INITIATION OF PROTEIN SYNTHESIS IN RABBIT RETICULOCYTES

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Summary

DEAE-Sephadex column chromatography of crude rabbit liver transfer RNA (tRNA) gives rise to three peaks of methionine acceptor activity (met-$tRNA_{MI}$, met-$tRNA_{MII}$, and met-$tRNA_F$). Only one of these species (met-$tRNA_F$) could be charged by E. coli synthetase and formylated by E coli formylase (1,2).

In a cell-free protein synthesizing system obtained from rabbit reticulocytes, [^{14}C] met-$tRNA_F$ transfers radioactivity to the N-terminal position of the polypeptide chain in response to native messenger RNA. In a similar experiment, [^{14}C] met-$tRNA_{MII}$ transfers radioactivity mainly into the internal positions of the synthesized polypeptide chains.

We previously reported (1,2) the fractionation and coding specificities of rabbit liver methionine t-RNA species. Three met-tRNA species (met-$tRNA_{MI}$, met-$tRNA_{MII}$, and met-$tRNA_F$) were separated by DEAE-Sephadex column chromatography of crude rabbit liver tRNA. Only one of these species (met-$tRNA_F$) could be charged by E. coli synthetase and could be formylated by E. coli formylase. The coding specificities of these met-tRNA species were studied in a cell-free amino acid incorporating system obtained from rabbit reticulocytes using polyribonucleotides of defined sequences as messengers (3). These studies indicated that with rabbit reticulocytes, met-$tRNA_F$ transfers methionine into polypeptides preferentially in response to terminal AUG codons and met-$tRNA_M$ transfers methionine into polypeptides preferentially in response to internal AUG or GUG codons. Similar roles of mammalian met-tRNA species in polypeptide chain synthesis with artificial messenger RNA's, in a cell-free amino acid incorporating system from ascites tumor cells, have also been described recently (4,5). However, attempts to demonstrate the incorporation of

methionine at the N-terminal positions of the proteins synthesized in response to native messengers in the same cell-free protein synthesizing system were unsuccessful (4).

We also reported (1,2) that in cell-free hemoglobin synthesizing system from rabbit reticulocytes, [^{14}C] met-tRNA$_{MII}$ efficiently transferred radioactivity into proteins. In a similar experiment, the transfer of radioactivity from [^{14}C] met-tRNA$_F$ into proteins was approximately 20 per cent of that for [^{14}C] met-tRNA$_M$. The possibility that such transfer of methionine from met-tRNA$_F$ was due to incorporation of methionine at the N-terminal positions of the synthesized proteins was indicated. In this communication, we present evidence that [^{14}C] met-tRNA$_F$ transfers methionine at the N-terminal positions of the synthesized proteins in a cell-free protein synthesizing system from rabbit reticulocytes in response to native messengers, while the transfer of radioactivity from [^{14}C] met-tRNA$_M$ was mainly at the internal positions of the synthesized proteins.

Materials and Methods

Washed, twice-pelleted, reticulocyte ribosomes and reticulocyte lysate, used in these experiments, were prepared from anemic rabbit blood by the procedure described previously (3). Crude E. coli aminoacyl-tRNA synthetases were prepared according to the procedure of Rajbhandary and Ghosh (6). Rabbit reticulocyte synthetases were prepared from 100,000 x g reticulocyte supernatant by the procedure described for the preparation of E. coli synthetases (6).

Crude rabbit liver tRNA was prepared by the procedure previously described (3). Radioactively labeled aminoacyl-tRNA's were prepared from the purified fractions of rabbit liver tRNA. E. coli synthetase was used for charging of met-tRNA$_F$ species and reticulocyte synthetase was used for charging of met-tRNA$_M$ species. Under the conditions of charging, E. coli synthetase did not recognize met-tRNA$_M$ species. Detailed descrip-

tions of the procedures for charging of the met-tRNA species have been described (1).

Results

DEAE-Sephadex column chromatographic procedure for the preparation of met-tRNA fractions has been described previously (1). Three major methionine acceptor peaks were detected when charged with reticulocyte synthetase. Only one of these peaks (met-$tRNA_F$) could be charged with E. coli synthetase. The other two met-tRNA species (met-$tRNA_{MI}$ and met-$tRNA_{MII}$) had similar coding specificities, though met-$tRNA_{MII}$ was somewhat more efficient than met-$tRNA_{MI}$ in translation of the artificial messengers, poly r(A-U-G) and poly r(U-G) (1). The met-$tRNA_{MII}$ fraction (DEAE-Sephadex fraction 82) used in the experiments contained substantial amounts of met-$tRNA_F$. Also the met-$tRNA_F$ fraction (DEAE-Sephadex fraction 88) was contaminated with met-$tRNA_M$. However, since E. coli enzyme recognizes only met-$tRNA_F$ species, this fraction, when charged with E. coli enzyme, should represent only met-$tRNA_F$ species.

