate the translation of R17 RNA (Table I, Expt. III). SI was previously shown to be present in crude preparations of IF-2β. In Expt. IV (Table I) it may
TABLE I

Initiation factor requirements for R17 RNA translation in an Sl-free system

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Initiation factor additions</th>
<th>Sl additions</th>
<th>[^{14}\text{C}]\text{Lysine incorporation}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pmol</td>
<td>pmol</td>
</tr>
<tr>
<td>I</td>
<td>IF-1 + IF-2-a + IF-3</td>
<td>-</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>IF-1 + IF-2-a + IF-3</td>
<td>65</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>IF-1 + IF-2-a + IF-3</td>
<td>200</td>
<td>45</td>
</tr>
<tr>
<td>II</td>
<td>IF-1 + IF-3</td>
<td>-</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>IF-1 + IF-3</td>
<td>65</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>IF-1 + IF-3</td>
<td>200</td>
<td>48</td>
</tr>
<tr>
<td>III</td>
<td>IF-1 + IF-3 + crude IF-2</td>
<td>-</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>IF-1 + IF-3 + crude IF-2</td>
<td>65</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>IF-1 + IF-3 + crude IF-2</td>
<td>200</td>
<td>40</td>
</tr>
<tr>
<td>IV</td>
<td>IF-1 + IF-2-a + IF-3</td>
<td>-</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>IF-1 + IF-3</td>
<td>-</td>
<td>91</td>
</tr>
</tbody>
</table>

The conditions of the R17 RNA-dependent assay were as in previous work\(^1\). Each reaction mixture contained 65 pmol each of 30S and of 50S subunits, 572 µg of Sl-free S150 fraction, 76 mM NaCl, 12 mM magnesium acetate, Sl as indicated and initiation factors (when present): IF-1, 1.8 µg; IF-2-a, 0.2 µg; crude IF-2, 78 µg; IF-3, 4.2 µg. In Expt. IV, E. coli MRE600 high-speed supernatant (495 µg) was used instead of the Sl-free S150 fraction. After incubation for 20 min at 37\(^\circ\)C, the amount of \[^{14}\text{C}]\text{Lysine} incorporated into hot trichloroacetic acid insoluble material was determined. The blank in the absence of factors, 14 pmol, was subtracted from each value.

It can be seen that optimum incorporation is attained with IF-1 and IF-3 and the regular E. coli supernatant which contains Sl.

Ribosomal binding of fMet-tRNA with R17 RNA or AUG. The requirement of Sl in the formation of the chain initiation complex with R17 RNA is illustrated in Table II. Maximal ribosomal binding of fMet-tRNA with R17 RNA is obtained in the presence of IF-1, IF-2, IF-3 and one copy of Sl per ribosome. The addition of excess Sl causes inhibition of initiation complex formation. As previously observed\(^4\), Sl has no effect on the AUG-dependent binding of fMet-tRNA with ribosomal subunits.

DISCUSSION

The results presented here indicate that, in an Sl-free
assay system, the 30S ribosomal protein S1 and IF-3 are required for the translation of all messages. Maximal stimulation of translation occurs when S1 is added in stoichiometric amounts to the ribosomes (Figs. 4 & 5), indicating that the effect of the protein is directly on the ribosome and not on the message. S1 was shown to have an affinity for pyrimidine-rich sequences as judged by its ability to inhibit poly(U) and poly(C), but not poly(A) translation when added in excess relative to the ribosomes. This affinity is further exemplified here (Figs. 4 & 5). S1 is required for maximal rates of poly(U) and poly(A) translation, but when it is added in excess of the ribosomes, only poly(U) translation is inhibited. The inhibition of R17 RNA translation at levels of S1 in excess of the ribosomes is likely to be caused by the additional protein binding to pyrimidine-rich sequences in the message which inhibits the movement of the ribosomes along the mRNA (Fig. 5).

Although S1 is required for the translation of all mRNAs, it has no effect on the AUG-dependent binding of fMet-tRNA (Table II). S1 may not play a direct role in the recognition of the initiator tRNA. However, it is essential for proper message binding\(^7,22-24\), probably because it comprises a part of the mRNA binding site on the ribosome.\(^25\)

Recently protein S1 was found by Van Diejen et al.\(^26\) to be indispensable for the translation of MS2 RNA. 30S particles lacking S1 were prepared\(^6\) by dialysis of the 30S subunits against 1 mM Tris-HCl, pH 7.6, and S1 present in the E. coli high speed supernatant was neutralized by titration with anti-S1 IgG. A stimulation by S1 of the translation of MS2 RNA was observed when this protein was not present in excess relative to ribosomes. However, the effects of S1 on poly(U) and poly(A) translation were found to be minimal. The results with synthetic polynucleotides are at variance with the data presented here. The discrepancy could be due to the absence of IF-3 in the translation system of Van Diejen et al.\(^26\). Amino acid incorporation in the latter system is at least 20-fold less than what is reported here.

