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Alcohol Induces RNA Polymerase III-dependent Transcription through c-Jun by Co-regulating TATA-binding Protein (TBP) and Brf1 Expression*

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Chronic alcohol consumption is associated with steatohepatitis and cirrhosis, enhancing the risk for hepatocellular carcinoma. RNA polymerase (pol) III transcribes a variety of small, untranslated RNAs, including tRNAs and 5S rRNAs, which determine the biosynthetic capacity of cells. Increased RNA pol III-dependent transcription, observed in transformed cells and human tumors, is required for oncogenic transformation. Given that alcohol consumption increases risk for liver cancer, we examined whether alcohol regulates this class of genes. Ethanol induces RNA pol III-dependent transcription in both HepG2 cells and primary mouse hepatocytes in a manner that requires ethanol metabolism and the activation of JNK1. This regulatory event is mediated, at least in part, through the ability of ethanol to induce expression of the TFIIIB components, Brf1, and the TATA-binding protein (TBP). Induction of TBP, Brf1, and RNA pol III-dependent gene expression is driven by enhanced c-Jun expression. Ethanol promotes a marked increase in the direct recruitment of c-Jun to TBP, Brf1, and tRNA gene promoters. Chronic alcohol administration in mice leads to enhanced expression of TBP, Brf1, tRNA, and 5S rRNA gene transcription in the liver. These alcohol-dependent increases are more pronounced in transgenic animals that express the HCV NS5A protein that display increased incidence of liver tumors. Together, these results identify a new class of genes that are regulated by alcohol through the co-regulation of TFIIIB components and define a central role for c-Jun in this process.

Extensive alcohol consumption can lead to the development of a spectrum of alcoholic liver disease. The early stage of alcoholic liver disease is characterized by steatosis, which, in a fraction of patients, progresses to steatohepatitis with a varying degree of fibrosis. In 15–20% of alcoholic liver disease patients, the disease advances to cirrhosis, which is an independent risk factor for liver cancer development (1). In response to alcohol exposure, multiple signaling pathways are activated by a variety of cell surface receptors, including the Toll-like and cytokine receptors, which lead to enhanced production of reactive oxygen species and inflammatory cytokines. The activation of signaling events leads to changes in transcription via the activation or alteration in various transcription factors, including NF-κB, peroxisome proliferator-activated receptor (PPAR), EGR-1, and AP-1. However, the specific signaling events and gene targets that lead to pro-oncogenic effects associated with alcohol are still poorly understood.

RNA polymerase (pol)[2] III transcribes a variety of untranslated RNAs, including tRNA and 5S rRNA, that control the translational and growth capacity of cells. Oncogenic proteins, such as Ras, c-Myc, and PI3-kinase, stimulate RNA pol III-dependent transcription, whereas tumor suppressors, such as Rb, p53, and phosphatase and tensin homolog (PTEN), repress transcription of this class of genes (2). RNA pol III transcription products are elevated in both transformed and tumor cells, suggesting that it plays a crucial role in tumorigenesis (3, 4). Consistent with this idea, enhanced RNA pol III transcription is required for oncogenic transformation (5, 6). The ability of these oncogenic and tumor suppressor proteins to deregulate RNA pol III-dependent transcription occurs through their capacity to regulate the TFIIIB complex. TFIIIB consists of the TATA-binding protein (TBP) and two associated factors, Brf1 and Bdp1. TFIIIB, together with TFIIIC and RNA pol III, are required to transcribe tRNA genes, whereas these components, together with TFIIIA, are needed to transcribe 5S rRNA genes.

TBP is a central transcription initiation factor, required for directing transcription from all three nuclear RNA polymerases. TBP can be limiting for the transcription of RNA pol I- and III-dependent promoters (7, 8), whereas RNA pol II-dependent promoters are differentially affected by changes in TBP expression (9, 10). TBP expression is induced through EGFR1 and Ras activation, requiring all three classes of MAPKs (7, 11, 12). This results in enhanced TBP transcription through increased phosphorylation of Elk-1 and its recruitment to the TBP promoter (11). TBP expression can also be induced by expression of a mutant variant form of EGFR1,

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[2] The abbreviations used are: pol, polymerase; TBP, TATA-binding protein; ADH, alcohol dehydrogenase; qPCR, quantitative PCR.

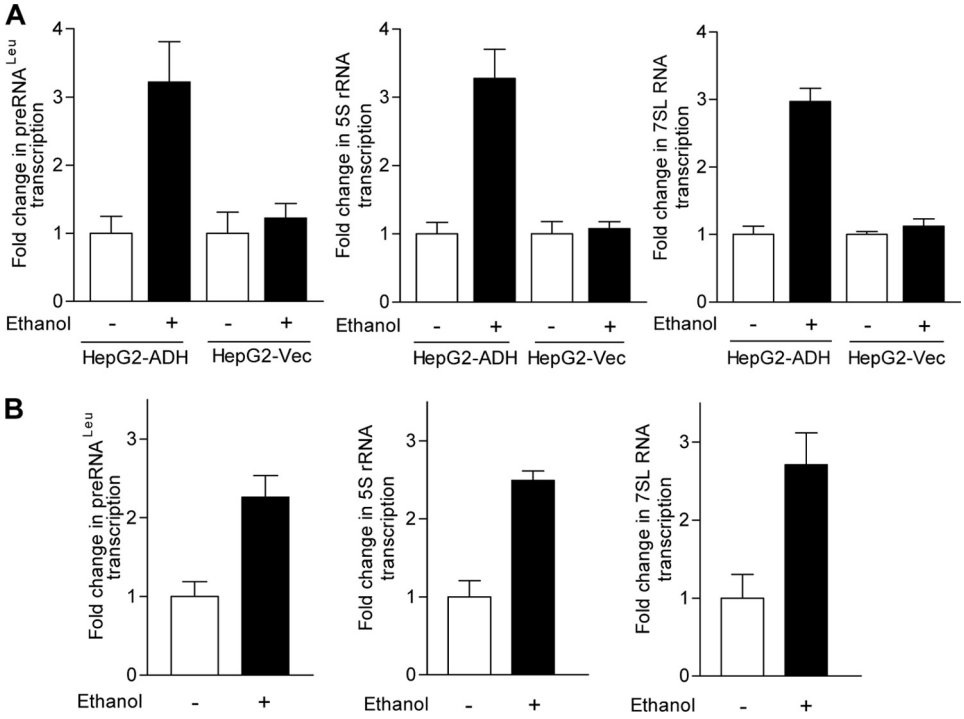


FIGURE 1. **Alcohol induces RNA pol III-dependent transcription.** *A*, ethanol enhances transcription in HepG2-ADH cells. HepG2-vector (*HepG2-Vec*) and HepG2-ADH cells were serum-starved overnight in 0.1% FBS/DMEM. Cells were treated with or without ethanol, RNA was isolated, and pre-tRNA^{Leu}, 7SL RNA, 5S rRNA, and GAPDH transcripts were measured by RT-qPCR. The -fold change was calculated by normalizing to the amount of GAPDH mRNA. *B*, ethanol stimulates transcription in primary mouse hepatocytes. Hepatocytes were treated with or without 50 mM ethanol, RNA was isolated, and the amounts of pre-tRNA^{Leu}, 5S rRNA, 7SL RNA, and GAPDH transcripts were measured by RT-qPCR. The bars represent means ± S.E. of at least three independent determinations.

