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Fluoxetine-Resistance Genes in *Caenorhabditis elegans* Function in the Intestine and May Act in Drug Transport

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ABSTRACT

Fluoxetine (Prozac) is one of the most widely prescribed pharmaceuticals, yet important aspects of its mechanism of action remain unknown. We previously reported that fluoxetine and related antidepressants induce nose muscle contraction of *C. elegans*. We also reported the identification and initial characterization of mutations in seven *C. elegans* genes that cause defects in this response (Nrf, *n*ose *r*esistant to *f*luoxetine). Here we present genetic evidence that the known *nrf* genes can be divided into two subgroups that confer sensitivity to fluoxetine-induced nose contraction by distinct pathways. Using both tissue-specific promoters and genetic mosaic analysis, we show that a gene from one of these classes, *nrf-6*, functions in the intestine to confer fluoxetine sensitivity. Finally, we molecularly identify *nrf-5*, another gene in the same class. The NRF-5 protein is homologous to a family of secreted lipid-binding proteins with broad ligand specificity. NRF-5 is expressed in the intestine and is likely secreted into the pseudocoelomic fluid, where it could function to transport fluoxetine. One model that explains these findings is that NRF-5 binds fluoxetine and influences its presentation or availability to *in vivo* targets.

UNDERSTANDING the molecular mechanisms of the action of drugs is essential for rational development and delivery of compounds with greater efficacy, greater specificity, and fewer adverse side effects. Genetic screens for mutants with altered responses to drugs constitute an approach to identifying drug mechanisms of action that is particularly promising for identifying novel targets and pathways (Schafer 1999). Genetic screens using *Caenorhabditis elegans* have been successful in identifying drug targets. For example, screens for resistance to the antihelminthic drug levamisole led to the identification of levamisole-sensitive acetylcholine receptors (Lewis *et al.* 1980; Fleming *et al.* 1997) and screens for resistance to another antihelminthic drug, ivermectin, led to the identification of a novel class of ivermectin-sensitive glutamate-gated chloride channels (Dent *et al.* 2000). In addition to identifying direct drug targets, genetic resistance screens can also identify components of signaling pathways that are affected by the drug of interest. For example, the acetylcholinesterase inhibitor aldicarb causes increased synaptic acetylcholine levels and aldicarb-resistant mutants have identified several novel components of synaptic transmission (Nguyen *et al.* 1995; Miller *et al.* 1996, 2000) Finally, such screens can identify genes involved in drug metabolism, transport, or localization, which are key factors in determining *in vivo* drug availability and efficacy.

We isolated mutations affecting seven Nrf genes that cause resistance to nose contraction by the antidepressant fluoxetine (Nrf: *n*ose *r*esistant to *f*luoxetine). Genetic analysis suggested that three of these genes, *nrf-5*, *nrf-6*, and *ndg-4*, function in a common pathway and that mutations in these genes confer their fluoxetine-resistant phenotype by a similar mechanism (Choy and Thomas 1999). Here, we describe further genetic and molecular characterization of the Nrf genes. We construct double mutants between two classes of Nrf mutants and demonstrate that these two classes confer fluoxetine resistance by different pathways. We have examined the site of action of *nrf-6* and found that it is required in the intestine for sensitivity to fluoxetine-induced nose contraction. We have also cloned *nrf-5* and found that it is homologous to a family of mammalian secreted lipid-binding proteins. A *nrf-5::gfp* fusion is expressed in the intestine, suggesting that *nrf-5* is secreted into the pseudocoelomic fluid where it could function in drug transport.

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under accession nos. AY491012–AY491013.

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MATERIALS AND METHODS

Genetics and pharmacology: General culturing and maintenance of strains was as described (Brenner 1974). For *nrf* (Peg: *p*ale *e*ggs)*; nrf* (non-Peg) double mutants, the Peg mutation was followed by the Peg phenotype and the non-Peg mutation was balanced in *trans* with flanking double-mutant

chromosomes (Choy and Thomas 1999). To construct *nrf-2; nrf-3* double mutants, both mutations were balanced in *trans*. All double mutants were confirmed by complementation testing. Nrf assays were performed as previously described (Choy and Thomas 1999).

