PROTEIN SYNTHESIS IN RABBIT RETICULOCYTES: FACTORS CONTROLLING TERMINAL AND INTERNAL METHIONINE CODON (AUG) RECOGNITION BY METHIONYL tRNA SPECIES

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Summary

In a cell-free amino acid incorporating system obtained from rabbit reticulocytes, poly r (A-U-G) directed the transfer of methionine from both [^{35}S]Met-tRNA$_m^{Met}$ and [^{35}S]Met-tRNA$_f^{Met}$ into polymethionine product (1). In each case, the transferred radioactivity was mainly into internal positions of the polypeptide chains. However, in these transfer reactions, the apparent K_m of poly r(A-U-G) was significantly higher with Met-tRNA$_f^{Met}$ than with Met-tRNA$_m^{Met}$. In the presence of excess crude rabbit liver tRNA, Met-tRNA$_f^{Met}$ transferred methionine mainly into terminal positions and Met-tRNA$_m^{Met}$ into internal positions of the polypeptide chains.

Recently, several laboratories reported that eukaryotic protein synthesizing systems, like the bacterial ones, use a specific methionyl tRNA species (Met-tRNA$_f^{Met}$) for initiation of peptide chains (1-10). This methionyl tRNA species preferentially recognizes the terminal methionine codon (AUG or GUG) in a genetic message and inserts methionine into the N-terminal position of the synthesized peptide. However, the reason for preferential recognition of the terminal methionine codon in eukaryotic protein synthesis is not clear. In bacterial protein synthesis, at least two factors are known to control terminal recognition of the initiator codons: (1) In this system, the Met-tRNA$_f^{Met}$ species is used in the formylated form (f-Met-tRNA$_f^{Met}$). The f-Met-tRNA$_f^{Met}$ species, with its blocked α-amino group, appears to the protein synthesizing machinery to be a peptidyl tRNA and binds to the peptidyl site on

the ribosome (11-13). (2) The peptide chain elongation factor (T), which forms an AAtRNA-T factor-GTP complex with the AAtRNA's other than Met-$tRNA_f^{Met}$ as a step leading to the transfer of an amino acid into the peptide chain, does not recognize Met-$tRNA_f^{Met}$, either in the formylated or nonformylated form (14).

The mechanism of peptide chain initiation in eukaryotic cells is not clear. The available evidence suggests that these cells probably use the Met-$tRNA_f^{Met}$ species in the nonformylated form (3, 4, 8, 9, 15). Several laboratories have reported that the mammalian peptide chain elongation factors, like the bacterial ones, do not recognize Met-$tRNA_f^{Met}$ (8, 10, 16, 17). However, using purified T factors from yeast and E. coli, Richter and Lipmann observed that both the formylatable and nonformylatable Met-$tRNA^{Met}$s species of yeast bind to the peptide chain elongation factor (18). A recent report indicates that the synthesis of puromycin peptides with Met-$tRNA_f^{Met}$, f-Met-$tRNA_f^{Met}$ and N-acetyl Phe-$tRNA^{Phe}$ in the rabbit reticulocyte system, unlike the bacterial ones, requires the participation of the peptide chain elongation factor in addition to the initiator proteins (19).

We previously reported that in a cell free system from rabbit reticulocytes, poly r(A-U-G) directed the transfer of methionine from both Met-$tRNA_f^{Met}$ and Met-$tRNA_m^{Met}$ into polymethionine, although the efficiency of these transfer reactions was different for these two $tRNA^{Met}$ species (1). In this communication, we present evidence that in each of the above transfer reactions, the methionine transferred into polypeptides was located exclusively at internal positions. However, in the presence of excess crude tRNA in the above system, the transfer of methionine from Met-$tRNA_f^{Met}$ was exclusively into the terminal positions of the peptide chains, and the transfer of methionine from Met-$tRNA_m^{Met}$ was exclusively into the internal positions of the peptide chains. These results suggest that the preferential recognition of the terminal and internal methionine codons by these two $tRNA^{Met}$ species is determined by the relative abundance of the two $tRNA^{Met}$ species, and the relative affinities of

these two $tRNA^{Met}$ species for the terminal and internal codons. Furthermore, the peptide chain elongation factors in the reticulocyte system recognize both the Met-$tRNA^{Met}$ species during protein synthesis.

