We previously showed that ethanol regulates dopamine \(\beta\)-hydroxylase (DBH) mRNA and protein levels in human neuroblastoma cells (Thibault, C., Lai, C., Wilke, N., Duong, B., Olive, M. F., Rahman, S., Dong, H., Hodge, C. W., Lockhart, D. J., and Miles, M. F. (2000) Mol. Pharmacol. 58, 1593–1600). DBH catalyzes norepinephrine synthesis, and several studies have suggested a role for norepinephrine in ethanol-mediated behaviors. Here, we performed a detailed analysis of mechanism(s) underlying ethanol regulation of DBH expression in SH-SY5Y cells. Transient transfection analysis showed that ethanol (25–200 mM) caused concentration- and time-dependent increases in DBH gene transcription. Progressive deletions identified ethanol-responsive sequences in the –262 to –142 bp region of the DBH gene promoter. Mutagenesis of cAMP-response element (CRE) sequences in this region abolished ethanol responsiveness while maintaining responsiveness to phorbol esters. Co-expression of dominant-negative CRE-binding protein greatly reduced ethanol induction of DBH. Inhibitors of protein kinase A, casein kinase II, and MAPK reduced ethanol induction of DBH promoter activity. Pharmacogenomic studies with microarrays showed that protein kinase A, MEK, and casein kinase II inhibitors blocked induction of DBH and a large subset of ethanol-responsive genes. These genes had diverse functional groupings, including multiple members of the MAPK and phosphatidylinositol signaling cascades. Real-time PCR analysis validated select microarray results. Taken together, these results suggest that ethanol regulation of DBH requires a functional CRE and its binding protein and may require interaction of multiple kinase pathways. This mechanism may also mediate ethanol responsiveness of a complex subset of genes in neural cells. These studies may have implications for behavioral responses to ethanol or mechanisms underlying ethanol-related neurological disease.

Acute and chronic exposure to ethanol can cause changes in signal transduction and gene expression in multiple cell types or organ systems (1, 2). In the nervous system, some of these changes in gene expression likely contribute to mechanisms underlying development of long-lasting behaviors such as tolerance, dependence, sensitization, and craving, as seen with other drugs of abuse (3). Thus, identifying ethanol-responsive genes and their cognate mechanism(s) of regulation might provide new targets for intervention in behaviors associated with ethanol abuse and alcoholism.

We (4, 5, 7, 9, 10) and others (6, 8, 11) have previously identified specific genes regulated by ethanol in neural cells or the intact nervous system. However, relating individual gene regulation events to complex phenotypic changes induced by ethanol in cells or the nervous system is a difficult task. To circumvent this difficulty, we recently utilized expression profiling with high density oligonucleotide arrays to identify patterns of gene regulation occurring with ethanol (12). These studies identified several distinct mRNA expression patterns occurring in SH-SY5Y neuroblastoma cells exposed to ethanol. Among these patterns were a group of genes involved in the production and metabolism of the neurotransmitter norepinephrine. Indeed, the most prominent mRNA induction occurred with dopamine \(\beta\)-hydroxylase (DBH), the enzyme responsible for conversion of dopamine to norepinephrine. We also found that ethanol increased DBH protein levels and norepinephrine production in SH-SY5Y cells and elevated DBH mRNA levels in mouse adrenal gland.

DBH is localized in neurosecretory vesicles of noradrenergic neurons of the central and peripheral nervous systems and in chromaffin granules of adrenal medullary cells (13, 14). Norepinephrine has been suggested to play an important role in several ethanol-related behaviors. Infusion of norepinephrine into the hypothalamic paraventricular nucleus increases ethanol consumption in rats (15). Some studies have also shown that acute administration of ethanol increases synthesis, turnover, and release of norepinephrine in rat brain (16–20). Recently, DBH knockout mice were shown to have reduced ethanol preference in a two-bottle choice paradigm (21). Thus, there is significant evidence suggesting a role of DBH in ethanol-related behaviors.

Defining how ethanol regulates DBH gene expression could have important implications for understanding ethanol-dependent long-term changes in central nervous system function. Our recent microarray studies suggested a role for CAMP
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signaling in ~30% of the ethanol-responsive genes, including DBH (12). One of the best characterized elements regulating DBH gene expression is the cAMP-response element (CRE) motif, which binds the CRE-binding protein (CREB) transcription factor (22–24). CREB can be phosphorylated by multiple kinases, including protein kinase A (PKA), Ca2+/calmodulin-dependent kinase (CaMK), casein kinase II (CKII), protein kinase C (PKC), and mitogen-activated protein kinase (MAPK) (25–30), thereby altering gene transcription (31, 32). Phosphorylation at Ser133 is required for CREB-induced gene transcription; however, CREB activity and specificity can be modulated by phosphorylation at additional sites on CREB or of proteins associated with CREB (for review, see Ref. 33).

Although there is significant evidence suggesting that cAMP signaling is an important target for ethanol action (34–40), there is little evidence actually linking ethanol-induced changes in expression of a native gene to the cAMP pathway. Therefore, we undertook this study to investigate the mechanism of ethanol regulation of DBH expression and, in particular, whether cAMP signaling plays a role in ethanol induction of DBH transcription. Here, we report that ethanol activates DBH gene expression at the transcriptional level. We also show that ethanol stimulates human DBH gene transcription through a CRE requiring CREB and multiple protein kinase pathways in SH-SY5Y cells. Importantly, these kinase pathways also seem to be required for a complex subset of ethanol-signaling in SH-SY5Y cells. Importantly, these kinase pathways also seem to be required for a complex subset of ethanol-responsive genes identified by microarray studies. Our results also suggest that ethanol-induced DBH gene expression may be related to the function of ethanol via CRE-mediated gene activation.

