Members of the sterol regulatory element-binding protein (SREBP) family of transcription factors control the synthesis and uptake of cholesterol, fatty acids, triglycerides, and phospholipids. Continuous intragastric infusion of ethanol-containing diets as part of total enteral nutrition generates well defined 6-day cycles (pulses) of urine ethanol concentrations (UECs) in rats. Pulsatile UECs are the result of cyclical expression and activity of the principal alcohol-metabolizing enzyme, hepatic Class I alcohol dehydrogenase (ADH), and this mechanism involves regulated CCAAT/enhancer-binding protein-β expression and binding to the ADH promoter. In this study, we further explore the molecular mechanism for ethanol-induced ADH expression during the UEC pulse in adult male rats fed ethanol by total enteral nutrition for 21–30 days. In hypophysectomized rats, in which the ADH protein increased by ~6-fold, the nuclear form of SREBP-1 decreased by ~7-fold. Because the ADH promoter contains two canonical sterol response element (SRE) sites (~63 to ~53 and ~52 to ~40 relative to the transcription start site), electrophoretic mobility shift assays were conducted using an ADH-specific SRE site. Hepatic nuclear protein binding decreased by 2.4-fold on the ascending limbs and by 3.6-fold on the descending limbs of UEC pulses (p < 0.05). The specificity of nuclear protein binding to the ADH-SRE site was confirmed using antibody and UV cross-link assays. The in vitro binding status of SREBP-1 to ADH-SRE sites, as measured by the chromatin immunoprecipitation assay, had a pattern very similar to the electrophoretic mobility shift assay results. Functional analysis of the ADH-SREs demonstrated these sites to be essential for ADH transcription. In vitro transcription assays demonstrated that depletion of the SREBP-1 protein from nuclear extracts increased transcription activity by ~5-fold and that the liver X receptor agonist T0901317 (a known activator of SREBP-1c transcription) reduced in vitro expression of ADH mRNA by 2-fold. We conclude that SREBP-1 is a negative regulator of the ADH gene and may work in concert with the CCAAT/enhancer-binding proteins to mediate ethanol induction of ADH in vivo.

Ethanol metabolism is of fundamental importance in understanding the health effects of alcohol. The alcohol dehydrogenase (ADH) EC 1.1.1.1 system is responsible for the majority of ethanol oxidation in mammals. Although multiple isozymes of Class I ADH exist in human liver, only the Class I ADH mRNA and protein appear to be significantly expressed in rat liver, and ethanol is metabolized predominantly in the liver (1, 2). ADH also catalyzes the oxidation and reduction of a variety of physiological steroidal and nonsteroidal substrates (3). The expression of the Class I ADH gene is tissue-specific and hormonally regulated throughout development (1, 4–8). We previously reported that continuous intragastric infusion of an ethanol-containing diet into rats results in unique and predictably recurring cyclic fluctuations in plasma and urine ethanol concentrations and that a similarly cyclic fluctuation in hepatic ADH activity is responsible (9).

Characterization of 1 kb of the 5′-flanking region of the rat Class I ADH gene and deletion analysis in transient transfection experiments revealed two positive elements in this flanking region, a proximal positive element from −241 to −12 and a distal positive element from −1327 to −977 (10). The proximal positive element has greater transcription activity than the distal positive element (10). We have reported that members of the CCAAT/enhancer-binding protein (C/EBP) family of transcription factors (particularly C/EBPβ) are involved in ethanol induction of rat Class I ADH expression by acting on the proximal positive element (11). Specifically, alcohol increases the ratio of liver-activating protein to liver inhibitory protein. A recent report indicates that overexpression of sterol regulatory element-binding protein-1c (SREBP-1c) decreases the relative ratio of full-length C/EBPβ (liver-activating protein) and truncated C/EBPβ (liver inhibitory protein) (12). This suggests a potential indirect linkage of SREBP-1 with regulated ADH gene transcription. Additionally, our preliminary computer analysis of the ADH promoter identified two perfect sterol response element sites, suggesting potential direct effects of SREBPs on this gene. Furthermore, ethanol has been reported to modulate SREBP-1 expression in mouse and rat hepatoma cell lines (13).

SREBPs directly activate the expression of genes dedicated to the synthesis and uptake of cholesterol, fatty acids, triglycerides, and phospholipids (14). Three members of the SREBP family are produced by two genes, designated SREBP-1 (isoforms 1a and 1c) and SREBP-2 (15). SREBP-1a and SREBP-1c are produced through the use of alternative promoters that give rise to alternative first exons that splice to a common

---

*The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

**To whom correspondence should be addressed: Arkansas Children’s Nutrition Center, 1120 Marshall St., Little Rock, AR 72202. Tel.: 501-364-2781; Fax: 501-364-2818; E-mail: badgerthomasm@uams.edu.

