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Genetic Analysis of Halothane Sensitivity in *Caenorhabditis elegans*

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The nematode *Caenorhabditis elegans* appears to be a useful model for studying the action of volatile anesthetics. A mutant strain that is hypersensitive to the widely used anesthetic halothane was described earlier. The mutation is now shown to be an allele of *unc-79*. Other alleles of *unc-79* are also associated with hypersensitivity to halothane. A strain with a mutation in a second gene, *unc-80*, is also hypersensitive to halothane. Nematodes bearing mutations in both *unc-79* and *unc-80* are slightly more sensitive to halothane than those bearing only one of these mutations. Mutations in a third gene, *unc-9*, suppress both *unc-79* and *unc-80*. Nematodes bearing the suppressor mutations alone have normal sensitivity to halothane. These results show that sensitivity to halothane can be altered by mutations in several different genes.

NO ONE KNOWS HOW THE VOLATILE anesthetics routinely used in operating rooms produce unconsciousness. Study of the mechanism of anesthetic action requires an appropriate yet tractable model system. One method currently used is to measure various biochemical and biophysical properties of anesthetics in vitro (*1*), but the relation of these measurements to each other and to consciousness is not clear. We have chosen instead to use the nematode *Caenorhabditis elegans* as a model for studying anesthesia. The anatomy of *C. elegans* can be described at the level of individual cells; the cell lineages are completely known and are largely invariant among individuals (*2*). There are 302 neurons in the adult hermaphrodite, organized in dorsal and ventral nerve cords, a nerve ring, and several ganglia. A complete wiring diagram of the synaptic connections has been compiled by reconstructions from serial electron micrographs (*3*). Furthermore, *C. elegans* has been extensively analyzed genetically, and a number of mutations are known that derange the structure and the function of the nervous system (*4*). Most important for our work, the response of *C. elegans* to volatile anesthetics is similar to that of mammals (*5*).

As the dose of an anesthetic gas increases, wild-type worms first become hyperactive, then become progressively more uncoordinated until they stop moving altogether and assume a straight posture. At this point, they no longer withdraw when tapped on the snout. All effects are reversed within minutes after removal from anesthetic. The potency of various anesthetics in producing this response in *C. elegans* parallels their potency in mammals. In addition, a mutant with an abnormal response to anesthetics has been isolated; this mutant is resistant to some anesthetics but is hypersensitive to the most lipid-soluble anesthetics, including halothane. The mutation associated with hypersensitivity to halothane previously was called HS1 (*5*); we have renamed this mutation *ec1* in accordance with standard nomenclature (*6*).

Worms were grown and handled by standard procedures, and the anesthetic response was assayed as described in detail (*5*). Briefly, worms are grown on petri plates filled with an agar-based growth medium. Plates of worms to be tested were placed in a glass dish, and a flat glass cover was clamped to the dish to create an airtight chamber. A volume of halothane, estimated to give an appropriate concentration based on the volume of the chamber, was injected into the sealed chamber by means of a glass syringe and a three-way stopcock. The worms were observed through the lid of the chamber with a dissecting microscope; they were judged to be anesthetized when they stopped moving and assumed a straight posture, as described (*5*). After 2 hours, a sample of the gas in the chamber was removed and its exact concentration was determined with a gas chromatograph.

Dose-response curves for the wild-type strain N2 and the strain bearing the mutation *ec1* are shown in Fig. 1. Our results are similar to those of Morgan and Cascorbi (*5*) for both N2 and the strain bearing the *ec1* mutation. We show below that *ec1* is an allele of the gene *unc-79*, and it is labeled as such in the figures. The effective dose at which 50% of the worms are immobile (the ED_{50}) is 1.1 ± 0.05% (SEM) for *ec1* and 3.2 ± 0.06% for N2. Virtually all animals with the *ec1* mutation are immobile in 2% halothane, but no wild-type animals are. The 2% dose could be used when scoring both halothane-hypersensitive and normal populations. Using halothane hypersensitivity as a marker phenotype, we mapped *ec1* to a region on linkage group III (*7*) and tested for allelism with several previously identified genes in that region (*8*); *ec1* did not complement *unc-79 (e1068)* for either halothane hypersensitivity or uncoordinated movement. In order to be sure that the altered anesthetic response was due to the defect in *unc-79*, we tested two other *unc-79* alleles, *e1068* and *e1291*, for hypersensitivity to halothane. All three *unc-79* alleles are associated with hypersensitivity to halothane, and animals with these genes do not differ among themselves to a statistically significant extent in their response to halothane. This result indicates that halothane hypersensitivity is a characteristic of *unc-79* mutants and not some special property of *ec1*.

