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The anesthetic action of ethanol analyzed by genetics in *Caenorhabditis elegans*

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Abstract

Acute exposure to ethanol causes paralysis at high concentrations in the nematode *Caenorhabditis elegans*. We set out to elucidate the mechanism of the anesthetic action of ethanol by genetic approaches. We identified nine mutations that conferred reduced sensitivity to ethanol after chemical, irradiation, or transposon insertion mutagenesis. Of these nine, we further characterized five mutations that defined four genes, *jud-1–jud-4*. Analysis of the phenotypes of the animals heterozygous for two unlinked genes revealed that *jud-1* and *jud-3* act synergistically in a gene dose-dependent manner. We cloned *jud-4* and found that it encodes a protein with limited homology to human Homer proteins. *jud-4* was expressed in the hypodermis and vulva muscles, suggesting that this gene acts in tissues directly exposed to the external environment. Characterization of the other mutations identified in this study will facilitate the elucidation of the molecular mechanism for the anesthetic action of ethanol.

Keywords: *C. elegans*; Ethanol; Genetics; Judang (*jud*) mutations; Transposon

Although ethanol is a chemically simple molecule, it exerts profound effects on living organisms. In multicellular organisms, ethanol causes hypersensitivity, uncoordination, and immobility when acutely delivered, and causes dependence, tolerance, adaptation, and craving when delivered chronically. The complex genetics of ethanol metabolism has been studied using various genetic animal models such as rodent, fly, and nematode. Studies using rodent models showed that specific subunits of the $GABA_A$ receptor, the 5-hydroxytryptamine $(5\text{-HT})_{1B}$ receptor, the protein kinase Cγ (PKCγ) isoform, TGF-α, dopamine receptors, and m-neu1, a mouse homolog of the Drosophila neuralized gene, have roles in ethanol action [1–10]. Rodent models, however, are limited by the lack of a comprehensive genetic analysis in terms of physiological context in vivo. Genetics using the fly model revealed that *cheapdate* (*chpd*), encoding a neuropeptide that activates the cAMP pathway, was important in ethanol sensitivity regulation in *Drosophila* [11].

The nematode *Caenorhabditis elegans* is another model organism used to study the genetics of ethanol action. Acute exposure to ethanol causes hyperactivity followed by uncoordination at low concentrations (exogenous dose of 100–500 mM ethanol) and paralysis at high concentration (exogenous dose of approximately 1.2 M ethanol) in *C. elegans*. Several mutations involved in ethanol sensitivity have been identified [12]. For example, *unc-79* encodes a novel protein that confers resistance to ethanol. *slo-1* was identified by isolating mutations that conferred ethanol resistance in terms of uncoordinated movement; s*lo-1* encodes a BK potassium channel protein that may be involved in the inhibition of neural activities [13,14]. Because at a high concentration of ethanol nematodes become paralyzed within 10 min and still reversibly recover

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even after a 6 h exposure [15], this complete paralysis caused by ethanol may be the result of the anesthetic action of ethanol. In this study, we wanted to identify new mutations that confer resistance to the action of high concentrations of ethanol in *C. elegans* in the hope that such mutations will allow the identification of specific molecules affecting the site of the anesthetic action of ethanol. Forward genetics can identify genes without bias; we therefore used three mutagenesis protocols available for *C. elegans*, ethylmethanesulfonate (EMS), psoralen/UV, and transposon mutagenesis, to isolate mutations that conferred resistance to the anesthetic action of ethanol.

Materials and methods

Strains and culture. Most *C. elegans* strains were obtained from the *Caenorhabditis* Genome Center (CGC). Basic techniques of genetics and worm culture were by standard methods at 20 °C, as previously described [16]. The following strains and alleles were used in this work. *C. elegans* Bristol strain N2 was used as wild-type. Mutant strains used for genetic mapping in this study are as follows: LGI, *unc-101*(*sy108*), *dpy-5*(*e61*); LGII, d*py-10*(*e128*)*unc-4*(*e120*), *sqt-2*(*sc31*)*lin-31*(*n301*), *lin-31*(*n301*)*clr-1*(*e1745*)*dpy-10*(*e128*), maDf4/*dpy-10*(*e128*)*unc-104*(*e1265*), ccDf1/*dpy-25*(*e817*), ccDf2/*dpy-25*(*e817*), ccDf5/*dpy25*(*e817*); LGIII, *dpy-18*(*e364*); LGIV, *dpy-20*(*e1282*); LGV, *dpy-11*(*e224*); LGX, *unc-1*(*e538*)*dpy-3*(*e27*), *lon-2*(*e628*)*unc-84*(*e1410*), *mnDP1*/+V;*mnDf4*, *mnDP*1/+V;*mnDf19*, *mnDP1*/+V;*mnDf20*; RW7000; and RW7097 [*mut-6*(*st702*) *unc-22*(*st192st527*)IV].

