Ethanol-Induced Upregulation of 10-Formyltetrahydrofolate Dehydrogenase Helps Relieve Ethanol-Induced Oxidative Stress

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Alcoholism induces folate deficiency and increases the risk for embryonic anomalies. However, the interplay between ethanol exposure and embryonic folate status remains unclear. To investigate how ethanol exposure affects embryonic folate status and one-carbon homeostasis, we incubated zebrafish embryos in ethanol and analyzed embryonic folate content and folate enzyme expression. Exposure to 2% ethanol did not change embryonic total folate content but increased the tetrahydrofolate level approximately 1.5-fold. The expression of 10-formyltetrahydrofolate dehydrogenase (FDH), a potential intracellular tetrahydrofolate reservoir, was increased in both mRNA and protein levels. Overexpressing recombinant FDH in embryos alleviated the ethanol-induced oxidative stress in ethanol-exposed embryos. Further characterization of the zebrafish *fdh* promoter revealed that the −124/+40 promoter fragment was the minimal region required for transactivational activity. The results of site-directed mutagenesis and binding analysis revealed that Sp1 is involved in the basal level of expression of *fdh* but not in ethanol-induced upregulation of *fdh*. On the other hand, CEBPα was the protein that mediated the ethanol-induced upregulation of *fdh*, with an approximately 40-fold increase of *fdh* promoter activity when overexpressed *in vitro*. We concluded that upregulation of *fdh* involving CEBPα helps relieve embryonic oxidative stress induced by ethanol exposure.

Fetal alcohol syndrome (FAS) is a severe congenital disorder that can develop in a fetus exposed to high levels of alcohol during pregnancy (1). The clinical symptoms include mental retardation and defects to the face and organs. The prevalence rate of 0.2 to 2 cases in 1,000 living births has brought public health concern and awareness of social and economic burdens. Currently, the pathomechanisms of FAS remain inconclusive, making prevention of FAS a more challenging task. Folate deficiency is proposed to be causally linked to FAS, since alcoholism induces folate deficiency and increases the risk for embryonic anomalies (reference 2 and references therein). Folate supplementation is considered a potential preventive intervention to FAS, although the underlying mechanism of this protective effect remains elusive (3).

Folate is a water-soluble vitamin (B₉) that serves as a one-carbon carrier in folate-mediated one-carbon metabolism (OCM) (Fig. 1). Folate participates in the biosynthesis of nucleotides, amino acids, and neurotransmitters. Folate is essential for rapidly growing tissues and proliferating cells, especially the fetus. Folate is involved in generating S-adenosylmethionine (SAM). SAM is the primary methyl donor for most intracellular methylations, including DNA/RNA methylation, endowing folate with the potential to modulate gene activity simply via dietary intervention. Therefore, understanding how ethanol exposure affects embryonic folate status and development is crucial for unraveling the etiology of FAS and developing effective preventive/therapeutic protocols against FAS.

In cells, folate is both reduced to dihydrofolate (DHF) and tetrahydrofolate (THF) and polyglutamylated to form biologically active folylpolyglutamates. One-carbon units of three different oxidative states are attached to these folylpolyglutamates on the N-5 and/or N-10 positions, forming different folate adducts. These folate adducts provide their one-carbon units to generate the biomolecules noted above (4). The interconversion between different folate adducts also occurs via several redox and synthetic

reactions catalyzed by folate enzymes. In general, each one-carbon adduct is important in one major pathway, for example, 5-methyl-THF in methylation of DNA and RNA, 5,10-methylene-THF in dTMP synthesis, and 10-formyl-THF in purine synthesis. Reduced folates are unstable but significantly stabilized by binding to folate binding proteins, mostly enzymes (5). Therefore, the intracellular concentration of folate enzymes is likely to affect the intracellular folate availability and to modulate folate-mediated functions.

Ethanol-induced toxicity has been reported in zebrafish, a prominent model organism for human diseases and drug/toxin research (6). The externally fertilized and developed embryos provide a “closed system,” concerning the folate pool, and allow for exclusion of interference from the maternal supply of folate during gastrulation. This “closed and isolated system” enables the intracellular fluctuation among different folate derivatives to be observed. Many zebrafish folate enzymes have been shown to be comparable to mammalian orthologs, supporting the appropriateness of modeling the impact of ethanol on folate-mediated OCM with zebrafish (7–11). Here, we report how the embryonic folate status and the expression of folate enzymes are altered when embryos are exposed to ethanol. The emphasis is focused on the regulation of expression of 10-formyltetrahydrofolate dehydrogenase, since this was the folate enzyme found to be upregulated in response to ethanol exposure in the current study.

Received 28 October 2013 Accepted 13 November 2013

Published ahead of print 25 November 2013

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doi:10.1128/MCB.01427-13

FIG 1 Reactions in folate-mediated one-carbon metabolic pathways. Reactions involving folate coenzymes and enzymes of OCM are responsible for the biosynthesis of purines, thymidylate, and SAM. The boxed compounds are folate derivatives carrying one-carbon units of various oxidative states. Numbers are enzymes that catalyze the reactions that generate THF: 1, cytosolic SHMT; 2, mitochondrial SHMT; 3, dihydrofolate reductase; 4, 10-formyltetrahydrofolate dehydrogenase; 5, γ -glutamyl hydrolase; 6, methionine synthase; 7, 5-aminoimidazole-4- carboxamide ribonucleotide formyltransferase and glycylamide ribonucleotide. SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; GAR, glycylamide ribonucleotide; AICAR, aminoimidazole carboxamide ribonucleotide.

MATERIALS AND METHODS

Materials. PCR primers were from MDBio, Inc. (Taipei, Taiwan). The Smart random amplification of 5' cDNA ends (RACE) amplification kit was obtained from Clontech, Inc. (Mountain View, CA), and the PCR master mix was from ABgene (Epsom, Surrey, United Kingdom). Leibovitz's L15 medium, fetal bovine serum (FBS), and trypsin-EDTA were from Invitrogen (Carlsbad, CA). The Bradford protein assay kit was from Pierce (Rockford, IL). Mouse monoclonal anti-His antibody was obtained from GenScript (Piscataway, NJ). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG was from Santa Cruz Biotechnology (Santa Cruz, CA). Morpholino oligonucleotides (MO) were obtained from Gene Tools, LLC (Philomath, OR). All other chemicals, including buffers, antibiotics, and protease inhibitors, were from Sigma-Aldrich (St. Louis, MO).

