Specific increase of a mitochondrial RNA transcript in chronic ethanol-fed rats

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An in vitro transcription system utilizing isolated mitochondria has been used to study the effect of chronic ethanol consumption on liver mitochondrial DNA transcription. The results obtained showed an overall increase of RNA synthesis and a dramatic accumulation of a discrete polyadenylated RNA species. This effect is a consequence of the chronic ethanol consumption since these changes do not occur when isolated control mitochondria are incubated in the presence of ethanol.

Mitochondrion; RNA synthesis; Ethanol; Isolated organelle; Rat liver

1. INTRODUCTION

The depression of oxidative phosphorylation observed in mitochondria from ethanol-fed rats may be a fundamental factor contributing to ethanol-induced liver damage [1–4]. This impairment of the liver mitochondria energy metabolism could in turn be due to the decrease of the mitochondrial translation ability elicited by chronic ethanol consumption [5,6]. Evidence has been presented suggesting that the ethanol-induced lesion in mitochondrial translation could be the result of a reduced number of functional ribosomal particles in the mitochondria from ethanol-fed rats [7].

We have initiated studies to establish the effect of chronic ethanol consumption on liver mitochondrial DNA (mtDNA) transcription as assayed in vitro, using isolated organelles. In this respect, it has been reported previously that incorporation of [α-^{32}P]UTP into total RNA of isolated mitochondria from ethanol-treated and control rats was not significantly different [7]. In this work we have undertaken a comparative study of the oligo(dT)-cellulose-bound and unbound discrete RNA species synthesized in isolated mitochondria from control and ethanol-fed rats. The results obtained showed a dramatic increase of a discrete RNA species separated into the oligo(dT)-bound fraction. This effect is a consequence of the chronic ethanol consumption since isolated control mitochondria incubated in the presence of ethanol did not increase the in vitro synthesis of that polyadenylated RNA.

2. MATERIALS AND METHODS

2.1. *Animal treatments*

Male Wistar rats initially weighing 150–250 g were fed for 31 days with an adequate diet in which ethanol provided 36% of the total calories [8]. Control rats were pair-fed with the same diet in which ethanol was isocalorically replaced with maltose/dextrin.

2.2. *Isolation of rat liver mitochondria*

The livers of rats killed by decapitation were rapidly removed, chilled in homogenization medium (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. The minced tissue was resuspended in the same medium (approximately 4 ml/g of tissue) and homogenized in a loose-fitting Potter-Elvejhem homogenizer by using 4 up-and-down strokes. The homogenate was spun at 1,000 × g for 5 min. The supernatant was aliquoted in microfuge tubes and centrifuged at full speed (13,000 × g) for 2 min in a MSE microfuge. The mitochondrial pellets were washed twice in homogenization buffer and pelleted. Finally, the mitochondrial pellet was resuspended in the appropriate incubation buffer (see below) and spun for 1 min. The number of washes employed in this preparation eliminate contaminating cytoplasmic RNA (estimated by the absence of 28S and 18S cytoplasmic rRNA in the electrophoretic patterns), and minimized the possibility that any ethanol present in the liver of the rat at the time of sacrifice remained in the mitochondria upon isolation.

2.3. *In vitro labeling and isolation of mitochondrial nucleic acids*

Samples of the mitochondrial fraction (1 mg of mitochondrial protein) were resuspended in 0.5 ml of incubation buffer which contained 25 mM sucrose, 75 mM sorbitol, 100 mM KCl, 10 mM K$_2$HPO$_4$, 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 microfuge tubes [9]. Incubation was at 37°C for 60 min in a rotary shaker.

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After the incubation, the mitochondrial samples were pelleted at 13,000 × g for 1 min and washed twice with 10% glycerol, 10 mM Tris-HCl pH 6.8, 0.15 mM $MgCl_2$. Lysis of mitochondria and nucleic acid extraction were carried out as described by Attardi and Montoya [10]. The total mitochondrial RNA fraction was fractionated by oligo(dT)-cellulose chromatography and analyzed by electrophoresis through 1.4% agarose slab gels in the presence of deionized CH_3HgOH [10,11]. After the run, for analytical purposes, the gels were first stained with ethidium bromide, photographed under UV light and then dried and exposed for autoradiography at −70°C. Quantification of the amount of mtDNA or RNA was carried out in the ethidium bromide stained gels and in the autoradiograms respectively, using a LKB Ultroscan XL laser densitometer and a Gel Scan XL software. Protein concentration was determined by the Waddel method [12].