EGFRvIII (13). In this case, Elk-1 is not required, and an EGFRvIII-mediated increase in c-Jun expression leads to the recruitment of AP-1 to the TBP promoter and enhanced activity. Brf1 can also be limiting for RNA pol III-dependent transcription (6), yet much less is known regarding the mechanisms by which its expression is regulated.

We examined the effects of alcohol on RNA pol III-dependent transcription in both the hepatocellular carcinoma HepG2 cell line and primary mouse hepatocytes. Alcohol induces the transcription of tRNA, 5S rRNA, and 7SL RNA genes and requires the metabolism of alcohol by ADH, as well as the activation of JNK1. This regulatory event produces an increase in the expression of the TFIIIB components, Brf1 and TBP. Increased expression of both TBP and Brf1 is driven by increased c-Jun expression and enhanced occupancy of c-Jun to TBP and Brf1 promoters. Intriguingly, Elk-1, c-Jun and c-Fos are found at tRNA genes, and alcohol stimulates a marked increase in c-Jun occupancy. Importantly, increases in TBP, Brf1, and RNA pol III gene expression can be recapitulated in mouse models. C57BL/6 mice or Hepatitis C virus (HCV) NS5A transgenic mice exhibit an increase in these transcripts after chronic alcohol administration. Furthermore, the HCV NS5A transgenic mice, which exhibit an increased incidence of liver tumors after chronic alcohol administration, display an even greater increase in tRNA, 5S rRNA, Brf1, and TBP expression. Together, these results have uncovered new gene expression processes that are regulated by alcohol and support the idea that enhanced c-Jun expression drives alcohol-mediated induction of RNA pol III-dependent transcription through multiple mechanisms.

EXPERIMENTAL PROCEDURES

Plasmids, Reagents, and Animals—HepG2-ADH and HepG2-vector cells were kindly provided by Dr. D. L. Clemens (14). Ethanol was from Sigma-Aldrich; cell culture medium (minimum Eagle's medium) and DMEM were obtained from Cellgro. Zeocin, RT-PCR kit, Lipofectin reagent, Lipofectamine 2000, and Opti-MEM were from Invitrogen. All antibodies were obtained from Santa Cruz Biotechnology with the exception of phospho-JNK and phospho Elk-1 antibodies that were from Cell Signaling. Mice expressing the HCV NS5A gene under control of the apoE promoter were obtained from Ratna Ray (St. Louis University). Wild-type and NS5A transgenic mice on the C57BL/6 strain were fed with Lieber-DeCarli diet containing 3.5% ethanol or isocaloric dextrin for long term alcohol feeding (15). All animal experiments were performed with age- and sex-matched mice from same littermates and conducted in accordance with the approved Institutional Animal Care and Use Committee protocol at the University of Southern California.

RT-qPCR and Transient Transfection Assays—Total RNA was isolated from engineered HepG2 cells, primary mouse hepatocytes, or liver tissues of mice fed alcohol using the single-step extraction method TRIzol reagent (Invitrogen). Precursor tRNA^{Leu} 5S rRNA transcripts were measured as described previously (16).

For transient transfection assays, cells were transfected with DNA or siRNAs as described previously (11). Serum-free medium was added to each dish with Lipofectin-DNA or Lipofectamine 2000-siRNA complexes, and cells were further

incubated at 37 °C for 4 h. The medium was changed, and cells were incubated overnight before harvesting. Protein concentrations of the resultant lysates were measured by the Bradford method, and luciferase activity was measured using a Promega luciferase assay system. Resultant luciferase activities were normalized to the amount of protein in each lysate. The -fold change in promoter activity was calculated by measuring the level of luciferase activity with empty vector and without ethanol treatment and setting this value at 1 for each independent experiment. Differences in TBP promoter activity are expressed as the means ± S.E. At least three independent experiments were conducted for each determination.

Immunoblot Analysis—Immunoblot analysis was carried out as described previously (7). Cells were grown to 80% confluency in 10% fetal bovine serum (FBS) in DMEM and then serum-deprived using 0.1% FBS in DMEM for 3 h. Where noted, cells were pretreated with 5 μM of the JNK inhibitor, SP600125, for 1 h and then incubated with or without 50 mM ethanol for 60 min. Lysates (50 μg of protein) were subjected to immunoblot analysis. Membranes were probed with specific antibodies as indicated. Hybond-P membrane was used for protein transfer. Bound primary antibody was visualized using horseradish peroxidase-conjugated secondary antibody (Vector Laboratories) and enhanced chemiluminescence reagents (Amersham Biosciences).

RESULTS

Alcohol Induces RNA pol III-dependent Transcription via the Activation of JNK1—To determine whether alcohol regulates RNA pol III-dependent transcription, we used the hepatocellular carcinoma cell lines HepG2, stably expressing ADH (HepG2-ADH), or vector alone (HepG2-vector). Previous analysis of these cells revealed that although the parental HepG2 cells do not exhibit detectable ADH activity, the HepG2-ADH cells effectively metabolize ethanol to acetaldehyde (14). These cells were treated with ethanol, and the amounts of precursor $tRNA^{Leu}$, 5S rRNA, and 7SL RNA transcripts were measured by RT-qPCR. Ethanol treatment resulted in a concentration-dependent increase in transcription where the maximum response for ethanol-mediated induction was observed at the ethanol concentration of 50 mM after 60 min of treatment (data not shown). Therefore, all experiments were performed using these conditions. This effect was dependent on the metabolism of ethanol as transcription induction occurred in cells that expressed the ADH gene but not the vector control cells (Fig. 1*A*). Primary mouse hepatocytes treated with ethanol also displayed an increase in transcription from all three genes (Fig. 1*B*). These results indicate that RNA pol III-dependent gene transcription is induced by ethanol through its ability to be metabolized.