Ethanol sensitivity assay. Exposure of *C. elegans* to ethanol was carried out by immersing worms in ethanol solutions of defined concentrations. Worms were washed off agar plates with known concentrations of ethanol in M9 buffer and placed in clear 55 mm petri dishes. Moving worms were scored in time courses. Another way was that about seventy worms were placed in clear wells containing known concentrations of ethanol and scored for motility in time courses until all individuals ceased moving.

Mutagenesis. Ethanol resistant mutants were isolated in screens for mobile worms in 7% (v/v) ethanol after mutagenesis of N2 with ethylmethanesulfonate (EMS) or trimethylpsoralen/UV or a mutator strain [16–18]. For the EMS mutagenesis, L4 animals were mutagenized for 4 h in 0.1 M EMS. About 20 nematodes were transferred to 90 mm NGM plates and allowed to reproduce by self-fertilization for two generations to obtain worms homozygous for recessive mutations. Worms were then tested for ethanol sensitivity. For the psoralen/UV mutagenesis, L4 animals were treated for 15 min in 3 μg/ml of trimethylpsoralen in the foil-wrapped tubes, and then poured into 90 mm plates. Worms were irradiated with UV (360 nm, 340 μW/cm^2) for 1 min and incubated at 20 °C for 5 h in the dark. Then the progeny (F2–F3 generation) were tested for their ethanol sensitivity. For the transposon insertion mutagenesis, we used the mutator strain RW7097. RW7097 contains 50–70 copies of Tc1 transposon and a mutator mutation, *mut-6* IV. Mutators can mobilize transposable elements in the genome to generate a form of rearrangement. Tc1 is a transposable element of 1610 bp with 54 bp terminal inverted repeats in *C. elegans*. *mut-6* is effective at activating Tc1 motility [19]. One worm was placed on each of 90 mm plate at 20 °C and the progeny (F2 and F3 generation) were tested for their ethanol sensitivity. After treatment with 7% (v/v) ethanol for 10 min, moving worms were picked and transferred to NGM plates. After growth, worms were retested in ethanol solution. If the offspring still showed resistance to ethanol, the strains were mated to the wild-type strain (N2), and mutant animals were collected from the second generation (F2) offspring. This backcrossing was repeated for more than three rounds of genetic backcrosses for each new mutation.

Gene mapping and cloning. Genetic mapping of mutants isolated from chemical and radiation mutagenesis was done by standard techniques previously described [16]. For the identification of Tc1-tagged genes responsible for ethanol sensitivity mutants from transposon insertion mutagenesis, we followed procedures previously described [20]. Tc1 insertion mutants were backcrossed with the low Tc1 copy number N2 strain more than 10 times. Genomic DNA was isolated from backcrossed mutants, N2, and RW7097 and used for Southern blot analysis. Southern blot analysis was performed with Tc1 probe and various Tc1 non-cutting six-cutter restriction enzymes. In the case of *jud-4*, a single Tc1-containing NarI (Roche) fragment of 3.3 kb, which co-segregated with the mutant phenotype, was detected. NarI-digested genomic DNA of each strain was self-ligated and used as a PCR template. A size-selected PCR product (1.7 kb) from inverse PCR using Tc1 external primer pairs (5′-CAT TTC GCT TTA TGC ACA CGG-3′ and 5′-GTT GAA CAT TTT TGA ATA TTG TG-3′) was subcloned into the pGEM-T easy vector (Promega, A1360) and sequences of DNA fragments flanking the Tc1 element were used to determine the Tc1 insertion site in the *C. elegans* genome. The corresponding ORF was found from the *C. elegans* genome database.