Precharged [^{14}C] met-$tRNA_{MII}$ and [^{14}C] met-$tRNA_F$ were used in in vitro protein synthesis using washed reticulocyte ribosomes and lysate. Such a protein synthesizing system actively incorporates amino acids into proteins and synthesizes mostly hemoglobin (7). As previously reported, the transfer of radioactivity into proteins from [^{14}C] met-$tRNA_F$ was low and was approximately 20 per cent of that for [^{14}C] met-$tRNA_{MII}$. Proteins thus synthesized were analyzed for the N-terminal amino acid by the procedure described previously (8,9). The results are shown in Fig. 1. Approximately 95 per cent of the incorporated radioactivity was released as PTH-met-SO_2 after the first cycle of Edman degradation when [^{14}C] met-$tRNA_F$ was used as the source of radioactivity (Fig. 1B). Under similar conditions, the majority of the radioactivity in proteins synthesized with [^{14}C] met-$tRNA_{MII}$ remained at the origin of the paper chromatogram (Fig. 1A).

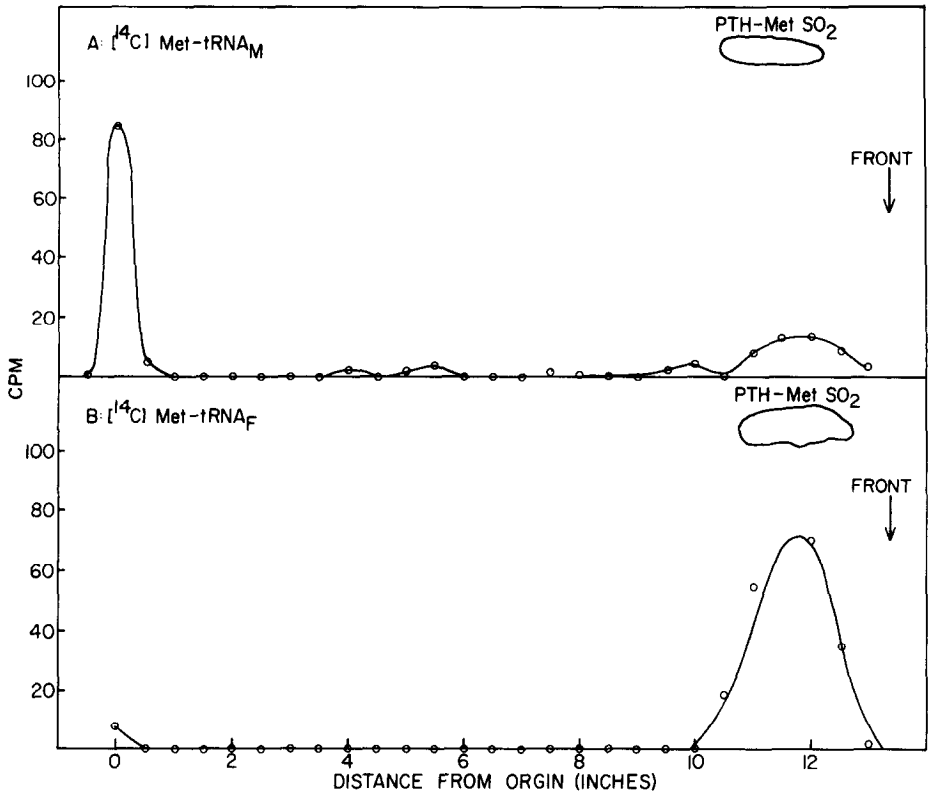

Fig. 1. End group analysis by Edman degradation of [^{14}C] methionine labeled proteins synthesized in a cell-free protein synthesizing system from rabbit reticulocytes. [^{14}C] methionine labeled proteins were synthesized using precharged [^{14}C] met-tRNA$_{MII}$ or [^{14}C] met-tRNA$_F$. Standard incubation mixture contained, in total volume of 2 ml, the following: 66 µmoles Tris-HCl (pH 7.5), 6 µmoles of $MgCl_2$, 24 µmoles of mercaptoethanol, 133 µmoles of KCl, 1.6 µmoles of ATP, 0.4 µmoles each of 19 unlabeled amino acids (minus methionine), 4 µmoles of unlabeled methionine, 5 A_{260} units of crude rabbit liver tRNA, 48 A_{260} units of reticulocyte ribosomes and 18 A_{260} units of reticulocyte lysate. In addition, in the experiment described in Fig. A, 112 µµmoles of met-tRNA$_{MII}$ (Fraction 82, charged with reticulocyte synthetase, 40,080 CPM) were added, and in the experiment described in Fig. B 360 µµmoles of [^{14}C] met-tRNA$_F$ (Fraction 88, charged with E. coli synthetase, 125,280 CPM) were added. Incubation was carried out at 37° for 10 minutes. The reaction mixture was then treated with 20 µl of 10 NaOH and the solution was allowed to stand at room temperature for 1 hour. An aliquot was spotted on a filter paper disc and was counted for radioactivity after washing 3 times with 5% trichloroacetic acid, once with ethanol:ether (1:1), and once with ether. The total amounts of radioactivity incorporated in the original incubation mixture were calculated from such results and were found to be approximately 2,240 CPM in experiment A and 400 CPM in experiment B. The total protein fraction in the original incubation mixture was precipitated by the addition of 100 µl of 100% trichloroacetic acid. The precipitate was washed 3 times with cold 5% trichloracetic acid, twice with performic

