

Modification of the Mitochondrial Proteome in Response to the Stress of Ethanol-dependent Hepatotoxicity*

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Mitochondria are particularly susceptible to increased formation of reactive oxygen and nitrogen species in the cell that can occur in response to pathological and xenobiotic stimuli. Proteomics can give insights into both mechanism of pathology and adaptation to stress. Herein we report the use of proteomics to evaluate alterations in the levels of mitochondrial proteins following chronic ethanol exposure in an animal model. Forty-three proteins showed differential expression, 13 increased and 30 decreased, as a consequence of chronic ethanol. Of these proteins, 25 were not previously known to be affected by chronic ethanol emphasizing the power of proteomic approaches in revealing global responses to stress. Both nuclear and mitochondrially encoded gene products of the oxidative phosphorylation complexes in mitochondria from ethanol-fed rats were decreased suggesting an assembly defect in this integrated metabolic pathway. Moreover mtDNA damage was increased by ethanol demonstrating that the effects of ethanol consumption extend beyond the proteome to encompass mtDNA. Taken together, we have demonstrated that chronic ethanol consumption extends to a modification of the mitochondrial proteome far broader than realized previously. These data also suggest that the response of mitochondria to stress may not involve non-discriminate changes in the proteome but is restricted to those metabolic pathways that have a direct role in a specific pathology.

Chronic ethanol consumption causes liver damage by a complex process thought to involve oxidative and nitrosative stress, hypoxia, up-regulation of proinflammatory cytokines, and defects in energy metabolism (1–3). As both a source for the

formation and target of modifications mediated by reactive oxygen and nitrogen species, the mitochondrion is recognized as a site critical in the cellular stress response induced by chronic ethanol exposure. Increased mitochondrial production of reactive oxygen species (4–6), oxidation of mitochondrial proteins (7–10), depressed oxidative phosphorylation activity (11–13), and disrupted fatty acid metabolism (14–16) occur following consumption of alcohol, indicating that ethanol induces significant changes in mitochondria physiology. These responses are also accompanied by a profound increase in the sensitivity of the respiratory chain to inhibition by NO, which we propose plays a key role in contributing to the hypoxia associated with ethanol-dependent hepatotoxicity (17). While mechanisms responsible for ethanol-induced mitochondrial dysfunction have been investigated, the impact of chronic ethanol consumption on the overall content of mitochondrial proteins, the “mitochondrial proteome,” has not been studied. Earlier studies by Cunningham and colleagues (18, 19) have demonstrated that ethanol consumption decreases the synthesis of the 13 mitochondrially encoded proteins that comprise respiratory complexes I, III, and IV and the ATP synthase. It is proposed that the inhibition of mitochondrial protein synthesis following chronic ethanol exposure is due to defects in mtDNA (20, 21) and a decline in the number of functional mitochondrial ribosomes resulting from chronic ethanol exposure (22, 23). It is important to note that the mitochondrial proteome involves over 100 proteins as components of oxidative phosphorylation, most of which are encoded by the nuclear genome. Little or no information is available on the effects of ethanol on these nuclear encoded proteins, and because they are thought to play an important regulatory role in mitochondrial function this is potentially important in understanding the basis of mitochondrial pathologies. Indeed the mechanisms by which chronic ethanol alters the mitochondrial genome or levels of other mitochondrial proteins and the role that these changes may play in the development of liver injury from chronic ethanol exposure are not known. Ethanol-dependent hepatotoxicity, particularly in the early stages that precede cirrhosis, is then an ideal model to examine the response of the mitochondrial proteome in an organ exposed to a metabolic stress.

Two-dimensional gel electrophoresis is the central tool for proteomic analysis. This technique combines isoelectric focusing (IEF)¹ in the first dimension where proteins are separated

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¹ The abbreviations used are: IEF, isoelectric focusing; BN, blue native; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; QPCR, quantitative PCR; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; Hsp, heat shock protein.

according to differences in net charge followed by the separation of proteins based on molecular weight in the second dimension using standard SDS-PAGE. This technique is capable of resolving hundreds to thousands of proteins in a complex biological sample on a single two-dimensional gel that can then be identified by mass spectrometry. A number of proteomic approaches have been used with mitochondria including sucrose density gradient fractionation and immunocapture techniques, and these have identified ~600 proteins as members of the mitochondrial proteome (24, 25). While conventional two-dimensional IEF/SDS-PAGE is well suited to identify changes in the levels of the more hydrophilic proteins of the mitochondrion, *e.g.* matrix proteins, analysis of membrane proteins is hampered by the fact that many of these proteins precipitate at the basic end of the IEF gels and are thus poorly resolved on conventional two-dimensional gels (26–28). Therefore, alternate protein separation techniques are required to define changes in the levels of membrane proteins. To address this issue we used the two-dimensional blue native (BN)-PAGE technique developed by Schagger and von Jagow (29, 30) and modified by Brookes and colleagues (31) to complement our two-dimensional gel analyses of the mitochondrial proteome. Using this approach changes in the levels of both mitochondrial and nuclear encoded proteins that comprise the respiratory complexes following chronic ethanol consumption can be assessed. Moreover because the first dimension BN-PAGE is done under non-denaturing conditions information regarding protein-protein interactions and the assembly of oxidative phosphorylation complexes is retained. This is particularly important because it will enable probing the molecular basis of the observation that chronic ethanol consumption decreases the functioning of the oxidative phosphorylation system (12, 13).

In this investigation, global alterations in mitochondrial protein levels and mtDNA damage were studied in a well characterized rodent model of chronic ethanol feeding (32) that is known to cause hepatic mitochondrial dysfunction (12, 13) and oxidative and nitrosative stress (5, 33) in the early stage of the disease process. Using both the proteomic approaches described above, alterations in the levels of proteins involved in β -oxidation of fatty acids and the oxidative phosphorylation system were found in mitochondria isolated from the livers of rats chronically exposed to ethanol. Moreover significant liver mtDNA damage was detected in response to ethanol consumption. This result is important because it demonstrates that ethanol exposure alters the mitochondrial genome during the very early stages of ethanol-induced liver injury, *i.e.* fatty liver stage, in young animals. These data are consistent with the hypothesis that chronic ethanol initiates reactions that induce mtDNA damage, which in turn negatively impacts mitochondrial protein synthesis and functioning of the oxidative phosphorylation system. Furthermore the results from these proteomic analyses reveal that although the response of liver to chronic ethanol exposure is a complex process the number of changes in proteins is limited to a specific group of metabolic pathways.

EXPERIMENTAL PROCEDURES

Materials—Lieber-DeCarli ethanol and control liquid diets were purchased from Bio-Serv (Frenchtown, NJ). Immobilized pH gradient strips, ampholytes, and molecular weight standards were purchased from Amersham Biosciences. Monoclonal antibodies specific for complex I iron-sulfur protein 5 and 7, complex III subunit I, complex IV subunit I, and ATP synthase α and β subunits were purchased from Molecular Probes (Eugene, OR). All other biochemicals were obtained from Sigma or Bio-Rad.

Animals and Diets—Male Sprague-Dawley rats (200 g) were individually housed in suspended cages and maintained under a 12-h light/dark cycle for the entire duration of the feeding protocol. Nutritionally adequate ethanol and control liquid diets were formulated and pre-

pared according to Lieber and DeCarli (32). The ethanol diet provides 36% of the total daily caloric intake as ethanol, 11% as carbohydrate, 18% as protein, and 35% as fat. Weight-matched control rats were pair-fed and received identical diets except that ethanol calories were substituted isocalorically by dextrin maltose. Animals were maintained on the diets for at least 31 days as described previously (5). These studies were approved by the local institutional animal care and use committee.