1 The abbreviations used are: ADH, alcohol dehydrogenase; C/EBP, CCAAT/enhancer-binding protein; SREBP, sterol regulatory element-binding protein; SRE, sterol response element; LXR, liver X receptor; UEC, urine ethanol concentration; RPA, ribonuclease protection assay; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation; TEN, total enteral nutrition; LDLR, low density lipoprotein receptor; CREB, cAMP-response element-binding protein.
second exon. In the liver, SREBP-1c is the predominately expressed form of SREBP-1 (16). SREBP-1c regulates the synthesis of fatty acids, and SREBP-2 has a major role in the synthesis of cholesterol (17). SREBPs are synthesized as precursors bound to the endoplasmic reticulum membrane and the nuclear envelope (18, 19). The transcriptionally active N-terminal portion is released from the membrane by proteolysis (20). The liver X receptor (LXR) enhances transcription of the SREBP-1c gene by binding to a consensus recognition sequence in the liver X receptor (LXR) enhances transcription of the SREBP-1c portion is released from the membrane by proteolysis (20). The RNeasy mini kit (QIAGEN Inc., Valencia, CA). cDNA was synthesized was treated with RNase-free DNase (Ambion Inc., Austin, TX) to remove any genomic DNA contamination. A 400-bp rat Class I ADH cDNA probe was used for detection of ADH Northern blot analysis was conducted as described previously (11, 26). A 400-bp rat Class I ADH cDNA probe was used for detection of ADH mRNA. 18 S ribosomal RNA antisense oligonucleotide was synthesized (Bio-Synthesis, Inc., Lewisville, TX) using published sequences (27). All primers were designed using the ECL Plus system (Amersham Biosciences). A 400-bp rat ADH cDNA probe was used for detection of ADH protein expression. The ADH cDNA was inserted into the pCR2.1 vector. Inserts were sequenced in their entirety to assess orientation and to eliminate any clones with mutations attributable to PCR subcloning. pTRI-RNA1AS (containing an 80-bp 18 S fragment) was purchased from Ambion Inc. Antisense probes for RPA were generated by linearizing the plasmid with HindIII and using a T7 promoter. A total of 9, 24, 25. Livers were collected and stored at 70 °C.

**Northern Blot Analysis and Real-Time Reverse Transcription-PCR**—Northern blot analysis was conducted as described previously (11, 26). A 400-bp rat Class I ADH cDNA probe was used for detection of ADH mRNA. 18 S ribosomal RNA antisense oligonucleotide was synthesized (Bio-Synthesis, Inc., Lewisville, TX) using published sequences (27). All filters were probed with the 18 S RNA oligonucleotide as an internal control. Bands were quantitated by densitometry of the autoradiogram. The SREBP proteins and mRNAs in rats fed an ethanol-containing diet by continuous intragastric infusion and explored the possible involvement of SREBPs in the regulation of ADH expression.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Reagents**—All chemicals were purchased from Sigma unless otherwise specified. Radionucleotides were purchased from PerkinElmer Life Sciences. T4 polynucleotide kinase was purchased from Promega (Madison, WI). Rabbit polyclonal antibodies against SREBP-1, SREBP-2, and calpain-2 and horseradish peroxidase-conjugated donkey anti-rabbit IgG were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Synthetic oligonucleotides were obtained from Integrated DNA Technologies, Inc. (Coralville, IA). The LXR agonist T0901317 was purchased from Alexis (San Diego, CA). SYBR Green PCR mixture and 18 S RNA primers were purchased from Applied Biosystems (Foster City, CA).

**Animal Experimental Protocols and Hypophysectomy**—Adult male Sprague-Dawley rats (intact or hypophysectomized) were purchased from Harlan Industries Inc. (Indianapolis, IN). The rats were surgically castrated with an intrastriga tube, allowed to recover, and infused with diets formulated to meet the nutritional requirements of the rat as described previously (22). The control rats were infused with the same diet, except that ethanol was isocalorically replaced with carbohydrate. The rats were killed following 21–30 days of continuous diet infusion when their urine ethanol concentrations (UECs) were high on the control day and low on the following day. The UECs were determined as described previously (9, 24, 25). Livers were collected and stored at 70 °C.

**Electrophoretic Mobility Shift Assays (EMSAs)**—EMSAs were performed as described previously (11). Double-stranded oligonucleotides were prepared by combining and heating equimolar amounts of complementary single-stranded DNA to 95 °C for 5 min in distilled H2O, followed by cooling to room temperature overnight. EMSAs were carried out in 20 µl containing 7 µM Tris-HCl (pH 7.5), 0.7 µM MgCl2, 0.35 µM dithiothreitol, 3% glycerol, 0.35 mM EDTA, and 1.5 µg of poly(dI-dC) (Roche Applied Science). The nuclear extracts were preincubated with poly(dI-dC) for 15 min on ice. End-labeled oligonucleotides (0.1 µM) were added, and the mixtures were incubated for another 15 min on ice, after which 3 µl of loading buffer was added, and samples were separated on 5% polyacrylamide gels in 0.5 TBE buffer containing 1% formaldehyde for 10 min at 37 °C. Fixation was stopped by adding glycerol to a final concentration of 0.125 M. Fixed tissue was washed twice with ice-cold phosphate-buffered saline containing protease inhibitor mixture (Sigma). The ChIP assay kit (Upstate Biotechnol-
Ethanol Regulation of ADH and SREBP-1