3. RESULTS

Isolated liver mitochondria from control and ethanol-fed rats were incubated in the presence of [α-^{32}P]UTP in a KCl-containing buffer with an energy supplying system consisting of 1 mM ADP plus oxidizable substrate. This incubation system has been proved to support in vitro DNA transcription and RNA processing of brain mitochondria in a way that closely resembles the in vivo process [9]. In this system, the incorporation of [α-^{32}P]UTP into total RNA was slightly but significantly higher in ethanol than in control mitochondria as estimated either by determination of the radioactivity incorporated or by densitometric analysis of the gels (Table I). On the other hand, the content of mtDNA, quantified from the ethidium bromide-stained gel (not shown), was significantly lower in ethanol than in control mitochondria (Table I). This observation would mean that the transcriptional activity per molecule of mtDNA would be very significantly increased in isolated ethanol mitochondria with respect to that of the control organelles.

The in vitro synthesized RNA was fractionated through oligo(dT)-cellulose and analyzed by electrophoresis in agarose-CH_3HgOH slab gels. Fig. 1 shows the electrophoretic patterns of the newly synthesized total (lanes a and f), oligo(dT)-cellulose-bound (lanes c and d) and -unbound RNA (lanes b and e) fractions of control and ethanol mitochondria incubated under the experimental conditions described above. In these patterns it is possible to recognize the characteristic set of transcripts previously described in HeLa cells [13,14] and brain mitochondria [9,15]. Therefore, the RNA species were designated according to Amalric et al. [13] and Montoya et al. [14]. The inspection of the gels clearly reveals the appearance of a very pronounced RNA band in the total RNA from ethanol mitochondria (lane f), located between the bands corresponding to RNA 5 and RNA 7–9 (the width and shape of the band would suggest that it could be a doublet). This band, named ES in Fig. 1 (ES, for ethanol stimulated) was mostly found in the oligo(dT)-cellulose-bound fraction (lane d), therefore corresponding to a polyadenylated RNA species. Densitometric quantitations of the autoradiogram
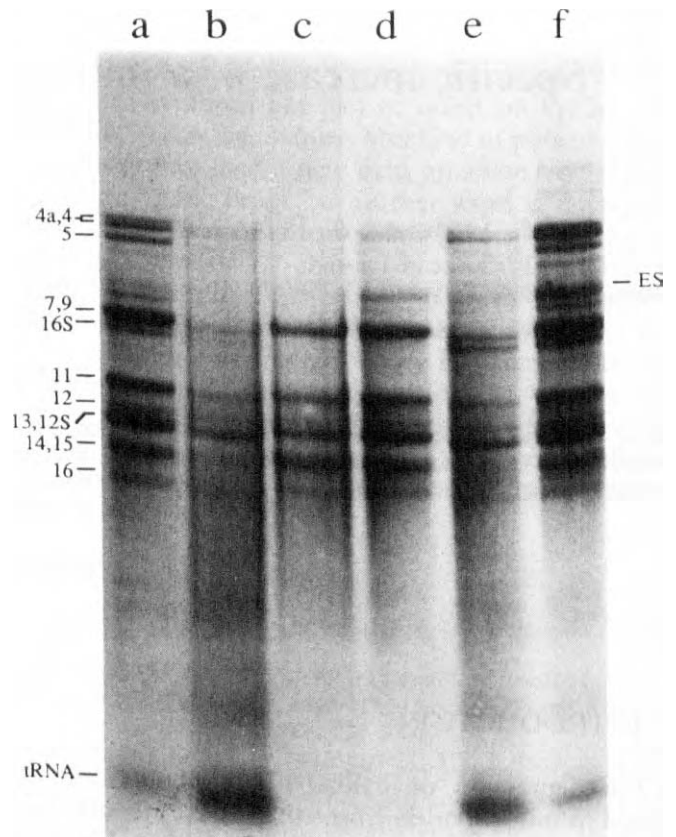

