A Novel Transcription Mechanism Activated by Ethanol

INDUCTION OF Slc7a11 GENE EXPRESSION VIA INHIBITION OF THE DNA-BINDING ACTIVITY OF TRANSCRIPTIONAL REPRESSOR OCTAMER-BINDING TRANSCRIPTION FACTOR 1 (OCT-1)

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Background: The regulatory role of ethanol on gene expression has not been fully defined.

Results: Ethanol reduces OCT-1 binding to the Scl7a11 promoter. Mutation of the OCT-1 binding motif abolishes ethanol-induced Scl7a11 promoter activity.

Conclusion: Ethanol increases Scl7a11 expression by reducing OCT-1 binding to its promoter.

Significance: Ethanol up-regulates gene expression by inhibiting the DNA binding activity of transcriptional repressor(s).

Excessive alcohol consumption causes a number of disorders, including liver cirrhosis (1). Emerging evidence suggests that activation of hepatic stellate cells is a central event in alcoholic fibrosis and cirrhosis (2). In addition, an increase in oxidative stress has been shown to affect cell proliferation and differentiation (3), and is thought to play a causal role in alcohol-induced liver cirrhosis (4).

Solute carrier family 7, member 11 (Slc7a11) is a plasma membrane cystine/glutamate exchanger that provides intracellular cystine to produce glutathione, a major cellular antioxidant. Oxidative and endoplasmic reticulum stresses up-regulate Slc7a11 expression by activation of nuclear factor erythroid 2-related factor 2 and transcription factor 4. This study examined the effect of ethanol on Slc7a11 expression and the underlying mechanism involved. Treatment of mouse hepatic stellate cells with ethanol significantly increased Slc7a11 mRNA and protein levels. Deletion of a 20-bp DNA sequence between -2044 to -2024 upstream of the transcription start site significantly increased basal activity and completely abolished the ethanol-induced activity of the Scl7a11 promoter. This deletion did not affect Slc7a11 promoter activity induced by oxidative or endoplasmic reticulum stress. DNA sequence analysis revealed a binding motif for octamer-binding transcription factor 1 (OCT-1) in the deleted fragment. Mutation of this OCT-1 binding motif resulted in a similar effect as the deletion experiment, i.e. it increased the basal promoter activity and abolished the response to ethanol. Ethanol exposure significantly inhibited OCT-1 binding to the Scl7a11 promoter region, although it did not alter OCT-1 mRNA and protein levels. OCT-1 reportedly functions as either a transcriptional enhancer or repressor, depending on the target genes. Results from this study suggest that OCT-1 functions as a repressor on the Scl7a11 promoter and that ethanol inhibits OCT-1 binding to the Scl7a11 promoter, thereby increasing Scl7a11 expression. Taken together, inhibition of the DNA binding activity of transcriptional repressor OCT-1 is a mechanism by which ethanol up-regulates Scl711 expression.

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2 The abbreviations used are: TSS, transcription start site; ER, endoplasmic reticulum; MHSC, mouse hepatic stellate cell; DEM, diethyl maleate; TM, tunicamycin; AhR, aryl-hydrocarbon receptor.
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Slc7a11 promoter region has been suggested to be responsible for the induction of Slc7a11 expression by amino acid deprivation (14) and by salubrinal (15). In addition, it has been suggested that ATF4 regulates the basal expression of Slc7a11 (15).

Ethanol has been shown to induce gene expression via various mechanisms, including oxidative as well as ER stress (16–19). In addition, ethanol has been shown to induce Slc7a11 expression in various cell types. For example, Slc7a11 was increased significantly, 1.54-fold, after exposure to 75 mM ethanol for 9 days using HepG2 cells (20). The mechanism(s) responsible for ethanol-induced Slc7a11 has not been defined.

In this study, we report that ethanol exposure significantly increases Slc7a11 expression in mouse hepatic stellate cells (MHSCs) and that octamer-binding transcription factor 1 (OCT-1) is a repressor of Slc7a11 transcription. Ethanol exposure inhibited OCT-1 binding to the Slc7a11 promoter in MHSCs and, therefore, up-regulated Slc7a11 expression.