Wild-type worms move almost constantly in the absence of anesthetic (*5*). Worms with

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mutations in *unc-79* were first identified by distinctive uncoordinated movements; well-fed worms move forward and backward normally but pause for long periods (*9*), resuming movement when prodded (unlike anesthetized worms). This phenotype has been nicknamed "fainter." A mutation in a second gene is associated with a similar, but less severe, fainter phenotype (*9*). We obtained worms with this mutation, *unc-80 (e1272)*, and tested their response to halothane. As shown in Fig. 1, *unc-80* mutants are also hypersensitive to halothane, with an ED_{50} of 1.28 ± 0.05%, a value higher than but close to that for *unc-79 (ec1)* mutants. The uncoordinated phenotype of *unc-80* had proved difficult to map genetically (*8, 9*), but the anesthetic response could be mapped readily (*10*) in 2% halothane to a region of linkage group V. Thus, mutations in *unc-79* and *unc-80* define two separate, unlinked genes associated with nearly identical responses to halothane.

In order to test the interaction between *unc-79* and *unc-80*, we constructed a double mutant strain *unc-79;unc-80* and assayed these mutants for halothane sensitivity. If the two genes affect different processes, both of which contribute to the response to halothane, the double-mutant strain may be even more hypersensitive to halothane than mutants bearing *unc-79* or *unc-80* alone. Indeed, the double-mutant strain is somewhat more sensitive to halothane than either of the single-mutant strains (Fig. 2). The ED_{50} for *unc-79;unc-80* is 0.8 ± 0.03%, slightly but significantly less than that of either *unc-79* or *unc-80* ($P < 0.01$). This result suggests that *unc-79* and *unc-80* may affect different processes, each contributing to halothane hypersensitivity. Although *unc-79* and *unc-80* mutants resemble each other in overall movement and anesthetic responses, there are subtle differences between these mutant strains; *unc-80* mutants tend to be less sluggish, are more potent in mating, and lay unusually small eggs. In these properties, the *unc-79;unc-80* double mutant resembles the *unc-79* mutant.

One method of identifying additional genes that affect anesthetic response is to identify suppressors of one or both known genes. We looked for such suppressors by treating *unc-79 (ec1)* hermaphrodites with the mutagen ethyl methanesulfonate (EMS) (*11*). A suppressor of *unc-79* was found by looking among the F_2 progeny for worms mobile in 2% halothane. The suppressor was named *ec27* after outcrossing. A worm with the *ec27* mutation is itself uncoordinated, although quite different from those bearing *unc-79* and *unc-80*. The uncoordinated phenotype was used to map *ec27* to the X chromosome and to show that it is allelic to *unc-9 (e101)* (*12*). Strains with the suppressor mutation alone have normal halothane sensitivity. However, the halothane hypersensitivity associated with *unc-79* or *unc-80* is suppressed (Table 1). Since *ec27* and *e101* differ in several ways [for example, *e101* is male-sterile (*13*) and *ec27* is male-fertile], we tested the ability of *unc-9 (e101)* to suppress *unc-79* and *unc-80* as well. Both *unc-79* and *unc-80* are effectively suppressed by *unc-9 (e101)* (Table 1). In fact, *e101* suppresses *unc-80* somewhat better than *ec27* does. Recall that neither *unc-9* allele was originally identified as a suppressor of *unc-80*.

The ability of *unc-9 (ec27)* to suppress *unc-79* and *unc-80* simultaneously was tested by constructing the triple mutant strain *unc-79 (ec1);unc-80;unc-9*. This strain behaves like the *unc-9* strain and is not hypersensitive to halothane. We have also shown that two other uncoordinated mutations, *unc-2 (e55)* and *unc-42 (e270)*, do not suppress either *unc-79* or *unc-80* and that *unc-36 (e251)* does not suppress *unc-79*. The suppression by *unc-9* is thus not a general property of all uncoordinated mutations.