Fig. 1. Electrophoretic patterns in agarose-CH_3HgOH slab gels of liver mitochondrial RNA synthesized in isolated organelles from control (lanes a–c) and ethanol-fed (lanes d–f) rats. Lanes a and f, total RNA; lanes b and e, oligo(dT)-cellulose-unbound RNA; lanes c and d, oligo(dT)-cellulose-bound RNA. Equivalent amounts of material were run in the lanes that correspond to the same RNA fraction of control and ethanol mitochondria.

in Fig. 1 showed that the relative labeling of this band was more than nine times higher in isolated ethanol than in control mitochondria. The labeling of the other RNA bands was only slightly higher in ethanol than in control mitochondria (data not shown).

In order to study if the increase in the labeling of RNA ES could be produced directly by ethanol, isolated mitochondria were incubated in the presence of various ethanol concentrations (0–0.5%) in the incuba-

Table I

Mitochondrial source	[^{32}P]UTP incorporated into RNA		Mitochondrial DNA d.u.
	cpm × 10^{-3}	d.u.	
Control	86.3 ± 3.2	26.8 ± 2.2	0.15 ± 0.019
Ethanol	100.6 ± 2.6	31.8 ± 1.5	0.08 ± 0.0010
% of control	117	118	58

d.u., densitometric units, determined from the autoradiograms (RNA) or the ethidium bromide stained gels (DNA). The values, expressed as × mg^{-1} of mitochondrial protein, are mean ± S.D. of thre experiments.
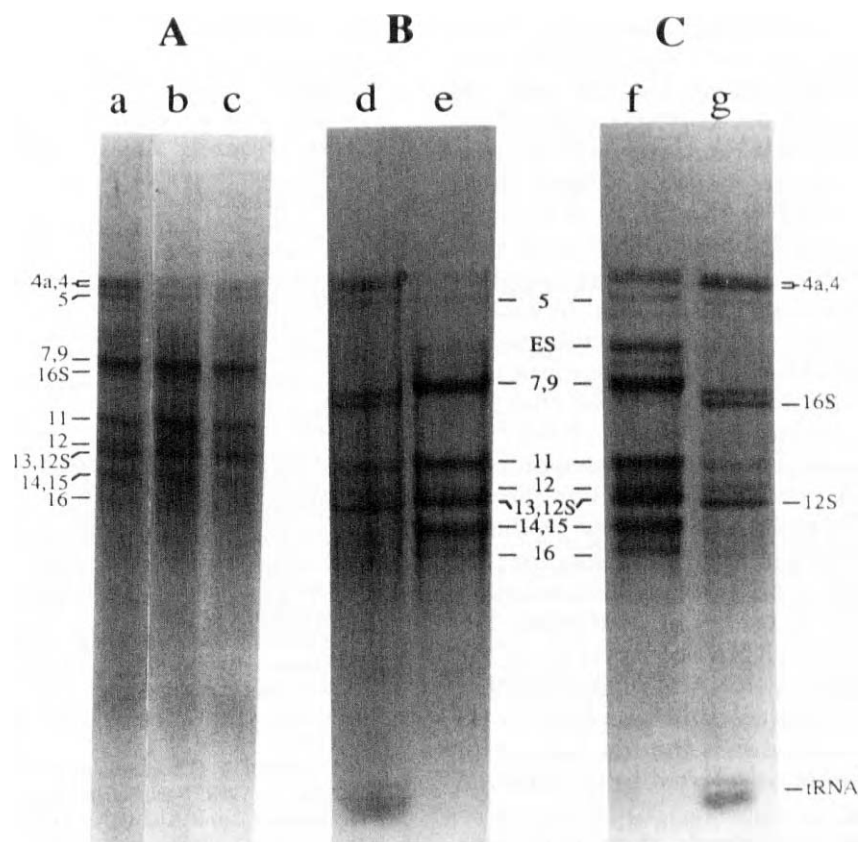
Fig. 2. Synthesis of RNA in isolated liver mitochondria from control (panels A and B) and ethanol-fed (panel C) rats in the presence of ethanol. Autoradiograms after electrophoresis through agarose-CH₃HgOH slab gels of the total RNA labeled with [α-³³P]UTP in the absence (lane a) or presence of 0.1 (lane b) and 0.5% (lane c) ethanol in the incubation medium. Panel B, electrophoretic patterns of the oligo(dT)-cellulose-unbound (lane d) and -bound (lane e) RNA synthesized in isolated control mitochondria in the presence of 0.5% ethanol. Panel C, oligo(dT)-cellulose-unbound (lane g) and -bound (lane f) RNA synthesized in isolated mitochondria from ethanol-fed rats in the presence of 0.5% ethanol. Equivalent amounts of material were run in lanes a–c, in lanes d and g, and in lanes e and f.

