eat-2 and *eat-18* Are Required for Nicotinic Neurotransmission in the *Caenorhabditis elegans* Pharynx

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ABSTRACT

Mutations in *eat-2* and *eat-18* cause the same defect in *C. elegans* feeding behavior: the pharynx is unable to pump rapidly in the presence of food. EAT-2 is a nicotinic acetylcholine receptor subunit that functions in the pharyngeal muscle. It is localized to the synapse between pharyngeal muscle and the main pharyngeal excitatory motor neuron MC, and it is required for MC stimulation of pharyngeal muscle. *eat-18* encodes a small protein that has no homology to previously characterized proteins. It has a single transmembrane domain and a short extracellular region. Allele-specific genetic interactions between *eat-2* and *eat-18* suggest that EAT-18 interacts physically with the EAT-2 receptor. While *eat-2* appears to be required specifically for MC neurotransmission, *eat-18* also appears to be required for the function of other nicotinic receptors in the pharynx. In *eat-18* mutants, the gross localization of EAT-2 at the MC synapse is normal, suggesting that it is not required for trafficking. These data indicate that *eat-18* could be a novel component of the pharyngeal nicotinic receptor.

THE *Caenorhabditis elegans* pharynx is a self-contained neuromuscular pump that is the feeding organ of the worm (Figure 1). Feeding consists of rapid cycles of contraction and relaxation, or pumping, of the pharyngeal muscle. The pumping action of the pharynx brings food into the worm, grinds it up, and transports it to the intestine. The pharynx in wild-type worms is capable of two pumping rates, depending on environmental conditions: in the absence of food, the pharynx pumps about once each second; in the presence of food, the pumping rate increases to about four pumps every second.

The excitatory pharyngeal motorneuron MC is responsible for rapid pumping (Figure 1; Avery and Horvitz 1989; Raizen *et al.* 1995). When MC is ablated with a laser, worms are incapable of rapid pumping regardless of the environmental conditions (Avery and Horvitz 1989). MC activity causes excitatory postsynaptic potentials (EPSPs) in the pharyngeal muscle that can be seen in electrical recordings of current flowing out of the worm's mouth (Raizen *et al.* 1995). During rapid pumping, every pharyngeal muscle action potential is preceded by an MC EPSP. Therefore, MC controls the rate of pharyngeal pumping.

To find genes involved in MC neurotransmission, Raizen *et al.* (1995) carried out a screen for mutants that were incapable of rapid pharyngeal pumping but had no other obvious defects. Mutations in two genes identified in that screen, *eat-2* and *eat-18*, were characterized in detail. Mutations in both *eat-2* and *eat-18* lack MC neurotransmission. This observation is based on behavioral criteria: the mutants are incapable of rapid pumping and on electrophysiological criteria: there are no MC EPSPs. Fourteen recessive alleles of *eat-2* were identified, and some of the alleles displayed complex intragenic complementation (Raizen *et al.* 1995). Two alleles of *eat-18* were identified, one recessive and one semidominant. Interestingly, there is allele-specific genetic interaction between *eat-2* and *eat-18*, indicating that the gene products might function together in the same protein complex (Raizen *et al.* 1995).

In this article we report the cloning of *eat-2* and *eat-18*. We show that *eat-2* encodes a non-α-nicotinic acetylcholine receptor subunit. EAT-2 functions postsynaptically in the pharyngeal muscle and is localized to the MC synapse. *eat-18* encodes a small, transmembrane protein with no similarity to previously characterized proteins. The EAT-2 channel is correctly targeted to the MC synapse in *eat-18* mutants, indicating that *eat-18* is not required for trafficking of the receptor. Finally, using an α-bungarotoxin (α-BTX)-binding assay, we show

Sequence data from this article have been deposited with the EMBL/GenBank Data Libaries under accession nos. 17537184 (*eat-2*) and 25145442 (*eat-18*).

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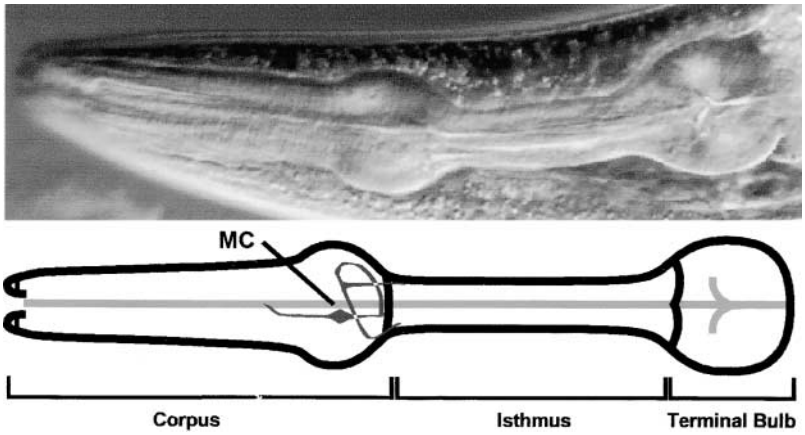

FIGURE 1.—The *C. elegans* pharynx is a neuromuscular pump used for feeding. The corpus brings food into the worm and concentrates it. The isthmus transports the food to the terminal bulb where it is ground up and then moved into the intestine. MC is the main excitatory motorneuron of the pharynx. It makes synapses with the pharyngeal muscle near the junction of the corpus and isthmus and is required for rapid pharyngeal pumping.

that *eat-18* affects most or all of the nicotinic receptors in the pharynx.

MATERIALS AND METHODS

Worm culture: Worms were grown at 20° using standard culture conditions (SULSTON and HODGKIN 1988) with slight modifications (AVERY 1993). The wild-type strain was N2. Worms were fed bacterial strain HB101 (BOYER and ROULLAND-DUSSIOX 1969).

YAC rescue: Yeast containing yeast artificial chromosome (YAC) Y48B6A were grown overnight in selective media. QIAGEN (Chatsworth, CA) yeast genomic DNA prep kit was used to isolate genomic DNA following the manufacturer's instructions. Yeast genomic DNA was injected into *eat-2(ad465)* at a concentration of 100 μg/ml with *rol-6* DNA at a concentration of 10 μg/ml as a co-injection marker using standard injection technique (MELLO and FIRE 1995).