Given that the JNKs play an important role in regulating RNA pol III transcription (17), we next examined the role of JNK activation in alcohol-mediated transcription induction. HepG2-ADH cells were treated with ethanol, and immunoblot analysis was performed to measure the amount of phosphorylated JNK1 and JNK2 (Fig. 2*A*). Ethanol induced a strong activation in JNK1 but a more modest activation of JNK2. No activation of these JNKs was observed in the

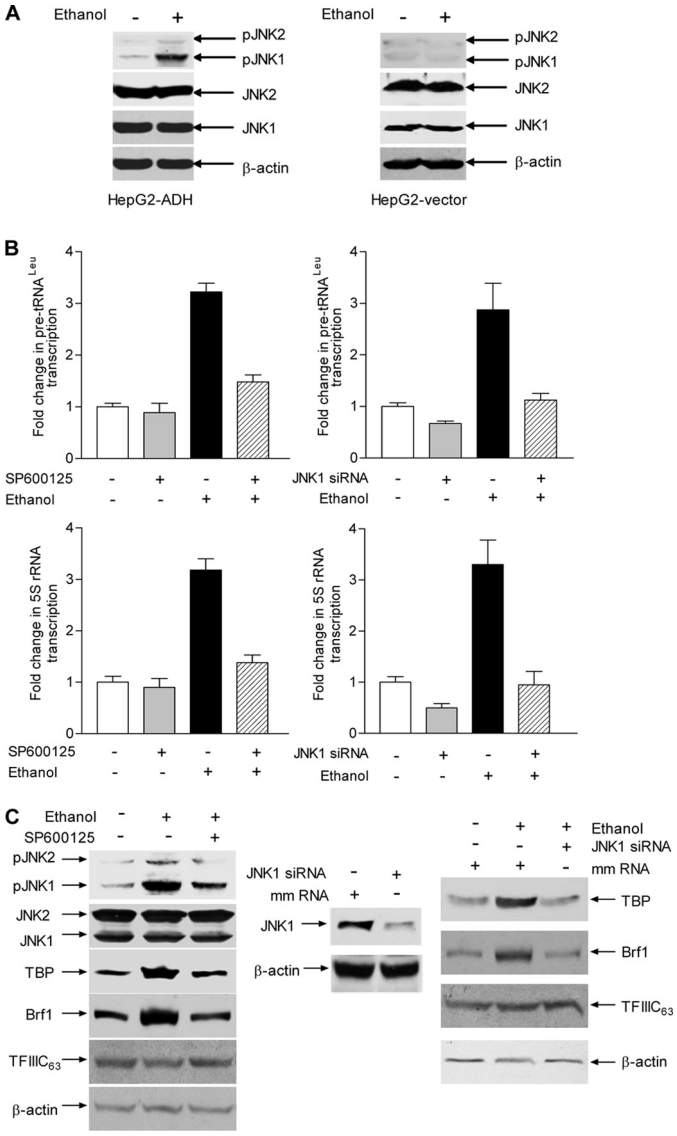
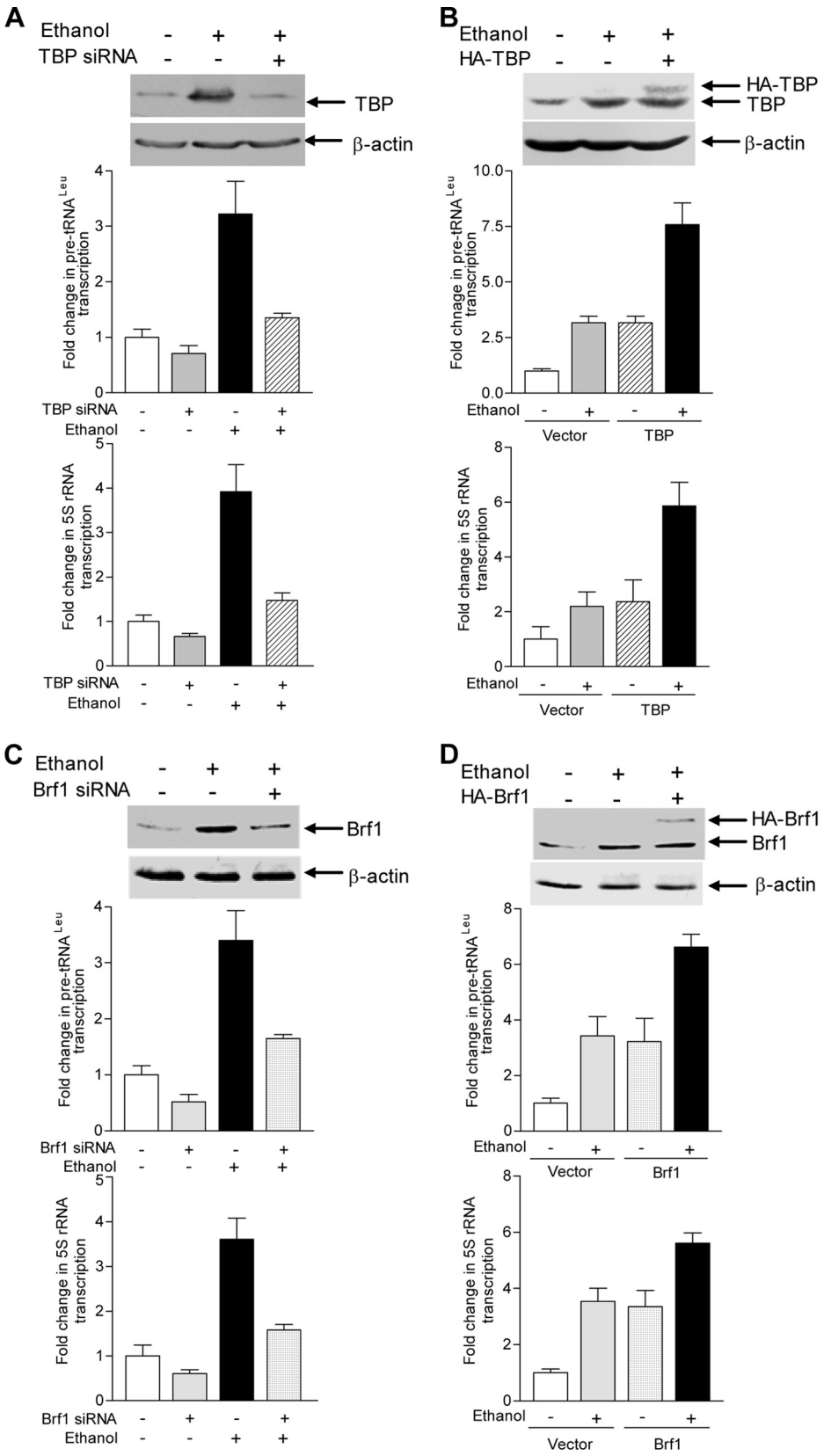


FIGURE 2. **Alcohol-mediated activation of JNK1 is required for induction of RNA pol III-dependent transcription.** *A*, ethanol induces JNK1 activation in HepG2-ADH cells. HepG2-vector and HepG2-ADH cells were treated with or without ethanol. Immunoblot analysis was performed using protein lysates derived from these cells and antibodies against phosphorylated JNK1 and -2 (*pJNK1* and *pJNK2*), JNK1, JNK2, and β-actin as designated. A representative blot from three independent determinations is shown. *B*, ethanol-mediated induction of RNA pol III-dependent transcription requires JNK1 activation. HepG2-ADH cells were pretreated with 5 μM SP600125 and then treated with or without ethanol (*left panel*). HepG2-ADH cells were transfected with either mismatch or JNK1-specific siRNA for 48 h and then treated with ethanol (*right panel*). RNA was isolated, and RT-qPCR was performed to measure the amounts of pre-$tRNA^{Leu}$, 5S rRNA, and GAPDH transcripts. The -fold change was calculated by normalizing to the amount of GAPDH mRNA. The values represent means ± S.E. of three independent determinations. *C*, ethanol-mediated JNK1 activation induces expression of TBP and Brf1. Immunoblot analysis was performed using lysates derived from HepG2-ADH cells pretreated with SP600125 (*left panel*) or transfected with either JNK1 siRNA or mismatch RNA (*middle* and *right panels*) as indicated in *B*. Antibodies against phospho-JNKs, JNKs, TBP, Brf1, $TFIIIC_{63}$, or β-actin were used as indicated.

