INITIATION OF PROTEIN SYNTHESIS IN EUKARYOTES

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Summary: The two major species of $tRNA^{Met}$ from wheat germ have been purified and studied for their role in protein synthesis. $tRNA_1^{Met}$ can be charged both by wheat embryo synthetase and E. coli synthetase and $tRNA_2^{Met}$ can be charged only by wheat embryo synthetase. Methionyl-$tRNA_1^{Met}$ fails to transfer methionine into a polypeptide chain while met-$tRNA_2^{Met}$ can transfer methionine into a polypeptide chain. At low Mg^{+2} concentration the transfer of methionine from met-$tRNA_2^{Met}$ is dependent on the incorporation of methionine from met-$tRNA_1^{Met}$ at the N-terminal position.

Initiation of protein synthesis in a prokaryotic system involves N-formylmethionyl-$tRNA_F^{Met}$ species (1,2,3,4). In eukaryotic systems the presence of at least two $tRNA^{Met}$ species has been shown (5,6,7,8,9,10,11). One of the species ($tRNA_1^{Met}$) from eukaryotes can be recognized by both the homologous synthetase and E. coli synthetase. $tRNA_1^{Met}$ from yeast or mammalian sources can also be formylated by E. coli transformylase (5,6,7,11). The second major species ($tRNA_2^{Met}$) can be charged only with the homologous enzyme.

Recent studies from a number of laboratories suggest that $tRNA_1^{Met}$ may be the initiator tRNA in eukaryotic system (5-10,12,13,14). In the present work, a main objective of which has been elucidation of the mechanism of polypeptide chain initiation in an eukaryotic system, the two major species of $tRNA^{Met}$ have been purified from wheat embryo and studied for their role in polypeptide synthesis using a cell-free system from wheat embryo. The salient findings were as follows. (1) Met-$tRNA_1^{Met}$ can not transfer methionine into polymethionine directed by poly r-(A-U-G) while met-$tRNA_2^{Met}$ can be utilized for polymethionine synthesis under identical conditions. (2) Poly r-(A-U-G) directed polymethionine synthesis at low Mg^{+2} concentration required both met-$tRNA_1^{Met}$ and met-$tRNA_2^{Met}$. Methionine from met-$tRNA_1^{Met}$ is present only in the N-terminal position. (3) In the presence of the triplet ApUpG met-$tRNA_1^{Met}$ can bind to ribosomes even at low Mg^{+2}

concentrations while met-$tRNA_2^{Met}$ can bind only at higher Mg^{+2} concentration. (4) Met-$tRNA_2^{Met}$ can form the ternary complex (T-GTP-Amino acyl tRNA) with both E. coli enzyme and wheat embryo enzyme but met-$tRNA_1^{Met}$ is not recognized by both E. coli and wheat embryo enzyme.

Materials and Methods:

Unfractionated tRNA was isolated from wheat embryos (15). The tRNA was fractionated on a BD-Cellulose column in presence of Mg^{+2} to separate the two major ($tRNA^{Met}$) species (16). $tRNA_1^{Met}$ was further purified on DEAE-Sephadex column (5). $tRNA_2^{Met}$ was further purified on a DEAE-Sephadex column followed by a BD-Cellulose column in absence of Mg^{+2}. The tRNA species were charged with ^{35}S-methionine (specific activity, 2000 to 5000 mCi per m mole) using a partially purified wheat embryo synthetase free from nucleic acids (5). Wheat embryo ribosom were isolated by a slight modification of Allende and Bravo's method (17). A protein fraction enriched in T and G and free from nucleic acids were isolated from the 150,000xg supernatant fraction by $(NH_4)_2SO_4$ precipitation followed by DEAE-Cellulose chromatography (18). The procedures for polypeptide synthesis using ribopolynucleotides of repeating sequences and binding of aminoacyl-tRNA to ribosomes were described previously (2,5,19).