Sequencing: We amplified *eat-2* and *eat-18* from mutant alleles and sequenced the PCR products. Mutations in *eat-2* were *ad451*, G-to-A change at nucleotide 627 resulting in an E-to-K change at amino acid 92; *ad453*, C-to-T change at nucleotide 2395 resulting in a P-to-S change at amino acid 295; *ad465*, G-to-A change at nucleotide 673 resulting in a stop codon at amino acid 107; *ad692*, T-to-G change at nucleotide 811 resulting in an M-to-R change at amino acid 153; *ad1093*, a C-to-T change at nucleotide 774 resulting in a P-to-S change at amino acid 141; *ad1113*, C-to-T change at nucleotide 2482 resulting in an R-to-T change at amino acid 324; *ad1114*, C-to-T change at nucleotide 796 resulting in an S-to-L change at amino acid 148; *ad1115*, G-to-A change at nucleotide 2395 resulting in a P-to-S change at amino acid 295.

Mutations in *eat-18* were *ad1110*, G-to-T change at nucleotide 46 of exon 1b resulting in a stop codon at amino acid 16; *ad820sd*, G-to-A change at nucleotide 179 of exon 1b resulting in a G-to-E at amino acid 60. *nu209* deletes nucleotides 1523–1965 of the Y105E8A.7 coding region.

***eat-2* cDNA:** We obtained cDNA yk108h12, corresponding to Y48B6A.4, from Yuji Kohara. The sequence of yk108h12 is shown in Figure 2. The sequence confirms that the splicing pattern predicted by Genefinder and shown in Wormbase (release WS102) is correct.

***myo-2::eat-2* cDNA fusion:** PCR was used to amplify 1000 bp of DNA directly upstream from the *myo-2* transcription start site. The sequence of the forward PCR primer for the *myo-2* promoter was GGGTTTTGTGCTGTGGACG. The sequence of the reverse PCR primer was AATGCGATTTTCAAGGTCAT TTCTGTGTCTGACGATCGA. The reverse primer contains 19 nucleotides just upstream of the ATG of *myo-2* and the first 20 nucleotides of the open reading frame (ORF) of *eat-2*, including the ATG. The reverse complement of this primer was used as a forward primer to amplify the *eat-2* cDNA using rtPCR. The reverse primer for the rtPCR reaction was CTGTTTATTCAATATCAACAATCGGAC. The two PCR products were fused using overlap extension PCR (HO *et al.* 1989) to create a fusion between the *myo-2* promoter and the *eat-2* cDNA. The fusion product was purified using a Qiaquick PCR purification kit and was injected into *eat-2(ad465)* at a concentration of 100 μg/ml with *rol-6* as a co-injection marker. Two stable transgenic lines were isolated. The slow-pumping phenotype was rescued in all transgenic worms that were analyzed.

EAT-2::GFP fusion: Green fluorescent protein (GFP) was inserted into the intracellular loop between the third and fourth transmembrane domains of *eat-2* between leucine 377 and leucine 378. This was done by a three-part fusion reaction. PCR was used to amplify from 4 kb upstream of the *eat-2* start site to nucleotide 2861 of the *eat-2* coding region. The primers used in this reaction were CAAATCGGCAAACCGGCAAAT ACA and CAATCCCGGGGATCCTCTAAGCAACTTCGTGCC ATCA. GFP was amplified from pPD95.77 from the 1995 Fire lab vector kit using primers GATGGCAACGAAGTTGCTTAG AGGATCCCCGGGATTG and TGGCTGATGTTGCTGGTTT TCAAGTTTGTATAGTTCATCCAT. The 3′ end of *eat-2* from nucleotide 2862 of the coding region to 400 bp downstream from the stop site was amplified using primers ATGGATGAAC TATACAAACTTGAAAACCAGCAACATCAGCCA and CGTG GTGTGGTGTCAGACTG. GFP was fused to the 3′ end of *eat-2* by overlap extension PCR (HO *et al.* 1989). This fusion product was gel purified and fused to the 5′ end of *eat-2* by overlap extension PCR. The fusion construct was gel purified and injected into *eat-2(ad465)* at a concentration of 100 mg/ml with *rol-6* as a co-injection marker.

***eDf7* breakpoint identification:** To find the breakpoint of *eDf7*, we designed primers to the right of the *eDf7* right breakpoint beginning in cosmid F47G4. We used these primers to amplify DNA from homozygous *eDf7* embryos. We continued to design primers moving to the left, toward *eat-18*, until we found a pair that did not result in a PCR product, indicating that the left primer binding site was missing because of the deficiency. We determined that the right breakpoint was in cosmid zk270. We cut genomic DNA from CB2773 (*eDf7/eDf3*) with *Eco*RI, ligated it, and then amplified it with primers close to the breakpoint: zk270.51205 (CAATTATGG CATGTCTGACTC) and zk270.50999 (GTCCACCAAACTTT CCC). We next TA cloned the PCR product into pGEM-T (Promega, Madison, WI). Sequence analysis showed that the left breakpoint was in Y105E8A.7. The sequence of Y105E8A.7 at the left breakpoint of *eDf7* is …….TTTTAGATCACA AAATCCGT. The sequence of the right breakpoint of *eDf7* in

zk270.1 is ACATTGCAATTTCGTG…….. The novel junction of *eDf7* is ….ATCCGTacattgc…. where the uppercase letter sequence is from Y105E8A.7 and the lowercase letter sequence is from zk270.1.

5′ RACE: RNA was isolated from wild-type worms using RNA Stat-60 (Tel-Test B, Friendswood, TX). We used a 5′/3′ rapid amplification of cDNA ends (RACE) kit from Roche according to the manufacturer's instructions. Gene-specific race primers from Y105E8A.7 were r2 (TCGGACCGTCGAATATCGT) and r3 (GTAGGTTGGTGAGTAGAGGGTT). By our analysis, exon 1b begins at nucleotide 1625 of the Y105E8A.7 genomic sequence and ends at nucleotide 1821.

