THE REGULATION OF PROTEIN SYNTHESIS BY TRANSLATIONAL CONTROL RNA

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SUMMARY

The mechanism by which translational control RNA (tcRNA) inhibits protein synthesis was investigated. In the presence of heme the inhibitory role of muscle tcRNA on hemoglobin synthesis was confirmed. Upon the addition of muscle tcRNA to a rabbit reticulocyte cell-free system the binding of [^{32}P]-globin mRNA to 40S ribosomal subunits and its subsequent incorporation into polysomes was inhibited. Furthermore, muscle tcRNA inhibits met-tRNA binding to polysomes and yet stimulates the formation of methionine-puromycin. These results suggest that muscle tcRNA blocks the binding of globin mRNA to ribosomes resulting in an abortive initiation complex that is, however, still capable of the methionine-puromycin reaction.

INTRODUCTION

A low molecular weight RNA has been isolated from initiation factors which appears to be involved in the regulation of protein synthesis at the translational level (1,2). This translational control RNA (tcRNA) inhibits the translation of heterologous mRNAs (1) and may stimulate the utilization of homologous mRNA (2). We have isolated and partially characterized this RNA from muscle and reticulocytes (1). Muscle tcRNA inhibits globin synthesis while reticulocyte tcRNA inhibits the translation of both myosin and myoglobin mRNAs. The tcRNA is not effective in blocking the utilization of these mRNAs when it is derived from the same cell type as the mRNA. This report is concerned with the mechanistic basis of the inhibition of protein synthesis in a reticulocyte lysate by muscle tcRNA. The results reported here suggest that muscle tcRNA inhibits the binding of globin mRNA to the 40S ribosomal subunit and its subsequent incorporation into polysomes.

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Erythroblast tcRNA has little effect on this reaction. In addition, muscle tcRNA decreases the amount of met-tRNA binding to polysomes in a reticulocyte lysate; however, in apparent contradiction, the addition of muscle tcRNA results in an increase in methionine-puromycin formation. These studies suggest that tcRNA inhibits protein synthesis by blocking the mRNA binding reaction resulting in an abortive initiation complex that is nevertheless still capable of the methionine-puromycin reaction.

MATERIALS AND METHODS

The preparation of rabbit reticulocyte lysates and the conditions for cell-free protein synthesis were as previously described (1). The radioactive label was obtained from New England Nuclear (Boston, Massachusetts) and the isotope and amount added to each 0.2 ml final incubation volume is stated in the figure legends. Incubations were normally done at 30° unless otherwise indicated. Radioactive [^{32}P] labelled globin mRNA was isolated from phenylhydrazine treated chickens (3) and translational control RNA (tcRNA) from muscle and reticulocytes were prepared as previously described (1). Each preparation was tested for activity prior to use. The preparations of muscle tcRNA used showed at least a 60% inhibition of protein synthesis. Methionine-puromycin formation was measured by the method of Leder and Burtsztyn (4) as modified by Anderson et al (5). The precipitation of met-tRNA from polysomes and ribosomes was done using cetyl trimethylammonium bromide (CTAB) as the precipitating agent (6). Following incubation, the reaction mixtures were rapidly chilled and 1.5 μM cycloheximide was added. Sucrose density gradient analysis of the incubation mixtures were performed after bringing the volume to 1 ml by the addition of buffer containing 0.1 M KCl, 0.003M $MgCl_2$, 0.02M Tris-HCl (pH 7.4), 0.006M mercaptoethanol and layering the sample onto a 24 ml, 10 to 30% sucrose gradient containing the same buffer. A 3 ml, 45% sucrose cushion was at the bottom of each gradient. Centrifugation was for 5 hrs at 25,000 RPM at 5° in an IEC SB 110 rotor. The gradients were analyzed by continuous monitor-