Results:

Wheat embryo tRNA on fractionation on a BD-cellulose or DEAE-Sephadex column resolved into at least two major methionine accepting species. $tRNA_1^{Met}$ species could be charged by synthetase from both wheat embryo and E. coli. $tRNA_2^{Met}$ species could be charged only by wheat embryo enzyme. Table 1 shows the properties of the two $tRNA^{Met}$ species purified and used in the subsequent experiments. $tRNA_1^{Met}$ is purified to 96% purity and $tRNA_2^{Met}$ is purified to 30% purity and is contaminated with about 1% of $tRNA_1^{Met}$. Both the species of methionyl-$tRNA^{Met}$ could not be formylated with E. coli transformylase. Fractionation of wheat embryo tRNA into the two species with similar properties have also been achieved by other workers (8,9,10).

The $tRNA^{Met}$ species were charged with ^{35}S-methionine using wheat embryo

TABLE 1
Properties of Wheat Embryo $tRNA^{Met}$ Species

Enzyme used	Met acceptor activity (pmoles/A_{260} Unit)		
	Unfractionated tRNA	$tRNA_1^{Met}$	$tRNA_2^{Met}$
Wheat embryo synthetase	43	1760	480
E. coli synthetase	25	1780	4.1

TABLE 2
Transfer of Methionine into Polypeptides directed by poly r (A-U-G).

^{35}S-met-tRNA	^{35}S-met polymerised (pmoles/ml).			
	11 mM Mg^{+2}		16 mM Mg^{+2}	
	-poly AUG	+poly AUG	-poly AUG	+poly AUG
^{35}S-met-$tRNA_1^{Met}$ (306pmoles).	1.17	9.25	0.62	9.75
^{35}S-met-$tRNA_2^{Met}$ (292 pmoles).	1.03	66.4	0.73	148.6

Reaction mixture contained, 0.05M Tris-Cl(pH7.5), 0.05M KCl, 0.5mM DTT, 2mM ATP, 50μM GTP, 5mM PEP, 1μg/ml PEPKinase, 67 nmoles of poly r (A-U-G), $45A_{260}$ Units/ml wheat embryo ribosomes and 0.6mg/ml purified wheat embryo T and G fraction and incubated at 30° for 30 min. (1 pmole ≡ 2800 cpm)

enzyme and the amounts of methionine polymerised as directed by poly r (A-U-G) were studied. (Table 2). At both 11mM and 16mM Mg^{+2} concentration only $tRNA_2^{Met}$ was able to transfer methionine into the polypeptide. On lowering the Mg^{+2} concentration to 4mM very little polymethionine was synthesised from met-$tRNA_2^{Met}$. Addition of met-$tRNA_1^{Met}$, however, stimulated the polymethionine synthesis by five-fold (Table 3). In an effort to determine the position of the methionine residues transferred from the two $tRNA^{Met}$ species, an experiment was performed using ^{35}S-met-$tRNA_1^{Met}$ and ^{3}H-met-$tRNA_2^{Met}$ and end group determinations on the poly-methionine synthesized were carried out by the Edman procedure (2). Over 90% of the ^{35}S-methionine incorporated was present as N-terminal amino acid. These results clearly demonstrate that $tRNA_1^{Met}$ acts only as an initiator tRNA in the

Table 3
Polymethionine Synthesis from Met-tRNA$_2^{Met}$ at varying Mg^{+2} concentration.