Cloning of GFP-fused constructs. The green fluorescent protein (GFP) gene was fused to the C02D4.1 ORF for observing their expression pattern. The sequences of the PCR primer sets were as follows: C02-1 (5′-GGC CTG CAG TTG AAA TCA ATC G-3′), C02-2 (5′-GGG ATC CAA TAC ATA TTG TGT TTA AAC-3′), C02-6 (5′-GGG CAT GCG CAT GTGTAA AGG TTG-3′), C02-7 (5′-CCC CTG CAG ACA TCT GAG ACA ACA C-3′), and C02-8 (5′-CCC CTG CAG ATG TCT GGC GTG TG-3′).

Microinjection and microscopy. Microinjection of DNA into the gonads of adult hermaphrodites was carried out according to standard procedures [21]. The pRF4 plasmid, which contains the dominant *rol-6* (*su1006*) gene [22], was used as an injection marker. The expression patterns of the transgenes were observed using an Axioplan2 microscope (Zeiss). Images were taken by an AxioCam (Zeiss) camera.

Results and discussion

Isolation of ethanol resistant mutants

We previously reported on the effects of ethanol on *C. elegans* behavior at high concentrations [23]. Briefly, ethanol exerts a reversible paralytic effect on *C. elegans*. Using the failure to respond to touch as an endpoint phenotype, we established the experimental system to identify genes crucial in controlling the sensitivity of *C. elegans* to ethanol. Based on the observation that 7% (v/v) was the highest concentration that can paralyze the worms for a prolonged exposure without any lethal effect, we decided to use 7% (v/v) ethanol for our genetic experiments. We have isolated new mutations that showed changed ethanol sensitivity by three mutagenesis methods. From screening 60,000 haploid genomes that had been exposed to EMS for resistance to ethanol, we found seven ethanol sensitivity mutants. In addition, one mutant from 15,000 haploid genomes exposed to psoralen/UV radiation and one mutant from approximately 50,000 haploid genomes of the mutator strain RW7097 was identified (Table 1). These ethanol resistant mutants were named '*jud*', the abbreviation of 'JUDANG' (a Korean word meaning "being tolerant to alcohol").

Phenotypes of ethanol resistance mutants

Nine *jud* mutant alleles were isolated from this study: *ys9*, *ys10*, *ys11*, *ys15*, *ys16*, *ys17*, and *ys20* from EMS mutagenesis, *ys19* from psoralen/UV mutagenesis, and *ys18*

Table 1
Mapping of the *jud* mutations

Gene or allele name	Mutagenesis method	Recombination mapping	Genetic location or CDS
jud-1(ys9), jud-1(ys10)	EMS	*ys9*(14)*clr-1*(24)*dpy-10*	II: −2.02
jud-2(ys11)	EMS	*unc-1*(34)*ys11*(15)*dpy-3*	X: −17.5
jud-3(ys19)	Psoralen/UV	*dpy-10*(17)*ys19*(74)*unc-4*	II: 0.32
jud-4(ys18)	Transposon insertion	N/P[a]	X C02D4.1
ys15	EMS	N/P	II
ys16	EMS	N/P	X
ys17	EMS	N/P	III
ys20	EMS	N/P	V

[a] N/P, not performed.

from transposon insertion mutagenesis. These *jud* mutants showed decreased sensitivity to ethanol to different degrees (Fig. 1, data not shown). All mutants were normally shaped on observation using a dissection microscope and moved normally in the absence of ethanol. These observations indicate that they have functional neuromuscular systems. Consistent with this, *ys9* animals were as sensitive to aldicarb as wild-type animals (data not shown). Aldicarb is an inhibitor of acetylcholine esterase, which can make animals hypercontract, and aldicarb resistance is a typical phenotype of presynaptic defects. Interestingly, most *jud* mutants did not survive after freezing and thawing for storage, even after extensive outcross with wild-type animals. This tight linkage between ethanol resistance and failure to survive freezing indicates that ethanol sensitivity is likely to be related to membrane properties such as fluidity. It would be very interesting to see what proteins the *jud* genes encode.