ogy, Inc.) was used following the steps recommended by the manufacturer. Formaldehyde-cross-linked cells were sonicated with the Sonic Disembrator (Fisher) set at 20% output. A series of sonication times were tested. Samples with DNA that was sheared to between 100 and 1000 bp (requiring ~4 min of sonication) were used for the subsequent immunoprecipitation reactions. Sonicates were diluted 10-fold in ChIP dilution buffer (final volume of 2 ml); a portion (20 µl) was kept as the input; and the remainder wasPrecleared with 80 µl of salmon sperm DNA (20 µg/ml) and protein A-agarose for 1 h at 4°C with rotation. Antibody against SREBP-1 was added to the clarified chromatin and incubated overnight at 4°C with rotation. Immunocomplexes were collected, washed, and eluted according to the protocol of Upstate Biotechnology, Inc., and fixation was reversed by addition of NaCl to a final concentration of 0.2 M and incubation at 65°C for 4 h. Samples were treated with protein kinase K. DNA was purified by phenol/chloroform extraction and ethanol precipitation and dissolved in 20 µl of 1 x M Tris and 0.1 x M EDTA. The input DNA was diluted 25-fold. Eluted DNA was amplified by PCR with a 5'-primer (5'-CTTGAGTGAGAAATGAGTGG-3', -108 to -85) and a 3'-primer (5'-GAAATTTGTGATCTTGTCG-3', +23 to +42) specific for the SRE-containing region in the ADH promoter. We optimized the PCR cycle number for the input DNA and immunoprecipitated to remain in the PCR linear range. PCRs contained 3 µl of immunoprecipitate and diluted input. PCR amplifications were performed (32 cycles for the input, 35 cycles for the immunoprecipitate, and the no-antibody precipitation negative control), and products were analyzed on 2.5% agarose gel.

In Vitro Transcription—The template containing the −241-bp promoter region and the downstream 450 bp was generated as described in our previous study (11). Table I lists the synthetic oligonucleotides used in competition assays. The Rep-C/EBP-5' and Rep-C/EBP-3' primers were used to generate the template containing a consensus C/EBP site, which replaced both SRE sites in the proximal region of the ADH promoter. In vitro transcriptions were carried out in 20 mM Hepes (pH 7.9), 100 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 4.5 mM MgCl2, 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, 20 µCi of [α-32P]UTP, and 100 ng of template DNA. The reactions were initiated by adding nuclear extract (90 µg for the competition assay and 15 µg of depleted nuclear extract to monitor for SREBP activity in ADH transcription initiation). For competition assays, the competitor oligonucleotides were added at the same time with template. Incubations were carried out for 60 min at 30°C, and the reaction mixture was extracted, precipitated, and analyzed on 5% denaturing acrylamide gels as described previously (11).

Cell Culture—Rat hepatoma FGG4 cells (obtained from Mary C. Weiss, Pasteur Institute, Paris, France) were seeded in T-25 flasks at 1.75 x 10⁶ cells/ml in Dulbecco’s modified Eagle’s medium containing 100 units/ml penicillin, 100 µg/ml streptomycin sulfate, and 5% fetal bovine serum and allowed to attach overnight in 5% CO₂ at 37°C. The next day, cells were washed twice with phosphate-buffered saline, and the medium was changed to glucose-free Dulbecco’s modified Eagle’s medium supplemented with 1 x M sodium pyruvate and 5% fetal bovine serum with same amount of antibiotics. Ethanol and/or different concentrations of T0901317 in 0.01% Me2SO were added at the same time. In the experiment to study the effects of T0901317, control cells received 0.01% Me2SO. After changing the medium, cells were allowed to equilibrate for 30 min in the incubator, and then caps were tightly closed for the remainder of the experiment to prevent the loss of ethanol from evaporation. After 24 h, cells were harvested directly into 1.0 ml of TRI reagent (Molecular Research Center Inc., Cincinnati, OH) to isolate RNA.

Nuclear Run-on—Nuclear run-on analyses were conducted as described in our previous study (9).

Statistics—Student's t test was used to determine whether group means differed at a significance level of p < 0.05.

