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Ethanol-response genes and their regulation analyzed by a microarray and comparative genomic approach in the nematode *Caenorhabditis elegans*☆

Jae Young Kwon,[a,1] Mingi Hong,[a,1] Min Sung Choi,[a] Sujin Kang,[b] Kyle Duke,[c] Stuart Kim,[c] Sunho Lee,[d] and Junho Lee[a,*]

[a] *National Research Laboratory, Department of Biology, Yonsei University, 134 Shinchon, Seodaemun-ku, Seoul 120-749, South Korea*
[b] *Department of Applied Statistics, Yonsei University, 134 Shinchon, Seodaemun-ku, Seoul 120-749, South Korea*
[c] *Department of Developmental Biology, Stanford University Medical Center, 279 Campus Drive, Stanford, CA 94305, USA*
[d] *Department of Applied Mathematics, Sejong University, 98 Kunja-Dong, Kwangjin-ku, Seoul, South Korea*

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Abstract

The nematode shows responses to acute ethanol exposure that are similar to those observed in humans, mice, and *Drosophila,* namely hyperactivity followed by uncoordination and sedation. We used in this report the nematode *Caenorhabditis elegans* as a model system to identify and characterize the genes that are affected by ethanol exposure and to link those genes functionally into an ethanol-induced gene network. By analyzing the expression profiles of all *C. elegans* ORFs using microarrays, we identified 230 genes affected by ethanol. While the ethanol response of some of the identified genes was significant at early time points, that of the majority was at late time points, indicating that the genes in the latter case might represent the physiological consequence of the ethanol exposure. We further characterized the early response genes that may represent those involved directly in the ethanol response. These genes included many heat shock protein genes, indicating that high concentration of ethanol acts as a strong stress to the animal. Interestingly, we identified two non-heat-shock protein genes that were specifically responsive to ethanol. *glr-2* was the only glutamate receptor gene to be induced by ethanol. T28C12.4, which encodes a protein with limited homology to human neuroligin, was also specific to ethanol stress. Finally, by analyzing the promoter regions of the early response genes, we identified a regulatory element, TCTGCGTCTCT, that was necessary for the expression of subsets of ethanol response genes.

Keywords: C. elegans; Ethanol; Microarray; Ethanol-response genes; Regulatory elements

Ethanol elicits short-term and long-term responses in organisms ranging from multicellular organisms down to single-celled yeast. On acute exposure, multicellular organisms show hypersensitivity, uncoordination, and sedation, whereas on chronic exposure, humans and mice show dependence, tolerance, adaptation, and craving. Single-celled yeast does not show these kinds of complicated responses, but nonetheless is inhibited in growth and other various functions. Although yeast cells and their responses to ethanol stress have been studied in detail (for example, [1–5]), the studies in a single-celled organism have limitations in application to complicated multicellular organisms. Therefore, to elucidate the mechanisms of alcohol action in multicellular organisms, the use of appropriate model systems is needed. Alcohol research up to the late 1990s largely relied on investigating changes rendered in various cell culture systems. As a result of these cell-culture-based studies, many molecular targets that are affected by ethanol were identified. These include *N*-methyl-*d*-aspartate (NMDA), γ-aminobutyric acid ($GABA_A$), and serotonin receptors, calcium and potassium channels, and adenosine transporters (for example, [6–10]). Some G-protein-coupled receptors, including dopamine, opioid, and adenosine receptors, were also shown to be up- or down-regulated on ethanol exposure in specific cell lines [11]. In most cell culture systems, increase in cAMP synthesis was found to occur on acute exposure to ethanol, whereas decrease in cAMP production was observed on chronic exposure. Although the individual studies described

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* Corresponding author. Fax: +82-2-312-5657.
E-mail address: leej@yonsei.ac.kr (J. Lee).

[1] These two authors equally contributed to this work.

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above presented clues to the molecular basis of the mechanisms of ethanol, there was continuous dispute over the relevance these in vitro studies would have in in vivo systems.

To understand the complex genetics of ethanol, various genetic animal models have been used. Studies have been conducted mainly using rodent models (for example, [12]). By mutating specific subunits of the $GABA_A$ receptor, specific sites crucial to the specific activities of each subunit have been identified [13–16]. Mice lacking the 5-hydroxytryptamine 1B receptor show decreased ataxic responses to ethanol [17], and mice lacking the protein kinase Cγ isoform are less sensitive to the hypnotic and hypothermic effects of ethanol [18]. Transgenic mice overexpressing TGF-β also show increased sensitivity to the sedative effects of ethanol [19]. On exposure to ethanol and other addictive drugs, dopamine is elevated in a portion of the brain known as the mesocorticolimbic dopamine system and is thought to act as a positive reinforcer to drugs. Dopamine receptor-deficient mice have been studied and show different responses to the various effects of ethanol, according to receptor type [20,21]. A recent study found that mice lacking m-neu1, a mouse homolog of the *Drosophila neuralized* gene, which is a neurogenic gene in the Notch receptor-mediated signaling pathway, exhibit hypersensitivity to ethanol [22]. Although observing the effects of various genes to ethanol exposure in vivo became possible with the use of mouse models, there was a lack of research connecting the individual genes into a comprehensive genetic network. This was due mainly to the lack of a model system easily manipulated in genetic analysis. In 1998, Moore et al. first laid the foundation for a genetic approach to dissect the acute, and possibly chronic, effects of ethanol in vivo using *Drosophila* as a model system [23]. Through P element mutagenesis and an ethanol-sensitivity screen, they found a mutant, *cheapdate (chpd)*, which showed increased sensitivity to ethanol. *chpd* was found to be an allele of the *amnesiac* gene, which encodes a neuropeptide that activates the cAMP pathway, demonstrating the importance of the cAMP pathway in ethanol-sensitivity regulation in *Drosophila*. The nematode *Caenorhabditis elegans* is another genetically tractable model organism with which to study ethanol action. Several mutations were described to be involved in ethanol sensitivity in this organism [24]. For example, *unc-79* mutation confers resistance to ethanol, and *fc20* and *fc21* confer hypersensitivity to ethanol. Recently, it was also reported that *gas-1*, a gene encoding a mitochondrial protein, is also important in ethanol response in *C. elegans* [25].

At the organismic level, several expression-profiling approaches can be used to identify functional changes occurring with chronic exposure to ethanol [26]. A recent approach to elucidating the in vivo ethanol action mechanism at the genomic level is to use microarrays. The measurements of the changes in multiple genes can be used to identify the causes and consequences of diseases and which gene products may be potential targets for therapy [27]. There are two reports that have utilized microarrays in alcohol research. One study focused on profiling global change in gene expression on chronic exposure to ethanol in a neuroblastoma cell line [28]. Genes involved in norepinephrine synthesis and reuptake, glutathione metabolism or transport, and protection against apoptosis were found to be up-regulated. The other study using microarrays in alcohol research conducted microarray analysis of the superior frontal cortex of alcoholics and nonalcoholics [29]. As a result, 163 of the 4000 genes analyzed were found to show at least a 40% change in expression. These genes were found to include myelin-related genes, cell cycle genes, and several neuronal genes.

In this report, we wanted to use a model organism and a genomic approach to study the gene network induced by ethanol stress. *C. elegans* shows responses to acute ethanol exposure that are similar to those observed in humans, mice, and *Drosophila*, namely hyperactivity followed by uncoordination and sedation [24]. *C. elegans* also has the advantage of having been completely sequenced and of having microarrays that correspond to the whole genome. Using such microarrays has the advantage of being more unbiased in observing expression profiles, and unexpected results can also be obtained. Therefore, in this study, we wanted to identify genes that show a difference in expression on ethanol exposure by microarray experiments and to link those genes functionally into an ethanol-induced gene network. We performed a comparative genomic analysis to identify the regulatory elements required for ethanol response. Our study on the global changes in expression profile and identification of regulatory elements can facilitate further studies, aiming at elucidating the functions of individual genes in the response to ethanol.

