eat-5 and *unc-7* Represent a Multigene Family in *Caenorhabditis elegans* Involved in Cell–Cell Coupling

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Abstract. The *Drosophila melanogaster* genes *Passover* and *l(1)ogre* and the *Caenorhabditis elegans* gene *unc-7* define a gene family whose function is not known. We have isolated and characterized the *C. elegans* gene *eat-5*, which is required for synchronized pharyngeal muscle contractions, and find that it is a new member of this family. Simultaneous electrical and video recordings reveal that in *eat-5* mutants, action potentials of muscles in the anterior and posterior pharynx are unsynchronized. Injection of carboxyfluorescein into muscles of the posterior pharynx demonstrates that all pharyngeal muscles are dye-coupled in wild-type animals; in *eat-5* mutants, however, muscles of the anterior pharynx are no longer dye-coupled to posterior pharyngeal muscles. We show that a gene fusion of *eat-5* to the green fluorescent protein is expressed in pharyngeal muscles.

unc-7 and *eat-5* are two of at least sixteen members of this family in *C. elegans* as determined by database searches and PCR-based screens. The amino acid sequences of five of these members in *C. elegans* have been deduced from cDNA sequences. Polypeptides of the family are predicted to have four transmembrane domains with cytoplasmic amino and carboxyl termini. We have constructed fusions of one of these polypeptides with β-galactosidase and with green fluorescent protein. The fusion proteins appear to be localized in a punctate pattern at or near plasma membranes. We speculate that this gene family is required for the formation of gap junctions.

THROUGH efforts to identify by mutational analyses factors important in neural development and establishment of neural circuitry, the *Caenorhabditis elegans* gene *unc-7* and the *Drosophila* genes *Passover* and *l(1)ogre* have been molecularly characterized (Starich et al., 1993; Watanabe and Kankel, 1990; Krishnan et al., 1993). Sequence comparison of the predicted protein products established that these three genes are related and represent members of a gene family (Krishnan et al., 1993). In *Drosophila melanogaster*, the *Passover* or *shaking B* gene encodes two polypeptides that arise by alternative splicing (Crompton et al., 1995; Krishnan et al., 1995). Mutations that affect the neural polypeptide of *Pas* result in the failure to form proper electrical synapses between the giant fiber cells and their neuronal targets (peripheral synapsing interneurons and tergotrochanteral motor neurons) and disrupt an escape–response pathway (Thomas and Wyman, 1983, 1984). A single viable mutation in the *Drosophila l(1)ogre* gene results in disorganization and reduction in the size of proliferative centers in the optic ganglia of the fly (Lipshitz and Kankel, 1985). Lethal alleles likely representing the null *l(1)ogre* phenotype result in degenerative effects throughout the central nervous system at late larval stages (Lipshitz and Kankel, 1985); however, synthesis of ogre protein appears not to be limited to the central nervous system (Watanabe and Kankel, 1992). In *C. elegans*, mutations in *unc-7* result in severely impaired forward locomotion (Starich et al., 1993), and there is some evidence that ectopic gap junctions form between a pair of interneurons and certain classes of motor neurons in *unc-7* mutants (White, J., E. Southgate, and N. Thomson, personal communication). It is not known what function the products of these related genes may share, but it had been speculated that they may represent novel channel proteins (Starich et al., 1993) or possibly adhesive molecules important in establishing synaptic specificity (Krishnan et al., 1993).

In addition to the genes defined originally by mutations, computer database searches identified *C. elegans* expressed sequence tags (ESTs)[1] with similarity to *Pas* (Krishnan et al., 1993; Barnes, 1994), referred to as the OPUS (Ogre, Passover, UNC-7, Shaking B) family (Barnes, 1994). We report here the identification and analysis of several additional members of this gene family in *C. ele-*

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1. *Abbreviations used in this paper*: EPG, electropharyngeogram; EST, expressed sequence tag; GFP, green fluorescent protein; Rol, roller; RT, reverse transcription; TM, transmembrane.

gans, including *eat-5*, which is required for synchronized pharyngeal muscle contractions (Avery, 1993). Our data indicate that members of the family function in cell–cell interactions and lead us to speculate that the polypeptides encoded by family members are structural components of gap junctions, analogous to vertebrate connexins. The connexins constitute a large vertebrate family of polypeptides that are the structural components of intercellular gap junction channels (for recent reviews see Bennett et al., 1991; Kumar and Gilula, 1992; Stauffer and Unwin, 1992). Gap junctions allow passage of small molecules (1 kD or less) and have been detected classically by their ability to transfer small dyes between cells or to couple cells electrically.

No invertebrate equivalent of a connexin has been identified molecularly, although the possibility that pas, ogre, UNC-7, and their homologs function as connexins has been independently proposed (Barnes, 1994). The data presented here offer support for the idea that this gene family encodes gap junction proteins.

Materials and Methods

Cloning of eat-5

The λ phage clone (TB3C) corresponding to *eat-5* was isolated from a *C. elegans* genomic library (Stratagene, La Jolla, CA) using digoxigenin-labeled cosmids B0332 and T21B9 as probes following the manufacturer's protocol (Genius kit; Boehringer Mannheim Biochemicals, Indianapolis, IN). All plasmid subclones in Fig. 1 were constructed by inserting genomic fragments derived from λTB3C into the multiple cloning site of pBluescript-KS(+) (Stratagene). The frame-shift mutant was made by inserting four bases (GCGC) into the unique KasI site of the shortest rescuing plasmid clone with DNA polymerase I (Klenow fragment). The insertion created a unique BssHII site. We reverted the insertional mutant back to wild type by cutting with BssHII and deleting the inserted bases with S1 nuclease. These clones were checked by restriction enzyme digestions. General cloning procedures followed Sambrook et al. (1989).

eat-5 rescuing activity of cosmids, λ phage, and plasmid clones was tested by germline transformation as described by Mello et al. (1991). Briefly, the clones to be tested were coinjected with either pRF4 (Kramer et al., 1990) or pRAK3 (Davis et al., 1995) plasmids, which contain the dominant marker *rol-6(su1006)*, into the gonads of *eat-5* mutant hermaphrodites. Expression of *rol-6(su1006)* in transformed animals results in a dominant roller (Rol) phenotype. F1 Rol progeny of injected hermaphrodites and/or transgenic Rol lines derived from heritably transformed F2 progeny were scored for their Eat phenotype.

The *eat-5* phenotype was scored in Rol animals at larval stage 2 or 3, since we found that the penetrance of *eat-5(ad464)* was ~97% during larval stage 3, but much lower in later stages (data not shown). The pumping phenotype was observed under a microscope (Axiophot; Carl Zeiss, Inc., Thornwood, NY) with a ×20 objective while the worms were feeding on a plate seeded with *Escherichia coli*. Scoring of rescue in the F1 generation was based on the Eat phenotype of individual Rol animals. When a germline-transmitting transgenic line was obtained, we scored the phenotype for the line rather than for individual animals. A line was considered rescued if we consistently saw an Eat^+ phenotype in Rol animals in F2 and subsequent generations.

The *eat-5* cDNA was isolated from a λgt10 library, constructed by S. Kim (Stanford University, Stanford, CA). The insert was cloned as a partially digested 1.7-kb EcoRI fragment into pBluescript, and both strands were sequenced using Sequenase version 2.0 (United States Biochemical Corp., Cleveland, OH). We also sequenced a single strand of the smallest rescuing genomic fragment (3.7-kb KpnI–XhoI fragment) to identify exon/intron boundaries.

Reverse Transcription (RT)–PCR Analysis of eat-5 Transcripts

The *eat-5* polypeptide product predicted from the cDNA sequence appeared to be truncated in comparison to the predicted products of other family members. Examination of sequences downstream of the predicted translational stop site suggested to us that sequences from a single intron might remain in this cDNA. To examine this possibility, we carried out RT-PCR analysis (Kawasaki, 1990) of *eat-5* transcripts from wild-type (N2) worms. Total RNA was isolated as previously described (Starich et al., 1993), and 5 μg representing all developmental stages was used for reverse transcription. Reverse transcription was carried out with a primer (5′-TCGTTCCACATCCTGCCTAC-3′) corresponding to a sequence 3′ of the proposed intron. The reaction was carried out for 1 h at 42°C, followed by 1/2 h at 52°C, using avian myeloblastosis virus reverse transcriptase (United States Biochemical Corp.). The initial primer was removed using a microconcentrator (Centricon 100; Amicon Corp., Danvers, MA).

