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THE COMPLEXITY OF MITOCHONDRIAL TRANSLATION PRODUCTS IN MAMMALIAN CELLS

Narayan K. Bhat*, Banavadi G. Niranjan, and Narayan G. Avadhani**

The Laboratories of Biochemistry, Department of Animal Biology,
School of Veterinary Medicine, University of Pennsylvania,
Philadelphia, Pennsylvania 19104

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SUMMARY: The complexity of mitochondrial translation products in mouse liver and Ehrlich ascites tumor cells have been studied using a mitoplast system active in ^{35}S methionine incorporation. Electrophoretic analysis on gradient polyacrylamide-SDS gels and urea-SDS gels under highly dissociating conditions show that both of the mitochondrial systems synthesize about 22 polypeptides. Many of these ^{35}S labeled products compare with the polypeptides predicted by the DNA sequence analysis data reported by Anderson et al. (1).

Mitochondria (mt)[1] from a variety of animal cells contain a closed circular genome of about 14 to 16 kbp duplex DNA. Recently, Anderson et al. (1) have reported the complete nucleotide sequence of human mt DNA. The DNA sequence information along with the studies of Attardi and coworkers (2,3) on the sequencing and mapping of mt Poly(A)+ RNA provide a wealth of information on the organization and expression of human mt genome. The human mt DNA contains information for coding about 13 polypeptides in addition to sequences coding for 2 mt specific rRNAs and 22 tRNAs. Information is also emerging to suggest that a similar pattern of sequence organization and mode of expression might exist in the bovine (4), mouse and rat mt systems (5,6). In contrast to the information on sequence organization, a number of reports using intact cells (7-9) as well as isolated mt particles (10,11) have suggested that the function of mt translation system is to contribute for only 6-8 polypeptides associated with the mt innermem-

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** To whom correspondance concerning this paper should be addressed.

1. Abbreviations: kbp, kilo base pair; mt, mitochondria; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin ; CAP, chloramphenicol.

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brane. In this report, using a mitoplast system highly active in ^{35}S methionine incorporation, we show that mouse ascites tumor cell mt as well as mouse liver mt synthesize over 20 polypeptides including almost all of the 13 products predicted by the DNA sequence analysis data.

MATERIALS AND METHODS

Cell Fractionation: Lettre Ehrlich ascites cells grown for 7 days in the peritoneal cavity of Swiss mice were used (12). Cells were homogenized in mt isolation buffer (5 mM Hepes, pH 7.4, 220 mM Mannitol, 70 mM sucrose, 1 mM EDTA, and 0.05% BSA) in a tight fitted teflon homogenizer and made to 15% (w/v) with mt isolation buffer. The crude mt fraction was separated by differential centrifugation (13). Mouse liver mt was isolated as described earlier (15). Mitoplasts were prepared by the digitonin procedure of Greenawalt (14) using 50 µg digitonin/mg mt protein.

Mitochondrial Protein Synthesis: The mitoplasts were washed once with mt isolation buffer and once with a buffer containing 0.25 M sucrose, 30 mM Tris-HCl (pH 7.4), 100 mM KCl, 10 mM $Mg(CH_3COO)_2$, 7 mM 2-mercaptoethanol and 5 mM potassium phosphate. Washed mitoplasts were incubated in a medium containing 15 mM Tris-HCl (pH 7.4), 60 mM KCl, 6 mM $Mg(CH_3COO)_2$, 4 mM 2-mercaptoethanol, 3 mM potassium phosphate (pH 7.4), 0.14 M sucrose, 2 mM ATP, 2 mM GTP, 4 mM pyruvate, 5 mM creatine phosphate, 0.2 mg/ml creatine phosphokinase, 80 uM each of 19 L-amino acids except methionine, 150 µCi/ml ^{35}S methionine (600 Ci/mmol), and 300 µg/ml cycloheximide at a final concentration of 10 mg protein/ml. Incubation was carried out for 60 min at 35°C. Chloramphenicol when added was at 500 µg/ml. Aliquots of 10 µl were used for determining hot CCl_3COOH insoluble radioactivity (15). At the end of incubation, mitoplasts were pelleted, washed two times with mt isolation buffer and mixed with 3 µg each of leupeptin and pepstatin to suppress proteolytic activity. The mitoplasts were sonic ruptured, incubated with 1 µg/mg each DNase and RNase for 15 min at room temperature and then lyophilized and stored at -70°C.