Results and discussion

Effects of ethanol on C. elegans behavior

We first confirmed the relevance of ethanol action in *C. elegans* to that in higher organisms, as previously reported [24]. When treated with 7% ethanol, the nematode responded to this stress by showing higher motility and then started to slow its movement and eventually stopped moving (Fig. 1A). This series of action occurred within 10 min. When retrieved from ethanol, the animals began to recover from ataxia within 3 min, fully recovering by 10 min, indicating that the ethanol effect was fully reversible. Other specific behavioral phenotypes included an egg-laying defect reflecting abnormal neuromuscular functions, a pharyngeal pumping defect, which again reflects neuromuscular disorders, and loss of touch sensitivity (J. Kwon, M. Hong, and J. Lee, unpublished observations). From these observations, we confirmed that the nematode could be used as a model organism for studying the acute action

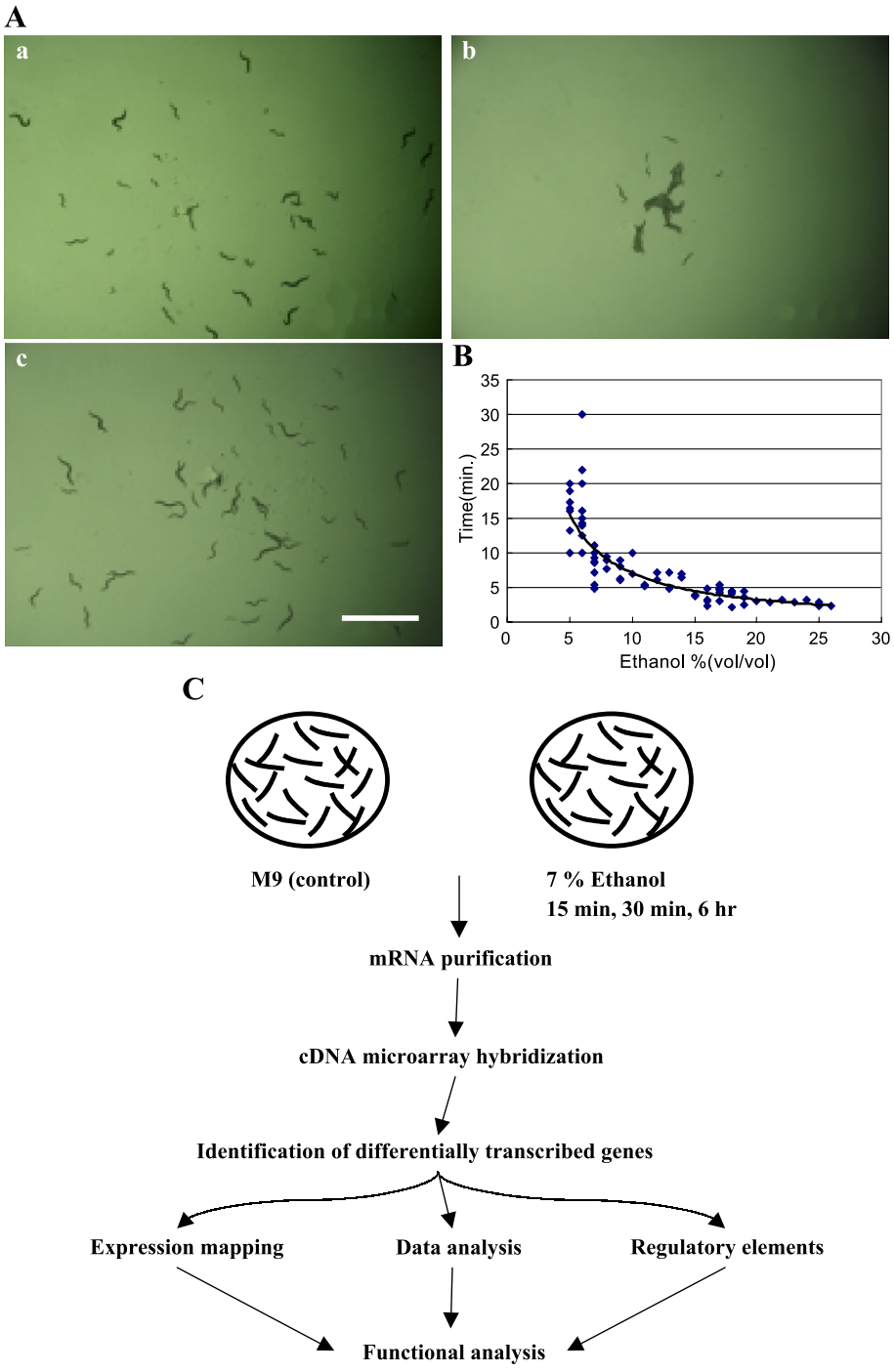


Fig. 1. Nematodes can be used for ethanol research. (A) Effects of ethanol on *C. elegans* motility. (a) The motility of wild-type N2 animals is normal 3 min after soaking in the M9 control buffer for 10 min. (b) Animals are paralyzed 3 min after soaking in 7 vol% ethanol for 10 min. (c) The animals shown in (b) were allowed to recover for 10 min. They regained motility. Scale bar, 3 mm. (B) Effects of different concentrations of ethanol on the *C. elegans* motility. Each spot represents the time point when the last animal among the examined animals ($n = 50$ for each spot) was paralyzed by ethanol at the given concentration. We chose 7% ethanol for further experiments because the animals responded to ethanol in the most consistent manner at this concentration. (C) A schematic of the procedure of this study. The steps for identification of ethanol-susceptible genes using the microarray technique followed by functional studies are shown.

of ethanol. We next examined how the animals responded to different concentrations of ethanol in M9 medium (Fig. 1B). At 7% ethanol, all animals stopped movement within 10 min and could recover to the normal state even after 6 h of exposure (data not shown). After exposure to ethanol longer than 6 h, some animals died, indicating that the damage caused by ethanol became lethal. At lower concentrations of ethanol, animals retained motility for much longer time, and showed higher fluctuation in the extent of responsiveness to ethanol. For example, at 6%, most

animals became paralyzed by 10 min, but some animals stopped movement as early as 2 min, and some animals still moved even after 30 min. At concentration higher than 7%, we observed a more rapid response from the animals, but we also detected that some animals could not survive after a slightly prolonged exposure. From these results, we decided to use 7% ethanol for our further experiments. At this concentration, the animals responded to ethanol with less variability and did not show any lethal effects for at least 6 h. We considered the 6-h time point as the endpoint of acute ethanol action in the nematode. It was reported that EC_{50} of the wild-type *C. elegans* is 1050 ± 30 mM [24], and the concentration used in this study is somewhat higher than the EC_{50} concentration to obtain more consistent results. It is also conceivable that the internal ethanol concentration may be much lower than that in the external environment.

Microarray experiments and identification of genes affected by ethanol

The response mechanism of the whole organism to ethanol would be a complicated one and may be best explained by elucidating all the spectra of genes affected by ethanol exposure. Microarray experiments would be an ideal approach to this purpose. We thus decided to utilize microarray experiments to examine ethanol action in *C. elegans* (Fig. 1C). We expected that when exposed to ethanol, the nematode would adapt to the situation by inducing or repressing genes involved in ethanol action as well as inducing general stress proteins. We performed microarray experiments using mRNAs from animals exposed to 7% ethanol for various periods of time: 15 min, 30 min, and 6 h. We reasoned that genes whose transcription level changed after 6 h treatment would reflect the physiological end results of ethanol effects, whereas genes whose transcription level change rapidly in response to ethanol would reveal direct targets of ethanol action, or genes playing important roles in the ethanol-induced signaling pathways. To identify genes whose transcription level was affected by ethanol, we set the initial microarray analysis point at 15 min of ethanol treatment. Fifteen minutes is technically the minimal time necessary for preparing animals for mRNA preparation. We analyzed the microarray results from seven independent experiment sets: four of 6-h exposure, two of 15-min exposure, and one of 30-min exposure.

An overall result from microarray experiments was that most genes were not affected by ethanol. Specifically, when 477 genes that were previously categorized by their cellular roles (WormPD [43]) were examined for a transcriptional response to ethanol, we found that almost no category of genes showed significant transcriptional increase (data not shown), implicating that genes in any given category of cellular function do not universally respond to ethanol, but that specific individual genes may respond to the stress of ethanol. The only exception was the heat shock protein family genes, which showed rapid and steady increase in transcription.

To identify systematically genes significantly affected by ethanol, we identified genes that showed at least a twofold average change in transcript level in early time point and late time point experiments, respectively (see Experimental procedures). We then selected genes that satisfied the *B* and *T* value criteria as described under Experimental procedures. We obtained 24 genes that showed statistically significant change from the early time point experiments and 219 genes from the late time point experiments, among which 13 were also identified in the early time point experiments. The total number of nonredundant genes identified is 230. We confirmed that the selected genes were authentic by performing Northern analysis on 50 randomly selected genes (data not shown). The Northern results for most of the genes were consistent with the microarray results. We also confirmed that the response of the nematode to ethanol was a reversible active process by analyzing the transcript levels of representative ethanol response genes from the microarray results after 6 h recovery from exposure to ethanol for 6 h. Transcript levels of the up- and down-regulated genes were back to the normal level after recovery (data not shown).