PCR amplification of the cDNA products was carried out using two primers (5′ of the RT primer) flanking the proposed intron (5′-TCAGGTCAGAGTGCTTGGAA-3′, spanning exons 4 and 5, and 5′-TACTCTTGGTCCTGATTGTC-3′, corresponding to exon 7). Primers were annealed at 52°C, and products were amplified through 35 cycles using 1-min extension times at 72°C. Expected product sizes were: a 500-bp fragment corresponding to unprocessed RNA (no introns removed), a 390-bp fragment corresponding to the sequence found in the cloned *eat-5* cDNA (after removal of the intron between exons 6 and 7), and a 220-bp fragment corresponding to the additional removal of the proposed intron in the *eat-5* cDNA (between exons 5 and 6). Only PCR amplification products of 500 and 220 bp could be detected by hybridization to a ^{32}P-labeled *eat-5* probe. The 220-bp product was isolated, and six independent clones were sequenced across the proposed intron/exon junction. The sequence of all clones confirmed the removal of the proposed intron. We therefore concluded that the *eat-5* cDNA we had previously isolated retained sequences from a single intron. All figures and analysis of the *eat-5* cDNA represent the cDNA sequence after removal of this intron.

Electropharyngeograms

Electropharyngeograms were recorded, synchronized with video recordings, and analyzed as described by Raizen and Avery (1994). The traces shown in Fig. 2 have been digitally filtered by convolution with a Gaussian of width $\sigma = 0.8$ ms.

Because signal size depends on the size of the worm, all electropharyngeograms were recorded from adults. Unfortunately, the *eat-5* phenotype is impenetrant in adults: many *eat-5* mutant worms pump normally. Even mutants whose pharyngeal contractions are predominantly unsynchronized occasionally have synchronized contractions. For the analysis in Fig. 2, therefore, we selected three adults that appeared starved (starved appearance correlates with unsynchronized contractions; Avery, 1993). A video mixer was used to produce a video recording showing both pharynx motion and an oscilloscope trace of the electropharyngeogram (Raizen and Avery, 1994). At the same time, a high resolution electrical recording was made on a computer.

All three electrical recordings showed the sorts of abnormalities depicted in Fig. 2. One 6-s period was chosen for detailed analysis because the electrical recording had low noise and the worm moved very little, allowing pharyngeal motions to be seen. Pharyngeal motions during these 6 s were correlated with the computer recording by comparing the electrical signals on the videotaped oscilloscope with those in the computer file.

Dye Injections

Terminal bulb muscles of dissected wild-type and *eat-5* adult pharynxes were impaled with sharp electrodes as described by Davis et al. (1995). The electrodes were filled with 500 mM potassium acetate + 10 mM carboxyfluorescein. Dye was injected with 40 ms–2.5 nA pulses repeated at 5 Hz for ~7.5 min, with occasional pauses to monitor membrane potential. We report results only from pharynxes that maintained a negative resting potential and continued to produce action potentials after injection. The pharynxes were then examined with epifluorescence optics, and the presence or absence of dye in the terminal bulb, isthmus, and corpus was noted. In most cases, they were also photographed. Photographs were taken on Fujicolor Super HG 1600 color negative film (Fuji Photo Film Co., Ltd., Tokyo, Japan). Fig. 3 was made by scanning color prints made from these negatives with an Apple OneScanner (Apple Computer, Inc., Cupertino, CA) and manipulating the scanned images with Adobe Photoshop. Although we did not attempt to accurately preserve relative brightness of fluorescence in preparation of the figure, it is approximately correct. In particular, the fluorescence in the isthmus of *eat-5* pharynxes was

consistently brighter than that in the corpus and isthmus of wild-type pharynxes, probably because the dye was diluted into a greater volume of muscle cytoplasm in wild type.

PCR Screening for Other Family Members

Two degenerate oligonucleotides (5'-CAA/GTAT/CGTNGGNNNNC-CNAT-3' and 5'-A/GAANNNNACCCAC/TTGA/GTAA/GTA-3', corresponding to the predicted peptide sequences QYVGXPI/M and YYQ-WVXF) were synthesized and used to amplify by PCR sequences related to *Pas*, *l(1)ogre*, and *unc-7*. To amplify from *C. elegans* genomic DNA, ~1 ng of DNA and 200 pmol of each degenerate oligonucleotide were used per 50 μl reaction. Samples were denatured at 94°, annealed at 46°, and extended at 72°C (1 min for each step) for 35 cycles. Amplification from a λZAP cDNA library (constructed by R. Barstead, Oklahoma Medical Research Foundation, Oklahoma City, OK) followed similar conditions except that the first denaturing step was carried out at 95°C for 5 min to denature the phage particles. 2 μl of the λZAP library (3×10^9 plaque-forming units per ml) were used per 50 μl reaction. Taq polymerase was used to carry out the reactions, and products were gel purified and cloned into pBluescript (Stratagene) that had been digested with EcoRV and tailed with thymidine triphosphate (Marchuk et al., 1991). Individual clones were sequenced and evaluated for sequence similarity to other family members.

The sequences of the PCR degenerate oligonucleotide primer binding sites for family members were obtained by sequencing the corresponding sites in either cDNA or genomic clones. The cDNAs corresponding to pcr32 and pcr55 were isolated from the λZAP library. No cDNA corresponding to pcr49 could be isolated, but the sequence of the 5' primer binding site was determined by sequencing a corresponding genomic restriction fragment.

Physical Mapping

To place unmapped family members on the physical map, genomic clones were isolated from an amplified λEMBL3 library (originally constructed by C. Link, University of Denver, CO) using gene-specific probes. For pcr32, pcr49, and pcr55, probes were derived from the original PCR clones. For wEST01007, wEST02207 (McCombie et al., 1992), and cm9d9 (Waterston et al., 1992), probes were derived from cDNA clones. Multiple positively hybridizing plaques were isolated for each specific probe, and DNA samples isolated from the appropriate phage were examined by restriction digestion (and in some cases crosshybridization) to verify that clones identified with a single specific probe all derived from the same locus. Although individual genes share similarity with other family members, we found no evidence of one gene-specific probe hybridizing to genomic clones representing another family member.

A representative clone for each gene was placed on the physical map by fingerprint analysis (courtesy of A. Coulson, Sanger Center, UK), or, if clones could not be placed unambiguously (wEST01007 and pcr49), by hybridizing gene-specific probes (entire λ clones) to duplicate nylon filters gridded with YAC clones representing the *C. elegans* genome (Coulson et al., 1988; kindly provided by A. Coulson).

Construction and Expression of eat-5 and wEST01007 Fusions

An *eat-5*::GFP gene fusion was constructed by ligating an *eat-5* genomic fragment from the rescuing clone pRE5-7 (Fig. 1) into the green fluorescent protein (GFP) expression vector pPD95.75 (kindly provided by A. Fire, Carnegie Institution of Washington, Baltimore, MD). This gene fusion contains all of the insert from pRE5-7 except the region encoding the carboxyl-terminal 64 amino acids of EAT-5, the 3' untranslated region and 3' genomic region. This *eat-5*::GFP gene fusion was coinjected (40 ng/μl) into *C. elegans* with the dominant marker *rol-6(su1006)* (80 ng/μl), and stably transformed lines were established from Rol progeny.

