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Effect of chronic ethanol consumption on hepatic mitochondrial transcription and translation

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Liver mitochondria from ethanol-fed rats display an impaired ability for protein synthesis in vitro. Studies were conducted to explore the possible mechanisms which might account for this impaired capacity of ethanol mitochondria for protein synthesis. The present studies did not demonstrate any significant ethanol-induced lesion in mitochondrial nucleic acid metabolism in organelles isolated from ethanol-fed rats for any of the parameters investigated (mtDNA content, steady-state mtRNA concentration, mtRNA polymerase activity, concentration of specific mRNAs and rRNAs, mtRNA processing). An investigation of ribosome function in isolated mitochondria demonstrated significant decreases in the number of active ribosomes (55% fewer) in mitochondria from ethanol-fed rats. Initiation of protein synthesis was also significantly depressed (46%) in ethanol mitochondria. In addition, the yield of ribosomal particles from ethanol mitochondria was decreased 32% as compared to the yield of ribosomal particles from control mitochondria. However, isolated ribosomes from ethanol mitochondria were determined to be fully functional in a poly(U)-directed phenylalanine polymerization system. Soluble translation factors from ethanol mitochondria were also found to support full activity of control ribosomes in a poly(U)-directed phenylalanine polymerization system. These results suggest strongly that the ethanol-induced depression of mitochondrial protein synthesis is due to a decrease in the number of competent ribosomes in hepatic mitochondria from chronically ethanol-fed rats.

Introduction

Chronic ethanol consumption adversely affects mitochondrial protein synthesis in vitro [1–4], resulting in reduced synthesis of all the mitochondrial gene products and decreases in the steady-state concentrations of these polypeptides in the organelle [4]. The effects of chronic ethanol consumption on mitochondrial protein synthesis may be responsible for the observed depressions of oxidative phosphorylation activity in mitochondria from ethanol-fed rats [5–12] since mitochondrial protein synthesis is required for the normal biogenesis of the oxidative phosphorylation system. The depression of oxidative phosphorylation activity elicited by chronic ethanol consumption may be a significant factor contributing to the decrease in hepatic tissue energy state previously observed in rats fed ethanol chronically [13,14]. This depression of hepatic energy state may in turn contribute significantly to the development of ethanol-induced liver pathology.

In the present investigation studies were initiated to establish the effects of chronic ethanol consumption on mitochondrial gene expression in order to determine the origin of the ethanol-induced lesion. The effects of chronic ethanol consumption on mitochondrial nucleic acid metabolism and the processes involved with mitochondrial translation are reported. These studies demonstrate that the effects of chronic ethanol consumption on mitochondrial biogenesis are confined to mitochon-

Abbreviations: ethanol mitochondria, mitochondria isolated from ethanol-fed rats; control mitochondria, mitochondria isolated from liquid diet control rats; ribosomes, ribosome-enriched fraction; ethanol ribosomes, ribosomes prepared from ethanol mitochondria; control ribosomes, ribosomes prepared from control mitochondria; soluble translation factors, protein fraction containing mitochondrial translation factors; *COI, COII, COIII*, mitochondrial genes encoding cytochrome oxidase polypeptide subunits I-III; *ATPase 6/8*, mitochondrial gene encoding polypeptide subunits 6 and 8 of the F_0F_1-ATPase; *ND3, ND4/4L*, mitochondrial genes encoding polypeptide subunits 3, 4 and 4L of the NADH dehydrogenase; 12S, the small mitochondrial rRNA; 16S, the large mitochondrial rRNA; SDS, sodium dodecyl sulfate; bicine, *N,N*-bis(2-hydroxyethyl)glycine; n.s., not significant.

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drial translation since no significant alterations were observed on mitochondrial nucleic acid metabolism. Evidence is presented which suggests that the ethanol-induced lesion in mitochondrial translation is the result of a reduced number of functional ribosomal particles in mitochondria from ethanol-fed rats.

Experimental procedures

Materials

Sprague-Dawley rats and several reagents were obtained from sources listed previously [15,16]. Oligodeoxyribonucleotide probes were synthesized by Dr. Brian Pollock of the Department of Microbiology/Immunology, Bowman Gray School of Medicine of Wake Forest University. RNase-free enzyme grade sucrose (Schartz-Mann), ultrapure urea (Schartz-Mann), [α-^{32}P]UTP (3000 Ci/mmol), [γ-^{32}P]ATP (4500 Ci/mmol), L-[*methyl*-^{14}C]methionine (61 mCi/mmol) were purchased from ICN Biomedicals. L-[2,6-^{3}H]Phenylalanine (60 Ci/mmol) was obtained from the Amersham Corporation. [8(n)-^{3}H]Puromycin (7 Ci/mmol) was from Moravek Biochemicals or from the Amersham Corporation (discontinued). Formamide, phenol (99 + %), bicine and sodium chloride were obtained from Aldrich Chemical Company. T4 polynucleotide kinase was from Bethesda Research Laboratories, and nylon hybridization membranes (Nytran, 0.1 μm) from Schliecher and Schuell. Fish sperm DNA and rifampicin were obtained from United States Biochemical Company. Tris, RNase-free deoxyribonuclease (Type IV from bovine pancreas), ribonuclease A (from bovine pancreas), tRNA (Type X from brewer's yeast), $tRNA_{Phe}$ (Type V from brewer's yeast) and 3,5-diaminobenzoic acid were purchased from Sigma Chemical Company. Mixed bed ion exchange resin (AG 501-X8), Triton X-100 and most electrophoresis reagents were from Bio-Rad. Superfine DNA grade Sephadex G-25, polyuridylic acid homopolymer and OptiPhase HiSafe II scintillation fluid were from Pharmacia/LKB. Budget-solv complete scintillation cocktail was from Research Products International and tissue solubilizer was from New England Nuclear. All other chemicals were reagent grade.