Databases were reviewed for known RNAi effects of the ethanol-affected genes, and it was found that most genes did not show any significant phenotype (Supplementary Table 1), indicating that these genes are not essential genes, but that they may be specifically responsive to certain stresses such as ethanol. It is also possible that RNAi phenotypes may have been undetected for the genes expressed in neurons. Not many mutations have been identified for the genes that we identified as ethanol-affected genes (data not shown), making it difficult to understand their biological roles. Most genes selected as ethanol-affected genes in this study, except for the stress-response proteins, are not affected by heat shock as determined by the examination of the microarray data deposited in the Stanford database, indicating that ethanol elicits responses from the nematode in a different way compared to heat shock (unpublished observation). The gene expression profile in the heat shock response in *C. elegans* has been studied in detail by Kim et al. [41] and GuhaThakurta et al. [40]. Some of the genes selected in our study are identical to the genes that were selected as heat shock response genes. For example, 7 of 28 genes from GuhaThakurta et al. were included in the selected genes in our study. Two genes encoding typical heat shock proteins, T27E4.2 and C12C18.1, were in class I. ZK1290.5 was in class III; F08G2.5, T27F2.4, C30C11.4, and W02D9.10 were in class II. C30C11.4 encodes a family of HSP70 proteins, and other genes encode novel proteins. Two novel genes, F26H11.3 and Y43F8B.2A, from Kim et al. [41] were also included in class III of the ethanol-response genes. It is conceivable

Table 1
Ethanol-response genes categorized into four classes

Name	Predicted identity or function	Expression mountain	Name	Predicted identity or function	Expression mountain
Class I: rapid induction					
B0280.12	*glr-2*	10	H24K24.5	*fmo-15*	21
C12C8.1	*hsp-70*	36	T08G5.3	Unknown	14
F08H9.3	Heat shock protein of the HSP16 class	15	T27E4.2	*hsp-16.11*	36
F08H9.4	Heat shock protein HSP16	15	T27E4.9	*hsp-16.49*	36
F26G1.2	Unknown	15	T28C12.4	Esterase	21
F39F10.1	Unknown	25	Y46H3D.6	Zinc finger protein	21
F47G4.3	Glycerol-3-phosphate dehydrogenase	21			
Class II: late induction					
AC3.7	UDP-glucuronosyltransferase	8	F59B1.8	Unknown	24
B0513.6	Unknown	4	F59C6.6	*nlp-4*	4
C01F6.8	*icl-1*	2	H01A20.1	*nhr-3*	1
C01G10.8	Unknown	20	H06H21.3	Translation initiation factor	5
C02B10.6	Protein tyrosine phosphatase	4	K01D12.12	Glutathione *S*-transferase	21
C03G6.14	Cytochrome P450	19	K02B12.7	GTPase activating protein	2
C04F12.3	Ortholog of human BCL3	15	K02F3.4	Basic-leucine zipper transcription factor	1
C06B3.6	Unknown	15	K06H7.4	*grp-1*, sec7	11
C07A12.4	*pdi-2*, protein disulfide isomerase	30	K07C6.5	Cytochrome P450	19
C10H11.3	UDP- glycosyltransferases	21	K08D10.3	*rnp-3*	2
C10H11.4	UDP- glycosyltransferases	21	K11H12.8	Unknown	30
C12D5.7	Cytochrome P450	24	M02B7.2	Exonuclease	11
C13G3.1	Unknown	15	M03E7.5	Vesicle transport v-SNARE	7
C16A3.4	RNA binding	2	M03F8.1	Unknown	14
C16C8.4	Ubiquitin family	2	M60.7	Intracellular signaling cascade	6
C17E4.5	RNP-1 like RNA binding protein	18	R04D3.2	Unknown	7
C17G10.4	*cdc-14*	5	R07E5.7	Unknown	18
C18H9.6	Unknown	24	R08F11.3	Cytochrome P450	24
C23G10.1	Protein phosphatase	4	R08H2.1	*dhs-23*	3
C25A1.1	Unknown	11	R09H10.4	*ptr-14*	–
C29F7.1	Unknown	24	R102.5	Unknown	15
C30C11.4	hsp70	5	R107.7	*gst-1*	2
C30E1.2	Unknown	3	R11G11.1	*nhr-132*	6
C31E10.7	Cytochrome *b5*	21	T10B9.4	Cytochrome P450	21
C33A12.3	Unknown	44	T11G6.5	Unknown	5
C33F10.7	Esterase and β-lactamase	1	T16G1.4	Unknown	8
C37A5.8	Unknown	15	T16G1.6	Unknown	19
C37C3.10	Unknown	0	T19C4.6	*gpa-1*	0
C37H5.2	α/β hydrolase	15	T19H12.1	UDP-glucuronosyltransferase	21
C37H5.3	α/β hydrolase	21	T20B5.3	*N*-acetyl-β-D-glucosaminidase	1
C39E9.8	Unknown	8	T21B10.1	Unknown	2
C47B2.8	*prx-11*	21	T24H10.3	*dnj-23*	11
C54D10.1	Glutathione *S*-transferase	24	T26A8.4	Nucleic acid binding	5
D1014.3	α-SNAP protein	11	T27F2.4	Basic-leucine zipper transcription factor	25
D2030.9	Lithosperm lec14B protein-like	5	T28C12.5	Carboxylesterase, type B	21
F01D5.10	Unknown	14	W02D9.10	Unknown	15
F08B1.1	*vhp-1*	1	W03B1.9	Unknown	4
F08C6.6	Unknown	24	W04G5.8	Unknown	3
F08F3.4	Unknown	27	Y110A7A.8	mRNA splicing	18
F08F3.7	Member of the cytochrome P450 family	24	Y37E11AR.2	Seven in absentia protein family	1
F08G2.5	Unknown	25	Y37E11AR.4	Unknown	6
F08G2.6	Unknown	25	Y38A10A.5	*crt-1*	5
F09E5.8	Alanine racemase	18	Y40C5A.4	Rhodopsin-like GPCR superfamily	6
F18H3.3	*pab-2*	1	Y44E3B.1	Unknown	11
F23B2.4	WD domain, G β repeats	9	Y45F10C.1	Unknown	4
F23B2.6	*aly-2*	7	Y46H3A.4	Triacylglycerol lipase	6

Table 1 (*continued*)