Electrophoretic Analysis: Labeled mt samples were dissolved in a buffer containing 125 mM Tris-HCl (pH 6.8), 4% SDS, 10% 2-mercaptoethanol and the proteins were dissociated by heating at 90°C for 3 min. Electrophoresis was carried out on 8 to 16% gradient polyacrylamide gel containing 0.2% SDS using the buffer system described by Laemmli (16). The mt proteins were also resolved on 15% polyacrylamide gels containing 7 M urea using a procedure modified from Shapiro et al. (17). The buffer system consisted of 0.1 M sodium phosphate (pH 7.2), 0.2% SDS. For urea gel analysis, proteins were dissociated in 10 mM sodium phosphate (pH 7.2), 7 M urea, 3% SDS, and 5% 2-mercaptoethanol by heating at 90°C for 3 min. The gels were processed for fluorography using En^{3}hance (New England Nuclear) and exposed to Royal X-omatic films at -70°C.

RESULTS

In the initial experiments, we observed that various published procedures for the incorporation of labeled amino acids into isolated mt particles yield only marginal activity with Ehrlich ascites and mouse liver mt preparations. The modification of the hypo-osmotic procedure of Fukamachi et al. (18) as de-

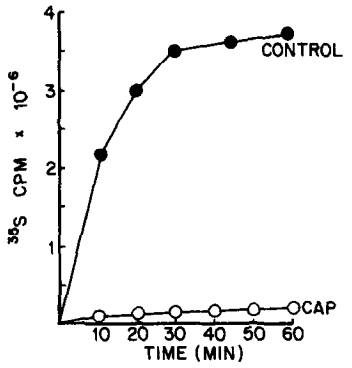

Fig. 1: The kinetics of ^{35}S methionine incorporation by isolated mitoplasts. Ehrlich ascites tumor cells grown for 7 days in the peritoneal cavity of Swiss mice were used for isolating the mt particles. The digitonin washed particles were incubated in the protein synthetic buffer containing 150 µCi/ml ^{35}S methionine (600 Ci/mmol) in the presence (CAP) or absence (Control) of 500 µg/ml chloramphenicol. Details of protein synthesis were as described under Materials and Methods. The values correspond to the extent of incorporation/mg protein.

tailed in the Materials and Methods allows an efficient incorporation of ^{35}S methionine into mt particles isolated from various animal tissues. The kinetics of ^{35}S methionine incorporation into Ehrlich ascites mitoplasts has been presented in Fig. 1. It is seen that using an isotope concentration of 150 µCi/ml, a specific activity of about 3.7 x 10^6 CPM/mg protein is obtained. Further, chloramphenicol, a specific inhibitor of mt specific ribosomes, causes over 90% inhibition of incorporation in this system.

The mt translation products labeled with ^{35}S methionine were electrophoresed on 8 to 16% gradient polyacrylamide gels. As shown in Fig. 2A and 2C, radioactive polypeptides synthesized in mouse liver and Ehrlich ascites mt, respectively, resolve into 20 to 22 species in the size range of 7K to >100K daltons. Almost all of these species are inhibited by chloramphenicol (see Figs. 2B and 2D). In order to determine if some of these polypeptides are precursors or processing intermediates, a pulse-chase experiment was carried out. As shown in Fig. 2E, all of the components labeled during 60 min incubation are detected in mt pulse labeled for 5 min. Further, most of these products are seen even after 50 min chasing (see Fig. 2F) suggesting that they may represent matured products rather than short lived precursors. There is, however, a quantitative variation after