EXPERIMENTAL PROCEDURES

Reagents—Cell culture medium, serum, and supplies were obtained from Invitrogen and Hyclone Laboratories (Logan, UT). Plasmid preparation kits were obtained from QIAGEN Inc. (Santa Clarita, CA). Inhibitors (H-89, GF 109203X, PD 98059, and KN62) were purchased from Calbiochem. Chrysin and other reagents were purchased from Sigma. Restriction endonucleases were obtained from Promega (Madison, WI). STAT-60 reagent was purchased from Tel-Test (Friendswood, TX).

Cell Culture—The human neuroblastoma cell line SH-SY5Y-AH1861 (passage 7) was obtained from Dr. Robert Messing (University of California, San Francisco). Cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 2% fetal bovine serum in a 10% CO2 atmosphere at 37 °C. Fresh medium was supplied every 2 days, and cultures were split after 1 week.

Plasmid Constructs—The DBH promoter deletion plasmids (2600CAT, 978CAT, 262CAT, 142CAT, 114CAT, 62CAT, and 38CAT) were generated in the laboratory of Dr. Kwang-Soo Kim and have been previously described (23). Constructs 978CAT and 262CAT were constructed by subcloning the SpI1-XhoI fragments from 978CAT and 262CAT into the pBLCAT3-1 plasmid (41). pBLCAT3-1 is a derivative of pBLCAT3 that was constructed by deleting the CRE-like and TATA-like sequences upstream of the multiple cloning site and derives significantly lower background CAT activity compared with the pBLCAT3 backbone (41). The original mutant constructs mAP1, mYY1, and mCRE (24) were similarly subcloned in the pBLCAT3-1 backbone to generate mAP1, mYY1, and mCRE, respectively. Dr. M. E. Greenberg (Harvard Medical School) kindly provided the dominant-negative construct pRbSVCreBEM1 expressing CREB-M1.

Transient Transfection Assays—DNA constructs used for transfections were purified by alkaline lysis using a QIAGEN Endo-Free Maxi prep kit. Cells were seeded in 6-well plates at a density of 0.3 × 106 cells/well 24 h prior to transfections. Transient transfections were performed with a total of 1 μg of plasmid DNA using FuGENE 6 reagent (Roche Applied Science) according to the manufacturer’s instructions. After overnight incubation, ethanol (0–200 mM) was added with fresh medium, and plates were sealed with Parafilm. Cells were harvested 24 h later in 1× reporter lysis buffer (Promega), and aliquots of cell extracts were assayed for protein and CAT activity (42). Preliminary studies with a β-galactosidase reporter plasmid indicated that ethanol reduced β-galactosidase activity in the presence of the DBH construct (30–50%) and therefore could not be used as an internal control. Furthermore, when basal CAT activity was normalized with β-galactosidase, no significant differences were observed in transfection efficiency between deletion constructs (see Fig. 2C). Therefore, CAT activity was normalized to total lysate protein (43), and transfactions were repeated three to nine times in triplicate to two to three independent preparations of plasmids. Results are expressed as CAT activity/μg protein. Plasmid DNA microinjection was performed according to the recommended procedures of Affymetrix (Santa Clara, CA) and have been described previously (12). In short, equivalent amounts (15 μg) of total RNA for each sample were reverse-transcribed into double-stranded cDNA using the Superscript choice system (Invitrogen), followed by biotin-labeled cRNA synthesis using a BioArray High Yield RNA transcription labeling kit (ENZO Diagnostics, Farmingdale, NY). Prior to hybridization, cRNA samples (10 μg) were fragmented randomly to an average size of 30–60 bases by incubation at 94 °C for 35 min in 40 μl Tris acetate (pH 8.1), 100 mM potassium acetate, and 30 μl magnesium acetate.

Array Hybridization and Scanning—Labeled cRNA samples were analyzed on Human Genome U95Av2 oligonucleotide array Set A (Affymetrix) representing 12,600 known genes and 6,500 expressed sequence tags, in which each gene is represented by a probe set consisting of 16–20 probes. Hybridizations and scanning were performed according to Affymetrix protocols and as described previously (12). Briefly, aliquots of fragmented cRNA (10 μg in a 200-μl master mixture) were hybridized to arrays at 45 °C for 16 h in a rotator oven set at 60 rpm. Following hybridization, arrays were washed and stained with streptavidin-phycocerythrin (Molecular Probes, Inc., Eugene, OR) using an Affymetrix fluids station. Hybridization signals were amplified by incubating arrays with biotinylated goat anti-streptavidin antibody (Sigma) in staining buffer. After washing, arrays were scanned using a confocal GeneArray scanner (Hewlett-Packard Co., Palo Alto, CA).