Mosaic analysis: *nrf-6(sa367); lin-15(n756ts)* mutants were injected with the *nrf-6*-rescuing cosmid C08B11 (5 ng/μl), pbLH98 *lin-15(+)* (60 ng/μl) (Clark *et al.* 1994), and pTJ1286 *sur-5::gfp* (100 ng/μl) (Yochem *et al.* 1998) to create the extrachromosomal array used for mosaic analysis. Animals that had lost the array completely were Peg due to *nrf-6(sa367)* and Muv (multivulva) due to *lin-15(n765ts)*. Mosaic animals that lost the extrachromosomal array in the E lineage (intestine) were picked by their Peg and non-Muv phenotypes. Mosaic animals that lost the array in the AB lineage (hypodermis, including nose hypodermis, neurons, etc.) were picked by their non-Peg and partial Muv phenotypes. Candidate mosaic animals were scored for GFP expression in the nose hypodermis and intestine using a dissecting microscope with a UV lamp attachment and were then scored for the Nrf phenotype after incubation in 1.0 mg/ml fluoxetine for 10 min. The GFP expression pattern was then confirmed using a compound microscope with Nomarski optics and epifluorescence.

Molecular biology: *nrf-5(sa513); lin-15(n756ts)* animals were rescued for both the Nrf and Peg phenotypes by cosmid pools containing F55B12, by F55B12 alone, or by pRC28, a 4.5-kb subclone containing the predicted gene F55B12.5 and 1.8 kb of promoter. Bulk PCR products of F55B12.5 from wild-type and *nrf-5(sa513)* genomic DNA were sequenced using an ABI automated sequencer and the *sa513* mutation was confirmed by sequencing on both strands.

nrf-5 cDNAs yk56b12 and yk32e7 were obtained from the DNA Data Bank of Japan and were sequenced to confirm intron-exon boundaries of the F55B12.5 Genefinder predicted gene. From these sequences, we determined that an earlier prediction was missing a 75-bp intron in predicted exon 8 that was spliced out in both yk32e7 and yk56b12. In addition, we determined that exon 4 is spliced as predicted in both cDNAs. The yk32e7 cDNA is almost full length; it terminates 7 bp downstream of the predicted ATG of F55B12.5. Subsequent to our sequencing efforts, additional overlapping ESTs (yk1134g07, yk1436a01, yk1440f07, etc.) sequenced by the Genome Consortium and documented on WormBase (release WS138) confirmed that the F55B12.5-predicted ATG codon corresponds to the true translation initiation site of *nrf-5*. Both yk32e7 and yk56b12 have 89 nt 3′-UTRs followed by a poly(A) tail.

The *nrf-5::gfp* plasmid pRC34 was created by inserting the GFP coding region from pPD95.67 (A. Fire) in a *Sna*BI site (generated by a silent mutation) at Val548 in the *nrf-5*-rescuing plasmid pRC28 (see above). *nrf-5(sa513); lin-15(n756ts)* animals were injected with pRC34 (25 ng/μl), pbLH98 (60 ng/μl), and pBluescript (Stratagene, La Jolla, CA) (78 ng/μl, carrier), and transgenic lines were analyzed for rescue of *nrf-5* and GFP expression. An intestinal-specific version of the *nrf-6::gfp*-rescuing plasmid pRC17 (Choy and Thomas 1999) was made by deleting a 1-kb *Not*I-*Eco*RI fragment of the promoter region followed by Klenow enzyme filling and religation.