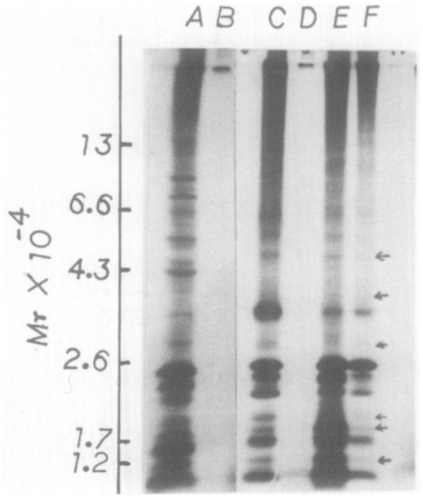

Fig. 2: Electrophoretic analysis of ^{35}S labeled proteins on gradient polyacrylamide gels. Mitoplasts from mouse liver and Ehrlich ascites cells were labeled with ^{35}S methionine as described in Fig. 1. Labeled proteins (250 µg) were dissociated at 90°C in a buffer containing 4% SDS, 10% 2-mercaptoethanol and electrophoresed on 8 to 16% gradient polyacrylamide gel containing 0.2% SDS. Lane A - mouse liver mt labeled for 60 min; B - mouse liver mt labeled in presence of chloramphenicol; C - Ehrlich ascites mt labeled for 60 min; D - Ehrlich ascites mt labeled in presence of chloramphenicol; E - Ehrlich ascites mt pulse labeled for 5 min; and F - Ehrlich ascites mt pulse labeled as in Lane E were washed and resuspended in cold medium and chased for 50 min with 200 fold excess of unlabeled methionine. Details of electrophoresis and fluorography were as described under Materials and Methods.

the chase, possibly reflecting variations in the turnover rates of different polypeptides. Relatively lower intensities of some of the bands in Fig. 2F indicated by the arrows suggest a faster turnover rates for these species.

In order to obtain a better resolution of mt translation products particularly in the lower molecular weight range, the ^{35}S labeled mt proteins were analyzed on 15% polyacrylamide gels containing 7 M urea using the buffer system described in the Materials and Methods. In addition to providing higher resolution (19), this system also permits an accurate estimation of molecular weights in the range of 5K to about 66K daltons. As shown in Fig. 3B, the Ehrlich ascites mt translation products resolve into 18 components in the range of 5.4K to 66K daltons in this gel system. Further, at least 13 of the ^{35}S labeled polypeptides in Fig. 3B exhibit size distribution comparable to the reading frames predicted in the human mt system (1,5).

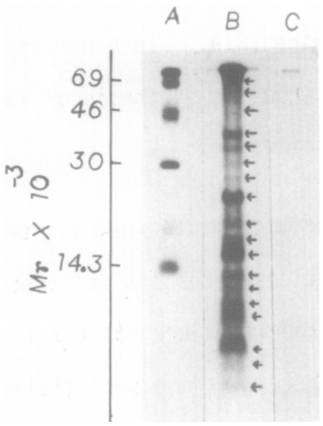

Fig. 3: Electrophoretic separation of ^{35}S labeled mt translation products on SDS-urea gels. Ehrlich ascites mt were labeled with ^{35}S methionine as described in Fig. 1. The mt proteins (250 µg) were dissociated by heating at 90°C (3 min) in presence of 7 M urea, 3% SDS and 5% 2-mercaptoethanol and electrophoresed on 15% polyacrylamide gels containing 7 M urea and 0.2% SDS as described under Materials and Methods. Lane A - ^{14}C labeled marker proteins; B - Ehrlich ascites mt labeled for 60 min; and C - Ehrlich ascites mt labeled in the presence of chloramphenicol. Other details were as in Fig. 2 and Materials and Methods.

DISCUSSION

In this paper we report the development of an incubation system for high specific activity labeling of mitoplasts from various animal tissues. Although not shown here, treatment of mt particles with digitonin is a critical factor in obtaining high levels of incorporation. Digitonin treatment helps removal of lysosomal fraction and associated hydrolytic enzymes. Also, removal of outermembrane may permit partial swelling of mt particles facilitating higher incorporation. Use of a buffer system containing low sucrose (0.14 M), addition of high levels of GTP (2 mM) and malate or pyruvate are also factors responsible for high stimulation.