**EXPERIMENTAL PROCEDURES**

**Isolation and Culture of Mouse Hepatic Stellate Cells—**Hepatic stellate cells represent only 5–8% of liver cells. The immortal cell line of MHSCs used in this study was established from H-2Kb-tsA58 transgenic mice, as we described previously (21). Briefly, under anesthesia of ketamine hydrochloride, mice were perfused through the portal vein with a perfusion solution containing 137 mM NaCl, 5.4 mM KCl, 0.6 mM NaH2PO4, 0.8 mM NaHPO4, 10 mM HEPES, 4.2 mM NaHCO3, 5.5 mM glucose, 3.8 mM CaCl2, and 180 mg/liter collagenase (pH 7.4) at 37 °C at a flow rate of 7 ml/min for 10 min. The liver was then excised and incubated in the perfusion solution supplemented with 400 mg/liter Pronase E and 20 mg/liter DNase I at 37 °C for 20 min. The mixture was then filtered through a mesh (pore size, 150 mm), and centrifuged at 1400 g for 20 min (22). Gey’s balanced salt solution/8.2% Nycodenz/17% Nycodenz) and centrifuged at 4500 g for 20 min. The mixture was then filtered through a mesh (pore size, 150 mm), and centrifuged at 1400 g for 7 min. The supernatant, enriched with stellate, Kupffer, and endothelial cells, was overlaid with a triple-layered density cushion (Gey’s balanced salt solution/8.2% Nycodenz/17% Nycodenz) and centrifuged at 1400 g for 20 min (22). Gey’s balanced salt solution contains 120 mM NaCl, 5 mM KCl, 0.84 mM NaH2PO4, 0.22 mM KH2PO4, 1.85 mM MgCl2, 1.53 mM CaCl2, 27 mM NaHCO3, and 5.5 mM glucose. Stellate cells in the upper white layer were resuspended in DMEM and cultured in 100-mm dishes at a density of 1 × 10³ cells/dish. The H-2Kb-tsA58 mice express a thermolabile SV40 tumor antigen (tsA58) driven by a mouse thermostable SV40 tumor antigen (tsA58) promoter (23). Cells obtained from these mice continuously divide at 34 °C (23).

**Quantitative Real-time RT-PCR Assay—**MHSCs grown to confluence in six-well plates were made quiescent in serum-free DMEM for 12 h and then treated with ethanol, diethyl maleate (DEM), tunicamycin (TM), or culture medium alone (control) for 6 h at the doses indicated in the figure legends. Total RNA was extracted using RNeasy plus mini kits (Qiagen, Valencia, CA) and subjected to quantitative real-time PCR using the iCycler system (Bio-Rad) as described previously (24). The specific primers used for amplification were as follows: Slc7a11, 5’-TTTATGGGACTATATTTAC-3’ (forward) and 5’-CGCTCAGGCGCACTAACCTGTTTT-3’ (reverse); OCT-1, 5’-GACCTCAGCAAAAACACCAT-3’ (forward) and 5’-CTACGATTCCAAGGCTTCTACT-3’ (reverse); GAPDH, 5’-GAGCCAAAAGGTTATCTATAC-3’ (forward) and 5’-TAAACGTTTGGTGGTCAGG-3’ (reverse). The expression levels of target mRNAs were normalized to GAPDH mRNA.

**Western Blot Analysis—**Quiescent MHSCs in serum-free DMEM were treated with ethanol, DEM, TM, or culture medium alone (control) at the doses indicated in the figure legends for 6 h. For whole cell protein extraction, cells were lysed in M-PER mammalian protein extraction reagent (Thermo Scientific, Rockford, IL). Samples containing 5 µg (for β-actin detection) or 40 µg of protein (for detection of proteins of interest) were resolved on 10% SDS-PAGE gels and then transferred to a PVDF membrane (Millipore, Billerica, MA). Antibodies against β-actin, Slc7a11, OCT-1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), followed by detection with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc.), were used as described previously (24). Immunoreactive bands were visualized using ECL plus chemiluminescence reagent (GE Healthcare/Amersham Biosciences, Piscataway, NJ) and analyzed using a GS-700 imaging densitometer (Bio-Rad).