***eat-18* Northern blot:** RNA was isolated from wild-type worms using Trizol (Invitrogen, San Diego). Poly(A)$^+$ RNA was purified using RNAeasy mini kit (QIAGEN). A total of 10 μg of poly(A)$^+$ RNA was run on a 1% agarose formaldehyde gel. The RNA was transferred to a nylon membrane by capillary action. The blot was probed with ^{32}P-labeled probe corresponding to exon 1b of Y105E8A.7.

***eat-18* promoter::*eat-18*cDNA fusion:** The *eat-18* promoter::*eat-18*cDNA fusion was made by overlap extension PCR. One kilobase of genomic DNA upstream of the start site of exon 1b was amplified using primers ATGGAAGAAGGGCAT TTTGAG and TCAAAACTAGCAAACAGGCAATGA. RT-PCR was used to amplify a cDNA consisting of exons 1b, 2, and 3 using primers GTCATTGCCTGTTTGCTAGTTTTG and TCG GACCGTCGAATATCGT. The PCR products were gel purified using a Qiaquick gel extraction kit from QIAGEN and fused using overlap extension PCR (Ho *et al.* 1989). Two independent transgenic lines were obtained. All transgenic worms that were analyzed had a wild-type pumping rate.

***eat-18*::GFP fusion:** The *eat-18::GFP* fusion was made by fusing GFP to exon 1b at nucleotide 1762 of Y105E8A.7. One kilobase of sequence upstream of the ATG of exon 1b was used as a promoter. The forward primer for the *eat-18* promoter was CCGACTATATCCGACTCACCTC. The reverse primer located in exon 1b was CAATCCCGGGGATCCTCTAGCAAA CAGGCAATGACAATAC. GFP, including 3′ untranslated region from *unc-54*, was amplified from promoterless GFP vector pPD95.75 from the 1995 Fire lab vector kit (Fire *et al.* 1990). The forward primer was CTATTGTCATTGCCTGTTTGCTA GAGGATCCCCGGGATTG and the reverse primer was CAA ACCCAAACCTTCTTCCGATC. The PCR products were gel purified using a Qiaquick gel extraction kit and fused using overlap extension PCR (Ho *et al.* 1989). The fusion product was injected into N2 worms at a concentration of 100 μg/ml with *rol-6* as a marker.

***eat-2*::GFP in *eat-18(ad1110)*:** *eat-2(ad465)* carrying the EAT-2::GFP transgene was crossed to *eat-18(ad1110)* males. Nonrescued transgenic F_2 progeny were singled. Complementation tests with *eat-2(ad465)* and *eat-18(ad1110)* were used to confirm that the genetic background was *eat-18(ad1110)*.

α-Bungarotoxin-binding assay: For staining of cell-surface-exposed nicotinic acetylcholine receptors, rhodamine-labeled α-bungarotoxin (Molecular Probes, Eugene, OR) was diluted to 1:200 in 1× injection buffer (20 mm K_3PO_4, 3 mm K citrate, 2% polyethylene glycol 6000, pH 7.5). This solution was injected into the pseudocoelom of young adult animals, which were mounted on dry agarose pads under halocarbon oil. To achieve consistent concentration of bungarotoxin from animal to animal, solution was injected until a few eggs were pushed out. Assuming that this is induced once a certain internal pressure is reached, roughly the same relative amount of bungarotoxin solution was injected. Animals were retrieved from the pads with M9 solution, transferred to nematode growth medium plates for 6 hr (which allowed the coelomocytes to take up excess bungarotoxin from the pseudocoelomic fluid), and subsequently analyzed by fluorescence microscopy.

RESULTS

***eat-2* encodes a nicotinic receptor subunit:** *eat-2* maps to the right arm of chromosome II, to the left of *unc-52* (Raizen *et al.* 1995). Previous work from our lab indicated that the MC neurotransmitter is acetylcholine and that it works by stimulating a nicotinic acetylcholine receptor (Avery and Horvitz 1990; Raizen *et al.* 1995). Therefore we searched this region of chromosome II for genes that were likely to be involved in nicotinic neurotransmission. One of the genes in this region is a nicotinic acetylcholine receptor subunit encoded by Y48B6A.4. When we injected the YAC clone Y48B6A (containing Y48B6A.4) into the *eat-2* mutant *ad465*, we found that the slow-pumping phenotype was rescued. To determine if Y48B6A.4 was responsible for rescuing the *eat-2* mutant, we used PCR to amplify genomic DNA that contained the nicotinic receptor coding region, plus 4 kb of upstream sequence and 500 bp of downstream sequence. We injected this PCR fragment into *eat-2(ad465)* and found that the slow-pumping phenotype was rescued in all transgenic animals. We then sequenced Y48B6A.4 from *eat-2(ad465*) and found an adenine-to-guanine change at nucleotide 673 (Figure 2). This change causes an early stop codon in the second exon that would result in the production of only the first 106 amino acids of the protein and it is unlikely that this truncated protein would have any activity. We therefore conclude that Y48B6A.4 is *eat-2* and that the reference allele *ad465* is a null allele.

EAT-2 protein: *eat-2* encodes a nicotinic acetylcholine receptor subunit with a predicted size of 474 amino acids (Figure 2). It has all the characteristics of a ligand-gated ion channel subunit: a large extracellular amino terminus containing a C loop (C128–C142), four transmembrane domains, and a large intracellular loop between the third and fourth transmembrane domains (Changeaux and Edelstein 1998). There are two broad categories of nicotinic receptor subunits, α and non-α. α-subunits are characterized by a pair of adjacent cysteines in the amino-terminal extracellular portion of the protein near amino acid position 190 (Changeaux and Edelstein 1998). The adjacent cysteines are connected by a strained disulfide bond and contribute to the acetylcholine-binding site (Sine 2002). Non-α-subunits lack the pair of adjacent cysteines (Changeaux and Edelstein 1998). Because *eat-2* lacks the adjacent cysteines, it is a non-α-nicotinic receptor subunit.