Genetic mapping of ethanol resistance mutants

As a first step toward molecular and genetic analysis of *jud* genes, linkage group mapping and recombination frequency test of *jud* mutants were performed using marker strains by standard techniques previously described [16]. We found that *jud-1* (*ys9*) was located at −2.02 map unit (m.u.) on chromosome II between *vab-1* and *clr-1* (Table 1). Analysis of heterozygous animals containing a single copy of *jud-1* (*ys9*) and deficiencies that uncover the region *ccDF2*, *ccDf5,* and *maDf4* showed that these trans-heterozygotes were as resistant to ethanol as *jud-1* (*ys9*) homozygous animals (data not shown), suggesting that *jud-1* (*ys9*) is a severe loss-of-function allele. *jud-2* (*ys11*) was mapped to −17.5 m.u. on chromosome X between *unc-1* and *dpy-3*. *jud-3* (*ys19*) was mapped to +0.32 m.u. on chromosome II between *dpy-10* and *unc-4*. *ys15*, *ys16, ys17*, and *ys20* were mapped to chromosome II, X, III, and V, respectively, but further three factor crosses have not been performed (Table 1). We then examined whether we had isolated the same mutations that had been identified in the previous other screens. Multiple *slo-1* alleles had been isolated from the genetic screen using the intoxication assay [13]. However, we did not isolate any *slo-1* mutations from our screen. This is not surprising because *slo-1* mutant animals were not resistant to 7% ethanol in terms of paralysis (unpublished observation), although they are resistant to ethanol at a lower concentration. Therefore, we have identified genes distinct from *slo-1* that are responsible for the anesthetic action of ethanol.

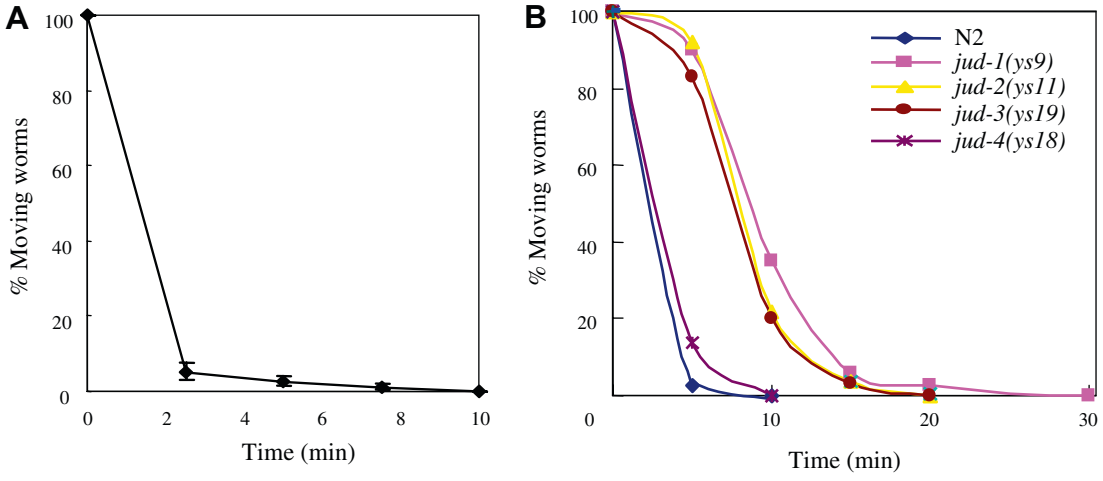

Fig. 1. Ethanol sensitivity of *jud* mutants. (A) A time course response of *C. elegans* to 7% ethanol. Within 1 min, worms start to cease movement. After 10 min, no moving animal can be seen. (B) The graph shows the time course sensitivity assay for ethanol action in the isolated mutants. The *x*-axis is the time after exposure to ethanol, and the *y*-axis, percentage of worms that were still mobile at the time.