HepG2-vector cells. Pretreatment of the HepG2-ADH cells with the JNK inhibitor, SP600125, or transfection with siRNA specific for JNK1 to reduce JNK1 expression (Fig. 2*C*) abrogated the ethanol-mediated increase in tRNA and 5S rRNA gene transcription (Fig. 2*B*). These results support the idea

that alcohol preferentially activates JNK1 and that alcohol-mediated induction of RNA pol III transcription requires the activation of JNK1.

Enhanced Expression of Both TBP and Brf1 Mediates Alcohol Induction of RNA pol III-dependent Transcription—To determine the mechanism by which alcohol enhances RNA



pol III transcription, we examined potential changes in the expression of TBP and its associated factor, Brf1. Ethanol treatment induced an increase in both TBP and Brf1 expression but not that of the $TFIIIC_{63}$ subunit in HepG2-ADH cells (Fig. 2*C*). These increases also required the activation of JNK1 as treatment with a JNK inhibitor or decreased expression of JNK1 prevented ethanol-mediated increases in TBP and Brf1 expression.

We next assessed whether the alcohol-mediated increase in TBP and/or Brf1 expression were required for enhanced RNA pol III transcription. Inhibiting the alcohol-mediated increase in either TBP (Fig. 3*A*) or Brf1 (Fig. 3*C*) abrogated alcohol-mediated induction of RNA pol III transcription. Conversely, enhanced TBP (Fig. 3*B*) or Brf1 (Fig. 3*D*) expression had a modest effect on RNA pol III transcription in the absence of alcohol. However, treatment with alcohol induced a robust increase in RNA pol III transcription when either TBP or Brf1 expression was increased. These results support the idea that alcohol mediates RNA pol III-dependent transcription induction through its ability to enhance the expression of both TBP and Brf1.

Alcohol Induces Both Elk-1 and AP-1 Activation, but Only Increased c-Jun Expression Is Required to Induce TBP, Brf1, and tRNA Gene Expression—The TBP promoter is transcriptionally regulated through the recruitment of either Elk-1 (11) or AP-1 (13) to the promoter. Therefore, we measured the affect of alcohol on Elk-1, c-Jun, and c-Fos. Immunoblot analysis indicated that alcohol increased Elk-1 phosphorylation and expression of c-Jun, but no change in c-Fos expression was observed (Fig. 4*A*). AP-1 activity was assessed by transfecting a human AP-1-dependent promoter-driven luciferase construct, or the same promoter containing a mutated AP-1 site, into HepG2-ADH cells (Fig. 4*B*). Expression of these constructs in HepG2-ADH revealed that only the promoter containing an intact AP-1 binding site was induced by alcohol. To determine Elk-1 activity, cells were cotransfected with a Gal4-responsive promoter-luciferase construct and an Elk-1-Gal4 DNA binding domain fusion protein expression vector (Fig. 4*C*). Ethanol treatment induced Elk-1 transactivation function. Together, these results demonstrate that alcohol induces c-Jun expression and Elk-1 phosphorylation and enhances the activities of both AP-1-dependent and Elk-1-dependent promoters.

We next assessed the potential role of Elk-1 and c-Jun in mediating the alcohol-dependent increases in TBP, Brf1, and tRNA gene expression. Cellular Elk-1 expression was decreased, and RT-qPCR was used to measure the amounts of TBP and Brf1 mRNAs as well as pre-$tRNA^{Leu}$ transcripts (Fig. 4*D*). No significant change in the expression of these transcripts was observed upon decreased expression of Elk-1. In contrast, down-regulation of c-*jun* expression abrogated the ethanol-mediated increases in all three of these transcripts (Fig. 4*E*). This can be compared with the expression of $TFIIIC_{63}$, which was unaffected by decreased c-Jun expression. These results support the idea that although Elk-1 is activated in alcohol-treated cells, increased expression of c-Jun alone drives the enhanced expression of TBP, Brf1, and tRNA genes.