Fish maintenance and preparation of zebrafish cDNA libraries. Zebrafish (*Danio rerio*, AB strain) were maintained under the standard conditions of a 14-h/10-h light/dark cycle as described by Westerfield (12). Embryos were staged by hours and days postfertilization (hpf and dpf) according to the methods described by Kimmel et al. (13). Total RNA isolation and cDNA library construction from zebrafish embryos were performed with TRIzol reagent (Invitrogen, CA) and the Smart random amplification of 5' cDNA ends (RACE) kit following the manufacturer's instructions. The animal studies and all procedures were approved by an Affidavit of Approval of Animal Use Protocol of National Cheng Kung University (IACUC approval number 96062).

Quantification of *fdh* expression levels. For determination of the mRNA and protein levels of FDH, reverse transcription-PCR (RT-PCR) and Western blot analysis were performed as previously described (9). The specific primers for *fdh* mRNA quantification were 5'-CGCTGAGCATATGAGGGTGGTG-3' (forward) and 5'-GGTATAGACTGCTCCCGAG-3' (reverse) for zebrafish *fdh*, 5'-AGACATCAAGGAGAAGCTGTG-3' (forward) and 5'-TCCAGCAGCGAGTATTTC-3' (reverse) for zebrafish β -actin, and 5'-GAGCCTGTTGGGGTTTGTGG-3' (forward) and 5'-CCACTCTCCGACGAAGTCA-3' (reverse) for human *fdh*.

Drug administration. For oxidative stress analysis, ethanol was added to water containing embryos at 24 hpf and incubated for 1.5 h before embryos were stained with 2',7'-dichlorofluorescein diacetate (DCFH-

DA). For gene expression characterization and folate content measurement, ethanol was added to 3-hpf embryos for 6 h before mRNA extraction. For promoter activity assays in cells, zebrafish liver epithelium cells (ZLE) were cultured in Leibovitz's 15 medium supplemented with 10% FBS at 28°C as previously described (8). Cells were seeded (1×10^6 cells/6-cm dish) and transfected with promoter constructs. The next morning, ethanol was added to culture medium to 1.5% and incubated for 1 h. Then, the cells were incubated in fresh medium without ethanol for another 24 h before analyzing with a dual-luciferase assay. Antioxidants *N*-acetylcysteine (NAC) and manganese(III) tetrakis(1-methyl-4-pyridyl)porphyrin (MnT) were freshly prepared and added to embryo medium simultaneously with ethanol to reach the final concentrations of 20 μ M and 10 μ M, respectively.

Identification of the transcriptional initiation site. The transcriptional initiation site of zebrafish *fdh* was identified with full-length, RNA ligase-mediated-RACE (RLM-RACE) by using the GeneRacer kit following the instructions provided by the manufacturer (Invitrogen). To obtain the 5' ends of the *fdh* transcript, the first-strand cDNA was PCR amplified by using the GeneRacer 5' primer and a reverse zebrafish *fdh*-specific primer, 5'-CCCTTCAGCCTCCAGCGGGG-3' (position +222 to +241 of the zebrafish *fdh* coding sequence). The PCR product of approximately 300 bp was cloned and sequenced to identify the transcription initiation site.

Construct preparation and site-directed mutagenesis. Approximately 1.7 kb of the zebrafish *fdh* promoter region (−1694/+40) was PCR amplified from zebrafish genomic DNA with a primer design based on the genomic sequence of zebrafish *fdh* available in GenBank (BX004829.12). This promoter fragment, with an immediate downstream enhanced green fluorescent protein (EGFP) coding sequence, was subcloned into the pGL3-basic reporter vector (Promega, Madison, WI) for activity analysis. The deletion constructs of the zebrafish *fdh* promoter were generated by restriction enzyme digestion or PCR cloning with the appropriate primer. Constructs with site-directed mutations were obtained using the primers containing the desired mutations (Table 1) and the QuikChange site-directed mutagenesis kit (Stratagene, Agilent Technologies, La Jolla, CA) as previously described (14). All successful constructs were confirmed by restriction enzyme digestion and DNA sequencing. The *Escherichia coli*

TABLE 1 Sequences of mutated sites for the zebrafish *fdh* promoter –124/+40 region

Mutated site	Sequence	
	Wild type	Site-directed mutation
Site 1	AAA GGG CAC	CCA TGG CAC
Site 2	CCA CCC C	CCA TGG C
Site 3	CAG TTC TGC	CAG AGC TCC

strains XL1 Blue and DH5α were used for transformation and selection of clones.

DAPA. Two complementary 5'-biotinylated oligonucleotides (MDBio, Inc., Taiwan) specific to the site of interest (Table 2) were annealed and incubated in DNA affinity precipitation assay (DAPA) buffer (20 mM Tris [pH 7.4], 0.5 mM EDTA, 2 mM MgCl₂, 60 mM KCl, 0.01% Nonidet P-40, 5% glycerol) at 4°C for 1 h with either 2 μg of purified recombinant zebrafish Sp1 protein in the presence of 100 μg of zebrafish liver nuclear extract or 100 μg of protein in total cell lysate containing overexpressed zebrafish Sp1-like protein (15). Then, 20 μl of a slurry of streptavidin-conjugated beads (Sigma-Aldrich) was added to the mixture and incubated at 4°C for another hour. The protein-DNA-streptavidin-bead complex was washed thoroughly with DAPA buffer, resuspended in 35 μl of double-distilled water containing 1× SDS loading dye, and then boiled. The protein released in the supernatant was analyzed by Western blotting using anti-His antibody (1:10,000) or anti-zebrafish Sp1-like antibody (1:600). Zebrafish liver nuclear extract was prepared following the manufacturer's protocol (nuclear extraction kit by Panomics Inc., CA).

Promoter activity analysis (in cultured zebrafish cells and embryos). ZLE cells were chosen for *in vitro* assay of promoter activity, since *fdh* is expressed most abundantly in both mammalian and zebrafish liver cells (16, 17; unpublished data). Cells were seeded in a 24-well culture plate the day before transfection. A total of 840 ng of plasmid DNA composed of 650 ng of zebrafish promoter-firefly luciferase construct, 150 ng of pcDNA3.1(+) *myc*-His encoding the tested transcription factor, or empty pcDNA3.1(+) *myc*-His vector, and 40 ng of reference plasmid pRL-TK was transfected into cells. Cells were incubated for 48 h before subjecting them to a luciferase activity assay with the dual-luciferase reporter assay system (Promega) on a Wallac 1420 Victor 2 multilabel counter (Beckman Coulter, Inc.) 48 h after transfection.