Table 1. The Inhibition of Globin Synthesis by tcRNA

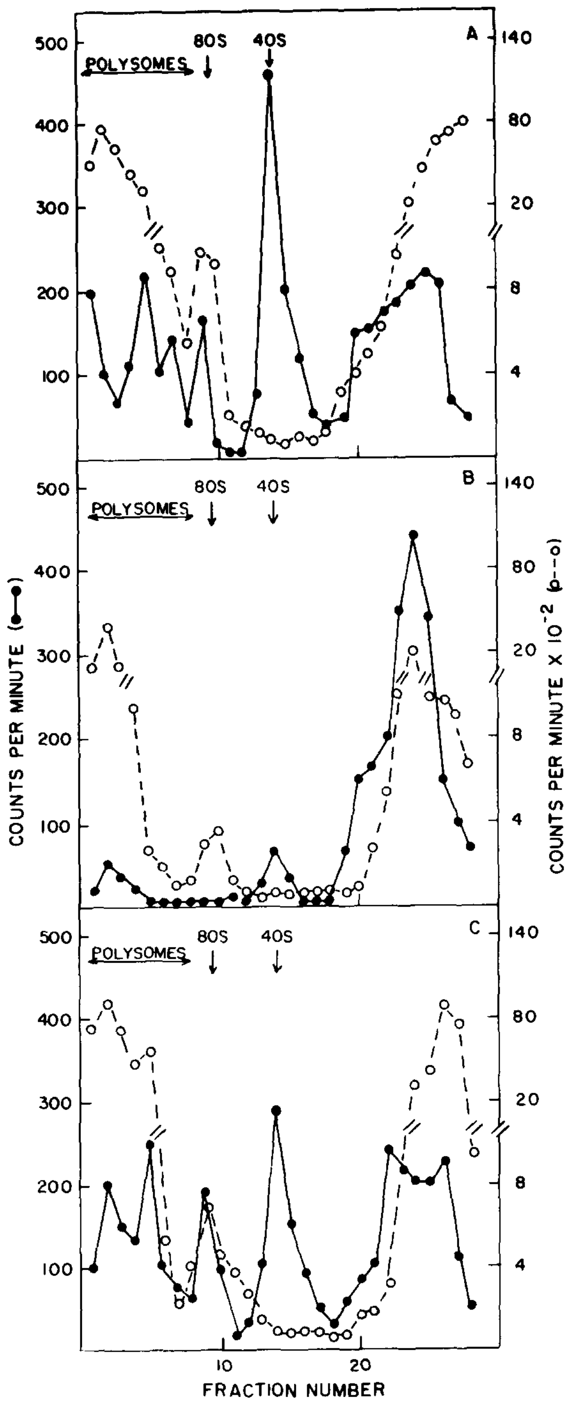
Additions	CPM	CPM α-globin	CPM β-globin
none	91,065		
50 μM hemin	130,045		
2 μg muscle tcRNA	30,340		
Hemin + muscle tcRNA	31,245		
2 μg reticulocyte tcRNA	86,455		
*none	462,860	257,840	203,960
*3 μg muscle tcRNA	36,480	11,730	23,850

Incubations were performed at 35° for 30 min. as described in Methods. Reactions contained 5 μC [^{35}S]-methionine except * which contained 20 μC per reaction. Isolation and characterization of α- and β-globin chains were as described by Dintzis (9). All radioactivity was determined after hot trichloroacetic acid precipitation.

ing on a Gilford Spectrophotometer. Radioactivity was determined by liquid scintillation counting.

RESULTS

Previous results have demonstrated that muscle tcRNA can inhibit the synthesis of hemoglobin (1) in the absence of added heme, a compound which is known to effectively prolong the initiating ability of reticulocyte lysate cell-free amino acid incorporating systems (7). As shown in Table 1, the addition of heme increases the amount of radioactivity incorporated into protein but has no effect on the ability of muscle tcRNA to inhibit globin synthesis. Also, in agreement with our previous results (1) reticylocyte tcRNA is ineffective in inhibiting globin synthesis. Bogdanovsky et al (2) has demonstrated a differential effect of reticulocyte initiation factor RNA on the stimulation of α- and β-chain synthesis. It was of interest, therefore, to determine if muscle tcRNA inhibits both α- and β-globin synthesis


A
B
C
POLYSOMES
80S
40S
COUNTS PER MINUTE (●—●)
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FRACTION NUMBER
500
400
300
200
100
140
80
20
8
4
10
20

to the same degree or in a differential manner. As shown in Table 1, both α- and β-globin synthesis are substantially inhibited by muscle tcRNA; however, α-globin chain synthesis is somewhat more susceptible to the inhibition by muscle tcRNA. The reason for this and its relationship to the finding of Bogdanovsky et al (2) that reticulocyte initiation factor RNA stimulates α-globin synthesis to a greater degree than β-globin synthesis is unclear.