Microarray Data Analysis—The Affymetrix GeneChip Data Analysis Software (MAS Version 4.0) was used for consistent changes in replicate experiments using a permutation technique (45). This statistical analysis of microarrays (SAM) method gives a more valid estimate of statistical significance when studying a large data set and identifies genes with changes in expression by assimilating a set of gene-specific t tests. The percentage of genes identified by this method is dependent on the set of genes used to set the false discovery rate. We utilized SAM with a multiclass analysis and selected 410 reliable genes using a false discovery rate of 20%. This somewhat relaxed stringency level was used so as not to exclude potentially informative genes from multivariate and functional group analyses.
S-scores from SAM analysis results were again filtered to select genes having an S-score \( \geq 1.5 \) or \( < -1.5 \) in at least two samples. This resulted in 390 final candidate genes.

To identify and graphically display groups of genes with correlated expression patterns, hierarchical clustering was applied using the centered correlation method in the program Cluster (46). Results were visualized with the Tree View program (46).

**Real-time Reverse Transcription (RT-PCR) Analysis**—Quantitative real-time RT-PCRs for selected genes were performed in the Nucleic Acid Research Facility of the Virginia Commonwealth University Massey Cancer Center using the ABI Prism 7900 sequence detection system (Applied Biosystems, Foster City, CA) according to the manufacturer’s protocol. Probes and primers were designed using Primer Express Version 2.0 (Applied Biosystems), and these primers span intron-exon sequences of the gene. TaqMan fluorogenic probes were labeled at the 5’-end with a reporter, 6-carboxyfluorescein, and at the 3’-end with a quencher dye, \( N,N',N'- \)tetramethyl-6-carboxytetramethylrhodamine. Probe and primer sequences were as follows: 1) for the \( DBH \) gene, sense primer (5’-GTGCTTATCAAGGGAGCTTCCAAAA-3’), antisense primer (5’-GCCCTATTGCCCTTTGGT-3’), and TaqMan probe (5’-TGGCAACACATTATCAAGTACCCAGGC-3’); 2) for the \( \beta \)-like kinase gene, sense primer (5’-CTCCTGCCGCGCTTCTTG-3’), antisense primer (5’-CACCCTGAAACATTGTGATCTCTG-3’), and TaqMan probe (5’-CACAGGACATTTGCAAGCTGTAATTTGATCT-3’); and 3) for the thioredoxin-interacting protein gene, sense primer (5’-GCCATTGTGGTTCCAGGCTTCATGA-3’) and antisense primer (5’-CTCATTGTGGTTCCAGGCTTCATGA-3’), and TaqMan probe (5’-ACCATTCTCATCTCACA-CGTGGTGGTTGGC-3’).

All quantitative RT-PCR analyses were performed in triplicate under conditions recommended by the manufacturer (TaqMan® One Step PCR Master Mix reagent kit, PN 4309169; ABI, Foster City, CA). The cycling conditions were 48 °C for 30 min, 95 °C for 10 min, and 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The threshold cycle was determined to provide optimal standard curve value (0.98–1.0). 18 S rRNA from the pre-developed TaqMan® assay reagents (PN 4310893E) was used as endogenous control. Input RNA amounts were calculated with relative standard curves for all mRNAs of interest and 18S rRNA. Transcript abundance was calculated as the ratio of control or experimental sample values normalized to 18S rRNA.

**RESULTS**

**Ethanol Causes Dose- and Time-dependent Increases in Human DBH Gene Transcription**—To determine whether ethanol regulates \( DBH \) gene expression at the transcriptional level, we performed transient transfection assays in SH-SY5Y cells. The 5’-flanking sequences (−978 to +9 bp) of the \( DBH \) promoter, containing defined regulatory elements such as glucocorticoid-response element, AP1, AP2, CRE, YY1, and TATA, were fused to a CAT reporter gene (Fig. 1A). Cells transfected with either 978CAT or pBLCAT3-1 plasmid DNA (vector control) were treated with increasing concentrations of ethanol (50–200 mM) for 24 h or with 100 mM ethanol for 6, 12, and 24 h. Ethanol increased \( DBH \) promoter activity in a concentration-dependent manner with a maximal induction at 150 mM ethanol (Fig. 1B). The fold increase in CAT activity was 2.5 ± 0.1 (mean ± S.D.) after a 6-h exposure to 100 mM ethanol (Fig. 1C). Maximal \( DBH \) promoter induction (4.3 ± 0.3-fold × mock-treated) was seen at 12 h post-ethanol exposure.

**Identification of an Ethanol-responsive Region in the DBH Gene Promoter**—Deletion analysis was performed to identify potential ethanol-responsive regulatory elements residing within the 5’-proximal region of the \( DBH \) gene (Fig. 2A). The basal CAT activity of the deleted constructs showed variable expression levels, depending upon the presence of various positive and negative elements, as previously reported by Ishiguro et al. (47). Deletions of the 5’-promoter sequences (−2600 to −262 bp) did not produce any significant change in ethanol responsiveness of the gene (Fig. 2B). However, further deletion from −262 to −142 bp markedly reduced ethanol responsiveness. These results indicate that the region between −262 and −142 bp contains sequence information necessary for ethanol regulation of \( DBH \) transcription.