Isolation of Rat Liver Mitochondria—Coupled liver mitochondria were prepared by differential centrifugation of liver homogenates (34, 35) using ice-cold mitochondria isolation medium containing 0.25 M sucrose, 1 mM EDTA, and 5 mM Tris-HCl, pH 7.5. Protease inhibitors were added to the isolation buffer to prevent protein degradation (10). The total mitochondria yield from control animals was 220 ± 20 mg of protein and from ethanol-treated animals was 260 ± 10 mg of protein ($n = 8$, $p = 0.19$).

Two-dimensional Gel Electrophoresis—First dimension IEF was performed using 11-cm immobilized pH gradient strips with pH range 3–10 using an in-gel rehydration method. Briefly mitochondrial protein from control and ethanol-fed rats were diluted in a rehydration solution containing 40 mM Tris-HCl, pH 8.8, 7 M urea, 2 M thiourea, 4% CHAPS, 2% ampholytes (pH 3–10), and 5 mM tributylphosphine and incubated for 30 min at room temperature to fully solubilize samples. After incubation, 180 μ g of protein was applied to IEF gels, and the gels were allowed to rehydrate overnight. IEF was performed for 1 min at 300 V, 1.5 h at a 300–3500-V gradient, and then held at 3500 V for 4.5 h using an Amersham Biosciences Multiphor II system. After IEF, the immobilized pH gradient strips were equilibrated for 20 min in 50 mM Tris-HCl, pH 8.8, 6 M urea, 20% glycerol, 2% SDS, 0.005% bromphenol blue, and 65 mM dithiothreitol followed by a 20-min incubation in the same buffer containing 2.5% iodoacetamide in place of dithiothreitol before SDS-PAGE. Two-dimensional SDS-PAGE was performed using 10% homogenous Tris-glycine acrylamide gels (Bio-Rad Criterion precast gels). After electrophoresis, gels were stained with Sypro Ruby Red (Molecular Probes), and gel images were captured with excitation and emission wavelengths of 480 and 620 nm, respectively, using the ProXpress imager (PerkinElmer Life Sciences).

Blue Native Gel Electrophoresis—BN-PAGE was performed using the method developed by Schagger and von Jagow (29, 30) and modified by Brookes *et al.* (31). All buffers and solutions were kept at 4 °C and pH 7.0. Mitochondrial pellets (1 mg of protein) were resuspended in 113 μ l of extraction buffer containing 0.75 M aminocaproic acid, 50 mM BisTris, and 1% *n*-dodecyl- β -D-maltoside and allowed to incubate on ice for 60 min. After incubation, samples were centrifuged at 14,000 rpm for 5 min, and the supernatant containing the extracted mitochondrial complexes was collected and kept on ice. Protein content was measured by the Bradford method, and 2.5 μ l of a 5% (w/v) suspension of Coomassie Brilliant Blue G-250 (Serva Blue G) in 0.5 M aminocaproic acid was added to 60 μ g of protein (~30 μ l by volume). Samples were then stored on ice for no more than 30 min before being loaded onto a non-denaturing 5–16.5% gradient gel to separate the individual oxidative phosphorylation complexes intact. Electrophoresis buffers and conditions are as described previously (31).

After native electrophoresis, the entire vertical lane containing all the mitochondrial protein complexes was cut from the gel, rotated 90°, and laid on top of a denaturing 5–15% Tris-Tricine SDS-PAGE gel to resolve the individual polypeptides of the complexes based on molecular weight (31). Samples from each pair of control and ethanol-treated mitochondria were resolved on one SDS-PAGE gel to control for intergel differences. In some experiments the individual oxidative phosphorylation complexes were excised from the first dimension BN-PAGE gel and applied to the top of a denaturing SDS-polyacrylamide gel to resolve individual proteins in that complex. For these experiments, each control and ethanol sample was run in duplicate so that one gel could be stained and the second gel could be immunoblotted to nitrocellulose membranes. Gels were stained using a mixture of Coomassie Blue R-250 and G-250 (0.05% (w/v) each in 25% isopropanol, 10% glacial acetic acid). For immunoblot analysis, proteins were transferred to 0.2- μ m nitrocellulose membranes and blocked with 5% nonfat milk. Levels of mitochondrial proteins were detected using a 1:10,000 dilution of primary antibodies (complex I iron-sulfur protein 5 and 7, complex III subunit I, complex IV subunit I, or ATP synthase α and β subunits) followed by incubation with a 1:10,000 dilution of horseradish peroxidase-conjugated anti-mouse IgG. Binding of the secondary antibody was visualized by enhanced chemiluminescence.

Image Analysis of Two-dimensional Gels—Mitochondrial proteins from control and ethanol-fed rats were separated by the proteomic methods described above. Gels were scanned, saved as TIFF files, and

TABLE I
Chronic ethanol consumption decreases the activity of respiratory complexes I, III, and IV in liver

Complex I activity was assessed by measuring the oxygen consumption of mitochondria isolated from control and ethanol-fed rats in the presence of glutamate/malate (5 mM) and ADP (0.5 mM). Antimycin-sensitive complex III activity was assessed indirectly by measuring the respiratory rate in the presence of succinate (15 mM), ADP (0.5 mM), and antimycin (2 nM). Complex IV (cytochrome c oxidase) and citrate synthase activities were measured using standard spectrophotometric methods (65, 66). Data are expressed as the mean \pm S.E. for five pairs of control and ethanol-fed rats, and statistical analysis was performed using a paired *t* test.

Activity	Control	Ethanol	<i>p</i> value
Complex I ($\mu\text{mol O}_2/\text{min}/\text{mg}$)	0.185 \pm 0.003	0.112 \pm 0.007	<0.0001
Complex III ($\mu\text{mol O}_2/\text{min}/\text{mg}$)	0.232 \pm 0.010	0.144 \pm 0.020	<0.05
Complex IV (units/mg)	23.08 \pm 1.14	12.04 \pm 1.22	<0.0001
Citrate synthase ($\mu\text{mol}/\text{min}/\text{mg}$ protein)	0.178 \pm 0.001	0.164 \pm 0.009	Not significant

analyzed for differences in protein expression using PDQuest Image Analysis software (Bio-Rad). Because the gels were stained with Sypro Ruby Red it was necessary to invert the data from the gels so that the fluorescent protein spots could be identified by the software as dark protein spot density against a light background. Protein spots were identified and a matched set containing five control gels with the corresponding ethanol gels was created. To compare protein spot density across different gels a reference gel (*i.e.* master image) was selected. To increase the likelihood that the highest possible number of protein spots in each gel could be matched to the corresponding protein spots in the master image, the selection of the master image was based on the highest number of detected proteins spots and the best protein spot resolution across the whole proteome. Thus, the control gel from pair 1 met all the requirements and was used as the master image for protein spot matching purposes. Automatic matching of protein spots in each gel to corresponding protein spots in the master image was performed, and the protein spots that were incorrectly matched to the reference gel were manually corrected. To correct for any possible intergel protein loading differences, the data for all protein spots in a given gel were normalized to the total density in valid protein spots for that gel. Normalized protein spot densities were transferred to Microsoft Excel, and -fold changes for each pair were determined. The -fold changes were then averaged resulting in a mean-fold change \pm S.E. Normalized protein spot densities from control gels were compared with normalized protein spot densities from ethanol gels using a two-tailed paired Student's *t* test. Scanned TIFF images for one-dimensional and two-dimensional BN-PAGE were analyzed using Scion Image Beta 4.02 (Scion Corp.) after removing the background uniformly across all the pairs. The areas under the curve for the individual spots obtained from mitochondria isolated from ethanol-fed animals were normalized to their respective pair-fed controls and represented as -fold change. A two-tailed paired Student's *t* test was performed on the BN-PAGE raw data.