Sucrose gradient analysis of reticulocyte lysate incubations, containing added [^{32}P]-labelled globin mRNA in the absence or presence of muscle or reticulocyte tcRNA is shown in Figure 1. In the absence of tcRNA the radioactivly labelled globin mRNA is found to sediment with the 40S ribosomal subunit as well as be incorporated into polysomes. Although, in the presence of reticulocyte tcRNA there is slightly less [^{32}P]-globin mRNA associated with the 40S ribosomal subunit compared to the control, a substantial proportion of the radioactivity is found in this fraction as well as in the polysome region of the gradient. In addition, the incorporation of [^{3}H]-amino acids into the nascent chains of the polysomes of both the control and reticulocyte tcRNA treated lysate are similar. On the other hand, when muscle tcRNA is added to the reaction mixture (Fig. 1B), both the binding of the [^{32}P]-globin mRNA to the 40S ribosomal subunit and its subsequent incorporation into polysomes is almost completely inhibited. Also, as expected, there is a substantial decrease in the radioactivity associated with the nascent chains in the polysome region of the sucrose gradient. It thus would appear that muscle tcRNA inhibits globin synthesis by blocking the entry of mRNA into ribosomal structures during the initiation of protein

Figure 1. The inhibition of globin mRNA binding to ribosomes by muscle tcRNA. The reactions were incubated for 5 min. at 30° with [^{32}P]-globin mRNA and subsequently terminated and analyzed by sucrose density gradient centrifugation as described in Methods. Fractions were collected on Millipore filters, dried, and the radioactivity determined. A, control, B 2 μg muscle tcRNA, and C, 2 μg reticulocyte tcRNA added to incubation mixtures. 50 μM hemin was present in all reaction mixtures. ●—● [^{32}P]-globin mRNA, O--O [^{3}H]-amino acid mixture (5 μC per reaction).