Genetic interactions between ethanol resistance mutations

Because three of the genes, *jud-1*, *jud-2*, and *jud-3*, had been accurately mapped, we focused our genetic analysis on these genes. We constructed the strains containing heterozygotes of two mutations for complementation tests (Fig. 2). Trans-heterozygotes of *jud-1* (*ys9*) and *jud-1* (*ys10*), two alleles mapped on the same chromosome, exhibited the same degrees of ethanol resistance compared with *ys9* and *ys10* homozygote mutants, therefore *ys9* and *ys10* are alleles of the same gene (Fig. 2A). Trans-heterozygotes of *jud-1* (*ys9*) and *jud-2* (*ys11*) showed a lesser degree of ethanol resistance than *ys9* and *ys11* homozygotes (Fig. 2B). Thus, *jud-1* (*ys9*) and *jud-2* (*ys11*) are different genes, consistent with the linkage mapping data. This is similar to the case of *jud-2* (*ys11*) and *jud-3* (*ys19*) (Fig. 2D). Interestingly, trans-heterozygotes of *jud-1* (*ys9*) and *jud-3* (*ys19*) showed more resistance to ethanol than each homozygote (Fig. 2C). *ys10* and *ys19* showed the same result as *ys9* and *ys19* (data not shown). We conclude from these data that *jud-1* and *jud-3* mutations are synergistic in conferring ethanol resistance. The fact that even in the presence of a single copy of wild-type *jud-1* and *jud-3* genes the loss of single copies of *jud-1* and *jud-3* caused a resistance phenotype suggests that the doses of *jud-1* and *jud-3* genes are collectively counted as a single parameter for ethanol resistance *in vivo* and that these two genes may encode proteins that have similar biochemical properties, such as similar enzyme activities.

The epistasis analysis of the mutations may clarify their position in a genetic pathway controlling this sensitivity. However, all *jud* mutants exhibited normal shape and behaviors without ethanol, making it difficult to construct double mutants containing two *jud* mutations. We could