RESULTS

Hepatic ADH mRNA Expression during the UEC Pulse—Rats were killed low on the ascending limb and high on the descending limb of the UEC pulse as indicated by the arrows in Fig. 1. A Northern blot of ADH mRNA in individual liver samples from rats fed diets without ethanol (total enteral nutrition (TEN)) or with ethanol, but processed at those two points of the UEC cycle (ascending and descending limbs of the UEC pulse), is presented in Fig. 2A. Fig. 2B depicts the means ± S.E. for the ADH mRNA, the relative order of which is descending limb > ascending limb > TEN control. These data are consistent with the results from our previous studies (9, 11).

Expression Levels of SREBP-1 in Rat Hepatic Tissue—C/EBPs regulate hepatic ADH expression (11), and SREBP-1 can induce a change in the ratio of the full-length C/EBPβ protein (termed liver-activating protein) versus the truncated C/EBPβ protein (termed liver inhibitory protein) (12). To study the effects of ethanol on the hepatic SREBPs, we examined the protein and mRNA expression levels of SREBP-1. Nuclear extracts from the livers of ethanol-fed rats had decreased amounts of the nuclear form of SREBP-1 as determined by SDS-PAGE and immunoblotting (p < 0.05) (Fig. 3A). When rats were killed on the ascending limb of the UEC pulse, the expression of the SREBP-1 protein decreased even more. The decrease in the nuclear form of SREBP-1 was paralleled by a decrease in the precursor form of SREBP-1 in samples from the ascending (but not descending) limb of the UEC pulse (Fig. 3A). The means ± S.E. for all rats (n = six rats/group) are shown in Fig. 3B. We used quantitative RPA to determine whether ethanol altered the amounts of mRNA for SREBP-1a and SREBP-1c. Six samples from each group (ascending limb, descending
limb, and TEN rats) were examined by RPA, and the data were normalized to 18S RNA. No significant differences in SREBP-1a and SREBP-1c mRNAs were found between the three groups (Fig. 3, C and D). These data demonstrate that ethanol regulation of the nuclear form of SREBP-1 occurs mainly at the post-transcriptional level.

Expression of SREBP-1 in Hypophysectomized Rats—ADH mRNA and ADH-specific activity were previously reported to be 2-fold greater in male hypophysectomized rats than in intact sham-operated rats (32). We examined the ADH protein in whole cell lysates and the SREBP-1 protein in nuclear extracts from hypophysectomized rats to determine whether there was an inverse relationship between ADH and SREBP-1. We found an ~6-fold greater level of the ADH protein in hypophysectomized rats, but an ~7-fold lower level of the nuclear form of SREBP-1 in hypophysectomized rats (Fig. 4).

Hepatic Nuclear Protein(s) Interact with Rat Class I ADH-specific SREs—As demonstrated above, the hepatic ADH mRNA and protein increased in rats fed an ethanol-containing diet or after hypophysectomy, and under either condition, the nuclear form of SREBP-1 decreased. To investigate the potential SREBP-1 involvement in the regulation of ADH gene expression, the promoter of the rat Class I ADH gene was analyzed for SREs using the TransFac Database (33). We identified two potential SREBP-binding sites separated by 1 bp in the proximal promoter sequence, one located at −63 to −53 and the other at −51 to −40 relative to the transcription start site. Footprint analysis of the rat Class I ADH promoter using rat liver nuclear extracts disclosed two protected regions corresponding to −60 to −52 and −44 to −36 relative to the transcription start site (34). The potential SREBP-binding sites lie within these protected regions. EMSAs were performed using the ADH-specific SREBP-binding site (ADH-SRE) (Table I) corresponding to −65 to −38 of the ADH promoter and hepatic nuclear extracts from ethanol-fed rats killed low on the ascending limb or high on the descending limb of the UEC pulse to determine 1) whether hepatic nuclear proteins bind to these sites and 2) whether ethanol alters this binding. Indeed, we found that nuclear proteins bound to these sites and that ethanol decreased (p < 0.05) the nuclear protein binding (Fig. 5). The sequence specificity of the DNA-protein interaction with nuclear extracts from the TEN control rat liver was determined by competition assay with labeled ADH-SREs in the presence of increasing amounts of unlabeled oligonucleotides.
Fig. 6A shows that formation of the complex of nuclear protein(s) with the labeled ADH-SRE oligonucleotide was competed against with the unlabeled ADH-SRE oligonucleotide. When the unlabeled low density lipoprotein receptor (LDLR)-SRE oligonucleotide was added to the reaction as a competitor, the shifted band was also competed against (Table I) (35); on the other hand, an unrelated CYP2E1-C/EBP oligonucleotide did not compete for binding (Fig. 6B) (11). The shifted band was confirmed to be due to bound SREBP-1 protein by incubating labeled and unlabeled ADH-SRE oligonucleotides with nuclear extracts from the TEN control rat livers and subjecting them to EMSA; the gel lanes containing the unlabeled oligonucleotide were transferred to nitrocellulose and subjected to immunoblotting with anti-SREBP-1 antibody. Fig. 6C shows that the SREBP-1 protein migrated to the same position as the shifted band. A protein of ~68 kDa bound to the ADH-SRE oligonucleotide in a UV cross-linking experiment, and addition of unlabeled ADH-SRE and LDLR-SRE oligonucleotides inhibited this binding (Fig. 6D). The molecular mass of the nuclear form of SREBP-1, which binds to the SRE site in the nucleus, is ~68 kDa, and the above data demonstrate that the protein bound to the ADH-SRE site is this protein.