**Mutation of the CRE Abolishes Ethanol Responsiveness of the DBH Promoter**—The \( DBH \) promoter sequences from −262 to −142 bp contain CRE, AP1, and YY1 cis-regulatory elements. CRE is an essential cis-regulatory element for cAMP-stimu-
lated transcription of DBH and also modulates basal expression of the gene (23). To evaluate the possible role of these promoter elements in ethanol induction of DBH transcription, transient transfection analysis of site-directed mutant constructs (Fig. 3A) was performed in SH-SY5Y cells. Mutations of putative sites for AP1 and YY1 did not alter ethanol responsiveness of the DBH promoter. However, mutation of the CRE site completely abolished ethanol induction (Fig. 3B). Even though the basal level of expression for the mCRE construct was greatly reduced, this was still 2-fold higher than the background of mock-treated cells, suggesting that decreases in basal activity did not account for loss of ethanol responsiveness. Furthermore, the mCRE construct was induced by phorbol 12-myristate 13-acetate treatment, thus confirming that it could still be activated (Fig. 3C). These results strongly suggest that a CRE cis-regulatory element mediates ethanol responsiveness of the DBH gene.

Coexpression of Dominant-negative CREB Attenuates Ethanol Induction of DBH Promoter Activity—To determine whether CREB, the cognate DNA-binding protein of the CRE site, is required for ethanol induction of the DBH promoter, we used a dominant-negative inhibitor of CREB function (48). Either empty or mutant CREB expression vector was transiently cotransfected along with 978CAT into SH-SY5Y cells. CREBM1 is a mutant form of CREB that can still bind to CRE, but cannot be activated by phosphorylation at Ser133, as this site has been mutated to alanine (48). Phosphorylation at this site is critical for CREB activation by various protein kinases such as PKA, MAPK, and CaMK (49, 50). As shown in Fig. 4, coexpression of CREBM1 greatly decreased ethanol- or forskolin-induced DBH promoter activity. Although ethanol still caused slight increases in DBH promoter activity in the presence of the CREBM1 construct, similar residual induction was seen with forskolin, a known activator of PKA-dependent Ser133 phosphorylation. This strongly suggests that the CRE site and CREB are likely to play a role in ethanol regulation of DBH.

Ethanol Induction of DBH Gene Transcription Involves Multiple Protein Kinases—Prior studies have shown that ethanol can activate the cAMP pathway, causing nuclear translocation of PKA with subsequent CREB phosphorylation (2, 34, 37, 38, 40, 51–54). Initially, to investigate whether ethanol regulation of DBH promoter activity requires the classical cAMP-dependent signal transduction pathway via PKA, we employed a selective PKA inhibitor (H-89) in transient transfection assays. No apparent morphological or viability changes were observed after 6 or 16 h of H-89 treatment. Pretreatment with H-89 greatly decreased DBH promoter activity caused by ethanol or the PKA activator Bt2cAMP (Fig. 5A). The basal CAT activity was also reduced with H-89 treatment, consistent with previous studies showing that PKA, via CRE/CREB, regulates basal DBH promoter activity (55). These results suggest a requirement for PKA-mediated signaling in the ethanol response.

Several other kinases such as CKII, MAPK, PKC, and CaMK are known to phosphorylate CREB and thereby activate CREB-dependent transcription (25, 26, 29, 30, 49, 56–59). We explored the possible involvement of these kinases in ethanol regulation of DBH. Cells were pretreated with inhibitors of MEK (PD 98059), CKII (chrysin), CaMK (KN62), and PKC (GF 109203X) for 30 min and incubated for an additional 6 h with 100 mM ethanol. Both chrysin and PD 98059 attenuated DBH CAT activity (Fig. 5), implying the involvement of CKII and MEK, respectively. GF 109203X and KN62 showed no effect on ethanol-induced DBH CAT activity, suggesting that PKC and CaMK are not required for ethanol regulation of DBH. PD 98059 and KN62 also reduced basal CAT activity, whereas GF 109203X and chrysin had either very little or no effect, respectively. This suggests the involvement of MEK and CaMK in regulating basal DBH gene expression. Importantly, the differing sensitivity of basal activity (CaMK, MEK, and PKA) versus ethanol induction (CKII, MEK, and PKA) to various kinase inhibitors reinforces the specificity of inhibitor effects on the ethanol response.

Pharmacogenomic Analysis: Inhibitors of PKA, CKII, and MEK Kinases Attenuate Induction of an Ethanol-responsive Expression Cluster—Our previous microarray studies (12) showed that ~30% of ethanol-responsive genes in SH-SY5Y cells, including DBH, are also cAMP-responsive. We hypothe-
nucleotide array analysis. Comparisons were generated between control versus ethanol-treated, control versus inhibitor-treated, and ethanol- versus inhibitor/ethanol-treated samples using the S-score analysis algorithm. The S-score produces a robust measure of expression changes by weighting multiple probe pairs according to an error model characteristic of oligonucleotide arrays (44). S-scores showing consistent results across replicate experiments were identified using a permutation-based statistical analysis (SAM) (45) and stringent filtering criteria as described under “Experimental Procedures.” Of 9644 genes selected as expressed using a low stringency filter, 323 (3.4%) showed significant increased or decreased expression with ethanol treatment. As observed previously (12), the majority of ethanol responses showed increased expression with ethanol (248/323). Correlated gene expression profiles were identified using hierarchical clustering (46) for ethanol and inhibitor/ethanol-treated. Several distinct patterns emerged, including genes that were up- or down-regulated with ethanol or inhibitor treatments (Fig. 6A). Strikingly, a very large proportion of ethanol responses (270/323) were blocked by all three inhibitors (cluster 1) (Fig. 6A). Virtually all ethanol responses blocked by H-89 were also inhibited to a similar degree by chrysin or PD 98059 (Fig. 6B).