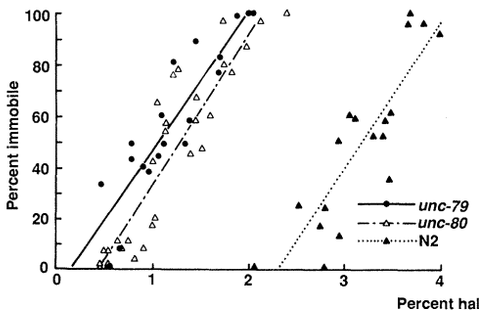
Finally, each of the mutations was tested for dominance. None shows a clear-cut dominance in uncoordinated movement, and heterozygotes for *unc-79* and *unc-80* have a response to halothane identical to that of the wild-type; the ED_{50} for the *unc-79*/+ is 3.37 and the ED_{50} for *unc-80*/+ is 3.40. The suppression of these mutants by *unc-9* is also recessive; that is, *unc-79;unc-9 (ec27)*/+ and *unc-80;unc-9 (ec27)*/+ are identical to *unc-79* and *unc-80*, respectively, in worms grown under similar conditions.

We conclude that single gene mutations can have significant effects on the response of *C. elegans* to a commonly used inhalation anesthetic. A mutation in either *unc-79* or *unc-80* increases the sensitivity of the worm to halothane. The hypersensitivity of both is suppressed by mutations in a third gene, *unc-9*. No interaction between *unc-79* or *unc-80* and *unc-9* was suspected on the basis of previous work, and *unc-80* was tested only because of its gross similarity to *unc-79*.

Because nothing is known about the neuroanatomy or molecular defects of the mutant strains with altered halothane response, we cannot infer how the response to halothane is controlled. It is possible that the halothane hypersensitivity of these mutations may be an indirect consequence of a grosser cellular defect such as altered membrane biogenesis. We also cannot predict how many other genes affect the worm's response to one or more anesthetics. Both *unc-80* and *unc-79* are associated with hypersensitivity to halothane but not to all volatile anesthetics (*14*). We have not yet used any of the other anesthetics to find other hypersensitive mutants, nor have we found resist-

Table 1. The ED_{50} of hypersensitive mutant and suppressor strains. The two alleles of the suppressor gene *unc-9* are *ec27* and *e101*. Each was tested with *unc-79 (ec1)* and *unc-80 (e1272)*. The wild-type strain N2 is included for comparison. The ED_{50} is expressed as mean ± SEM for the volume percent of halothane.

Gene (allele)	ED_{50}
N2	3.2 ± 0.06
unc-79	1.1 ± 0.05
unc-80	1.28 ± 0.04
unc-9 (ec27)	3.1 ± 0.11
unc-79;unc-9 (ec27)	3.3 ± 0.07
unc-79;unc-9 (e101)	3.3 ± 0.06
unc-80;unc-9 (ec27)	3.0 ± 0.06
unc-80;unc-9 (e101)	3.4 ± 0.06



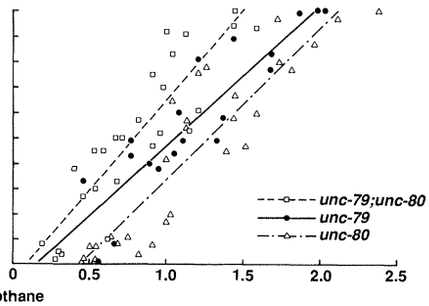


Fig. 1 (left). Dose-response curves of wild-type N2 and mutant *unc-80* and *unc-79 (ec1) C. elegans* in halothane. Synchronous cultures of young adult hermaphrodites were exposed to different concentrations of halothane for 2 hours. Worms immobile for 10 seconds were scored as anesthetized. Halothane concentration was determined by gas chromatography. **Fig. 2 (right).** The double mutant *unc-79;unc-80* compared to *unc-79* and *unc-80*. Dose-response curves were obtained as described for Fig. 1. The *unc-79;unc-80* strain was constructed by mating *unc-79 (ec1)* males to *dpy-18 (e364);unc-80* hermaphrodites, and picking the non-Dpy non-Unc F_1 hermaphrodite progeny. These were allowed to self-fertilize, and halothane-hypersensitive non-Dpy F_2 hermaphrodites were picked individually. From plates segregating Dpy F_3 progeny, non-Dpy F_3 hermaphrodites were picked individually and allowed to self-fertilize. From these, strains that gave no Dpy F_4 progeny were initiated. The genotype of these strains was confirmed by complementation tests with *unc-79* and *unc-80* to be sure the strain was *unc-79;unc-80*.

ant mutants. It may be that mutant analysis will show that there is more than one mechanism of anesthetic action in *C. elegans*.