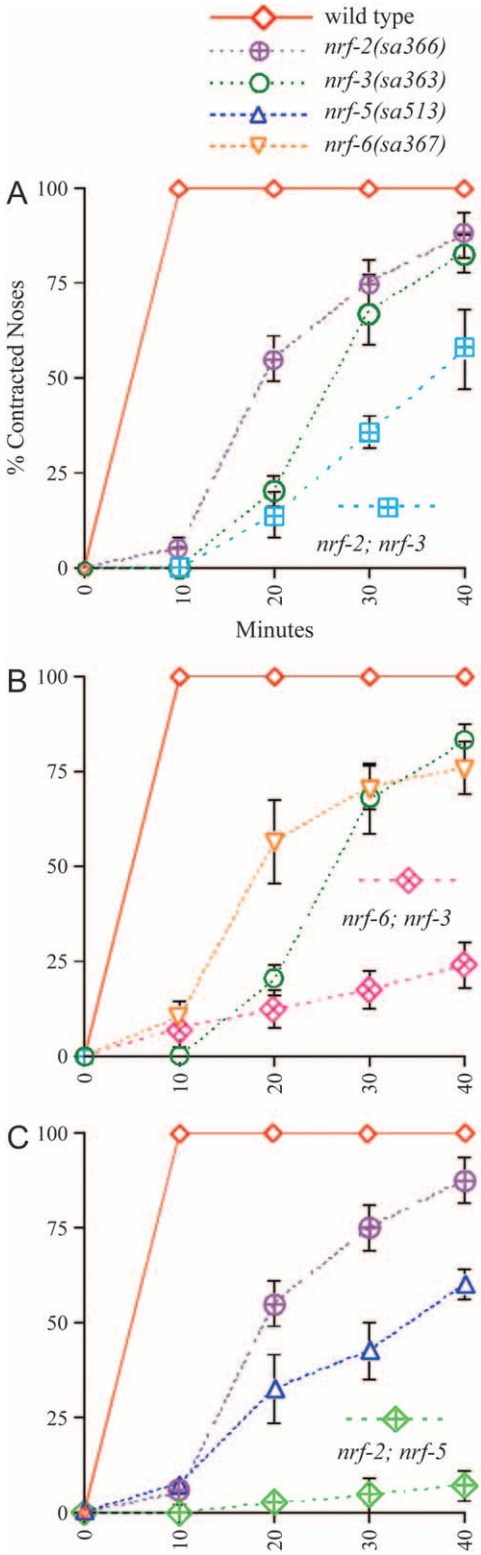
RESULTS

Nrf–Nrf double mutants: We previously found that Nrf mutants are only partially fluoxetine resistant (Choy and Thomas 1999). One explanation for this observation is that they play similar roles in conferring sensitivity to fluoxetine-induced nose contraction, but act in parallel. If this were the case, then Nrf–Nrf double mutants should have increased resistance to fluoxetine-induced nose contraction when compared to the single mutants. Alternatively, the Nrf genes could act in a common signaling pathway. In this case, Nrf–Nrf double mutants should have similar resistance as single null mutants. To distinguish between these hypotheses, we constructed various Nrf–Nrf double mutants and assayed their response to fluoxetine.

We previously found that all pairwise combinations of double mutants between null alleles of the three Nrf Peg genes, *nrf-5*, *nrf-6*, and *ndg-4*, were indistinguishable from the respective single mutants for both the Nrf and the Peg phenotype (Choy and Thomas 1999). These results suggest that *nrf-5*, *nrf-6*, and *ndg-4* function in the same pathway to confer sensitivity to fluoxetine-induced nose contraction and that elimination of the function of any of these three genes is sufficient to eliminate the function of the entire pathway. To test whether the Nrf non-Peg genes *nrf-2* and *nrf-3* act in a common pathway, we constructed *nrf-2; nrf-3* double mutants and assayed their Nrf phenotype. We found that the *nrf-2; nrf-3* double mutant was slightly more resistant to fluoxetine-induced nose contraction than either of the single mutants (Figure 1A). Finally, to determine whether the Nrf Peg genes and the Nrf non-Peg genes act to confer fluoxetine sensitivity by different pathways, we constructed double mutants between these two classes. We constructed all pairwise combinations among Nrf Pegs *nrf-5*, *nrf-6*, and *ndg-4* with the Nrf non-Pegs *nrf-2* and *nrf-3* (with the exception of *nrf-2 nrf-6*, because these genes are closely linked to each other on chromosome II). We found that all these double mutants were much more resistant to fluoxetine than the respective single mutants (Figure 1, B and C; data not shown). This suggests that the Nrf Peg genes and the Nrf non-Peg genes act in different pathways to confer fluoxetine sensitivity. The common secondary phenotypes among these two classes of Nrf mutants (Peg or non-Peg) further supports this interpretation.

***nrf-6* site of action:** *nrf-6* and *ndg-4* mutant animals have multiple phenotypes (Nrf and Peg) and *nrf-6::gfp* and *ndg-4::gfp* fusions are expressed in hypodermis and intestine (Choy and Thomas 1999). Because the mutants were isolated for a phenotype that affects the nose muscles and the *nrf-6* and *ndg-4* hypodermal expression is highest in the hypodermal cells surrounding those muscles (hyp 4 and hyp 5), we hypothesized that expression of these genes in the nose hypodermal cells was required for fluoxetine-induced nose contraction. In contrast, the pale egg phenotype of *nrf-6* and *ndg-4* results from a defect in yolk protein transport. In *C. elegans*, yolk proteins are normally synthesized in the intestine, secreted into the pseudocoelomic space, and then taken up into oocytes prior to fertilization (Kimble and Sharrock 1983; Grant and Hirsh 1999). *nrf-6* and *ndg-4* mutants accumulate yolk globules in the pseudocoelomic fluid, suggesting that yolk transport into

oocytes, rather than yolk synthesis, is defective. However, this observation is also consistent with a defect in some aspect of yolk synthesis or transport from the intestine that decreases efficiency of yolk protein uptake into oocytes. It therefore seemed reasonable that *nrf-6* and *ndg-4* expression in the intestine is required for efficient yolk transport into oocytes.