It is beyond the scope of this study to describe a detailed functional analysis of all differential expression profiles identified by microarray studies. We focused mainly on those ethanol-induced genes (213/248) whose expression was blocked by all inhibitors (cluster 1) (Fig. 6A). To assess functional similarities, we manually categorized genes into 11 functional classes based on either known functions or structural homology to genes of known function (data not shown). Similar distributions were obtained using software approaches to map expression data on biological pathways (60). In general, genes involved in signal transduction, defense/stress, and transcription/translation were the most highly represented categories. In particular, analysis showed increased expression of multiple members of the MAPK and phosphatidylinositol signaling cascades (Table I).

Validation of Microarray Results by Quantitative RT-PCR Analysis—To validate select microarray results on ethanol-responsive genes, we performed quantitative RT-PCR on the DBH, S-like kinase, and thioredoxin-interacting protein genes.
Even though δ-like kinase did not pass stringent criteria for the current microarray studies, it was previously identified as an ethanol-responsive gene by our laboratory (12). Thioredoxin-interacting protein was selected because of its role in cellular responses to oxidative stress (61), a pathway implicated by our previous microarray results (12). Fig. 7 shows that ethanol responses from quantitative RT-PCR results were consistent with microarray data.

**DISCUSSION**

Here, we have shown that ethanol regulates DBH gene expression at the transcriptional level and identified promoter elements essential for ethanol responsiveness. We provide data suggesting an important role for cAMP signaling in ethanol regulation of DBH: 1) a CRE was required for ethanol induction of the DBH promoter; 2) a dominant-negative CREB construct greatly reduced ethanol responsiveness of the DBH promoter; and 3) an inhibitor of PKA blocked ethanol regulation of DBH gene transcription. Inhibitor studies also suggested that CKII and MEK kinases are required for ethanol regulation of DBH.

We used a pharmacogenomic approach with microarray studies to extend these findings and showed that PKA, CKII, and MEK kinases are indeed required for a large, diverse subset of ethanol-responsive genes in SH-SY5Y cells. These results increase our understanding of molecular mechanism(s) underlying ethanol-regulated gene expression and could have functional implications for cellular and behavioral responses to ethanol.

**Fig. 5. Inhibitors of PKA, CKII, and MEK kinases greatly reduce ethanol induction of the DBH promoter.** SH-SY5Y cells were transfected with the 978CAT construct 24 h prior to drug treatments. All kinase inhibitors were added 30 min prior to other drug treatments. Cells were harvested for CAT activity 6 h after the last drug additions.

A, cells were pretreated with H-89 (10 μM) for 30 min, followed by a 6-h incubation in the absence or presence of Bt2cAMP (db; 0.5 mM), forskolin (Fk; 1.0 μM), or ethanol (Et; 100 mM). B, cells were pretreated with saline, PD 98059 (PD; 10 μM), KN62 (KN; 5 μM), or GF 109203X (GF; 100 nM) 30 min prior to the addition of saline or ethanol (100 mM). C, cells were pretreated with saline or chrysin (10 μM) prior to the addition of saline or ethanol (100 mM). CAT activities for all experiments are the mean ± S.D. of triplicate determinations. Similar results were obtained in experiments repeated two to three times. Data without error bars have an S.D. too small to be visible.

**Fig. 6. Inhibitors of PKA, CKII, and MEK kinases block ethanol induction of a large subset of ethanol-responsive genes.** SH-SY5Y cultures were mock-treated or treated with H-89, chrysin, or PD 98059 for 30 min, followed by a 16-h incubation with or without ethanol (100 mM). RNA was then isolated and processed for microarray analysis. Triplicate experiments were performed and analyzed by S-score algorithm, SAM, and hierarchical clustering as described under “Experimental Procedures.” S-scores were generated for the following pairs of treatment conditions: ethanol versus control (E/C), H-89 + ethanol versus ethanol (H-89/E/E), H-89 alone versus control, chrysin + ethanol versus ethanol (CE/E), chrysin alone versus control, PD 98059 + ethanol versus ethanol (PDE/E), and PD 98059 alone versus control. A, hierarchical clustering analysis of S-score gene expression data from triplicate experiments is depicted. Only the four identified comparisons were clustered for clarity. Cluster 1 identifies ethanol-induced genes blocked by H-89, PD 98059 (PD), and chrysin (Chr). Red and green depict positive (increased) and negative (decreased) S-scores for pairwise comparisons, respectively. B, graphical representation of average S-scores ± S.D. for cluster 1 is shown.
Previous studies by our laboratory found increased DBH mRNA abundance in neural cell cultures and mice exposed to ethanol (12). Prior work from our laboratory and others showed that ethanol can cause selective increases in mRNA abundance by either increasing gene transcription (4, 5, 62–64) or altering mRNA stability (65). In this study, transient transfection analysis showed that ethanol treatment increased DBH gene transcription. The ethanol concentration-response curves for DBH transcription (Fig. 1B) and increases in DBH mRNA abundance (12) were similar, suggesting that increased transcription is a major factor in the ethanol response. This does not preclude possible concurrent changes in DBH mRNA stability.