Mass Spectrometry—Protein spots found by the image analysis to have altered levels of expression as a result of chronic ethanol feeding were excised from gels and subjected to matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry essentially as described in Brookes *et al.* (31) to identify these proteins. Gel pieces were washed with 5% acetonitrile to remove Coomassie Blue, SDS, and salts. After destaining, the gel pieces were dried, rehydrated with a trypsin-containing solution, and allowed to digest for 16–20 h. The in-gel tryptic digest was extracted several times with 50% acetonitrile, 5% formic acid. An aliquot of the peptide extract was mixed with an equal volume of the matrix α -cyano-4-hydroxycinnamic acid in 50% acetonitrile, and 1.0 μl of this mixture was spotted onto a gold target plate for MALDI-TOF analysis using a PE-Biosystems Voyager Elite instrument (Framingham, MA) equipped with a nitrogen laser (337 nm) and operated using a delayed extraction mode. The peptide masses were entered into the MASCOT search engine,² and the National Center for Biotechnology Information data base was searched to match the tryptic peptide fingerprint with a parent polypeptide.

Mitochondrial DNA Damage Assessment by Quantitative PCR (QPCR)—Genomic DNA was extracted from liver homogenates, quantified using PicoGreen (Molecular Probes) on a Cytofluor 4000 Series fluorometer, and 15 ng was used for QPCR. QPCR assesses generalized DNA damage in a gene-specific manner in which DNA lesions block rTth polymerase and lead to a decrease in amplification (36, 37). Sensitivity of the assay is increased through amplification of large targets that increases the probability of encountering a DNA lesion. A 16,059-bp QPCR product, which encompasses all but 236 bp of NADH5/6 genes in the mitochondrial genome was amplified using

primer set M13597 FOR (bp 13597–13620) and 13361 REV (bp 13361–13337). Copy number differences in mitochondrial DNA were normalized using a short QPCR, which yields products directly related to gene copy number using primer pair 13597F/13713R (5'-CCAGCTACTAC-CATCTTCAAGT/GATGGTTTGGGAGATTGGTTGATGT-3') for mitochondrial DNA.

Mitochondrial DNA damage was quantified by comparing the relative efficiency of amplification of large (>15-kb) fragments of DNA and normalizing this to gene copy numbers by the amplification of smaller (<250-bp) fragments, which have a statistically negligible likelihood of containing damaged bases. Hence, to measure mtDNA damage, long (*L*) and short (*S*) QPCRs were performed to determine DNA damage and number of gene copies present, respectively. To calculate lesion frequencies, the long QPCR values (*L*) were divided by the corresponding short QPCR results (*S*) to account for potential copy number differences between samples (*L/S*). Normalized values from damaged ($L_d/S_d = A_d$) samples were compared with non-damaged controls ($L_o/S_o = A_o$) resulting in a relative amplification ratio (A_d/A_o). Assuming a random distribution of lesions and using the Poisson equation ($f(x) = e^{-\lambda} \lambda^x / x!$ where λ = the average lesion frequency) for the non-damaged templates (zero class; $x = 0$), the average lesion frequency per DNA strand was determined: $\lambda = -\ln A_d/A_o$.

RESULTS

For these experiments, animals were fed a diet containing 36% total calories as ethanol for 5–6 weeks. Under these conditions a number of changes in mitochondrial function occur including inhibition of mitochondrial protein synthesis, increased formation of reactive oxygen and nitrogen species, and increased sensitivity to the regulation of respiration by NO (5, 17, 18). This exposure of ethanol decreased the activity of mitochondrial complexes I, III, and IV (Table I) in agreement with previous studies (12). Chronic ethanol consumption had no effect on citrate synthase activity (Table I) and total mitochondria protein yield, indicating that ethanol feeding had little effect on overall mitochondrial content or purity. This preparation does contain some minor contamination from other nonmitochondrial membrane sources but is functionally viable for the periods needed for respiratory measurement and proteomics.

Conventional two-dimensional gel electrophoresis, *i.e.* two-dimensional IEF/SDS-PAGE, was performed on mitochondria to determine global changes in the levels of mitochondrial proteins resulting from chronic ethanol feeding. Mitochondrial protein (180 μg) from the livers of control and ethanol-fed rats was separated in the first dimension by using broad range pH 3–10 IEF gels followed by separation in the second dimension using 10% acrylamide gels. Gels were stained with Sypro Ruby Red to visualize proteins (Fig. 1). Using PDQuest Image Analysis software 194 individual protein spots were detected in common from gels generated from five pairs of control and ethanol-fed animals (Fig. 1A). Image analysis revealed that 37 individual protein spots were differentially expressed in response to chronic ethanol. For example, Fig. 1B shows magnified images of selected proteins identified by the analysis software as having either increased or decreased levels of expression in mitochondria from ethanol-fed rats as compared

² See www.matrixscience.com.

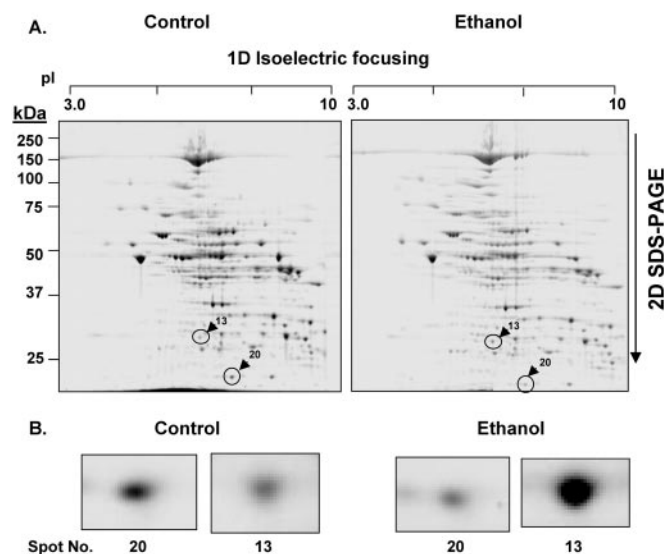


FIG. 1. Two-dimensional gel electrophoresis of whole liver mitochondria from control and ethanol-fed rats. *A*, representative high resolution separation of mitochondrial proteins from one pair of control and ethanol-fed rats using two-dimensional IEF/SDS-PAGE by the procedures given under "Experimental Procedures." *B*, close-up of protein spots that demonstrate significant decreases (*spot 20*) or increases (*spot 13*) in these proteins in response to chronic ethanol consumption. Note that while the protein spot images were transformed to increase contrast, identical transformation values were applied to both control and ethanol images. *1D*, one-dimensional; *2D*, two-dimensional.

with their corresponding pair-fed control. A schematic "master map" image was generated from these data that illustrates the position of each of the 194 protein spots that were common to all gels (Fig. 2). Of these, the levels of 157 protein spots were unchanged (*gray circles*), 23 protein spots were decreased (*white circles*), and 14 protein spots were increased (*black circles*) as a consequence of chronic ethanol consumption. All 37 protein spots that showed differences in response to ethanol feeding were analyzed by MALDI-TOF mass spectrometry, and 25 individual protein spots were successfully identified (Table II). Of these 25, 18 were identified as unique proteins. Proteins identified include enzymes involved in β -oxidation of saturated and unsaturated fatty acids, nuclear encoded subunits of the oxidative phosphorylation system, mitochondrial chaperones, and enzymes of amino acid metabolism.