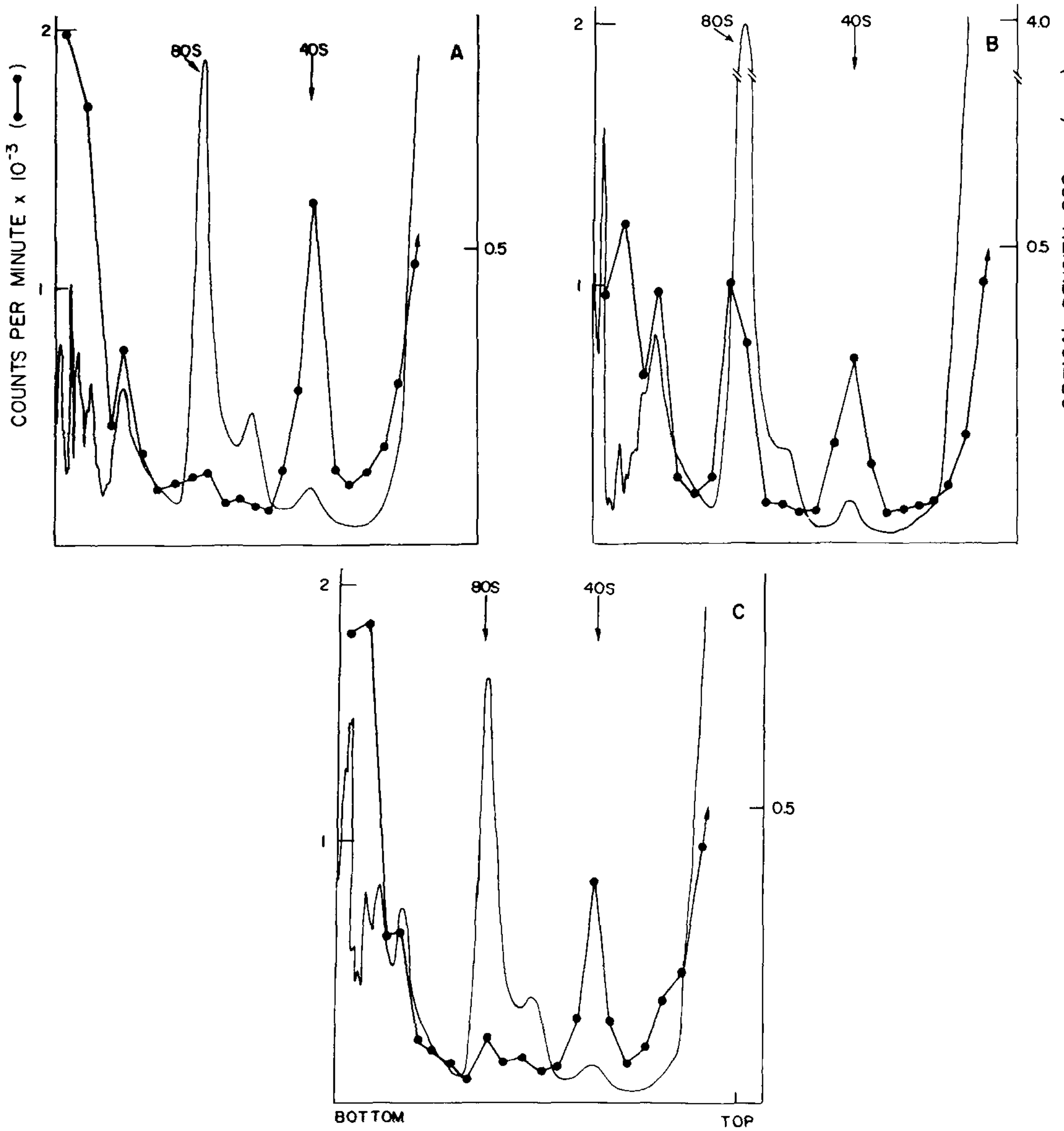

Figure 2. The labelling of ribosomes with [^{35}S]-met-tRNA in the presence and absence of tcRNA. Reactions were incubated for 10 min. at 30° with 10 μC [^{35}S]-methionine per sample. Analysis of the reactions was performed as described in Methods. 50 μM hemin was present in all reactions mixtures. A, control having a total of 201,300 acid precipitable CPM after incubation, B, 1 μg muscle tcRNA added with 68,000 acid precipitable CPM; C, 1 μg reticulocyte tcRNA added with 214,400 acid precipitable CPM.

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synthesis while reticulocyte tcRNA is ineffective. The precise manner by which the mRNA binding reaction is inhibited by tcRNA or if it is the only mode of action of tcRNA is not known.

In order to determine the presence of met-tRNA and peptidyl-tRNA in the various ribosomal structures, incubations of reticulocyte lysates containing [^{35}S]-methionine were carried out. The analysis of these reaction mixtures on sucrose density gradients and the subsequent precipitation of the various fractions with CTAB (6) is shown in Figure 2. If a comparison is made between the control (Fig. 2A) and the reaction containing muscle tcRNA (Fig. 2B), it can be seen that there is an increase in the 80S ribosome peak, a decrease in the amount of [^{35}S]-met-tRNA bound to the 40S ribosomal subunit and the polysomes, and the appearance of a peak of [^{35}S]-met-tRNA associated with the single ribosomes (80S) when muscle tcRNA is present in the incubation mixture. Although there is less [^{35}S]-met-tRNA found associated with the 40S ribosomal subunit when reticulocyte tcRNA is added (Fig. 2C), the other differences noted in comparing the results obtained with muscle tcRNA with the control are not observed.

The appearance of a radioactive peak of [^{35}S]-met-tRNA sedimenting with the 80S ribosomes when muscle tcRNA is added suggested that even though the binding of mRNA is inhibited an 80S ribosomal complex is formed containing met-tRNA. This possibility was tested using the met-puromycin reaction (4). As shown in Figure 3, when increasing amounts of muscle tcRNA are added to the lysates there is an increase in the amount of [^{35}S]-met-puromycin formed. Although muscle tcRNA inhibits globin synthesis presumably by blocking the binding of mRNA to the ribosomes during the formation of the initiation complex, these results show that upon the addition of muscle tcRNA there is an actual increase in the amount of met-tRNA bound to 80S ribosomes which is capable of reacting with puromycin.

