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Saturation of the processing of newly synthesized rRNA in isolated brain mitochondria

J.A. Enriquez, M.J. López-Pérez and J. Montoya

Departamento de Bioquímica y Biología Molecular y Celular, Facultad de Veterinaria, Universidad de Zaragoza, Miguel Servet 177, 50013 Zaragoza, Spain

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Isolated rat brain mitochondria, when incubated in the presence of [α-^{32}P]UTP in an appropriate incubation buffer, in which the energy requirements are provided by exogenous ADP in the presence of an oxidizable substrate, are able to support mitochondrial DNA transcription and RNA processing in a way faithfully resembling the in vivo process. Furthermore, we have strikingly found a saturation of the synthesis of mature 16 S and 12 S rRNA under conditions in which their RNA precursor as well as the mature mRNAs continue being synthesized. This suggests that synthesis of mature rRNAs could be regulated at the level of processing of their precursor rather than at the level of transcription.

Mitochondria; RNA synthesis; RNA processing; Isolated organelle; Rat brain

1. INTRODUCTION

The detailed analysis of the transcription process of the mammalian mitochondrial genome has led to the characterization of their transcription products [1–4], the identification of the main initiation sites for transcription [5] and has revealed the existence of two transcriptional events for the heavy (H) strand and one for the light (L) strand of the mitochondrial DNA (mtDNA) [6], starting at closely located promoters. Of the two distinct transcription units that take place in the H-strand, one is responsible for the synthesis of the majority of rRNA whereas the second results in the synthesis of a polycistronic molecule, corresponding to almost the entire H-strand, that is processed to produce the mRNAs and tRNAs encoded in this strand [3,6]. In vitro transcription systems utilizing isolated HeLa cell mitochondria have been developed to further study the mechanisms that regulate the expression of the mtDNA [7–9]. In these systems, a different dependence on ATP [9] concentrations for rRNA synthesis versus mRNA synthesis and a sensitivity to intercalating drugs and temperature [7] have been described.

Most of the knowledge about the transcription of the mammalian mitochondrial genes has been obtained in cell cultured systems. However, very little is known about the mechanisms that regulate the mtDNA transcription and RNA processing in differentiated tissues. Quantitative analysis of mitochondrial RNAs in rat liver and cerebellum has shown that the level of the mRNAs relative to that of the rRNAs is higher than in HeLa cells [10,11]. Recently, it has also been described that isolated rat liver mitochondria are able to synthesize RNA, utilizing the same incubation buffer of HeLa cell mitochondria [12]. However, the efficiency of the labeling was much lower than in Hela cells and nothing is known about the energy requirements, polyadenylation of in vitro synthesized mRNAs, and regulation of transcription in isolated mitochondria of differentiated mammalian tissues.

In this work we have undertaken the study of mtDNA transcription and RNA processing in isolated rat brain mitochondria under optimized metabolic conditions in which the energy requirements for RNA synthesis are provided by different concentrations of exogenous ADP in the presence of an oxidizable substrate. In this incubation medium, mitochondrial RNA is synthesized and processed in a way faithfully resembling the in vivo process. Furthermore, we have strikingly found a saturation of the synthesis of mature 16 S and 12 S rRNA under conditions in which their RNA precursors as well as the mature mRNAs continue being synthesized.

Correspondence address: J. Montoya, Departamento de Bioquímica y Biología Molecular y Celular, Facultad de Veterinaria, Miguel Servet 177, E-50013 Zaragoza, Spain

2. MATERIALS AND METHODS

2.1. *Purification of free mitochondria from rat brain*

Wistar rats weighing approximately 200 g were killed by decapitation. The brains were rapidly removed, chilled in medium A (0.32 M sucrose, 1 mM K-EDTA, 10 mM Tris-HCl, pH 7.4) and finally chopped with scissors. All further operations were carried out at 2–4°C using sterile solutions and glassware. Homogenization of the tissue,

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preparation of a crude mitochondrial fraction, which contains synaptosomes, by differential centrifugations and purification of free mitochondria by partitioning in an aqueous two-phase system composed of Dextran T 500 and poly(ethylene glycol) 4000, were carried out as previously described [13,14].