We used mosaic analysis to determine the site of action of *nrf-6* for each of the two *nrf-6* mutant phenotypes. *C. elegans* extrachromosomal arrays are lost at a low frequency during mitosis and can therefore be used to generate mosaic animals (Stinchcomb *et al.* 1985). We expressed an extrachromosomal array composed of *sur-5::gfp* and a genomic cosmid containing *nrf-6(+)* in a *nrf-6(sa367)* mutant strain. *sur-5::gfp* is expressed in nearly every cell in *C. elegans* and can therefore be used as a cell-autonomous marker (Yochem *et al.* 1998). The intestinal and hypodermal cell types are conveniently separated in the *C. elegans* cell lineage: the nose hypodermal cells, including hyp 4 and hyp 5, are derived from the AB blastomere, whereas the intestinal cells are derived from the E blastomere (Figure 2; Sulston *et al.* 1983).

We identified 10 mosaic animals that had lost the extrachromosomal array in all intestinal cells but were still expressing GFP in most or all of the nose hypodermal cells hyp 4 and hyp 5 (see materials and methods). All 10 of these mosaics were both Nrf and Peg. We also isolated the reciprocal class of mosaic animals that had lost the extrachromosomal array in nose hypodermal cells but not in the intestine. Of 10 of these mosaics, 9 were non-Nrf and non-Peg (Figure 2). Taken together, the data from these two classes of mosaic animals indicate that, contrary to our expectations, *nrf-6* expression is required in the intestine for fluoxetine-induced nose contraction as well as for efficient yolk transport. The significance of *nrf-6* expression in the hypodermis remains unclear. We also performed additional experiments expressing *nrf-6(+)* under either an intestinal-specific truncated *nrf-6* promoter or a hypodermal-specific *dpy-7* promoter (Gilleard *et al.* 1997) in *nrf-6(sa367)* mutants. We found that intestinal-specific expression of *nrf-6(+)* rescued both the Nrf and the Peg defect of *nrf-6* mutants, whereas hypodermal-specific expression did not rescue either of the defects (data not shown). The results of these tissue-specific expression experiments support the conclusions of our mosaic analysis. Although we did not perform mosaic analysis of *ndg-4*, *nrf-6* and *ndg-4* have identical mutant

Figure 1.—Nrf–Nrf double mutants are enhanced for their Nrf phenotype. Time courses of nose contraction for various double mutants and their respective single-mutant controls in 1.0 mg/ml fluoxetine. (A) Double mutant between Nrf non-Peg mutants *nrf-2* and *nrf-3*. The double mutant is slightly more resistant than the respective single mutants. (B) Double mutant between Nrf Peg mutant *nrf-6* and Nrf non-Peg mutant *nrf-3*. The double mutant is clearly more resistant than either of the single mutants. (C) Double mutant between Nrf non-Peg mutant *nrf-2* and Nrf Peg mutant *nrf-5*. The double mutant is again clearly more resistant than the single mutants. Similar results were found with other Nrf Peg–Nrf non-Peg double-mutant combinations as described in the text. Each plot represents the average of at least four trials of 10 animals each. Some error bars are smaller than the plot symbols.

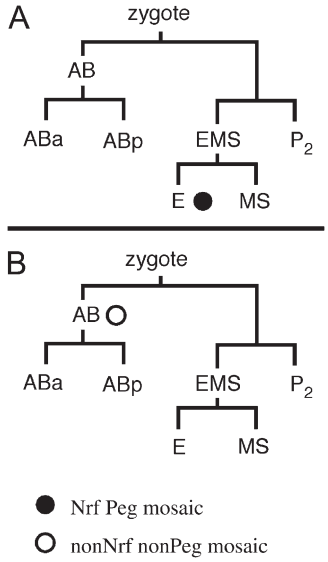


FIGURE 2.—*nrf-6* mosaic analysis. Circles indicate the cell division in which the *nrf-6*(+) extrachromosomal array was lost in the mosaic animals. The solid circle indicates Nrf Peg mosaics; the open circle indicates non-Nrf non-Peg mosaics. (A) Lineage diagram of Nrf Peg mosaics. All intestinal cells (and only intestinal cells) are derived from the E blastomere. Ten mosaics of this class were isolated. (B) Lineage diagram of non-Nrf non-Peg mosaic animals. All nose hypodermal cells including hyp 4 and hyp 5, as well as many other hypodermal and neuronal cells, are derived from the AB blastomere. Ten mosaics of this class were isolated: all were non-Peg and 9 were non-Nrf.

phenotypes and act in the same pathway by genetic criteria. Moreover, *nrf-6* and *ndg-4* encode homologous proteins and have the same expression pattern based on GFP fusions (CHOY and THOMAS 1999). We conclude that *ndg-4* very likely also acts in the intestine to confer sensitivity to fluoxetine-induced nose contraction and to mediate efficient yolk uptake.