Materials and Methods

Reticulocyte ribosomal preparations used in these experiments have been described previously (1, 20). Crude reticulocyte ribosomal preparations (obtained by pelleting reticulocyte lysate at 100,000 x g for 2 hours) presumably contain all the peptide chain initiation factors and synthesize hemoglobin at low Mg^{++} (3mM) (6). Preincubated and washed ribosomal preparations were prepared by incubating the crude reticulocyte ribosomes in a complete cell-free system to free the ribosomes from endogenous messenger RNA and were then diluted in Buffer B containing 0.25 M sucrose and pelleted to remove endogenous amino acids and tRNAs. Such ribosomal preparations retain most of the enzymic activities necessary for polypeptide synthesis, and efficiently direct amino acid incorporation into polypeptides in response to added polyribonucleotide messengers, amino acids, and tRNA's (20).

Rabbit liver $tRNA_f^{Met}$ and $tRNA_m^{Met}$ preparations used in these experiments were the same as previously described (1). Pure yeast $tRNA_f^{Met}$ was kindly provided by Dr. U. L. Rajbhandary, Massachusetts Institute of Technology and pure E. coli $tRNA_f^{Met}$ was a generous gift of NIGMS. As before, E. coli synthetase was used for charging the $tRNA_f^{Met}$ species (rabbit liver, yeast and E. coli) and reticulocyte synthetase was used for charging of rabbit liver $tRNA_m^{Met}$ (1).

[^{35}S] Methionine (5-10C/m mole) used in these experiments was purchased from Amersham/Searle. Other materials and methods used in these experiments were the same as previously described (1).

Results

Poly r(A-U-G) directed transfer of methionine from [^{35}S] Met-$tRNA_m^{Met}$ or [^{35}S] Met-$tRNA_f^{Met}$ into polymethionine product was studied using crude reticu-

locyte ribosomes at 3mM Mg^{++}. The transfer reaction was studied in the presence of an excess of unlabelled methionine to prevent the incorporation of radioactively labelled methionine by transacylation between the two Met-$tRNA^{Met}$ species. As reported previously, the transfer of methionine from both tRNA species was observed, although with different efficiencies (1). When an excess of crude tRNA (which presumably contains both $tRNA_f^{Met}$ and $tRNA_m^{Met}$) was added, the transfer of methionine from [^{35}S] Met-$tRNA_f^{Met}$ was considerably lowered (Fig. 1A). Under similar conditions, the transfer of methionine from [^{35}S] Met-$tRNA_m^{Met}$ was slightly stimulated (Fig 1 B).

