INCORPORATION OF METHIONINE FROM MET-$tRNA_F^{MET}$ INTO INTERNAL POSITIONS OF POLYPEPTIDES BY MOUSE LIVER POLYSOMES.

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SUMMARY: Two methionine accepting tRNA species corresponding to $tRNA_F^{Met}$ and $tRNA_M^{Met}$ from mouse ascites tumor cells were tested for their ability to donate methionine into internal positions of growing polypeptide chains on mouse liver polysomes. Both tRNA species can function in the elongation of polypeptide chains as judged by their ability to incorporate methionine into protein in the absence of chain initiation. The insertion of methionine into internal positions of polypeptide chains from Met-$tRNA_F^{Met}$ was confirmed by Edman degradation and CNBr cleavage. When both $tRNA^{Met}$ species were present in saturating concentrations in the cell-free system a strong preference for the incorporation of methionine from Met-$tRNA_M^{Met}$ became apparent.

INTRODUCTION: The cytoplasm of several eucaryotic cells contains two methionine accepting tRNA species: $tRNA_F^{Met}$ and $tRNA_M^{Met}$ (1). The former species, which can be formylated by transformylase from E. coli, has been demonstrated to respond to an AUG or GUG codon located at or close to the 5' end of synthetic polynucleotides (2) and to initiate the formation of hemoglobin in rabbit reticulocytes (3). On the other hand, $tRNA_M^{Met}$ was shown to donate methionine only into internal positions of nascent peptides in response to the AUG triplet (2). These results as well as studies on the enzymatic binding of both tRNA species to ribosomes (4,5) led to the concept that $tRNA_F^{Met}$ can only function as a chain initiator in eucaryotic cells whereas $tRNA_M^{Met}$ is utilized in the elongation of peptide chains.

We report here that $tRNA_F^{Met}$ inserts methionine into internal positions of new peptide chains on mouse liver polysomes with high efficiency as long as Met-$tRNA_M^{Met}$ is only present in limiting concentrations. When both Met-$tRNA^{Met}$ species are introduced into the cell-free system in saturating concentrations, however, Met-$tRNA_M^{Met}$ is considerably more active in donating methionine into internal positions of new peptide chains than Met-$tRNA_F^{Met}$.

Abbreviations: BD-cellulose: benzoylated diethyldiaminoethyl cellulose
PTH: phenylthiohydantoin.

PREPARATION OF METHIONINE ACCEPTING tRNAs: Crude tRNA extracted from ascites tumor cells with water-saturated phenol and further purified on DEAE cellulose, was deacylated by exposure to 1.8 M tris.HCl, pH 8.0 at room temperature for 60 min., reprecipitated with NaCl and C_2H_5OH, and fractionated on a column of BD-cellulose. Methionine accepting tRNA species were charged with ^{35}S-methionine (14-29 Ci/mmole), ^{3}H-(methyl) methionine (6-3 Ci/mmole), or uniformly labeled ^{3}H-methionine (150 mCi/mmole) depending on the type of experiment to be performed.

FORMYLATION OF MET-tRNA^{MET}: N-10-formyl-tetrahydrofolate was prepared according to Jones et al. (7) and incubated with ^{35}S-Met-tRNA and a transformylase preparation from E. coli for 30 minutes. The extent of formylation was checked by treatment of the respective Met-tRNA with NH_4OH at pH 9.0 and by subsequent separation of the released formyl-^{35}S-methionine and ^{35}S-methionine by high voltage paper electrophoresis. Authentic formyl methionine and methionine served as markers.

Aminoacylsynthetase from mouse liver was isolated as described by Nishimura and Weinstein (8), the corresponding enzyme from E. coli was prepared according to Muench and Berg (9).

PROTEIN SYNTHESIS: The isolation of mouse liver polysomes followed the instructions given by Falvey and Staehelin (10). A 'pH-5-supernatant' and a crude transfer factor preparation were prepared from mouse liver as described by Moldave (11). Incubations were carried out in duplicate at 37°. Each tube contained, in a total volume of 0.5 ml: 3-7 OD units of polysomes, 0.1 ml 'pH-5-supernatant' or 25 µl of crude transfer factors, 0.2 mM GTP, 4 mM glutathione, 6 mM $MgCl_2$, 80 mM NH_4Cl, 20 µg of total aminoacyl tRNA, and labeled Met-tRNA$_M^{Met}$ or Met-tRNA$_F^{Met}$. Incubations were terminated by adding 0.5 ml of 0.4 M NH_4OH to each tube. The tubes were then maintained at room temperature for 1 hour. Subsequently 5 ml of ice-cold CCl_3·COOH containing 10^{-2} M methionine was added to each tube. The precipitates were washed twice with CCl_3.COOH and applied to nitrocellulose filters, the fil-

ters were rinsed with CCl_3.COOH and ethanol and counted in a model 3320 Packard scintillation counter.

