THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 46, Issue of November 15, pp. 43572–43577, 2002
Printed in U.S.A.

Ethanol Induction of Class I Alcohol Dehydrogenase Expression in the Rat Occurs through Alterations in CCAAT/Enhancer Binding Proteins β and γ*

Received for publication, May 8, 2002, and in revised form, July 19, 2002
Published, JBC Papers in Press, September 3, 2001, DOI 10.1074/jbc.M204535200

Ling He, Martin J. J. Ronis, and Thomas M. Badger‡

From the Arkansas Children's Nutrition Center, Little Rock, Arkansas 72202

Alcohol dehydrogenase (ADH) is the principal ethanol-metabolizing enzyme. Ethanol induces rat Class I ADH mRNA and activity by an as yet unknown mechanism. In the current study, adult male rats were fed an ethanol-containing diet by continuous intragastric infusion for 42 days. Hepatic Class I ADH mRNA, protein, and activity levels in the ethanol-infused rats increased 3.9-, 3.3-, and 1.7-fold, respectively ($p < 0.05$). Cis-acting elements within the proximal promoter region of the ADH gene were studied by electrophoretic mobility shift assay (EMSA). Hepatic nuclear extract (HNE) binding to either the consensus or ADH-specific CCAAT/enhancer binding protein (C/EBP) sites was >2.4-fold greater in ethanol-fed rats ($p < 0.05$) than controls. Antibody-specific EMSA assays demonstrated binding of the transcription factor C/EBPβ to the C/EBP site. Western blot immunoblot analysis of HNEs demonstrated 3.5- and 2.3-fold increases in C/EBPβ (LAP) and C/EBPδ ($p < 0.05$), respectively, in ethanol-fed rats compared with controls, whereas levels of the truncated C/EBPβ (LIP) and C/EBPγ were lower in ethanol-fed rats ($p < 0.05$). HNE from ethanol-fed rats increased (3-fold) the *in vitro* transcription of rat Class I ADH ($p < 0.05$), and mutation of the C/EBP element in the proximal promoter region blocked this effect. Antisera against LIP or C/EBPγ enhanced transcription efficiency ($p < 0.05$). These data provide the first evidence for the mechanism by which ethanol regulates rat hepatic Class I ADH gene expression *in vivo*. This mechanism involves the C/EBP site and the enhancer binding proteins β and γ.

Ethanol is metabolized predominantly in the liver. The alcohol dehydrogenase (ADH,[1] EC1.1.1.1) system is responsible for the majority of ethanol oxidation. It is abundant in the liver, although lesser amounts are expressed in most tissues (1, 2). ADH also catalyzes the oxidation and reduction of a variety of physiological steroidal and nonsteroidal substrates (3). Although multiple isozymes of Class 1 ADH exist in human liver, only Class I ADH mRNA and protein appear to be significantly expressed in the rat liver. The expression of the Class I ADH gene is tissue-specific and hormonally regulated throughout development (1, 4–10). Continuous intragastric infusion of an ethanol-containing diet to rats results in unique and predictably recurring cyclic fluctuations in plasma and urine ethanol concentrations, and a recent report suggests that a similar cyclic fluctuation in hepatic ADH activity is responsible (11).

Genomoic DNA clones encoding the three copies of human (12) and the single copy each of rat (13) and mouse (14) Class I ADH genes have been isolated. Analysis of the intron-exon boundaries and portions of the proximal 5′-promoter region reveals these genes are well conserved among the three species. Characterization of the first kb of the 5′-flanking region of the rat Class I gene and deletion analysis in the transient transfection experiments exposed two positive elements in this flanking region, a proximal positive element from −241 to −12 and a distal element from −1327 to −977 (15). The proximal positive element has greater transcription activity than the distal element (15). At least four important regions have been identified within the proximal positive element: the CCAAT/enhancer-binding protein site (−22 to −11); the E-box-Upstream Stimulatory Factor-binding site (−60 to −54); the G3T-Sp1-binding site (−87 to −78); and the HNF-5 site (−35 to −28) (15). An EDBP site is located −10 to 0. The CCAAT/enhancer-binding protein (C/EBP), the E-box, and the G3T sites are highly conserved among the rat, mouse, and human (13, 14, 16).

In primary rat hepatocyte cultures, growth hormone (17, 18), insulin-like growth factor I (16), glucagon (19), and cyclic AMP (20) have been reported to increase ADH enzyme activity, as well as its mRNA. Regulation of ADH gene expression is thought to occur through transcription factors binding to cis-acting elements, and C/EBPs belong to a family of transcription factors implicated in this process (6, 20, 21). The C/EBPs are thought to be critical for cellular differentiation and function in a variety of tissues. So far, more than five members (gene products) of C/EBP family have been described, including C/EBPα, -β, -γ, -δ, and -ϵ (22, 23). Specificity of gene control by C/EBPs is ensured through their ability to homo- and heterodimerize and to interact with other transcription factors. Two isoforms of C/EBPβ are generated from a single mRNA, the full-length 36-kDa protein termed "liver activating protein" (LAP) and the truncated protein termed "liver inhibitory protein" (LIP). Heterodimerization of LIP with the full-length LAP attenuates transcriptional activity, suggesting that LIP inhibits transcription (24). C/EBPγ heterodimerization with C/EBPα and -β also attenuates transcription activation of target genes, suggesting dominant negative regulation of C/EBP transactivation by this factor (25). C/EBPδ can readily form heterodimers with C/EBPα and -β, and the transactivating efficiency is comparable with that of C/EBPα and -β homodimers

* This work was supported in part by National Institutes of Health Grant AA0845. 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.: 501-364-2785; Fax: 501-364-2818; E-mail: badgerthomasm@uams.edu.