The detection of changes in the steady-state level of the 13 mitochondrial encoded polypeptides is not feasible using conventional two-dimensional IEF/SDS-PAGE due to the high hydrophobic nature of these proteins and as such requires the application of other techniques such as two-dimensional BN-PAGE. In this approach, mitochondria are solubilized using the non-ionic detergent dodecylmaltoide to gently extract intact oxidative phosphorylation complexes from the inner membrane. Following extraction, equal concentrations of protein (60 μ g) from control and ethanol-fed mitochondria samples were separated on non-denaturing 5–15% gradient gels to separate the respiratory complexes intact and in native form (Fig. 3A). As shown in Fig. 3B, densitometry performed on the entire one-dimensional BN-PAGE lane for both control and ethanol samples demonstrates an overall decrease in the content of complexes I, III, IV, and V in mitochondria isolated from liver of ethanol-fed rats. Image analysis of complex II was not possible due to the high level of Coomassie Blue-stained background at the bottom of the gel. Quantification of the densitometry data revealed a 50% decrease in the content of complexes I, III, and IV, whereas complex V was decreased ~30% by chronic ethanol feeding (Fig. 3C). In these experi-

ments the same amount of protein was loaded on the gels to be consistent with the normalization process used for the two-dimensional IEF/SDS-PAGE analysis. Under conditions where significant changes in the proteome occur this will be reflected in changes in the relative proportion of each spot or band to the total band intensity on the gel. Using this approach significant changes in the amounts of the respiratory complexes were observed that were in reasonable agreement with the changes observed in the specific activities measured spectrophotometrically (Table I).

A two-dimensional proteomic approach can also be used in combination with BN-PAGE to resolve the individual polypeptides present in each of the oxidative phosphorylation complexes. To minimize intergel differences, samples from each control and ethanol pair were resolved adjacently on a single SDS-polyacrylamide gel. A representative set of two-dimensional BN-polyacrylamide gels from one pair of control and ethanol-fed rats is shown in Fig. 4A. Using this technique, we observed that the levels of several polypeptides of the respiratory complexes were decreased in mitochondrial samples from ethanol-fed rats (Fig. 4A). Image analysis allowed us to successfully identify 63 individual protein spots in common between all control and ethanol-fed samples (Fig. 4B). Identification of proteins present within complexes III, IV, and V were facilitated by the comparison of the reference proteome map with data published previously (31, 38). As expected the mitochondrially encoded polypeptides of cytochrome *c* oxidase, subunits I–III, were decreased in mitochondria from ethanol-fed rats (Table III). However, significant decreases in several nuclear encoded polypeptides of complex IV as well as complexes III and V were observed in mitochondria from ethanol-fed rats (Table III). Unfortunately the resolution of the individual subunits within complex I was not sufficient to allow quantification of the more than 43 polypeptides present in this complex by gel densitometry. Fig. 4B illustrates the master map image of the two-dimensional BN-PAGE data. Protein levels that were unchanged by ethanol feeding are represented by *gray circles*, whereas proteins that were decreased are represented by *white circles*.

Alterations in of some of the respiratory complex subunits in response to chronic ethanol consumption were demonstrated using immunoblot techniques. For immunoblot analysis, the intact protein band for complexes I, III, IV, and V were cut from the one-dimensional BN-polyacrylamide gel (Figs. 5A–7A) and transferred to a denaturing gel to separate the individual proteins of each complex. Proteins were then transferred to nitrocellulose and probed for changes in expression with monoclonal antibodies directed against the individual proteins. Chronic ethanol consumption decreased the levels of the mitochondrial encoded 30- and 17-kDa polypeptides of the NADH dehydrogenase complex (Fig. 5, *B* and *C*) and subunit I of cytochrome *c* oxidase (Fig. 6, *B* and *C*). Moreover the non-significant decrease in complex III core protein 1 (Table III) was confirmed with immunoblotting (Fig. 6, *B* and *C*). Interestingly the overall decrease in α and β subunits of the F_1 portion of the ATP synthase (Table III) appears to be due to a significant decrease in the α and not the β subunit (Fig. 7, *B* and *C*). In contrast, levels of both α and β subunits of complex V were found to be decreased in response to chronic ethanol consumption when conventional two-dimensional IEF/SDS-PAGE was used to resolve mitochondrial proteins (Table II). While the reason for this discrepancy is not known, differences in sample preparation, especially in the solubilization efficiency of the different detergents used, is likely to be responsible for these results. These results highlight the need to use multiple approaches like immunoblotting for *post hoc* analyses to verify gel densi-

FIG. 2. **Schematic master map of spots detected by two-dimensional IEF/SDS-PAGE.** This master map image was generated using PDQuest Image Analysis software and is a composite image of all gels generated with control and ethanol samples. *Ellipses* depict locations of proteins that had no significant changes (*gray*), significant increases (*black*), or decreases (*white*) in content with respect to chronic ethanol consumption. Significant changes were defined by $p \leq 0.05$ as shown in Table II. Numbers are shown for 37 individual protein spots that were significantly different with chronic ethanol consumption as compared with control.

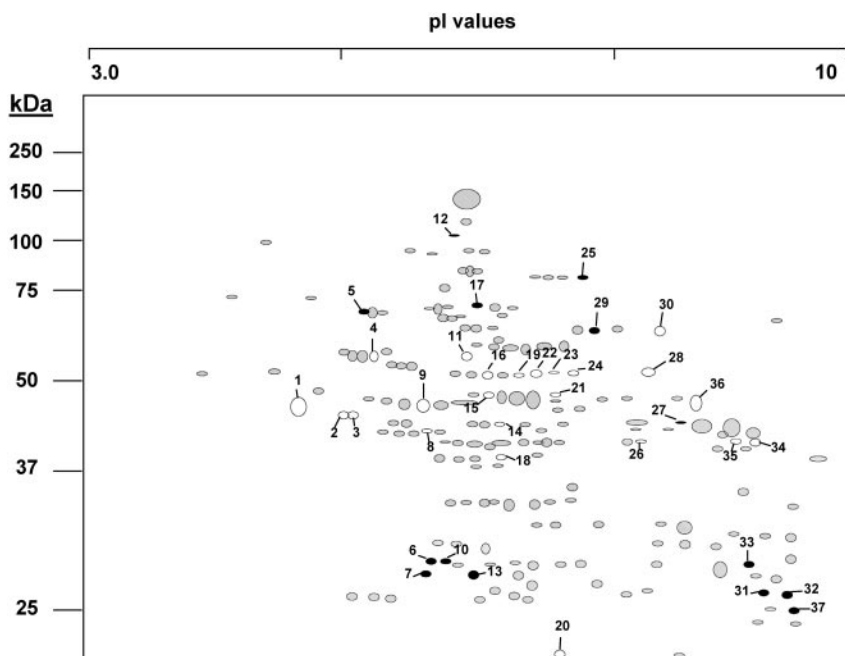


TABLE II
Proteins differentially expressed in liver mitochondria following chronic ethanol consumption: identification of proteins from two-dimensional IEF/SDS-polyacrylamide gels

Twelve additional protein spots (2, 3, 6, 10, 11, 14, 17, 23, 25, 27, 31, and 33) shown in Fig. 2 were also found to be differentially expressed in response to ethanol treatment; however, these proteins could not be identified as generated peptide fragments did not match those present within the protein data base due to either insufficient peptide coverage or sequence variation. MOWSE, molecular weight search; FDR, false discovery rate.

