The Presence of N-Formyl-Methionyl-tRNA in HeLa Cell Mitochondria[1]

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Introduction

The discovery of the role of N-formyl-methionyl-tRNA (f-met-tRNA; Marker and Sanger, 1964; Marker, 1965) in the initiation of protein synthesis in bacteria (Clark and Marker, 1966; Adams and Capecchi, 1966) raised the question of the existence of a similar initiation system in animal cells. Previous experiments, utilizing the techniques which had been successful in demonstrating f-met-tRNA in Escherichi coli failed to detect f-met-tRNA in HeLa cells (Summers and Maizel, 1967). In the present experiments larger amounts of high specific activity methionine C^{14} have revealed the presence of low levels of formyl-methionyl-adenosine (f-met-A) in RNAse digests of whole HeLa cell RNA. However, the subsequent observation, that inhibition by aminopterin of the formation of f-met-A, had no effect on the rate of total cellular protein synthesis, suggested

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that only a special class of cellular proteins might utilize this mode of initiation. The relative independence of mitochondria from nuclear control and the somewhat bacteria-like nature of the mitochondrion, evidenced by the existence of mitichondrial DNA (Kroon, Borst, Van Bruggen and Ruttenberg, 1966) specific tRNA's (Barnett and Brown, 1967) and a protein synthesis system (Roodyn, 1966) bearing strong similarities to that in bacteria led to a search for f-met-tRNA in the mitochondrion. It appears that all of the f-met-tRNA of HeLa cells is located in the mitochondrion.

METHODS

HeLa cells were grown in suspension culture, in Eagle's medium (Eagle, 1959) supplemented with 7% horse serum. In all experiments cells were collected at 37°, resuspended and incubated in the same volume of methionineless medium containing 5% dialyzed horse serum. After 5 minutes the cells were again centrifuged and resuspended at 4×10^6 cells/ml for 2' before the addition of uniformly labeled methionine (1.5-3 μc/ml; 205 μc/μmole). For the growth of cells in the presence of aminopterin, the medium was supplemented with thymidine, adenine, glycine and serine after the conditions of Hakala and Taylor (1959). Isolation of RNA by cold phenol extraction has been described (Pene, Knight & Darnell, 1968). All RNA preparations were either dialyzed overnight against 0.01M Na acetate-EDTA, pH 5.1, or precipitated with ethanol three times before RNAse treatment. RNAse digestion and identification by electrophoresis of the products of RNAse digestion involved the procedures of Marker and Sanger (1964). The electropherograms were cut into 1 cm strips, treated with 1 ml of concentrated NH_4OH followed by 20 ml of Bray's solution and counted in a liquid scintillation spectrometer. Preparation of E. coli extracts and charging of E. coli f-met-tRNA was performed according to Marker (1965).

Cytochrome c reductase was assayed in phosphate buffer, 0.01 M,

pH 7.4; containing 0.5 μmoles of cytochrome c; 0.2 μmoles of KCN; 0.025-0.05 ml of enzyme sample and 15 μmoles of Na succinate in a total volume of 1.1 ml. The change in optical density at 550 mμ was followed with time. (We thank Dr. Cyril Moore for instruction in this assay and use of his equipment.) Gradient separation of mitochondria from whole cytoplasm was a modification of a method of Attardi and Attardi (1967). SET buffer used in the mitochondrial isolation procedure contained 0.25 M tris, pH 7.4; 0.25 M sucrose; and 0.01 M EDTA.

RESULTS

Identification of N-formyl-Methionyl-tRNA in HeLa Cells:

When total cell RNA from HeLa cells, which had been incubated with high specific activity methionine C^{14} was subjected to RNAse digestion and electrophoretic analysis, the pattern shown in Figure 1a resulted. Radioactive materials with identical electrophoretic behavior (Figure 1d) were obtained from RNAse digests of *E. coli* tRNA which had been incubated with a bacterial extract in the presence of C^{14} methionine and a formyl donor (Marker, 1965). Marker and Sanger, 1964 have identified the more positively charged species (faster migrating toward cathode) in the *E. coli* pattern as methionyl adenosine (met-A) and that nearer the origin as N-formyl-methionyl-adenosine (f-met-A).