For wEST01007, we determined the approximate transcriptional start site by isolating additional corresponding cDNAs, one of which included sequences derived from the trans-spliced leader SL1 (data not shown). A 4.0-kb HindIII fragment encoding the first 334 amino acids of the predicted product of wEST01007 plus 3 kb of sequences upstream of the SL1 splice site was ligated into the expression vectors pPD21.28 (*lacZ*) and pPD95.75 (GFP), kindly provided by A. Fire. Constructs were coinjected (2 ng/μl pPD21.28; 0.5 ng/μl pPD95.75) into *C. elegans* with 80 ng/μl of the dominant marker *rol-6(su1006)* (Kramer et al., 1990). Stably transformed lines were identified, and extrachromosomal arrays were integrated by irradiating worms with ~3,300 roentgens from a ^{137}Cs source. Individual F1 progeny were screened for those producing a high percentage of F2 Rol worms, and integration of the array was confirmed by isolating F2 worms giving rise to all Rol progeny.

Sequence Analysis

Sequence analysis was carried out using programs from the GCG Sequence Analysis Software Package (Genetics Computer Group, Inc., Madison, WI). The multiple sequence alignments shown derive from the Pileup program, and all similarity scores cited derive from the BestFit program. Topology predictions were generated following the method of Jones et al. (1994).

Results

eat-5 Encodes a Protein Similar to UNC-7, ogre, and pas

We had previously shown by three-point recombination mapping that *eat-5* is between *let-75* and *unc-13* (Avery, 1993), and much closer to *let-75*. *unc-13* has been cloned (Maruyama and Brenner, 1991) and located on the *C. ele-*

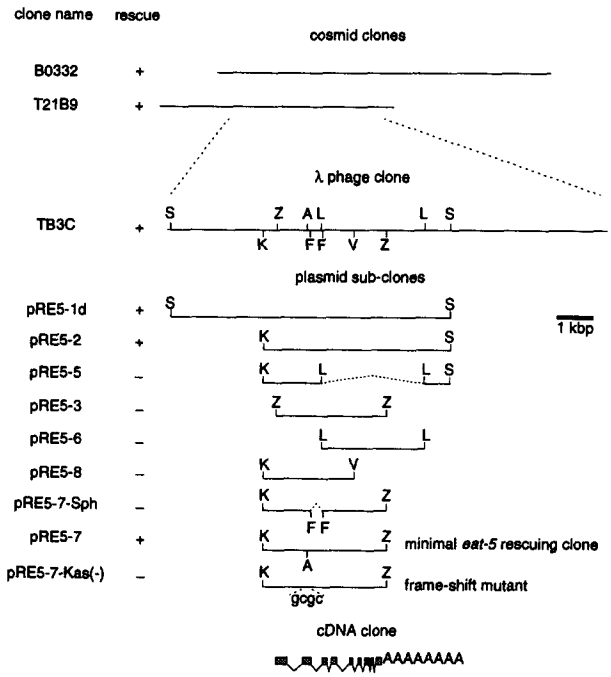


Figure 1. Molecular localization of *eat-5*. The figure summarizes our strategy and results in defining the minimal genomic fragment that contains *eat-5*(+). The top two lines represent cosmid clones. The third line represents a λ phage clone (orientation shown is arbitrary with respect to the cosmids) that appeared to overlap with both cosmids (see Materials and Methods). Subsequent lines represent plasmid subclones derived from the insert in the lambda clone. Rescuing activity of transgenes was assessed by scoring the Eat phenotype of transgenic animals (see Materials and Methods for details). The frame shift mutant is a derivative of the minimal rescuing clone. Four bases (GCGC) have been inserted in a unique KasI site (see Materials and Methods for details). The cDNA clone is aligned to the plasmids to show the intron–exon relationship. Restriction site symbols: *F*, SphI; *K*, KpnI; *L*, SalI; *S*, SacI; *V*, EcoRV; *Z*, XhoI. The 1-kbp scale bar does not apply to the cosmid clones, which are ~40 kb long.

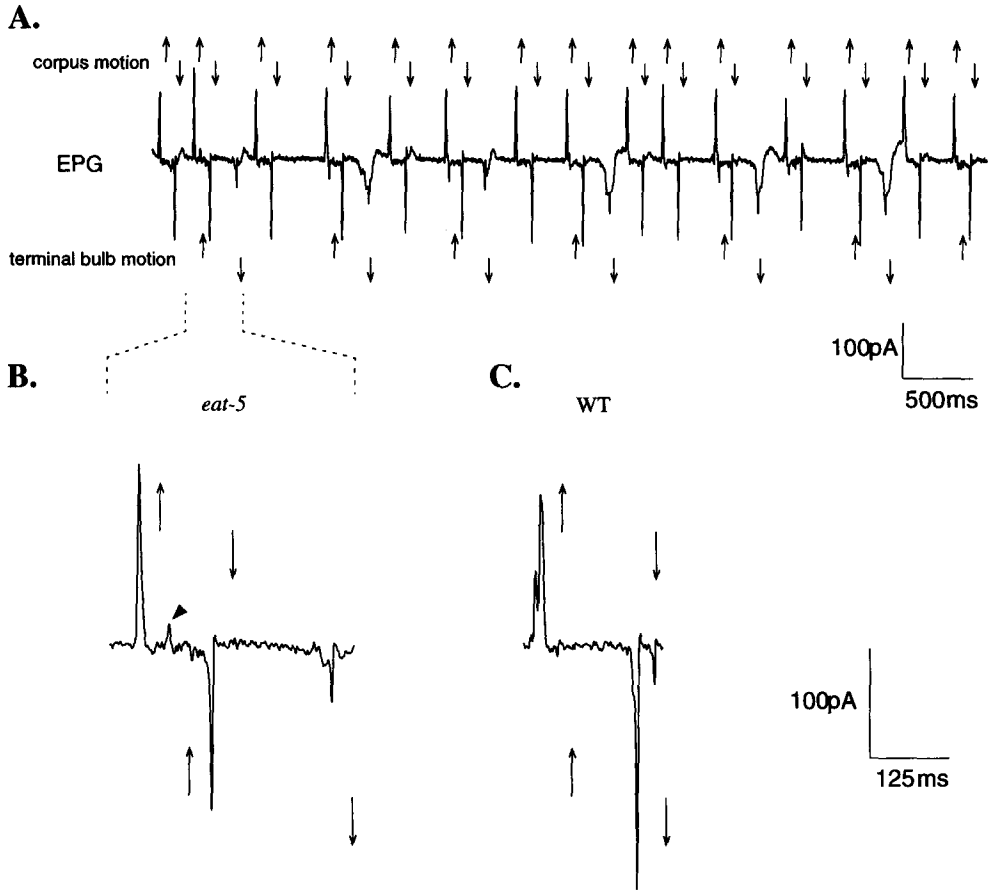


Figure 2. Electrical recording from an *eat-5* mutant pharynx. The timing of pharyngeal muscle motion was correlated with the EPG record. An upward arrow marks the time when the first sign of muscle contraction was perceived. A downward arrow marks the time when the first sign of muscle relaxation was seen. (*A*) A continuous record of *eat-5(ad464)* over a period of 15 pumps. (*B*) An expanded view of a pump in which a small depolarization peak (*arrowhead*) can be clearly seen during the plateau phase. (*C*) A representative record of a wild-type pump. Note that there is no depolarization peak during the pump. The upper calibration applies to *A*, the lower to *B* and *C*.

gans physical map (Coulson et al., 1986, 1988). *let-75* is probably identical to the cloned pharyngeal myosin heavy chain gene *myo-1* (Miller et al., 1986; Albertson, 1985; Hoppe, P., A. Rose, and R. Waterston, personal communication). Two cosmids (B0332 and T12B9) adjacent to *myo-1* were able to rescue the *eat-5* mutant phenotype in transgenic worms. By isolating a hybridizing clone, λTB3C, from a λ genomic library and subcloning, we narrowed the rescuing activity down to a 3.7-kb genomic fragment contained in pRE5-7 (Fig. 1).