For analysis of activity in embryos, a total of 985 pg of the above-mentioned plasmid mixture (composed of 800 pg promoter constructs, 150 pg transcription factor-expressing construct, or empty vector) dissolved in 1× Danieau's buffer was injected into each embryo at the stage of 1 to 8 cells. After 24 h, the injected embryos with green fluorescence were washed with phosphate-buffered saline (PBS) and removed to a new tube (3 embryos for each tube). The embryos were incubated in 50 μl 1× passive lysis buffer for 30 min at room temperature before homogenization and centrifugation. The supernatants were used for luciferase activity assays.

ROS detection. Embryos were incubated in 0.1 mM H₂-dichlorofluorescein diacetate in the dark for 30 min. After a thorough wash with water, embryos were directly observed and photographed under a fluorescence microscope. Embryos injected with MO or synthesized mRNA at the 1- to 8-cell stage were grown to 24 hpf and then incubated in ethanol water for 1.5 h. After washing thoroughly, embryos were subjected to reactive oxygen species (ROS) detection as described above.

Folate detection. Embryonic total folate was quantified in a *Lactobacillus casei* assay (18). Individual folate adducts in embryos were measured following the protocols previously described (19). In brief, approximately 50 embryos were homogenized in 0.3 ml of extraction buffer flushed with nitrogen and heated in boiling water for 5 min before centrifugation. Conversion of folyl polyglutamates in the supernatant to folate monoglutamates was achieved by incubating the embryo extracts with 5.2 μg of purified recombinant zebrafish γ-glutamyl hydrolase (GH) and incubation

TABLE 2 Sequences of DAPA probes used for the zebrafish *fdh* promoter –124/+40 region

DAPA probe	Sequence (5'–3')
Site 1 (probe 1)	Biotin-CTTTGGAAGGGCACCTTTGGAAAGGGCAC
Site 1-SDM (probe 1 ^{sdm})	Biotin-CTTTGGCCATGGGCACCTTTGGACCATGGAC
Site 2 (probe 2)	Biotin-GTTCCACCCCTAGGTTCCACCCCTAG
Site 2-SDM (probe 2 ^{sdm})	Biotin-GTTCCATGGCCTTTAGTTCCATGGCCTAG

tion at 37°C for 5 min. After centrifugation and filtration, 50 μl of the clear supernatant was injected into an Aquasil C₁₈ column of a high-performance liquid chromatography (HPLC) system (Agilent 1100) for folate detection. The potential folate peaks in extracts were identified by overlapping the retention times between the prospective folate peaks and folate standards.

Cloning of CEBP expression constructs. Primers were designed based on the zebrafish CCAAT/enhancer binding protein α (zCEBPα) sequence available in GenBank (NM_131885) to PCR amplify the complete zCEBPα coding sequence from zebrafish 5'-RACE-ready cDNA libraries. The primer sequences were the following: 5'-GAGCAAGCAAACCTCTACGA-3' (forward) and 5'-GTTAAGCGCAGTTGCCCATG-3' (reverse). The PCR fragments of 864 bp were cloned into the expression vector p3×-Flag-cMyc-CMV26, generating zCEBPα/p3×-Flag-cMyc-CMV26 for expression in 293T cells. Successful cloning was confirmed by restriction enzyme digestion and DNA sequencing.

ChIP. Chromatin immunoprecipitation (ChIP) was performed following the protocol adapted from the ZFIN database (http://zfin.org/cgi-bin/webdriver?Mlval=aa-ZDB_home.apg) with a modification. In brief, embryos injected with CEBPα-Flag-capped RNA were incubated in 1.5% ethanol at 6 hpf for 16 h. After cross-linking with 2.2% formaldehyde, approximately 100 embryos were lysed in cell lysis buffer (10 mM Tris-Cl [pH 8.0], 10 mM NaCl, 0.5% NP-40, protease inhibitor cocktail) and centrifuged. The pellet of nuclei was incubated in nucleus lysis buffer (50 mM Tris-Cl [pH 8.0], 10 mM EDTA, 1% SDS, protease inhibitor cocktail) for 20 min on ice and then frozen at –80°C. After shearing with sonication, chromatin extracts were diluted in IP dilution buffer (16.7 mM Tris-Cl [pH 8.0], 167 mM NaCl, 1.2 mM EDTA, 1.1% Triton X-100, 0.01% SDS) and incubated with anti-Flag antibody at 4°C overnight. The chromatin-antibody complexes were precipitated with preblocked protein G beads and washed with dialysis buffer (50 mM Tris-Cl [pH 8.0], 2 mM EDTA) and with IP wash buffer (100 mM Tris-Cl [pH 8.0], 500 mM LiCl, 1% NP-40, 1% deoxycholic acid). DNA-protein complexes were eluted with elution buffer (50 mM NaHCO₃, 1% SDS), de-cross-linked, and digested with proteinase K. After phenol-chloroform extraction, the obtained DNA fragments were PCR amplified with the following zebrafish *fdh* promoter primers: 5'-GTTTGCACGACGCTCATGCTTC-3', forward; 5'-GCACGTCCCAATAGGATCCGTTCTCTGC-3', reverse.

CEBP knockdown with shRNA. The plasmid containing short hairpin RNA (shRNA)-human CEBPα (TRCN0000007305) was obtained from the National RANi Core Facility (Academia Sinica, Taipei, Taiwan). Purified plasmid was electroporated into 293T cells and incubated for 24 h. Cells were then treated with FBS-free Dulbecco's modified Eagle's medium (DMEM) or 2% ethanol in FBS-free DMEM for 1 h and allowed to recover in 10% FBS-DMEM for 24 h before being subjected to further analysis. Knockdown efficacy was confirmed by Western blotting with anti-human CEBPα antibody (D56F10; Cell Signaling).

Statistical analysis. Data are presented as mean values ± standard errors of the means for at least three independent experiments performed in triplicate. The probability value (*P* value) was calculated by using Student's *t* test, and a *P* value less than 0.05 was considered statistically significant.