***eat-2* functions in pharyngeal muscle:** Previously, we showed that *eat-2* mutants are defective in MC neurotransmission (Raizen *et al.* 1995). *eat-2* could function either presynaptically in MC or postsynaptically in the pharyngeal muscle. If *eat-2* functions postsynaptically, we should be able to rescue *eat-2* mutants by expressing the channel subunit specifically in the muscle. To test this, we fused a full-length *eat-2* cDNA to the pharyngeal muscle-specific *myo-2* promoter (Okkema *et al.* 1993)

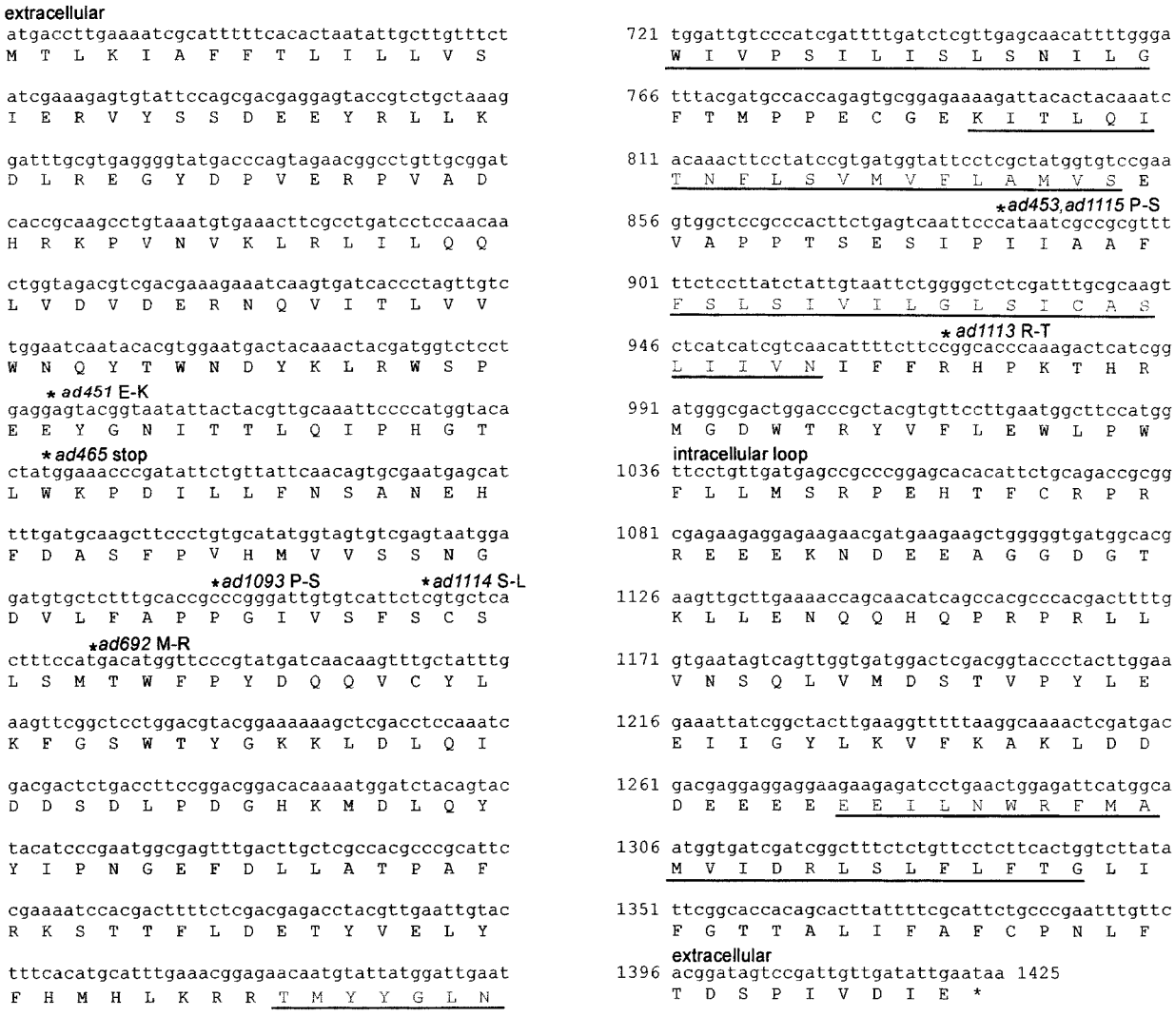

FIGURE 2.—*eat-2* cDNA sequence and predicted protein sequence. Horizontal bars below the sequence indicate transmembrane domains. Asterisks indicate nucleotides affected in *eat-2* mutants. Resulting amino acid changes are indicated.

and injected *eat-2* mutant worms with the fusion product. We found that in *eat-2* mutants containing the fusion construct, the slow-pumping phenotype was rescued. This result demonstrates that *eat-2* functions postsynaptically in the pharyngeal muscle.

EAT-2 is localized to the MC synapse: To analyze the subcellular localization of EAT-2, we made a translational GFP fusion in which GFP is inserted in frame into the intracellular loop between the third and fourth transmembrane domains (Figure 3). We injected this GFP fusion into *eat-2(ad465)* and found that transgenic worms carrying the GFP fusion had wild-type pumping rates, showing that it is able to provide wild-type *eat-2* activity. We examined the transgenic worms by fluorescence microscopy and observed that the EAT-2::GFP fusion protein is localized to small dots near the junction of pharyngeal muscles pm4 and pm5 (Figure 3). On the basis of electron microscope reconstruction of the *C. elegans* pharynx, this is the location of the most-posterior MC processes and of the MC synapse (ALBERTSON and THOMSON 1976; data not shown). This localization, combined with the observation that EAT-2 functions in pharyngeal muscle, is consistent with *eat-2* being the postsynaptic receptor for the MC motor neuron.

Cloning *eat-18*: *eat-18* maps to the right arm of chromosome I, left of *unc-54*, and was found to interact genetically with the breakpoint of deficiency *eDf7*: *eDf7* fails to complement *eat-18(ad820sd)* but does complement *eat-18(ad1110)* (RAIZEN *et al.* 1995). These results suggested that the *eDf7* breakpoint was close to or in *eat-18*. We cloned the left breakpoint of *eDf7* and found that it is in the gene Y105E8A.7 (Figure 4A). To determine if Y105E8A.7 is *eat-18*, we used PCR to amplify DNA containing the coding region of this gene, 1 kb of upstream sequence and 200 bp of sequence downstream of the stop codon. We injected the PCR product into *eat-18(ad1110)* and found that it rescued the slow-pumping phenotype in all transgenic animals that were analyzed (two transgenic lines were established).