DISCUSSION

Translational control RNA has been shown to be specific in its ability to inhibit the utilization of different mRNAs (1). The studies reported here suggest that tcRNA inhibits the binding of mRNA to the ribosome during the initiation of protein synthesis. This could be accomplished by a site

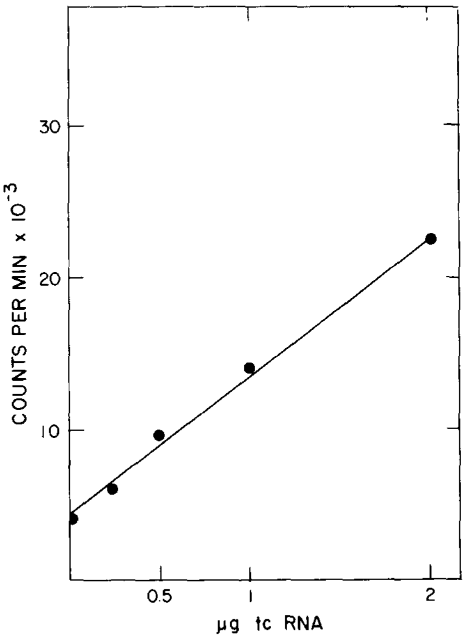

Figure 3. The formation of [^{35}S]-methionine-puromycin. Incubations were for 5 min. at 30° as described in Methods. 50 μM hemin was present in all reaction mixtures. The tcRNA was prepared from muscle. Methionine-puromycin formation was measured as described by Leder and Burtsztyn (4).

recognition of muscle tcRNA to globin mRNA forming a double stranded region at or near the ribosomal binding site. An abortive initiation complex may then form, lacking mRNA, but still capable of the methionine-puromycin reaction. Schreier and Staehelin (8) have suggested the possibility of such a complex occurring in eukaryotic systems. Alternatively, the muscle tcRNA may contain an AUG codon and bind to ribosomes thereby blocking mRNA binding but allowing the binding of met-tRNA and subsequent methionine-puromycin formation. Further experiments are required to test these alternatives; however, the latter possibility seems less likely due to the specificity of the reaction.

It is not clear how our previous results (1) and those reported here relate to those of Bogdanousky et al (2). We have studied the inhibitory effect of muscle tcRNA on globin synthesis and have shown that while reticulocyte tcRNA is not an effective inhibitor of globin synthesis it does indeed inhibit myosin and myoglobin synthesis. Recent findings from this laboratory suggest that polysomal tcRNA preparations from muscle stimulate the translation of polysomal mRNA while tcRNA preparations from non-ribosomal bound mRNA in muscle inhibits the utilization of these non-functional mRNAs (to be published elsewhere). Therefore, the stimulating activity of the reticulocyte RNA as reported by Bogdanousky et al (2) may be due to the possibility that different populations of tcRNA are present in the cell.

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REFERENCES

1. Heywood, S. M., Kennedy, D. S., and Bester, A. J. (1974) Proc. Nat. Acad. Sci., U.S., 71, 2428-2431.
2. Bogdanousky, D., Hermann, W., and Schapira, G. (1973) Biochem. Biophys. Res. Comm., 54, 25-32.
3. Heywood, S. M. (1970) Proc. Nat. Acad. Sci., U.S., 67, 1782-1788.
4. Leder, P. and Bursztyn, H. (1966) Biochem. Biophys. Res. Comm., 25, 233-245.
5. Anderson, W. F. and Crystal, R. G. (1972) Proc. Nat. Acad. Sci., U.S., 69, 706-711.
6. Darnbrough, C., Hunt, T. and Jackson, R. J. (1972) Biochem. Biophys. Res. Comm., 48, 1556-1564.
7. Gross, M. and Rabinovitz, M. (1972) Proc. Nat. Acad. Sci., U.S., 69, 1565-1571.
8. Schreier, M. H. and Staehelin, T. (1973) Regulation of Transcription and Translation in Eukaryoties, pp. 335-349, Springer-Verlag, New York.
9. Dintzis, H. M. (1961) Proc. Nat. Acad. Sci., U.S., 47, 247-261.