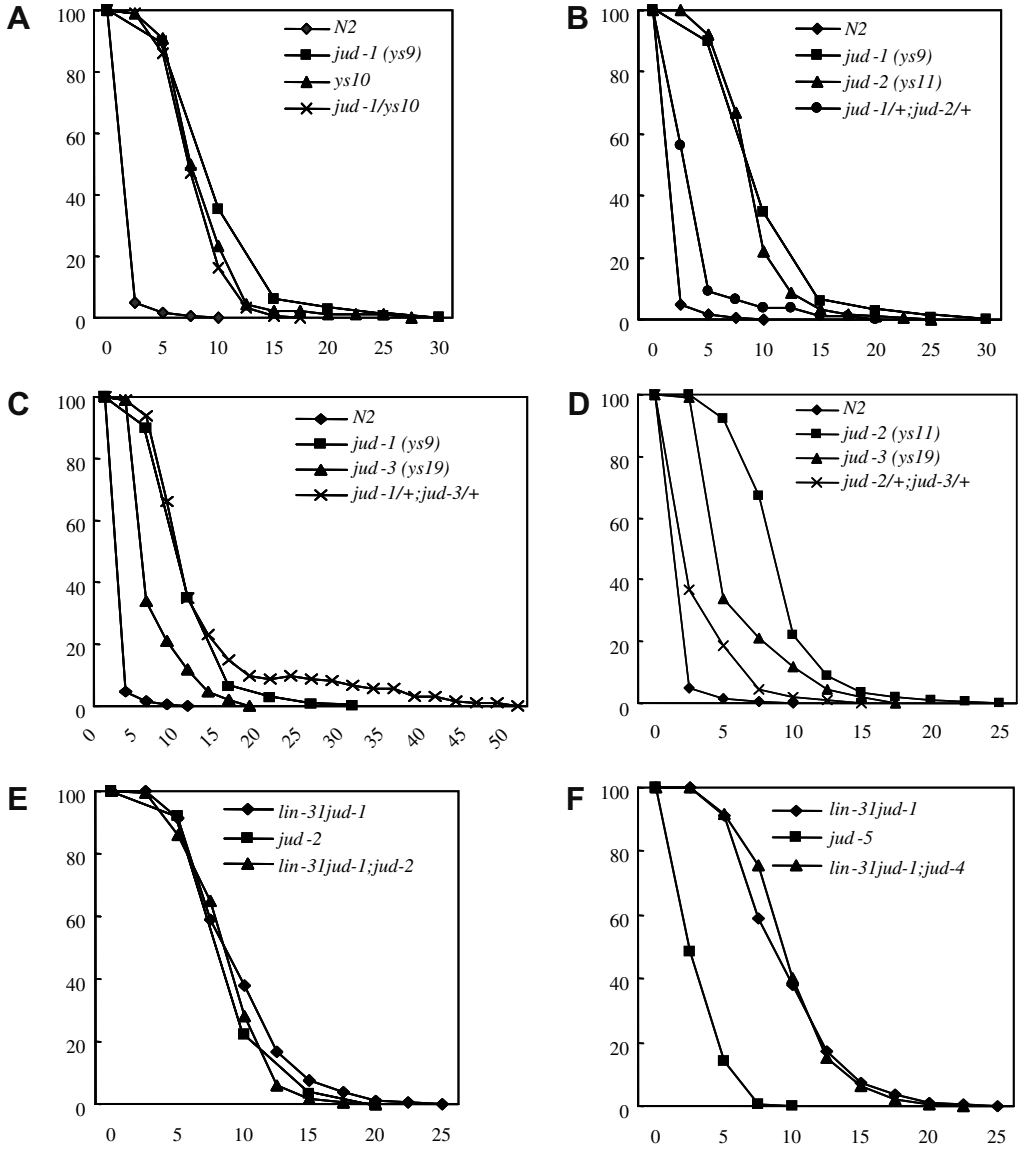

Fig. 2. Genetic interaction between *jud* mutations. (A–D) Complementation test of *jud-1*, *jud-2*, and *jud-6*. Trans-heterozygotes for two of the mutations were analyzed for their sensitivity to ethanol exposure. (A) *ys9* and *ys10* are allelic. (B) *jud-1*/+, *jud-2*/+ trans-heterozygotes; (C) *jud-1*/+, *jud-3*/+ trans-heterozygotes; (D) *jud-2*/+; *jud-3*/+ trans-heterozygotes. (E and F) Double mutant analysis between *jud-1*, *jud-2*, and *jud-4*.

not make double mutants of the genotype *jud-1* (*ys9*) *jud-3* (*ys19*) because these two genes are on the same chromosome. Therefore, we only tested the double mutants that we managed to construct. We constructed the double mutants of *jud-1*; *jud-2* and *jud-1*; *jud-4*. *jud-1* (*ys9*) is one of the most resistant mutants, and *jud-4* (*ys18*) has a