***nrf-5* is a member of a family of secreted lipid-binding proteins:** *nrf-5* mutants have the same Nrf and Peg phenotypes as *nrf-6* and *ndg-4*. We had previously shown, on the basis of genetic analysis, that *nrf-5*, *nrf-6*, and *ndg-4* all act in the same pathway (CHOY and THOMAS 1999). Because *nrf-6* and *ndg-4* encoded novel proteins of unknown function, we sought to elucidate the function of the Nrf Peg genes by cloning *nrf-5*. We found that a cosmid subclone containing a single predicted gene (F55B12.5) rescued both the Nrf and the Peg phenotype of *nrf-5* mutants (see MATERIALS AND METHODS). We sequenced F55B12.5 genomic DNA from *nrf-5(sa513)* mutants and found a stop mutation at Ser159 (Figure 3). This early stop mutation is predicted to be a null allele. On the basis of these results, we conclude that *nrf-5* is F55B12.5. We obtained two *nrf-5* EST cDNAs from the *C. elegans* Genome Project and sequenced them to determine the intron-exon boundaries of F55B12.5 (see MATERIALS AND METHODS). Using BLAST searches, we found that NRF-5 is homologous to four mammalian-secreted lipid-binding proteins: bactericidal permeability-increasing (BPI) protein (GRAY *et al.* 1989; BEAMER *et al.* 1997; BEAMER 2003), lipopolysaccharide-binding protein (LBP; FENTON and GOLENBOCK 1998; IOVINE *et al.* 2002; MULERO *et al.* 2002), cholesteryl ester transfer protein (CETP; BRUCE *et al.* 1998a; KAWANO *et al.* 2000), and phospholipid transfer protein (PLTP; HUUSKONEN *et al.* 1999; VAKKILAINEN *et al.* 2002). These proteins have diverse functions, but share the ability to bind various lipids. NRF-5 is also similar to a number of predicted *C. elegans* proteins of unknown function (with corresponding orthologs in the related nematode *C. briggsae*) as well as several to predicted fish proteins (Figure 4). Interestingly, no convincing reciprocal match to *nrf-5* was found in the Drosophila genome.

Expression pattern of *nrf-5*: To determine the expression pattern of *nrf-5*, we inserted GFP (CHALFIE *et al.* 1994) at the C-terminal end of *nrf-5* in the genomic subclone used to rescue *nrf-5* mutants (see MATERIALS AND METHODS). We observed GFP fluorescence throughout the entire intestine of transgenic animals expressing this *nrf-5::gfp* fusion (Figure 5; data not shown). No GFP expression was observed in any other tissue. Furthermore, the *nrf-5::gfp* fusion rescued the Nrf and Peg phenotypes of *nrf-5* mutants (data not shown). We found that *nrf-5::gfp* fluorescence was concentrated at the basal membrane of the intestine (Figure 5). On the basis of its similarity to secreted lipid-binding proteins and the fact that it contains an N-terminal signal sequence (NIELSEN *et al.* 1997), we conclude that NRF-5 is secreted. The concentration of *nrf-5::gfp* fluorescence at the basal membrane of the intestine suggests that NRF-5 protein is secreted from the intestine into the pseudocoelomic fluid. We did not observe GFP fluorescence in the pseudocoelomic fluid itself; however, one possibility is that the level of secreted *nrf-5::gfp* is too low to detect in this manner, particularly against the background of fluorescence from the intestine.

DISCUSSION

Nrf Peg and Nrf non-Peg genes act in distinct pathways: We found that double mutants between Nrf Peg genes (*nrf-5*, *nrf-6*, and *ndg-4*) and Nrf non-Peg genes (*nrf-2* and *nrf-3*) had dramatically increased fluoxetine resistance when compared to respective single mutants. This result suggests that the Nrf Peg genes and the Nrf non-Peg genes act in distinct pathways to confer fluoxetine sensitivity in *C. elegans*. We also found that *nrf-2*; *nrf-3* double mutants had slightly increased fluoxetine resistance when compared to *nrf-2* and *nrf-3* single mutants. The simple interpretation of this result is that *nrf-2* and *nrf-3* also function in different pathways to confer fluoxetine sensitivity. However, an alternative possibility is that *nrf-2* and *nrf-3* are both partial loss-of-function mutations and that the small enhancement in the *nrf-2; nrf-3* double mutant is explained by an additive effect of two mutations in the same pathway. Without