**Recombinant Plasmid Construction and Cell Transfection—**For functional analysis of the Slc7a11 promoter, four Slc7a11 promoter fragments (P1–P4) were generated by PCR from wild-type C57BL/6 mouse genomic DNA using platinum TaqDNA polymerase (Invitrogen). The 5’ end of these fragments starts at nucleotide +22 downstream from the TSS of the Slc7a11 gene. The 3’ end of the P1, P2, P3, and P4 fragments start at nucleotides −2044 to −2024, −306, and −134 upstream from the TSS of the Slc7a11 gene, respectively. The following primers were used for generation of these DNA fragments: the shared reverse, atcccagcttgccctcatcattacacaccag; P1 forward, atgcctcggagcctgcaattgctcattc; P2 forward, atgcctcggagcctgcaattgctcattc; P3 forward, atgccgaggatctgcattaaaactc; and P4 forward, atgcctcggagattgtggcccatttctcagag.

The PCR products were cloned into the XhoI/HindIII sites of the pGL2 luciferase vector (Promega, Madison, WI). Mutations in the binding site for OCT-1 or for ATF4 in the Slc7a11 promoter were generated by site-directed mutagenesis of the pGL2 luciferase vector (pGL2−actin). The specific primers used for amplification were as follows: OCT-1, 5’-TGCTAGCTGAGGCTGAGTGTGTTCTCTGAGAATTATG-3’ (forward) and 5’-CTATGAGCCAATGAATCTCGAGTA-GAAGACTATG-3’ (reverse); and ATF4, 5’-AGATTCTCTCAGGCGACATGTCGAGAGATCTTC-3’ (forward) and 5’-ATGTCGACATGTCGAGAGATCTTC-3’ (reverse). Mutated nucleotides are underlined. The mutants were selected by analysis of DpnI digestion (25). The PCR products were cloned into the XhoI/HindIII sites of the pGL2 luciferase vector (Promega, Madison, WI). Mutations in the binding site for OCT-1 or for ATF4 in the Slc7a11 promoter were generated by site-directed mutagenesis of the wild-type pGL2−Slc7a11 promoter plasmid P1. The following primers were used for induction of mutations: OCT-1, 5’-TGCTAGCTGAGGCTGAGTGTGTTCTCTGAGAACATTATG-3’ (forward) and 5’-CTATGAGCCAATGAATCTCGAGTAGAATGTCGAGATCTTC-3’ (reverse); and ATF4, 5’-AGATTCTCTCAGGCGACATGTCGAGAGATCTTC-3’ (forward) and 5’-ATGTCGACATGTCGAGAGATCTTC-3’ (reverse). Mutated nucleotides are underlined. The mutants were selected by analysis of DpnI digestion (25).

The recombinant plasmid constructs were transfected into MHSCs using a Lipofectamine 2000 kit (Invitrogen) according to the protocol of the manufacturer. Briefly, MHSCs grown in
six-well plates to ~60–70% confluency were incubated with 2 ml/well serum-free DMEM containing 10 μl of Lipofectamine 2000 and 4 μg of pGL2 plasmid encoding either the wild-type or mutated Slc7a11 promoter fragments. At 6 h post-transfection, cells were replenished with fresh medium containing 10% FBS and then cultured for an additional 24 h. The transfected cells were treated for 12 h with ethanol, DEM, TM, or culture medium alone (control) at the doses indicated in the figure legends prior to reporter gene analysis.

Luciferase Assay—For the luciferase assay, transfected cells were lysed with 100 μl of reporter lysis buffer provided by the luciferase assay kit (luciferase assay system, Promega). Lysate (10 μl) was incubated in a 96-well plate at room temperature for 2 min with 100 μl of luciferase assay reagent (Promega). Luminescence was measured using the BL10000 LumiCount (Packard BioScience, Meriden, CT). The protein level in the lysate was determined using a BCA protein assay kit (Thermo Scientific). The luciferase activity was expressed as the luminescence intensity relative to the protein level.