Further study of the HeLa extracts revealed:

1) The small peak in the position of f-met-A was found to be consistently about 5% of the larger peak at -42 cm.

2) Material from the presumptive f-met-A region was eluted and subjected to mild base hydrolysis at low temperature after which the majority of the radioactivity migrated toward the anode identically with an N-formyl-methionine standard (stained by the method of Toennies and Kolb, 1951). A similar change in mobility was observed after treatment of the presumptive f-met-A peak from the *E. coli* extracts (Fig. 1e) in agreement with Marker and Sanger (1964).

The base hydrolysis does not yield perfect recovery of all the radioactivity as f-met. Some cleavage of the formyl group occurs leaving free methionine, while air-oxidation produces some formyl methionine sulfoxide. Both of these reactions were described by Marker and Sanger and are evident in Fig. 1b and 1e.

3) Material eluted from the f-met-A (or formyl-methionine sulfoxide) position of either the HeLa or E. coli sample was heated in HCl and gave a product which migrated toward the cathode together with a methionine standard (stained with ninhydrin). The methionine peak was not split because under these conditions the separation of free methionine and methionine sulfoxide is less than one cm (Fig. 1c and 1f). These results indicate the presence of low levels of N-formyl-methionyl-tRNA in HeLa cells.

The possibility of some contaminant of bacterial origin as the source of the f-met-A was considered. Since, however, all labelling studies were performed under sterile conditions with sterile cultures in the presence of penicillin and streptomycin, bacterial contamination could not have contributed to these results. Tests for the presence of PPLO in the HeLa cell cultures were made by two laboratories experienced in the isolation of PPLO and no such organisms were isolated. In addition, HeLa cells grown for 24 hrs. in medium containing 25γ/ml of aureomycin, an antibiotic effective against many PPLO, showed no decrease of N-formyl-methionine.

Rate of Protein Synthesis during Inhibition of F-Met-A Formation

The ratio of N-formyl-methionyl-tRNA to methionyl-tRNA in E. coli extracts is 60:40 (Marker, 1965 and Fig. 1d); in the HeLa cell extracts the ratio was 1:20 suggesting that although present, the f-met might not be involved in the initiation of HeLa cell proteins. If mammalian cell culture medium is supplemented with glycine, serine, adenine and thymine (Hakala and Taylor, 1959), it is possible to grow cells in the presence of aminopterin, a competitive inhibitor of dihydrofolic acid reductase (Hakala, Zakrzewski and Nikol 1961; Zakrzewski and Nikol, 1958). These growth conditions seemed

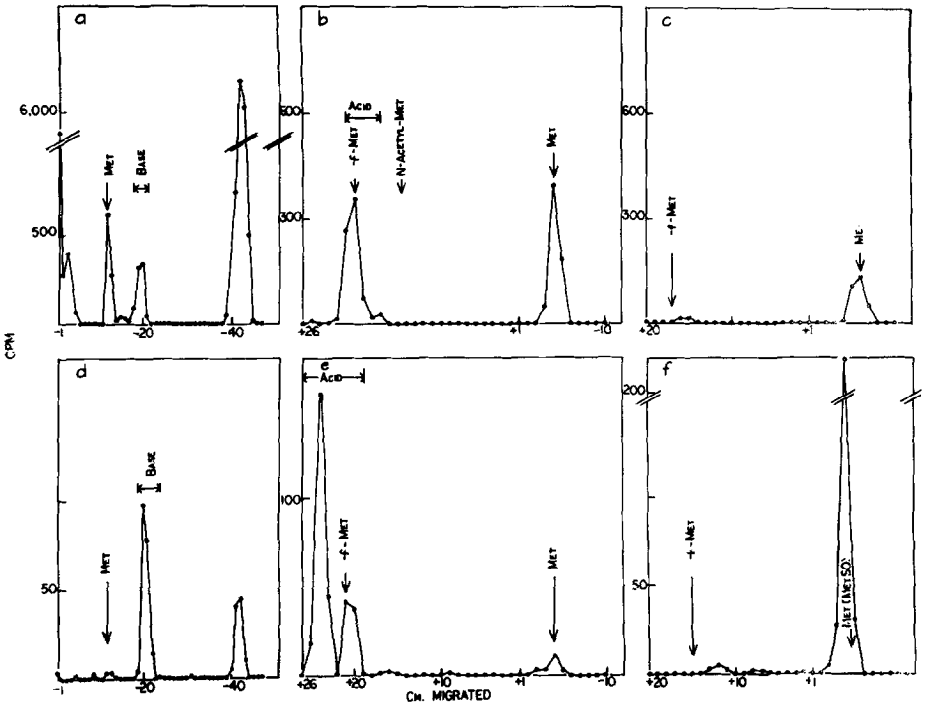

Fig. 1. Identification of Products of RNAse Digests of HeLa Cell RNA.