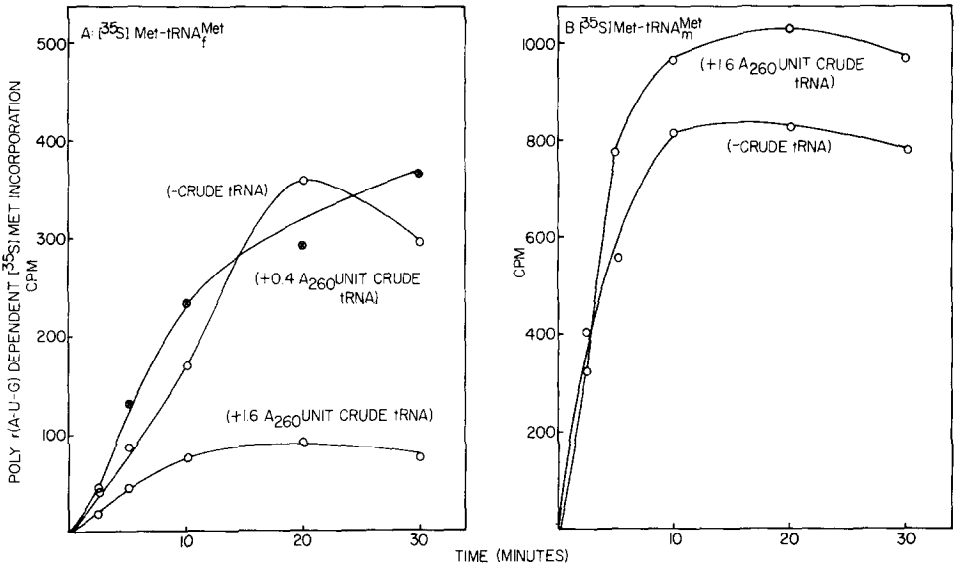

Fig. 1. Kinetics of poly r(A-U-G) directed [^{35}S] methionine transfer from [^{35}S] Met-$tRNA_f^{Met}$ and [^{35}S] Met-$tRNA_m^{Met}$ into polypeptides. Experimental conditions were the same as described previously (1). The incubation mixture in Stage II contained (in a total volume of 0.15 ml), in addition to the usual components of protein synthesis, 1.3 A_{260} units of crude reticulocyte ribosomes, 3 pmoles of either [^{35}S] Met-$tRNA_f^{Met}$ or [^{35}S] Met-$tRNA_m^{Met}$, 10 nmoles of unlabelled methionine, and where indicated, uncharged crude rabbit liver tRNA. The Mg^{++} concentration used in these experiments was 3mM. At the indicated times, 0.01 ml of the reaction mixture was assayed for hot trichloroacetic acid (5 percent) precipitable radioactivity by the filter paper disc assay method as described previously (1).

TABLE 1

End Group Determination of [^{35}S] Polymethionine Product Synthesized in Response to Poly r(A-U-G)

[^{35}S] Met-tRNA^{Met} Added	Crude tRNA Added A_{260} Units	[^{35}S] Methionine Incorporation In Polymethionine			
		Terminal		Internal	
		Cpm	Percent	Cpm	Percent
Met-tRNA$_f^{Met}$	None	78	10	664	90
Met-tRNA$_f^{Met}$	0.4	76	31	169	69
Met-tRNA$_f^{Met}$	1.6	751	88	103	12
Met-tRNA$_m^{Met}$	None	166	16	926	89
Met-tRNA$_m^{Met}$	1.6	63	10	543	90

Polymethionine products synthesized as described in Fig. 1 were used for Edman degradation by the procedure described previously (1). A paper chromatographic procedure was used to separate PTH-Met SO_2 fromthe undegraded polymethionine product after one cycle of Edman degradation (1).

Polymethionine products synthesized in the above reactions were analyzed for radioactivity at the terminal and internal positions by Edman degradation (1, 21) (Table 1). In the absence of any added crude tRNA, most of the radioactivity transferred into polymethionine from either Met-tRNA^{Met} species was located at internal positions of the peptide chain. In the presence of increasing amounts of added crude tRNA, methionine transfer from Met-tRNA$_f^{Met}$ into the terminal position of the peptide chain, increased. Approximately 90 percent of the methionine was transferred into the terminal positions when 1.6 A_{260} units of crude tRNA was added to the incubation mixture. Under similar conditions, the transfer of methionine from [^{35}S] Met-tRNA$_m^{Met}$ was mainly into the internal positions.

The above results clearly show that although both Met-tRNA$_m^{Met}$ and Met-tRNA$_f^{Met}$ can transfer methionine into the internal positions of the polypeptide

chain, in the presence of a relative excess of the other, the transfer of methionine from Met-$tRNA_f^{Met}$ was mainly into the terminal positions and the transfer of methionine from Met-$tRNA_m^{Met}$ was mainly into the internal positions. Apparently, under these conditions, the affinity of Met-$tRNA_f^{Met}$ for the terminal AUG codon is greater than that of Met-$tRNA_m^{Met}$ and the affinity of Met-$tRNA_m^{Met}$ for the internal AUG codon is greater than that of Met-$tRNA_f^{Met}$. The results presented in Fig. 2 are in agreement with this interpretation.