Deletion (Fig. 2) and site-directed (Fig. 3) mutagenesis studies on the DBH promoter strongly implicated the CRE site and its cognate DNA-binding protein (CREB) in ethanol regulation of DBH gene transcription. Importantly, even though mutation of the CRE site decreased both basal and ethanol-induced promoter activities, the mCRE construct was still responsive to cAMP (cluster 1) (Fig. 6A). Thus, the CRE site mutation specifically altered ethanol responsiveness of the DBH gene transcription. Importantly, even though mutation of the CRE site decreased both basal and ethanol-induced promoter activities, the mCRE construct was still responsive to cAMP (cluster 1) (Fig. 6A). Thus, the CRE site mutation specifically altered ethanol responsiveness of the DBH gene transcription.

**Table I**

List of genes for cell defense and signal transduction categories

| Accession no. | Gene name | Putative function |
|---------------|-----------|------------------|
| M90863        | HLA-G     | Histocompatibility antigen, class I |
| M58603        | Nuclear factor of α-light gene enhancer (p105) |
| U84457        | Chemokine (CX-C motif) ligand-1 |
| M14758        | ATP-binding cassette, subfamily B (MDR/TAP), member 1 |
| D28118        | Zinc finger protein-161 |
| L76191        | Interleukin-1 receptor-associated kinase-1 |
| A011896       | TNFAIP3-interacting protein-1 |
| AB007935      | Immunoglobulin superfamily, member 3 |
| AL080181      | Immunoglobulin superfamily, member 4 |
| AF010513      | Etoposide-induced mRNA |
| L12723        | Heat shock 70-kDa protein-4 |
| A180675       | Dnaj (Hsp40) homolog, subfamily C9 |
| X15183        | Heat shock 90-kDa protein-1s |
| L19185        | Human natural killer cell-enhancing factor |
| L36503        | Glutathione S-transferase Theta2 |
| M33764        | Ornithine decarboxylase |
| X16277        | Ornithine decarboxylase |
| S73591        | Thioredoxin-interacting protein |
| R01383        | Metallothionein I-A gene |
| M10943        | Metallothionein gene (hMT-If) |
| D11139        | Tissue inhibitor of metalloproteinases |
| A1557064      | NADH dehydrogenase (ubiquinone) flavoprotein-2, 24 kDa |
| U950875       | Interferon-γ receptor-2 |
| U51336        | Inositol-1,3,4-triphosphate 5/6-kinase |
| AF002715      | MAPKKK-4 |
| L36670        | MAPK kinase-4 |
| U09578        | MAPK-activated protein kinase-3 |
| U52522        | Partner of Rac1 (arfaptin-2) |
| M24194        | G protein, β-polypeptide-2-like 1 |
| AB00231       | PDZ domain-containing guanine nucleotide exchange factor-1 |
| M64572        | Tyrosine phosphatase, non-receptor type 3 |
| Y17169        | Tyrosine kinase-9-like (Aβ-related protein) |
| U14603        | Protein-tyrosine phosphatase type IVA, member 2 |
| M14333        | fyn oncogene related to src, fgr, yes |
| Y13620        | B-cell CLL/lymphoma-9 |
| X12791        | Signal recognition particle, 19 kDa |
| U59305        | Ser/Thr protein kinase related to myotonic dystrophy protein kinase |
| M97287        | AT-rich sequence-binding protein-1 (binds to nuclear matrix/scaffold-associating DNAs) |
| AF024635      | Ser/Thr kinase-24 (STE20 homolog) |
| X57346        | Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, β-polypeptide |
| U73377        | Shc (Src homology 2 domain-containing)-transforming protein-1 |
| U51336        | Inositol-1,3,4-triphosphate 5/6-kinase |
| M68941        | Protein-tyrosine phosphatase, non-receptor type 4 (megakaryocyte) |
| AA126515      | Retinol dehydrogenase-11 (all-trans and 9-cis) |
| AB018314      | Protein phosphatase-1, regulatory (inhibitor) subunit 13B |
| L11329        | Dual-specificity phosphatase-2 |
| Y18004        | Sex comb on midleg-2 like 2 (Drosophila) |
| U55647        | Small optic lobes homolog (Drosophila) |
| M34441        | FGF receptor-1 (fms-related tyrosine kinase-2, Pfeiffer's syndrome) |
| Y12711        | Progesterone receptor membrane component-1 |
| M23379        | Ras p21 protein activator (GTPase-activating protein)-1 |
| D65097        | Tousled-like kinase-1 |
| M16908        | v-yes-1 virus related oncogene homolog |

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not induced by ethanol in neuroblastoma cell cultures (see data on lactate dehydrogenase in Ref. 4). Fig. 6A further supports this supposition, showing that H-89 did not alter ethanol down-regulation of some gene clusters (cluster 2) and that H-89 treatment caused strong induction of some genes unresponsive to ethanol (cluster 3). This raises the possibility that a functional CRE site is necessary, but not sufficient, for ethanol regulation of genes such as DBH. A similar conclusion was also recently reached by Constantinescu et al. (65), who showed that phosphorylation of CREB by type II PKA is necessary, but not sufficient, for ethanol regulation of an artificial CRE-luciferase construct.