[1] The abbreviations used are: ADH, alcohol dehydrogenase; C/EBP, CCAAT/enhancer-binding protein; LAP, liver-activating protein: LIP, liver inhibitory protein; UEC, urine ethanol concentration; IVT, *in vitro* transcription; EMSA, electrophoretic mobility shift assay; EDBP, enhancer-site downstream binding protein.

(26). Because previous reports using cell cultures suggest that the C/EBP site (6, 20) is important in the hormonal regulation of the Class 1 ADH gene, the C/EBP family of transcription factors could also play a role in the mechanisms of ethanol regulation of ADH.

In vivo, the mechanisms underlying the regulation of ADH gene expression by ethanol are still unclear. In the present study, we have investigated the expression of the hepatic Class 1 ADH gene in rats fed an ethanol-containing diet by continuous intragastric infusion and the possible involvement of cis-acting elements in the proximal promoter region of the gene.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—All chemicals unless otherwise specified were purchased from Sigma Aldrich. Radionucleotides were purchased from PerkinElmer Life Sciences. T4 polynucleotide kinase was purchased from Promega (Madison, WI). Rabbit polyclonal antibodies against C/EBPα, -β, -γ, -δ, -ϵ, and horseradish peroxidase-conjugated donkey anti-rabbit IgG were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All synthetic oligonucleotides were obtained from Integrated DNA Technologies, Inc. (Coralville, IA).

Animals and Experimental Protocol—Experiments conformed to ethical guidelines for animal research established by our institution and received prior approval by our animal welfare committee. Adult male Sprague-Dawley rats were purchased from Harlan Industries (Indianapolis, IN). The rats were surgically cannulated with an intragastric tube, allowed to recover, and infused with an ethanol-containing diet (13g/kg/day) as described previously (27). The control rats were infused the same diet except that ethanol was isocalorically replaced with carbohydrate. The rats were sacrificed following 38 to 42 days of continuous diet infusion and when their urine ethanol concentrations were high on the descending limb of an ethanol pulse, as previously described (11, 27, 28). Liver was collected and stored at −70 °C.

Northern Blot Analysis—Northern blot analysis was conducted as described previously (22). A 400-bp rat Class 1 ADH cDNA probe was used for the detection of ADH mRNA (2, 29). 18 S ribosomal RNA antisense oligonucleotides were synthesized (Bio Synthesis, Inc., Lewisville, TX) using the published sequences (30). All filters were probed with the synthetic 18 S rRNA anti-sense oligonucleotide as an internal control. Bands were quantitated by densitometry of the autoradiograph, and the ratio of ADH message to 18 S rRNA in the same sample was determined and expressed as relative RNA units or as percentage of that for the control.

Preparation of Antibody Against Rat Class 1 ADH—The amino acid sequence of alcohol dehydrogenase (EC 1.1.1.1) was used for the preparation of anti-peptide antibody targeted against a specific region of rat Class 1 ADH. The amino acid sequence ^{225}INKDKFAKAKELG^{238} is encoded in this gene. This peptide was checked for matching sequences in the Entrez protein sequence data library (NCBI) and found to be unique. An antisera against this sequence was produced in rabbits by Bioworld (Dublin, OH).

Western Immunoblot Analysis—Liver homogenates were resolved on 12% polyacrylamide gel and transferred to an Hybond-P membrane (Amersham Biosciences). Membranes were blocked overnight at 4 °C with gentle shaking in TBST (10 mM Tris-buffered saline, 0.13 M NaCl, pH 7.6, 0.05% (v/v) Tween-20) plus 5% (w/v) milk powder. Membranes were incubated with primary antibody diluted to 1:1000 (ADH), 1:2500 (C/EBPs) in TBST plus 5% milk powder for 1.5–3 h (1.5 h for ADH, 3 h for C/EBPs) at room temperature with shaking. After washing three times in TBST, the membranes were incubated for 1 h at room temperature in TBST plus 5% milk powder containing horseradish peroxidase-conjugated secondary IgG (1:2500 for ADH, 1:5000 for C/EBPs). Membranes were washed three times in TBST and proteins visualized using the enhanced chemiluminescence plus system (ECL Plus; Amersham Biosciences) and detected by autoradiography. Immunoquantitation was obtained by densitometric scanning of the resulting autoradiographs using a Bio-Rad GS525 molecular imager (Richmond, CA).

ADH Activity—ADH activities were measured in homogenates prepared from liver frozen in liquid nitrogen, essentially as described previously (11).

Oligonucleotides—Table I lists the synthetic oligonucleotides used in this study. The ADH-C/EBP probe contains two cis-acting elements (C/EBP-binding site −22 to −11 and EDBP-binding site −10 to −1). ADH-EDBP contains only the EDBP-binding site (−10 to −1); CYP2E1-EBP contains the C/EBP-binding site.

Preparation of Rat Liver Nuclear Extracts—The nuclear extracts were isolated from livers frozen at −70 °C using the nuclear extraction kit from Sigma. Briefly, 100 mg of liver tissue were added into 1 ml of 1× lysis buffer containing 10 mM Hepes, pH 7.9, 1.5 mM MgC_{12}, 10 mM KCl, 1 mM dithiothreitol, and protease inhibitors. The tissue was homogenized for 40 s and centrifuged for 20 min at 11,000 × *g*. The pellet was resuspended in 140 μl of extraction buffer containing 20 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 0.42 M NaCl, 0.2 mM EDTA, 25% glycerol, 1 mM dithiothreitol, and protease inhibitor. Incubation occurred while shaking for 30 min followed by centrifugation at 20,000 × *g* for 5 min and snap freezing of the supernatant.