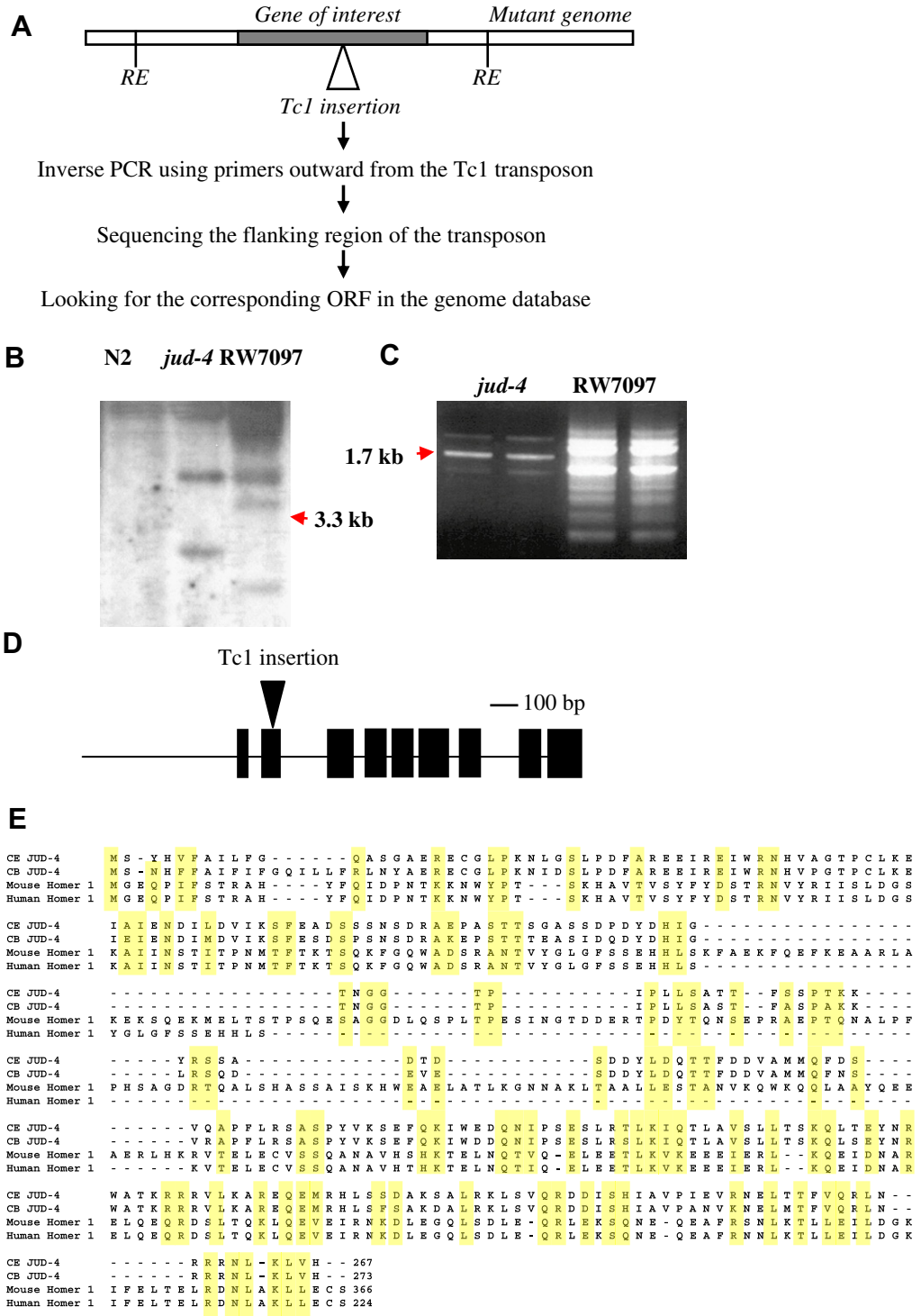

Fig. 3. *jud-4* encodes a novel protein with a limited homology with Homer proteins. (A) An experimental scheme for positional cloning of *jud-4* by transposon tagging. RE indicates a restriction enzyme site. (B) Southern blot analysis of genomic DNAs from *jud-4* and RW7097 strains digested with NarI. The probe was made from Tc1 sequences. A 3.3 kb fragment (the arrow) presents a unique insertion in *jud-4* mutant animals. (C) The result of the inverse PCR with Tc1 external primer pairs. The 1.7 kb fragment was excised and sequenced to determine the insertion site on the genome. (D) The genomic structure of the *jud-4* gene. The insertion site of the *jud-4* mutation is indicated. (E) The amino acid sequence alignment of JUD-4 with its homologs from various species.

weaker phenotype compared with *jud-1*. *jud-2* shows the intermediate degree of ethanol resistance. Double mutants of the genotype *jud-1*; *jud-2* showed ethanol resistance similar to that of *jud-1* (Fig. 2E) and double mutants of *jud-1*; *jud-4* also showed ethanol resistance similar to *jud-1* (Fig. 2F), indicating that these genes may act in the same pathway, not in separate parallel pathways, and that *jud-1* is the most downstream gene in this pathway.

jud-4, defined by a transposon insertion mutation, encodes a novel protein with a limited homology to Homer proteins

jud-4 is one of the ethanol resistance mutants generated from the mutator strain RW7097. Positional mapping of *jud-4* was performed, and the Tc1 insertion that caused resistance to ethanol of *jud-4* was located within the coding region of C02D4.1 on chromosome X (Fig. 3). Complementation tests with strains containing deficiency at the corresponding regions revealed that this region contained the *jud-4* loss-of-function mutation (data not shown). *jud-4* encodes a predicted novel protein that shows a limited homology to the mammalian Homer proteins (Fig. 3E). The C terminal region of the proteins is known to be involved in the oligomerization of the proteins, suggesting that JUD-4 may act as oligomers. The RNA*i* phenotype of *jud-4* is reported to be wild-type [24], suggesting that this gene may not be essential for survival under normal conditions. To examine the in vivo expression pattern of *jud-4*, we constructed GFP fusion constructs of C02D4.1 full length ORF using its own promoter with and without the Tc1 insertion, and generated transgenic animals. In the transgenic line containing the wild-type *jud-4* promoter, but not in the lines with the Tc1 insertion promoter, GFP was expressed in the vulva muscles and the hypodermis in the