Spot no.	Protein	Mass		MOWSE score	Accession no. (NCBI)	Mean -fold change ^a	<i>p</i> value ^b	FDR
		Calculated	Observed					
<i>kDa</i>								
1	ATP synthase β subunit	51.1	46.3	144	gi1374715	0.65	0.0395	0.22
4	Hsp60	60.9	56.2	115	gi3219998	0.84	0.0376	0.21
5	dnaK-type molecular chaperone grp75 (Hsp70/GRP75)	73.7	68.6	121	gi2119726	1.80	0.0148	0.14
7	3-Mercaptopyruvate sulfurtransferase	32.9	28.5	78	gi20304123	1.82	0.0034	0.08
8	Aldehyde dehydrogenase	48.2	42.9	75	gi16073616	0.44	0.0233	0.18
9	Aldehyde dehydrogenase	48.2	46.5	152	gi16073616	0.71	0.0130	0.13
12	Oxoglutarate dehydrogenase (lipoamide); α -ketoglutarate dehydrogenase	116.0	105.5	64	gi33563270	2.87	0.0004	0.09
13	3-Hydroxyisobutyrate dehydrogenase	35.3	28.4	67	gi27710396	1.41	0.0005	0.04
15	Glutamate dehydrogenase	61.4	48.0	101	gi118543	0.56	0.0248	0.18
16	Methylmalonate-semialdehyde dehydrogenase	57.8	51.6	78	gi13591997	0.77	0.0479	0.26
18	Acyl-CoA dehydrogenase, short chain-specific	44.9	39.5	67	gi584714	0.81	0.0361	0.21
19	Methylmalonate-semialdehyde dehydrogenase	57.8	51.5	74	gi13591997	0.65	0.0135	0.13
20	Ubiquinol-cytochrome <i>c</i> reductase iron-sulfur subunit	27.7	22.8	108	gi136708	0.48	0.0111	0.13
21	Glutamate dehydrogenase	61.4	48.1	72	gi118543	0.69	0.0346	0.22
22	Methylmalonate-semialdehyde dehydrogenase	57.8	51.9	68	gi13591997	0.77	0.0049	0.11
24	Methylmalonate-semialdehyde dehydrogenase	57.8	52.0	83	gi13591997	0.76	0.0011	0.05
26	Acyl-CoA dehydrogenase, medium chain	46.5	41.5	94	gi8392833	0.78	0.0210	0.18
28	Methylmalonate-semialdehyde dehydrogenase	57.8	52.3	166	gi13591997	0.74	0.0027	0.08
29	Acyl-CoA dehydrogenase, very long chain	70.7	63.0	72	gi6978435	1.31	0.0536	0.28
30	Acyl-CoA dehydrogenase, very long chain	70.7	62.8	142	gi6978435	0.85	0.0285	0.20
32	2,4-Dienoyl-CoA reductase (NADPH)	36.1	26.9	78	gi111287	1.70	0.0345	0.22
34	β -Ketoacyl-CoA thiolase	41.8	41.4	84	gi18426866	0.12	0.0019	0.07
35	Ubiquinol-cytochrome <i>c</i> reductase, core protein II	48.3	41.5	67	gi418146	0.21	0.0069	0.10
36	Chain A, rat liver F ₁ -ATPase (α subunit)	55.2	46.8	156	gi6729934	0.71	0.0072	0.10
37	Δ^3,Δ^2 -Enoyl-CoA isomerase	32.2	25.7	100	gi8393243	1.58	0.0315	0.21

^a Mean -fold change was determined by averaging the -fold change observed from five pairs of control and ethanol-fed animals.

^b *p* values were determined using a two-tailed paired Student's *t* test on the normalized protein spot densities obtained using PDQuest.

tometry data. In agreement with previous studies (12), levels of cytochrome *c* are not affected by chronic ethanol consumption (Fig. 7B).

Previously it has been shown that the levels of 8-hydroxydeoxyguanosine, a marker of oxidative damage, are increased in mtDNA isolated from liver of animals fed ethanol for long periods of time, e.g. 6–12 months (20, 21). However, it is not known whether mtDNA damage occurs following shorter

exposures to ethanol (*i.e.* 1 month) when significant decreases in oxidative phosphorylation are already present. To assess mtDNA damage, QPCR was used. The basis of this assay is that DNA lesions will block DNA polymerase and therefore lead to a decrease in amplification of the PCR product, which in this case is a large segment of the mitochondrial genome. Thus, decreased PCR product formation is indicative of mtDNA damage. Fig. 8 illustrates that mtDNA damage was significantly

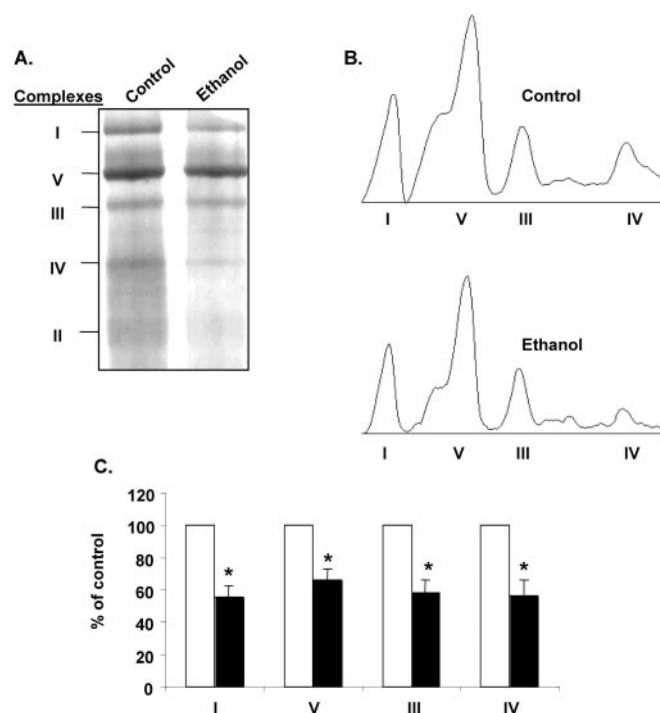


FIG. 3. Chronic ethanol consumption decreases the overall level of the intact oxidative phosphorylation complexes in liver mitochondria. *A*, intact oxidative phosphorylation complexes from 60 μg of liver mitochondria from control and ethanol-fed rats were separated on a non-denaturing 5–15% polyacrylamide gradient gel using one-dimensional BN-PAGE techniques. The positions of the respiratory chain complexes are indicated by the *Roman numerals* on the left. *B*, representative traces showing the densitometry analyses of lanes for control and ethanol samples shown in *A*. Complex II was not included in the analysis due to low signal to noise ratio. *C*, comparison of the relative quantities of complexes I, V, III, and IV in liver mitochondria from control (*white bars*) or ethanol-fed (*black bars*) rats. Results represent the mean \pm S.E. for five pairs of control and ethanol-fed animals; *, $p < 0.05$.

increased in ethanol-fed rats as compared with controls as indicated by a decreased amplification of the 16-kb fragment in ethanol samples. Thus, short term exposure to ethanol before the development of overt pathology caused significant damage to liver mtDNA that was accompanied by significant alterations to the mitochondrial proteome.

DISCUSSION

Chronic exposure to moderate levels of ethanol is known to induce metabolic defects in mitochondrial function including changes in fatty acid metabolism and oxidative phosphorylation. The extent of both the adaptive and pathological responses of mitochondria to ethanol-dependent hepatotoxicity has not been analyzed up to now, however, using a proteomic approach. In the mitochondrion this offers a particular challenge due to the highly hydrophobic nature of some of the proteins in the respiratory chain complexes. In this study changes in the mitochondrial oxidative phosphorylation subproteome in response to ethanol are clearly far more extensive than previously realized. The major findings are discussed in detail below.