Demonstration of SREBP-1 Protein Binding to the SRE Site of the Class I ADH Promoter in Vivo by ChIP—We probed the in vivo binding status of the ADH-SRE sites using the ChIP technique. Fig. 7 illustrates the results from ChIP analysis. Ethanol reduced SREBP-1 protein association with the ADH-SREs in the proximal promoter. In addition, the decrease in SREBP-1 associated with the ADH-SREs was greater in the descending limb compared with the ascending limb of the UEC.

Fig. 5. EMSA of the ADH-SRE oligonucleotide corresponding to region −63 to −40 of the rat ADH gene promoter. A, shown is a diagram of the proximal region of the promoter of the rat Class I ADH gene. B, hepatic nuclear extracts from ethanol-treated rats killed on the ascending and descending limbs of the UEC pulse or with no ethanol (TEN) were incubated with the labeled ADH-SRE site. C, shown are the means ± S.E. of relative amounts of DNA-protein binding complex. Rats fed diets with ethanol were killed on the ascending (A) and descending (D) limbs of the UEC pulse or with no ethanol (TEN). *, p < 0.05. F, free probe; ADU, arbitrary densitometric units.

**Table I**

| Oligonucleotides used in in vitro transcription competition experiments |
|---------------------------------|
| ADH-SRE | −63 | −53 | −51 | −40 |
|        | TAGATCACATGTGGGATCAGCTGACAC | ATCTAGTGTACACCCTAGTCGACTGTG |
| SRE2   |      |      |      |      |
| SRE1   |      |      |      |      |
| ADH-SRE1 | GGGATCAGCTGACAC | CCCTAGTCGACTGTG |
| ADH-SRE2 | TAGATCACATGTGGG | ATCTAGTGTACACCCTAGTCGACTGTG |
| mut-SRE1 | GGGTGAATCTGACAC | CCCACTAATGACTGTG |
| mut-SRE2 | TAGTGAAATGTGGG | ATCTAGTGTACACCCTAGTCGACTGTG |
| LDLR-SRE | TAGATCACATGTGGG | ATCTAGTGTACACCCTAGTCGACTGTG |
| Rep-C/EBP-5' | TTTGAAATTCGACCACCACCGTCCA | AAACTTATTGTTTGGGCCAGTGTT |
| Rep-C/EBP-3' | GGGATCAGCTGACAC | CCCTAGTCGACTGTG |

**Fig. 6** 

**Fig. 5.** EMSA of the ADH-SRE oligonucleotide corresponding to region −63 to −40 of the rat ADH gene promoter. A, shown is a diagram of the proximal region of the promoter of the rat Class I ADH gene. B, hepatic nuclear extracts from ethanol-treated rats killed on the ascending and descending limbs of the UEC pulse or with no ethanol (TEN) were incubated with the labeled ADH-SRE site. C, shown are the means ± S.E. of relative amounts of DNA-protein binding complex. Rats fed diets with ethanol were killed on the ascending (A) and descending (D) limbs of the UEC pulse or with no ethanol (TEN). *, p < 0.05. F, free probe; ADU, arbitrary densitometric units.

**Table I**

| Oligonucleotides used in in vitro transcription competition experiments |
|---------------------------------|
| ADH-SRE | −63 | −53 | −51 | −40 |
|        | TAGATCACATGTGGGATCAGCTGACAC | ATCTAGTGTACACCCTAGTCGACTGTG |
| SRE2   |      |      |      |      |
| SRE1   |      |      |      |      |
| ADH-SRE1 | GGGATCAGCTGACAC | CCCTAGTCGACTGTG |
| ADH-SRE2 | TAGATCACATGTGGG | ATCTAGTGTACACCCTAGTCGACTGTG |
| mut-SRE1 | GGGTGAATCTGACAC | CCCACTAATGACTGTG |
| mut-SRE2 | TAGTGAAATGTGGG | ATCTAGTGTACACCCTAGTCGACTGTG |
| LDLR-SRE | TAGATCACATGTGGG | ATCTAGTGTACACCCTAGTCGACTGTG |
| Rep-C/EBP-5' | TTTGAAATTCGACCACCACCGTCCA | AAACTTATTGTTTGGGCCAGTGTT |
| Rep-C/EBP-3' | GGGATCAGCTGACAC | CCCTAGTCGACTGTG |