Despite the fact that about half of the gene is missing

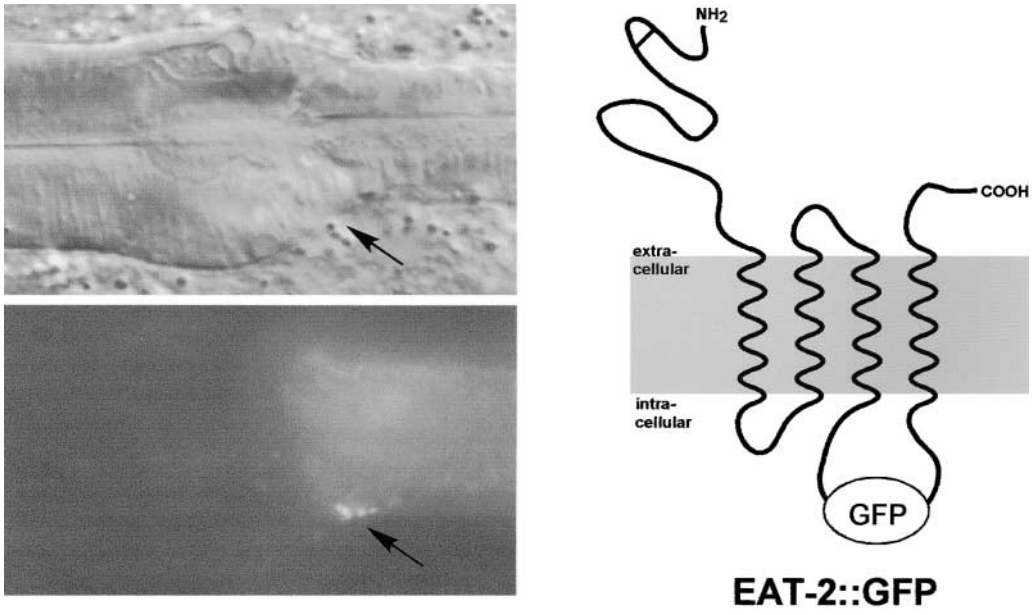

FIGURE 3.—Localization of an EAT-2::GFP fusion. GFP was inserted into the intracellular loop between transmembrane segments 3 and 4. This GFP fusion rescues the slow-pumping defect of *eat-2(ad465)*. The GFP fusion is localized at the MC synapse (arrows).

from the *eDf7* chromosome, it is able to complement *eat-18(ad1110)*. Because of this observation, we wanted to determine what part of the gene is required for *eat-18* activity. Using PCR, we generated a series of genomic clones that contained deletions at the 5′ or 3′ end of Y105E8A.7 (Figure 4B). We injected the PCR products into *eat-18(ad1110)* to see which ones could rescue the slow-pumping phenotype. The smallest rescuing piece begins 731 bp downstream of the ATG of Y105E8A.7 and includes DNA to 3188 bp downstream from the start codon (Figure 4B). This 2458-bp fragment rescued the slow-pumping phenotype of *eat-18(ad1110)* in all transgenic animals that were analyzed. This result is consistent with the observation that the *eDf7* chromosome is able to complement *eat-18(ad1110)* and indicates that only a portion of the 5′ end of Y105E8A.7 is needed for *eat-18* activity.

The rescuing fragment does not contain the first exon of Y105E8A.7, suggesting that there might be one or more additional exons in the intron between the first two exons. We used 5′ RACE to identify any additional exons that might be present. This analysis revealed an additional exon that begins 1625 bp downstream from the predicted start codon of Y105E8A.7 (Figure 4A). There is an ATG at the beginning of this exon and there are no good splice sites at the 5′ end of this

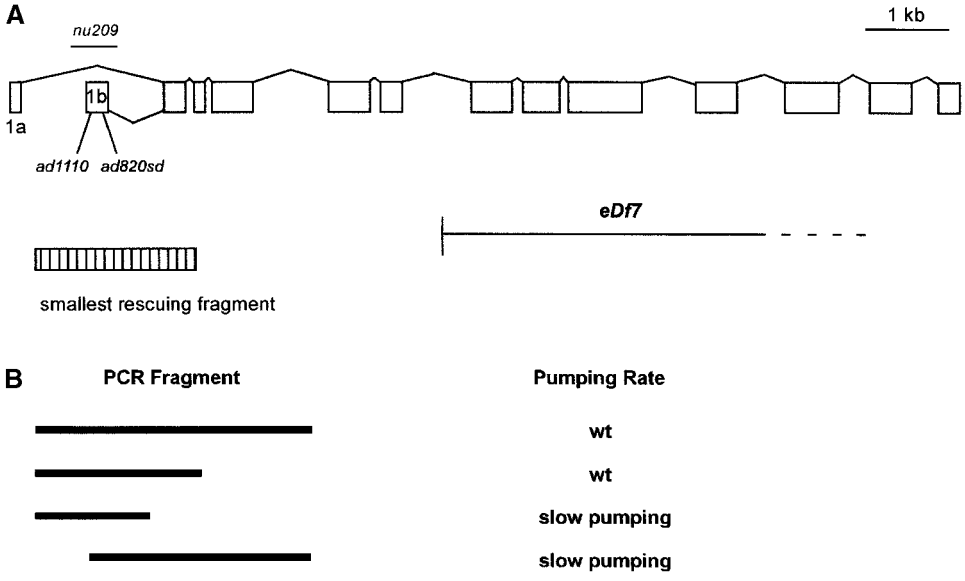

FIGURE 4.—(A) The *eat-18* coding region. *eat-18* is encoded by Y105E8A.7. The left breakpoint of *eDf7* is in the middle of Y105E8A.7 and removes the 3′ end of the gene. The deficiency is represented by the horizontal bar below *eDf7* and the left breakpoint is represented by the vertical bar. The *eat-18* transcript consists of the splice variant that includes exon 1b. Mutations in the three *eat-18* alleles affect exon 1b: *ad1110* introduces an early stop codon in exon 1b, *ad820sd* changes a glutamate to a glycine at amino acid 60, and *nu209* deletes exon 1b. (B) Genomic fragments containing parts of the *eat-18* coding region were generated by PCR. They are represented by horizontal black bars and are aligned with the coding region in A. The left end of the first three fragments is 731 bp downstream of the ATG of exon 1a. The right ends are at base pairs 4170, 3188, and 2782, respectively. The last PCR fragment begins 1.7 kb downstream of the exon 1a ATG and ends at base pair 4170. The PCR products were injected along with *rol-6* as an injection marker. At least two transgenic lines were established for each fragment. Rescuing fragments restored pumping rates to wild-type levels in all transgenic animals analyzed. The smallest rescuing fragment begins at position 731 and ends at 3188.