Methods

Male Sprague-Dawley rats initially weighing 150–250 g were fed for 31 days a nutritionally adequate diet in which ethanol provided 36% of the total calories [17]. Pair-fed control rats received the same diet, but with maltose/dextrin isocalorically substituted for ethanol. Coupled mitochondria were prepared by differential centrifugation of liver homogenates as described by Thayer and Rubin [6] with modifications described by Coleman and Cunningham [4]. The characteristics of these mitochondria from ethanol-fed and control animals have been described previously [4,12,15]. Their contamination with other cell organelles is quite low as was previously established with marker enzymes [18]. This was also confirmed by phospholipid analyses where essentially no sphingomyelin was detected [19]; this lipid is found in other hepatic membranes. Protein concentrations were determined by the method of Lowry et al. [20].

Total mitochondrial RNA was prepared by hot phenol/chloroform extraction of isolated mitochondria in the presence of SDS [21], DNase treatment of the crude nucleic acid fraction [22], and subsequent ethanol precipitation [23]. Mitochondrial RNA concentrations were determined spectrophotometrically [24]. Mitochondrial RNA prepared in this manner from both ethanol and control mitochondria was of consistently high quality (A_{260}/A_{280} = 1.9–2.0). Studies conducted with [α-^{32}P]UTP-prelabeled mtRNA showed the efficiency of extraction by this method to be 87% $\pm$ 5 and 88% $\pm$ 4 for ethanol and control mitochondrial sources, respectively (n = 8 pairs, n.s.). The activity of the mitochondrial RNA polymerase was determined through the incorporation of [α-^{32}P]UTP into isolated mitochondria as described by Gaines and Attardi [25]. In these latter two experiments mitochondria (10 mg protein) were incubated with 150 μCi [α-^{32}P]UTP at 37°C for 30 min in a solution containing 10% glycerol, 35 mM Tris-Cl (pH 7.8), 20 mM NaCl, 5 mM $MgCl_2$, 11 mM ATP and 1 mg/ml BSA in a final volume of 2 ml. Mitochondrial DNA was prepared by ribonuclease treatment of the crude nucleic acid fraction (see above) with subsequent ethanol precipitation [23]. Quantitation of mitochondrial DNA was accomplished by the fluorometric technique of Fiszer-Szafarz et al. [26].

Urea-polyacrylamide gel electrophoresis of mtRNA and agarose gel electrophoresis of mtDNA were performed as described by Ogden and Adams [27]. Mitochondrial RNA was electrophoretically transferred from urea-polyacrylamide gels to nylon hybridization membranes, as follows. Gels were equilibrated in 40 mM Tris-acetate/1 mM EDTA (pH 8.0) for 10 min. Electrophoretic transfer was carried out in the same buffer at 10 V/cm for 5 h at 4°C. For slot blot analyses mtRNA was applied directly to nylon hybridization membranes utilizing a slot blot apparatus (Hybri-slot manifold, Bethesda Research Laboratories) following denaturation of the RNA in 1.2 M NaCl, 120 mM sodium citrate (pH 7.0), 37.5% (v/v) formaldehyde, at 60°C for 15 min. Following slot blot or Northern transfer, mtRNA samples were immobilized on hybridization membranes through covalent crosslinking with ultraviolet irradiation [28]. Oligonucleotide probes were 5′ end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase [29]. Hybridization conditions for Northern blots and slot blots were modified from those described by Thomas [30] as follows. Membranes were prehybridized for 3–4 h at 42°C in a buffer containing 50% (v/v) formamide,

0.75 M NaCl, 75 mM sodium citrate (pH 7.0), 50 mM sodium phosphate (pH 6.5), 0.5% (w/v) SDS and 100 μg/ml low-molecular-weight phenol extracted fish-sperm DNA. Hybridization of [^{32}P]oligonucleotide probes with immobilized mtRNA was carried out in the same buffer for 12–16 h at 42°C. Membranes were washed subsequently in five changes of 0.9 M NaCl, 50 mM sodium phosphate (pH 7.7), 5 mM EDTA at 42°C with a final wash at the T_H of the specific hybrid [31]. The membranes were exposed at −70°C to Kodak X-Omat AR film with a Lightning Plus intensifying screen (Sigma Chemical Company). Autoradiograms were analyzed by densitometry utilizing a Zenieh Model SL-TRFF scanning laser densitometer and an electrophoresis reporting program (ERIP-V3A) from Biomed Instruments.