FIG 2 Effect of ethanol treatment on folate distribution. (A) Zebrafish embryos at 3 hpf were exposed to ethanol for 6 h before being subjected to folate content analysis via HPLC. (B) Embryos of 3 hpf were exposed to ethanol and collected at the indicated stages for folate content analysis. Embryos that were incubated in water were used as the control. Reported are the averaged results of eight independent repeat experiments. Arrow, the time ethanol was added to water. **, $P < 0.01$; ***, $P < 0.001$.

RESULTS

Embryonic folate composition was altered in embryos exposed to ethanol.

We found that THF and 5-methyl-THF, the two most stable and abundant folate species in mammals, were increased and decreased, respectively, in 9-hpf embryos that had been exposed to ethanol for 6 h (Fig. 2A). Detailed analysis revealed that embryonic THF was increased and 5-methyl-THF was decreased, both in a time- and dose-dependent manner, in response to ethanol exposure (Fig. 2B). The significant increase and decrease of THF and 5-methyl-THF, respectively, were observed 4 h after exposure and became more evident at 6 h postexposure. These data suggest that the folate-mediated OCM in zebrafish embryos exposed to ethanol is altered in a way that leads to increased THF and decreased 5-methyl-THF.

FDH was upregulated in ethanol-exposed embryos. To investigate why THF increased, we examined the mRNA levels of five folate enzymes: dihydrofolate reductase (DHFR), cytosolic- and mitochondrial-serine hydroxymethyltransferase (cSHMT and mSHMT), γ GH, and FDH. All these enzymes catalyze the generation of THF except for γ GH, which converts polyglutamyl folate to monoglutamyl folate and facilitates the export of intracellular folate from cells. We found that the mRNA levels of DHFR, cSHMT, and mSHMT were not significantly changed, whereas γ GH mRNA was decreased and *fdh* mRNA was increased

in a dose-dependent manner (Fig. 3A). These findings were in agreement with Western blotting results that showed significant and dose-dependent increases of FDH protein in ethanol-exposed embryos (Fig. 3B). Together, these results imply a role for FDH in the affected embryonic OCM response to ethanol exposure.

Increased FDH expression and additional folate alleviate ethanol-induced oxidative stress. Why would embryos increase *fdh* expression when exposed to ethanol? By using DCF staining, we showed that ethanol induced a dose-dependent increase of oxidative stress in embryos. DCF staining is a method commonly used to detect oxidative stress in biological samples, in which green fluorescence is a measure of oxidative stress (20, 21). Submerging 24-hpf embryos in ethanol-containing water for 1.5 h led to a dose-dependent increase of oxidative stress (Fig. 4A). Both THF and 5-methyl-THF possess strong anti-oxidative stress effects (22). We found that ethanol-induced oxidative stress was alleviated when THF or 5-methyl-THF was added to embryo medium simultaneously with ethanol, although the effects were not as significant as those exerted by adding MnT, an antioxidant often used to reduce oxidative stress and ROS-mediated cell death (Fig. 4B, middle panel). Reduced folates, especially THF, are unstable but can be significantly stabilized by binding to folate enzymes, such as FDH (5). We found that the oxidative stress in embryos was substantially affected when the *fdh* expression level

FIG 3 The expression of folate enzymes fluctuates in embryos exposed to ethanol. (A) Zebrafish embryos at 3 hpf were immersed in egg water containing ethanol at the indicated concentrations for 6 h and harvested for mRNA quantification. (B) Embryos at 6 hpf were incubated in ethanol for 66 h and harvested for Western blot analysis with anti-zebrafish FDH antibodies. For quantification, the band intensities of both mRNA and protein were determined with densitometry and normalized against β -actin. Data are presented as the ratio between ethanol-exposed embryos and control embryos incubated in water. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

FIG 4 The oxidative stress and folate content of ethanol-exposed embryos are responsive to altered *fdh* expression and antioxidant. (A) Embryos of 24 hpf were exposed to 1% and 2% ethanol for 1.5 h before DCF staining. (B) Embryos with or without the indicated treatments were evaluated for oxidative stress based on DCF staining at 24 hpf after exposure to ethanol for 1.5 h. For the embryos with nucleotides injected, both *fdh* MO (9.6 ng) and mRNA (1.24 ng) were injected into embryos at the 1- to 8-cell stages. Embryos were grown until 24 hpf and then incubated in ethanol for another 1.5 h before they were subjected to DCF staining. For the groups exposed to tested compounds, folate and MnTMPyP (MnT), the antioxidant used as a control for antioxidative activity, were added to embryo water at 24 hpf with ethanol. (C) Embryos injected with *fdh* mRNA (mRNA) or *fdh* MO (morphant) or without any injection (control) were analyzed for folate content at 24 hpf. (D) FDH MO-injected embryos were exposed to 1.5% ethanol at 24 hpf for 1.5 h (EtOH-MO) before they were subjected to folate analysis. Results were compared with those for exposure to ethanol only (EtOH), those for injection with MO only (MO), and those for embryos without any treatment (control). (E) The effectiveness of *fdh* knockdown and overexpression in embryos injected with *fdh*-specific MO and mRNA, respectively, were examined with Western blot analysis at 24 hpf. C, control embryos without any injection; 1, *fdh* MO at 4.8 ng; 2, *fdh* MO at 9.6 ng; 3, *fdh* mRNA at 1.24 ng. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

was changed. Increasing *fdh* expression, by injecting *fdh* mRNA into embryos 24 h before exposure to ethanol, decreased ethanol-induced oxidative stress in comparison to the uninjected control group (Fig. 4B, third panel, and E). In contrast, the oxidative stress increased when embryos were injected with antisense MO to knock down *fdh* expression before exposure to ethanol. Knocking down *fdh* expression also increased the basal level of embryonic free radicals in the control group, in which embryos were not exposed to ethanol (Fig. 4B, first panel). These results suggest antioxidative properties for FDH. Examination of embryonic folate content revealed that overexpression of *fdh* increased THF, whereas knocking down *fdh* decreased THF (Fig. 4C). Knocking down *fdh* also counteracted the increased THF level in embryos exposed to ethanol (Fig. 4D). These results showed that increasing FDH expression helps conserve embryonic THF and relieves oxidative stress induced by ethanol exposure.

Ethanol-induced upregulation of FDH was not due to oxidative stress. Since increased expression of *fdh* relieved ethanol-induced oxidative stress, it seemed reasonable that the expression of

fdh was a response to oxidative stress. However, adding antioxidants did not prevent the increase of *fdh* mRNA in the presence of ethanol (Fig. 5). These results suggest that the upregulation of *fdh* is not due to ethanol-induced oxidative stress.