Conditions	^{35}S-met polymerised (pmoles/ml)			
	4mM Mg^{+2}		13mM Mg^{+2}	
	10 min	30 min	10 min	30 min
-poly (A-U-G) + met-tRNA$_1^{Met}$ + met-tRNA$_2^{Met}$	0.72	1.01	0.90	1.26
+poly (A-U-G) + met-tRNA$_2^{Met}$	3.15	7.05	103.0	166.0
+poly (A-U-G) + met-tRNA$_1^{Met}$	1.10	2.78	3.80	7.95
+poly (A-U-G) + met-tRNA$_1^{Met}$ + met-tRNA$_2^{Met}$	13.2	27.0	127.2	221.4

^{35}S-met-tRNA$_1^{Met}$ and ^{35}S-met-tRNA$_2^{Met}$ used were 197 and 361 pmoles/ml respectively.

wheat embryo system. Studies with E. coli system showed that the initiator tRNA$_F^{Met}$ binds better to ribosomes at lower Mg^{+2} than the noninitiating tRNA$_M^{Met}$ (1,2,3,). Using wheat embryo ribosomes and the triplet ApUpG we also observed that met-tRNA$_1^{Met}$ can bind at a lower Mg^{+2} concentration and with a higher efficiency than met-tRNA$_2^{Met}$ (Table 4). Addition of ribosomal washings or a partially purified fraction containing T and G did not stimulate the amount of met-tRNA's bound to

Table 4
Binding of Met-tRNA's to Wheat Embryo Ribosomes at varying Mg^{+2} concentration.

Mg^{+2} Conc. (mM)	^{35}S-Met-tRNA bound (pmoles)			
	^{35}S-Met-tRNA$_1^{Met}$		^{35}S-Met-tRNA$_2^{Met}$	
	-AUG	+AUG	-AUG	+AUG
2	0.04	0.75	0.01	0.04
4	0.02	2.77	0.02	0.04
6	0.06	3.3	0.01	0.14
10	0.28	3.43	0.01	0.73
20	1.13	3.63	0.04	2.9

Reaction mixture (.05ml) contained 0.05A$_{260}$ Units of ApUpG, 1.4 A$_{260}$ Units of ribosomes and 4.1 pmoles of ^{35}S-met-tRNA$_1^{Met}$ or 3.5 pmoles of ^{35}S-met-tRNA$_2^{Met}$(19). (1 pmole ≡ 6800 cpm)

ribosomes. Tarrago et al. have also observed an increased binding activity of met-tRNA$_1^{Met}$ (9).

The supernatant transfer factor T from E. coli fails to form a ternary complex with the initiator tRNA$_F^{Met}$ but forms the ternary complex with the noninitiator tRNA species (20), Similar discrimination by the eukaryotic T protein of the eukaryotic initiator tRNA would also prevent met-tRNA$_1^{Met}$ from transfering methionine into the internal position. Using the Sephadex G-100 chromatographic technique to separate the ternary complex ^{3}H-GTP-T-^{35}S-Met-tRNA we have observed that only met-tRNA$_2^{Met}$ can form the ternary complex with ^{3}H-GTP and T protein from E. coli and wheat embryo. Both E. coli T and wheat embryo T failed to recognize the initiator met-tRNA$_1^{Met}$ (Figure 1.). Tarrago et al. also failed to detect any complex formation between GTP, wheat embryo met-tRNA$_1^{Met}$ and T factor from E. coli or wheat embryo (9). These findings, however, do not agree with the observations by Richter and Lipmann that eukaryotic initiator tRNA^{Met} (yeast) can form ternary complex with T factor and GTP (21).