Electrophoretic Mobility Shift Assays (EMSA)—EMSA were performed as described previously (31). Double-stranded oligonucleotides were prepared by combining and heating equimolar amounts of complementary single-stranded DNA to 95 °C for 5 min in dH_2O and cooling to room temperature overnight. The annealed oligonucleotides were diluted to a concentration of 10 μM and stored at −20 °C. EMSA were carried out in 20 μl containing 100 mM KCl, 20 mM Tris-HCl, pH 8.0, 1.5 mM $MgCl_2$, 1.0 mM dithiothreitol, 0.3 μg of bovine serum albumin, 7% glycerol, and 1.5 μg of poly(dI-dC) (Roche Molecular Biochemicals). The nuclear extracts were blocked with poly(dI-dC) for 15 min on ice. 0.1-μm end-labeled oligonucleotides were then added to the reactions and incubated for another 15 min on ice, after which 3 μl of loading buffer was added. The samples were loaded on a 4% nondenaturing polyacrylamide gel (acrylamid:bisacrylamide = 39:1) in low ionic strength Tris borate EDTA (unless otherwise specified). Serial amounts of nuclear extracts were tested in the experiment; in each reaction 10 μg was the ideal concentration. For the competition experiments, the unlabeled and labeled oligonucleotides were added to the reaction at the same time. For the supershift experiments, antibodies were added to the reaction, incubated 20 min at room temperature, and then the oligo was added to the reaction and incubated on ice for 15 min.

In Vitro Transcription—The rat genomic DNA was extracted with the Wizard genomic DNA purification kit (Promega). The PCR primers A−241F, A−34R, A−5F, and A+450R were synthesized based on the published rat Class1 ADH 5′-flanking sequence and coding region sequence (GenBank™ accession nos. M29516 and U10900) (Table I). The A−34R primer contains a 4-bp mutation in the C/EBP element. A−241F and A+450R primers were used to amplify the DNA template containing the proximal promoter region and subsequent sequence including the exon 1 region (−241 bp and subsequent 450 bp). A−34R and A−5F were used to construct the same length template containing the mutated C/EBP element. All of the cloned and mutated DNA fragments were sequenced. *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 $MgCl_2$, 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, 20 μM unlabeled UTP, 10 μCi of a [^{32}P]UTP. The mixture was made, and the reactions were initiated by adding 90 μg of nuclear extract (with the antibody assay, 2 μl of the serum was added to the reaction at the same time with the nuclear extract). Incubations were carried out for 60 min at 30 °C, and reactions were terminated by adding 100 μl of a stop buffer containing 0.3 M Tris-HCl (pH7.4), 0.3 M sodium acetate, 0.5% SDS, 10 mM EDTA, 10 μg/ml tRNA. The mixture was extracted twice with 0.3 M Tris-HCl(pH 7.4)-10 mM EDTA-saturated phenol. The aqueous layers were made to 0.5 M NH_4OAc, and 2.5 volumes of ethanol were added to precipitate the transcripts. The pellets were suspended in 95% formamide, 18 mM EDTA, 0.025% SDS. The samples were heated at 95 °C for 5 min and then analyzed in 5% denaturing acrylamide gel.

Statistics—Student's *t* test was used to determine whether group means differed at $p < 0.05$.

RESULTS

Effects of Ethanol-containing Diet on Hepatic ADH mRNA Protein and Activity Levels—Fig. 1 shows the Northern (*panel A*) and Western (*panel B*) blots of individual liver samples from rats fed diets with either no ethanol (*TEN*) or ethanol. *Panel C* depicts the means ± S.E. for the mRNA, protein, and activities for ADH. As can be seen, feeding an ethanol-containing diet to rats resulted in increased liver ADH mRNA (3.9-fold), protein (3.28-fold), and activity (1.7-fold) levels when compared with rats fed diets with no ethanol.

Expression Levels of Five C/EBP Family Members in Rat Nuclear Extracts—To examine the effects of ethanol on the hepatic expression of C/EBP family members, Western immunoblot analysis was employed by using antisera (described

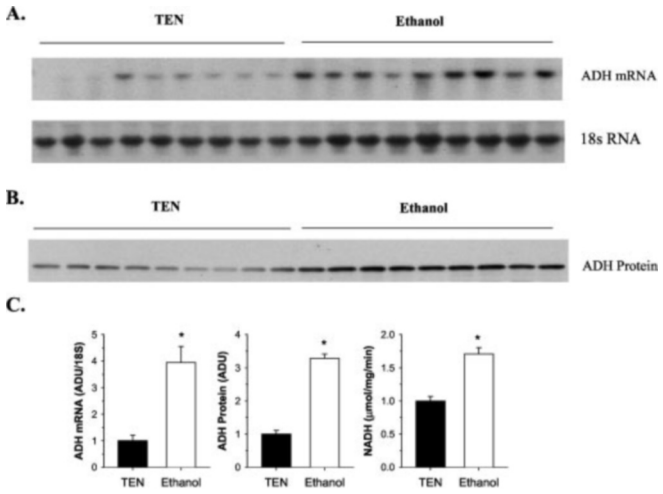

FIG. 1. **Ethanol-dependent induction of hepatic ADH mRNA, protein, and activity in rats.** *A*, Northern blot for hepatic mRNA encoding Class 1 ADH of rats fed ethanol-containing diets (*Ethanol*, $n = 9$) or diets without ethanol (*TEN*, $n = 9$) and hybridization with 18 S rRNA oligonucleotide is shown. *B*, a Western blot of hepatic ADH was probed with rabbit serum immunized against an ADH-specific peptide. *C*, densitometric analysis of the blots was conducted, and the means ± S.E. are shown for hepatic Class 1 mRNA levels, Class 1 ADH protein, and ADH activity for rats infused diets without ethanol (*TEN*, $n = 9$) or rats infused ethanol-containing diets (*Ethanol*, $n = 9$). * indicates that mean levels of the ethanol-treated rats and the control rats differed ($p < 0.05$). *ADU*, Arbitrary Densitometric Units.