Promoter methylation is probably not involved in zebrafish *fdh* transcriptional regulation. To investigate the mechanism underlying ethanol-induced upregulation of *fdh*, we cloned and characterized the zebrafish *fdh* promoter. The transcription start site was determined by cloning and sequencing zebrafish *fdh* mRNA via RLM-RACE with the GeneRacer kit (Invitrogen). This allowed us to obtain the sequence information for the full-length 5' ends of the mRNA, since only full-length transcripts with a 5'-capping structure can be amplified by this method. Our results showed that exon 1 of zebrafish *fdh* is 58 bp in length (GenBank accession number [NM_001198772](#)). Comparison with the online *fdh* gene sequence revealed that the entire exon 1 is the 5'-untranslated region of *fdh* mRNA, and the translation initiation site is located at the beginning of exon 2. Excess alcohol ingestion is suggested to affect gene expression via perturbation of gene-spe-

FIG 5 Addition of antioxidants to ethanol-treated embryos did not prevent the ethanol-induced increase of *fdh* mRNA. Embryos at 3 hpf were incubated in 1.5% ethanol and antioxidant. Embryos were harvested at 9 hpf and examined for *fdh* mRNA level. Presented values are averaged results of at least three independently repeated experiments with different batches of embryos. NAC, N-acetylcysteine; MnT, manganese(III) tetrakis(1-methyl-4-pyridyl)porphyrin. Statistics were calculated by comparison with control embryos without any treatment (Ctl). **, $P < 0.01$.

cific promoter methylation. Potential methylation sites are found in the mammalian *fdh* promoter (23, 24). However, we did not find any potential CpG islands after examining the $-5000/+1000$ region of the zebrafish *fdh* gene sequence with the online software for potential methylation sites (CpG Islands Searcher; <http://CpGislands.usc.edu>). These results suggest that altering the promoter methylation pattern may not be the mechanism responsible for ethanol-induced *fdh* upregulation in zebrafish.

The promoter elements responsible for ethanol-induced *fdh* upregulation are different from those involved in basal level expression. For a more detailed characterization, the $-1694/+40$ fragment in the 5'-flanking region of the zebrafish *fdh* promoter was subcloned upstream of a luciferase coding sequence for activity analysis. Serial deletion on this $-1694/+40$ fragment showed that $-124/+40$ was the critical region required for transactivational activity (Fig. 6A). Deleting most of exon 1 ($+1/+40$) completely abolished transactivational activity, suggesting that exon 1 is essential. Deleting the $-124/-49$ region also abolished the transcriptional activity, suggesting that there is a regulatory element(s) critical for the basal level activity of the *fdh* promoter residing within this region.

Analysis of the sequence of the $-125/+25$ region, using online software (TFSEARCH version 1.3; <http://www.cbrc.jp/research/db/TFSEARCH.html>) for potential transcription factor identification, revealed consensus sequences for several transcription factors (Fig. 6B). A potential CEBP α binding site was predicted at the $-105/-90$ region (designated site 1). An overlapping ADR/STRE (aldehyde dehydrogenase/stress response element) consensus sequence was predicted at the $-77/-69$ region (designated site 2). A possible HSF/cap site was predicted at the $-24/-17$ region (designated site 3). To further characterize the roles of site 1, site 2, and site 3, we introduced mutations into these three sites on the $-124/+40$ promoter constructs. Site-directed mutation at site 1 (SDM-1) on the $-124/+40$ fragment resulted in decreased transactivational activity, suggesting a positive regulatory element in the site 1 region (Fig. 6C). Mutation at site 2 (SDM-2) completely abolished promoter activity, suggesting that a critical element essential for the basal activity is located in this region. Mutation at site 3 (SDM-3) resulted in increased activity, suggesting a negative

regulatory role for the $-24/-17$ region. The clone with mutations at both site 1 and site 3 (SDM-1,3) manifested a compromised and comparable transactivational activity to the wild type, supporting the above-proposed regulatory activities of site 1 and site 3. These results confirmed the essential role of the $-124/-49$ region to *fdh* promoter activity, as well as the regulatory activities of the three predicted regulatory elements.

Further dissection of the transactivational activity of the $-124/+40$ fragment revealed that both site 1 and site 3 are involved in the response to ethanol-induced upregulation of *fdh*. The $-124/+40$ fragment, which was sufficient to mediate the basal activity for *fdh* transcription, was also responsive to the presence of ethanol. However, the ethanol-induced activation on the *fdh* $-124/+40$ fragment was abolished in the three mutants, SDM-1, SDM-3, and SDM-1,3, since no significant difference between the transactivational activities in the presence or absence of ethanol was observed for any of the three constructs, based on the averaged results of seven independent repeat experiments (Fig. 6D). These data suggest that both site 1 and site 3 are required for the ethanol-induced upregulation of *fdh*. The activities of SDM-2 and $-49/+40$ constructs were too low to determine whether they responded to the presence of ethanol. Nevertheless, the observation of no change in the promoter activity of the SDM-1,3 construct in the presence of ethanol indicated that site 2 alone is not sufficient to mediate the upregulation of *fdh* in response to ethanol. These results suggest that the elements in site 1 and site 3 are required for regulation of the response to ethanol, whereas site 2 is crucial for basic transcriptional activity, albeit it is not involved in ethanol-induced *fdh* upregulation.

Sp1 is involved in the transcriptional regulation of zebrafish *fdh* but not in ethanol-induced *fdh* upregulation. Sp1 has been reported to regulate a group of "ethanol-responsive genes" (25). Sp1 also participates in the transcriptional regulation of several folate-requiring enzymes, including serine hydroxymethyltransferase (SHMT) and dihydrofolate reductase (DHFR) (24, 26, 27). All three enzymes, including FDH, catalyze the production of THF. Previously, we had characterized a zebrafish Sp1-like protein and showed that this protein was structurally and functionally similar to human Sp1 (15). To examine the role of Sp1 in regulating zebrafish *fdh* expression, we coexpressed this Sp1-like protein in zebrafish embryos, which were injected with the *fdh* $-1694/+58$ promoter constructs simultaneously, for the promoter activity assay. A 4-fold increase in transactivational activity of the $-1694/+58$ fragment was observed (Fig. 7A). The endogenous *fdh* mRNA was also significantly increased in these coinjected embryos, supporting a positive regulatory effect of Sp1 for the zebrafish *fdh* promoter (Fig. 7B).