2.2. In vitro labeling and isolation of nucleic acids

Samples of the free mitochondria fraction were resuspended at a final concentration of 2 mg of mitochondrial protein/ml in 0.5 ml of incubation buffer which contained 25 mM sucrose, 75 mM sorbitol, 100 mM KCl, 10 mM K_2HPO_4, 0.05 mM EDTA, 5 mM $MgCl_2$ 1 mM ADP, 10 mM glutamate, 2.5 mM malate, 10 mM Tris·HCl, pH 7.4, 1 mg of bovine serum albumin (BSA) per ml and 20 μCi of [α-^{32}P]UTP (400–600 Ci/mmol) in Eppendorf tubes. Incubation was at 37°C for 60 min. This incubation buffer differs from that of the RNA synthesizing system with isolated HeLa cell [7–9] and rat liver mitochondria [12].

After incubation, the isolation of total nucleic acids from free mitochondria of brain oligo(dT)-cellulose chromatography and electrophoresis through 1.4% agarose-CH_3HgOH slab gels were carried out as described before [8,15]. Quantification of the autoradiograms were carried out using a LKB Ultroscan XL laser densitometer and a Gel Scan XL software.

The preparation of cold mitochondria RNA from 16 rat brains was carried out as previously described [13].

3. RESULTS AND DISCUSSION

3.1. *MtDNA transcription in isolated rat brain mitochondria*

In order to establish the appropriate incubation condition for transcription in isolated brain mitochondria, we have carried out a comparative study of the incorporation of [α-^{32}P]UTP into newly synthesized RNA utilizing either the incubation medium previously employed in HeLa cell [7,8] and liver [12] mitochondria ('glycerol medium' containing ATP), or the highly efficient respiratory ('100 mM KCl') medium for brain mitochondria described by Lai and Clark [16] in the presence of ADP and an oxidizable substrate. In this case, glutamate plus malate was chosen as the respiratory substrate since they have been described to be the most efficient in supporting brain mitochondrial protein synthesis [17]. Incorporation of labeled [α-^{32}P]UTP into total mitochondrial RNA was linear during the first 60 min in the '100 mM KCl' medium with 1 mM ADP, while in the glycerol medium the plateau was reached after 30 min. The specific incorporation in the '100 mM KCL' medium was about 100-fold higher than in the 'glycerol' one (data not shown). These results clearly showed that the incubation medium used for isolated HeLa cells mitochondria was not the most appropriate to maintain in vitro transcription of isolated brain mitochondria.

Fig. 1 shows a comparison between the electrophoretic patterns of the in vitro labeled RNA in the presence of 1 mM ADP, glutamate, malate and phosphate (lanes a–c), and those of a cold mitochondrial RNA preparation (lanes d–f) from rat brain. As shown, isolated brain mitochondria synthesized RNA in a way that closely resembles the in vivo process. In these patterns it is possible to recognize the characteristic set of transcripts previously described in the mitochondria of HeLa cells [6,8,18], sheep brain [13], and rat liver and muscle [12,19,20]. Therefore, the RNA species were designated according to Amalric et al. [18] and Montoya et al. [6]. The RNA species 5, 7, 9 and 11–17 of the oligo(dT)-cellulose-bound RNA fraction represent the mRNAs encoded in the H-strand. The proportion of the total in vitro labeled RNA which was retained by the oligo(dT)-cellulose column was approximately 2%. This value is comparable with those found in vivo and in sheep mitochondria [13]. Polyadenylation of the mRNAs appears to be as efficient as in vivo, as shown by comparison of the RNA content of the total, oligo(dT)-cellulose unbound and bound RNA fractions (Fig. 1). The extent of polyadenylation reached in this work is much higher than that obtained in isolated HeLa cell mitochondria where more than 80% of the mRNAs did not bind to oligo(dT)-cellulose [8]. This could be a consequence of the different media used for the incubation in both cases. In our system the ATP required for transcription is endogenously produced through oxidative-phosphorylation, maintaining therefore constant the ATP concentration within the mitochondria. In fact, in isolated HeLa cell mitochondria the supply of high concentrations of ATP (>1 mM) increased the extent of polyadenylation [8,9] although it never reached the level observed in vivo.