Name	Predicted identity or function	Expression mountain	Name	Predicted identity or function	Expression mountain
Class II: late induction					
F25D1.5	Dehydrogenase	19	Y47D3B.10	*dpy-18*	1
F30A10.5	*stl-1*	18	Y53C12A.6	Unknown	7
F36F12.8	Zinc finger protein	7	Y57A10C.6	3-Keto-acyl-CoA thiolase	24
F41E6.6	Cysteine protease and a protease inhibitor	15	Y5H2B.3	Unknown	12
F43A11.1	G-protein-coupled receptor	0	Y71H2AM.21	Potassium channel	1
F43E2.8	hsp-4, member of the hsp70 gene class	20	Y73C8C.10	NADH oxidase	3
F43H9.4	Unknown	15	Y75B8A.23	Unknown	4
F45E4.2	plp-1	18	ZC395.10	Unknown	18
F47G4.1	Unknown	3	ZK20.1	Unknown	6
F52F12.7	Cholesterol transporter	18	ZK686.4	RNA binding	2
F53A9.6	Unknown	8	ZK909.3	Pyrophosphohydrolase	14
F54C9.2	*stc-1*	40			
Class III: transient induction					
C04F5.7	UDP-glucosyltransferase	19	H25K10.1	Serine/threonine protein phosphatase	19
C17C3.5	Unknown	4	K04A8.10	UDP-glucuronosyltransferase	21
F23F1.2	Calcium ion binding	17	T27F6.8	Protein containing an F box	6
F26H11.3	DNA binding	36	Y43F8B.2A	Unknown	36
F38E1.8	Rhodopsin-like GPCR superfamily	10	ZK1290.5	Aldo/keto reductase	8
F38E11.2	*hsp-12.6*	15			
Class IV: late repression					
C05C10.4	Acid phosphatase	8	F39E9.2	Protein binding	8
C08E8.4	Unknown	17	F40F9.9	MIP transmembrane protein	6
C08F11.12	Unknown	22	F41E6.5	Glycolate oxidase	8
C08F11.3	Unknown	17	F45D3.3	Unknown	8
C09B8.1	*ipp-5*	1	F46C5.1	Unknown	8
C14C6.2	Unknown	8	F48G7.5	Unknown	16
C15A11.5	*col-7*, member of the collagen superfamily	35	F53C3.2	Homolog of yeast Skp-1p	12
C15C8.3	Cathepsin-like protease	24	F53E10.4	Unknown	17
C16H3.2	*lec-9*, member of the galectin gene class	31	F54D5.3	Unknown	8
C17F4.3	Unknown	14	F54D8.3	*alh-1*	8
C18A3.6	*rab-3*	6	F55H12.4	Unknown	8
C23G10.11	Unknown	8	F56G4.1	Acyltransferase 3 family	8
C24B9.9	Unknown	15	F56G4.2	*pes-2*	12
C25H3.10	Unknown	15	F57B1.5	Unknown	8
C35C5.8a	Unknown	8	F58B3.2	*lys-5*	25
C38D9.4	Unknown	17	F59E11.12	Zn-finger, C4-type steroid receptor	31
C45G7.3	Unknown	15	H12C20.3	*nhr-68*	19
C49F5.7	Unknown	8	H16D19.1	Lectin C-type domain short and long forms	17
C50F4.1	Unknown	8	H16D19.2	Lectin C-type domain short and long forms	17
C50F4.8	Unknown	8	K01A2.2	*far-7*	15
C52D10.8	*skr-13*	12	K02G10.7	Transmembrane channel protein	8
C52D10.9	*skr-8*	12	K04E7.2	*opt-2*	24
D1025.4	Unknown	8	K07A1.6	Trypsin inhibitor-like cysteine rich domain	22
D1053.1	*gst-42*	8	K07E8.3	Unknown	8
DH11.2	Unknown	8	K08F9.1	Glucose transporter	17
E01G6.3	Serine esterase	17	K10H10.2	β-Synthase	1
EGAP4.1	Unknown	17	R06F6.11	Unknown	6
F07C4.6	Unknown	17	R07B1.10	*lec-8*	8
F07F6.5	Unknown	16	R09B5.6	3-Hydroxyacyl-CoA dehydrogenase	22
F08D12.2	Unknown	19	T04A11.1	Phenazine biosynthesis-like protein	8
F08D12.3	Unknown	8	T04A11.2	Phenazine biosynthesis-like protein	8

(continued on next page)

Table 1 (*continued*)

Name	Predicted identity or function	Expression mountain	Name	Predicted identity or function	Expression mountain
Class IV: late repression					
F09A5.1	Tetracycline resistance-like protein	8	T04A11.4	Phenazine biosynthesis-like protein	8
F11A5.9	Sodium/phosphate transporter	8	T07H3.6	Unknown, meprin/TRAF-like MATH	8
F11E6.5	*elo-1*, a palmitic acid elongase	19	T12B5.2	Unknown	8
F16H6.1	Lectin C-type domain short and long forms	27	T22A3.2	Hsp20/α crystallin family	14
F21A3.3	EGF-domain protein	16	T22F3.11	DNA binding, transporter	8
F21C10.10	Unknown	8	W09C5.4	*ins-33*, insulin-like peptide	17
F22B7.9	Unknown	15	Y110A2AL.9	Unknown	8
F22D6.10	*col-60*, member of the collagen gene class	35	Y11D7A.11	*col-120*	35
F25B4.9	C-type lectin	8	Y11D7A.3	Unknown	21
F27C8.4	*spp-18*	8	Y22F5A.5	*lys-2*, member of the lysozyme gene class	19
F28A12.4	Peptidase	19	Y39B6A.5	Unknown	8
F28F8.2	Long-chain-fatty-acid-CoA ligase	8	Y73F4A.3	Dopamine-β-monooxygenase	15
F31F7.1	Unknown	8	ZC395.5	Unknown	15
F32A5.5	Transporter	8	ZK250.5	F-box domain, FTH domain	8
F37C12.10	Unknown	15	ZK512.7	Unknown	22

Expression mountain for each gene is shown according to Kim et al. [41].

that the proteins with no apparent homology to known heat shock proteins may indeed be new members of general stress-response proteins.

Classification and characterization of ethanol-affected genes

We were now in a position to classify the ethanol-affected genes identified by microarray. We classified 230 nonredundant ethanol-affected genes into four groups according to their expression profiles (Table 1, Fig. 2A).

Class I

Class I consists of genes whose transcription levels increased rapidly at a very early stage and were maintained at high levels up to 6 h exposure. Among the 24 genes selected as up-regulated genes in the early time point microarray results, 13 genes were assigned to class I. Among the class I genes, T28C12.4 encodes a novel carboxyl esterase-like protein with similarity to neuroligin, a protein shown to be involved in axon guidance. B0280.12 encodes one of the 10 glutamate receptors in *C. elegans* [44,45]. Five of the genes in this class are heat shock protein genes. The remaining genes encode novel proteins.

Class II

Class II genes are those showing increase in transcript abundance at 6 h, but not at early time points. Class II is composed of 115 genes. The functions of roughly half of the genes classified into class II are not known, and the remaining genes encode diverse proteins such as a ribosomal protein, an RNA binding protein, a translation initiation factor, a few UDP-glycosyl transferases, and GST. These genes are not represented by the 15-min microarray because their expression was not significantly altered at early time points. T28C12.5, a paralog of T28C12.4, a class I gene, is grouped in this class.

Class III

Class III consists of genes whose transcription level increased significantly at early time points, but not after longer exposure to ethanol. Eleven genes were grouped in this class. Among them, F26H11.3a encodes a protein with weak similarity to the yeast GCN5. This gene, although not encoding a conventional heat shock protein, was also confirmed by Kim et al. to be induced by heat shock [41], indicating that it may be a general stress-response gene, responding to ethanol by changing its activity at the transcriptional events. It would be of interest to examine the biological function of this gene. Another class III gene, F38E11.2, a member of the small heat shock protein family, was found to be clustered not on the mountain 36, but on the mountain 15. This gene is reported not to show chaperone activity in vitro, and both its mRNA and its protein are reported not to be induced under stress conditions. ZK1290.5, encoding an aldo/keto reductase, was previously identified as a heat shock-induced gene [40], indicating that this gene may function in response to general stresses.

Class IV

Class IV genes are down-regulated at 6 h, but not at earlier time points. Ninety-one genes were categorized in this class. About two-thirds of the class IV genes encode

novel proteins. Some of the remaining genes encode metabolic enzymes such as a peptidase, a fatty acid CoA ligase, an acyltransferase, and a fatty acid desaturase. These genes are not represented by the 15-min microarray because their expression was not significantly altered at early time points.

Class I, II, and III genes are in the category of up-regulated genes, and class IV genes are in the category of down-regulated genes. It is conceivable that the genes in classes I and III may represent the immediate response of the nematode to the acute exposure to ethanol. The class II and IV genes may represent the physiological results from ethanol exposure because they were not induced or reduced at early time points, but were altered at a late time point. To functionally categorize the ethanol response genes, we mapped the genes onto the gene expression map [41] as described under Experimental procedures. We found that each class of ethanol response genes was significantly clustered (with 99% confidence) in distinct subsets of expression mountains (Table 1, Fig. 2B). Class I genes were found to be clustered on mountains 15, 21, and 36; class II, on 15, 18, 21, 24, 30, and 36; class III, 19, 21, and 36; and class IV, 8, 15, 17, 19, 22, 31, and 35. Mountain 36, which contained 3 of 13 class I genes and 2 class III genes, is enriched in heat shock protein genes. The fact that heat shock protein genes enriched on mountain 36 were rapidly induced is consistent with the previous reports that heat shock proteins are induced by exposure to ethanol in other model systems (for example, [46–49]). Mountain 21, which contains 4 class I genes, 9 class II genes, and 1 class III gene, is enriched in genes related to lipid metabolism, indicating that these genes may be involved in the plasma membrane restructuring to cope with the ethanol stress. Mountain 18, which contained 8 of 114 class II genes, was suggested to correspond to late germline genes [41], indicating that the animal may direct its available resources toward reproduction, resulting in an up-regulation of these germ-line-expressed genes. Many of the class IV genes were found to be clustered on mountain 8, corresponding to enrichment in genes expressed in the intestine. The descriptions of the genes clustered on mountain 8 show a diverse assembly of genes, ranging from alcohol dehydrogenases to energy generation-related genes. This may imply a role for these genes in the intestine for the response to ethanol stress. That these genes are down-regulated by ethanol may reflect a global lowering of metabolism in the animal on ethanol exposure. Because most genes in the intestine are involved in metabolism, it is conceivable that ethanol affects the metabolism of organisms. However, it is not clear whether ethanol affects the metabolism level directly or whether the drop in metabolism reflects yet another defensive response. Class IV genes were also significantly clustered in the mountains enriched with collagen genes. At this point it is difficult to explain why the nematode responds to ethanol by reducing transcription of collagen-related genes.