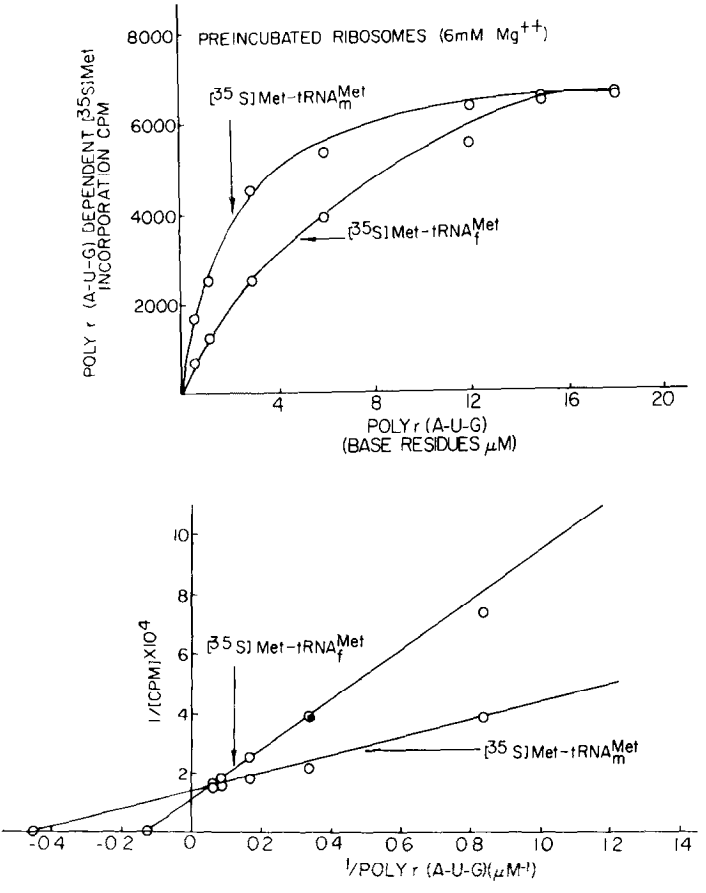

Fig. 2. Effects of poly r(A-U-G) concentrations on methionine transfer from [^{35}S] Met-$tRNA_f^{Met}$ and [^{35}S] Met-$tRNA_m^{Met}$. Approximately 0.65 A_{260} units of washed and preincubated reticulocyte ribosomes were used in a total volume of 0.15 ml. The Mg^{++} concentration used in these experiments was 6 mM. The concentrations of poly r(A-U-G) were varied as described in the figure. The incubation was at 37° for 10 minutes. A 0.05 ml aliquot was used for assay for polypeptide synthesis. See the text for further details.

We studied the transfer of methionine from Met-$tRNA_m^{Met}$ and Met-$tRNA_f^{Met}$ in the presence of varying concentrations of poly r(A-U-G). Washed and preincubated reticulocyte ribosomes were used and the transfer reactions were studied at 6mM Mg^{++}. Under these conditions, the transfer reactions increased linearly with time for at least 10 minutes and the transfer of methionine into polymethionine product was mainly into the internal positions. However, in these reactions, the saturating concentrations of poly r(A-U-G) required for the transfer of methionine from Met-$tRNA_m^{Met}$ was much less than that required for the transfer of methionine from Met-$tRNA_f^{Met}$ (Fig. 2, top fig.). In the bottom figure, the data are presented in the form of double reciprocal plots. The apparent K_m of poly r(A-U-G) obtained from this plot was 2μM for Met-$tRNA_m^{Met}$ and 8μM for Met-$tRNA_f^{Met}$.