The in vitro oligo(dT)-cellulose unbound RNA fraction contains the main mitochondrial RNA species found in vivo (rRNAs 12 S and 16 S and the tRNAs) and a doublet which is composed of a fairly pronounced band and a more slowly moving one, that correspond to RNAs u4a and u4, precursors of the rRNAs [6]. The absence of any trace of cytoplasmic rRNA in the RNA synthesized in isolated mitochondria indicates that there was not any contamination of the mitochondrial preparation with active nucleolar transcription complexes.

As estimated by densitometric measurements, the ratio of labeling of the two rRNA species is approximately of 1, which corresponds to the value most frequently found in vivo, either in rat (Fig. 1) or HeLa cells. This ratio is substantially higher than that found in isolated HeLa cell mitochondria [8]. This could indicate that in this system the whole rDNA region of the mtDNA is always transcribed completely and the termination signal recognized correctly.

3.2. *Synthesis of mitochondrial rRNA at different ADP concentrations*

Incubation of isolated brain mitochondria in the presence of increasing concentrations of exogenous ADP (from 0 to 2 mM), maintaining constant the amount of glutamate, malate and phosphate, had an overall stimulatory effect upon mitochondrial DNA

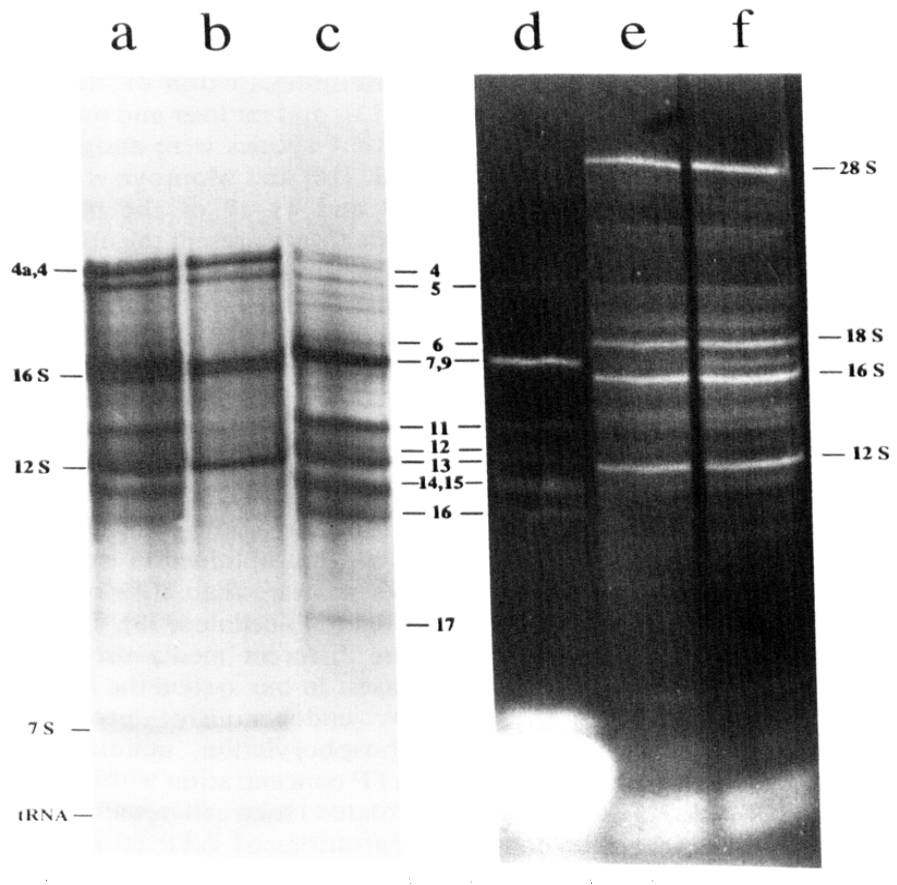

Fig. 1. Comparison of the electrophoretic patterns in agarose-CH_3HgOH lab gels of the rat brain mitochondrial RNA synthesized in isolated organelles (autoradiogram) (lanes a-c) and in vivo (ethidium bromide staining) (lanes d-f). (Lanes a and f) Total RNA; (lanes b and e) oligo(dT)-cellulose-unbound-RNA; (lanes c and d) oligo(dT)-cellulose-bound RNA. The sample run in lane c was derived from 7 times the amount of mitochondria of the samples in lanes a and b; lane c contains the totality of the oligo(dT)-cellulose-bound RNA and lanes e and f correspond to 3% of the preparation. (The very intense band at the bottom of lane d corresponds to tRNA carrier.)