ChIP—Quiescent MHSCs were treated with 0, 50 mM, 100 mM, or 200 mM ethanol for 4 h. The binding of OCT-1 to the Slc7a11 promoter region was determined by ChIP as described (24). Briefly, ethanol-treated cells were cross-linked by 1% formaldehyde at room temperature and then lysed in 500 μl of cell lysis buffer (5 mM PIPES, 85 mM KCl, 0.5% Nonidet P-40, 1 mM PMSF, and protease inhibitor mixture (pH 8.0)). Nuclei were isolated and homogenized in 300 μl of nuclear lysis buffer (50 mM Tris-HCl, 1% SDS, 10 mM EDTA, and protease inhibitor mixture (pH 8.1)). The resulting nuclear lysis was sonicated until cross-linked chromatin was sheared to an average length of 0.3–1.0 kb. Supernatant (5 μl) was used as an input control. The remaining lysate was diluted 10-fold with ChIP dilution buffer (16.7 mM Tris-HCl, 167 mM NaCl, 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, and protease inhibitor mixture (pH 8.1)), and precleared with salmon sperm DNA/protein A-agarose (Santa Cruz Biotechnology, Inc.). The precleared sample was incubated with OCT-1 antibody followed by salmon sperm DNA/protein A-agarose. Bound protein-DNA complexes were eluted with a solution containing 0.1M NaHCO3 and 1% SDS. Following reversion of the protein-DNA cross-links, DNA fragments in the eluate and input controls were purified using the QIAquick PCR purification kit (Qiagen) and then subjected to quantitative real-time PCR using an iCycler system (Bio-Rad). The PCR primers used were as follows: 5’-TTAGTGTTGTTAGCTGTTGTT-3’ (forward) and 5’-CTCTTGGTACAAAATGGA-3’ (reverse). A 245-bp DNA fragment contains a putative OCT-1 binding site (GGCTGATTATGTA) (M00137) in the NQO1 promoter.

Measurement of Intracellular ATP Levels—The cellular ATP levels were determined using a bioluminescence ATP detection kit (Promega). MHSCs grown in a white-bottom, clear 96-well plate at confluence were incubated at 37 °C in serum-free DMEM (100 μl/well) with the indicated concentrations of ethanol or culture medium alone as a control for 6 h. In the experiments for studying the cytotoxicity resulting from the combinational treatment of DNA transfection and ethanol exposure, MHSCs were transfected with empty pGL2 plasmid as described above and then treated with the indicated concentrations of ethanol. Thereafter, 100 μl of kit reagent was added to each well. After a 10-min incubation, luminescence was quantified using a BL10000 Lumicount luminometer (Packard BioScience, Downers, IL). Cellular ATP contents were expressed as the luminescence intensity (arbitrary unit) generated in the assay. At the end of the experiments, cells were lysed in M-PER mammalian protein extraction reagent (Thermo Scientific). Protein levels in the lysate were determined using a BCA protein assay kit (Thermo Scientific). The levels of ATP were normalized by the protein levels.

Statistical Analysis—For experiments using the microplate reader, the mean value for each experiment was averaged from triplicate wells in the same plate. Data are reported as the mean ± S.E. of the mean for at least five independent experiments. Differences between the control and treatment groups were analyzed by one-way or multiple factor analysis of variance followed by Tukey’s post-hoc tests. Statistical significance was considered when p was less than 0.05. Statistix software (Statistix, Tallahassee, FL) was used for statistical analyses.

RESULTS

Ethanol Exposure Up-regulates Slc7a11 Expression in MHSCs—Data from previous studies using various cell types indicate that Slc7a11 plays a protective role against injuries induced by a variety of factors (26–29), including those induced by ethanol (20). This report studied the effect of ethanol on Slc7a11 expression in MHSCs. The data in Fig. 1A show that the basal mRNA level of Slc7a11 is relatively low, i.e. about 0.02% of the GAPDH mRNA level, in MHSCs. Ethanol exposure resulted in a dose-dependent increase in Slc7a11 mRNA levels. Specifically, the Slc7a11 mRNA levels in MHSCs treated with 50, 100, and 200 mM ethanol were 2.8-, 5.1-, and 20.0-fold higher, respectively, than that in untreated control cells (Fig. 1A). Similarly, the same doses of ethanol elevated Slc7a11 protein levels in MHSCs 3.6-, 4.9-, and 8.8-fold, respectively (Fig. 1B and C). These data suggest that ethanol is able to induce Slc7a11 expression at both the mRNA and protein level.