acid (8). The performic acid treated polypeptide product was used for Edman degradation by the microscale procedure of Gray and Hartley (9). An aliquot of the reaction mixture after one cycle of Edman degradation was applied to a paper chromatogram. The chromatogram was developed in butanol:acetic acid:H_2O solvent system (4:1:5). The paper chromatogram was then cut into one-half inch pieces and was counted for radioactivity. The figure above represents the chromatographic patterns of the Edman degradation products of the proteins synthesized using [^{14}C] met-tRNA$_{MII}$ (Fig. A) and [^{14}C] met-tRNA$_F$ (Fig. B).

Discussion

The experimental procedures described in this paper clearly demonstrate the transfer of methionine from met-tRNA$_F$ into the terminal positions of proteins (presumably hemoglobin) synthesized *in vitro* in response to physiological messenger RNA. The role of the other met-tRNA species (met-tRNA$_M$) in the transfer of methionine into the internal positions of the native protein is also clear. This is the first demonstration of the roles of the mammalian met-tRNA species in protein synthesis in response to physiological messenger RNA in a completely homologous *in vitro* protein synthesizing system.

Present work was done with unformylated met-tRNA$_F$ and the experimental results clearly indicate that the preference for the terminal position of the genetic message still exists in the mammalian system. As noted before, this met-tRNA$_F$ species can be formylated by *E. coli* formylase (3). Attempts to demonstrate the formylase activity in reticulocyte lysate have been unsuccessful. A recent report indicates the presence of met-tRNA formylase activity in extracts of Ehrlich ascites tumor cells (10). Further work is in progress to determine the effect of formylation of met-tRNA$_F$ on polypeptide chain initiation in the reticulocyte system.

Our results indicate that the roles of the met-tRNA species in the mammalian system are similar to those described in the bacterial system, namely, one met-tRNA species (met-tRNA$_F$) recognizes the terminal position and initiates the peptide chain synthesis and the other met-tRNA species (met-tRNA$_M$) recognizes the internal codon and inserts methionine in the middle of the polypeptide chain. Two methionine tRNA species have also been described

in yeast (6,11), wheat germ (12), and guinea pig liver (13). In each case, only one met-tRNA species could be formylated by E. coli formylase. In an E. coli amino acid incorporating system, yeast met-$tRNA_F$ was found to be capable of initiating protein synthesis in a manner similar to that described for E. coli met-$tRNA_F$ (6,11,14). Recently, Takeishi, Sekiya, and Ukita (15) reported the specificity of the [^{14}C] met-$tRNA_M$ and [^{14}C] met-$tRNA_F$ species from both E. coli and yeast in the synthesis of hemoglobin in a cell-free amino acid incorporating system from rabbit reticulocytes. Only the met-$tRNA_M$ species from both sources actively transferred methionine into hemoglobin, presumably at the internal positions. While the present work was in progress, we learned that Rajbhandary and Lodish (16) also observed the transfer of methionine from radioactively labeled yeast met-$tRNA_F$ into the terminal position of in vitro synthesized hemoglobin.

Acknowledgments

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References

1. Gupta, N. K., Chatterjee, N. K., Bose, K. K., Bhaduri, S., and Chung, A., Submitted to J. Mol. Biol.
2. Chatterjee, N. K., Bose, K. K., Bhaduri, S., and Gupta, N. K., Abstract, American Chemical Society Meeting, Chicago (1970) (in press).
3. Gupta, N. K., J. Biol. Chem., 243, 4959 (1968).
4. Smith, A. E. and Marcker, K. A., Nature, 226, 607 (1970).
5. Brown, J. C. and Smith, A. E., Nature, 226, 610 (1970).
6. Rajbhandary, U. L. and Ghosh, H. P., J. Biol. Chem, 244, 1104 (1969).
7. Schweet, R. S., Lamfrom, H., and Allen, E. H. Proc. Natl. Acad. Sci., 49, 122 (1958).
8. Hirs, C.H.W., J. Biol. Chem., 219, 611 (1956).
9. Gray, W. R. and Hartely, B. S., Biochem. J., 89, 379 (1963).
10. Li, Chien-Chung and Yu, Chuan-Tao, Biochim. Biophys. Acta, 182, 440 (1969).
11. Takeishi, K., Ukita, T., and Nishimura, S., J. Biol. Chem., 243, 5761 (1968).
12. Leis, J. P. and Keller, E. B., Fed. Proc., 29, 468 (1970).
13. Caskey, C. T., Redfield, B., and Weissbach, H., Arch. Biochem. Biophys., 120, 119 (1967).
14. Ghosh, H. P., Söll, D., and Khorana, H. G., J. Mol. Biol., 25, 275 (1967).
15. Takeishi, K., Sekiya, T., and Ukita, T., Biochem. Biophys Acta, 199, 559 (1970).
16. Rajbhandary, U. L., Personal Communication.