transcription, as shown in the electrophoretic patterns of the in vitro labeled mitochondrial RNA (Fig. 2) and as determined by the radioactivity incorporated into RNA (Fig. 3a). (The pattern obtained in the absence of ADP was omitted in Fig. 2 since only a faint smear was visible after long exposure of the autoradiogram.) Densitometric measurements of the autoradiogram indicate that all RNA species were affected, although the behaviour of the synthesis of the mature rRNA species differed from the synthesis of the other RNAs. Thus, the synthesis of 12 S and 16 S rRNA increased rapidly (approximately 10-fold) in the range of concentrations of 0.1–0.5 mM ADP and then a plateau was reached (Fig. 3b). This unexpected saturating effect in the synthesis of mature rRNA contrasts with the progressive stimulation of the labeling of the different mRNA species over the whole range of ADP concentrations (0–2 mM) (Fig. 3c) and was accompanied by an especially pronounced accumulation of the band containing the rRNA precursors u4a and u4 (Fig. 3b). These results suggest that the synthesis of mature 12 S and 16 S rRNA is limited by the processing of their common precursor rather than by the transcription rate in isolated rat brain mitochondria. Thus, at ADP concentrations below 0.25 mM, the rRNA precursors were mostly processed originating the mature rRNAs. However, as the transcription rate increases, at higher ADP concentrations (from 0.5 mM ADP), processing becomes saturated with the consequent progressive accumulation of the rRNA precursors. Therefore, the synthesis of mature rRNAs seems to be regulated at the level of processing rather than at the level of transcription in isolated rat brain mitochondria. It is noteworthy that the saturation of the processing step that originates the mature rRNAs occurred precisely when the ratio of labeling 16 S/12 S reached the value found in vivo (approximately 1).

As indicated above, the mature mRNAs continued being synthesized over the whole range of ADP concentrations. This fact suggests that the requirements for the processing of their precursors are different from those of the rRNAs. The cessation of import of ribosomal proteins, or at least of some critical ones, from the cytoplasm could be the factor responsible for the