Detailed genetic analysis and biochemical studies on cytoplasmic mutants of Yeast and Neurospora have ascribed a functional role for 6 to 7 of the mitochondrially translated products (20.21), although additional products of unknown function have been detected in Yeast mt (22). Similarly, experiments with isolated mt from rat liver, beef heart, and Drosophila (10,11) suggested the synthesis of only 6-8 polypeptides. Furthermore, some of the recent studies showed the syn-

thesis of only 5-7 polypeptides resistant to cycloheximide and sensitive to chloramphenicol in intact hepatocytes (8,9). In contrast to these observations, results reported in this paper show the synthesis of about 20 to 22 polypeptides in mouse liver and Ehrlich ascites mt.

Control experiments using mitoplasts pre-digested with pancreatic RNase and Ca^{+2} dependent micrococcal nuclease (results not presented) suggest that the radioactive products seen in our experiment are not due to the activity of contaminating cytoplasmic polysomes. Similarly, extreme sensitivity of these products (see Fig. 2B and 2D; and Fig. 3C) to chloramphenicol suggests that they are translated on mt specific ribosomes. Further, because of the hydrophobic nature of mt proteins, the membranes were dissociated in the presence of high levels of 2-mercaptoethanol (10%) and SDS (4%) as recommended (23,24). Even under such highly reducing conditions, a number of high-molecular weight components ($>$ 70K daltons) are resolved on gradient polyacrylamide gels (Fig. 2). These high molecular weight components do not appear to be the aggregates in the usual sense since they remain undissociated even in the presence of 7 M urea at 90°C. However, unusual dimer formation of cytochrome b subunit involving covalent bonding has been reported in Yeast mt system (25). We cannot therefore, rule out the possibility that some of these high molecular weight components may represent such aggregation.

In order to minimize aggregation and improve the resolution, the mt translation products were analyzed on 15% polyacrylamide gel containing 7 M urea (Fig. 3). The results show that the mt translation system used in the present study is able to synthesize 18 polypeptides of 5K daltons to about 66K daltons. A recent report using intact cells suggested that about 17-19 polypeptides of $<$ 51K daltons may be synthesized in the HeLa mt system (26). An important aspect of the present finding is that the majority of the products of 5K to 66K daltons resolved on the urea gel system (Fig. 3 and Table 1) show close similarity with mt polypeptides predicted by the DNA sequence analysis (1-5) with respect to size.

TABLE 1: Size distribution of mitochondrial translation products.

Product	Molecular weight (daltons)	Comparable polypeptides predicted by the sequence data (daltons)
1	67,000	66,600
2	57,000	57,000
3	50,000	51,400
4	42,000	42,700
5	37,000	38,900
6	34,000	35,600
7	31,000	31,000
8	26,000	25,500
9	21,500*	24,800
10	19,500	18,600
11	17,000	--
12	15,000	--
13	13,000	13,200
14	10,500	10,700
15	8,100	7,900
16	6,500	--
17	5,100	--
18	3,800	--

The molecular weights of ^{35}S bands resolved on 15% polyacrylamide gel containing 7 M urea from Fig. 3B were determined. The numbering of products indicated by the arrows on Fig. 3B was from top to bottom of the gel. The molecular weights presented in column 3 are those predicted in the human mt system by Anderson et. al. (1). Product indicated with an asterisk suggest possible variation between the mouse and human system or that it may be processed from a larger precursor.

In view of the fact that mt genome is fully packed leaving no room for additional information, resolution of over 20 polypeptides in our studies raises questions on the nature as well as the genetic origin of the additional polypeptides. Although our pulse-chase results fail to indicate extensive processing, it is still possible that some of these additional products may represent unusual processing intermediates. Another possibility is that they may represent matured products coded by extramitochondrial genetic information. In summary, we report a mt translation system capable of synthesizing almost all of the predicted mt translation products.

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