This fragment was used as a hybridization probe to identify a single cDNA clone from a λgt10 library (provided by S. Kim). Additional cDNA sequences were obtained by RT-PCR. Comparison of the genomic and cDNA sequences allowed us to identify intron/exon boundaries (Fig. 1). To verify that the open reading frame predicted by the cDNAs is necessary for *eat-5*(+) activity, we tested whether a frame-shift mutation in the second exon would abolish the rescuing activity of the 3.7-kb genomic fragment. A 4-bp insertion was introduced at a unique KasI site to alter the predicted amino acid sequence from WAPFIMAIEA to WARAIYYGN(opal). We found that the mutant fragment indeed showed no rescuing activity. Rescuing activity was restored when the mutation was reverted back to wild type by deleting these 4 bp. The conceptual translation product of the *eat-5* gene, 423 amino acids in size, was found to share extensive sequence similarity to UNC-7 (38% identity, 62% similarity), ogre (25% identity, 55% similarity), and pas (26% identity, 50% similarity) (see Fig. 6). A single low abundance *eat-5* transcript, ~1.7 kb in size, can be detected on Northerns at roughly equivalent levels in all developmental stages (data not shown).

Pharyngeal Muscle Action Potentials Are Unsynchronized in eat-5 Mutant Pharynxes

Muscle contractions of the pharyngeal corpus (the anterior pharynx) and terminal bulb (posterior pharynx; see Fig. 3 *A*) are normally tightly synchronized. Avery and Horvitz (1989) proposed that contractions are synchronized by electrical coupling of pharyngeal muscle cells. The gene *eat-5* is defined by a single allele (*ad464*), which causes unsynchronized contractions (Avery, 1993). In addition to synchronized corpus and terminal bulb contractions like those of wild-type worms, *eat-5* mutants have corpus contractions that occur without terminal bulb contraction, and terminal bulb contractions that are delayed with respect to corpus contractions. We therefore proposed that *eat-5* might affect electrical coupling of pharyngeal muscle. To test this hypothesis, we made simultaneous electrical recordings (electropharyngeograms [EPGs]) (Raizen and Avery, 1994) and video recordings from *eat-5* mutant pharynxes.

Fig. 2 shows results from such comparative recordings. Every muscle action potential produces two current transients in the EPG, a positive one caused by the beginning of the action potential, followed by a negative one caused by the end of the action potential. In wild-type pharynxes, corpus and terminal bulb motions are synchronized, and,

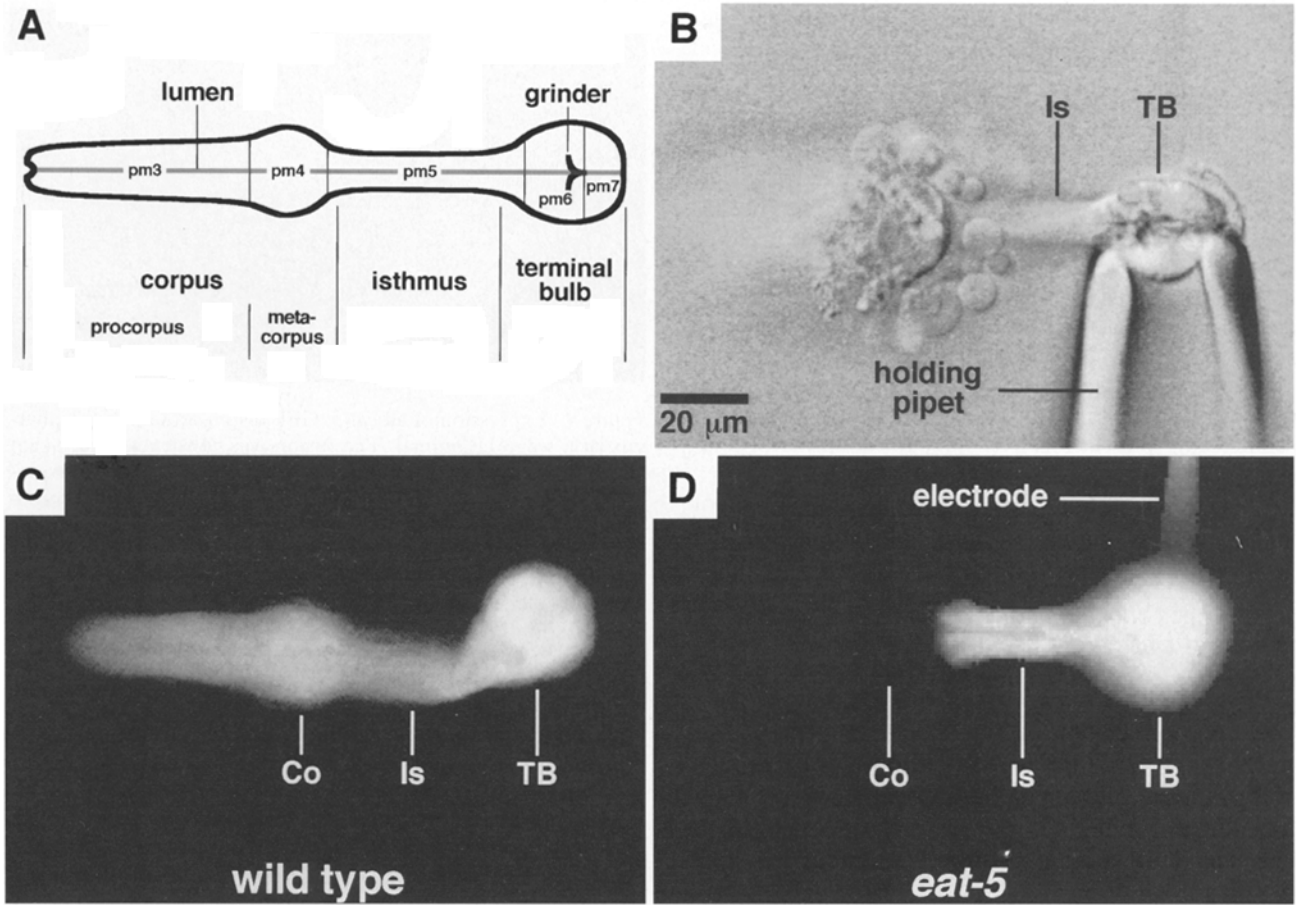


Figure 3. Dye-coupling of wild-type and *eat-5* mutant pharynxes. (*A*) Anatomy of the pharynx. Anterior is to the left. The pharynx is divided into three functional parts: the corpus, the isthmus, and the terminal bulb. The corpus is further subdivided into the procorpus and metacorpus. There are five types of large muscles in the pharynx, arranged from anterior to posterior: pm3 in the procorpus, pm4 in the metacorpus, pm5 in the isthmus, and pm6 and pm7 in the terminal bulb. Each cell runs the entire length shown. (For instance, each pm5 cell runs the entire length of the isthmus.) There are three cells of each type, arranged with triradiate symmetry around the central lumen (Albertson and Thomson, 1976). (*B–D*) Terminal bulb muscles of dissected pharynxes were impaled with sharp electrodes containing a solution of fluorescent dye, and the dye was injected by iontophoresis. Anterior is to the left. Bar in *B* also applies to *C* and *D*. (*B*) This Nomarski differential interference contrast micrograph shows a pharynx being injected. The microscope was focused on the terminal bulb; the corpus is not visible. A dimple can be seen on the upper side of the terminal bulb where the electrode penetrates, but the electrode itself is invisible. (*C*) A wild-type pharynx. Dye spread throughout the terminal bulb (*TB*), isthmus (*Is*), and corpus (*Co*). (*D*) An *eat-5* mutant pharynx. Dye spread through the terminal bulb and isthmus (muscles pm5, pm6, and pm7), but did not cross into the corpus (muscles pm3 and pm4). The microscope was focused on the isthmus/corpus boundary. The terminal bulb is out of focus in this picture. This pharynx was still impaled at the time the picture was taken—the electrode filled with fluorescent dye can be seen at the upper right. This is the same pharynx as in *B*. *A* is reproduced and adapted from *Neuron*, 1989, Vol. 3, pp. 473–485 by copyright permission of Cell Press.

as expected, the current spikes indicating the start and end of the muscle action potentials occurred close together. A pair of positive transients preceded the contractions by ~30 ms, and two negative transients preceded the relaxations by ~30 ms (Fig. 2 *C*). Previous work showed that the first large negative transient corresponds to the end of the corpus action potential, and the second, smaller one corresponds to the end of the terminal bulb action potential (Raizen and Avery, 1994).