As expected, the dominant-negative CREBM1 construct blocked ethanol induction of DBH transcription (Fig. 4). This supports involvement of CREBM in ethanol induction of DBH. CREBM1 cannot be activated by PKA phosphorylation at Ser133, but can still bind to the CRE. Thus, CREBM1 occupies the CRE, preventing access by CREB and other CRE-binding factors (33). However, overexpression of CREBM1 could also competitively interfere with the activity of other bZIP proteins such as activating transcription factor-1 and CREM-τ (33, 66–68). Nevertheless, when combined with the results of our promoter mapping (Figs. 2 and 3) and kinase inhibitor (Figs. 5 and 6) studies, the CREBM1 results greatly strengthen a role for CREB in ethanol regulation of DBH transcription. Ongoing studies of CREB phosphorylation and DNA binding could provide more direct evidence of CREB involvement in ethanol induction of DBH. However, in light of the multikinase mechanism of ethanol action suggested by our studies here (see discussion below), it is possible that multiple sites of CREB phosphorylation will have to be investigated to fully understand ethanol actions on CREB. It has been shown that acute ethanol induces CREB phosphorylation at Ser133 in rat cerebellum (40) and in NG108-15 neuroblastoma cells (65, 69).

Our transfection studies discussed above clearly suggest a role for CRE/CREB in ethanol regulation of DBH. Our prior microarray studies showed that a CAMP analog (Bt2cAMP) also induces DBH and a number of other ethanol-responsive mRNAs in SH-SY5Y cells (12). Those data and our current studies suggest that ethanol might act through cAMP signaling to induce DBH gene expression. In support of this idea, we showed here that Bt2cAMP and forskolin increased DBH promoter activity and that H-89, a well characterized inhibitor of PKA, greatly reduced ethanol induction of DBH transcription (Fig. 5A). Taken together, these studies strongly suggest a requirement for PKA in ethanol induction of DBH.

There is significant literature suggesting that cAMP signaling is an important target for ethanol action (2, 34, 37, 38, 40, 51–54, 70, 71). Ethanol perturbs membrane components of the adenyl cyclase signal transduction system and can alter neuronal CAMP generation (35, 72, 73). Acute ethanol exposure in vivo has been shown to activate the CAMP pathway in rodent brain regions, with consequent increases in CREB phosphorylation (40, 51, 52). Recent studies indicate that various post-receptor events of the cAMP signal transduction cascade (i.e. Gs protein, PKA, and CREB) are also modulated by chronic ethanol exposure in rodent brain tissue (74). Perhaps most importantly, several recent studies using gene targeting and transgenic animals have clearly shown an important role for both DBH (21) and CAMP signaling (75, 76) in ethanol-mediated behavioral responses in mice.

![Graph](image-url)

**Fig. 7. Real-time RT-PCR verification of representative ethanol responses from microarray analysis.** Results obtained for ethanol induction of the DBH, DLK, and thioredoxin-interacting protein (TXNIP) genes are shown from control and ethanol-treated samples. Real-time RT-PCR assays were performed as described under “Experimental Procedures.” Relative product abundance was assessed using co-amplified internal standard 18 S RNA. Data are expressed as the mean ± S.D. of triplicate determinations from a representative experiment. Data without error bars have an S.D. too small to be visible.

| Accession no. | Gene name       | CRE motifs          | Score* |
|---------------|-----------------|---------------------|--------|
| U34252        | Aldehyde dehydrogenase-9A1 | TGACCTCA         | 87     |
| AF037335      | Carboxic Anhydrase XII          | TGACATCA*        | 87     |
| U36341        | Creatine transporter       | TGACATCA         | 87     |
| AF035812      | Dynnein, light polypeptide-2 | NF               |        |
| D31661        | EphB2                   | NF               |        |
| U60060        | Fasciculation-1         | NF               |        |
| L37882        | Frizzled homolog-2       | NF               |        |
| M14333        | fyn oncogene            | TGGAGTGG         | 86     |
| L21723        | Heat Shock 70-kDa protein-4 | TGGAGTGGAT*     | 93     |
| X53002        | Integrin-β5             | NF               |        |
| D195629       | Kinectin-1              | TGGAGTGG         | 87     |
| U70322        | Karyopherin-β2          | TGGAGTGG         | 93     |
| M58603        | NF-κB                  | NF               |        |
| L19185        | Natural killer factor    | TGGAGTGG         | 86     |
| M690023       | Tetraspan-3             | TGGAGTGG         | 86     |
| S73594        | Thioredoxin interacting  | TGGAGTGG         | 87     |
| U29195        | Neuronal pentraxin II    | TGGAGTGG         | 87     |
| AB0111131     | Piccolo                 | TGGAGTGG         | 87     |
| D11428        | Peripheral myelin protein-22 | NF               |        |
| AF035287      | Stromal cell-derived factor receptor-1 | NF     |        |

* Multiple CRE motifs.
Yang et al. (51) have previously shown that, whereas acute ethanol exposure increases cAMP signaling to CREB in cerebellar granule cells, chronic ethanol exposure (3 weeks) actually causes a decrease in CREB phosphorylation. Their results are consistent with the well described heterologous desensitization that occurs with the adenyl cyclase system exposed to chronic ethanol (35). Our previous studies strictly showed an induction of DBH mRNA and protein following up to 3 days of ethanol exposure (12). We also continue to see increased DBH gene transcription after 3 days of ethanol treatment. It is possible, however, that more prolonged exposure to ethanol will result in adaptations of DBH induction.