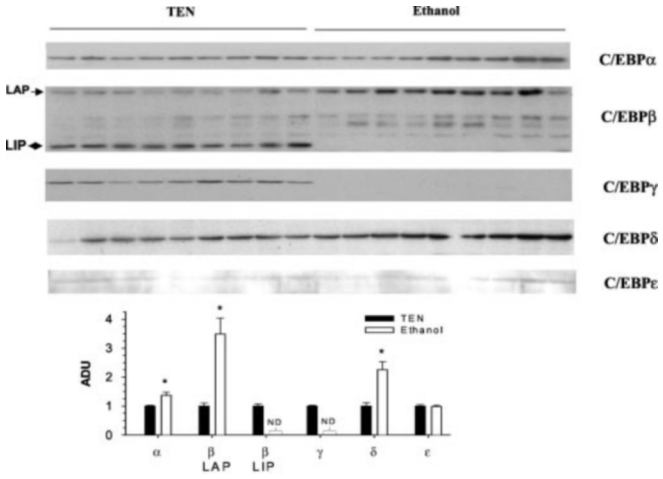

FIG. 2. **The expression levels of five C/EBP family members in rat liver nuclear extracts.** Western immunoblot analysis was conducted on hepatic nuclear extracts from rats infused diets with no ethanol (*TEN*) or with diets with ethanol (*Ethanol*) using anti-C/EBPα, -β, -γ, -δ, and -ε rabbit sera. In the immunoblot with anti-C/EBPβ, rabbit serum shows two major bands: the *top* one is the full-length C/EBPβ protein, LAP, and the *bottom* one is the truncated C/EBPβ protein, LIP. *, $p < 0.05$ compared with TEN controls. *N.D.*, not detected.

above) against C/EBPα, -β, -γ, -δ, and -ε. As shown in Fig. 2, extracts from control (*TEN*) and ethanol-treated rats did not differ significantly in immunoreactive C/EBPε. However, immunoblotting with anti-C/EBPβ produced two major bands. The top band migrated with the 36-kDa full-length C/EBPβ LAP, and the bottom band migrated with the 21-kDa truncated isoform LIP. Ethanol increased LAP 3.5-fold ($p < 0.05$) and decreased LIP to non-detectable levels as compared with the control rats. Ethanol treatment also reduced the expression of immunoreactive C/EBPγ to non-detectable levels and increased the immunoreactive C/EBPα and -δ ($p < 0.05$) as compared with controls.

Hepatic Nuclear Extracts Interact with the Consensus C/EBP Site and with Rat Class 1 ADH-specific C/EBP Oligonucleotide—EMSA was performed using the end-labeled consensus C/EBP or ADH-specific C/EBP oligonucleotides (Table I) and hepatic nuclear extracts prepared from rats fed ethanol or control diets. The first 22 bp of the proximal promoter of the rat Class 1 ADH contains the C/EBP and EDBP sites (16). The mean increase in nuclear protein binding to the consensus C/EBP site was 2.5-fold ($p < 0.05$) greater in ethanol-fed rats than in rats fed no ethanol (data not shown). In EMSA using an ADH-specific C/EBP oligonucleotide, DNA-protein complexes were also detected as bands with retarded motility (Fig. 3*A*.) The nuclear protein binding fast migrating complex (*LIP binding?*) was lowered to be undetected when rats were fed ethanol diets. The mean nuclear protein binding to the C/EBP site (the medium migrating complex) increased 2.4-fold ($p < 0.05$) in rats fed ethanol-containing diets compared with control rats (*panel B*). Not shown are similar nuclear protein binding experiments using the consensus Sp1 oligonucleotide and upstream stimulatory factor (USF) oligonucleotide where no ethanol effects were observed.

The sequence specificity of the DNA-protein complexes formed with nuclear extracts from ethanol-treated rat livers was determined by competition experiments with labeled ADH-C/EBP oligonucleotide and labeled EDBP oligonucleotide in the presence of increasing amounts of unlabeled oligonucleotides. Fig. 4*A* shows that labeled C/EBP forms two complexes that could be competed away with the unlabeled ADH-C/EBP oligo (*lanes 1–3*). Using the EDBP site oligonucleotide as labeled probe in the EMSA, there is only one DNA-protein complex formed at the same position as the slow migrating complex, and this complex could be competed away by increasing amounts of unlabeled EDBP oligonucleotide (*lanes 4–6*). Together, these data suggest that the slow migrating complex contains EDBP and the medium migrating complex contains the C/EBPs. The difference in the mobility of the complexes formed with the C/EBP and EDBP sites is consistent with the difference in the molecular weight of the proteins binding to these two sites (16, 33, 34). In the antibody-specific EMSA, the C/EBP binding complex was completely supershifted by C/EBPβ antisera (*lane 8*), whereas antiserum against C/EBPα had no effect on the nuclear protein binding activity (*lane 7*).