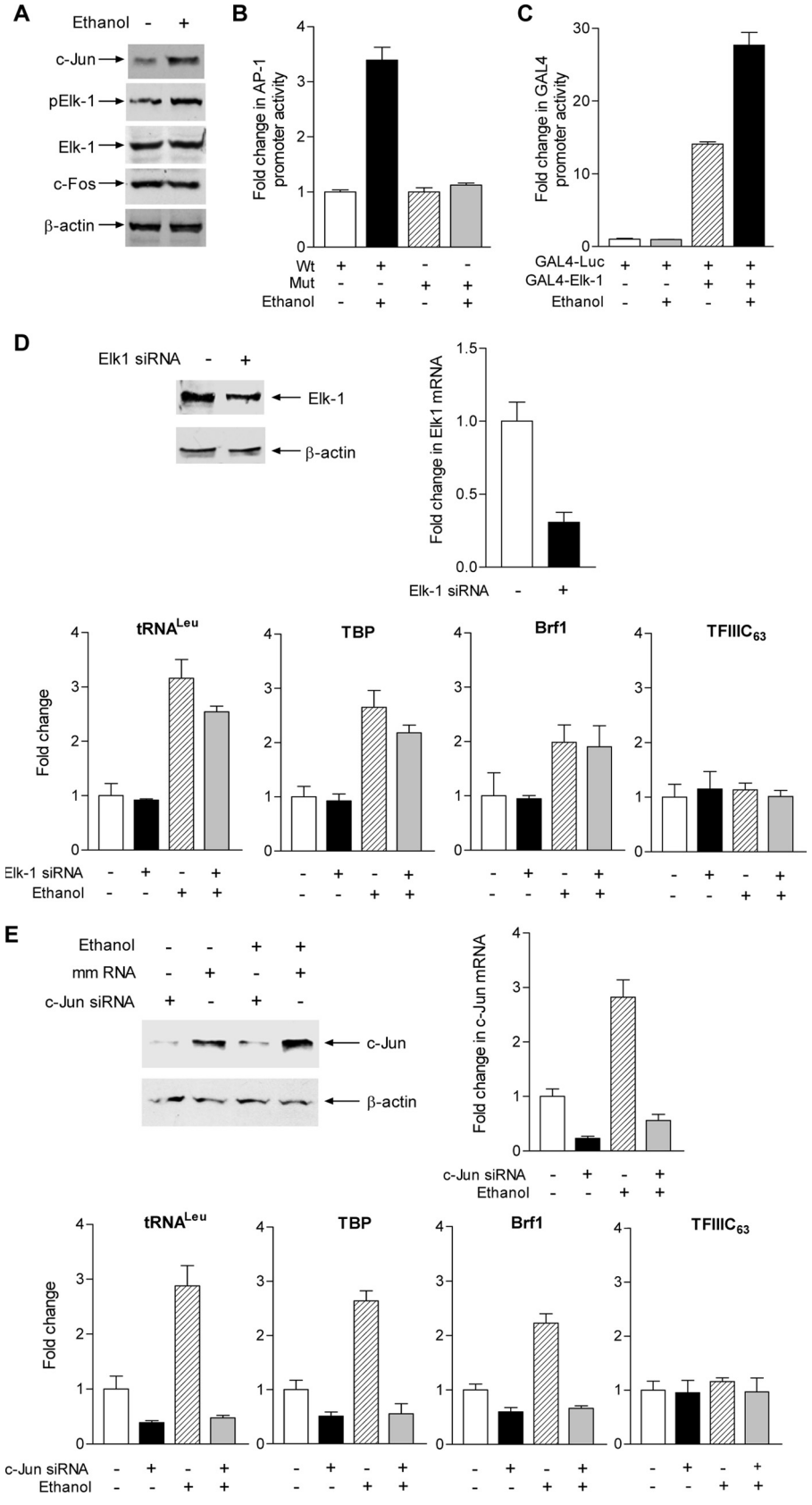
Alcohol Induces Enhanced Recruitment of c-Jun to TBP, Brf1, and tRNA Gene Promoters—To determine whether the alcohol-mediated increases c-Jun required to stimulate TBP, Brf1, and tRNA gene expression were due to enhanced occupancy of c-Jun at these promoters, chromatin immunoprecipitation (ChIP) assays were performed. Ethanol induced a marked increase in the recruitment of c-Jun to the TBP promoter, whereas a modest increase in the occupancy of both c-Fos and Elk-1 was observed (Fig. 5*A*). These results support the idea that alcohol mediates an increase in TBP expression primarily through enhanced expression of c-Jun and its recruitment to the TBP promoter.

Sequence analysis revealed both putative AP-1 and putative Elk-1 binding sites in regions 5′ of the transcription start site of the Brf1 gene (Fig. 5*B*). ChIP analysis revealed that ethanol induced an increase in the occupancy of c-Jun to the region containing an AP-1 binding site, whereas no significant increase in binding of Elk-1 to the Elk-1 binding site was observed. Interestingly, c-Jun, c-Fos, and Elk-1 were also found to occupy the $tRNA^{Leu}$ gene (Fig. 5*C*). Upon ethanol treatment, an increase in TBP recruitment to the $tRNA^{Leu}$ gene was observed, as well as a marked increase in the occupancy of c-Jun. However, alcohol did not induce any apparent change in recruitment of either c-Fos or Elk-1 to the tRNA gene. These results suggest that c-Jun may function directly at sequences near tRNA genes to induce transcription. Together, these results support the idea that alcohol enhances expression of TBP, Brf1, and tRNA gene by facilitating c-Jun recruitment to these promoters.

TBP, Brf1, and RNA pol III-dependent Gene Expression Are Increased in Alcohol-fed Mice—We further assessed whether the observed alcohol-mediated increases in gene expression occur *in vivo*. C57BL/6 mice were chronically fed with a control diet or 3.5% ethanol in the Lieber-DeCarli liquid diet for

FIGURE 3. **Enhanced TBP and Brf1 expression is required for alcohol-mediated induction of RNA pol III-dependent transcription.** *A*, inhibiting ethanol-induced expression of TBP abrogates enhanced RNA pol III-dependent transcription. HepG2-ADH cells were transfected with TBP siRNA (+) or mismatch RNA (−) for 48 h and then treated with ethanol. Resultant protein lysates were subjected to immunoblot analysis using antibodies directed against TBP and β-actin as indicated, and a representative blot is shown (*top*). RT-qPCR was performed on RNA isolated from these cells to measure pre-$tRNA^{Leu}$, 5S rRNA, and GAPDH transcripts (*bottom*). The -fold change was calculated by normalizing to the amount of transcript in cells transfected with control mismatch RNA. *B*, ethanol and enhanced TBP expression cooperate to stimulate RNA pol III-dependent transcription. HepG2-ADH cells were transiently transfected with an HA-tagged TBP expression plasmid or control vector for 48 h. Resultant protein lysates were subjected to immunoblot analysis using antibodies directed against TBP and β-actin as indicated (*top*). RT-qPCR was performed on RNA derived from these cells to measure pre-$tRNA^{Leu}$, 5S rRNA, and GAPDH transcripts (*bottom*). The -fold change was calculated by normalizing to the amount of transcript in cells transfected with vector control plasmid and without ethanol treatment. *C*, inhibiting ethanol-mediated increases in Brf1 expression abolishes RNA pol III-dependent transcription induction. Experiments were carried out as described in *A* except that Brf1 siRNA (+) or mismatch RNA (−) was transiently transfected to reduce Brf1 expression, and antibodies against Brf1 were used for the immunoblot analysis shown (*top*). *D*, ethanol and enhanced Brf1 expression work together to increase RNA pol III-dependent transcription. Experiments were conducted as described in *B*, except that an HA-tagged Brf1 expression construct was used to increase Brf1 expression. The values represent means ± S.E. of three independent determinations.

12 months. These mice display mild fatty liver disease (15). RNA was subsequently isolated from the livers of these mice, and RT-qPCR was used to measure the amounts of pre-tRNA^{Leu} and 5S rRNA (Fig. 6*A*), as well as TBP, and Brf1 mRNA (Fig. 6*B*). Alcohol feeding resulted in a modest induction of all of these transcripts.



To further evaluate potential alterations in these transcripts by alcohol, a transgenic mouse model expressing the hepatitis C NS5A protein was examined. Although C57BL/6 mice fed alcohol over a 12-month period do not develop liver tumors, previous studies demonstrated that alcohol feeding of C57BL/6 NS5A transgenic mice for 12 months induces liver tumors in ~23% of these mice (15). In addition, these mice develop more advanced fatty liver disease and hepatomegaly and have significantly higher serum TNFα levels than wild-type mice fed alcohol. Comparison of non-alcohol-fed wild-type and NS5A transgenic mice revealed that the livers of NS5A transgenic mice exhibited ~2–3-fold higher levels of pre-tRNA^{Leu}, 5S rRNA, TBP, and Brf1 transcripts (Fig. 6). Upon alcohol feeding of the NS5A transgenic mice, further induction of RNA pol III transcription as well as TBP and Brf1 expression was observed in the livers of these mice without tumors. More pronounced induction of these genes occurred in liver tumors of the same mice. When compared with the control livers in non-alcohol-fed NS5A transgenic mice, there was a robust increase in pre-tRNA^{Leu} and 5S rRNA, as well as TBP, and Brf1 mRNA in the liver tumors of the alcohol-fed mice. Together, these results demonstrate that both RNA pol III-dependent transcription and TBP and Brf1 expression are induced in animals that are chronically fed alcohol. In addition, these results support the idea that these gene expression processes are further enhanced in the oncogenic state.