The number of active mitochondrial ribosomes in mitochondria isolated from ethanol-fed and control rats was determined utilizing radioactive puromycin essentially as described by Unsworth and Pain [32]. Mitochondria were suspended to a protein concentration of 5 mg/ml in a buffer consisting of 100 mM KCl, 10 mM magnesium acetate, 1 mM dithiothreitol, 10 mM Bicine (pH 7.5). Assay mixtures (100 μl) were preincubated at 30°C for 5 min prior to addition of 10 μCi [^{3}H]puromycin and then for an additional 30 min. The reaction was terminated with 200 μl 2 M NaOH and after 10 min the NaOH was neutralized by addition of HCl. The radiolabeled puromycin incorporated was then measured as described by Unsworth and Pain [32]. Measurement of the formation of initiator peptides in isolated liver mitochondria was accomplished as described by Bianchetti et al. [33] for yeast mitochondria. However, the translation buffer utilized was modified from that described [33] to possess optimal characteristics for protein synthesis in isolated rat liver mitochondria [4].

Liver mitochondrial ribosomes were prepared as described by Denslow and O'Brien [34] with modifications as follows. Mitochondria were suspended in a buffer containing 500 mM KCl, 20 mM magnesium chloride, 6 mM 2-mercaptoethanol, 10 mM Tris-HCl (pH 7.6) to 5 mg/ml mitochondrial protein. Mitochondrial suspensions were lysed by the addition of 20% (v/v) Triton X-100 to a final concentration of 2% (v/v). Mitochondrial lysates were stirred for 30 min and layered onto 1 M sucrose cushions prepared in the same buffer. Ultracentrifugation was carried out at 200000 × g in a Beckman 50.2 Ti rotor for 4 h (4°C). Ribosome enriched fractions were collected and measured quantitatively by ultraviolet spectroscopy [35]. Soluble translation factors were prepared as described by Denslow and O'Brien [34]. Isolated mitochondrial ribosomes were assayed for activity in a poly(U)-directed phenylalanine polymerization system modified from Hosokawa et al. [36]. Ribosomes (2 pmol) were suspended in a solution containing 50 mM KCl, 50 mM Tris-HCl (pH 7.8), 20 mM magnesium chloride, 1 mg/ml poly(U), 500 μg/ml tRNA, 50 μg/ml $tRNA_{Phe}$, 100 μg/ml pyruvate kinase, 2 mM phosphoenolpyruvate, 5 mM ATP, 0.3 mM GTP and soluble mitochondrial translation factors (0.2–0.4 mg/ml), in a final volume of 50 μl. Mixtures were preincubated at 37°C for 5 min prior to the initiation of the reaction by the addition of 5 μCi [^{3}H]phenylalanine. The progress of the reaction was monitored by the periodic withdrawal of the reaction mixture for scintillation counting. These aliquots (5 μl) were pipetted onto filter paper, precipitated with ice-cold 5% (w/v) trichloroacetic acid, and extensively washed with 5% (w/v) trichloroacetic acid. The samples were placed in tissue solubilizer before being analyzed for radioactivity in LKB OptiPhase HiSafe II scintillation fluid.

The values reported in the tables and figures are averages of multiple data acquisitions ± S.E. Statistical analyses were performed using the paired t-test, employing the Statview 512 + program from Brainpower.

Results

The DNA contents of ethanol and control mitochondria were determined in order to examine the effects of chronic ethanol consumption on the mitochondrial genetic material. The concentrations for ethanol and control mitochondria were 0.94 ± 0.04 and 0.98 ± 0.03 μg DNA/mg mitochondrial protein, respectively (n = 10, n.s.). These results show that chronic ethanol consumption has no effect on the DNA content of rat liver mitochondria, consistent with observations made in baboons [37]. In addition, agarose gel electrophoresis of mtDNA demonstrated no difference in the size of the DNA molecule between ethanol and control mitochondrial sources (data not shown). This result suggests that chronic ethanol consumption does not elicit significant deletions in the mtDNA.

Determination of the time course for incorporation of radiolabeled UTP into mitochondrial RNA in vitro provides a measure of the relative abilities of ethanol and control mitochondria to transcribe the endogenous DNA template into RNA products. The results shown in Fig. 1 for the incorporation of [α-^{32}P]UTP into isolated ethanol and control mitochondria demonstrate that there is no significant difference in the mtRNA polymerase activities of these mitochondria when transcribing the endogenous DNA template in vitro. Furthermore, the recoveries of total extractable mtRNA for ethanol and control mitochondria were determined to be 5.08 ± 0.22 and 5.27 ± 0.27 μg RNA/mg mitochondrial protein, respectively (n = 8, n.s.).