Fig. 6A shows that formation of the complex of nuclear protein(s) with the labeled ADH-SRE oligonucleotide was competed against with the unlabeled ADH-SRE oligonucleotide. When the unlabeled low density lipoprotein receptor (LDLR)-SRE oligonucleotide was added to the reaction as a competitor, the shifted band was also competed against (Table I) (35); on the other hand, an unrelated CYP2E1-C/EBP oligonucleotide did not compete for binding (Fig. 6B) (11). The shifted band was confirmed to be due to bound SREBP-1 protein by incubating labeled and unlabeled ADH-SRE oligonucleotides with nuclear extracts from the TEN control rat livers and subjecting them to EMSA; the gel lanes containing the unlabeled oligonucleotide were transferred to nitrocellulose and subjected to immunoblotting with anti-SREBP-1 antibody. Fig. 6C shows that the SREBP-1 protein migrated to the same position as the shifted band. A protein of ~68 kDa bound to the ADH-SRE oligonucleotide in a UV cross-linking experiment, and addition of unlabeled ADH-SRE and LDLR-SRE oligonucleotides inhibited this binding (Fig. 6D). The molecular mass of the nuclear form of SREBP-1, which binds to the SRE site in the nucleus, is ~68 kDa, and the above data demonstrate that the protein bound to the ADH-SRE site is this protein.

Demonstration of SREBP-1 Protein Binding to the SRE Site of the Class I ADH Promoter in Vivo by ChIP—We probed the in vivo binding status of the ADH-SRE sites using the ChIP technique. Fig. 7 illustrates the results from ChIP analysis. Ethanol reduced SREBP-1 protein association with the ADH-SREs in the proximal promoter. In addition, the decrease in SREBP-1 associated with the ADH-SREs was greater in the descending limb compared with the ascending limb of the UEC.
Ethanol Regulation of ADH and SREBP-1

**Fig. 6. Identification of binding of SREBP-1 to the ADH-SRE site.** A, hepatic nuclear extracts from TEN control rats were incubated with the labeled ADH-SRE oligonucleotide and increasing amounts of the unlabeled ADH-SRE oligonucleotide (lanes 1–3). B, competition of the labeled ADH-SRE site with the unlabeled LDLR-SRE and CYP2E1-C/EBP sites is shown in lanes 4–7. C, the unlabeled ADH-SRE oligonucleotide was incubated with nuclear extracts from the TEN control rat livers and subjected to EMSA. The gel lanes from the unlabeled oligonucleotide were cut – 3

**Fig. 7. SREBP-1 protein binds to the ADH-SREs, and ethanol reduces this binding as determined by ChIP.** Liver tissues were minced and homogenized, followed by formaldehyde fixation and immunoprecipitation with or without anti-SREBP-1 antibody (SREBP-1Ab) as indicated. The DNA in the immunoprecipitates was amplified using primers encompassing 150 bp including the ADH-SREs in the promoter of the rat Class I ADH gene. Three livers from rats fed diets pulse and transferred to nitrocellulose and subjected to Western blotting with anti-SREBP-1 antibody (lanes 8). D, after UV cross-linking of the labeled ADH-SRE oligonucleotide and hepatic nuclear extracts with or without either excess molar unlabeled ADH-SRE or LDLR-SRE oligonucleotide, samples were subjected to SDS-PAGE. An autoradiogram of the gel after exposure to x-ray film is shown in lanes 9–11.

pulse. These data confirmed the EMSA results and indicate that SREBP-1 binds to the ADH-SREs in vivo and that ethanol alters this binding pattern.

**Functional Analysis of the ADH-SREs in the Proximal Promoter Region**—We used in vitro transcription to directly examine the function of the SRE sites in the proximal ADH promoter region. The template contained the proximal promoter region up to −241 relative to the transcription start site and included the SRE1 and SRE2 sites. Fig. 8 demonstrates that when a 100-fold molar excess of oligonucleotide (Table I) containing ADH-SRE1 and ADH-SRE2 was added to the in vitro transcription assay, the transcription activity decreased by −2.5-fold. With a 5-bp mutation in the SRE site (Table I), the transcription-inhibiting activity of the oligonucleotide was much lower. When a consensus C/EBP sequence replaced both SRE sites in the proximal promoter, the transcription activity was restored to 84% of the intact template.

**Delineating the Function of the SREBP-1 Protein in ADH Gene Expression**—The above in vitro transcription data show that SRE sites in the proximal ADH promoter are essential elements for efficient mRNA transcription. Because the nuclear form of SREBP-1 was decreased in rats fed ethanol and in hypophysectomized rats, we hypothesized that SREBP-1 acts as a negative regulator of ADH gene expression. To directly assess this postulated function, we immunodepleted SREBP-1 and SREBP-2 proteins from nuclear extracts of TEN rats. An antibody against calpain-2 was used as control in the immunodepletion experiment. After depletion, we used Western blotting to examine for SREBP-1 protein presence; −90% of the SREBP-1 protein was removed from the nuclear extracts (data not shown). When the SREBP-1 protein was removed, the ADH transcription activity increased by −5-fold compared with the control antibody reactions (Fig. 9). These data confirm that SREBP-1 is a negative regulator of ADH gene transcription in vitro.