To analyze the *eat-5* recordings (Fig. 2, *A* and *B*), we first looked at corpus contraction–relaxation cycles that occurred without any terminal bulb motions. In each case, the electrical record showed a large (>100 pA) positive spike ~30 ms before the contraction, and a large negative spike ~30 ms before the relaxation. Next, we looked at terminal bulb contraction–relaxation cycles in which the terminal bulb motions were not synchronized with corpus motions. In each case, a single small (<100 pA) spike preceded the contraction by ~30 ms, and a small negative spike preceded the relaxation by ~30 ms (Fig. 2 *B*). Every corpus motion corresponded to a large spike, and every terminal bulb motion to a small spike, except when corpus signals obscured the terminal bulb signals.

We conclude that the unsynchronized motions in *eat-5* mutant pharynxes are caused by unsynchronized muscle action potentials. This is particularly clear in cases where the corpus and terminal bulb action potentials overlapped as in Fig. 2 *B*—the beginning and end of each of the two action potentials can be identified and correlated with the contraction and relaxation of the corresponding muscle. These

results are consistent with the hypothesis that *eat-5* is necessary for normal electrical coupling of pharyngeal muscles.

Dye-coupling of Pharyngeal Muscles Is Reduced in eat-5 Mutants

To test whether the cytoplasms of pharyngeal muscle cells (Fig. 3 *A*) are functionally coupled by gap junctions, we injected carboxyfluorescein into terminal bulb muscles of four wild-type pharynxes (Fig. 3 *B*). In every case, the dye spread from a single terminal bulb muscle cell into all the large muscle cells of the pharynx (Fig. 3 *C*). Dye injected into the terminal bulbs of four *eat-5* mutant pharynxes spread through the terminal bulb and isthmus, but it failed to spread into the corpus (Fig. 3 *D*). These results support the conclusion that *eat-5* is necessary for coupling of pharyngeal muscle cells.

The expression pattern of a translational gene fusion of *eat-5* to the GFP (Chalfie et al., 1994) is consistent with *eat-5* playing a role in coupling pharyngeal muscle cells. This fusion gene is derived from the *eat-5* rescuing genomic fragment (pRE5-7; Fig. 1) with the GFP sequences replacing the COOH-terminal 64 amino acids and 3′ untranslated region of *eat-5*. The polypeptides expressed from this gene fusion can be detected in the pharyngeal corpus (primarily metacorpus) and isthmus at all larval and adult stages (Fig. 4).

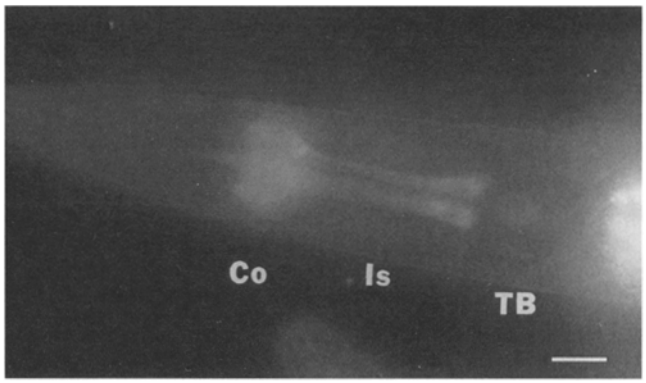


Figure 4. Expression of an *eat-5*::GFP fusion protein in the pharynx of a stage L3 animal. The fusion was constructed such that GFP replaces the COOH-terminal 64 amino acids of the predicted EAT-5 sequence. Autofluorescence due to metabolic products in the intestine (gut granules) is visble on the far right and is unrelated to expression of the fusion polypeptides. *Co*, corpus; *Is*, isthmus; *TB*, terminal bulb.

PCR Amplification of Other Family Members in C. elegans

A comparison of the UNC-7, pas, and ogre amino acid sequences allowed us to design oligonucleotide primers to screen for other family members in *C. elegans* using PCR methods. Two highly conserved regions ~60 amino acids apart were chosen, and degenerate oligonucleotides corresponding to the predicted peptide sequences QYVGXPI/M (5′ primer) and YYQWVXF (3′ primer) were used in the PCR amplification procedure.

We initially carried out PCR amplification of genomic DNA to avoid biased representation of particular genes in cDNA libraries. The products resulting from this amplification comprised a collection of fragments of ~250 bp, as well as a less abundant collection ranging in size up to ~600 bp. We concentrated our efforts on the smaller size fragments, which more closely corresponded to the expected fragment size of family members. Four family members were identified based on their sequence similarity to *unc-7*. One of these members corresponded to the EST cm10a8 (Waterston et al., 1992), already present in the database. The other three members were previously unidentified and were designated pcr32, pcr49, and pcr55. The sequences obtained for three of these clones predicted an uninterrruped open reading frame; for the fourth, pcr55, a small intron appeared to interrupt the open reading frame. The presence of this intron was confirmed by sequencing a cDNA corresponding to pcr55 (data not shown).

Since family members that might have large introns in this region would be excluded under our amplification conditions, we extended our screen by amplifying by PCR a cDNA library (provided by R. Barstead) using the same set of degenerate primers. The products of PCR amplification, in this case, were restricted to a collection of fragments of ~250 bp. From this collection, we isolated an additional family member and found it to be identical to another previously identified EST, wEST02207 (McCombie et al., 1992).

Computer database searches revealed that two other EST sequences shared similarity throughout the region amplified by our PCR experiments: cm9d9 (Waterston et al., 1992) and wEST01007 (McCombie et al., 1992). One other EST sequence in the database (cm18f10; Waterston et al., 1992) appeared to be similar to this region as well (Barnes, 1994), although the available DNA sequence has several ambiguities and does not extend through the region corresponding to our 3′ primer. The presence in public databases of these EST cDNAs related to *Pas*, *l(1)ogre*, and *unc-7* has been reported previously (Krishnan et al., 1993; Barnes, 1994).

Of those family members that had not been placed on the physical map, we mapped physically pcr32, 49, and 55, and the ESTs wEST01007, wEST02207, and cm9d9 (Fig. 5). Family members were mapped either by fingerprint analysis of corresponding genomic clones (courtesy of A. Coulson) or by hybridization of gene-specific probes to a filter gridded with YAC clones (Coulson et al., 1988). Recently, the *C. elegans* genomic sequencing project (Waterston et al., 1993) has determined the genomic sequences corresponding to pcr32 and pcr55, and the ESTs wEST01007 and cm9d9. Additional members of this gene family have been identified by the sequencing project, and we now know of at least 16 family members in *C. elegans*. Their positions on the physical map are indicated in Fig. 5. Further work is needed to determine whether or not any of these family members corresponds to genes with known mutant phenotypes.