tion medium. As shown in Fig. 2, lanes a–c, this addition produced an overall decrease of the labeling of the newly synthesized RNA (mainly at 0.5% ethanol) and did not increase the labeling of the RNA ES. In Fig. 2, panel B the electrophoretic patterns are also shown of the oligo(dT)-cellulose-bound (lane e) and unbound (lane d) RNA of isolated control mitochondria incubated in the presence of 0.5% ethanol. On the other hand, the addition of 0.5% ethanol to the in vitro incubation system of isolated ethanol mitochondria did not diminish the labeling of the RNA ES (Fig. 2, lanes f and g).

4. DISCUSSION

The results presented here are somehow contradictory to those of Coleman and Cunningham [7] who reported that there were no differences in the in vitro transcription of isolated liver mitochondria from control and ethanol-fed rats. In such a report, in vitro mitochondrial transcription was carried out under the incubation conditions previously employed with isolated mitochondria from HeLa cells [16]. However, we

have recently shown that these incubation conditions result in an inefficient in vitro RNA synthesis by isolated mitochondria from mammalian organs [9]. The stimulation of the synthesis of RNA ES by isolated liver mitochondria of ethanol-fed rats appears to be due to an in vivo effect elicited by chronic ethanol consumption. This conclusion can be drawn from the observation that the presence of ethanol in the in vitro transcription system did not stimulate the synthesis of RNA by isolated control mitochondria. Moreover, at the same ethanol concentration an important decreasing effect on the overall transcriptional activity of control mitochondria was observed (Fig. 2). In this respect the increased transcriptional ability observed in ethanol mitochondria (Table I) could therefore be interpreted as an adaptation response to the chronic ethanol consumption.

Judging from the estimated size of RNA ES (approximately 1940 nt), this RNA could correspond to the well characterized polyadenylated RNA 6, precursor of the mRNA 9 that codes for the subunit I of cytochrome c oxidase (COI). However, previous hybridization studies carried out to identify the RNA species synthesized in

isolated mitochondria (unpublished results) have shown that this band hybridized not only with the COI gene probe but with the genes coding for 12S and 16S rRNAs. Judging from the size corresponding to the position of the band, this RNA cannot contain the three genes; therefore, as mentioned before, the band could be a doublet consisting of RNA 6 and an RNA containing the 12S rRNA and part of the 16S rRNA sequences. A premature termination of the mtDNA transcription within the 16S rRNA gene could account for the synthesis of such an RNA species. A similar premature termination event has been described in HeLa cells mitochondria producing a polyadenylated RNA (RNA 16a) that correspond to part of the sequence of 16S rRNA [17]. This suggestion would imply that the reduction of functional liver mitochondrial ribosomes elicited by chronic ethanol consumption [7] could be somehow produced by a defective transcription and processing of the rRNAs. Whether the RNA ES band could correspond to a polyadenylated RNA transcript containing 12S rRNA and part of the 16S rRNA sequences or to the precursor of the COI mRNA (RNA 6) requires further investigation. In any case, the accumulation of RNA ES in isolated ethanol mitochondria is the consequence of an in vivo event that cannot be reverted by the addition of ethanol in vitro (Fig. 2).

The results described here clearly show that some impairment of the liver mitochondrial transcription takes place in ethanol-fed rats. This impairment could to some extent be responsible for the liver damage observed in chronic ethanol consumption.

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