The concentrations of specific mtRNAs among the total extractable mtRNA pool were investigated through slot blot hybridization analyses utilizing oligonucleotide probes to specific mtRNA sequences. These probes

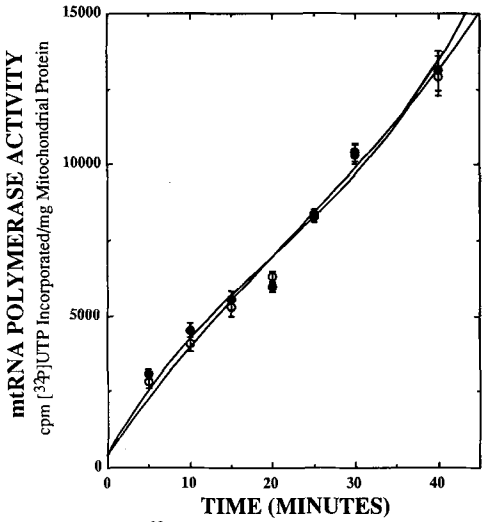

Fig. 1. Incorporation of [α-^{32}P]UTP into isolated mitochondria from ethanol-fed and control rats. Each point represents the average acid-insoluble radioactivity incorporated per mg mitochondrial protein from eight animals ± S.E. No significant differences were found between ethanol and control values at any time point by paired *t*-test analysis. Ethanol-fed (○) and control (●).

were designed according to the reported sequence of the mtDNA for polypeptide encoding genes *COI, COII, COIII, ATPase 6/8, ND3* and *ND4/4L* [38], and the 12S and 16S rRNAs [39]. The sequence of these probes are shown in Table I. Characterization of these probes by Northern blot analysis [40] showed single RNA specificity for each probe (data not shown). The sensitivity of the slot blot system was verified by probing various amounts of mtRNA isolated from chow-fed rats under conditions identical to those employed in actual analyses of mtRNA from pair-fed rats. The results of these analyses with probes directed against *COI, COIII, ATPase 6/8* and *ND4/4L* demonstrated linear concentration dependent responses to the amount of RNA applied to the membrane with a sensitivity to differences in RNA concentration of 10–20% (data not shown). In analyses with mtRNA from pair-fed rats, RNA samples were applied to hybridization membranes at 5 μg per slot, in triplicate for each sample obtained from an individual rat. Samples from pair-fed partners were applied to the same membrane to eliminate differences in the conditions of hybridization and washing between samples. Autoradiograms derived from the hybridization membranes were analyzed by densitometry to quantitate the relative abundance of the mtRNA under investigation. The results of these studies for the six mitochondrial mRNAs and the two rRNAs, shown in Fig. 2, demonstrate no significant difference between ethanol and control mtRNA preparations for any of the RNA species investigated.

Northern blot analyses of mtRNA prepared from ethanol and control mitochondria were carried out to determine whether chronic ethanol consumption affects mtRNA processing. In these studies, 10 μg of total mtRNA from ethanol and control mitochondria were separated by electrophoresis, electrophoretically transferred to hybridization membranes and hybridized to oligonucleotide probes. Representative autoradiograms from these analyses are shown in Fig. 3. These analyses demonstrated no difference between the apparent molecular weight of the hybridized RNA from ethanol and control preparations for any of the gene sequences investigated. In panels 1, 2 and 6 of Fig. 3 there are faint signals in each lane which are of higher molecular weight than the major hybridizing RNA. These bands may represent high-molecular-weight primary RNA transcripts or residual undigested mtDNA. In those cases where secondary hybridizing bands were observed, the bands were seen in both ethanol and control lanes.

Incorporation of radiolabeled puromycin into elongating polypeptide chains was utilized to assess the number of active ribosomes in ethanol and control mitochondria. The conditions employed for the formation of peptidyl-puromycin in isolated, intact, mitochondria would not allow for reinitiation of mitochondrial ribosomes since a complete translation system was not used. Therefore, the amount of radiolabel incorporated into the acid-insoluble fraction is a direct measure of the number of ribosomes actively participating in translation at the time of introduction of the puromycin [32]. The time course for incorporation of radiolabeled

TABLE I

Design of oligonucleotide probes directed against mitochondrial mRNAs and rRNAs

Gene	Sequence of oligonucleotide	DNA sequence [a]
COI	5′-GGCTAGGTTTCCGGCTAAGGG-3′	(414– 435)
COII	5′-GGCGGCCGGGGATTGCGTCGG-3′	(2221–2242)
COIII	5′-GCCTTGGTATGTTCCTTCACGG-3′	(3485–3507)
ATPase 6/8	5′-GGGTGAGGGAGGTGCAGG-3′	(2558–2576)
ND3	5′-TGAGGCAATCAGAATGC-3′	(4212–4229)
ND4/4L	5′-TGCCTCATCGTGTAATG-3′	(5256–5272)
16S	5′-GGGCAACCAGCTATCACCAAGCTCG-3′	(29– 53)
12S	5′-CGTATGACCGCGGTGGCTGGCAC-3′	(1142–1165)

[a] Numbers correspond to the relative numbering of the DNA sequences from the original literature [38,39].