These experiments will help define the genetic control of anesthetic response but will not in themselves determine the mechanism of anesthetic action. To do that, we hope to use the variety of genetic and molecular tools available with *C. elegans*, such as making genetic mosaics (*15*) and cloning the genes (*16*).

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11. A population of worms was soaked in a solution containing 25 μl of EMS in 5 ml of M9 buffer (*5*) for about 4 hours, with occasional agitation; final EMS concentration was 25 m*M*. The worms were then picked individually onto plates where they were allowed to self-fertilize for two generations before scoring.
12. We mapped *ec27* to the X chromosome by showing that mutant F_1 males resulted when wild-type males were mated to *ec27* hermaphrodites. The location of *ec27* on the X was determined by using the duplications *mnDp8, mnDp10*, and *mnDp25* (*8*). A complementation test was done by mating *mnDp25*/+; *unc-9 (e101)*/0 males to *unc-9(ec27) dpy-6* hermaphrodites. The resulting non-Dpy hermaphrodites were uncoordinated.
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Tissue-Specific Expression of Functionally Rearranged λ1 Ig Gene Through a Retrovirus Vector

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To explore the potential use of retrovirus vectors for the transfer of genomic DNA sequences into mammalian cells, recombinant retroviral genomes were constructed that encode a functionally rearranged murine λ1 immunoglobulin gene. Several of these genomes could be transmitted intact to recipient cells by viral infection, although successful transmission depended both on the orientation of the λ1 sequences and on their specific placement within vector sequences. The λ1 gene transduced by viral infection was expressed in a cell lineage–specific manner, albeit at lower levels than endogenous λ1 gene expression in cells from the B-lymphocyte lineage. Vectors yielding integrated proviruses that lacked viral transcriptional enhancer sequences were used to show that neither viral transcription nor the viral transcriptional sequences themselves had any effect on the tissue specificity of λ1 gene expression or the absolute amount of λ1 transcription. Vector transcription did, however, dramatically decrease the amount of λ1 protein that could be detected in transduced cells. These results suggest that retrovirus vectors may be useful reagents not only for the expression of complementary DNA sequences but also for studies of tissue-specific transcription in mammalian cells.

STUDIES OF THE TISSUE-SPECIFIC expression of cloned genes depend on both the means for introducing the desired sequences into appropriate cell types and the placement of those sequences in a chromosomal (or intranuclear) context compatible with the mechanisms that normally regulate gene expression. Although much success has been achieved in the development of methods for introducing genes into different kinds of mammalian cells, little is known about whether particular methods of gene transfer per se can influence the expression of inserted sequences. Since retrovirus vectors represent a versatile gene transfer system for introducing sequences into a wide range of recipient cells, we asked in this study whether retroviral-mediated gene transfer could be used to transfer genomic DNA sequences to cells and whether expression of such sequences within the context of an integrated provirus might differ in a qualitative fashion from the expression of sequences transferred by conventional DNA transfection techniques. A functionally rearranged gene encoding a murine λ1 immunoglobulin light chain was examined in the study presented here. Although endogenous λ1 genes are expressed at extremely high levels in plasma cells or myeloma cell lines, we and others have been unable to obtain the regulated expression of cloned λ1 genes after their transfer to cells by conventional gene transfer methods (*1*). In addition, no evidence for enhancer-like elements in the intron between the Jλ and Cλ gene segments has been found in contrast to those found in the κ and heavy-chain immunoglobulin genes (*1*).

Because previous reports have described difficulties in constructing retrovirus vectors with complete transcriptional units encoded within a provirus (*2*), we initially tested four constructs in which the λ1 sequences were introduced in both orientations into each of two sites in the vector pZIP-NeoSV(X)1 (*3*) (Fig. 1). In each of these constructs the insert was a 4.4-kb rearranged λ1 gene, containing the leader, joined V-J gene segments, and C gene segment with the normal two λ1 introns as well as approximately 250 bp of 5′ untranslated nucleotides and 2000

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