To examine whether the Sp1-mediated activation involves sites 1, 2, and 3, zebrafish Sp1 protein was coexpressed in cells transfected with *fdh* $-124/+40$ promoter SDM constructs for promoter activity analysis. Again, the activity of SDM-2 was too low to judge the effect of Sp1. However, we found that mutations at either or both site 1 and site 3 (i.e., SDM-1, SDM-3, and SDM-1,3) did not affect Sp1-mediated activation, as an approximately 4-fold increase was observed in all the examined mutants (Fig. 7C). These results indicated that site 2 alone is sufficient for Sp1-mediated activation of the zebrafish *fdh* promoter, whereas site 1 and site 3 are not involved in this regulation. Biotinylated oligonucleotides encompassing two repeats of the site 2 sequence (designated probe2 [Table 1]) were synthesized and used in a DNA

FIG 6 Genomic structure and transcriptional activity of the zebrafish *fdh* 5' upstream flanking region. (A) pGL3-basic plasmids containing the serial deleted fragments of the *fdh* promoter -1694/+40 region were transfected into ZLE cells for transactivation activity determinations. The transcription starting site, determined by using the RLM-RACE GeneRacer kit, is designated +1. E1 represents exon 1 of the *fdh* gene. *luc*, luciferase coding sequence. (B) The consensus sequence for the potential transcription factor binding sites predicted on the zebrafish *fdh* promoter region are underlined, and the potential factors involved are listed beneath (predicted by use of the online software TFSEARCH [version 1.3; www.cbrc.jp/research/db/TFSEARCH.html]). The letters in bold are the residues that were mutated in subsequent site-directed mutation experiments. (C) The activities of -124/+40 constructs with site-directed mutations were analyzed in ZLE cells by using a dual-luciferase assay system. The mutated sites, indicated by X, and their corresponding potential transcription factors are designated as follows: site 1 (SDM-1) for CEBP with the sequence AAAGGGCAC converted to CCATGGCAC; site 2 (SDM-2) for ADR1/STRE with sequence CCACCCCC converted to CCATGGC; site 3 (SDM-3) for HSF/Cap with sequence CAGTTCTGCT converted to CAGAGCTCC. SDM-1,3 refers to the construct containing mutations at both C/EBP and HSF/Cap sites. The number in parentheses also corresponds to the consensus sequences for potential transcription factor binding sites indicated in the panel. WT, wild type. (D) ZLE cells, transfected with the -124/+40/pGL3-basic constructs, either with or without the indicated site-directed mutation, were incubated in medium containing 2% ethanol for 1 h before dual-luciferase analysis for promoter activity determination. Obtained relative activity was normalized with the relative activity of the same construct without ethanol exposure (ex; -124/+40/SDM-1 with ethanol treatment/-124/+40/SDM-1 without ethanol treatment), yielding the fold change in relative activity. Data are presented as the relative ratios of the activity, with the -124/+40 control group activities designated 1. **, $P < 0.01$.

affinity precipitation assay to examine the binding of Sp1 to the site 2 sequence. A clear signal at the molecular weight corresponding to Sp1 was detected when anti-zebrafish Sp1-like antibodies and the cell extract containing overexpressed Sp1-like protein were used for the DAPA (Fig. 7D). The signal was significantly decreased when a 3-base mutation was introduced to probe 2 (probe 2^{sdm}). The mutation on probe 2^{sdm} is identical to that introduced into the SDM-2 clone (CCC → TGG) (Fig. 6B). These data suggest that Sp1 binds to the site 2 sequence specifically and is critical to the basal transactivational activity of the zebrafish *fdh* promoter. We noticed that the molecular weight of the DAPA signal was slightly higher than that of purified recombinant zebrafish Sp1-like protein, implying a possible posttranslational modification on Sp1. This hypothesis was supported by the observation that no appreciable DAPA signal was detected when purified

Sp1 protein was used in the place of Sp1-containing cell extract for DAPA (data not shown). Our results suggested that Sp1 binds at site 2 and upregulates *fdh* promoter activity, for which posttranslational modification of Sp1 might be necessary.

CEBPα is involved in the ethanol-induced upregulation of *fdh*. Sequence analysis on the *fdh* -124/+40 promoter fragment revealed a potential CEBPα binding site in site 1 of the *fdh* promoter region (Fig. 6B). To confirm the possibility of CEBPα regulating *fdh* expression and mediating ethanol-induced *fdh* upregulation, the complete cDNA encoding zebrafish CEBPα was cloned and cotransfected with *fdh* promoter -124/+40 wild-type and mutant plasmid constructs into ZEL cells. Overexpressing CEBPα increased *fdh* promoter activity of the -124/+40 fragment up to 37-fold (Fig. 8A). Again, the basal activity of SDM-2 was too low to judge the effects of CEBPα on *fdh* promoter activ-

FIG 7 Sp1 participates in *fdh* transcriptional regulation. (A and B) The pGL3-basic-1694/+40 construct and pcGlobin2 plasmids with (zSp1) or without (mock) the zebrafish Sp1 coding sequence were coinjected into zebrafish embryos before the 2-cell stage. The injected embryos at 24 hpf were subjected to promoter activity determinations with luciferase activity analysis (A) and endogenous *fdh* mRNA quantification by RT-PCR (B). Embryos of the same stage without any injection were used as a background control (control). (C) The -124/+40 construct with mutations at potential transcription factor binding sites (SDM-1, SDM-2, SDM-3, or SDM-1,3) was cotransfected with pcDNA3.1 containing the zebrafish Sp1 coding sequence into ZLE cells. Cells were grown for 48 h and subjected to promoter activity determinations in a dual-luciferase activity assay. (D) Whole extracts from cells overexpressing zebrafish Sp1 were used in a DAPA for monitoring of binding between Sp1 protein and FDH bait probes. M, molecular mass marker. Lanes: 1, purified recombinant zebrafish Sp1 (300 ng); 2 and 3, whole-cell extracts prepared from cells overexpressing Sp1 (input of DAPA; 3 μg); 4, cell extracts incubated with beads but no probe; 5, cells extract incubated with FDH probe 2 and beads; 6, cells extract incubated with FDH probe 2^{sdm} and beads. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