Nuclear Extracts Obtained from Rat Liver Tissue Interact with CYP2E1-specific C/EBP Oligonucleotide—Because ethanol treatment significantly increased the binding of C/EBPs to a consensus C/EBP oligonucleotide, we studied binding to the putative C/EBP site of another gene previously reported to be regulated by ethanol, the *CYP2E1* gene (35). The CYP2E1-specific C/EBP oligonucleotide was studied using EMSA, and no differences between the ethanol-treated and control groups were detected (Fig. 4*B*). In other experiments, 100-fold excess unlabeled putative CYP2E1 C/EBP was unable to compete for hepatic nuclear proteins binding to ADH-C/EBP oligos (data not shown).

Effect of the C/EBP Element in the ADH Proximal Promoter on in Vitro Transcriptional Activity—To determine the role of the C/EBP element in the transcriptional activity of the ADH proximal promoter, *in vitro* transcription was conducted using a template containing the −241 bp of the proximal promoter region and the subsequent sequence including exon 1 (450 bp). A second template was mutated only at the C/EBP site, and *in vitro* transcription activity was studied in the intact and mutated templates in the presence and absence of hepatic nuclear extracts from control and ethanol-fed rats. The transcripts generated from the *in vitro* transcription were 450-bp long RNA. Data in Fig. 5 demonstrates that mutation of the C/EBP site blocked the ethanol-induced increase in i*n vitro* transcription activity when compared with the intact template

TABLE I
Sequence of oligonucleotides

Oligo	Sequence (5′ to 3′)
Consensus C/EBP	TGCAGATTGCGCAATCTGCA ACGTCTAACGCGTTAGACGT −22 −1
ADH-C/EBP	TGTGTGCAACTTTGGCCCAGTT ACACACGTTGAAACCGGGTCAA −10 −1
ADH-EDBP	gatc TGGCCCAGTT g ACCGGGTCAA cctg −68 −54
CYP2E1-EBP	TGATTATTGGCTGAT ACTAATAACCGACTA
A−241F	ctgcAGATGCATAAACAGATTCAATTGGC
A−34R	CTTCCCTCCTGCAGTGAACTGGGCCAAACTCGAGCACAGATTTTATTTGC[a]
A−5F	CAGTTCACTGCAGGAGGGAAGAA
A+450R	CTAAATTCCCTGAAGCAAATTCCTC

[a] The mutated C/EBP site is underlined.

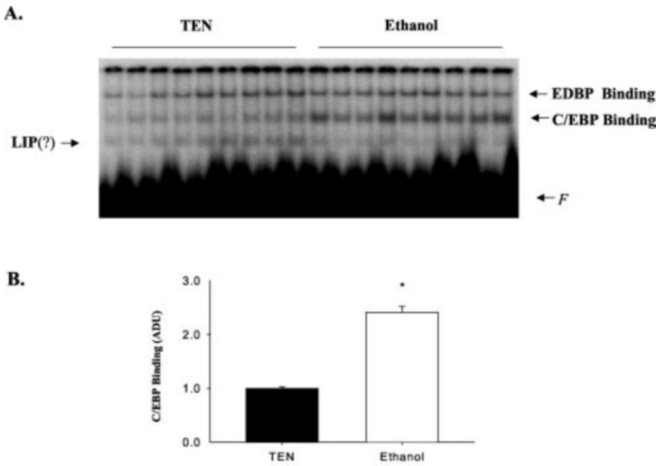

FIG. 3. **EMSA of oligonucleotide ADH-C/EBP and EDBP-binding site of the rat ADH promoter (−22 to −1).** Three DNA-protein complexes were formed in the EMSA. The fast migrating complex is a lower molecular weight nuclear protein (*LIP?*) binding to the C/EBP-binding site, the medium migrating complex is the nuclear protein binding to the C/EBP site, and the slow migrating complex is the nuclear protein binding to the EDBP-binding site (see Fig. 3*A*). The means ± S.E. following image scanning of C/EBP binding are shown in the bottom half of Fig. 5. *, $p < 0.05$ compared with TEN controls. No differences were observed with EDBP binding. (← *F*) indicates the free probe. *ADU*, Arbitrary Densitometric Units.

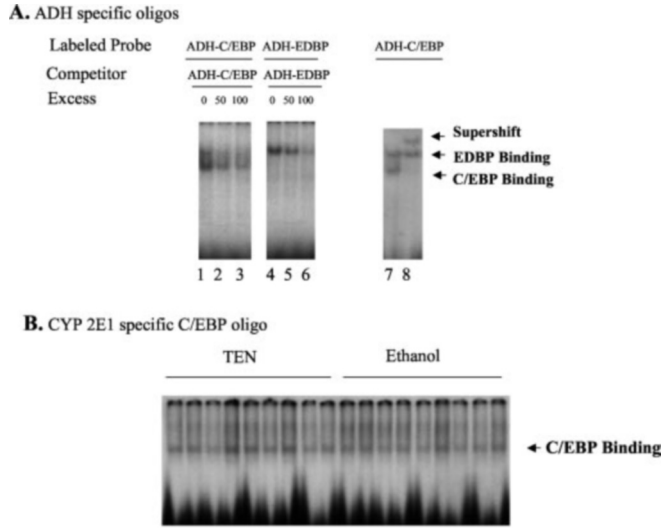

FIG. 4. **EMSA of competition experiments with oligonucleotides ADH-C/EBP, ADH-EDBP, and EMSA with CYP2E1-EBP oligo.** Hepatic nuclear extracts from ethanol-treated rats were incubated with labeled ADH-C/EBP oligonucleotide and increasing amounts of unlabeled ADH-C/EBP (*panel A*, *lanes 1–3*). Labeled ADH-EDBP with various unlabeled ADH-EDBP competition is shown in *lanes 4–6*. Antibody-specific EMSA was performed using ethanol-fed rat hepatic nuclear extracts and the labeled ADH-C/EBP in the presence of anti-C/EBPα (*lane 7*), -β (*lane 8*). In the competition and antibody *supershift* assay, the DNA-protein complexes were subjected to electrophoresis through a 5.5% nondenaturing polyacrylamide gel. EMSA was also performed with CYP2E1-specific C/EBP oligo (*panel B*). Complexes are marked (→). The supershifted complex is shown as ← *supershift*. Excess indicates -fold molar excess.