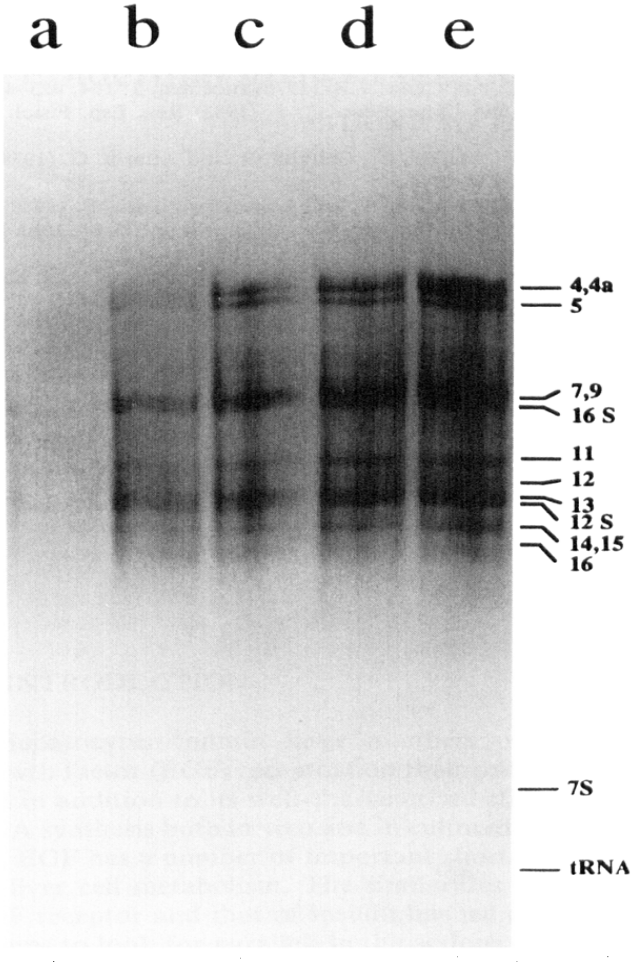

Fig. 2. Synthesis of RNA in isolated rat brain mitochondria in the presence of different concentrations of ADP. Autoradiogram after electrophoresis through an agarose-CH_3HgOH slab gel of the total RNA labeled with [α-^{32}P]UTP in the presence of 0.1 (a), 0.25 (b), 0.5 (c), 1.0 (d) and 2.0 (e) mM ADP. The concentrations of the other components of the incubation buffer were as indicated in Materials and Methods. Equivalent amounts of material were run in the 5 lanes.

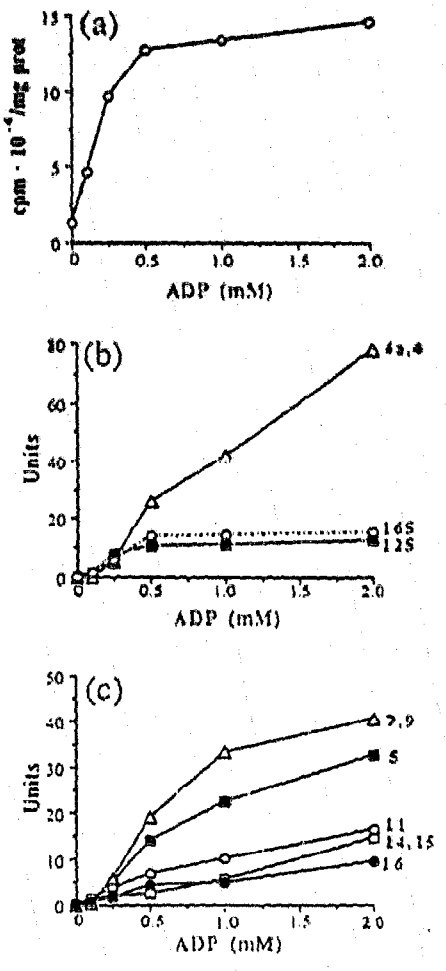

Fig. 3. Quantitation of the effect of ADP concentration on the synthesis of RNA in isolated rat brain mitochondria. (a) Radioactivity incorporated into total RNA at different ADP concentrations. (b and c) Relative labeling of individual RNA species as determined from densitometric tracings of the autoradiogram of Fig. 3, after normalization to the total amount in each sample.

limited efficiency of the processing step that leads to the formation of the mature rRNAs [8]. The saturation of the processing step could also explain the accumulation of rRNA precursors observed in isolated HeLa cell mitochondria in the presence of proflavine and at low temperatures [7].

From the results described in this paper it can be concluded that isolated rat brain mitochondria are able to support DNA transcription and RNA processing with an efficiency and fidelity equivalent to that of intact cells and makes this system of great value for the study of the control and interrelationships of mtDNA transcription and RNA processing in differentiated cells.

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