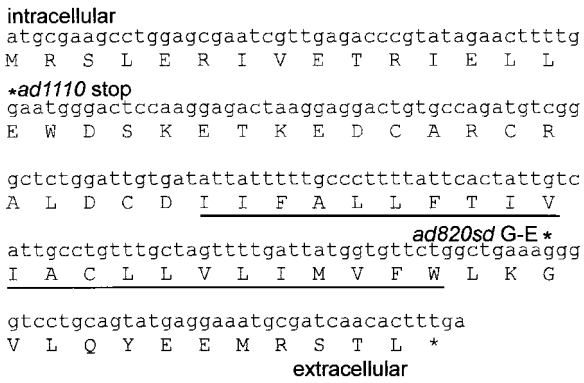

FIGURE 5.—*eat-18* cDNA and protein sequence. Eat-18 encodes a predicted 70-amino-acid protein with an intracellular amino terminus, a transmembrane domain, and a short extracellular carboxy-terminal end. The transmembrane domain is indicated by a bar above the sequence. Asterisks above the sequence indicate nucleotides affected in *eat-18* mutants.

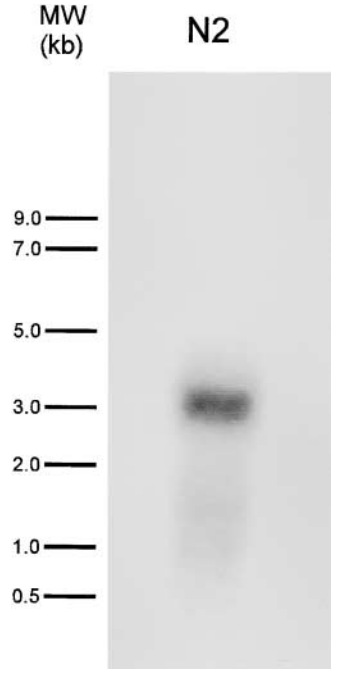

FIGURE 6.—Northern analysis of the *eat-18* transcript. A probe corresponding to exon 1b hybridized to a transcript of ~3 kb.

exon. We believe that this is an alternative first exon for Y105E8A.7 and that it is required for *eat-18* activity. We refer to the upstream first exon as exon 1a and to the downstream first exon as exon 1b (Figure 4A). Analysis of RT-PCR products from Y105E8A.7 shows that transcripts are produced using either of the alternative first exons.

We sequenced cDNAs isolated from transcripts that use the alternative first exon 1b. When a transcript is made using this exon, a stop codon is generated in the second exon, resulting in an open reading frame that would produce a 70-amino-acid protein. Translation initiation from exon 1b is in a different frame from exon 1a, resulting in the stop codon in the second exon. To determine if this small open reading frame was *eat-18*, we fused a cDNA composed of exons 1b, 2, and 3 to 1 kb of genomic sequence upstream of exon 1b (Figure 4). We injected this fusion product into *eat-18(ad1110)* and found that it rescued the slow-pumping defect. All transgenic animals from two independent lines analyzed were rescued. This shows that *eat-18* is encoded by this open reading frame and that the genomic sequence just upstream of exon 1b is the *eat-18* promoter. Sequence analysis of the predicted protein from this transcript suggests that it contains a 40-amino-acid intracellular domain at the amino-terminal end, a transmembrane domain, and a 10-amino-acid, carboxy-terminal, extra cellular domain and has no similarities to previously described proteins (Figure 5; SONNHAMMER *et al.* 1998).

A Northern blot was performed to determine the size of the *eat-18* transcript. A sequence corresponding to exon 1b was used to probe RNA isolated from wild-type worms. This probe hybridized to a transcript of ~3 kb (Figure 6). This is the same size as the full-length cDNAs that we isolated by RT-PCR from Y105E8A.7. This result shows that the small *eat-18* ORF is part of a larger transcript that comes from Y105E8A.7.

We have three mutant alleles of *eat-18*. Two, *ad1110* and *nu209*, are recessive, and one, *ad820sd*, is semidominant (RAIZEN *et al.* 1995). We sequenced the coding region of Y105E8A.7 in all three mutant alleles. Mutations in all three affect exon 1b (Figure 4). *ad1110* results in a stop codon early in the exon, at amino acid 16. *ad820sd* is a G-to-E change at amino acid 60, and *nu209* is a deletion that removes exon 1b. On the basis of the nature of the mutations, we conclude that *ad1110* and *nu209* are both null alleles. The fact that all three mutant alleles affect exon 1b is consistent with *eat-18* activity being contained in the 5′ end of the gene.

EAT-18 is expressed in pharyngeal muscle: To determine where *eat-18* is expressed, we fused GFP in frame to exon 1b and injected the fusion product into wild-type worms. We examined the transgenic worms and observed GFP expression in pharyngeal muscle and pharyngeal neuron M5 (Figure 7). There is also very faint GFP expression in five to six unidentified neurons in the extrapharyngeal nervous system (not shown). The expression pattern of EAT-18::GFP supports the conclusion that the pharyngeal muscle is the main site of *eat-18* function.