($p < 0.05$). Similarly, mutation of the C/EBP site decreased the *in vitro* transcription activity observed with hepatic nuclear extracts from control rats ($p < 0.05$).

To further investigate the effects of LIP and C/EBPγ on transcriptional activity, hepatic nuclear extracts from the control rats were treated with antisera against LIP and C/EBPγ. Extracts from control rats were used because of the greater concentrations of LIP and C/EBPγ compared with ethanol-fed rats (Fig. 2). As can be seen in Fig. 6, the transcription activity was enhanced when the inhibitory influences of LIP and C/EBPγ were removed by β and γ antisera ($p < -0.05$).

DISCUSSION

Blood and urine ethanol concentrations (UECs) cycle between 0 and 500 mg/dl, and these cycles reoccur about every 6 days in rats infused continuously with ethanol-containing diets (27, 36). Expression of ADH Class I mRNA and ADH activity are induced when the UECs reach levels greater than 300 mg/dl, and the UECs are abolished in rats treated with the ADH inhibitor, 4-methylpyrazole (11). These results suggest that ethanol cycles were caused by cyclic expression of ADH, which occurs in response to increasing ethanol levels, perhaps an example of time-dependent pharmacokinetics. Two important questions about the existence of ethanol pulses in the rat model concern the central cause of the pulses and the mechanism underlying induction of ADH by ethanol. The rat model of intragastric infusion of ethanol-containing diets offers an excellent opportunity to study the latter question.

The genes encoding the Class 1 ADH are mainly expressed in the liver. There is only a single Class 1 ADH gene expressed in the rat liver. In this report, we studied hepatic Class I ADH mRNA, protein, and activity levels from rats fed an ethanol-containing diet by continuous intragastric infusion. Using Northern hybridizations under very stringent experimental conditions, we demonstrated a 4-fold increase in the level of rat Class I ADH mRNA in ethanol-infused rats compared with the control rats. Western immunoblot analysis was performed by using a rabbit antiserum raised against a peptide sequence that is highly specific for rat Class I ADH. Using this probe,

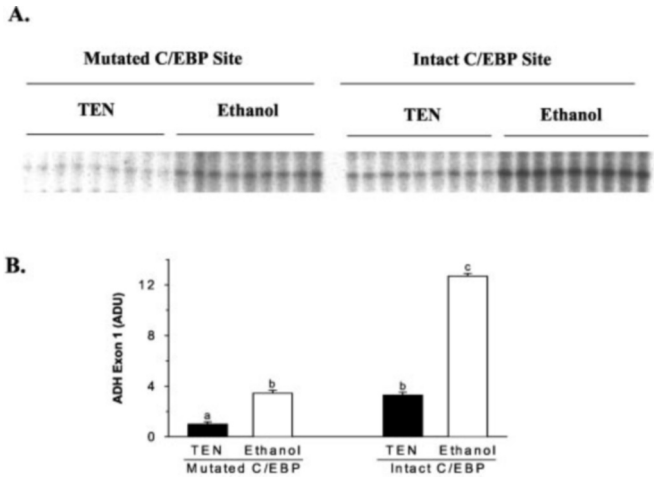

FIG. 5. **In Vitro Transcriptions (IVT) of ADH proximal promoter (−241 bp) plus subsequent 450-bp ADH sequence including exon 1.** Hepatic nuclear extracts from rats infused with non-ethanol-containing diets (*TEN*, $n = 9$) and rats infused with ethanol-containing diets (*Ethanol*, $n = 9$) were incubated with the ADH proximal promoter DNA containing either the mutated C/EBP site or the intact C/EBP site. The autoradiographs are shown at the top in *panel A*. *B*, the means ± S.E. of each group following image scanning are shown. *Bars* with different lowercase letters differed by $p < 0.05$. In IVT with either the mutated C/EBP site or the intact C/EBP site, the ethanol-treated rats compared with TEN controls sharing a lowercase letter differ $p < 0.05$ (*a*, *b*); within the TEN groups in the IVT with the mutated C/EBP site or the intact C/EBP site, promoters sharing a lowercase letter differ $p < 0.05$ (*b*); within the ethanol-treated groups in the IVT with the mutated C/EBP site or the intact C/EBP site, promoters sharing a lowercase letter differ $p < 0.05$ (*c*). *ADU*, arbitrary densitometric units.