**Effects of the LXR Agonist T0901317 on ADH Gene Expression in Vivo**—FGC4 cells are highly differentiated rat hepatoma cells (36) that respond to increasing amounts of ethanol (0, 1, 3, 10, 30, and 100 mM) by producing a dose-dependent increase in ADH mRNA (Fig. 10, A and B). Nuclear run-on assay demonstrated that the elevated Class I ADH mRNA levels correlated with increased transcription when FGC4 cells were incubated with 50 mM ethanol for 24 h (Fig. 10C). Because LXR activation is well known to increase transcription of the SREBP-1c gene (21, 22), we studied the effects of the LXR agonist T0901317 to determine whether activated LXR reduces ADH gene expression. As shown in Fig. 11, in the presence of 1 and 10 μM T0901317, ADH mRNA abundance decreased by 30 and 51%, respectively (p < 0.05), and this effect was completely abolished by 50 mM ethanol. These in vitro data are consistent with SREBP-1 being a negative regulator of ADH gene transcription and with ethanol acting as a negative regulator of ADH gene transcription and participating in the post-transcriptional processing of SREBP.

**mRNA Expression of SREBP-2, Fatty-acid Synthase, and Cholesterol 7α-Hydroxylase in Rat Hepatic Tissue**—We used quantitative RPA to determine whether ethanol altered the amounts of mRNA for SREBP-2, and real-time quantitative reverse transcription-PCR was conducted to examine the mRNA levels of fatty-acid synthase and cholesterol 7α-hydroxylase in rat hepatic tissues. Six samples from each group (ascending limb, descending limb, and TEN rats) were examined, and the data were normalized to 18 S RNA. As shown in Fig. 12, all three genes were induced by ethanol and exhibited the same pattern of change. Inductions of mRNA were observed to be greater in rats processed on the descending limb of the UEC pulse (p < 0.05). Compared with the non-alcohol-fed group,
fatty-acid synthase mRNA was 4.03/11006 1.29-fold greater, and cholesterol 7/H9251-hydroxylase mRNA was 10.13/11006 2.24-fold greater.

**DISCUSSION**

Alcoholism is a major public health and social concern throughout the world. Alcohol use and abuse results in a number of pathophysiological consequences, including cirrhosis. Ethanol enhances the accumulation of fatty acids in the liver and induces fatty liver or steatosis (37, 38). SREBPs are transcription factors that regulate many genes involved in cholesterol and fatty acid synthesis (14). Because recent reports found that ethanol induces SREBP-1 protein expression in mouse liver (13, 39), we were interested in the relative expression profiles of SREBPs and Class I ADH.

An intriguing feature of the TEN rat model is the dynamic "pulsatile or cyclic" pattern of blood and urine ethanol concentrations observed upon constant intragastric infusion of ethanol (9, 40). In this model, ethanol use and abuse results in a number of unaccounted for losses of ethanol and because ethanol intake is constant, the pulsatile blood and urine ethanol concentrations must be produced by cyclical ethanol metabolism. We have shown that this cyclic ethanol metabolism occurs via cyclic regulation of Class I ADH (9).

In this study, rats were evaluated when their UECs were high on the descending limb (where ethanol metabolism is high) and low on the ascending limb (where ethanol metabolism is low) of an ethanol pulse. We documented lower hepatic protein levels of the precursor and nuclear forms of SREBP-1 in ethanol-treated rats. We also observed decreased in vitro hepatic nuclear protein binding to the Class I ADH-specific SRE at −63 to −40 using hepatic nuclear extracts from ethanol-fed rats (ascending and descending limbs of the UEC pulse) compared with control rats. Specificity was studied in several ways. First, unlabeled ADH-SRE and LDLR-SRE oligonucleotides effectively competed for binding to the ADH-SRE site. Second, binding complexes of unlabeled ADH-SRE oligonucleotide with nuclear extract were confirmed to contain SREBP-1. Third, a 68-kDa protein bound to the ADH-SREs in a UV-cross linking assay, and this is the molecular mass of the nuclear form of SREBP-1. The discrepancy between the results of EMSA and Western blotting for the SREBP-1 protein may be caused by post-translational modification of SREBPs and/or interactions with other co-regulatory transcription factors such as Sp1 and CREB binding protein/nuclear factor-Y that affect the binding of SREBPs to promoters of SREBP target genes by forming complexes with SREBPs (41, 42).