Effect of Chronic Ethanol Consumption on Mitochondrial β -Oxidation Enzymes—Impaired mitochondrial β -oxidation is recognized as a key factor contributing to the accumulation of triglycerides in hepatocytes (39–41) leading to the development of fatty liver (for a review, see Ref. 40). One mechanism thought to contribute to the decrease in β -oxidation in ethanol-treated animals is the decrease in the redox state (*i.e.* NAD^+/NADH ratio) that results from the metabolism of ethanol and

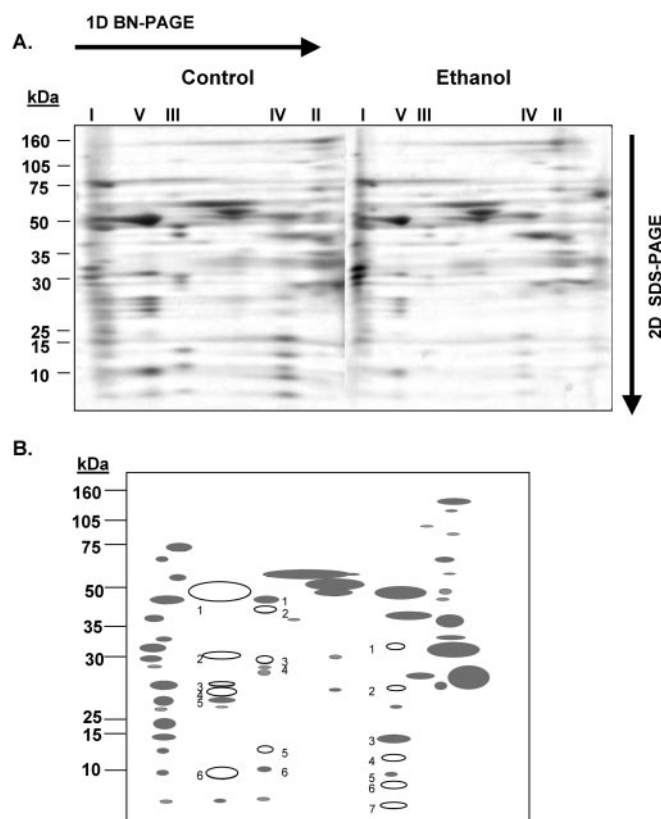


FIG. 4. Chronic ethanol consumption decreases the levels of individual polypeptides present within the oxidative phosphorylation complexes. *A*, intact oxidative phosphorylation complexes from 60 μg of liver mitochondria from control and ethanol-fed rats were separated under non-denaturing conditions using the one-dimensional BN-PAGE techniques described for Fig. 3. Intact one-dimensional BN-PAGE lanes for both control and ethanol samples were rotated 90° and laid across the top of a Tris-Tricine SDS-PAGE gel to resolve the individual polypeptides that comprise each oxidative phosphorylation complex. The individual polypeptides that make up each complex appear as vertically aligned spots on the two-dimensional BN-PAGE gel. *B*, schematic master map image of two-dimensional BN-polyacrylamide gels. This map is a composite image of all gels generated with control and ethanol samples. *Ellipses* depict locations of proteins that had no significant changes (*gray*) or decreases (*white*) in content with respect to chronic ethanol consumption. Significant changes were defined by $p \leq 0.05$ as shown in Table III. Numbers are shown for 19 protein “spots” that were analyzed for differences in response to chronic ethanol treatment. *1D*, one-dimensional; *2D*, two-dimensional.

acetaldehyde. Increased levels of NADH will inhibit the NAD^+ -dependent steps of β -oxidation thereby inhibiting fatty acid oxidation (39) and stimulate the activity of various lipogenic enzymes in liver (41). However, ethanol-related alterations in the hepatic redox state have been shown to normalize during continued exposure to ethanol without a concomitant decrease in hepatocyte triglyceride levels (42). Thus, alternative mechanisms for the continued decreased activity in β -oxidation and the maintenance of fatty liver must be considered.

In our studies chronic ethanol consumption decreased the levels of several of the acyl-CoA dehydrogenase isoforms that catalyze the first step of β -oxidation. Similarly the protein level of β -ketoacyl-CoA thiolase, which catalyzes the last step of β -oxidation, was dramatically reduced in mitochondria from ethanol-fed rats. Recent studies have also demonstrated that chronic ethanol feeding decreases the expression of several enzymes involved in fatty acid metabolism including the acyl-CoA dehydrogenase isomers due to impaired peroxisome proliferator-activated receptor α function (43–45). Whether decreased levels of these enzymes play a critical role in the control of mitochondrial β -oxidation flux is not known. Histor-

TABLE III
Oxidative phosphorylation proteins differentially expressed in liver mitochondria following chronic ethanol consumption: identification of proteins from two-dimensional BN-polyacrylamide gels

Spot no.	Protein	Mass		Mean -fold change ^a	<i>p</i> value ^b
		Calculated	Observed		
<i>kDa</i>					
ATP synthase (complex V)					
1	α and β chain	59.7	62.1	0.726	0.04
2	γ chain	32.9	37.2	0.501	0.01
3	B-chain	28.9	28.6	0.420	0.003
4	OSCP ^c	23.2	27.1	0.339	0.02
5	D-chain	18.3	25.4	1.028	NS ^d
6	F-subunit	10.7	13.5	0.303	0.003
Ubiquinol-cytochrome <i>c</i> reductase (complex III)					
1	Core protein I	52.6	58.2	0.611	NS
2	Core protein II	48.4	54.0	0.606	0.04
3	Core protein II	48.4	35.1	0.556	0.03
4	Heme protein	35.3	33.7	2.186	NS
5	Iron-sulfur subunit	29.6	16.6	0.364	0.008
6	14-kDa protein	13.3	14.1	0.781	NS
Cytochrome <i>c</i> oxidase (complex IV)					
1	Subunit I	57.0	41.0	0.556	0.04
2	Subunit II	25.5	27.4	0.591	0.01
3	Subunit III	29.9	18.3	0.646	NS
4	Subunit IV	19.5	15.2	0.555	0.02
5	Subunit V	16.8	13.3	0.593	NS
6	Subunit VI	10.0	12.0	0.453	0.004
7	Subunit VII	9.4	10.9	0.184	0.038

^a Mean -fold change was determined by averaging the -fold change observed in mitochondria samples isolated from livers of five pairs of control and ethanol-fed rats.

^b *p* values were determined using a two-tailed paired Student's *t* test.

^c OSCP, oligomycin sensitivity conferring protein.

^d NS, not significant.