ity. On the other hand, mutation at site 1 (SDM-1) significantly decreased the promoter activity. DAPA using probes containing the site 1 sequence (probe 1) and a cell extract containing overexpressed CEBPα revealed a clear binding signal (Fig. 8B). This binding signal was significantly diminished when the probe sequence was mutated (probe 1^{sdm}). The binding of CEBPα to the *fdh* promoter was further confirmed by ChIP assay, where a PCR product of approximately 200 bp corresponding to the zebrafish *fdh* promoter -124/+40 region was obtained (Fig. 8C). Unexpectedly, mutations at site 3 (SDM-3) and site 1,3 (SDM-1,3) also diminished the transactivational activity of CEBPα on the -124/+40 fragment. These results suggest that CEBPα directly binds to site 1 and participates in the transcriptional regulation of zebrafish *fdh*. Also, site 3 contributes to CEBPα-mediated regulation of *fdh* expression.

The similar regulatory patterns observed for *fdh* promoter activity in responses to overexpressed CEBPα and to ethanol exposure prompted us to hypothesize that CEBPα is involved in ethanol-induced *fdh* upregulation. We observed increased CEBPα mRNA in ethanol-treated embryos (Fig. 8D). Overexpressing CEBPα not only increased *fdh* expression but also alleviated the oxidative stress in ethanol-exposed embryos (Fig. 8E and F). In addition, this oxidative stress-relieving effect of overexpressed CEBPα was diminished by knocking down *fdh* expression (Fig. 8G). These results indicated that CEBPα participates in ethanol-

induced *fdh* upregulation, which helps relieve ethanol-induced oxidative stress.

The endogenous *fdh* mRNA was increased in 293T human kidney cells in response to ethanol exposure, elevated CEBPα, and Sp1. We found that endogenous *fdh* mRNA in 293T human kidney cells was increased dose dependently when ethanol at increasing concentrations was added to the culture medium (Fig. 9). Overexpression of Sp1 and CEBPα also increased endogenous *fdh* mRNA. These results suggest a possible similarity between the expressional regulation of human and zebrafish *fdh*. In addition, knocking down CEBPα blunted the increased expression of *fdh* in the presence of ethanol, supporting a direct involvement of CEBPα in ethanol-induced upregulation of *fdh*.

DISCUSSION

In the current study, we have reported the interplay among ethanol toxicity, oxidative stress, and folate-requiring OCM through FDH, a very abundant folate enzyme in the liver. We showed that FDH was upregulated in zebrafish embryos exposed to ethanol, which helped relieve ethanol-induced oxidative stress and maintain the intracellular THF level. The crucial element responsible for the basal *fdh* promoter activity was identified and shown to be modulated by Sp1 but not in response to ethanol exposure. On the other hand, the transcription factor CEBPα was involved in ethanol-induced upregulation of *fdh*, which could only occur when

FIG 8 CEBPα is involved in *fdh* transcriptional regulation in response to ethanol exposure. (A) The -124/+40 construct with mutation at potential transcription factor binding sites (SDM-1, SDM-2, SDM-3, or SDM-1,3) were cotransfected with p3×-Flag-c-Myc/CMV26 containing the zebrafish CEBPα coding sequence to ZLE cells. Cells were grown for 48 h and subjected to a promoter activity assay via a dual-luciferase activity assay. (B) Whole extracts prepared from cells overexpressing zebrafish CEBPα with Flag tag were used in a DAPA for examining the binding between CEBPα protein and FDH probe 1. Antibodies against the Flag tag were used for precipitated protein identification. Lanes: 1, whole-cell extracts prepared from cells overexpressing CEBPα (input of DAPA; 5 μg); 2, cells extract incubated with beads but no probe; 3, cell extract incubated with beads and FDH probe 1; 4, cell extract incubated with FDH probe 1^{sdm}. (C) ChIP assay results with chromatin extract prepared from ethanol (1.5%)-exposed embryos with zCEBPα-Flag mRNA injection. Lanes: 1', input; 2', control for no anti-Flag antibody; 3', anti-Flag antibody and input. (D) Zebrafish embryos at 3 hpf were incubated in ethanol at the indicated concentrations for 6 h and analyzed for CEBPα mRNA. (E) Plasmid containing CEBPα cDNA was injected into embryos at the 1- to 8-cell stages and analyzed for the endogenous *fdh* mRNA level at 24 hpf. (F) Embryos injected with zCEBPα mRNA (zCEBPα) or vector plasmid (mock) at the 1-cell stage were exposed to ethanol at 24 hpf for 1.5 h and analyzed with DCF staining for oxidative stress. Controls were embryos of the same stage that did not receive any injection. (G) The rescuing effect of CEBPα overexpression on ethanol-induced oxidative stress was monitored by coinjecting *fdh* MO and zCEBPα mRNA into embryos at the 1- to 8-cell stages and DCF staining at 24 hpf. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

the basal transcription activity of *fdh* was retained. The current study provides *in vivo* evidence for the antioxidative activity of folate and FDH. Our results also support the beneficial effects of folate supplementation to developing embryos, especially for those at risk of FAS (28, 29).

By overexpressing and knocking down *fdh*, we observed that the embryonic THF level was modulated by the FDH level. An intriguing yet unexplainable observation documented in the literature is that THF the main folate moiety carrying one-carbon units seems to always increase regardless of how total folate and other folate derivatives are altered in response to ethanol exposure (30–33). Although not yet confirmed, maintaining a steady and sufficient level of THF does provide the cells with the possible benefit of more flexibility to switch between statuses with different metabolic demands. For instance, more 10-formyl-THF and 5,10-

methylene-THF are needed during cell proliferation (for nucleotide synthesis), whereas sufficient 5-methyl-THF is required for cells that undergo both proliferation and differentiation when modulation of gene activity is required (for S-adenosylmethionine formation). Ethanol induces oxidative stress and decreases intracellular antioxidants, such as reduced glutathione (34). As THF and 5-methyl-THF are active antioxidants, it is conceivable there is a decrease in both THF and 5-methyl-THF in the presence of ethanol (22, 35). As reported previously, the THF level is not decreased in ethanol-treated zebrafish embryos. With a noncatalytic site to bind and stabilize THF and with its abundance in liver, FDH is proposed to act as a THF reservoir and play a crucial role in regulating intracellular THF availability (5, 36). The data provided in the current study support the THF stabilization activity of FDH *in vivo*.