DISCUSSION

Our studies demonstrate that RNA pol III-dependent transcription of tRNA and 5S rRNA is up-regulated by alcohol both *in vitro* and *in vivo*. This is consistent with earlier work revealing that mice given a sublethal dose of ethanol exhibit a transient increase in RNA pol III-transcribed short interspersed element RNA (18). We have also identified transcription factor targets that are responsible for this regulatory event. ADH-mediated metabolism of alcohol is required to stimulate RNA pol III-dependent transcription. Alcohol metabolism by ADH produces endoplasmic reticulum stress (19). Consistent with this idea, activation of the stress kinase, JNK1, is necessary for alcohol to induce RNA pol III-dependent gene expression. JNK1 activation has been shown to promote steatosis and liver damage (20), whereas JNK1 deficiency reduces the susceptibility of diethylnitrosamine-induced hepatocarcinogenesis in IκB kinase-$\beta^{\Delta hep}$ mice (21, 22). Alcohol-mediated activation of JNK1 leads to an increase in c-Jun expression and the subsequent enhanced activity of AP-1. This pathway induces an increase in the expression of Brf1 and TBP, subunits of the TFIIIB complex. Both TBP and Brf1 are limiting components for RNA pol III-dependent transcription in hepatocytes, and enhanced expression of these proteins is required for alcohol to induce transcription. However, given that TBP is also used by RNA pols I and II, it is likely that the alcohol-mediated increases in cellular TBP amounts produce other effects on cellular gene expression.

Our studies demonstrate a new mechanism by which TBP and Brf1 expression are co-regulated through increased expression of c-Jun. We find that the previously observed alcohol-mediated induction of AP-1 activity (23) occurs through the enhanced expression of c-Jun, but not c-Fos. We further identify a new alcohol target, Elk-1, yet enhanced Elk-1 activity did not significantly contribute to the induction of TBP, Brf1, or RNA pol III transcription. Although our previous studies demonstrated that JNK1 mediates an increase in cellular TBP and Brf1 amounts through the activation and recruitment of Elk-1 to the TBP and Brf1 promoters (11, 17), these studies were performed by depletion of JNK1. It is likely that the type of stimuli that activates JNK1 and the activation of other signaling events ultimately determine the relative importance of Elk-1 and c-Jun in driving both TBP and Brf1 transcription. Thus, although alcohol induces the activation of both Elk-1 and AP-1, its ability to stimulate TBP and Brf1 expression is primarily driven by enhanced c-Jun expression and its recruitment to their promoters. However, as initial studies showed that decreased expression of Elk-1 suppresses the tumorigenicity of hepatocellular carcinoma cells (24), Elk-1 activation is likely to regulate at least some subset of genes that play an important role in alcohol-mediated liver disease.

Intriguingly, we found that Elk-1, c-Jun, and c-Fos all occupy sites in close proximity to the tRNA^{Leu} gene. Recent genome-wide studies demonstrated that many RNA pol II transcription factors are associated with RNA pol III transcribed genes and overlap the occupancy of RNA pol III (25). Importantly, alcohol induces a marked increase in c-Jun occupancy on the tRNA gene, suggesting that it may play a direct role in regulating RNA pol III-dependent genes. Thus, it is possible that alcohol-dependent increases in c-Jun may not only drive enhanced expression of the TFIIIB subunits but also act di-

FIGURE 4. **Alcohol-mediated induction of TBP, Brf1, and tRNA gene expression requires increased c-Jun expression.** *A*, ethanol induces an increase in Elk-1 phosphorylation (*pElk-1*) and c-Jun expression. HepG2-ADH cells were treated with or without ethanol. Immunoblot analysis was performed using protein lysates derived from these cells, and antibodies were used to probe the proteins shown. *B*, ethanol induces AP-1-dependent promoter activity. HepG2-ADH cells were transfected with a human AP-1-dependent promoter-luciferase construct or the same promoter containing a mutated (*Mut*) AP-1 site. All cells were cotransfected with a CMV-driven β-galactosidase reporter construct. Resultant cell lysates from cells treated or untreated with ethanol were analyzed for luciferase and β-galactosidase activities. The -fold change in AP-1-dependent promoter activity was calculated relative to untreated control. *C*, ethanol stimulates Elk-1 transactivation function. HepG2-ADH cells were cotransfected with a Gal4-responsive promoter-luciferase construct containing a vector and either an Elk-1-Gal4 fusion protein expression vector or an empty vector. Cells were treated or untreated with ethanol, and resultant cell lysates were analyzed for luciferase and β-galactosidase activities. -Fold change was calculated based on normalization to luciferase activity in untreated Gal4 reporter cells. All values shown are the means ± S.E. of at least three independent experiments. *D*, Elk-1 activation is not required for ethanol-mediated induction of TBP, Brf1, and tRNA gene expression. Elk-1-specific siRNAs or mismatch RNAs were transfected into HepG2-ADH cells. Resultant protein lysates derived from cells treated or untreated with ethanol were used for immunoblot analysis to detect Elk-1 and β-actin (*top left*). RT-qPCR was performed using RNA isolated from these cells to measure Elk-1 mRNA (*top right*) and tRNA^{Leu}, TBP, Brf1, and TFIIIC$_{63}$ transcription (*bottom*). *E*, enhanced c-Jun expression is required to drive ethanol-mediated increases in TBP, Brf1, and tRNA gene expression. *Top left*, same as in *D* except that HepG2-ADH cells were transfected with c-*jun* siRNA or mismatch RNA, and immunoblot analysis was used to detect c-Jun and β-actin. RT-qPCR was performed to measure c-Jun mRNA (*top right*) and tRNA^{Leu}, TBP, Brf1, and TFIIIC^{63} transcription (*bottom*). The values represent means ± S.E. from three independent experiments.