1.6×10^7 cells were labeled for 15' with methionine C^{14}. Total cell RNA was prepared by phenol extraction and RNAse treated as described in Methods.

a) RNAse digest analyzed by high voltage (3000 V, 2 hours, pH 3.5) electrophoresis. Chromatogram was divided into 1 cm strips, counted and data plotted as centimeters migrated. Minus (-) indicates migration toward cathode; plus (+) migration towards anode. Arrows within other figures indicate mobilities of standard compounds run parallel to radioactive samples.

b) Material from region marked "base" in (a) was eluted with water, the pH brought to 10.5 with NH_4OH and incubated for one hour at 37 C in an N_2 atmosphere. The sample was then neutralized and subjected to electrophoresis (80 minutes, 3000 V).

c) Region of (b) marked "acid" was eluted with 1N HCL, incubated 15' at 100°C in an N_2 atmosphere, and analyzed by electrophoresis (80 min., 3000 V).

d) E. coli tRNA labeled with C^{14} methionine was prepared, RNAse digested and analyzed by electrophoresis.

e) Base hydrolysis in air of presumed f-met-A peak from (d) and subsequent electrophoresis were performed as in (b).

f) Acid hydrolysis in air of f-met from (e) and subsequent electrophoresis were performed as in (c).

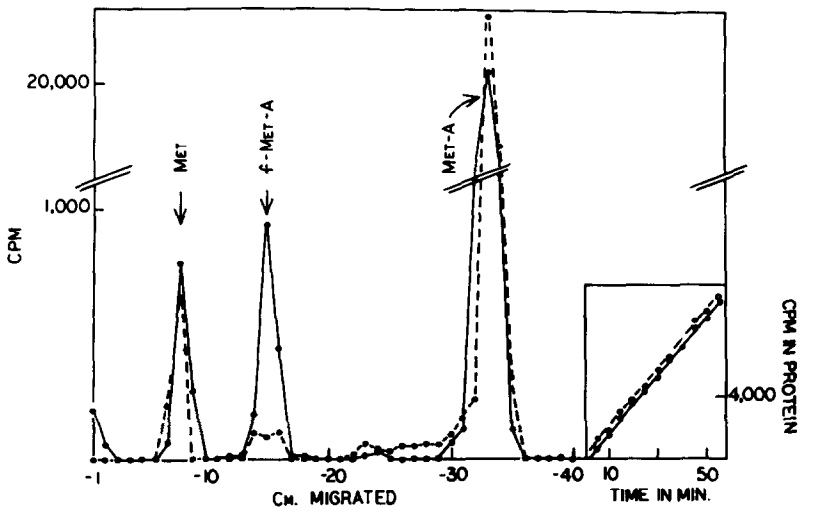

Fig. 2.

8 x 10^7 cells were collected and resuspended in Eagle's medium lacking methionine supplemented with 3 x 10^{-5}M thymidine, 3 x 10^{-5}M adenine, 3 x 10^{-5}M glycine and serine and 2 x 10^{-5}M inositol. Aminopterin (1 x 10^{-6}M) was added to one-half the culture while the other half served as control. After 15 minutes at 37° both cultures were labeled with methionine and RNA prepared and tested for the presence of f-met-A as in Fig. 1. (--o-- aminopterin treated; —●— control).

Insert: HeLa cells were collected as above except they were resuspended in medium lacking valine. One-half was treated with aminopterin for 15 min. and the other half served as control. C^{14} valine was then added and total acid precipitable radioactivity in protein determined at 5 minute intervals (Penman, Becker, Scherrer, and Darnell, 1963).

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to offer a functional test of the role of HeLa cell f-met in general protein synthesis since folic acid was a likely intermediate in the formation of f-met.