EAT-2 is correctly localized in *eat-18* mutants: One possible role for *eat-18* is that it is required for folding or trafficking of the nicotinic receptor. To test this, we introduced the functional EAT-2::GFP fusion into *eat-18(ad1110)*. We examined the transgenic worms and found that the EAT-2::GFP fusion is correctly localized in *eat-18* mutants (Figure 8). This result indicates that

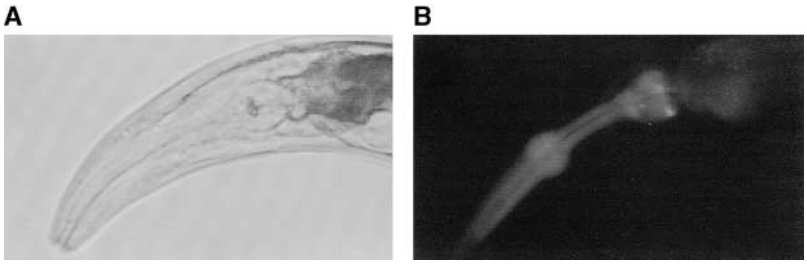

FIGURE 7.—*eat-18* is expressed in pharyngeal muscle. (A) A bright-field image of the pharynx. (B) Fluorescent image of the pharynx. The EAT-18::GFP fusion protein is expressed in pharyngeal muscle and pharyngeal neuron M5. Fluorescence posterior to the pharynx is auto-fluorescence from the intestine.

eat-18 is not required for folding or trafficking of the EAT-2 channel.

***eat-18* is required for α-bungarotoxin-binding in the pharynx:** RAIZEN *et al.* (1995) reported that *eat-2* and *eat-18* mutants differ significantly in their response to nicotine. Pharynxes that have been dissected from either wild-type or *eat-2* mutant worms hypercontract in 100 μM nicotine. Pharynxes from *eat-18* mutants, however, are resistant to this concentration of nicotine. A possible explanation for this result is that *eat18* is required for the function of other nicotinic receptors in the pharynx in addition to *eat-2*. We used an α-BTX-binding assay to look at the expression of nicotinic receptors throughout the pharynx. α-BTX is a competitive inhibitor of nicotinic acetylcholine receptors and, when bound to the receptor, occupies part of the acetylcholine-binding site (SAMSON *et al.* 2002). Fluorescently labeled α-bungarotoxin injected into the pseudocoelom of *C. elegans* binds to nicotinic receptors throughout the body. The location of the receptors can be seen by using fluorescence microscopy. In wild-type worms, there is extensive labeling of the pharynx (Figure 8). When α-BTX was injected into two alleles of *eat-2*, *ad453* and *ad1113*, there was extensive labeling of the pharynx similar to wild type (Figure 9). However, when α-BTX was injected into *eat-18(ad1110)* mutants, staining of the pharynx was almost completely abolished (Figure 9). α-Bungarotoxin still labeled *eat-18(ad1110)* worms outside the pharynx, suggesting that *eat-18* is required for α-bungarotoxin-binding to most or all pharyngeal nicotinic receptors.

FIGURE 8.—EAT-2::GFP is correctly localized in *eat-18 (ad1110)*. The functional EAT-2::GFP fusion was crossed into the *eat-18(ad1110)* mutant background. (Top) A differential interference contrast image of a worm pharynx. Arrow points to the location of the MC synapse. (Bottom) A fluorescence image showing the localization of the fusion protein at the MC synapse (arrow).

DISCUSSION

MC mechanism: Previous work in our lab led to the proposal that the MC neurotransmitter is acetylcholine (AVERY and HORVITZ 1990; RAIZEN *et al.* 1995). This is supported by the finding that *eat-2* encodes a nicotinic acetylcholine receptor subunit. The observation that EAT-2 functions in the pharyngeal muscle demonstrates that MC stimulates the muscle directly, using fast synaptic transmission to control pharyngeal pumping rate. Stimulation by MC causes the EAT-2 channel to open, allowing current to flow in. This leads to the opening of a voltage-activated calcium channel and subsequent muscle contraction (LEE *et al.* 1997). The rate of MC firing controls the rate of pharyngeal pumping.

The role of *eat-18* in nicotinic neurotransmission: Mutations in *eat-18* cause the same defect in pumping as mutations in *eat-2*: worms are incapable of rapid pharyngeal pumping and EPSPs from the excitatory motor neuron MC are not present (RAIZEN *et al.* 1995). *eat-18* encodes a small transmembrane protein that does not have any similarity to previously described proteins, including proteins known to be involved in the function of nicotinic acetylcholine receptors. Several lines of evidence suggest that *eat-18* could be a component of the pharyngeal nicotinic receptor. First, an *eat-18*::GFP fusion is expressed in pharyngeal muscle, suggesting that this may be the site of *eat-18* activity. Also, we showed previously that pharynxes dissected from *eat-18* mutants were resistant to bath-applied nicotine, indicating that nicotinic receptors in the pharyngeal muscle are defective (RAIZEN *et al.* 1995). We have observed allele-specific genetic interactions between *eat-2* and *eat-18*, indicating that they could be members of the same protein complex. Worms heterozygous for the semidominant *eat-18* mutant *ad820sd* have an intermediate slow-pumping