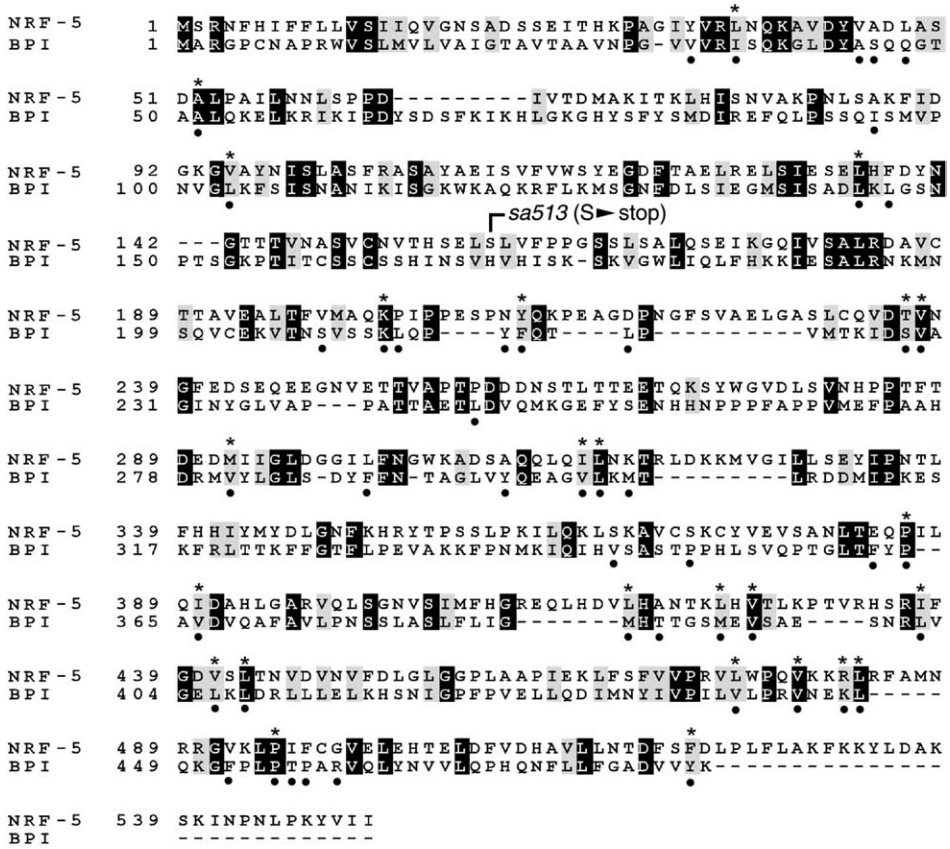


FIGURE 3.—Alignment of *nrf-5* and human BPI protein. Solid boxes denote identical amino acids; shaded boxes denote similarities. The position of the mutation S159 to stop in *sa513* is indicated. Solid dots below the alignment indicate 47 phospholipid contact residues based on BPI crystal structure (BEAMER *et al.* 1997). Asterisks above the alignment indicate the 25 identical or similar residues in *nrf-5* among these 47 contact residues.

information about the molecular nature of the single alleles of *nrf-2* and *nrf-3*, we cannot distinguish between these two interpretations.

***nrf-5* may encode a secreted lipid-binding protein:** We found that *nrf-5* is homologous to a family of secreted lipid-binding proteins including BPI, LBP, CETP, and PLTP. Members of this family have been identified in a wide range of organisms, including humans, chickens, and fish (BLAST; BEAMER *et al.* 1998). BPI and LBP both bind to lipopolysaccharide, a component of bacterial cell walls, and function in the immune system during bacterial infection (BEAMER *et al.* 1999; IOVINE *et al.* 2002; WEISS 2003). CETP and PLTP are involved in metabolism and transport of cholesterol and other lipids in the bloodstream (BRUCE *et al.* 1998b; KAWANO *et al.* 2000; DESRUMAUX *et al.* 2001). The only common function among members of this diverse family is the ability to bind to large hydrophobic molecules such as lipids and cholesterol (BRUCE *et al.* 1998a; GUYARD-DANGREMONT *et al.* 1999).

The structure of BPI at atomic resolution has been determined by X-ray crystallography (BEAMER *et al.* 1997). One molecule of the phospholipid phosphatidyl choline was bound to BPI in each of two hydrophobic-binding pockets. It was proposed that other phospholipids would be able to bind in this pocket as well, consistent with the relatively low specificity of lipid substrate binding among members of the family (BEAMER *et al.* 1997). From the BPI crystal structure, 47 of 456 amino acids were predicted to interact with the bound phospholipids. *nrf-5* is 35% similar to BPI overall, but it is 53% similar among these 47 predicted lipid contact residues (Figure 3). This degree of conservation is similar to other family members such as CETP and PLTP (36% and 42% similar to BPI overall and 49% and 57% similar among lipid contact residues, respectively). Therefore, it is likely that *nrf-5* binds large hydrophobic molecules in a similar binding pocket.