An interesting fact that can be seen from the mountain maps was that both up- and down-regulated genes showed clustering on mountain 15, an uncharacterized mountain (Fig. 2B) [41]. These genes may show a consistent expression profile regarding ethanol, either up or down. Therefore the genes on mountain 15 may reflect genes specifically responsive to ethanol. However, the possibility that those genes may be genes responsive to general stress or changes in metabolism cannot be ruled out.

After identifying over 200 ethanol-response genes by microarray, it is still difficult to explain in terms of molecular mechanisms how animals become uncoordinated by ethanol exposure. It would be of interest to examine the effects of overexpression, RNAi, and knockout of ethanol-response genes, in particular genes in class I and III, because these genes represent the immediate response of the animal to ethanol exposure. For example, if a gene is critical for the animal to be affected by ethanol, then overexpression of this gene may make the animal resistant to ethanol exposure and a knockout mutant would make the animal uncoordinated like ethanol-affected animals, without ethanol exposure.

Glr-2 is the only glutamate receptor gene responding to ethanol

We further characterized the early response genes because these genes would represent the immediate early transcriptional response to ethanol. While three of the class I genes are heat shock protein genes, we found that a few others encode proteins other than heat shock proteins. Among them *glr-2* and T28C12.4 attracted our attention. B0280.12 encodes a glutamate receptor, GLR-2. It has been shown that during mouse embryogenesis ethanol causes neuronal cell death and fetal alcohol syndrome, in which process NMDA receptors are repressed and GABA receptors activated [50]. Among the 10 glutamate receptor genes in *C. elegans* [44,45], *glr-2* was the only gene whose transcription level was significantly induced by ethanol (Fig. 3A). Other glutamate receptor genes were not induced at any time point of ethanol exposure, indicating that none of the glutamate receptor genes except for *glr-2* respond to ethanol. Time-course Northern analysis showed that *glr-2* transcript was increased at 15 min exposure to ethanol and was maintained high until 6 h (Fig. 3B). After retrieval from ethanol, the transcript level of *glr-2* was back to normal, indicating that the response of *glr-2* to ethanol was reversible (data not shown). Examination of other microarray data deposited in the Stanford database revealed that *glr-2* is not significantly affected by other conditions such as heat shock or starvation (Fig. 3C). *glr-2* was expressed in a subset of head neurons, the dorsal and ventral cords, and a few neurons in the tail, consistent with previous

A

Class	Property	Number of genes
I	Up at the early time point, maintained until the late point	13
II	Up-regulated at the late time point	115
III	Up at the early time point, but not at the late point	11
IV	Down-regulated at the late time point	91

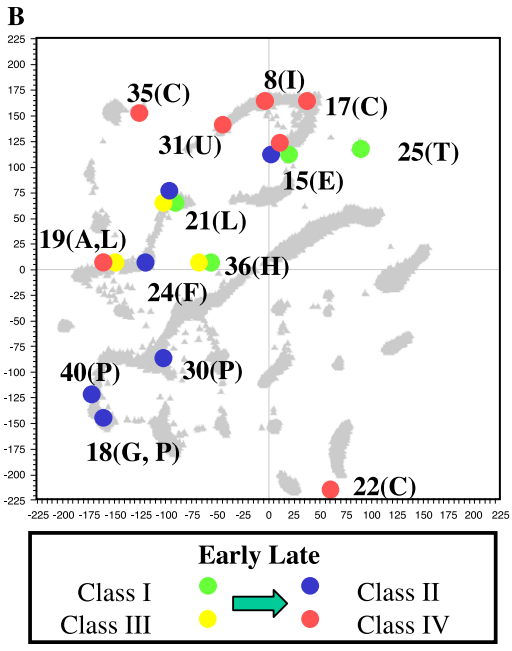
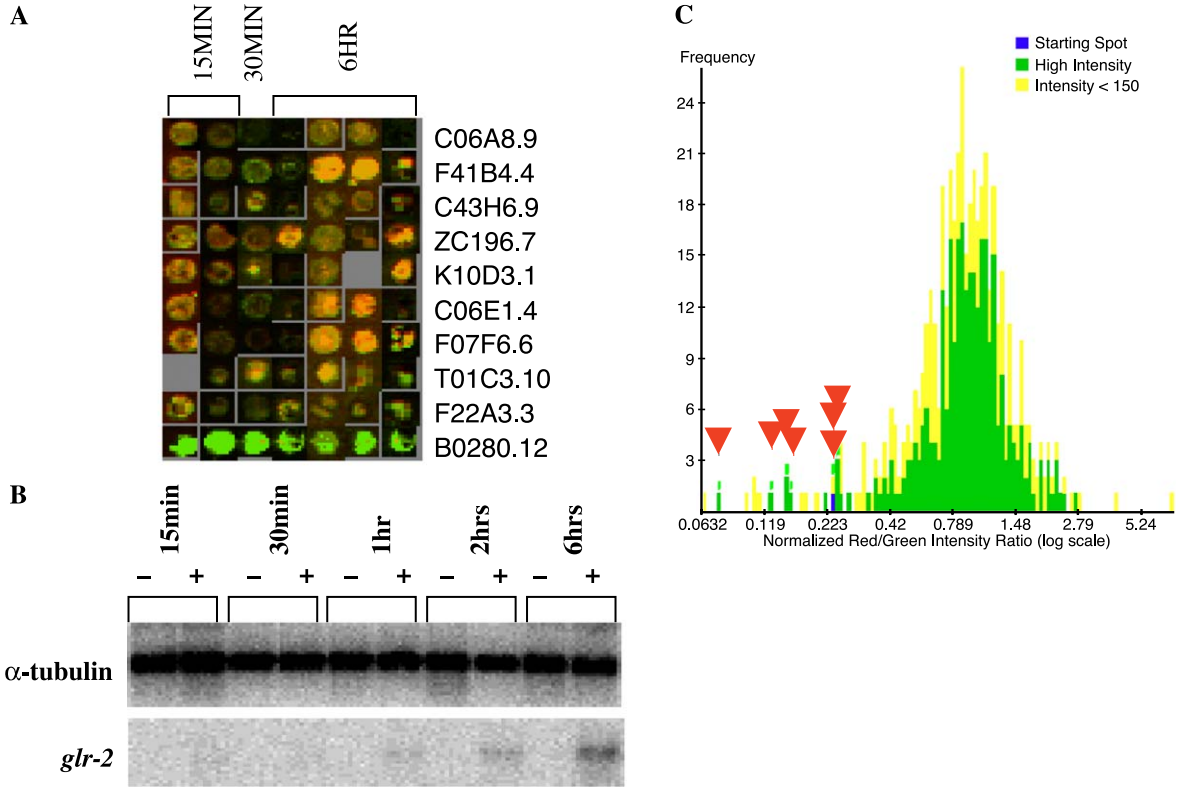


Fig. 2. Classification and expression mapping of ethanol response genes. (A) Genes that responded to ethanol were classified into four classes, I, II, III, and IV, by their expression profile properties. (B) Plotting ethanol response genes on the gene expression map. The genes were plotted on the published gene expression map [41]. Circles indicate the locations of the clustered genes on each expression mountain. Class I genes are indicated by green, class II genes blue, class III genes yellow, and class IV genes red. A indicates amino acid metabolism; C, collagen; F, fatty acid metabolism; G, germ line; H, heat shock; I, intestine; L, lipid metabolism; P, protein synthesis or expression; T, transposase; and U, unknown mountains. Mountain 15 was designated "E" (indicating an ethanol mountain) as discussed in the text.

reports (data not shown, [44]). Our results imply that *glr-2* is specifically responsive to ethanol and that the *glr-2*-containing glutamate receptors may be the target of ethanol action. It would be of interest to examine the role of GLR-2 in the physiological response to ethanol in *C. elegans*.



