TRANSLATION OF MITOCHONDRIAL PROTEINS IN DIGITONIN-TREATED RAT HEPATOCYTES

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1. Introduction

Although it is now clear that up to 13 peptides may be encoded in mammalian mitochondrial DNA [1], there is little agreement concerning the numbers of stable translation products detectable in these mitochondria [2–5]. Part of this uncertainty is due to the low rates of labeling of mammalian mitochondrial translations products resulting from the relatively slow growth rates of mammalian cells. Indeed, it is often necessary to isolate mammalian mitochondria in order to analyze their translation products [2,4], and the isolation procedures could conceivably lead to artifacts from proteolysis or from the early release of nascent peptides.

To circumvent this problem, it would be desirable to have available a mammalian system which combines the advantages of high rates of labeling of mitochondrial proteins with rapid preparation times. Here, we report the novel use of digitonin-treated rat hepatocytes [6], which provide such a system. This preparation, which is complete in <10 min, does not carry out cytosolic protein synthesis, but labels mitochondrial translation products at rates much higher than intact cells or isolated, in vitro labeled mitochondria.

2. Materials and methods

Hepatocytes were isolated from male Sprague-Dawley rats (180 g) as in [3,7]. Hepatocytes were depleted of cytosol by a 3 min incubation in 0.005% (w/v) digitonin as in [6]. These preparations will be referred to as hepatocyte ghosts. After the 3 min incubation, the suspension was immediately diluted with 10 vol. cold 0.125 M sucrose, 60 mM KCl, 1 mM Hepes (pH 7.1). The diluted ghosts were pelleted by a 2 min centrifugation at 2000 × *g* and then suspended to 15 mg protein/ml in the above solution. The ghosts were used immediately for labeling studies.

Isolated mitochondria, hepatocyte ghosts, and intact hepatocytes (1.5–4 mg protein/ml) were labeled in vitro with 50 μCi/ml of [^{35}S]methionine (1000 Ci/mmol) in a sterile medium containing 50 mM bicine, 90 mM KCl, 1 mM EGTA, 5 mM KH_2PO_4, 15 mM $MgCl_2$, 1 mM cycloheximide, 50 μg/ml of an unlabeled amino acid mixture [8] lacking methionine, 20 mM succinate and 2 mM ADP. The final pH was 7.4 and the temperature was 30°C. In some cases intact hepatocytes were labeled in cell maintenance media [7] using the same concentrations of [^{35}S]-methionine as above.

Mitochondria were isolated from labeled hepatocytes and hepatocyte ghosts as in [7]. Radioactivity was determined in the trichloroacetic acid-insoluble material. Electrophoresis was done on 12.5% polyacrylamide gel slabs containing SDS, using separation systems either lacking [9] or containing [2,4] urea. After electrophoresis, the gels were soaked for 30 min in 7% acetic acid and impregnated with sodium salicylate [10] for fluorographic analysis. Protein was measured by the biuret method.

3. Results and discussion

Hepatocyte ghosts incorporate [^{35}S]methionine into mitochondrial translation products at rates far

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Table 1
Comparison of the rates of labeling of mitochondrial translation products in isolated hepatocytes, digitonin-treated hepatocytes, and mitochondria

Preparation labeled	cpm/mg protein × 10^{-3} in:	
	Cells	Isolated mitochondria[a]
Isolated mitochondria	–	250–400
Hepatocytes + cycloheximide	7	50
Digitonin-treated hepatocytes + cycloheximide	800	2700

[a] Mitochondria from hepatocytes and digitonin-treated hepatocytes were isolated from pre-labeled preparations

All incubations were carried out for 30 min in the presence of 1 mM cycloheximide

exceeding those in either intact hepatocytes or isolated mitochondria (table 1). Protein synthesis in ghosts is due entirely to mitochondrial translation. Cycloheximide does not inhibit the incorporation of [^{35}S]methionine nor does it alter the nature of the translation products separated by electrophoresis (fig.1, tracks 2,3). Thus, the release of cytosol is sufficient to prevent cytoplasmic protein synthesis, and the ghosts behave as highly intact, isolated mitochondria. The high rates of labeling eliminate the need for isolating mitochondria in order to analyze the translation products electrophoretically. This is a particularly important advantage for studies with cultured cells in which large amounts of cells are needed for isolation of mitochondria, and in which the purification of mitochondria can be difficult. Thus, the use of ghosts provides similar advantages for mammalian systems as spheroplasts provide for yeast.