Predicted Polypeptide Sequence and Topological Model

Hydrophobicity analyses of ogre and pas led to predictions of a single transmembrane domain for each protein (Watanabe and Kankel, 1990; Krishnan et al., 1993). Hydrophobicity analysis of UNC-7, however, suggested as many

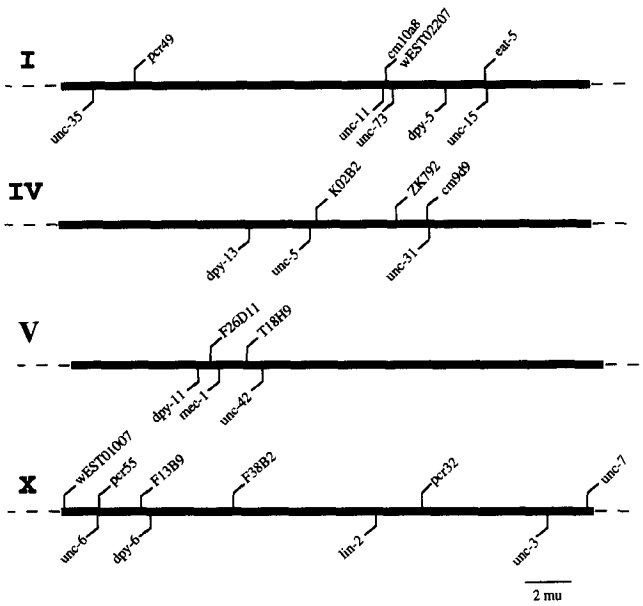


Figure 5. Genetic map positions of family members in *C. elegans*. Two family members are located on the cosmid ZK792.

as four transmembrane domains for this polypeptide (Starich et al., 1993). To compare a more complete set of family members over their entire coding regions, we sequenced cDNAs corresponding to three other *C. elegans* family members in addition to *eat-5*.

Fig. 6 shows an alignment of the predicted amino acid sequences corresponding to these cDNAs, as well as the sequences for pas, ogre, and UNC-7. Several strongly conserved regions are evident. The greatest similarity among the *C. elegans* sequences is between UNC-7 and PCR55 (59% identity, 78% similarity), while the least among these family members is between PCR55 and CM9D9 (30% identity, 53% similarity). For comparison, using the same parameters, UNC-7 shares 28% identity and 59% similarity with pas, and 24% identity and 55% similarity with ogre; ogre and pas share 47% identity and 69% similarity. The UNC-7 sequence differs in having a much longer (~125–amino acid) predicted amino terminus, although a second methionine aligns well with the predicted starts of the other polypeptides. The cm9d9 cDNA appears not to be full length, perhaps missing 10–20 amino acid residues as predicted from comparison to the other sequences.

Application of the program of Jones et al. (1994) for predicting the topology of integral membrane proteins leads to a remarkably consistent model for most family members, in contrast to the results obtained using programs based solely on hydrophobicity. Whereas the latter methods rely on hydrophobicity values assigned to individual amino acids, the method of Jones et al. relies on values derived from an analysis of the frequency of occurrence of particular amino acids believed to lie in intracellular, extracellular, or transmembrane domains in proteins for which some experimental evidence indicates a transmembrane location.

For all of the polypeptides except EAT-5, four transmembrane (TM) domains are predicted, with intracellular amino and carboxyl termini (Figs. 6 and 7 *B*). These TM domains are well aligned when comparing the polypeptide sequences to one another. Although only three TM domains are predicted for EAT-5, the EAT-5 amino acid sequence shows similarity to the other family members in all four predicted TM domains. Furthermore, the program used for predicting topology has a slight tendency to underpredict TM domains (Jones et al., 1994). We therefore think it is likely that EAT-5 has the same topology as the other family members.

The topological model predicts two extracellular loops, which together with the TM domains account for virtually all of the sequences conserved among family members. The first extracellular loop is predicted to be flanked by the regions used to design the degenerate oligonucleotides used for PCR amplification and includes two invariant cysteines. The second extracellular loop also contains two invariant cysteines as well as several highly conserved residues.

Subcellular Localization of wEST01007 Fusions

We attempted to determine the subcellular location of a family member protein to see whether it would be consistent with a role in establishing gap junctions between cells. We determined by Northern analyses that the family member wEST01007 (McCombie et al., 1992) is abundantly expressed in embryos (data not shown). Because of the abundance of transcripts and because cells are relatively large in early embryos, we examined the subcellular location of proteins produced from translational fusions of wEST01007 sequences to *lacZ* and GFP.

We transformed worms with constructs that included ~3 kb of genomic sequence upstream of the splice acceptor site for the trans-spliced leader SL1 (Krause and Hirsh, 1987), and most of the coding region of the gene corresponding to wEST01007 (data not shown) fused to *lacZ* or GFP reporter sequences. The reporter gene sequences were positioned ~30 amino acids downstream of the fourth predicted TM domain (after the sequence DEKAMIAS; Fig. 6). Constructs with these two reporter genes gave similar results for fusion protein expression and localization. Expression of these fusion genes is first detectable at about the 28-cell stage and becomes abundant in apparently most or all cells until the early stages of morphogenesis (Fig. 8, *A* and *B*). The fusions appear to be highly expressed in the anterior of embryos during morphogenesis (although still expressed posteriorly), and abundant expression eventually becomes limited primarily to the isthmus and terminal bulb regions of the pharynx in L2 and later larval stages (Fig. 8 *C*). In addition, expression is seen in the sex muscle cells during the L4 stage (Fig. 8 *D*). During all these stages, the fusion proteins appear to be located at or near the plasma membrane and are localized in striking punctate or disk-shaped patterns. In the sex muscle cells, which are linearly arranged during the L4 stage, the fusion proteins appear to be concentrated at cell–cell interfaces.

Discussion

We have identified the *C. elegans* gene *eat-5* as a homolog

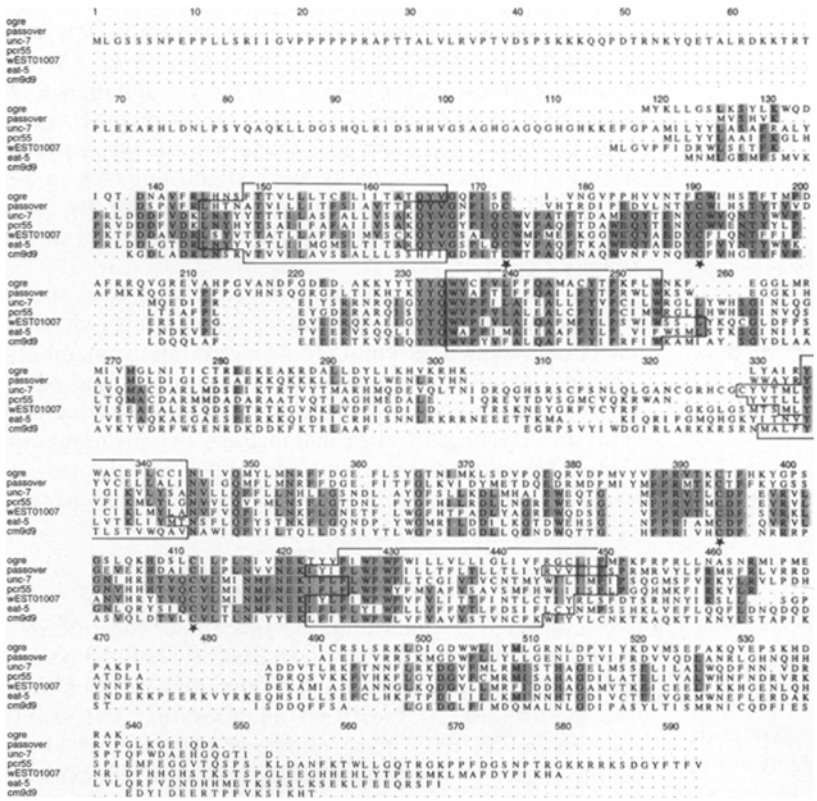


Figure 6. Sequence alignment of the predicted products of the Drosophila genes *Pas* and *l(1)ogre*, and the *C. elegans* family members represented by *unc-7*, *eat-5*, *cm9d9*, wEST01007, and pcr55. Predicted transmembrane domains are outlined. The positions of invariant cysteines are starred. Identical residues in four or more family members are shaded. The alignment was derived from the published sequences of *Pas* (Krishnan et al., 1993; EMBL/GenBank/DDBJ accession number L13306), *l(1)ogre* (Watanabe and Kankel, 1992; EMBL/GenBank/DDBJ accession number X61180), and *unc-7* (Starich et al., 1993; EMBL/GenBank/DDBJ accession number Z19122). The sequence data for the remaining family members are available from EMBL/GenBank/DDBJ under accession numbers U59212 (pcr55), U59211 (wEST01007), U59210 (*eat-5*), and U59213 (cm9d9).

of *unc-7*, *l(1)ogre*, and *Pas* and have established that *eat-5* and *unc-7* are just two of at least 16 members of this gene family in *C. elegans*. It is very likely that more family members remain to be identified in *C. elegans*, since the set of family members detected by PCR screens and the set identified by database searches are not completely overlapping.