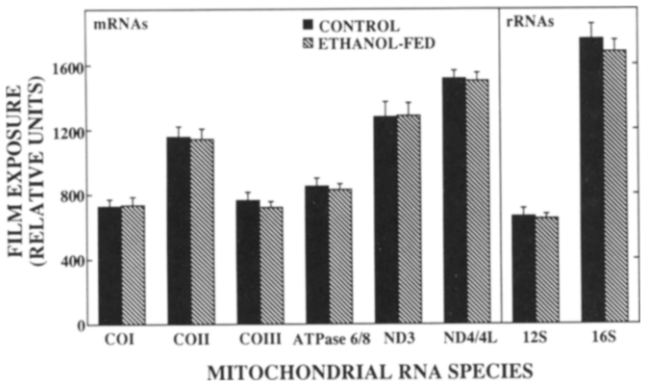

Fig. 2. Relative concentrations of liver mitochondrial mRNAs and rRNAs in mtRNA preparations from pair-fed rats. Radiolabeled oligonucleotide probes were hybridized with mtRNA preparations from ethanol and control mitochondria which had been applied to membranes using a slot blot apparatus. Membranes were subsequently washed and used for autoradiography and analyzed by densitometry. Each bar represents the average densitometrically determined abundance of each mtRNA sequence from 10 RNA preparations isolated from ethanol or control mitochondria ± S.E. Paired *t*-test analyses demonstrated no significant difference between ethanol and control values for any of the mtRNAs investigated.

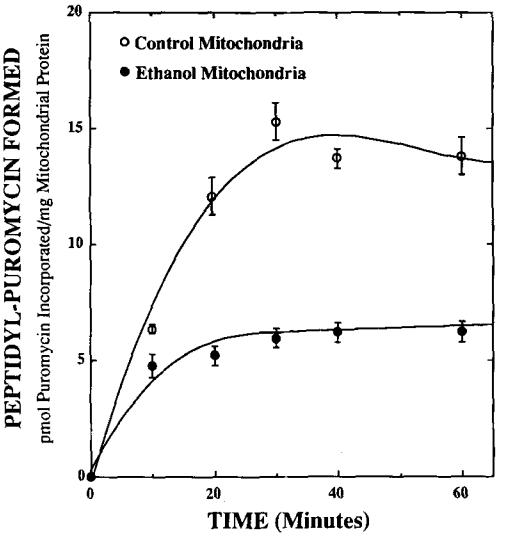

Fig. 4. Time course for incorporation of [^{3}H]puromycin into nascent polypeptides of ethanol and control mitochondria in vitro. Mitochondria were radiolabeled with [^{3}H]puromycin as described in Methods. Incubations were terminated at the indicated time points and acid insoluble material monitored for radioactivity. Each point represents the average pmol [^{3}H]puromycin incorporated per mg mitochondrial protein included in the assay for four preparations of mitochondria ± S.E. Differences between ethanol and control values at each time point were significant by paired *t*-test ($P \leq 0.01$). Control mitochondria (○) and ethanol mitochondria (●).

puromycin into peptidylpuromycin of ethanol and control mitochondria in vitro is shown in Fig. 4. The formation of peptidyl-puromycin was very fast for the first 10 min and had reached a full plateau by 30 min with both ethanol and control mitochondrial preparations. This result verifies that no reinitiation of mitochondrial ribosomes occurs under the assay conditions employed. In addition, Fig. 4 shows that the formation of peptidyl-puromycin had reached the steady-state condition within 30 min following the addition of puromycin to the assay mixture with both ethanol and control mitochondrial sources. The concentrations of