Ethanol Activates the Slc7a11 Promoter via a Mechanism Involving the OCT1-Binding Motif—To explore the DNA motif(s) responsible for ethanol-induced Slc7a11 transcription, a series of promoter-reporter constructs were generated by progressive 5’ deletion of the Slc7a11 promoter (Fig. 2A). As the data in Fig. 2B show, ethanol exposure significantly increased the reporter gene activity of the P1 construct, which includes 2044 bp upstream of the TSS of the Slc7a11 gene. We also
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![Graph](image)

FIGURE 1. The effect of ethanol exposure on Slc7a11 expression. A, MHSCs were incubated with 0, 50, 100, or 200 mM of ethanol for 6 h. The level of Slc7a11 mRNA was determined by quantitative real-time RT-PCR and normalized to GAPDH mRNA. B, Western blot analysis and quantitated relative to β-actin. Values represent the mean ± S.E. of five independent experiments. Differences among cells treated with different concentrations of ethanol or culture medium alone were analyzed by one-way analysis of variance followed by Tukey’s post hoc tests. *, p < 0.05 versus cells without ethanol treatment; †, p < 0.05 versus cells treated with 50 mM ethanol; ‡, p < 0.05 versus cells treated with 100 mM ethanol.

observed that prolongation of the P1 construct by 126 bp (using MluI/BglII as the cloning sites) at the 5’ end of the Slc7a11 promoter did not significantly alter the basal and ethanol-induced reporter gene activities (data not shown). In contrast, deletion of 20 or more bp from the 5’ end significantly increased the basal activity while completely abolishing the ethanol-induced activity of the Slc7a11 promoter. Specifically, the basal activities of the reporter gene in cells transfected with the P2, P3, and P4 constructs were 72, 71, and 38% higher, respectively, than those observed in cells transfected with the P1 construct. Ethanol did not induce a significantly higher reporter gene activity in cells transfected with the P2, P3, and P4 constructs. These data suggest that the 20 nucleotides spanning from −2024 to −2044 upstream of the TSS are indispensable for ethanol-induced Slc7a11 promoter activity.

A computer database search of the Slc7a11 promoter showed that an OCT1-binding site (ATGCTCAT) located between 2029 and 2040 nucleotides upstream of the TSS is included in the deleted 20-nucleotide promoter region. The Slc7a11 promoter OCT-1 binding site is highly homologous to the octamer motif (ATGCCTAT) found in the human H2B gene (30). We assessed the impact of this DNA motif on Slc7a11 transcription using a mutated promoter-reporter construct (P1-OM), as shown in Fig. 3A. The data in Fig. 3B show that mutation of the OCT1-binding site abolished ethanol-induced activity but significantly increased the basal activity of the Slc7a11 promoter when compared with that derived from the wild-type Slc7a11 promoter counterpart, P1. These findings are consistent with the data obtained from the deletion experiments (Fig. 2B) and support the view that the OCT1-binding motif plays an inhibitory role on the basal activity of the Slc7a11 promoter and a stimulatory role on ethanol-induced Slc7a11 promoter activity.