We also studied the transfer activities of pure yeast and E. coli Met-$tRNA_f^{Met}$ using reticulocyte ribosomes. Washed and preincubated reticulocyte ribosomes were used and the transfer reaction was performed at 6mM Mg^{++}

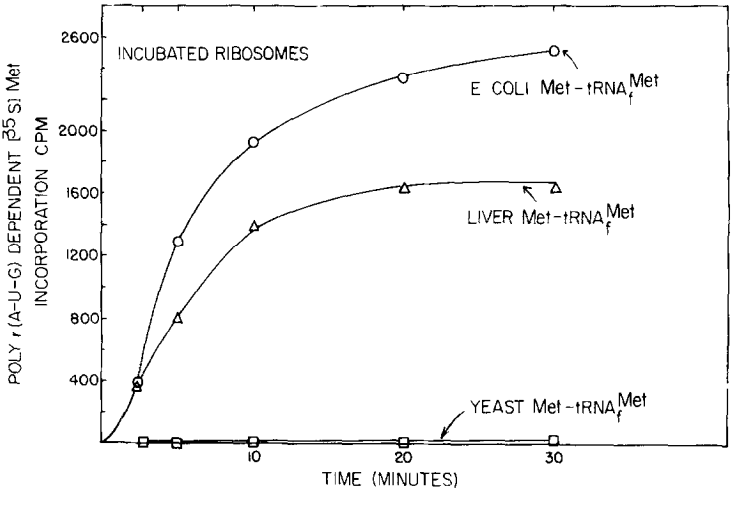

Fig. 3. Comparison of rabbit liver, yeast and E. coli [^{35}S] Met-$tRNA_f^{Met}$ for transfer activities in response to poly r(A-U-G). The experimental conditions were the same as in Fig. 1, except that the incubation mixtures contained 0.65 A_{260} units of preincubated reticulocyte ribosomes and 6 mM Mg^{++} in a total volume of 0.15 ml. Approximately 3 pmoles of each Met-$tRNA_f^{Met}$ species was used in these experiments.

(Fig 3). Like the rabbit liver Met-tRNA$_f^{Met}$, E. coli Met-tRNA$_f^{Met}$ was also active in the transfer reactions, and in each case, the transfer of methionine was mainly into the internal positions of the polypeptide chains (not shown here). Under similar experimental conditions, yeast Met-tRNA$_f^{Met}$ was inactive in the transfer reaction.

Discussion

The results presented clearly demonstrate that in rabbit reticulocyte protein synthesis both Met-tRNA$_f^{Met}$ and Met-tRNA$_m^{Met}$ can recognize the internal methionine codon (AUG), although with different efficiencies. The specificity for internal and terminal methionine codon (AUG) is probably determined by the relative affinity of these two Met-tRNA^{Met} species for the internal and terminal methionine codons and the relative abundance of each species. Studies with pure E. coli and yeast Met-tRNA$_f^{Met}$ species in the rabbit reticulocyte amino acid incorporating system show that the E. coli Met-tRNA$_f^{Met}$ species can also recognize the internal AUG codon in this system, whereas yeast Met-tRNA$_f^{Met}$ was inactive. The reason for the lack of activity of the yeast Met-tRNA$_f^{Met}$ species in these transfer reactions is not clear. Similar results with yeast Met-tRNA$_f^{Met}$ species in the E. coli cell-free system were also observed by Rajbhandary and Kumar (22).

It is reasonable to assume that the transfer of methionine from Met-tRNA$_f^{Met}$ into the internal positions of the polypeptide product uses the same protein synthesizing machinery as is used in the transfer of other amino acids in this system. The failure to detect the Met-tRNA$_f^{Met}$-T factor-GTP complexes with certain Met-tRNA$_f^{Met}$ species may be related to the instability of these complexes under the experimental conditions.

It should be pointed out that these studies were done with unformylated Met-tRNA$_f^{Met}$ species. This Met-tRNA$_f^{Met}$ species can be formylated with E. coli transformylase. Such a formylated species (f-Met-tRNA$_f^{Met}$) should only be incorporated into the terminal positions of the peptide chain. As previously

pointed out, available evidences suggest that such a f-Met-$tRNA_f^{Met}$ species may play no role in eukaryotic protein synthesis (3, 4, 8, 9, 15).

Acknowledgments

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