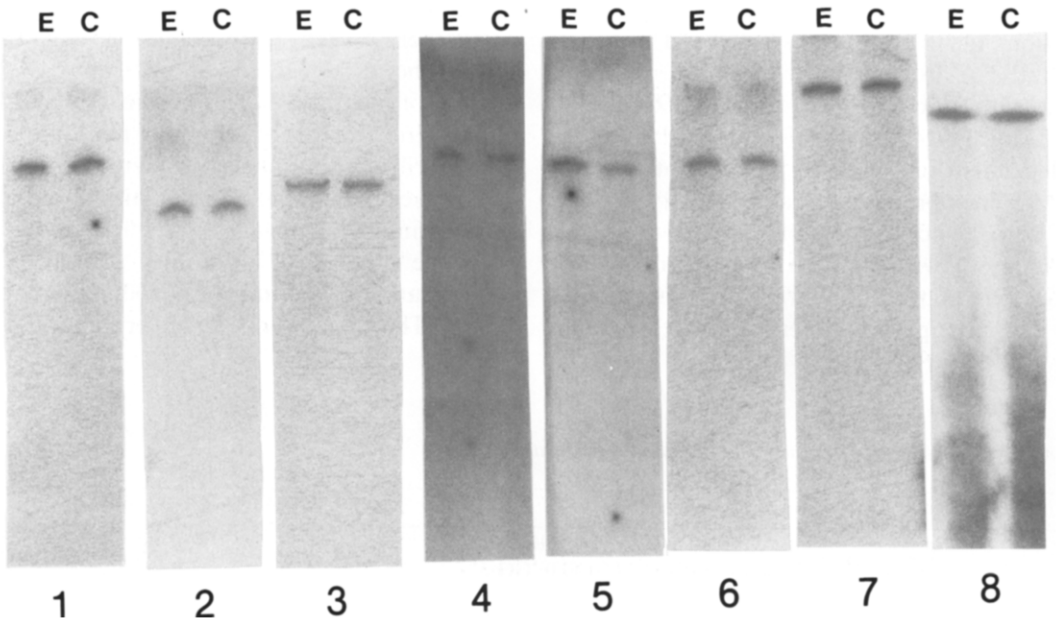

Fig. 3. Northern blot analysis of ethanol and control mitochondrial RNA preparations. RNA (10 μg) from ethanol and control mitochondria was separated on 5% (w/v) polyacrylamide gels containing 7 M urea, electrophoretically transferred to nylon hybridization membranes and analyzed with [^{32}P]oligonucleotide probes. Shown are the representative autoradiograms from these hybridizations. Lanes designated E contain RNA from ethanol mitochondria and lanes designated C contain RNA from control mitochondria. Hybridizations were carried out with the following oligonucleotide probes: Panel 1, *COI*; Panel 2, *COII*; Panel 3, *COIII*; Panel 4, *ATPase 6/8*; Panel 5, *ND3*; Panel 6, *ND4/4L*; Panel 7, 12S rRNA; Panel 8, 16S rRNA.

active ribosomes in isolated ethanol and control mitochondria as indicated by this method are shown in Table II. These results demonstrate a significant decrease (55%) in the number of active ribosomes in ethanol mitochondria.

The incorporation of radiolabeled methionine into mitochondrial peptides synthesized in the presence of high concentrations of puromycin, under conditions appropriate for reinitiation of mitochondrial ribosomes, allows the measurement of ribosome initiation activity in isolated, intact, mitochondria [33]. Table II gives the results of ribosome initiation assays with ethanol and control mitochondria in vitro. The results of the determination of initiator peptide formation with mitochondria from pair-fed rats demonstrates a significant decrease in the ribosome initiation activity of ethanol mitochondria. The ethanol-related decrease in ribosome initiation activity (46%) is comparable to the ethanol-induced reduction in the number of active ribosomes in ethanol mitochondria (55%). A value for initiation activity expressed per active ribosome was calculated using the number of active ribosomes as measured by formation of the peptidyl-puromycin complexes (Table II). The values for initiation activity expressed per active ribosome were not significantly different between ethanol and control mitochondria (Table II).

Mitochondrial ribosomes and soluble translation factors were prepared from isolated ethanol and control mitochondria in order to assess mitochondrial ribosome function in a cell-free system. As shown in Table III, the recovery of ribosomes from ethanol mitochondria was considerably diminished from that of control mitochondria (based on absorbance at 260 nm). The protein associated with the ribosome enriched fraction was also found to be significantly decreased in the preparations from ethanol mitochondria (Table III). However, no significant difference was obtained between ethanol and control values when the amount of protein per pmol of ribosomes in the fraction was calculated.

Preparations of ribosomes and translation factors from ethanol and control mitochondria were reconstituted in the poly(U)-directed phenylalanine polymerization system for determination of ribosome function. Activity measurements were made with ethanol and control ribosomes reconstituted with factor preparations from each mitochondrial source. Fig. 5A shows the results of the reconstitution of ethanol and control ribosome sources with control translation factor preparations. This reconstitution provides an indication of the competence of ethanol mitochondrial ribosomes for translation activity. As shown in Fig. 5A, no significant difference was found between the control translation factor stimulated activity of ethanol and control ribosomes at any time point. Fig. 5B illustrates the reconstitution of control ribosomes with ethanol and control mitochondrial translation factors. The results of this reconstitution demonstrate no significant difference in the activity of the control ribosomes during the course of the 30 min incubation period with either ethanol or control mitochondrial translation factors. Thus, at the concentrations employed in the assays (0.2 mg/ml, Fig. 5B; 0.4 mg/ml, data not shown) the soluble translation factors from ethanol mitochondria support full activity of control ribosomes. Preparation of the factors from ethanol and control mitochondria produced yields of 7.70 ± 0.62 and 7.74 ± 0.76 mg of translation factor containing protein from 75 mg mitochondrial protein starting material ($n = 8$, n.s.), respectively. These results provide no evidence that would suggest a