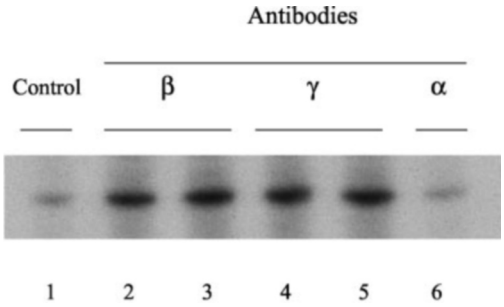

FIG. 6. **IVT of ADH proximal promoter (−241 bp) with anti-C/EBP*β*, -*γ*, and -*α* sera.** IVT were performed using hepatic nuclear extracts from rats infused with non-ethanol-containing diets (Control) and the ADH proximal promoter in the absence of antibody (*lane 1*) or in the presence of anti- C/EBPβ serum (*lane 2–3*), anti-C/EBPγ serum (*lane 4–5*), and anti-C/EBPα serum (*lane 6*).

there is only a single band on the Western blot, and it migrates at the same molecular weight as calculated for ADH. The mean level of rat Class 1 ADH enzyme increased 3.3-fold in ethanol-infused rats compared with the control rats. The ADH activity levels of ethanol-treated rats were 1.7-fold greater than controls. These results confirm our previous findings on ethanol induction of Class I ADH mRNA and activity and provide essential new data on the ADH protein not available in the original report. These data strengthen the argument that significant induction of ADH occurs at high UECs but beg the question as to the mechanisms underlying these effects.

Because nuclear run-on assays suggested that ethanol increased ADH Class I mRNA by elevation of gene transcription (11), we studied possible mechanisms of ethanol-induced transcription of the ADH gene. The first 3 kb of the 5′-flanking region of rat Class I ADH gene has been characterized. Within the 241 bp upstream of the start site of transcription (the proximal positive element), there are several known regulatory sites, including the C/EBP site (−22 to −11) and the EDBP site (−10 to 0) (15). The C/EBP family of transcription factors has been implicated in cell differentiation and energy metabolism (37, 38). These factors are expressed predominantly in cells with high gluconeogenesis and lipogenesis, most notably in liver and adipose tissue (38). The C/EBPs are basic/leucine zipper transcription factors that interact with each other and with other protein families to regulate cellular transcription (33, 34). C/EBP dimerization is a prerequisite to DNA binding (40), and heterodimerization of C/EBPs can either enhance or attenuate transcriptional activity. C/EBPβ and -δ enhance transcriptional activity, but LIP, as well as C/EBPγ, are dominant negative regulations of C/EBP transactivation (22, 26). Previous reports using cell cultures suggest that the C/EBP site (6, 20) is important in the expression of Class 1 ADH genes. However, there was no evidence available from *in vivo* studies to confirm these findings.

In the present studies, protein expression levels in liver nuclear extracts from ethanol-fed rats demonstrated significant increases in C/EBPβ and -δ and decreases in LIP and C/EBPγ to undetectable levels as compared with rats not fed ethanol. Furthermore, antibody-specific shift assays confirmed binding of C/EBPβ to the ADH-C/EBP site. *In vitro* transcription assays confirmed that the hepatic nuclear extracts of ethanol-fed rats significantly enhanced transcriptional activity as compared with extracts from rats fed no ethanol. These data suggest that ethanol can maximize ADH expression by elevating the concentrations of proteins that enhance transcription while simultaneously reducing negative regulators of transcription. Taken together, these results provide a potential mechanism for ethanol-induction of ADH expression.

Greater *in vitro* hepatic nuclear protein binding to Class I ADH-specific C/EBP sites was observed using hepatic nuclear extracts from ethanol-fed rats than those from control rats. This is a relatively specific event. The specificity was studied in several ways. First, the binding results using the consensus C/EBP oligonucleotides demonstrated significantly greater interactions with hepatic nuclear extracts from ethanol-treated rats than controls, whereas when the consensus upstream stimulatory factor and consensus Sp1 oligonucleotides were used, the nuclear protein binding of the ethanol-fed rats and control rats did not differ. Second, hepatic nuclear protein binding to the CYP2E1-specific C/EBP-binding site was equal in ethanol-treated and control groups. These results suggest that increased C/EBP-related transcription factors only bind and activate specific hepatic genes. Further evidence for specificity is provided by a study demonstrating lower promoter activity when there is a 4-bp mutation in the C/EBP-binding site (located between nucleotides −22 and −11 of rat Class 1 ADH gene) (15). Our results are consistent with this latter report.

As far as we are aware, this is the first report to provide evidence that C/EBP-related transcription factors interact with the C/EBP site in the proximal promoter region of the Class I ADH gene in the liver of rats fed an ethanol-containing diet. Importantly, the liver tissue studied in this report was collected at a time following chronic ethanol feeding when the ethanol metabolism is predicted to be maximal. Thus, these ADH regulatory events are temporally linked to *in vivo* ethanol metabolism, thus providing valuable insights into the functional regulation of the principal ethanol metabolizing enzyme. The mechanism underlying the ethanol-induced expression of hepatic Class I ADH most likely involves regulation of these transcription factors. It is also important to note that similar effects were observed in rats infused with ethanol-containing

diets for short periods (13 days), well before any demonstrated inflammation, necrosis, or fibrosis, suggesting that the effects on C/EBPs and ADH are not occurring secondary to chronic ethanol exposure (data not shown). Further studies into ethanol regulation of C/EBPs and other genes coordinately regulated by them are underway.