DETERMINATION OF AMINO END-GROUPS AND CNBr CLEAVAGE: The modified Edman procedure as described by Schroeder (12) was used for determination of amino end-groups. The ethanol-washed CCl_3.COOH-precipitate of the incubation mixture was dissolved in hexafluoroacetone (trihydrate), applied to glass fibre strips and thoroughly dried. After completion of the degradation cycle one µmole of unlabeled PTH-methionine was applied to each strip. The eluted PTH amino acids were separated by thin-layer chromatography using $CHCl_3$ or $CHCl_3$:MeOH (20:1) as solvent systems and the radioactivity of spots corresponding to PTH-methionine was measured. In order to check the efficiency of the Edman degradation 1 µmole of the tripeptide NH_2-Met-Leu-Gly-COOH was included in the reaction mixture in two experiments. In these cases the PTH-methionine released after the Edman degradation served as a carrier for any radioactive PTH-methionine. Cyanogen bromide cleavage was performed in 70 % formic acid. After 20 hours the solvent was evaporated and the remaining material was dissolved in 50 % acetic acid and chromatographed on Sephadex G-10 in 50 % acetic acid. 1.500 cpm of ^{14}C-homoserine and unlabeled tyrosine were used as radioactivity and optical density markers for ^{3}H-homoserine. The collected fractions were evaporated to dryness, the residues were dissolved in 200 µl of formic acid, transferred to scintillation vials containing Instagel®, and counted with an efficiency of 34 % for tritium and 96 % for ^{14}C.

RESULTS AND DISCUSSION: The elution profile of the two methionine accepting tRNA species is shown in figure 1. The first methionine accepting peak can be charged with homologous aminoacyl synthetase as well as with the corresponding enzyme from E. coli. In addition, it can be formylated by E. coli transformylase. The tRNA species eluting with the second peak is not recognized by the E.coli enzyme and its aminoacyl derivative cannot be formylated enzymatically. Both tRNA species can effect the incorporation of ^{35}S-methionine into protein by mouse-liver polysomes (table 1). The cell-free system depends completely

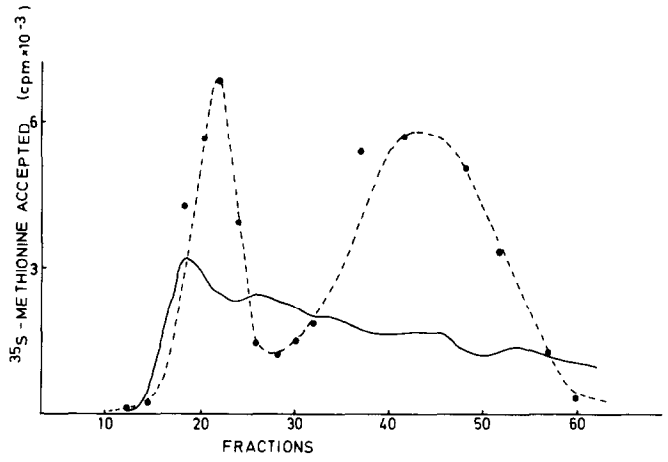

Fig. 1: Elution profile of tRNA from ascites cells on benzoylated DEAE cellulose. The column (0.9 x 70 cm), charged with 20 mg of tRNA was eluted with a linear gradient of 0.45 and 0.9 M NaCl (700 ml of each) in the presence of 10 mM $MgCl_2$ and 1 mM ß-mercaptoethanol. 5 ml fractions were collected tested for methionine acceptor activity as described by Cherayil et al. (6), and pooled according to their content in $tRNA_F^{Met}$ and $tRNA_M^{Met}$. Dotted line: ^{35}S-methionine accepted (cpm).

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on aminoacyl tRNA for the formation of protein and cannot incorporate free methionine. NaF and aurintricarboxylic acid, both inhibitors of chain initiation (13,14), do not inhibit the incorporation of methionine from either $tRNA_M^{Met}$ or $tRNA_F^{Met}$ to a significant degree. On the other hand, cycloheximide and fusidic acid, inhibitors of protein chain elongation (15,16), reduce the incorporation of methionine from both tRNA species. As shown in table 1, the incorporation of methionine from formylated Met-$tRNA_F^{Met}$ into protein is quite low. Moreover, the optical density profile of the polysomes as measured in sucrose gradients changes into a single 80 S ribosomal peak during translation. These findings indicate that our system is predominantly active in chain elongation and that the initiation of new chains is almost negligible. We therefore propose that both species of $tRNA^{Met}$ can insert methionine into internal positions of new protein chains.