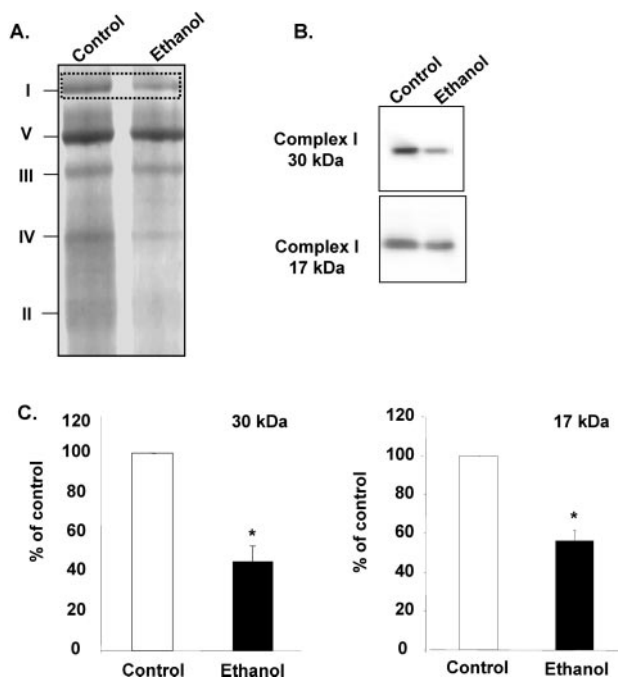


FIG. 5. Chronic ethanol consumption decreases levels of complex I proteins. A, liver mitochondria from control or ethanol-fed rats were separated using one-dimensional BN-PAGE techniques as described in Fig. 3. B, the band containing intact complex I was excised from the gel, and complex I was separated into its individual polypeptides using standard SDS-PAGE. Protein was transferred to nitrocellulose membranes and probed with antibodies against the mitochondrial encoded 30- and 17-kDa subunits of complex I. C, comparison of relative quantities of 30- and 17-kDa proteins from mitochondria of control and ethanol-fed rats. Results represent the mean \pm S.E. for five pairs of control and ethanol-fed animals; *, *p* < 0.05.

ically control of β -oxidation flux in liver has been ascribed to the entry of fatty acyl groups into mitochondria via carnitine palmitoyltransferase I (46). However, experimentally deter-

mined flux control coefficients of carnitine palmitoyltransferase I over β -oxidation indicated that some of the control of β -oxidation must reside elsewhere (46). Studies by Wood and colleagues (47, 48) have demonstrated that targeted disruption of the acyl-CoA dehydrogenase genes in mice decreases β -oxidation, which induces fatty liver. Moreover β -ketoacyl-CoA thiolases have been proposed as the rate-limiting step of β -oxidation in several tissues as well as critical for feedback control of β -oxidation and control by the acetyl-CoA/CoASH ratio (46). Another possible mechanism by which ethanol exposure might negatively impact fatty acid oxidation is via depressed activity of the tricarboxylic acid cycle due to inhibition of NAD⁺-dependent steps of this metabolic pathway (39). Our observation of a significant decrease in the level of glutamate dehydrogenase, an enzyme closely linked to the tricarboxylic acid cycle, supports this idea. These results taken together with our proteomic findings suggest that decreased expression of several key enzymes of fatty acid oxidation via ethanol-mediated inhibition of peroxisome proliferator-activated receptor α -mediated pathways contributes to the development of fatty liver.

In addition to these changes chronic ethanol consumption altered the levels of two auxiliary β -oxidation enzymes critical for the metabolism of poly- and unsaturated fatty acids. Chronic ethanol increased the levels of 2,4-dienoyl-CoA reductase and Δ^3, Δ^2 -enoyl-CoA isomerase, which are enzymes involved in the cis-trans isomerization of β -oxidation intermediates and required for β -oxidation to continue (49, 50). Linoleic and oleic acid are increased in liver mitochondria by chronic ethanol feeding (51–53), but whether these fatty acids act via a “peroxisome proliferator-activated receptor α -like” mechanism to increase the levels of these key proteins involved in β -oxidation of unsaturated fatty acids in liver is currently not known.

Effect of Chronic Ethanol on the Oxidative Phosphorylation System—Chronic ethanol consumption also negatively affects oxidative phosphorylation in liver mitochondria resulting in depressed hepatic energy metabolism. Previous studies have reported that chronic ethanol decreases the activities of all the

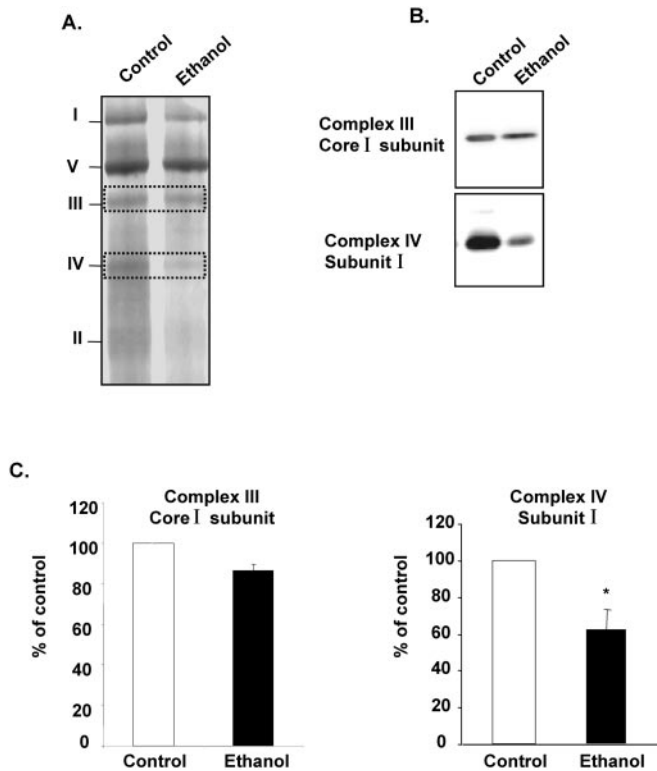


FIG. 6. Effect of chronic ethanol consumption on complex III and IV proteins. *A*, liver mitochondria from control or ethanol-fed rats were separated using one-dimensional BN-PAGE techniques as described in Fig. 3. *B*, the bands containing intact complex III and IV were excised from the gel, and the individual polypeptides of these complexes were separated using standard SDS-PAGE. Protein was transferred to nitrocellulose and probed with antibodies directed against the core I subunit of complex III or subunit I of complex IV. *C*, comparison of relative quantities of the core I subunit of complex III and subunit I of complex IV from mitochondria of control and ethanol-fed rats. Results represent the mean \pm S.E. for five pairs of control and ethanol-fed animals; *, $p < 0.05$.

oxidative phosphorylation complexes except complex II (12, 13). Similar decreases were observed in the present study for complex I, III, and IV activities. These observations strongly suggest that chronic ethanol exposure might impair the synthesis of the protein subunits that are encoded by the mitochondrial genome. Indeed studies by Coleman and Cunningham (18) demonstrate that the steady-state levels of all 13 mitochondrial encoded polypeptides were decreased in liver mitochondria from animals exposed to ethanol due to defects in mitochondrial ribosomes (22, 23), and these defects are proposed to contribute to decreased expression of the mitochondria gene products.