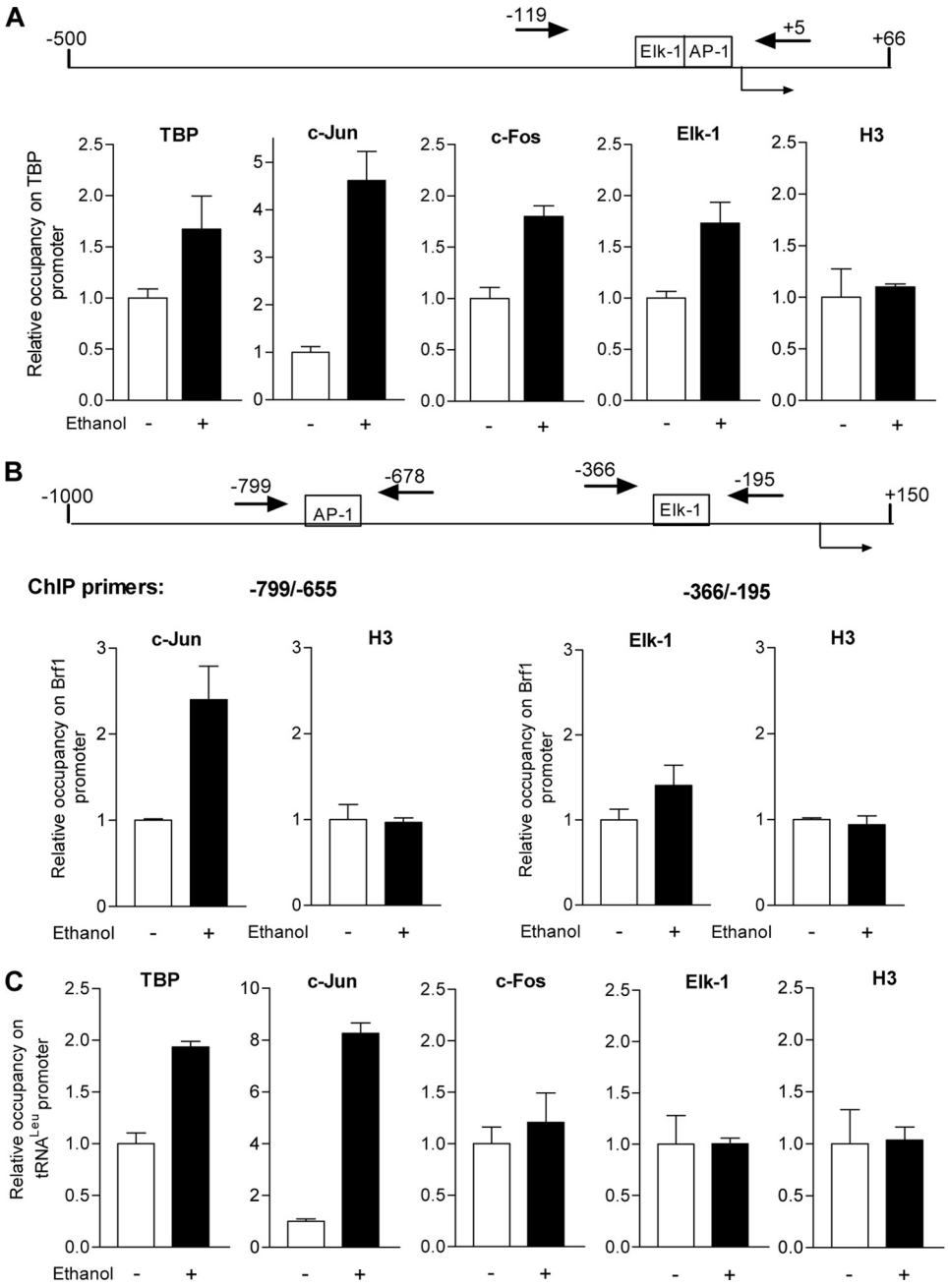


FIGURE 5. **Ethanol induces recruitment of c-Jun to TBP, Brf1, and tRNA gene promoters.** *A*, ethanol-mediated binding of transcription components to the TBP promoter. A schematic of the TBP promoter and primers used for ChIP assays is designated relative to the Elk-1 and AP-1 binding sites (*top*). HepG2-ADH cells were treated with or without ethanol, and ChIP assays were performed using TBP, c-Jun, c-Fos, Elk-1, and H3 antibodies and qPCR to quantify the amplified DNA. The relative occupancy of the proteins was calculated based on the control (no ethanol treatment). All values shown are the means ± S.E. of at least three independent chromatin preparations. *B*, ethanol enhances c-Jun recruitment to the Brf1 promoter. *Top*, schematic showing Elk-1 and AP-1 binding sites and the primers used for ChIP assays. Chromatin was isolated from HepG2-ADH cells treated with or without ethanol using antibodies of c-Jun, Elk-1, or H3, and qPCR was used to quantify the DNA. *C*, ethanol induces c-Jun recruitment to the tRNA^{Leu} gene. Cells were treated with or without ethanol, and ChIP assays were performed to measure the recruitment of TBP, c-Jun, c-Fos, Elk-1, and H3 on the tRNA^{Leu} gene promoter. The relative occupancy of the proteins was calculated based on the control (no ethanol treatment). All values shown are the means ± S.E. of at least three independent chromatin preparations.

rectly at RNA pol III promoters to facilitate transcription of these genes.

Although TBP is a central transcription initiation factor, it is regulated by oncogenic stimuli and it possesses oncogenic properties. Ras activation induces TBP expression, and this increase in TBP is required for Ras to promote cellular transformation (26). Small increases in TBP expression in rat1a fibroblasts induce anchorage-independent growth and tumorigenesis in mice (26). Importantly, the ability of TBP to promote cellular transformation requires enhanced RNA pol III-dependent transcription (5). Further supporting its role in oncogenesis, increased TBP expression has been observed in a clinically significant number of human colon cancers (26).[3]

[3] D. L. Johnson and S. S. Johnson, unpublished results.

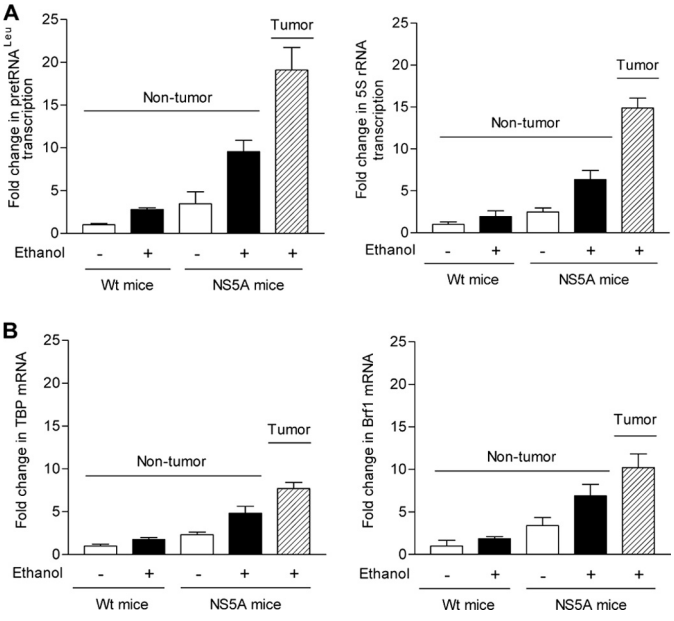


FIGURE 6. **Chronic alcohol administration in mice induces both TBP-dependent and RNA pol III-dependent transcription.** Wild-type C57BL/6 mice or C57BL/6 transgenic mice harboring the NS5A gene that is selectively expressed in hepatocytes were chronically fed with control diet or 3.5% ethanol in the Lieber-DeCarli liquid diet for 12 months. Liver tissues were harvested from these mice, RNA was extracted from non-tumor or tumor portions of the livers, and RT-qPCR was used to measure the amount of pre-tRNA^{Leu} and 5S rRNA transcripts (*top*) and TBP and Brf1 transcripts (*bottom*) relative to GAPDH. The values represent means ± S.E. from three independent experiments. Each group of mice includes at least three mice. The -fold change was calculated in each group by normalizing to wild-type mice fed with control diet.