Ethanol Activates the Slc7a11 Promoter but Not through Nrf2, ATF4, AP1, and NF-κB—A computer database search showed that the eight putative AP1 binding sites in the mouse Slc7a11 promoter region are distributed from 141 to 133 and from 94 to 86 nucleotides upstream of the TSS, whereas the putative NF-κB binding site is located 438 nucleotides upstream of the TSS (Fig. 2A) (31). The schematic in Fig. 2A also shows that the mouse Slc7a11 promoter contains a functional Nrf2-binding site and an ATF4-binding site, respectively, which span from 141 to 133 and from 94 to 86 nucleotides upstream of the TSS, respectively. The data in Fig. 2 also show that ethanol did not increase the reporter gene activity in cells transfected with the P2 construct, which contains all the aforementioned binding motifs for Nrf2, ATF4, AP1, and NF-κB. Collectively, these data suggest that the induction of Slc7a11 by ethanol is not regulated by Nrf2, ATF4, AP1, and/or NF-κB.

The Nrf2 and ATF4 binding motifs have been reported to be involved in the induction of Slc7a11 promoter activity (29, 32). The schematic in Fig. 2A also shows that the mouse Slc7a11 promoter contains a functional Nrf2-binding site and an ATF4-binding site, respectively, which span from 141 to 133 and from 94 to 86 nucleotides upstream of the TSS, respectively. The data in Fig. 2 also show that ethanol did not increase the reporter gene activity in cells transfected with the P2 construct, which contains all the aforementioned binding motifs for Nrf2, ATF4, AP1, and NF-κB.
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FIGURE 3. The effect of OCT-1 binding motif mutation on Slc7a11 promoter activity. A, the wild-type (P1) and mutated (P1-OM) DNA sequences of the OCT-1 binding motif in the mouse Slc7a11 promoter. Luc, luciferase. B, plasmids containing the wild-type or mutant Slc7a11 promoter were transfected into MHSCs. The transfected cells were treated with 100 mM of ethanol, 100 μM of Nrf2 inducer (DEM), 2 μg/ml of ATF4 inducer (TM), or culture medium alone (control) for 12 h. Luciferase activity was measured using a luminescence assay and expressed relative to total protein level. Values represent the mean ± S.E. of five independent experiments. Differences among cells transfected with different DNA constructs and treated with different reagents were analyzed by multiple factor analysis of variance followed by Tukey’s post hoc tests. *, p < 0.05 versus control cells transfected with the same plasmid; †, p < 0.05 versus cells transfected with wild-type plasmid P1 and treated with the same reagent.

Ethanol Exposure Reduces OCT-1 Binding to the Slc7a11 Promoter but Does Not Alter OCT-1 Expression—Having demonstrated the regulatory role of OCT-1 in ethanol induced Slc7a11 transcription (15), the data in Fig. 4B also indicate that ethanol increased reporter gene activity in cells transfected with the P1-AM construct, although the activity is significantly lower than in cells transfected with the wild-type P1 construct. Thus, mutation of the ATF-binding motif abolished the induction role of tunicamycin in Slc7a11 promoter activity but did not alter the induction of ethanol.

In contrast to the lack of changes in OCT-1 expression as a function of ethanol concentrations, ethanol exposure reduced OCT-1 binding to the Slc7a11 promoter in a dose-dependent manner (Fig. 6A). As the data show, the amount of OCT-1 bound to the Slc7a11 promoter region was 28.2, 36.8, and 44.6% lower in cells treated with 50, 100, and 200 mM ethanol, respectively, than that seen in untreated control cells. These results suggest that ethanol inhibits OCT-1 binding to the Slc7a11 promoter. Taken together, it is highly likely that OCT-1 functions as a transcription repressor of the Slc7a11 promoter under normal conditions. Ethanol exposure, however, reduces OCT-1 binding to the Slc7a11 promoter and, therefore, increases Slc7a11 expression.
Ethanol Reduces OCT-1 DNA Binding in a Sequence-specific Manner—It has been reported that oxidative modification of OCT-1 nonspecifically inhibits its DNA binding activity, i.e. oxidative stress reduces the binding of OCT-1 not only to the octamer motif but also to other OCT-1 binding elements (32). Ethanol is able to induce oxidative stress (16, 18). This study, therefore, also monitored the binding of OCT-1 to the promoters of the AhR and the AhR target gene, NQO1. The AhR and NQO1 promoter OCT-1 binding elements contain nucleotides TAATGTGGC and GCTGATTATGT, respectively. They differ greatly from the octamer motif. We reported previously that acute ethanol exposure induced a dose-dependent decrease in AhR mRNA and protein levels in MHSCs (21). In contrast, ethanol did not affect the expression of NQO1 (data not shown). The data in Fig. 6B indicate that treatment of MHSCs with 50 and 100 mM ethanol did not significantly alter the amount of OCT-1 bound to the AhR promoter, whereas 200 mM ethanol diminished OCT-1 binding to the AhR promoter by ~25%. In contrast, ethanol induced a dose-dependent increase in OCT-1 binding to the NQO1 promoter (Fig. 6C). These results suggest that the effect of ethanol on the DNA binding activity of OCT-1 varies among genes and that the reduction in OCT-1 binding to the Slc7a11 promoter in the presence of ethanol is a gene-specific effect.