REFERENCES

1. Crabb, D. W., Bosron, W. F. & Li, T. K. (1989) *Pharmacol. Ther.* **34,** 59–73
2. Estonius, M., Danielesso. O., Karlsson. C., Persson, H., Jornavall, H. & Hoog, J. O. (1993). *Eur. J. Biochem.* **215,** 497–503
3. Mezey, E. & Potter, J. J. (1993) *Arch. Biochem. Biophys.* **225,** 787–794
4. Boleda, M. D., Farres, J., Guerri, C. & Pares, X. (1992) *Biochem. Biophys.* **43,** 1555–1561
5. Qulali, M., Ross, R. A. & Crabb, D. W. (1991) *Arch. Biochem. Biophys.* **288,** 406–413
6. Potter, J. J., Yang, V. W. & Mezey, E. (1993) *Biochem. Biophys. Res. Commun.* **191,** 1040–1045
7. Duster, G., Shean, M. L., Mcbride, M. S. & Stewart, M. J. (1991) *Mol. Cell. Biol.* **11,** 1638–1646
8. Mezey, E., Potter, J. J., Mishra, L., Sharma, S. & Janicot, M. (1990) *Arch. Biochem. Biophys.* **280,** 390–396
9. Felder, M. R., Watson, G., Huff, M. O. & Ceci, J. D. (1988) *J. Biol. Chem.* **263,** 14531–14537
10. Winter, L. A., Stewart, M. J., Shean, M. L., Dong, Y., Poellinger, L., Okret, S. & Gustafsson, J. A. (1990) *Gene* **91,** 233–240
11. Badger, T. M., Hoog, J. O., Svensson, S., McGehee, R. E., Fang, C., Ronis, M. J. J. & Ingelman-Sundberg, M. (2000) *Biochem. Biophys. Res. Commun.* **274,** 684–688
12. Duester, G., Smith, M., Bilanchone, V. & Hatfield, G. W. (1986) *J. Biol. Chem.* **261,** 2027–2033
13. Crabb, D. W., Stein, P. M., Dipple, K. M., Hittle, J. B., Sidhu, R., Qulali, M., Zhang, K. & Edenberg, H. J. (1989) *Genomics* **5,** 906–914
14. Zhang, K., Bosron, W. F. & Edenberg, H. J. (1987) *Gene (Amst.)* **57,** 27–36
15. Cortese, J. F., Majewski, J. L., Crabb, D. W., Edenberg, H. J. & Yang, V. W. (1994) *J. Biol. Chem.* **269,** 21898–21906
16. Potter, J. J., Mezey, E., Cornelius, P., Crabb, D. W. & Yang, V. W. (1992) *Arch. Biochem. Biophys.* **295,** 360–368
17. Mezey, E., Potter, J. J. & Rhodes, D. L. (1986) *Hepatology* **6,** 1386–1390
18. Potter, J. J., Yang, V. W. & Mezey, E. (1989) *Arch. Biochem. Biophys.* **274,** 548–555
19. Mezey, E., Potter, J. J. & Rhodes, D. L. (1986) *Gastroenterology.* **91,** 1271–1277
20. Potter, J. J., MacDougald, O. A. & Mezey, E. (1995) *Arch. Biochem. Biophys.* **321,** 329–335
21. Edenberg, H. (2000) *Prog. Nucleic Acid Res. Mol. Biol.* **64,** 295–342
22. Lekstrom-Himes, J. & Xanthopoulos, K.G. (1998) *J. Biol. Chem.* **273,** 28545–28548
23. Roesler, W. J., Park, E. A. & McFie, P. J. (1998) *J. Biol. Chem.* **273,** 14950–14957
24. Descombes, P. & Schebler, U. (1991) *Cell* **67,** 569–579
25. Cooper, C., Henderson, A., Artandi, S., Avitahi, N. & Calame K. (1995) *Nucleic Acids Res.* **23,** 4371-4377
26. Cao, Z., Umek, R. M. & McKinght, S. L. (1991) *Genes Dev.* **5,** 1538–1552
27. Badger, T. M., Crouch, J., Irby, D., Hakkak, R. & Shahare, M. (1993) *J. Pharm. Exp. Ther.* **264,** 938–943
28. Ronis, M. J. J., Huang, J., Crouch, J., Mercado, C., Irby D., Valentine, C. R., Lumpkin, C. K., Ingelman-Sundberg, M. & Badger, T. M. (1993) *J. Pharmacol. Exp. Ther.* **264,** 944–950
29. Crabb, D. W. & Edenberg, H. J. (1986) *Gene* **48,** 287–291
30. Buetler, T. M., Gallagher, E. P., Wang, C., Stahl, D. L., Hayes, J. D. & Eaton, D. L. (1995) *Toxicol. Appl. Pharmacol.* **135,** 45–57
31. Chen, B., He, L., Savell, V. H., Jenkins, J. J. & Parham, D. M. (2000) *Cancer Res.* **60,** 3290–3298
32. Bagchi, M.K., Tsai, S. Y., Weigel, N. L., Tsai, M. J. & O'Malley, B. W. (1990) *J. Biol. Chem.* **265,** 5129–5134
33. William, S. C., Cantwell, C. A. & Johnson, P. F. (1991) *Genes Dev.* **5,** 1553–1567
34. Davydov, I. V., Bohmann, D., Krammer, P. H. & Li-Weber, M. (1995) *Gene* **161,** 271–275
35. McGehee, R. E., Jr., Ronis, M. J. J. & Badger, T. M. (1997) *DNA Cell Biol.* **16,** 725–736
36. Tsukamoto, H., French, S. W., Reidelberger, R. D. & Largman, C. (1985) *Alcohol. Clin. Exp. Res.* **9,** 31–37
37. Landschulz, W. H., Hohnson, P. F., Adashi, E. Y., Graves, B. J. & McKnight, S. L. (1988) *Genes Dev.* **2,** 786–800
38. McKnight, S. L., Lane, M. D. & Gluechsohn-Waelsch, S. (1989) *Genes Dev.* **3,** 2021–2024
39. Deleted in proof
40. Landschulz, W. H., Johnson, P. F. & McKnight, S. L. (1989) *Science* **243,** 1681–1688