T28C12.4 is a novel gene specifically responsive to ethanol

Another interesting gene was T28C12.4, encoding a putative carboxyl esterase, which shows limited homology to neuroligin (Fig. 4A). Neuroligins were previously described as proteins involved in neuron–glial cell communication during synapse formation between pre- and postsynapses in embryonic neurogenesis [51]. Time-course Northern analysis showed that without ethanol exposure the normal transcript level was quite low and that after ethanol exposure T28C12.4 was rapidly induced and maintained at high transcript level even after 6 h exposure (Fig. 4B). T28C12.4 was expressed in the hypodermis, including hyp-7 cells, seam cells, and head and tail hypodermis (Fig. 4C). When treated with ethanol, the transgenic animals containing a T28C12.4::GFP construct showed higher level of fluorescence. When treated with other stress such as heat or salt, the green fluorescent protein (GFP) level did not increase, indicating that T28C12.4 is specific to ethanol (Fig. 4C). Consistent with this, no other microarray experiments deposited in the Stanford database showed its up-regulation (data not shown). We propose that T28C12.4 is the ethanol-response gene in the hypodermis. T28C12.5, a paralog of T28C12.4, which was grouped in class II, was expressed in neurons (data not shown), raising the possibility that these two genes may be involved in ethanol response in the hypodermis and neurons, respectively.

Identification of an ethanol-responsive element

To identify regulatory elements that are responsible for ethanol response, we analyzed the promoter sequences of early response genes (Fig. 5A). We used the MEME software to identify all possible motifs conserved in a subset of the class I genes (see Experimental procedures for details). Next, we searched for their homologs in *C. briggsae*, a species closely related to *C. elegans*. It is known that noncoding sequences are not conserved among *C. elegans* and *C. briggsae* genes except for functionally conserved regulatory elements [40]. Among several candidate motifs identified by the MEME software, we found one conserved motif that was also conserved in the corresponding *C. briggsae* sequences. The sequence of this putative regulatory element was TCTGCGTCTCT, which we named ethanol and stress response element (ESRE) (Fig. 5B and C). Seven of 13 class I genes contained this motif within 500 nucleotides upstream of the translational start sites. We then found that 3 of the class III genes also contained the motif in their promoter sequences (Fig. 5C). To exclude the possibility that this motif was found randomly in the promoter regions of any genes, we examined 10 randomly selected class IV genes and found that none of them contained the motif within 500 nucleotides upstream of the translational start sites.

To examine whether this sequence is indeed involved in the ethanol response, we generated an intact or a deleted construct of the T28C12.4 gene, an ethanol-specific response gene, that either contains or deletes, respectively, the ESRE in its upstream region (Fig. 6A). Only the transgene having the ESRE sequence was activated in vivo after exposure to ethanol (Fig. 6A). We also constructed and examined a series of deletion derivatives of two *hsp-16* genes that contain two copies of this conserved motif (Fig. 6B). We found that the induction levels of the ESRE-deleted transgenes were significantly reduced after ethanol stress. Concerning the orientation of the motif, it is probable that the ESRE motif acts in both orientations because deletion of either of the two inverted repeats of the ESRE sequences resulted in considerable decrease in GFP signals (Fig. 6B). Unexpectedly, this reduction also appeared in response to other stress as well. Deletion of the ESRE sequences resulted in the loss of the heat shock response even though the construct contained intact heat shock elements (data not shown), indicating that the ESRE may be required for general stress response. Our results suggested the role of the ESRE as a point of convergence at which general stress signals, including ethanol, meet. Supporting this notion, reduction of *hsf*, encoding a heat shock factor in *C. elegans*, by RNAi abolished the response of *hsp-16* genes to heat shock, but not to ethanol (J. Kwon and J. Lee, unpublished observation). However, one cannot rule out the possibility that the ESRE is an essential promoter element, without which no gene expression is possible under any condition. Consistent with the possibility that the ESRE may be a point of convergence for transcriptional response to general stress signals, the ESRE motif was initially identified by Candido et al. as an inverted repeat in the promoter region of the *hsp-16* gene pairs [52]. The position of the proximal ESRE partially overlaps the HSAS (heat shock-associated sequence), GGGTGTC, identified by GuhaThakurta et al. [40]. In addition, the ESRE complement was also specifically identified by as a candidate element based on its presence upstream of heat shock genes [40], although the function of the elements was not addressed. Our deletion analysis of the proximal ESRE seems to be inconsistent with the data reported by GuhaThakurta et al. in that they reported that deletion of the

Fig. 3. *glr-2* is specifically responsive to ethanol. (A) Among 10 glutamate receptor genes in *C. elegans, glr-2* is the only gene whose transcription level was significantly induced by ethanol. B0280.12 on the bottom row is the ORF encoding *glr-2.* (B) Time-course Northern analysis of the endogenous *glr-2* transcript levels after exposure to ethanol. *glr-2* is induced after 15 min of ethanol exposure and higher at 6 h exposure. α-Tubulin was used as the loading control. (C) *glr-2* is specifically responsive to ethanol. Shown is a history diagram of the log(base 2) values of the R/G ratio for *glr-2* in all available microarray experiments on the nematode deposited in the Stanford database. The *x* axis is the fold change of the given gene presented as normalized R/G ratio. Smaller numbers on the *x* axis indicate that the gene was up-regulated in those specific experiments. The *y* axis is the frequency of the experiments that show the given fold change in expression. All the green arrowheads pointing to individual experiments turned out to be the results from microarray experiments related to ethanol.

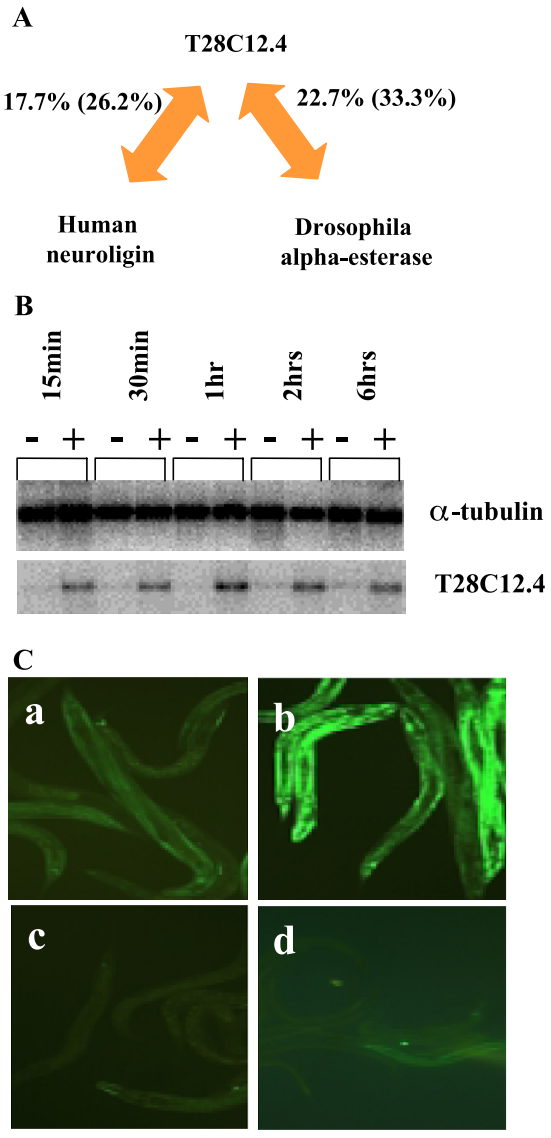


Fig. 4. T28C12.4 is specifically responsive to ethanol. (A) Sequence similarity among T28C12.4 and its putative homologs. The numbers indicate sequence identities, and the numbers in parentheses are sequence similarities. T28C12.4 encodes a protein with amino acid sequence similarity to neuroligin. (B) Time-course Northern analysis of the endogenous T28C12.4 transcript levels after exposure to ethanol. T28C12.4 is induced after 15 min of ethanol exposure and maintained high at 6 h exposure. α-Tubulin was used as the loading control. (C) T28C12.4 is specific to ethanol. When treated with ethanol for 6 h, the transgenic animals containing a T28C12.4–GFP construct showed high level of fluorescence (b). When treated with other stress such as heat (c) or salt (d), the GFP level was not increased. (a) Control.

HSAS caused no decrease in GFP reporter expression and our data showed that deletion of the proximal ESRE resulted in significant decrease in ethanol response. One possible explanation for this apparent inconsistency is that our analysis was based on the intensity of GFP fluorescence and theirs on the number of cells that expressed GFP at any level. For example, the animals that showed less intense GFP fluorescence in every cell would have been counted as positive in their analysis, but not in ours. Further studies such as identifying the *trans*-acting factors would provide more insight on the molecular mechanism of the ESRE action.