Models for *nrf-5*, *nrf-6*, and *ndg-4* function: The Nrf Peg mutants *nrf-5*, *nrf-6*, and *ndg-4* represent a specific subclass of the mutants isolated in a screen for Nrf mutants (CHOY and THOMAS 1999). The *nrf-6* and *ndg-4* gene products, as with all members of this protein family, contain a consensus acyl transferase domain identified by clusters of orthologous groups analysis (National Center for Biotechnology Information). Interestingly, one Drosophila member of the NRF-6/NDG-4 family has been reported to be expressed in embryonic and adult nervous tissue (DZITOYEVA *et al.* 2003). On the basis of mutant phenotypes and molecular identities of the *C. elegans* genes, several models are possible to explain how *nrf-5*, *nrf-6*, and *ndg-4* function in wild-type animals to confer sensitivity to fluoxetine-induced nose contraction and to mediate uptake of yolk proteins. We outline the details of two such models in the following sections.

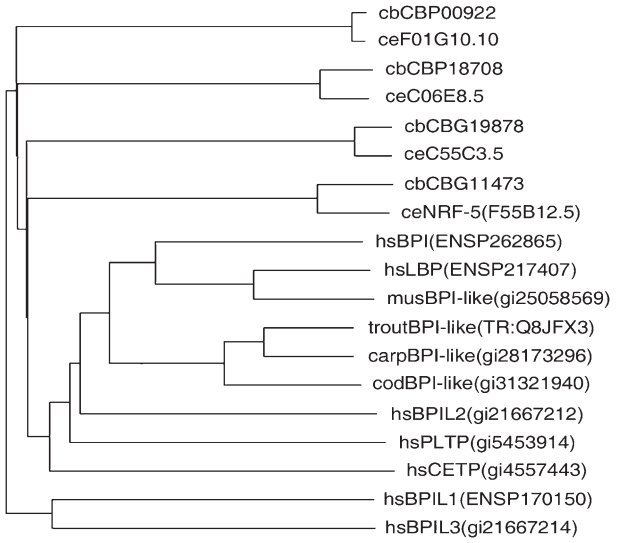


Figure 4.—*nrf-5* is homologous to a family of mammalian lipid-binding proteins. Dendrogram of *nrf-5* and related proteins. NRF-5 and its three closest relatives in *C. elegans* and their reciprocal-best-match orthologs in *C. briggsae* are compared with a variety of relatives in vertebrates. In each name, the first small letters indicate the organism (ce, *C. elegans*; cb, *C. briggsae*; hs, *Homo sapiens*; mus, *Mus musculus*; trout, *Oncorhynchus mykiss*; carp, *Cyprinus carpio*; cod, *Cadus morhua*), the next segment is a commonly used literature name if it exists, and the final segment is a unique database identifier (in parentheses when it follows a common name).

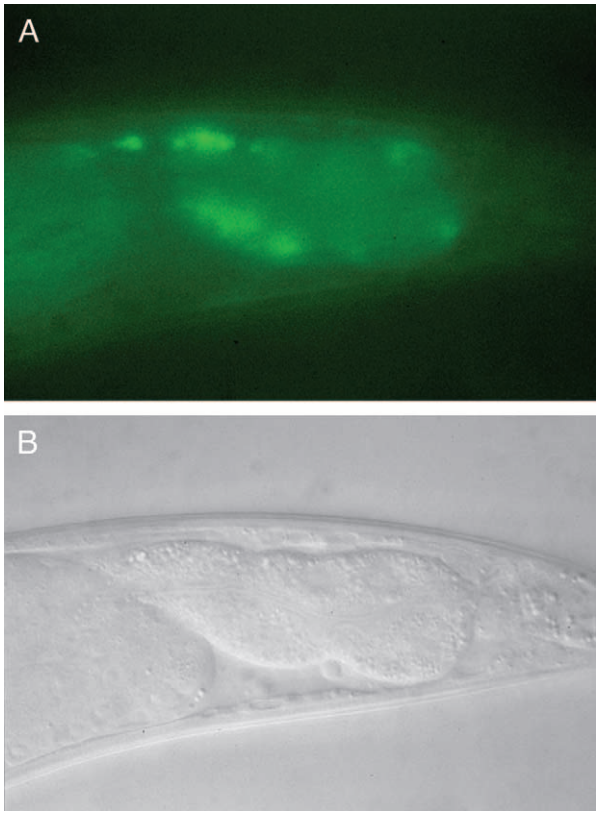


Figure 5.—Expression pattern of *nrf-5::gfp* fusion. (A) Fluorescence photograph of posterior intestine of adult hermaphrodite expressing *nrf-5::gfp* fusion. Anterior is to the left. Fluorescence is concentrated at the basal membrane of intestinal cells, consistent with the homology of NRF-5 to secreted proteins. (B) Nomarski photograph corresponding to A.