Fig. 2 shows that in cells grown in the presence of aminopterin, formylation of methionine was greatly depressed. Under the same conditions, however, the rate of incorporation of C^{14} valine into acid precipitable material was unchanged relative to the control and the cells could be grown for at least 4 generations.

Localization of N-formyl-methionyl-tRNA in the cell

The normal rate of overall protein synthesis in the absence of f-met

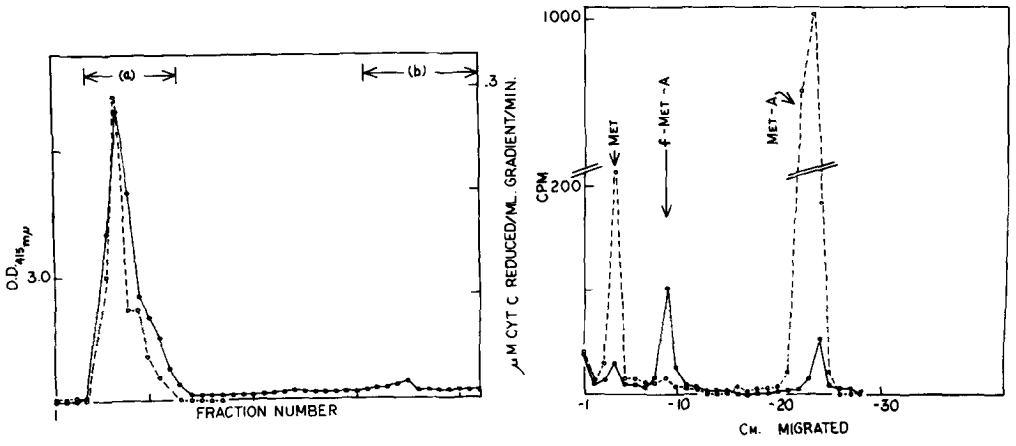

Fig. 3.

Upper Panel: 1.6 x 10^7 cells were labeled with C^{14} methionine as in Figure 1. After washing, cells were resuspended in SET buffer and homogenized. The cytoplasmic fraction was layered directly onto a 15-30% (w/w sucrose gradient containing 0.001 M $MgCl_2$; .005 M tris, pH 7.4) prepared over 5 ml of the same buffer containing 55% sucrose and centrifuged for 80 min. at 4°, 25,000 RPM. The gradient was collected in 0.8 ml fractions. O.D. at 415 mu recorded (—●—), and each tube assayed for cytochrome c reductase activity (--o--) open circles.
Lower Panel: RNA was phenol extracted from fractions marked a & b in upper panel and tested for f-met-A by RNAse digestion and electrophoresis. Region a (—●—); Region b (--o--).

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synthesis led us investigate the possibility that the f-met might be localized in the mitochondrion. A search for f-met-A in the RNA of mitochondria purified by several cycles of differential centrifugation and isopycnic banding (Attardi & Attardi, 1967) revealed that a large fraction of the total cellular f-met-A was, in fact, present in this organelle. However, some could still be found in the extra-mitochondrial portion of the cytoplasmic extract. Since incomplete sedimentation of the mitochondria or disruption during isolation might have accounted for the f-met-tRNA in the supernatant, the whole cytoplasmic extract was layered directly

onto a gradient as described in Figure 3. The fractions of the gradient containing mitochondria (those which showed both adsorption at 415 mμ and cytochrome c reductase activity) were pooled, RNA extracted and analyzed for f-met-A. Fractions from the top 10 ml of the gradient were treated similarly.

All of the N-formyl-methionyl-tRNA was found in the mitochondrial fraction, while only 2% of the total cellular methionyl tRNA was in these fractions. The ratio of f-met-A in the mitochondrion was approximately 60:40 which agrees well with the figures given by Marker (1965) for E. coli. These finding sare strong evidence against any random association of t-RNAs with the mitochondrial fraction during isolation. Also as in previous experiments, the total f-met-A was approximately 5% of the total met-A which argues against differential losses of either of the two RNA species during the experiment.

Discussion

The detection and chemical characterization of f-met-A from RNAse digests of HeLa cell material (Fig. 1) have been patterned after the original techniques of Marker and Sanger and seem to unambiguously indicate that f-met is attached to terminal adenosine in RNA of HeLa cells. The only known species of RNA to attach amino acids terminally to adenosine is tRNA.