In order to test this possibility, the radioactive products synthesized by polysomes in vitro in the presence of the complete spectrum of unlabeled

Table 1

Incorporation of methionine from different Me-tRNA^{Met} species into protein.

Conditions of incubation	Met-tRNA$_M^{Met}$	Met-tRNA$_F^{Met}$	f.Met-tRNA$_F^{Met}$
Complete system	135	127	7.0
- transfer factors	3.2	1.5	-
- GTP and Mg^{++}	82.5	-	-
2 mM Mg^{++}	104	93	-
+ aurintricarboxylic acid (10^{-4} M)	120	119	-
+ NaF (2 x 10^{-4} M)	121	98	-
+ cycloheximide (10^{-4} M)	75	78	-
(10^{-3} M)	43	51	-
+ fusidic acid (10^{-4} M)	74	63	-
(10^{-3} M)	47	33	-

1.3 picomoles of the ^{35}S-labeled Met-tRNA species were introduced into each tube. Numbers (averages from 6 experiments) represent femtomoles of ^{35}S-methionine incorporated per A_{260} unit of polysomes.

Table 2

Edman degradation of ^{35}S-methionine labeled proteins

Expt. No.	tRNA	Total radioactivity applied	Radioactivity after one cycle of degradation	Radioactivity of PTH-methionine
1	^{35}S-Met-tRNA$_F^{Met}$	19680	19864	18
2		4506	4310	11
3		10730	10613	2
1	^{35}S-Met-tRNA$_M^{Met}$	19100	18931	19
2		7790	7655	9

Numbers represent counts per minute. Results from three experiments.

aminoacyl tRNAs and either ^{35}S-Met-tRNA$_F^{Met}$ or ^{35}S-Met-tRNA$_M^{Met}$ were subjected to a single cycle of Edman degradation. As shown in table 2, all radioactivity remained in the protein moiety after this procedure in several experiments. In addition, no ^{35}S-labeled PTH-methionine could be detected by thin-layer chromatography. These findings demonstrate that no ^{35}S-methionine was incorporated into the NH_2-terminal position of nascent peptide

chains and that the incorporation of methionine from Met-$tRNA_F^{Met}$ into protein did not represent chain initiation. Further proof for the idea that $tRNA_F^{Met}$ can function in chain elongation was obtained by the following experiment: $tRNA_F^{Met}$ was charged with uniformly labeled ^{3}H-methionine (150 mC/mmole) and introduced into the mouse liver polysome system for in vitro protein synthesis. The products from several incubation mixtures representing a total of 4.000 cpm were pooled and treated with CNBr. Chromatography of the cleavage products on Sephadex G-10 yielded the elution profile depicted in figure 2. The major part of the tritium label was eluted as a sharp peak almost within the void volume. Only approximately 10 % of the tritium counts appeared together with ^{14}C-homoserine. Again, these results indicate that most of the methionine from $tRNA_F^{Met}$ was incorporated into internal positions of the newly synthesized polypeptides. A small amount of ^{3}H-activity eluting together with ^{14}C-homoserine can be attributed either to some incorporation of methionine into N-terminal positions or more likely to unknown degradation products of the resulting ^{3}H-methyl thiocyanate. Experiments to discriminate between these possibilities are in progress. However,

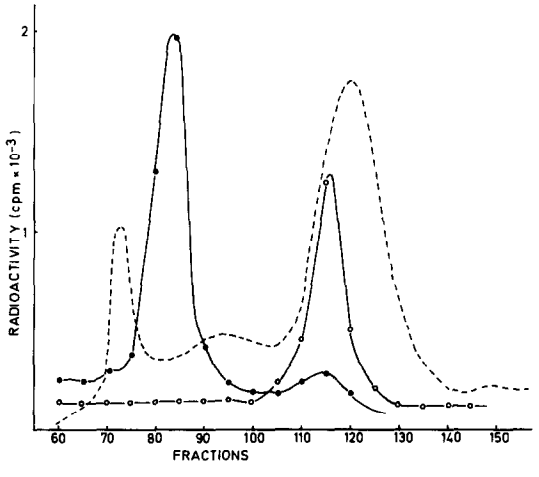

Fig. 2: Elution profile of CNBr cleavage products of ^{35}S-labeled proteins synthesized in vitro in the presence of ^{35}S-Met-$tRNA_F^{Met}$. Broken line: absorption at 280 nm. Closed circles: ^{3}H-radioactivity. Open circles: ^{14}C-label (homoserine marker).