Drug and yolk transport model: In this model, NRF-6 and NDG-4 functions in the intestine are required for the function of NRF-5, a protein that is secreted into the pseudocoelomic fluid. This is consistent with the common phenotypes of mutants in these three genes, as well as with our finding that NRF-6 functions in the intestine. In this model, NRF-5 binds and transports two different substances, each of which explains one of the mutant phenotypes (Nrf and Peg). First, NRF-5 binds to yolk proteins and is required to transport or present them to oocytes for endocytosis. *C. elegans* yolk proteins are lipoproteins that are composed of ~15% lipid (Sharrock *et al.* 1990). NRF-6 and NDG-4 may participate in transport or fatty acylation of yolk proteins at the site of the basal intestinal membrane. Since NRF-5 is homologous to lipid-binding proteins, one possibility is that it binds to secreted yolk lipoproteins via their lipid moieties. This could explain the yolk accumulation that is seen in Nrf Peg mutants. However, *nrf-5::gfp* and *nrf-6::gfp* were expressed in males, which do not produce yolk, and both *nrf-5* and *nrf-6* males are Nrf, indicating that the Nrf Peg genes have additional functions other than mediating yolk transport in hermaphrodites. The second function of NRF-5 in this model is to bind to fluoxetine and transport it to target tissues where the drug induces nose contraction. This function is consistent with the homology of NRF-5 to the mammalian lipid transfer proteins CETP and PLTP (see above). Both the selective serotonin reuptake inhibitors and tricyclic antidepressants to which Nrf mutants are resistant are hydrophobic. NRF-5 may bind both types of molecules in its putative hydrophobic binding pocket, consistent with the low binding specificity observed for mammalian members of this family (Bruce *et al.* 1998a). This model does not strictly require fluoxetine to bind to NRF-5 to induce nose contraction, since presumed *nrf-5* null mutants are only partially resistant. One possibility is that NRF-5 binding and transport of fluoxetine expedites a process that happens at a slower rate in Nrf Peg null mutants. Alternatively, other proteins, perhaps other members of the putative NRF-5 lipid-binding protein family, may be partially redundant with NRF-5.

Lipid metabolism defect model: In this second model, NRF-5, NRF-6, and NDG-4 mediate some aspect of lipid metabolism and a defect in this metabolic process is responsible for both the Nrf and the Peg phenotype of these mutants. For example, NRF-5, NRF-6, and NDG-4 could be involved in the transport or modification of particular lipids from the intestine, affecting their availability to other tissues through the pseudocoelomic fluid. An example is given by the human acyl transferase ACAT, which influences the availability of cholesterol by reversible esterification to fatty acids (reviewed in

Chang *et al.* 1997). This enzyme is unrelated to NRF-6 and NDG-4 (not shown). The yolk uptake defect of these mutants could be explained by the requirement of a particular lipid component in yolk proteins for their efficient uptake into oocytes. In the mutants, this lipid component is absent or aberrant, thereby leading to accumulation of yolk in the pseudocoelomic space. This is consistent with work demonstrating that yolk protein uptake by oocytes requires the lipoprotein receptor encoded by *rme-2* (Grant and Hirsh 1999).

A defect in lipid metabolism could also explain the Nrf mutant phenotype of *nrf-5*, *nrf-6*, and *ndg-4*. Changes in the lipid composition of a muscle or neuronal cell membrane could directly or indirectly alter its excitable properties. For example, several neurotransmitter receptors, including nicotinic acetylcholine receptors and glutamate receptors, have been shown to be modulated by the lipid composition of the membranes in which they are imbedded (Sunshine and McNamee 1994; Gagne *et al.* 1996). Alternatively, changes in lipid composition could result in changes in lipid secondary messenger pools (Graber *et al.* 1994; Leevers *et al.* 1999) in a signaling pathway that is responsible for fluoxetine-induced nose contraction. It is important to note that Nrf mutants do not have a nonspecific decrease in nose muscle excitability because they are fully sensitive to several nonantidepressant drugs that induce muscle contraction (Choy and Thomas 1999).

Although the suggestion that changes in lipid metabolism could result in neuromuscular defects is unusual, there are at least two precedents for similar mutant phenotypes. First, *C. elegans fat-3* fatty acid desaturase mutants are defective in the biosynthesis of long chain polyunsaturated fatty acids and have uncoordinated movement defects (Lesa *et al.* 2003; Watts *et al.* 2003). Second, Drosophila *easily shocked* mutants were isolated on the basis of a bang-sensitive paralytic phenotype, and *easily shocked* encodes an ethanolamine kinase, an enzyme involved in the biosynthesis of the ubiquitous phosphatidyl ethanolamine (Pavlidis *et al.* 1994). The exact mechanisms by which mutations in these two genes confer these mutant phenotypes have not yet been fully elucidated, but these examples clearly demonstrate that defects in lipid metabolism can result in unexpected neuromuscular defects and support the idea that the Nrf phenotype of *nrf-5*, *nrf-6*, and *ndg-4* mutants could be explained by similar defects.

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