Effect of Ethanol on Cellular ATP Content—Ethanol has been shown to reduce viability and functionality and induce cytotoxicities in liver parenchymal cells (33). However, hepatic stellate cells are relatively resistant to ethanol-induced cytotoxicity as compared with the liver parenchymal cells (34, 35). This study determined the effect of ethanol on the metabolic viability of MHSCs by measuring cellular ATP content. The data in Fig. 7 show that ethanol at concentrations of 50, 100, and 200 mM did not reduce the ATP levels in MHSCs incubated for 6 h.

A preliminary study from our laboratory suggested that transfection of MHSCs with PGL2 plasmids reduced ethanol-induced Slc7a11 expression. Specifically, the Slc7a11 mRNA levels increased by 50, 100, and 200 mM ethanol in PGL2-transfected MHSCs were ~50% less than in non-transfected cells (data not shown). This study, thus, determined the effect of ethanol on cellular ATP contents in MHSCs transfected with PGL2 plasmids. Fig. 7 shows that the ATP level in PGL2-transfected MHSCs was ~10% lower than in non-transfected cells. However, ethanol treatment did not significantly reduce ATP content in the transfected cells. These data, together with the results presented in Figs. 1–6, suggest that the reduced OCT-1 binding to the Slc7a11 promoter region, and the increased Slc7a11 expression and promoter activity in the MHSCs treated with ethanol, are not due to a reduced cell viability.

DISCUSSION

Data from this study demonstrated that ethanol exposure significantly increased Slc7a11 expression at the mRNA and protein level in MHSCs. This finding is consistent with a previous report from our laboratory that ethanol up-regulates...
Slc7a11 expression in HepG2 cells (20). The main function of the Slc7a11 cystine/glutamate antiporter is to maintain the intracellular level of glutathione and protect cells from oxidative damage (8, 12). Thus, up-regulation of Slc7a11 expression might be a compensatory effect in response to ethanol-induced oxidative stress.

Changes in gene expression could result from a changed activity of transcription factors. Indeed, ethanol has been reported to regulate the activity of many transcription factors (17, 18, 36–39). For example, ethanol has been shown to reduce or increase the binding activity of NF-κB to an NF-κB oligonucleotide probe in rat pancreatic acinar cells (36) and gastric mucosal epithelia, respectively (37). In addition, it has been suggested that ethanol increases the activity of transcription factors and STAT3, specificity protein-1 (Sp1), and AP1, thereby up-regulating IL-10 expression in human monocytes (38). Moreover, maternal ethanol exposure was found to increase both Nrf2 protein levels and Nrf2-antioxidant response element binding in mouse embryos, resulting in an increase in the expression of a number of Nrf2 target genes (18). Furthermore, ethanol was shown to increase Nrf2 and ATF4 expression (17, 18, 39). The Slc7a11 promoter contains binding motifs for many transcription factors, including AP1, Nrf2, NF-κB, and ATF4 (13, 14, 31). It has been reported that ATF4 controls the basal expression of Slc7a11 and that activation of Nrf2 (13) and ATF4 (15), in response to oxidative and ER stresses, up-regulates Slc7a11 expression. The data from this study confirmed the role of ATF4 in the basal and ER stress-induced expression of Slc7a11. However, our data clearly indicate that induction of Slc7a11 by ethanol in MHSCs is neither through ATF4 nor AP1, NF-κB, or Nrf2 because ethanol could not activate the Slc7a11 promoter-reporter constructs that contained the binding motifs for these transcription factors unless the OCT-1 binding motif completely abolished OCT-1 binding motif was also present.