A second important advantage of the ghosts is the speed of preparation, i.e., the transition from intact cells to ghosts is completed in <10 min, compared with 1–1.5 h preparation time for mitochondria. This results in a better quality mitochondria in ghosts which is reflected in a decrease of the non-specific background radioactivity and in the number of discrete labeled bands present on fluorographs of in vitro labeled mitochondria (fig.1). The presence of background radioactivity in in vitro labeled mitochondria is partially due to the release of nascent chains from the ribosomes. This is shown by kinetic experiments in which this background material, which is present in ghosts after a 5 min pulse with [^{35}S]methionine (fig.2A, track 1), is collected into discrete peptides corresponding to the major mitochondrial translation products after a 10 min chase (fig.1).

The data in fig.1 and 2 are consistent with the presence of a small number of mitochondrial translation

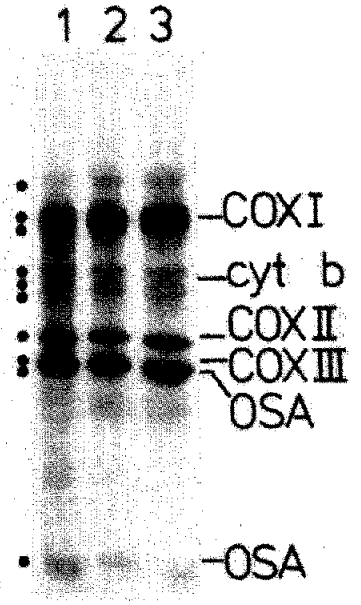

Fig.1. Comparison of the translation products labeled in isolated mitochondria- and digitonin-treated hepatocytes: (1) mitochondria labeled in vitro; (2) digitonin-treated hepatocytes labeled in vitro in the presence of 1 mM cycloheximide; or (3) in the absence of cycloheximide. All preparations were labeled with [^{35}S]methionine for 30 min as in section 2. The labeled preparations were diluted with 10 vol. cold 0.25 M sucrose containing 10 mM Tris–HCl (pH 7.4), 1 mM EDTA, 2 mM chloramphenicol, 2 mM cycloheximide and 20 mM unlabeled methionine. The pellet, obtained by centrifugation, was solubilized in 4% SDS, 10 mM Tris–HCl (pH 6.8), 20% glycerol, and heated for 1 min at 100°C. Mercaptoethanol was then added to 5% final conc. Equal amounts of radioactivity (20 000 cpm) were applied to each track. Electrophoresis was carried out on 12.5% polyacrylamide gels in a buffer system lacking urea [9]. The gels were impregnated with sodium salicylate without pre-soaking in 7% acetic acid. The radioactive band under band 9 in tracks 2 and 3 is non-protein material which is removed by acetic acid. The dots on the left indicate those peptides known to be bonafide, mature, mitochondrial translation products. The identities of some of these are given on the right. COX I, II and III (cytochrome oxidase subunits I–III, respectively), cyt *b* (cytochrome *b*), OSA (oligomycin-sensitive ATPase).