The ChIP assay results obtained with anti-SREBP-1 antibody were consistent with the EMSA results and further substantiate that the SREBP-1 protein binds to the ADH-SRE site and that, as a whole, ethanol decreases the SREBP-1 protein binding. Furthermore, the SREBP-1 protein binding fluctuates according to the urine ethanol cycle, with binding being higher on the ascending limb and lower on the descending limb of the UEC pulse, just the opposite of ADH mRNA expression. Consistent with these results, we found that the ADH protein increased by >6-fold in hypophysectomized rats and that the nuclear form of SREBP-1 decreased by 7-fold. In addition, the LXR agonist T0901317 (a known inducer of SREBP-1c transcription) reduced ADH gene expression in these cells, an effect that could be inhibited by ethanol. Together, these results strongly suggest that the SREBP-1 protein negatively regulates rat Class I ADH expression.

We further delineated the function of the ADH-SRE sites in the proximal promoter region using in vitro transcription, and the results demonstrate that both SRE sites in the proximal promoter region are essential for ADH gene transcription.
When the SREBP-1 protein was depleted from the nuclear extract, the transcription activity increased by ~5-fold. This clearly demonstrates that the SREBP-1 protein negatively regulates rat Class I ADH expression and that ethanol suppresses SREBP-1 to produce disinhibition and consequent ADH induction. The upstream stimulatory factor can also binding to region -46 to -73 of the ADH promoter and transactivate ADH gene expression (43). SREBP-1 and the upstream stimulatory factor may compete for binding to these sites and regulate ADH gene transcription. This may explain why ADH-SRE oligonucleotides decreased the transcription activity in the in vitro transcription assay.

Ethanol effects on SREBPs appear to differ in mice and rats, as other investigators have reported ethanol-induced increases in hepatic SREBPs in mice (13, 39). It is not entirely clear why this species difference occurs, but there are many metabolic and endocrinologic differences between rats and mice, and mice are well known to have greater ethanol metabolism than rats, which can result in lower blood ethanol levels even at high ethanol doses (39, 44). This is important because ethanol reduces SREBP-1 in a dose- and time-dependent manner, and differences in these two variables appear to explain some of the discrepancy between species. Moreover, in vitro studies with the rat H4IIEC3 hepatoma cell line, SREBP-1 expression was reported to increase acutely (30 min) and then to decrease below control levels after 60 min with ethanol (13). Thus, with longer ethanol exposure times, such as those in our chronic experiments, SREBP-1 was decreased. The latter time point in
that experiment more closely resembles the effects we observed for SREBP-1 in rats chronically treated in vivo with ethanol in the present study.

We previously reported increased liver weight and steatosis in rats fed ethanol-containing diets (45, 46). SREBP-2 are major transcription factors that activate the genes involved in fatty acid biosynthesis and cholesterol 7α-hydroxylase were the same as that of SREBP-2 mRNA. Fatty acid synthase and cholesterol 7α-hydroxylase mRNAs were examined by real-time reverse transcription-PCR; the data were also normalized to 18S RNA. The data are presented as the means ± S.E. from six samples for each group. α and β indicate differences in mean levels for a transcript between groups (p < 0.05). Ethanol-treated rats versus the TEN control group of rats differed (p < 0.05).

that experiment more closely resembles the effects we observed for SREBP-1 in rats chronically treated in vivo with ethanol in the present study.

We previously reported increased liver weight and steatosis in rats fed ethanol-containing diets (45, 46). SREBP-2 are major transcription factors that activate the genes involved in fatty acid and cholesterol biosynthesis. Whereas SREBP-1 protein expression decreased in ethanol-fed rats, SREBP-2 expression increased. SREBP-2 mRNA was lower on the ascending limb of the UEC pulse, where the blood ethanol concentration was low, and SREBP-2 mRNA was higher on the descending limb of the pulse, where the blood ethanol concentration was higher. Notably, the expression profiles of mRNAs encoding fatty-acid synthase and cholesterol 7α-hydroxylase were the same as that of SREBP-2 mRNA. Fatty acid biosynthesis and cholesterol biosynthesis are both activated in SREBP-2-overexpressing transgenic mice (17); and in SREBP-1c–/– mice, SREBP-2 can substitute partially for SREBP-1c in permitting an insulin-mediated increase in fatty-acid synthase mRNA (47). Thus, we speculate that, although hepatic SREBP-1 is decreased in ethanol-fed rats, hepatic SREBP-2 is increased and is likely responsible for elevated fatty acid and cholesterol biosynthesis. Furthermore, at this time, we cannot rule out the possibility that C/EBPs are involved in fatty acid biosynthesis because ethanol increases the expression of certain members of this family (11).

In summary, we previously presented evidence that ethanol induction of Class I ADH occurs via changes in levels of the C/EBP family of transcription factors (11). The new data presented in this study demonstrate that SREBP-1 inhibits ADH expression and that ethanol alters SREBP-1 expression post-transcriptionally. Together, our data suggest that concerted actions of ethanol on SREBP and C/EBP expression and ADH promoter binding are crucial for ADH induction by ethanol and formation of the UEC pulse.