TABLE III

Ribosome recovery from ethanol and control mitochondria

Mitochondrial source	Ribosome-enriched fraction		
	Ribosome yield [a] (pmol/mg mitochondria)	Total protein [b] (mg/mg mitochondria)	Protein content per ribosome [c] (μg/pmol ribosome)
Control	13.1 ± 0.50	0.30 ± 0.02	21 ± 2
Ethanol-fed	8.8 ± 0.56	0.22 ± 0.02	24 ± 3
%Decrease	32	27	–
P	0.0006	0.0011	N.S.
n	8	8	8

[a] Determined from absorbance measurements at 260 nm utilizing conversion factor from the literature [35].
[b] Measured by Lowry protein assay [20].
[c] Calculated from the values for ribosome yield and total protein.

TABLE II

Determination of active ribosome number and initiation activity in isolated ethanol and control mitochondria

Mitochondrial source	[^{3}H]Puromycin incorporated (pmol/mg mitochondria)	[^{14}C]Methionine incorporated (pmol/mg mitochondria)	Initiation activity per active ribosome (pmol ^{14}C/pmol ribosome)
Control	15.8 ± 0.82	0.232 ± 0.023	0.016 ± 0.003
Ethanol-fed	7.1 ± 0.4	0.126 ± 0.014	0.019 ± 0.002
%decrease	55	46	−16
P	0.0001	0.004	n.s.
n	10	7	7

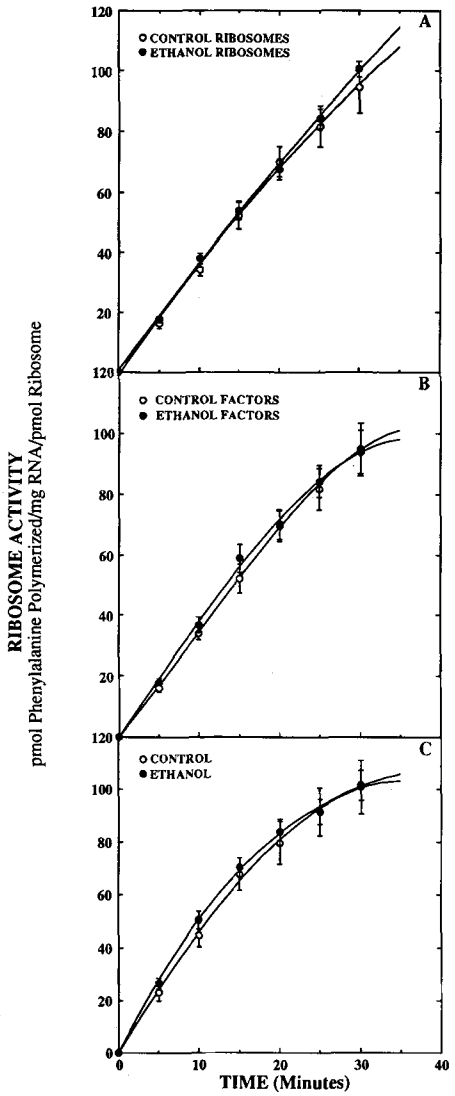

Fig. 5. Reconstitution of mitochondrial ribosomes with soluble translation factors. (A) Ribosomes (2 pmol) from control and ethanol mitochondria were reconstituted in the poly(U)-directed phenylalanine polymerization system with impure translation factors (0.2 mg/ml) from control mitochondria. (B) Ribosomes (2 pmol) from control mitochondria were reconstituted in the poly(U)-directed phenylalanine polymerization system with impure translation factors (0.2 mg/ml) from control or ethanol mitochondria. (C) Ribosomes (2 pmol) prepared from control and ethanol mitochondria were reconstituted in the poly(U)-directed phenylalanine polymerization system with impure translation factors (0.4 mg/ml) prepared from the same mitochondrial source. Each point in (A), (B) and (C) represents the average activity value determined from the acid-insoluble dpm incorporated at each time point ± S.E. ($n = 8$). Paired t-test analyses demonstrated no significant differences between values for ethanol and control samples at any time point.

decrease of mitochondrial translation factors within the preparations derived from ethanol mitochondria.

The results shown in Figs. 5A and B suggest that the isolated ribosomes and soluble translation factors from ethanol mitochondria are fully competent in the poly(U)-directed phenylalanine polymerization system when compared to control preparations. Fig. 5C illustrates the activity of ethanol and control ribosomes when reconstituted with the homologous translation factor preparations. The data in Fig. 5C demonstrate that ethanol mitochondrial ribosomes possess normal activity in this cell-free translation system when reconstituted with the homologous factor preparation. Similar results to that of Fig. 5C were obtained when this reconstitution was carried out at a translation factor concentration of 0.2 mg/ml (data not shown).