FIG 9 Expression of human *fdh* in response to ethanol exposure and altered expression of Sp1 and CEBPα. The mRNA levels of endogenous *fdh* were analyzed in human 293T cells with different treatments: (A) cultured in medium containing 1% or 2% ethanol, (B) transfected with plasmids carrying human Sp1, or (C) transfected with plasmids carrying zebrafish CEBPα. (D) Cells were transfected with sh-Luciferase (sh-Luc) were used as a nonspecific control for comparison. All statistical analyses were based on comparisons to the control (the first bar) of each experiment. *, $P < 0.05$; **, $P < 0.01$.

FDH catalyzes the reaction accompanied by the generation of NADPH. We observed no significant difference in total NADP and NADPH levels between embryos with or without ethanol exposure (data not shown), suggesting that the antioxidative activity exerted by FDH is not exerted through alterations of the NADP/NADPH ratio. We had also injected the mRNA of an FDH site-directed mutant, which displayed a significantly diminished binding activity to THF, into embryos before ethanol exposure. No significant rescuing effect against ethanol-induced oxidative stress was found in these embryos (unpublished data). These results further support the antioxidative activities of THF and FDH.

Similarities have been found between human and zebrafish *fdh* promoter structures and activities. The first exons of both human and zebrafish *fdh* genes are entirely nontranslated and serve as part of the minimal promoter for zebrafish *fdh* (24). Despite the difference of lacking a potential methylation site in the zebrafish *fdh* promoter, both zebrafish and human *fdh* mRNA are increased in response to ethanol exposure, increased Sp1, and CEBPα, supporting the conservation of the mechanisms regulating *fdh* transcription during evolution (9). Distinct roles for the regulatory elements in site 1, site 2, and site 3 in the region $-124/+40$ were found in controlling zebrafish *fdh* expression. The ethanol-induced response, mediated by site 1/3, occurs only when the basal activity, mediated by site 2, is sustained. Figure 10 depicts the hypothesized transcriptional complex in the zebrafish *fdh* promoter, based on these results. In the proposed scenario, Sp1 binds mainly to site 2 and partly to site 1 (probably assisted by an accessory protein X), which initiates basal-level transcription of *fdh*. Mutation at site 3, which contains a negative regulatory element,

will prevent the binding of a hypothetical inhibitor, Y, and increase the transcription activity. The binding of Sp1 to site 2 is a prerequisite for the subsequent activation mediated by CEBPα, since the binding of Sp1 might result in a conformational change that brings site 1 close to site 3 and facilitates subsequent activation. CEBPα at site 1, together with site 3 and another accessory protein, Z, form a complex that will prevent the binding of inhibitor Y to site 3, thus helping secure the binding of assistant protein X and enhance the transactivational activity of the *fdh* promoter. The reason that assistant protein X is included in the picture is because no potential Sp1 site was predicted in the site 2 sequence, despite DAPA resulting in clear binding between Sp1 and probe 2. The possibility of an indirect binding between Sp1 and probe 2 that involves other proteins cannot be excluded. Further studies to identify the hypothesized proteins and the role of site 3 will help clarify the mechanisms involved. One other important and intriguing question that remains unanswered is whether the signal(s) responsible for triggering the cascade leads to *fdh* upregulation. Although antioxidants did not prevent the increase of *fdh* mRNA in ethanol-exposed embryos, examination of the effect of oxidative stress sensors, such as the Nrf2-Keap1 system, in ethanol-induced *fdh* upregulation should help clarify the link between ethanol-induced oxidative stress and the function and regulation of *fdh* (37, 38).

The justifications for the choice of compound-exposure protocols and the timing for sample collection in different experiments should be noted. The variations between the adapted protocols and the timing for different experiments were mainly to overcome the technical difficulties due to the intrinsic differences

FIG 10 The hypothetical mechanism and molecules involved in the transcriptional regulation of zebrafish *fdh*. In the absence of ethanol, the binding of Sp1 to site 2 is sufficient to exert basal *fdh* promoter activity. A negative regulator resides in site 3, which does not completely block but will hinder the promoter activity. In the presence of ethanol, CEBP α is upregulated and binds to site 1 with help from site 3, leading to the formation of a transcription complex and increased *fdh* expression. X, Y, and Z symbolize the hypothesized molecules that potentially participate in this transcriptional regulation.

among samples (cultured cells versus embryos of different stages) and properties of examined targets (RNA, protein, and oxidative stress). The increase of *fdh* mRNA could be observed in embryos as early as 9 hpf with a 6-hour exposure to ethanol. The embryos at stages before 24 hpf were chosen as representative of the fetus at early developmental stages. However, for FDH protein analysis, the embryos at 3 dpf were used, in order to avoid interference from vitellogenin, a very abundant yolk protein that migrates together with FDH. Ethanol was added at 6 hpf, instead of 3 hpf, to avoid causing massive death of embryos at 24 hpf. For oxidative stress evaluation, embryos of 24 hpf were used to allow time for the injected nucleotides (either mRNA or MO) to function and exert their effects, since mRNA and MO need to be injected before 1 hpf. A 1.5-h exposure in ethanol was sufficient to induce appreciable oxidative stress that was detectable in 24-hpf embryos. Despite the different protocols used for different experiments, the trend and phenomenon that ethanol-induced upregulation of FDH helps relieve ethanol-induced oxidative stress still persist.

Conclusion. We showed here that the expression of 10-formyltetrahydrofolate dehydrogenase, a folate enzyme proposed to serve as an intracellular reservoir of tetrahydrofolate, was increased in ethanol-exposed zebrafish embryos at both the both mRNA and protein levels. The increased FDH alleviated ethanol-induced oxidative stress. Sp1 was involved in the basal level of expression of *fdh*. However, the Sp1-mediated regulation did not respond to the presence of ethanol. This ethanol-induced upregulation of *fdh* involved CEBP α and occurred only when the basal transactivational activity of the *fdh* promoter was sustained.

ACKNOWLEDGMENTS

We thank Verne Schirch, Virginia Commonwealth University, for valuable advice and assistance. We also thank the Taiwan Zebrafish Core Facility at ZeTH (supported by NSC grant 101-2321-B-400-014) for materials and technical support.

This work was supported by grant NSC 99-2320-B-006-013-MY3 from the National Science Council, Taiwan.

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