Results from the current study using both conventional and BN-PAGE proteomic techniques extend these earlier studies (18). BN-PAGE in combination with immunoblotting demonstrated that chronic ethanol consumption significantly decreased the levels of mitochondrial encoded gene products, and interestingly decreased expression of several nuclear encoded polypeptides that comprise some of the respiratory chain complexes was also observed. While previous investigations have reported decreased levels of mitochondrial gene products in response to chronic ethanol exposure (18), these studies were never fully extended to the nuclear encoded polypeptides. Moreover the use of low resolution one-dimensional SDS-PAGE in these early studies was insufficient to detect changes in nuclear encoded proteins as many of these proteins would have been masked by the co-migration of proteins with similar molecular weights. However, by using high resolution two-dimen-

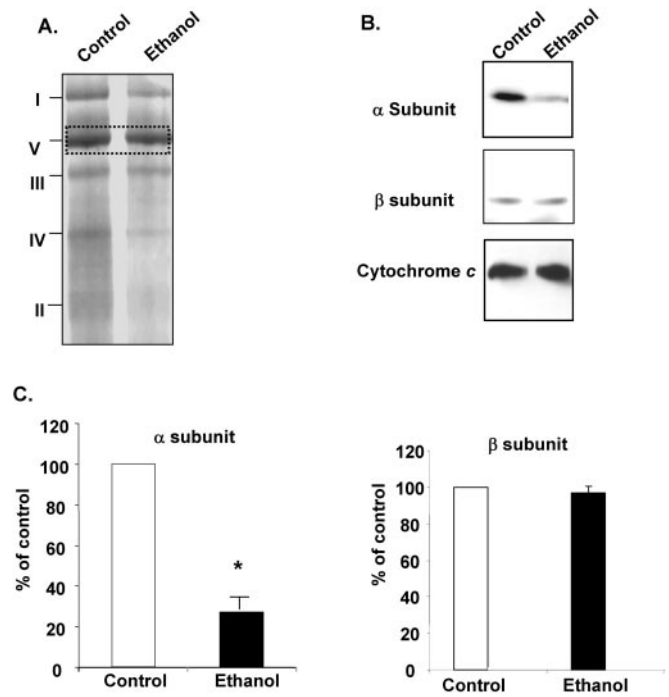


FIG. 7. Effect of chronic ethanol consumption on the α and β subunits of complex V and cytochrome *c*. *A*, liver mitochondria from control or ethanol-fed rats were separated using one-dimensional BN-PAGE techniques as described in Fig. 3. *B*, the band containing intact complex V was excised from the gel, and the individual polypeptides of ATP synthase were separated using standard SDS-PAGE. Protein was transferred to nitrocellulose and probed with antibodies directed against the α and β subunits of complex V. Levels of cytochrome *c* protein (*B*) were also determined using standard one-dimensional SDS-PAGE and immunoblotting techniques. *C*, comparison of the relative quantities of the α subunit and β subunit of the F_1 portion of the ATP synthase from mitochondria of control and ethanol-fed rats. Results represent the mean \pm S.E. for five pairs of control and ethanol-fed animals; *, $p < 0.05$.

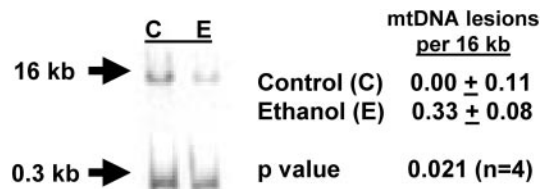


FIG. 8. Chronic ethanol consumption induces hepatic mitochondrial DNA damage. Genomic DNA was prepared from liver tissues of control (*C*) and ethanol (*E*)-fed rats, and mtDNA damage was assessed by QPCR as described under "Experimental Procedures." The 16-kb bands are mtDNA QPCR products from 15 ng of genomic DNA template. The bottom series of bands are from a separate short QPCR that was used to normalize for differences in the amount of mtDNA copies between individual samples. Results represent the mean \pm S.E. for four pairs of control and ethanol-fed animals.

sional proteomics, both two-dimensional IEF/SDS-PAGE and BN-PAGE, changes in mitochondrial as well as nuclear gene products were evident in animals chronically fed ethanol. Several of these results were confirmed when proteins were subjected to immunoblot analysis.

While previous studies provided evidence that decreased functioning of mitochondrial ribosomes is responsible for the down-regulation of the mitochondrial encoded gene products following ethanol exposure (22, 23), reasons to explain decreases in the nuclear encoded subunits of the oxidative phosphorylation system are not as clear. These changes could result from decreased import or increased rates of protein degradation following import during conditions of ethanol-induced ox-

idative stress in mitochondria (54). Moreover degradation of nuclear encoded proteins may be linked to the low levels of mitochondrial gene products observed in mitochondria from ethanol-fed rats. Studies have shown that inhibition of mitochondrial protein synthesis leads to increased proteolytic degradation of the unassembled nuclear encoded gene products of cytochrome *c* oxidase following import (55). In addition, it is possible that chronic ethanol exposure may disrupt cross-talk between the nuclear and mitochondrial genomes and down-regulate the nuclear encoded respiratory subunits at either transcriptional or translational levels. However, the effect of chronic ethanol exposure on the pathways involving the novel transcription factors NRF-1 and GABP/NRF-2, which are involved in mitochondria-nucleus cross-talk, is not known at present (56, 57).

Another site for ethanol exposure to influence the functioning of the oxidative phosphorylation system is at the level of the mitochondrial chaperones that are responsible for the assembly of the respiratory complexes. The mitochondrial chaperone protein 60-kDa heat shock protein (Hsp60) was decreased ~20% in mitochondria from ethanol-fed animals. In contrast, levels of Hsp70/GRP75 were increased. Hsp70/GRP75 and Hsp60 cooperate in the folding of nuclear encoded proteins imported into mitochondria (58). A disruption in the coordination in functioning of these two important chaperone proteins would be expected to impair the assembly of the respiratory complexes and negatively affect mitochondrial function following chronic ethanol consumption.

These disturbances in structure and loss of function of hepatic mitochondria seem to be associated with increased reactive oxygen species production and oxidative injury to mitochondria as a result of chronic ethanol consumption (5, 6). Moreover it is well known that mtDNA is more susceptible to oxidative damage than nuclear DNA (59–61). Indeed recent studies have shown that a single, large dose of ethanol depletes mtDNA (62). Interestingly these effects are transient with normal levels of mtDNA restored within 4 h of ethanol exposure. While the effects of acute, high dose ethanol exposure on mtDNA have been studied, less is known about the effect of chronic ethanol on mtDNA.

In the present study, QPCR was used to assess mtDNA damage (36, 63). Using this highly sensitive technique, we observed that mtDNA damage was significantly increased in liver from rats maintained on ethanol-containing diets for only 5–6 weeks. These results are important because they demonstrate for the first time that moderate ethanol exposure induces mtDNA damage in the very early stages of ethanol-induced liver injury (*i.e.* fatty liver stage) in young animals (3–4 months of age). Using less sensitive techniques, ethanol-related mtDNA damage has only been observed in rodents maintained on ethanol-containing diets for 6–12 months or in old animals (>1 year of age) (20, 21). While the role of mtDNA damage in the development of ethanol-induced mitochondrial dysfunction is not clear, it is predicted that defects in mtDNA would further compromise the functioning of the oxidative phosphorylation system due to a loss of the mitochondrial gene products that make up segments of the respiratory chain; this effect was evident in the present study.

Other Findings—In addition to the effects described above, ethanol consumption altered the levels of two enzymes involved in the catabolism of the branched amino acid valine, 3-hydroxyisobutyrate dehydrogenase and methylmalonate-semialdehyde dehydrogenase. What effect these alterations would have on the overall metabolism of valine is not clear. However, studies have shown that the catabolism of branched chain amino acids is disrupted from a variety of liver diseases (64).

Conclusions—In this first analysis of the mitochondrial proteome in response to stress we observed that chronic ethanol consumption altered the levels of several key proteins involved in β -oxidation of fatty acids and oxidative phosphorylation, two critical functions of liver mitochondria. Moreover the studies reported here succeeded in identifying novel changes in several other mitochondrial proteins including molecular chaperones and proteins involved in amino acid metabolism. These studies also demonstrate that moderate consumption of ethanol for a short period of time induced mtDNA damage in liver. Overall it is clear that the response of hepatocyte mitochondria to a complex metabolic stress such as chronic ethanol exposure involves multiple changes in the components of a pathway, not a single modification of a specific enzyme. These data suggest that our understanding of the responses of cells to stress must encompass an analysis of the changing control over metabolic processes that occur and may result in loss of regulation of the pathway concerned.

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