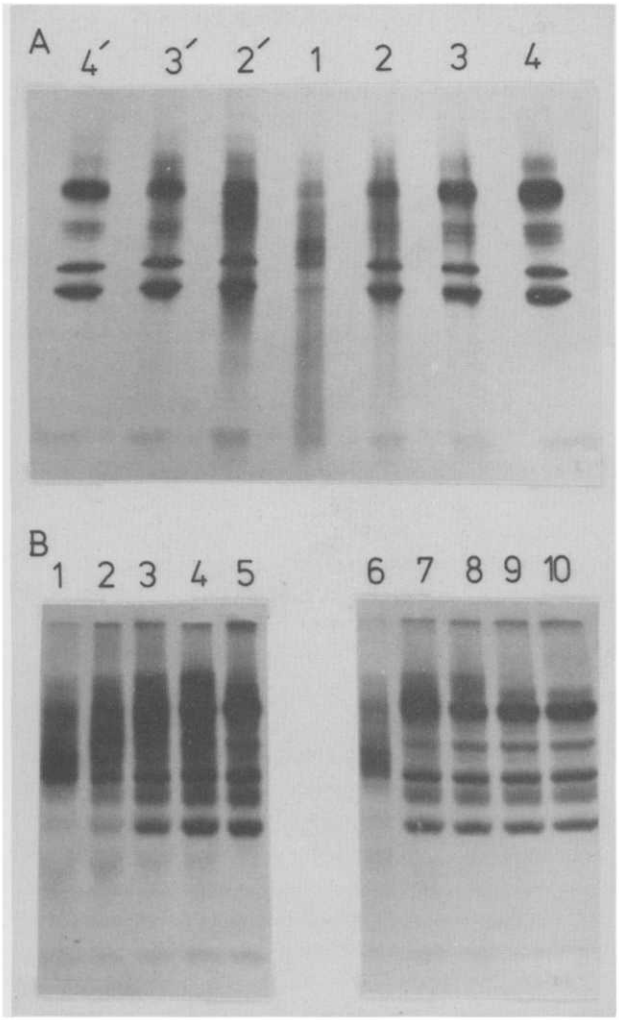

Fig.2. Labeling kinetics of mitochondrial translation products in digitonin-treated hepatocytes. Digitonin-treated hepatocytes were labeled with [^{35}S]methionine as detailed in section 2. After 5 min labeling the suspension was divided into 2 portions. To 1 portion 20 mM unlabeled methionine (chase) was added and to the second the corresponding volume of water (continuous labeling) was added. Incubations were continued for the times indicated below, after which the samples were removed and processed as in fig.1. Equal amounts of radioactivity (17 000 cpm) were added to each track. (A) Samples separated by electrophoresis in the absence of urea [9]. Samples 1–4 were continuously labeled 5, 10, 15 and 20 min, respectively. Samples 2′–4′ were chased for 5, 10 and 15 min, respectively after a 5 min pulse (sample 1). (B) Samples separated by electrophoresis in the presence of urea [2,4]. Samples 1–5 were continuously labeled for 5, 7, 10, 15 and 30 min, respectively. Samples 7–10 were chased for 5, 10, 15 and 25 min, respectively after a 5 min pulse (sample 6).

products in rat liver, corresponding approximately to the number of reading frames identified on human and bovine mitochondrial DNA [1]. This agrees with the findings in several human cell lines [5] but not with recent reports [2,4] that 25 translation products can be separated in Hela cell mitochondria using two-dimensional electrophoresis, and as many as 18 products can be separated in a single dimension [2,4]. Separation of ghost translation products in the single dimension using the buffer system in [2] resulted in the visualisation of ⩽10–11 bands (fig.2B). Since the amount of radioactivity placed on the gels was similar in these experiments and in those with Hela cells, the smaller number of ghost translation products is not due to a lower detection sensitivity. In view of the possibility that some of the apparent translation products observed in Hela cells might arise from the preparation of mitochondria [2,4], it would be of interest to label these products in ghosts prepared from Hela cells.

Several lines of evidence indicate that some mitochondrial transcripts are short-lived [2,11,12]. This raises the possibility that certain translation products might also be short lived and, perhaps, not detectable under steady-state labeling conditions. Due to the high specific labeling of ghosts, it is now possible to investigate the early kinetics of labeling in these liver preparations. Fig.2 shows that the 2 peptides which dominate the labeling pattern after a 5 min pulse rapidly disappear during a chase with unlabeled methionine or during continuous labeling. The apparent removal of these peptides during continuous labeling might be explained by dilution due to relative increases in the other products. However, disappearance during a chase suggests that they are rapidly removed either by breakdown or by relocation to another area of the gel. Although chloramphenicol inhibits their disappearance (not shown), it is difficult to envisage a mechanism by which growing chains appear so reproducibly as such discrete products with the same M_r-values from experiment to experiment. It should be mentioned, however, that a similar observation has been made regarding the translation of a maltose-binding protein in *Escherichia coli* [13]. Here, the accumulation of translation intermediates was attributed to a pause in translation at a specific point in the growing chain. The reason for this pause is not known, but it might be associated with co-translational processing of the growing nascent chains or to a membrane assembly event [13].

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