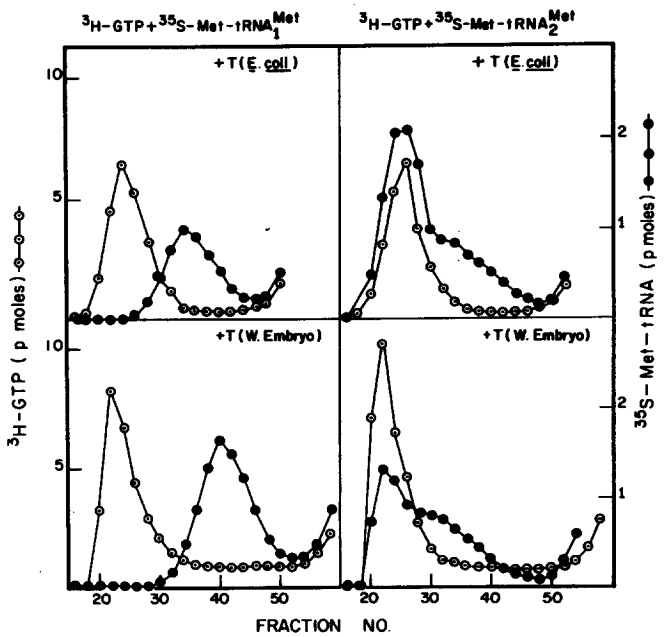

Fig. 1. Complex formation between GTP, met-tRNA^{Met} species and T factors from E. coli or wheat embryo as assayed by the Sephadex G-100 chromatographic technique (19,20).

In E. coli system the binding of $tRNA_F^{Met}$ and $tRNA_M^{Met}$ to ribosomes at low Mg^{+2} concentration are stimulated by initiation factors and T factor respectively (3,20). Wheat embryo $tRNA^{Met}$ species were tested for binding to E. coli ribosomes at low Mg^{+2} concentration. The results showed that E. coli initiation factors and T factor can not stimulate the binding of met-$tRNA_1^{Met}$ to ribosomes. Met-$tRNA_2^{Met}$ binding to ribosomes, however, is specifically stimulated by T factor (Table 5).

TABLE 5

Effect of E. coli T and Initiation Factors on Binding of Wheat Embryo Met-tRNA's to E. coli Ribosomes

Mg^{+2} Conc. mM	^{35}S-met-tRNA bound (pmoles)							
	^{35}S-met-$tRNA_1^{Met}$				^{35}S-met-$tRNA_2^{Met}$			
	-AUG	+AUG	+AUG +I.F.	+AUG +T	-AUG	+AUG	+AUG +I.F.	+AUG +T
5	0.05	0.50	0.46	0.33	0.03	0.04	0.07	0.36
10	0.09	1.88	1.74	1.06	0.04	0.18	0.30	0.71

1.15 A_{260} Units of 4-times NH_4Cl washed ribosomes from E. coli MRE 600 and 20 µg of crude initiation factors or 10 µg of purified T factor used (2,5,19).

Discussion:

Results presented in this communication lends further support to the universality of polypeptide chain initiation. Results obtained with mammalian systems have shown that a species of $tRNA^{Met}$ which can be both charged and formylated by E. coli enzyme is involved in initiation of polypeptide synthesis (5, 6,7,12,13,14). The initiator methionyl-residue, however, appeared to be unmodified in mammalian systems. Using plant system we have also confirmed the presence of a initiator $tRNA^{Met}$ species in higher plants. This species of $tRNA^{Met}$ ($tRNA_1^{Met}$) unlike the mammalian initiator $tRNA^{Met}$ can be charged by E. coli enzyme but can not be formylated. Our results show that met-$tRNA_1^{Met}$ is required for polymethionine synthesis from met-$tRNA_2^{Met}$ at low Mg^{+2} concentration. Also methionine donated by $tRNA_1^{Met}$ is present only at the N-terminal position. These data firmly establish

the initiator role of $tRNA_1^{Met}$ in higher plant system. The initiation of polypeptide synthesis, however, did not require any additional protein factor(s) (initiation factors). Possibly the ribosomes used still contain the "initiation factors". The presence of initiation factors on the ribosomes is also suggested by the binding of met-$tRNA_1^{Met}$ even at a low Mg^{+2} concentration. Experiments are in progress to demonstrate the presence of protein factor(s) involved in initiation of peptide synthesis in wheat embryo system using both synthetic messengers and natural mRNA.

The incorporation of unblocked methionine in N-terminal position is also possible due to the inability by wheat embryo T factor to form a complex with met-$tRNA_1^{Met}$.

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