Our data suggest that ethanol regulates DBH transcription through a PKA/CRE/CREB mechanism. However, CREB can also be phosphorylated by multiple other protein kinases such as CaMK, CKII, glycogen synthase kinase-3, PKC, and MAPK (see review in Ref. 33), in addition to the classical PKA phosphorylation. Our studies using selective kinase inhibitors implicate CKII, MEK, and PKA in the modulation of DBH promoter activity (Fig. 5). The lack of any effect by inhibitors of PKC and CaMK (Fig. 5B) supports the specificity of these results. However, in some cases, these inhibitors also decreased basal DBH promoter activity, thus complicating interpretation of the results (see data with PD 98059 in Fig. 5B). We feel that the striking decrease in absolute magnitude of the CAT activity in the presence of ethanol + PD 98059 is likely indicative of a requirement for this kinase in the ethanol response. For example, forskolin, a known activator of PKA, still showed residual stimulation of DBH promoter activity in the presence of the PKA inhibitor H-89 (Fig. 5A), although H-89 caused a marked decrease in the absolute magnitude of forskolin-stimulated CAT activity.

Importantly, microarray analysis of inhibitor studies also showed a requirement for PKA, CKII, and MEK signaling in a large subgroup of ethanol-responsive genes (Fig. 6), including DBH. This striking commonality in the mechanism for ethanol regulation of a diverse set of genes might be due to direct (e.g. via CREB) or indirect actions of these inhibitors. Further studies with combinations of selective pharmacological or genetic (dominant-negative) inhibitors of specific kinases are needed to verify the exact role of PKA, CKII, and MEK in ethanol regulation of DBH and other genes in cluster 1 of Fig. 6A. Future studies on CREB phosphorylation, as mentioned above, are likely to be complex, but are needed to show direct modulation of CREB activity by particular kinases. In particular, the role of CKII might be particularly complicated since this kinase has been shown to activate CREB by phosphorylation at Ser^{108} Ser^{111}, or Ser^{142} (77).

The role of genes identified by expression profiling appears to be complex, with multiple functional classes identified, as seen with our earlier microarray studies on ethanol-responsive gene expression in SH-SY5Y cells (12). A detailed comparison between the current microarray results and our previous data was not done because of the large number of differences in microarray platforms and analysis methods between the two experiments. However, we did note that DBH, 5-like kinase, neuronal pentraxin II, monocyte chemotactic protein-1, and a number of genes involved in oxidative stress were again ethanol-responsive in our current microarray studies (see Fig. 1 in Ref. 12, Table I, and Fig. 7).

In addition, we found that multiple members of the MAPK and phosphatidylinositol signaling pathways were contained in the group of genes with ethanol responses blocked by H-89, chrysin, or PD (Table I). Changes in MAPK or phosphatidylinositol signaling mRNA levels do not directly implicate involvement of phosphorylation events mediated by these kinase pathways in the responses to ethanol. However, ethanol has previously been shown to alter MAPK signaling, and there are numerous reports on interactions between MAPK signaling and cAMP (78). Importantly, MAPK activation has been shown to be required for PKA-dependent CREB phosphorylation in models of hippocampal synaptic plasticity (28). The microarray results and inhibitor studies thus support an important role for MAPK signaling in ethanol induction of DBH and other genes.

Rather than focusing on identifying functions of ethanol-responsive genes, a major reason for performing the microarray studies in this work was to determine the proportion of ethanol-responsive genes requiring PKA, CKII, and MEK. A very high percentage of ethanol-responsive genes were blocked by H-89 (270/323). Surprisingly, a very large percentage of all responses to ethanol and virtually all genes requiring PKA (i.e. blocked by H-89) for the ethanol response were also dependent on CKII and MEK (Fig. 6, A and B). Promoter analysis of the proximal 1000 bp upstream of transcription start sites for a random subset of these genes showed the presence of a CRE-like motif in 13 of 20 genes (Table II). Additionally, other investigators have shown that several other genes in this table have interactions with cAMP signaling or CREB (79–81). This does not prove involvement of CRE/CREB in ethanol regulation of these genes or that genes without an identified CRE might not also be regulated by CREB. However, this analysis of promoter motifs in these ethanol-responsive genes, the DBH promoter analysis data (Figs. 3–5), and the microarray data with H-89 (Fig. 6) are further evidence supporting a requirement for CRE/CREB in ethanol regulation of a substantial group of genes in SH-SY5Y cells. The mechanism whereby PKA, CKII, and MEK are all required for the ethanol response is currently unknown. As mentioned above, in addition to possibly directly regulating CREB activity, these kinase pathways could interact indirectly, as in PKA activation of MAPK signaling (78).

In summary, our results indicate that CRE site and the cognate DNA-binding protein (CREB) are crucial for ethanol induction of DBH transcription in SH-SY5Y cells. Our results also suggest that ethanol regulates DBH gene transcription through a PKA/CKII/MEK-dependent mechanism. Importantly, our pharmacogenomic analysis with microarrays suggests that this mechanism may modulate a substantial portion of ethanol-responsive genes in SH-SY5Y cultures. Since our previous work has shown that ethanol also regulates DBH in mouse adrenal gland, studies are clearly warranted to determine whether ethanol utilizes mechanisms identified here to regulate expression of DBH or other genes in intact animals. These studies could thus have important implications for understanding the mechanisms of ethanol-induced end-organ toxicity or behavioral adaptations seen with alcoholism.

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