We have previously established that IF-3 stimulates R17 RNA, poly(U) and poly(A) translation\(^14\) maximally at 12-14 mM
TABLE II
Conditions for maximal ribosomal binding of f\[^{14}\text{C}]\text{Met-tRNA} with R17 RNA or AUG as messenger
in an S1-free system

<table>
<thead>
<tr>
<th>Factor additions</th>
<th>S1 additions</th>
<th>R17 RNA copies/30S</th>
<th>AUG pmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>IF-1 + IF-2</td>
<td>-</td>
<td>0.23</td>
<td>0.67</td>
</tr>
<tr>
<td>IF-1 + IF-2 + IF-3</td>
<td>-</td>
<td>0.38</td>
<td>3.78</td>
</tr>
<tr>
<td>IF-1 + IF-2 + IF-3</td>
<td>0.5</td>
<td>1.50</td>
<td>3.72</td>
</tr>
<tr>
<td>IF-1 + IF-2 + IF-3</td>
<td>1.0</td>
<td>2.16</td>
<td>3.87</td>
</tr>
<tr>
<td>IF-1 + IF-2 + IF-3</td>
<td>5.0</td>
<td>0.81</td>
<td>3.68</td>
</tr>
</tbody>
</table>

The ribosomal binding of f\[^{14}\text{C}]\text{Met-tRNA} with R17 RNA as messenger was determined as previously described\(^{19}\). Each reaction mixture contained 158 pmol of 30S and 147 pmol of 50S subunits, S1 as indicated, and initiation factors (when present): IF-1, 1.8 \(\mu\)g; IF-2-a, 0.2 \(\mu\)g; IF-3, 2.1 \(\mu\)g. Binding values were calculated from the radioactivity under the 70S ribosomal peak. A blank in the absence of factors, 0.33 pmol, was subtracted from each value. For the AUG-dependent binding, each incubation mixture contained 20 pmol of 30S and 21 pmol of 50S subunits, S1 as indicated and initiation factors (when present): IF-1, 0.35 \(\mu\)g; IF-2-a, 0.06 \(\mu\)g; IF-3, 0.43 \(\mu\)g. After 15 min at 25\(^\circ\), the reaction was terminated as previously described\(^{19}\). A blank in the absence of factors, 0.18 pmol, was subtracted from each value.

Mg\(^{2+}\). The addition of IF-1 to IF-3 results in a slight increase in amino acid incorporation\(^{14}\). We have also shown here that at 12 mM Mg\(^{2+}\) R17 RNA is translated efficiently without added IF-2. This factor is required at 3-5 mM Mg\(^{2+}\) for the binding of fMet-tRNA\(^{19,27}\) on the ribosome and for subsequent GTP hydrolysis\(^{28}\).

The fact that IF-3 is required for efficient translation at 12-14 mM Mg\(^{2+}\) strongly suggests that it interacts with 70S particles. Indeed, an interaction of IF-3 with 70S ribosomes was demonstrated in this laboratory by two different methods. Radioactive IF-3 was covalently crosslinked in place on 70S particles using dimethylsuberimidate\(^{29}\). We have also demonstrated direct binding of IF-3 to 70S ribosomes by analyzing \[^{14}\text{C}]\text{IF-3-70S mixtures on polyacrylamide gel electrophoresis}^{30}\). From the stoichiometry of crosslinking of IF-3 to 30S, 50S and 70S particles, we proposed that the IF-3 binding site on the 70S ribosome is composed of contributions from each subunit\(^{29}\). Recently ribosomal proteins S9 and S12 were
identified in the binding site of IF-3 in 30S subunits. Some evidence was also obtained to indicate that S1 is located near IF-3 in intact 30S particles. Ribosomal proteins L2, L17 and possibly L5 were also found to be in the binding site of IF-3 in 50S subunits. The recent finding of Van Duin et al. that IF-3 can be crosslinked to both 16S and 23S rRNAs supports our observations on the interaction of the chain initiation factor with 30S and 50S ribosomal subunits.

In conclusion, we have presented evidence to show that IF-3 and S1 are required for the translation of both synthetic and natural messenger RNA. IF-3 may interact with 70S ribosomes to promote the binding of message, perhaps by loosening the conformation of the ribosome. S1 may align the message by promoting base pairing between the 3' end of the 16S rRNA and a purine-rich sequence on the 5' side of the initiator codon. The molecular mechanism by which the ribosome is able to recognize certain initiation signals is as yet unknown. However, the basis of selectivity in translation may reside in IF-3, S1 or other ribosomal proteins like S12.

ACKNOWLEDGEMENTS

The authors are indebted to Andrée Leblanc for her excellent technical assistance. This work was supported by a grant from the Medical Research Council of Canada.

References