In summary, we identified and characterized ethanol-affected genes in the nematode by microarray experiments. We found that most of the nematode genes were not affected at the transcriptional level even after long exposure to ethanol and that there were specific genes that were up- or down-regulated by ethanol. The expression profile of these ethanol-specific genes in the animals itself may be used for an index of the extent of ethanol exposure or the state of alcohol-related diseases of a given individual. Further studies of the biological functions of these genes

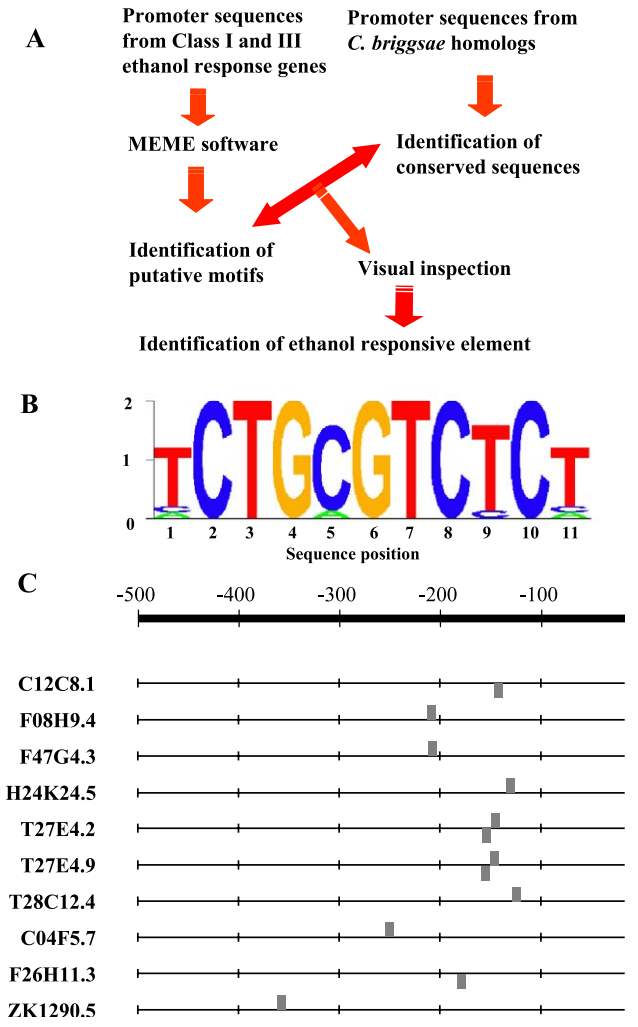


Fig. 5. Identification of the ethanol and stress response element (ESRE). (A) A schematic illustrating the steps for identification of a regulatory element from ethanol-response genes. (B) The consensus sequence derived from the predicted promoters of subsets of early response genes. A score of 2 bits means a perfect match. Larger letters indicate higher sequence conservation. (C) Positions of ESRE in the upstream regions (up to − 500 nt) of the class I and III genes that contained putative ESREs. Bars shown underneath the lines represent elements running in the opposite direction.

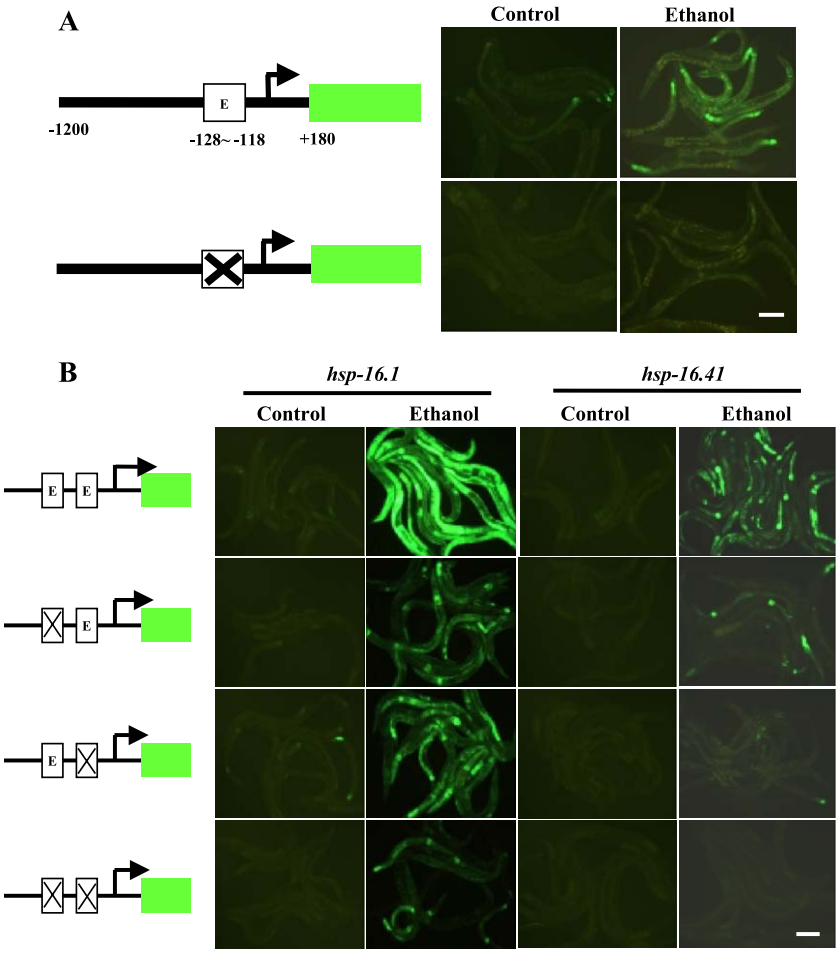


Fig. 6. The ESRE is necessary for the expression of some ethanol-response genes. (A) The effect of ESRE on the T28C12.4 transgene expression. Schematics of the T28C12.4 promoter region and derivatives containing ESRE mutant versions are shown on the left. The promoter and the first exon of the T28C12.4 gene were translationally fused to the GFP reporter gene. Corresponding expressions in the transgenic animals containing each reporter are shown. The left column contains controls and the right, GFP patterns after exposure to ethanol. The ESRE was necessary for T28C12.4 to be induced by ethanol. (B) The effect of the ESRE on the expression of *hsp-16* genes. Schematics of the *hsp-16.1* (encoded by T27E4.2) and *hsp-16.41* (encoded by Y46H3A.2) promoter regions and ESRE mutant derivatives are shown on the left. The upstream regions of the small heat shock protein genes *hsp-16.1* and *hsp-16.41* contain two ESRE candidates. The *hsp-16.1* and *hsp-16.41* promoters lacking ESREs showed reduced induction by ethanol. The scale bars represent 200 μm.

would help in establishing the global action mechanism of ethanol at the organismic level. Many of the genes identified in our microarray have human homologs, indicating that the conclusions drawn in this study may be extended to the biology of humans (Supplementary Table 2). Studies of these human genes may be helpful in elucidating ethanol action in humans in terms of alcoholism and the fetal alcohol syndrome.

Experimental procedures

Strains and culture

C. elegans Bristol strain N2 was used as wild type. *C. briggsae* was obtained from the *Caenorhabditis* Genome Center. Worms were maintained by standard methods at 20°C, as previously described [30].

Ethanol microarray experiments of worms

To extract mRNA for use as probes for microarray analysis, wild-type worms were grown on roughly 80 NGM-Lite plates with 100 mm diameter, which were seeded with OP50-1, a streptomycin-resistant strain of *Escherichia coli*. The worms were harvested with S basal buffer, divided equally, and treated in liquid culture in 1-L flasks, shaking at 250 rpm at 20°C. For controls, worms were cultured in 200 ml of S basal, and the test worms were cultured at a final concentration of 7% (v/v) ethanol in 200 ml of S basal. The mouths of the flasks were securely sealed with Parafilm, to block the diffusion of ethanol. Seven independent experiments were performed in which worms were exposed to ethanol for different time periods: four sets of 6-h exposure, two sets of 15 min exposure, and one set of 30 min exposure. After treatment, mRNAs were isolated from total RNA of each sample of worms using oligo(dT) selection.