Table 3

Incorporation of ^{3}H-methionine and ^{35}S-methionine from ^{3}H-Met-tRNA$_{M}^{Met}$ and ^{35}S-Met-tRNA$_{F}^{Met}$, present in saturating concentrations.

Met-tRNA^{Met}	femtomoles incorporated/A_{260} unit of polysomes	
	^{3}H-methionine	^{35}S-methionine
^{3}H-Met-tRNA$_{M}^{Met}$	525	-
^{35}S-Met-tRNA$_{F}^{Met}$	-	311
^{3}H-Met-tRNA$_{M}^{Met}$ + ^{35}S-Met-tRNA$_{F}^{Met}$	430	48

the insertion of methionine from Met-tRNA$_{F}^{Met}$ into internal positions of proteins does not necessarily mean that both methionine accepting tRNA species are equivalent in this function. In most of our experiments the supply of Met-tRNA was limiting for the rate of protein synthesis. We therefore determined the saturating concentration of each Met-tRNA^{Met} in our system. The amounts beyond which no further increase in methionine incorporation could be observed, were found to be 3.3 pmoles of Met-tRNA$_{F}^{Met}$ and 7.5 pmoles for Met-tRNA$_{M}^{Met}$ per A_{260} unit of ribosome. When these concentrations of ^{3}H-Met-tRNA$_{M}^{Met}$ and ^{35}S-Met-tRNA$_{F}^{Met}$ were used in a double-label experiment a 10 fold incorporation of ^{3}H-methionine over ^{35}S-methionine was observed whereas the ratio of incorporated activities was 1:0.6 when the Met-tRNA^{Met} species were tested separately (table 3). Thus, it appears that in the presence of saturating quantities of both Met-tRNA^{Met} species, Met-tRNA$_{M}^{Met}$ is the major donor of methionine into internal positions of the growing polypeptide chain whereas Met-tRNA$_{F}^{Met}$ is a 'second choice' molecule for this function. Nevertheless, it is quite clear that in contrast to other recent findings (2,3, 17), Met-tRNA$_{F}^{Met}$ can be used as a very effective donor for the insertion of methionine into internal positions of polypeptides.

REFERENCES

1. Clark, B.F.C., and Marcker, K.A., J. Mol. Biol., 17, 394 (1966)
2. Smith, A.E., and Marcker, K.A., Nature 226, 607 (1970)
3. Housman, D., Jacobs-Lorena, M., Rajbandary, V.L., and Lodish, H.F., Nature 227, 913 (1970)
4. Kerwar, S.S., Spears, C., and Weissbach, H., Biochem. Biophys. Res. Comm. 41, 78 (1970)
5. Moon, H.M., Collins, J.F., and Maxwell, E.S., Biochem. Biophys. Res.Comm. 41, 170 (1970)
6. Cherayil, J.D., Hampel, A., and Bock, R.M. in 'Methods of Enzymology', Vol. XII B, L. Grossman and K. Moldave eds., Academic press, New York and London, 1968, p. 166
7. Jones, K.M., Guest, J.R., and Woods, D.D., Biochem. J. 79, 566 (1961)
8. Nishimura, S., and Weinstein, I.B., Biochemistry 8, 832 (1969)
9. Muench, K.H., and Berg, P. in 'Procedures in Nucleic Acid Res.' Cantoni, G.L. and Davies, D.R. eds., New York and London 1966, p. 375
10. Falvey, A.K. and Staehelin, T., J. Mol. Biol. 53, 1 (1970)
11. Moldave, K. in 'Methods in Enzymology', Vol XII B, p. 721
12. Schroeder, W.A., 'Methods in Enzymology', Vol. XI, p. 445
13. Vesco, C., and Colombo, B., J. Mol. Biol. 47, 335 (1970)
14. Grollman, A.P., and Stewart, M.L., Proc. Natl. Acad. Sci. 61, 719 (1968)
15. Colombo, B., Felicetti, L., and Baglioni C., Biochem. Biophys. Res. Comm. 18, 389, (1965)
16. Tanaka, N., Kinoshita, T., and Masukava, H., Biochem. Biophys. Res. Comm. 30, 278 (1968)
17. Gosh, K., Grishko, A. and Gosh, H.P., Biochem. Biophys. Res. Comm. 42, 462 (1971)