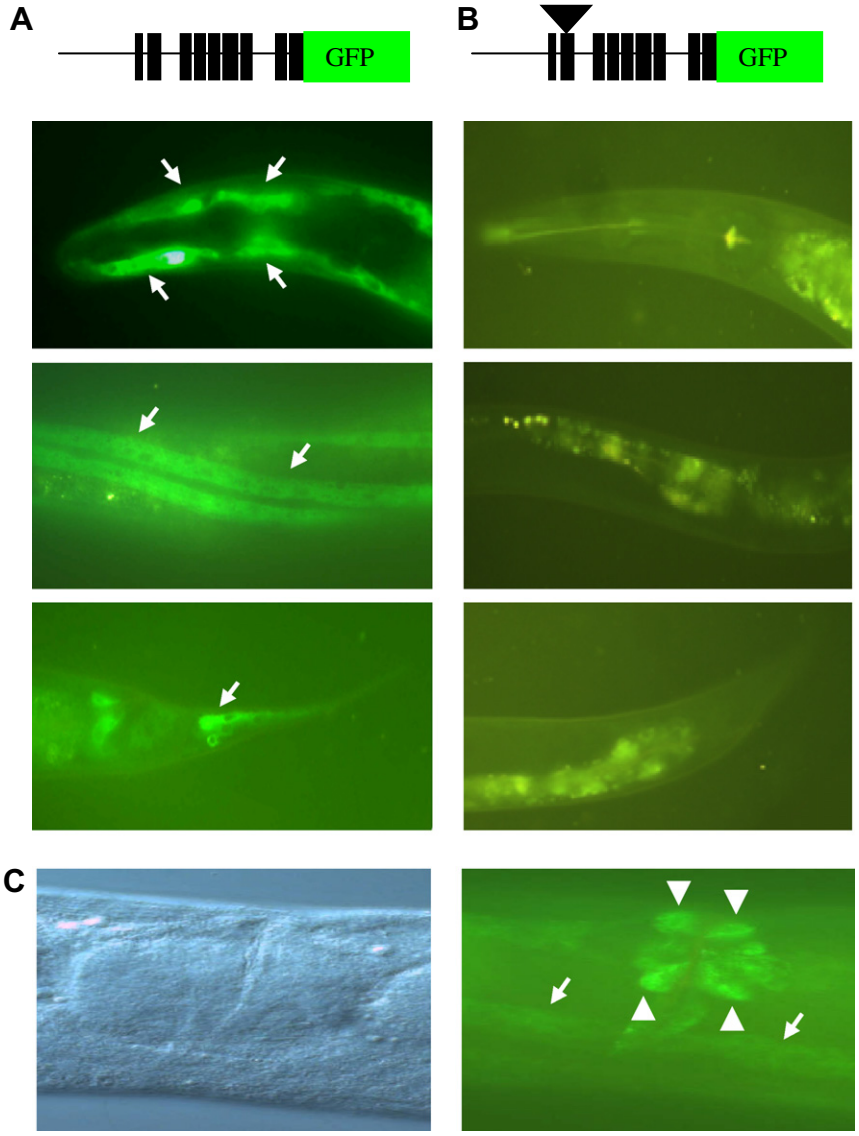

Fig. 4. *jud-4* is expressed in hypodermis and vulva muscles. (A) GFP reporter expression in the hypodermis for the wild-type *jud-4* GFP construct. (B) No GFP expression for the Tc1 insertion *jud-4* GFP construct. (C) *jud-4* is also expressed in the vulva muscles. The arrowheads are the vulva muscles expressing *jud-4*. The left panel is the Nomarski image of the right panel. The arrows indicate the hypodermal cells expressing *jud-4*.

entire body at most stages from L1 to adult (Fig. 4, and data not shown). Hypodermis is one of the first tissues able to interact with ethanol and JUD-4 can work for the ethanol sensitivity of nematode in this tissue. Since Homer proteins are known to be important in glutamate receptor functions and cocaine addiction [25], it is possible that JUD-4 may be functionally related to Homer proteins. It is also intriguing that the *Drosophila* Homer is involved in ethanol sensitivity [26]. It would be interesting to examine whether the mammalian homolog can rescue the phenotypes caused by the mutations in the nematode. Homer proteins may act in the neurons, but that JUD-4 is not expressed in the neurons in the nematode, therefore, we can not rule out the possibility that JUD-4 may have yet-to-be identified functions other than those of Homer proteins.

In summary, we performed unbiased genetic analysis to identify genes that play an important role in the anesthetic pathway initiated by ethanol, either as direct targets of ethanol or as intermediate effectors, and identified nine independent mutations, defined the genetic map positions of three genes, and cloned one of the mutations by transposon tagging. Further analysis of the genes identified in this study will facilitate the elucidation of the molecular mechanism underlying the anesthetic action of ethanol and possibly other general anesthetics.

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