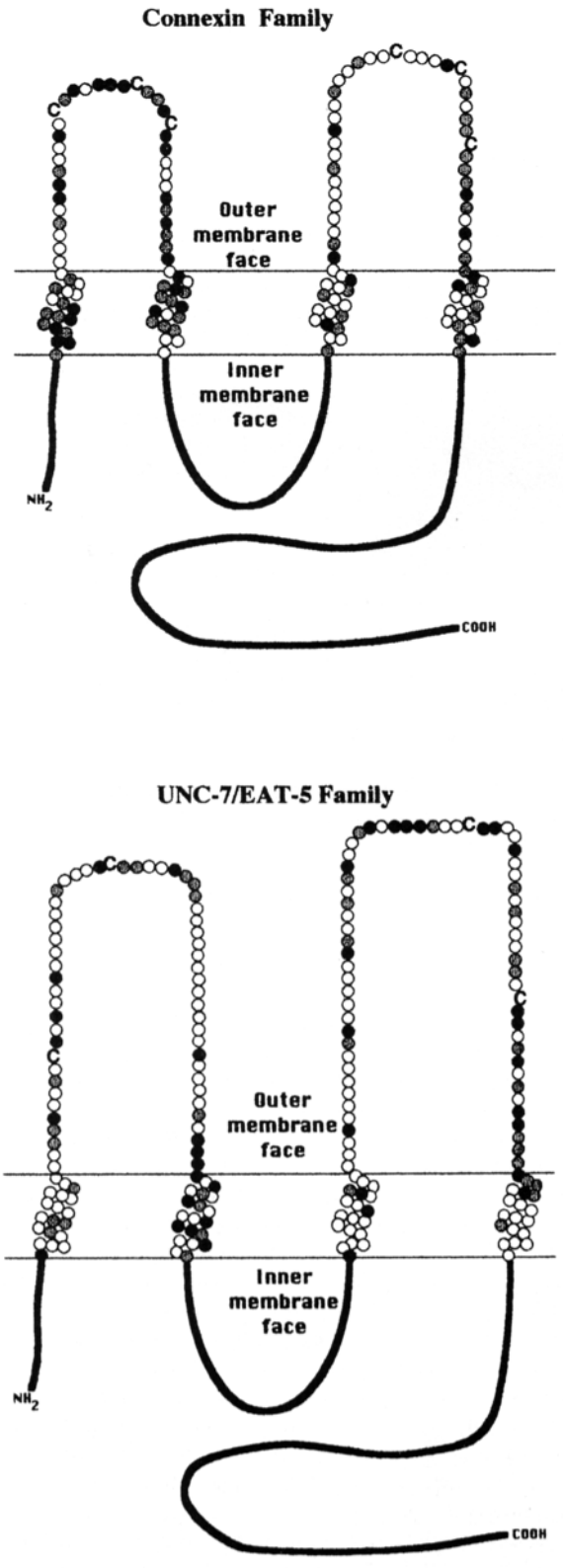
Do the Pas-related Genes Encode Gap Junction Channel Proteins?

The function of the proteins related to pas is still uncertain; however, it is clear from the analysis of mutants that these proteins are involved in cell–cell interactions and that they affect the formation or function of gap junctions. As described more fully below, our data support the hypothesis that proteins of this family are gap junction proteins. In summary: (*a*) there are predicted structural similarities between the proteins homologous to pas and proteins of the connexin family of vertebrate gap junction proteins; (*b*) fusion polypeptides of the family member wEST01007 are localized in a punctate pattern at or near plasma membranes, a location expected for gap junction plaques; and (*c*) mutations in family members result in defects in cell–cell coupling via gap junctions. Previously, it was known that *Pas* mutants fail to make functional gap junctional synapses between specific neurons and that an *unc-7* mutant forms ectopic gap junctions between particular classes of neurons. Our data show that in *eat-5* mutants, there is a loss of functional coupling between muscle cells in the posterior and anterior pharynx of *C. elegans.*

Comparison of Proteins Homologous to pas with Connexins

Although there is no obvious amino acid sequence similarity between polypeptides related to pas and the connexins, there appear to be structural similarities between proteins of these two families. The program of Jones et al. (1994) predicts that the polypeptides encoded by *Pas*-related genes share a common membrane topology of four transmembrane domains with cytoplasmic amino and carboxyl termini. Proteins of the vertebrate connexin family are predicted to have the same topology (Fig. 7). Comparison of pas-related polypeptides shows that within the family, there is extensive amino acid sequence conservation in the predicted transmembrane domains and the two extracellular loops; there is little, if any, conservation found outside of these regions. The highest degree of amino acid sequence conservation among connexins is also in their proposed extracellular loops and transmembrane domains (Fig. 7), although there is some sequence conservation in their amino termini. Therefore, for both families it appears that the same structural domains of the proteins, i.e., transmembrane domains and extracellular loops, are very important to the general function of family members.

In the connexin family, sequence conservation in the extracellular loops is thought to reflect the requirement for recognition and docking between gap junction hemi-channels in apposed cells. Comparisons of the extracellular domains of the connexins with proteins related to pas show no apparent amino acid sequence conservation; however, within each family, there are invariant cysteine residues in the two extracellular loops. The extracellular loops in the



vertebrate connexin family are smaller (35 residues in the first loop, 39–41 residues in the second) than the loops in pas-related polypeptides (for *C. elegans*, 58 residues and 67 residues; slightly longer in the *Drosophila* members). Vertebrate connexins have three invariant cysteines per extracellular loop, whereas there are two cysteines per predicted extracellular loop in pas-related proteins. Barnes (1994) has recently proposed a model for the family of pas homologs in which four conserved cysteines are found in a second extracellular loop. However, in the alignment presented here, which includes more full-length sequences, two of these cysteines do not appear to be conserved. In addition, the location of the third TM domain predicted by the program of Jones et al. differs from the model proposed by Barnes. The topology predicted by Crompton et al. (1995) for family members is in close agreement with the model that we present.

Several properties of invertebrate and vertebrate gap junctions appear to be different. Therefore, it may not be surprising that there is no obvious amino acid sequence similarity between the vertebrate connexins and proteins related to pas. Ultrastructural differences have been noted in the gap junction plaques examined in vertebrates and invertebrates by EM techniques (for review see Lane and Skaer, 1979). Upon freeze fracture, gap junction particles examined in vertebrate tissue tend to fracture onto the P face; in invertebrate preparations, the junction particles tend to fracture onto the E face (inverted gap junctions; Flower, 1972). Additionally, invertebrate junctions have larger ultrastructural dimensions and may also have pore diameters larger than those estimated for vertebrate junctions (Schwarzmann et al., 1981; Zimmerman and Rose, 1985).

Subcellular Location of wEST01007 Fusion Proteins

Gap junctions form in plasma membranes where cells contact each other, and junctional particles often cluster as tightly packed aggregates of membrane channels. These aggregates are readily visualized as a plasma membrane plaque by immunocytochemistry using anti-connexin antibodies on most vertebrate cells. The localization pattern of proteins produced from fusions of wEST01007 to *lacZ* or GFP is consistent with this organization. Because we observe the same punctate pattern of membrane localization with both *lacZ* and GFP tags, it is unlikely that the pattern is due to the reporter domains. Cells of *C. elegans* embryos are coupled by gap junctions early in embryogenesis (Bossinger and Schierenberg, 1992), and it seems possible that the gene corresponding to wEST01007 encodes a channel subunit of these gap junctions.