This report is the first to demonstrate the regulatory role of transcription factor OCT-1 in ethanol-induced Slc7a11 expression. Specifically, we observed that ethanol reduced the amount of OCT-1 bound to the Slc7a11 promoter but did not significantly affect the expression of OCT-1. Deletion or mutation of the OCT-1 binding motif completely abolished ethanol-induced Slc7a11 promoter activity. Previous reports demonstrated that OCT-1 can function as either a transcription enhancer or a repressor, depending on the target genes involved (40–43). For example, it has been reported that OCT-1 up-regulates the expression of snRNA genes (40). In contrast, binding of OCT-1 to the octamer motif in the mouse μ-opioid receptor gene negatively modulates its expression (42). Furthermore, OCT-1 can functionally interact with the retinoid X receptor. This interaction interferes with the contact of the retinoid X receptor with the thyroid hormone receptor, which, in turn, reduces the binding of the thyroid hormone receptor/retinoid X receptor to the promoter region of thyroid hormone receptor target genes and, therefore, negatively regulates the expression of these genes (43). Our data clearly demonstrate that OCT-1 functions as a repressor of the Slc7a11 promoter. It is highly likely that binding of OCT-1 to the Slc7a11 promoter constitutively inhibits its transcriptional activity. Ethanol exposure reduces OCT-1 binding to this motif, and, therefore, Slc7a11 expression increases.

The OCT-1 binding motif ATGCTCAT in the mouse Slc7a11 promoter region is highly homologous to the octamer element originally found in the human H2B gene (30). Besides this octamer motif, several variants of DNA elements are able to bind OCT-1 protein (44). Data from this study demonstrated that the effect of ethanol on OCT-1 binding differs among genes. Specifically, ethanol increased OCT-1 binding to the NQO1 promoter in a dose-dependent manner. In the case of the AhR promoter, only a high concentration of ethanol (200 mM) had a significant effect on OCT-1 binding to that promoter. These observations suggest that a reduction in OCT-1 binding to the Slc7a11 promoter is a sequence-specific effect rather than a nonspecific effect of ethanol on the DNA binding activity of OCT-1.

Several kinases and phosphatases have been shown to regulate the binding activity of OCT-1 to the cognate octamer motif (32). For example, PKC has been shown to inhibit the binding of OCT-1 to the octamer motif oligonucleotide probe in vitro (32) and to the promoter in vivo (45). Besides PKC, other protein kinases, such as PKA, have been shown to inhibit the binding of OCT-1 to the octamer motif. In contrast, casein kinase 2 (CK-II) enhances the binding of OCT-1 to this octamer motif (32). In addition, the calcium/calmodulin-activated phosphatase calcineurin is effective in increasing OCT-1-dependent transcription (46). Moreover, phosphatase 2A has been suggested to augment the binding of OCT-1 to the octamer motif (47). Further studies are required to investigate the mechanism underlying the inhibitory effect of ethanol exposure on OCT-1 binding to the Slc7a11 promoter, including the study of the involvement of the above-mentioned kinases and phosphatases in ethanol-induced changes in the OCT-1 DNA binding activity.

In summary, data from this report demonstrated that ethanol exposure up-regulates Slc7a11 expression, increases Slc7a11 promoter activity, and diminishes OCT-1 binding to the Slc7a11 promoter. Deletion or mutation of the Slc7a11 promoter OCT-1 binding motif completely abolished ethanol-in-
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duced Slc7a11 promoter activity. In addition, mutation of the Slc7a11 promoter ATF4 binding motif remarkably reduced the basal level of Slc7a11 promoter. These data suggest that under normal conditions, bound OCT-1 inhibits, whereas ATF4 enhances, Slc7a11 promoter activity. Ethanol exposure removes OCT-1 from the Slc7a11 promoter and, therefore, increases expression of Slc7a11.

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