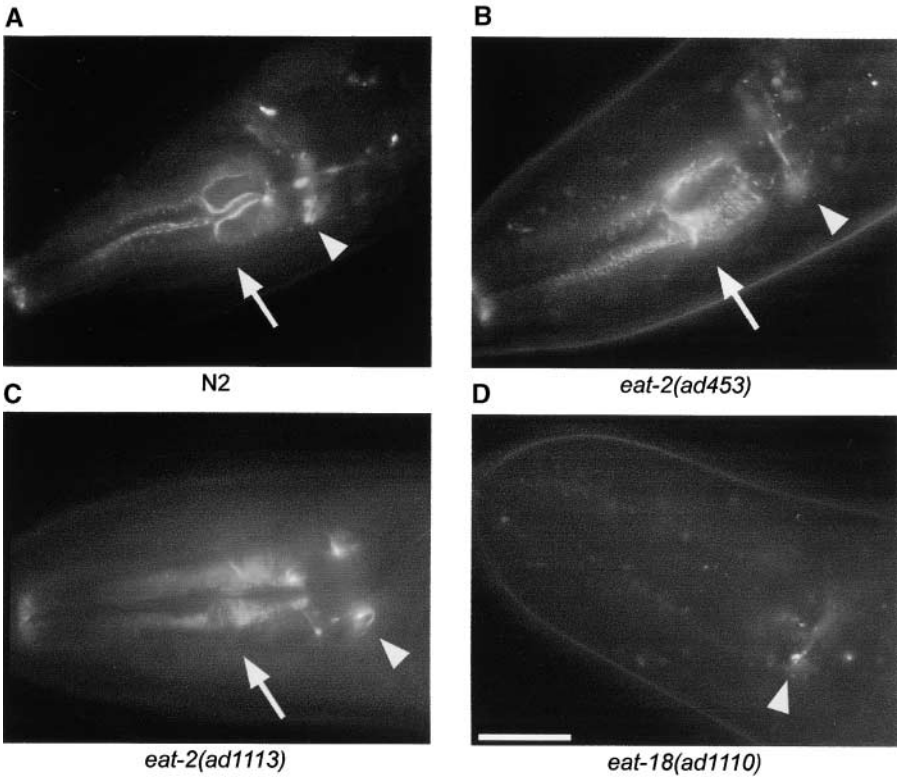

FIGURE 9.—α-Bungarotoxin-binding assay. Fluorescently labeled α-bungarotoxin injected into the body cavity of worms binds to many sites in the pharynx of (A) wild-type worms and (C) *eat-2 (ad1113)* and (B) *eat-2(ad453)* mutant worms. (D) α-Bungarotoxin-binding sites are absent in the pharynx in *eat-18(ad1110)* mutants. Arrows point to staining in the pharynx and arrowheads point to staining outside the pharynx.

phenotype of ~90 pumps/min. Wild-type worms pump >200 times/min and worms completely defective in MC neurotransmission pump at ~40 pumps/min. Worms that are *trans*-heterozygous for *eat-18(ad820sd)* and two alleles of *eat-2*, *ad453* and *ad1115*, have a wild-type pumping rate. In this case, the *eat-2* mutants are able to suppress the intermediate slow-pumping phenotype of *eat-18(ad820)*. Another *eat-2* allele, *eat-2(ad1113)*, when *trans*-heterozygous with *eat-18(ad820sd)*, enhances the intermediate slow-pumping phenotype. One interpretation of these allele-specific genetic interactions is that EAT-2 and EAT-18 physically interact. A functional EAT-2::GFP fusion appears to be correctly localized in *eat-18* mutants, indicating that it is not required for trafficking of EAT-2. Taken together, these data indicate that EAT-18 could be a component of pharyngeal nicotinic receptor. This interpretation is supported by results from the α-bungarotoxin-binding experiment. α-Bungarotoxin is a competitive inhibitor of acetylcholine and has been shown to occupy the acetylcholine-binding site of nicotinic receptors (SAMSON *et al.* 2002). We found that α-bungarotoxin bound to several sites on pharynxes from wild-type worms or *eat-2* mutants, but its binding was greatly reduced in pharynxes from *eat-18(ad1110)* mutants. A possible reason for the lack of acetylcholine binding to *eat-18* mutant pharynxes is that EAT-18 is required for the formation of the acetylcholine-binding site. This would also be consistent with the inability of MC to cause excitatory postsynaptic potentials in *eat-18* mutants.

There are alternative possibilities for the role of *eat-18* in the function of the nicotinic receptor. One alternative is that *eat-18* could be required for inserting the nicotinic receptor into the postsynaptic membrane. In this model, the nicotinic receptor would be targeted correctly to the location of the synapse, but it would remain in a subsynaptic pool of receptors. The role of *eat-18* could be to move the receptor from the subsynaptic pool and insert it into the postsynaptic membrane. This model is consistent with the apparent proper localization of the EAT-2::GFP fusion in *eat-18* mutant worms. The resolution of the light microscope could not distinguish between receptors in the subsynaptic pool and those that had been inserted into the membrane. This model would also explain the inability of MC to activate the receptor: acetylchololine released by MC would not have access to receptors in the subsynaptic pool. Ultrastructural analysis of the MC synapse would be required to determine the exact location of EAT-2 in wild-type and *eat-18* mutant worms.

***eDf7* chromosome:** The *eDf7* chromosome was an important tool in cloning *eat-18*. It is interesting that *eDf7* complements *eat-18(ad1110)* but does not complement *eat-18(ad820sd)* although the deficiency breakpoint does not overlap the *ad820sd* lesion. We think several things are responsible for this. Although *eat-18* is encoded by a small ORF, it is part of a large transcript. The 3′ half of the transcript is removed by *eDf7*. We think that this reduces the amount of EAT-18 protein that is made because the shortened transcript from the deficiency

chromosome is likely to be less stable than the full-length wild-type transcript. The decreased *eat-18* expression from the deficiency chromosome is still enough to supply wild-type levels of *eat-18* activity in the presence of the recessive allele *ad1110*. The semidominant allele, *ad820sd*, behaves like a dominant negative mutation (Raizen *et al.* 1995). If *eat-18* expression from the *eDf7* chromosome is reduced, there might not be enough activity to overcome the dominant negative effect of *ad820sd*, resulting in the slow-pumping phenotype.

Other genes required for ion channel function in *C. elegans*: RIC-3 and MEC-6 are two other proteins involved in ion channel function that have recently been identified through genetic screens in *C. elegans*. *ric-3* was identified in a screen for worms that are resistant to cholinesterase inhibitors and in a screen for suppressors of a dominant mutation in the nicotinic acetylcholine receptor subunit DEG-3 (Halevi *et al.* 2002). *ric-3* encodes a small protein with two transmembrane domains that localizes to the cell body of neurons and muscles. *ric-3* appears to be required for receptor assembly or trafficking and affects the function of several nicotinic acetylcholine receptors in *C. elegans*, including *eat-2* (Halevi *et al.* 2002). Although EAT-18 is also a small transmembrane protein, in contrast to RIC-3, it appears to be required for formation of the acetylcholine-binding site. Additionally, although *ric-3* is involved in nicotinic neurotransmission in several cell types, *eat-18* appears to be specifically required for pharyngeal nicotinic acetylcholine receptors. MEC-6 is a single pass transmembrane domain protein required for the maturation or function of the degenerin/epithelial sodium channels (DEG/ENaC) in *C. elegans* (Chelur *et al.* 2002). Chelur *et al.* (2002) used biochemical methods to show that MEC-6 interacts with several DEG/ENaC in *C. elegans* and that coexpression of MEC-6 with the DEG/ENaC channels greatly increases current flow through them. Biochemical and electrophysiological experiments will be needed to further define the role that *eat-18* plays in regulating pharyngeal nicotinic receptors.

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