Figure 7. Topological family models for connexins and the *C. elegans* proteins related to UNC-7 and EAT-5. (*A*) Model for connexin family members (derived from Kumar and Gilula, 1992). Invariant (*black*) and conserved (*gray*) residues predicted to lie in transmembrane or extracellular locations are indicated, as are the individual invariant cysteines located in the extracellular loops. (*B*) Corresponding model for *C. elegans* proteins similar to UNC-7 and EAT-5. The extracellular loops represented here are predicted to be slightly larger for the *Drosophila* homologs.

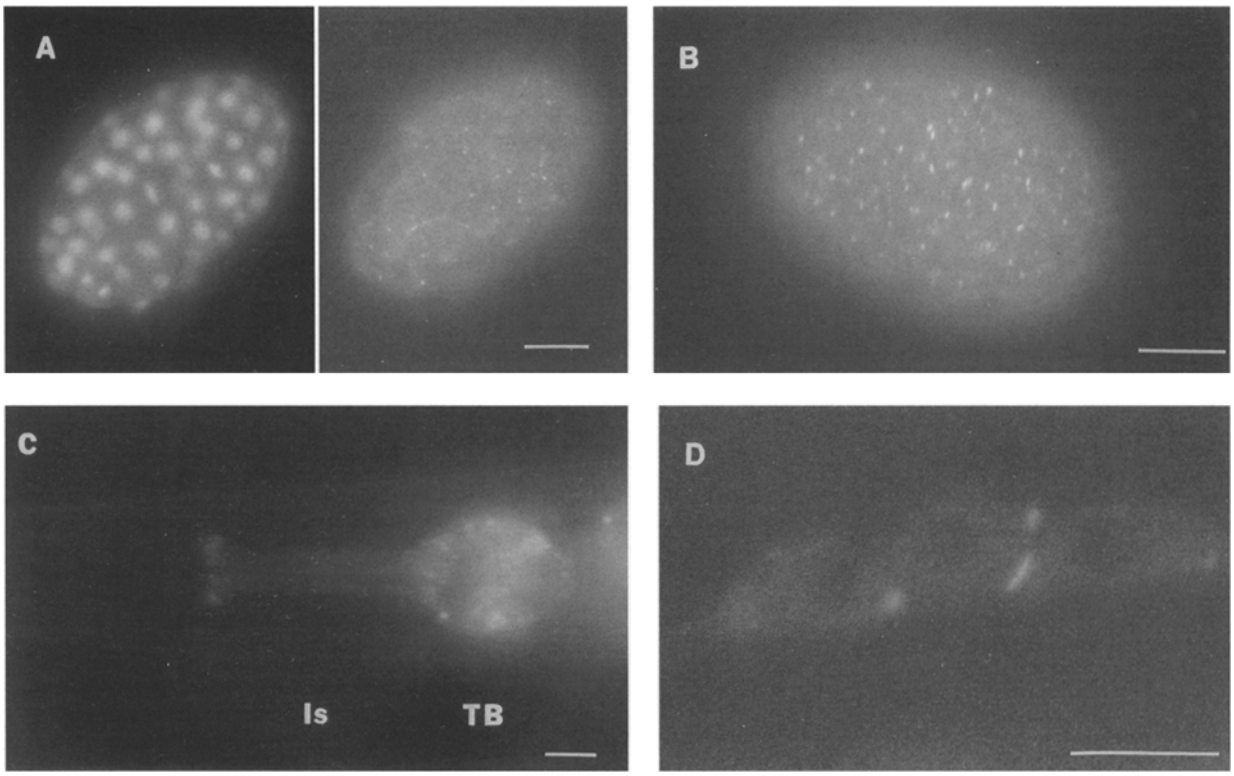


Figure 8. Localized expression of a wEST01007::GFP fusion polypeptide. Most of the coding region of wEST01007 (including the four predicted TM domains) plus 3 kb of sequences upstream of the transcriptional initiation site were fused to GFP. The construct was used to transform *C. elegans* and the extrachromosomal array integrated using gamma-irradiation. (*A*, *left*) Embryo stained with 4′,6-diamidino-2-phenylindole to visualize nuclei; (*A*, *right*) GFP fluorescence in same embryo; (*B*) GFP fluorescence in ~300-cell stage embryo; (*C*) pharyngeal expression at L4 stage (*Is*, isthmus; *TB*, terminal bulb; autofluorescence in anterior portion of intestine, visible on far right, is due to gut granules); (*D*) three adjacent sex muscle cells at L4 stage. Bar, 10 μm.

Phenotypes of Pas, unc-7, and eat-5 Mutants

Previous examination of *unc-7* and *Pas* mutant animals revealed defects in specific gap junctional connections in the nervous system. In neuronal *Pas* mutants, functional electrical synapses fail to be made between the giant fiber cells and their targets, the peripherally synapsing interneurons and the tergotrochanteral motor neurons. This defect does not seem to be due to mispositioning of the neurons since the neuronal processes still appear to come into close contact. Instead, the neurons appear to be unable to establish or to maintain their connections (Baird et al., 1993). This mutant phenotype would fit well with a loss of gap junctions. In an *unc-7* mutant, ectopic gap junctions between AVA interneurons (required for backward locomotion) and VB and DB motorneurons (which function in forward locomotion) have been observed by EM. If the *unc-7* mutant that was analyzed exhibits a null phenotype, then it is not straightforward to explain how the formation of ectopic gap junctions might result from a loss of gap junction subunits. One possibility is suggested by the observation in vertebrates that the second extracellular domain of connexins can determine whether intercellular connexon channels are formed (White et al., 1994). One could imagine, for example, that in wild-type worms the gap junctions in VB and DB motorneurons contain hemi-channels composed of mixed subunits encoded by *unc-7* and a different family member. These mixed subunit hemi-channels do not readily interact with the gap junction subunits present on the AVA internueron, and thus gap junctional connections are not established (or maintained). However, with the loss of UNC-7 from the VB and DB motorneurons, the remaining gap junction subunits are now compatible with those on AVA, and ectopic gap junctions form.

Our analysis of *eat-5* mutants demonstrates the loss of functional electrical coupling and dye-coupling between muscles of the pharyngeal terminal bulb and corpus. The pharyngeal muscle is an epithelium in which individual muscle cells are connected by desmosomes (Albertson and Thomson, 1976), and its integrity is necessary to separate the ionically controlled pseudocoelom from the fluid outside the worm. Since the epithelial functions of pharyngeal muscle are normal in *eat-5* mutants, we know that the loss of coupling is not caused by loss of contact between the muscle cells. Even though action potential synchrony is not completely lost in *eat-5* mutant adults, there was no detectable dye-coupling between corpus and terminal bulb. It may be that a small amount of residual gap junction coupling, too weak to allow detectable dye-coupling, is sufficient for partial synchronization of action potentials. It is also possible that pharyngeal neurons, although not necessary for synchronization of corpus and terminal bulb muscle contractions (Avery and Horvitz, 1989), are responsible for partial synchronization of action potentials in the absence of coupling between muscle cells.

The expression of an *eat-5*::GFP fusion protein in the metacorpus and isthmus of the pharynx is consistent with *eat-5* being necessary for coupling muscles of the corpus to the posterior pharynx. It is also possible that dye injected into the terminal bulb can diffuse into the isthmus via junctions formed by expression of wEST01007 or another family member (Fig. 8 *C*), whereas dye transfer from the isthmus into the corpus requires expression of *eat-5*(+).

Other Possible Functions of Family Members

It is possible that this family of molecules does not encode structural components of gap junctions, but instead affects the formation or function of gap junctions by some other mechanism. For instance, cell adhesion molecules have been shown to be important in facilitating the formation of gap junctions (Mege et al., 1988; Keane et al., 1988; Jongen et al., 1991; Meyer et al., 1992). The protein localization that we have observed would be consistent with a cell adhesion molecule, and mutations in cell adhesion molecules could also account for the mutant phenotypes described here. Functional assays involving expression of these genes in heterologous systems, such as *Xenopus* oocytes or tissue-culture cells (Werner et al., 1985; Swenson et al., 1989; Zhu et al., 1991), could be useful in determining whether members of this family can function as gap junction proteins to couple cells electrically or allow dye transfer between cells.

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