Discussion

Chronic ethanol consumption significantly affects the activity of specific segments of the hepatic mitochondrial oxidative phosphorylation system [5–12]. The altered portions of the oxidative phosphorylation system contain polypeptides which are encoded by the mitochondrial genome [38,41–46] and are assembled with cytoplasmically synthesized polypeptides to produce functional enzyme complexes. Other portions of the oxidative phosphorylation system which are composed entirely of cytoplasmically synthesized polypeptides are unaffected by chronic ethanol consumption [5–8,18]. The observation that chronic ethanol consumption specifically affects those complexes of the system which depend upon mitochondrial protein synthesis for normal biogenesis led to the hypothesis that the effects of chronic ethanol consumption on mitochondrial oxidative phosphorylation are mediated through a lesion in mitochondrial protein synthesis. Studies of the effects of chronic ethanol consumption on mitochondrial protein synthesis demonstrated a significant decrease in the incorporation of radiolabeled amino acids into mitochondrial protein in vitro [1–4]. This depression was shown to reflect a lowered rate of synthesis of all thirteen mitochondrial gene products in vitro, resulting in a reduction of the steady-state concentration of these polypeptides in mitochondria from ethanol-fed rats [4]. These observations [4] provide one explanation for the ethanol-induced depression of mitochondrial oxidative phosphorylation, since a reduction in the polypeptide subunits of the system would limit the number of functional complexes which could be assembled.

The present studies were undertaken to determine which portion of the mitochondrial protein synthesizing system was affected by chronic ethanol consumption. Mitochondrial nucleic acid metabolism was investigated in ethanol-fed rats since alteration of these processes of mitochondrial biogenesis could produce deleterious effects on the downstream processes in mitochondrial translation. These studies examined the content of mtDNA in ethanol mitochondria as well as all the major points in mtRNA metabolism in these mitochondria: RNA synthesis, steady-state RNA concentration and RNA processing. Since all these aspects of

mitochondrial biogenesis were normal in mitochondria from ethanol-fed rats, it was concluded that the effects of chronic ethanol consumption on mitochondrial protein synthesis must be due to a lesion at the level of translation.

All the results of the present studies on mitochondrial translation are in agreement with the suggestion that the effects of chronic ethanol consumption on mitochondrial protein synthesis are due to a reduction in the number of mitochondrial ribosomes in the ethanol mitochondrion. The observation that ethanol mitochondria possess 55% fewer active ribosomes than control mitochondria suggests strongly that the decreased incorporation of radiolabeled amino acids into ethanol mitochondrial protein in vitro is due to the participation of fewer ribosomes in the translation process. The reduced number of active mitochondrial ribosomes in ethanol mitochondria was not due to decreases in either mRNA or rRNA, since the concentrations of each RNA investigated were found to be unchanged by chronic ethanol consumption. Nevertheless, the recovery of ribosomal particles (A_{260}-absorbing material) from ethanol mitochondria was significantly decreased from that of control when these particles were prepared from isolated mitochondria. These observations suggest that the reduced concentration of active ribosomes in ethanol mitochondria reflects a decrease in the number of fully assembled ribosomal particles.

Isolated ribosomes from ethanol mitochondria were found to be competent for elongation of polypeptides when reconstituted with either ethanol or control soluble translation factors. In addition, soluble translation factors from ethanol mitochondria supported full activity of control ribosomes. These observations also support the view that the reduced ribosome activities in ethanol mitochondria were due to a diminished number of ribosomal particles contained in the ethanol mitochondrion.

The lesion responsible for the ethanol-induced reduction of mitochondrial ribosomes remains to be elucidated. It is possible that the reduction of ribosome number in ethanol mitochondria is due to faulty assembly of the mitochondrial ribosomes which might result from either a lack of adequate quantities of key ribosomal proteins in ethanol mitochondria or direct interference due to the presence of ethanol. Studies of the assembly of yeast mitochondrial ribosomes have shown that certain polypeptides are absolutely essential for the proper assembly of the ribosomal subunits [47,48] and that in the absence of these polypeptides mature ribosomal subunits do not accumulate. It has been established that all the proteins of mammalian mitochondrial ribosomes are cytoplasmically synthesized and imported into the organelle for assembly [49]. Therefore, chronic ethanol consumption might interfere with the synthesis of these proteins, their importation into the organelle or their assembly with the rRNAs to form mature ribosomal subunits.

The reduced levels of functional ribosomes appear to be sufficient to account for the depressions in the rate of synthesis and steady state levels of mitochondrial gene products [4]. However, the possibility remains that chronic ethanol consumption also influences the charging of tRNAs with amino acids. An ethanol-related lesion in the charging of mitochondrial tRNAs would also affect the translation process in the organelle. This possibility will be evaluated in a subsequent investigation, since a lesion in the charging of tRNAs would also effect the translation process while having no influence on DNA replication and transcription.

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