Previous attempts to find f-met-tRNA in mammalian cells were not designed to detect small amounts of this material and the small amount found here suggested that it might not be used as a general initiator of protein synthesis. This was confirmed using aminopterin, an inhibitor of folic acid reductase which blocked f-met-tRNA formation without affecting overall cell growth and protein synthesis. Experiments are now in progress to investigate the effect on mitochondrial synthesis of cell growth in the presence of aminopterin.

Although it seems clear that mitochondrial enzymes and f-met-tRNA are in structures of similar size and density it remains to be shown that

the f-met-tRNA has a role in the initiation of protein synthesis in mitochondria. However, there is evidence that protein synthesis systems in mitochondria employ "70S" ribosomes, as contrasted to "80S" ribosomes of the cytoplasm (Kuntzel and Noll, 1967; Rifkind, Wood and Luck, 1967). In addition, the synthesis of cytochromes b and c in yeasts (Huang, Briggs, Clark-Walker, Linnane, 1966) as well as *in vitro* mitochondrial protein synthesis (Kroon, 1965) are sensitive to chloramphenicol, an inhibitor of bacterial, "70S", protein synthesis, but insensitive to cycloheximide (Clark-Walker and Linnane, 1966), an inhibitor of animal cell and yeast "80S" protein synthesis. Therefore it may be well that f-met-tRNA is also "ribosome specific" and is a universal initiator for "70S" ribosomes. One of the negative but seemingly allowable conclusions that comes from these considerations is that general protein synthesis in animal cells employs some starting mechanism other than f-met-tRNA.

References

1. Adams, J. and Capecchi, M. R. (1966). Proc. Nat. Acad. Sci. Wash. 55, 147.
2. Attardi, B. and Attardi, G. (1967). Proc. Nat. Acad. Sci. Wash. 58, 1051.
3. Barnett, W. E. and Brown, D. H. (1967). Proc. Nat. Acad. Sci. Wash. 57, 452.
4. Clark, B. F. C. and Marker, K. A. (1966). Nature, 211, 378.
5. Clark-Walker, D. G. and Linnane, A. W. (1966). Bioch. Biophys. Res. Comm. 25, 8.
6. Eagle, H. (1959). Science, 130, 432.
7. Hakala, M. T. and Taylor, E. (1959). J. Biol. Chem. 234, 126.
8. Hakala, M. T., Zakrzewski, S. F. and Nikol, C. A. (1961). J. Biol. Chem. 236, 952.
9. Huang, M., Briggs, D. R., Clark-Walker, G. D., Linnane, A. W. (1966). Bioch. Biophys. Acta. 114 (1966) 434.
10. Kuntzel, H. and Noll, H. (1967). Nature 215, 1340.
11. Kroon, A. M. (1965). Biochim. Biophys. Acta., 108, 275.
12. Kroon, A. M., Borst, P., Van Bruggen, E. F. J., Ruttenberg, G. J. C.M. (1966). Proc. Nat. Acad. Sci. Wash. 56, 1836.
13. Marker, K. (1965). J. Mol. Biol. 14, 63.
14. Marker, K. and Sanger, F. (1964). J. Mol. Biol. 8, 835.
15. Pene, J. J., Knight, E. and Darnell, J. E. (1968). J. Mol. Biol. 33, 609.
16. Penman, S., Scherrer, K., Becker, Y. and Darnell, J. E. (1963). Proc. Nat. Acad. Sci. 49, 654.

17. Roodyn, D. B. (1966). In Regulation of Metabolic Processes in Mitochondria, Ed. J. H. Jager, S. Papa, E. Quagliaririella and E. L. Slater (B.B.A. Library, Vol. 7), Elsevier, Amsterdam, pp. 383-396.
18. Summers, D. F. and Maizel, J. V. (1967). Virology 31, 550.
19. Toennies, G. and Kolb, J. J. (1951). Analyt. Chem. 23, 823.
20. Zakrzewski, S. F. and Nichol, C. A. (1958). Biochim. Biophys. Acta, 27, 425.
21. Rifkin, M. R., Wood, D. D. and Luck, J. L. (1967). Proc. Nat. Acad. Sci. 58, 1025.