Overexpression of Brf1 has also been shown to transform mouse embryo fibroblasts (6), and Brf1 is overexpressed in cervical cancers (27). These results, together with our current studies, support the idea that alcohol-mediated increases in TBP and Brf1 drive alcohol-induced liver disease.

It has long been observed that neoplastic cells contain increased levels of rRNAs and tRNAs and that a high rate of RNA pol III transcription is a general feature of transformed and tumor cells (3). Regulating the synthesis of tRNAs and rRNAs is a fundamental determinant of the oncogenic potential of cells. Elevated RNA pol III transcription has been shown to be essential for cellular transformation and tumorigenesis (5, 6). Our current results support the idea that alcohol induces this class of genes, which is consistent with its role as a pro-oncogenic agent.

Although enhanced RNA pol III-dependent gene activity is required for cellular transformation and TBP and Brf1 can promote oncogenic transformation, the role that these genes might play in determining other cellular phenotypes and disease states in animal models has not yet been examined. Our findings support the idea that in the livers of alcohol-fed mice, expression of TBP, Brf1, and RNA pol III-dependent transcription increases with the severity of liver disease and hepatocellular transformation. Upon alcohol feeding, the transgenic animals expressing the NS5A protein, which exhibit more advanced fatty liver disease when compared with the wild-type mice, also exhibit higher RNA pol III-dependent gene activity and TBP and Brf1 expression than the wild-type animals. For those NS5A transgenic mice that do form liver tumors, gene activity is further enhanced. These results suggest that induction of RNA pol III transcription contributes to alcohol-associated liver disease. Given these results, TBP-, Brf1-, and RNA pol III-dependent transcription could be considered as biomarkers for the progression of alcoholic liver disease.

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REFERENCES

1. Wilfred de Alwis, N. M., and Day, C. P. (2007) *Semin. Liver Dis.* **27,** 44–54
2. Johnson, D. L., and Johnson, S. A. (2008) *Science* **320,** 461–462
3. White, R. J. (2004) *Oncogene* **23,** 3208–3216
4. Winter, A. G., Sourvinos, G., Allison, S. J., Tosh, K., Scott, P. H., Spandidos, D. A., and White, R. J. (2000) *Proc. Natl. Acad. Sci. U.S.A.* **97,** 12619–12624
5. Johnson, S. A., Dubeau, L., and Johnson, D. L. (2008) *J. Biol. Chem.* **283,** 19184–19191
6. Marshall, L., Kenneth, N. S., and White, R. J. (2008) *Cell* **133,** 78–89
7. Zhong, S., Zhang, C., and Johnson, D. L. (2004) *Mol. Cell. Biol.* **24,** 5119–5129
8. Zhang, C., Comai, L., and Johnson, D. L., (2005) *Mol. Cell. Biol.* **25,** 6899–6911
9. Colgan, J., and Manley, J. L. (1992) *Genes Dev.* **6,** 304–315
10. Sadovsky, Y., Webb, P., Lopez, G., Baxter, J. D., Fitzpatrick, P. M., Gizang-Ginsberg, E., Cavailles, V., Parker, M. G., and Kushner, P. J. (1995) *Mol. Cell. Biol.* **15,** 1554–1563
11. Zhong, S., Fromm, J., and Johnson, D. L. (2007) *Mol. Cell. Biol.* **27,** 54–64
12. Johnson, S. A., Mandavia, N., Wang, H. D., and Johnson, D. L. (2000) *Mol. Cell. Biol.* **20,** 5000–5009
13. Fromm, J. A., Johnson, S. A., and Johnson, D. L. (2008) *Mol. Cell. Biol.* **28,** 6483–6495
14. Clemens, D. L., Forman, A., Jerrells, T. R., Sorrell, M. F., and Tuma, D. J. (2002) *Hepatology* **35,** 1196–1204
15. Machida, K., Tsukamoto, H., Mkrtchyan, H., Duan, L., Dynnyk, A., Liu, H. M., Asahina, K., Govindarajan, S., Ray, R., Ou, J. H., Seki, E., Deshaies, R., Miyake, K., and Lai, M. M. (2009) *Proc. Natl. Acad. Sci. U.S.A.* **106,** 1548–1553
16. Crighton, D., Woiwode, A., Zhang, C., Mandavia, N., Morton, J. P., Warnock, L. J., Milner, J., White, R. J., and Johnson, D. L. (2003) *EMBO J.* **22,** 2810–2820
17. Zhong, S., and Johnson, D. L. (2009) *Proc. Natl. Acad. Sci. U.S.A.* **106,** 12682–12687
18. Li, T., Spearow, J., Rubin, C. M., and Schmid, C. W. (1999) *Gene* **239,** 367–372
19. Nishitani, Y., and Matsumoto, H. (2006) *FEBS Lett.* **580,** 9–14
20. Sakurai, T., Maeda, S., Chang, L., and Karin, M. (2006) *Proc. Natl. Acad. Sci. U.S.A.* **103,** 10544–10551
21. Sakurai, T., He, G., Matsuzawa, A., Yu, G. Y., Maeda, S., Hardiman, G., and Karin, M. (2008) *Cancer Cell* **14,** 156–165
22. Singh, R., Wang, Y., Xiang, Y., Tanaka, K. E., Gaarde, W. A., and Czaja, M. J. (2009) *Hepatology* **49,** 87–96
23. Yeligar, S. M., Machida, K., Tsukamoto, H., and Kalra, V. K. (2009) *J. Immunol.* **183,** 5964–5976
24. Ying, T. H., Hsieh, Y. H., Hsieh, Y. S., and Liu, J. Y. (2008) *Cell Biol. Int.* **32,** 210–216
25. Raha, D., Wang, Z., Moqtaderi, Z., Wu, L., Zhong, G., Gerstein, M., Struhl, K., and Snyder, M. (2010) *Proc. Natl. Acad. Sci. U.S.A.* **107,** 3639–3644
26. Johnson, S. A., Dubeau, L., Kawalek, M., Dervan, A., Schönthal, A. H., Dang, C. V., and Johnson, D. L. (2003) *Mol. Cell. Biol.* **23,** 3043–3051
27. Cabarcas, S., Jacob, J., Veras, I., and Schramm, L. (2008) *BMC Mol Biol.* **9,** 74