The isolated mRNAs were dissolved in 10 mM Tris–Cl, pH 7.4. cDNA synthesis, microarray hybridization, and microarray scanning were performed as described [31]. Briefly, 5–10 μg of mRNA was used in a cDNA reaction as described in DeRisi et al. [32]. The probes were purified with a Qiagen purification kit and 28 μl was subsequently hybridized to the near full-genome *C. elegans* microarrays [33]. The hybridization was performed at 65°C in a waterproof hybridization chamber containing the labeled probe, 8.3 mM Tris, 2× SSC, 0.17% SDS, and 0.67 μg yeast tRNA. After hybridization, the hybridization chamber was removed from the 65°C water bath, washed with 3× SSC/0.2% SDS, 0.2× SSC, and then 0.1× SSC. Scanning was performed with an Axon scanner. The intensity of each pixel in each spot was calculated for each channel, and the background of each spot was measured in a 2-pixel area outside of the spot. The background was subtracted from the foreground intensity. For each microarray hybridization, the Cy5 channel was normalized such that the total Cy5 signal equals the total Cy3 signal. Then the normalized Cy3/Cy5 ratio for each gene was calculated. The microarray data used in this report are available at the Stanford Microarray database Web site [34] (http://genome-www.stanford.edu/microarray). Green spots on the microarray data represent genes up-regulated by ethanol, whereas red spots represent down-regulated genes.

Statistics

There are many statistics that can provide a ranking of genes corresponding to the evidence of differential expression: a Bayes log posterior odds, *B* [35], a slightly modified *t* statistic for small sample comparison, *t** [36], and the log intensity ratio, M = log 2 (Cy3/Cy5). But a small number of replicates in this study made it difficult for us to decide their cutoffs for determining differentially expressed genes. As suggested by Speed [37], we decided cutoffs informally and selected the genes that satisfied all of the above three criteria as main candidates for differential expression. Briefly, M_{gj} was calculated as log ($ch1_{gj}$/$ch2_{gj}$)for gth gene of the jth array. B_g was calculated as log $[(p/1 - p) \times \Pr(M_g \mid I_g = 1)/\Pr(M_g \mid I_g = 0)]$, where M_g is the vector of the n measurements (No. of arrays), $I_g = 1$ if gene g is differentially expressed, and p is the proportion of differentially expressed genes in the experiments. Since the estimation of p is not possible, p is recommended to be fixed at some sensible value such as 0.01 or 0.001. The software calculating this B statistic is available at http://cran.r-project.org/src/contrib/PACKAGES.html#sma. $t^*_g = M_g/s_g + s_o$, where $M_g = \sum M_{gj}/n$, $s_g = [\sum(M_{gj} - M_g)^2/n(n-1)]^{1/2}$, and s_o is the 90th percentile of the standard errors of all the genes. By adding a constant term s_o to the denominator of the usual t statistic, the dependency of the statistic on the gene expression level can be avoided. The value of t^* can be easily obtained by running SAM (Significance Analysis of Microarrays) from http://www-stat-class.stanford.edu/SAM/SAMServlet. We selected genes that showed at least twofold difference in the intensity of the array spots by comparing the average M values of each gene and the same number of genes selected by the M value that ranked highest among the genes in terms of the t^* value. We selected genes that survived both of the selection processes and then discarded genes among them whose B value was smaller than 0. In this way, we obtained 26 genes from the early time point experiments and 220 genes from the 6-h exposure experiments. Among the 220 genes from the 6-h exposure experiment, 13 were also identified in the early time point experiments, indicating that these genes are induced early and maintained at high level until 6 h.

Northern analysis

mRNA was prepared and electrophoresed on a 1.0% agarose gel containing 2.2 M formaldehyde, according to the standard methods. α-tubulin was used as a loading control. We confirmed that α-tubulin was not affected by ethanol treatment by examining its microarray data (data not shown). The templates for probes were prepared by PCR using appropriate primers. The Northern blots were performed with radiolabeled probes and the signals observed with a Bioimaging analyzer (Fuji). The density of the Northern bands of the control and test worms was measured using the Image Gauge version 1.0 program, and their ratios were calculated. For confirmation of the microarray results, 50 randomly selected genes from among the up- or down-regulated genes were used as probes to perform Northern analysis. Most of the tested genes showed results comparable to the microarray results (data not shown). For the time-course Northern analysis of *glr-2* and T28C12.4, mRNAs purified from the animals treated with ethanol for 15 min, 30 min, 1 h, 2 h, and 6 h were used.

GFP constructs

Green fluorescent protein was fused to the first exon of the T28C12.4 ORF. The promoter and coding region were amplified from N2 genomic DNA by PCR, using appropriate primer pairs. The amplified product was cloned into the GFP-containing pPD95.77 vector (a gift from Dr. A. Fire). The cloned hybrid plasmid was amplified using the bacterial strain DH5α. The promoter region of the T28C12.4 gene contains the putative ESRE. We generated the GFP-fused construct with or without this site using the pPD95.77 vector and investigated whether the regulation of T28C12.4 caused by the ethanol was affected by this element. Some heat shock protein genes have one or more putative ESREs in their regulatory regions. We selected two of them, T27E4.2 and Y46H3A.2. These genes are members of the heat shock protein 16 gene family in *C. elegans*. Several GFP-fused constructs containing variable promoter regions of *hsp-16* genes were generated using pPD95.77 to verify the effects of the ESRE. The sequence

information of the primers used in this study is available upon request.

Microinjection and microscopy

Microinjection of DNA into the gonads of adult hermaphrodites was carried out according to standard procedures [38]. The pRF4 plasmid, which contains the dominant *rol-6(su1006)* gene [39], was used as an injection marker at the concentration of 100 ng/μl. To see the effects of ethanol on the transgenes we harvested and left the worms in M9 containing 7% ethanol with shaking for 6 h, as described above. For heat shock, worms were incubated at 30°C for 6 h. The salt stress was administered with M9 containing 200 mM NaCl. After treatment, control worms and worms under stress conditions were allowed to recover on NGM plates at 20°C for 10 h, as described [40]. Allowing the treated animals to recover gave a more consistent result than observing the animals immediately after the treatment. The animals were then mounted on agar pads, and the fluorescence was observed using an Axioplan2 microscope (Zeiss). Images were taken using an AxioCam (Zeiss) camera.

Assimilating data on genes grouped into the five classes and expression profile plotting on gene expression maps

Using Web-based databases such as the Stanford Microarray Database (http://genome-www.stanford.edu/microarray), Wormbase (http://www.elegans.swmed.edu), or WormPD (http://www.proteome.com/databases/WormPD/WormPDsearch-quick.html), the information on the genes regulated by ethanol was collected. The plotting program in the supplemental data of Kim et al. [41] was used (http://cmgm.stanford.edu/~kimlab/topomap/c_elegans_topomap.htm). The ORF names of each group of genes that we wished to see, namely genes grouped into the four classes, were entered into the program, and their positions were plotted on a 2D gene expression map. The 2D gene expression scatter plot depicts genes positioned relative to each other on an $x-y$ scatter plot under the influence of attractive and repulsive forces, the attractive forces representing a similarity in expression profiles in 553 independent microarray experiments [41].

Identification of the ethanol and stress response element

Analysis of the promoters of each class of genes was carried out utilizing the Web-based tool MEME (Multiple EM for Motif Elicitation; http://meme.sdsc.edu/meme/website/meme.html). The MEME software is a tool developed by Bailey et al. [42] for discovering motifs in a group of related DNA or protein sequences. A motif is defined by MEME as a sequence pattern that occurs repeatedly in a group of related protein or DNA sequences. The algorithm of this software discovers motifs by using the technique of expectation maximization to fit a two-component finite mixture model to the set of sequences. We provided the sequence information to the MEME software and set the width of the motifs at 6 to 30 nucleotides. In cases in which the promoter exceeded 500 bp in length, only the sequences up to 500 bp upstream of the start codon were used, as in a previous report [40]. MEME automatically defined the most probable candidate motifs shared by the set of sequences. Next, the corresponding regions from the *C. briggsae* genome were compared by visual inspection with the selected putative motif sequences. The promoter sequences of the *C. briggsae* homologs were obtained and assembled from the Web sites http://www.sanger.ac.uk/Projects/C_briggsae/blast_server.shtml and http://trace.ensembl.org/perl/ssahaview, respectively. Only one putative motif, which we named ESRE, was identified. The positions of the ESRE in each upstream region were visualized using a Web-based tool (http://rsat.ulb.ac.be/rsat/), and the consensus motif logo was generated by the procedure provided in the Web site http://ep.ebi.ac.uk/EP/SEQLOGO/.

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