The *Caenorhabditis elegans unc-93* Gene Encodes a Putative Transmembrane Protein That Regulates Muscle Contraction

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Abstract. *unc-93* is one of a set of five interacting genes involved in the regulation or coordination of muscle contraction in *Caenorhabditis elegans.* Rare altered-function alleles of *unc-93* result in sluggish movement and a characteristic "rubber band" uncoordinated phenotype. By contrast, null alleles cause no visibly abnormal phenotype, presumably as a consequence of the functional redundancy of *unc-93.* To understand better the role of *unc-93* in regulating muscle contraction, we have cloned and molecularly characterized this gene. We isolated transposon-insertion alleles and used them to identify the region of DNA encoding the unc-93 protein. Two unc-93 proteins differing at their NH_2 termini are potentially encoded by transcripts that differ at their 5′ ends. The putative unc-93 proteins are 700 and 705 amino acids in length and have two distinct regions: the NH_2 terminal portion of 240 or 245 amino acids is extremely hydrophilic, whereas the rest of the protein has multiple potential membrane-spanning domains. The *unc-93* transcripts are low in abundance and the *unc-93* gene displays weak codon usage bias, suggesting that the unc-93 protein is relatively rare. The unc-93 protein has no sequence similarity to proteins listed in current databases. Thus, *unc-93* is likely to encode a novel membrane-associated muscle protein. We discuss possible roles for the unc-93 protein either as a component of an ion transport system involved in excitation–contraction coupling in muscle or in coordinating muscle contraction between muscle cells by affecting the functioning of gap junctions.

ANIMAL behavior is produced through a complex series of events that result in specific muscle contractions and relaxations. Neuronal inputs to a muscle cell can cause membrane excitation, which is coupled to the mechanical sliding of the thick and thin filaments of the myofilament lattice (Shepherd, 1988). Although much is known about the structure and function of the myofilaments (Leavis and Gergely, 1984; Kamm and Stull, 1985; Wang, 1985; Warrick and Spudich, 1987) and of some of the proteins involved in excitation–contraction coupling (Catterall et al., 1988; Fill and Coronado, 1988; Jan and Jan, 1989), a complete description of the components of this signal transduction pathway has not yet been achieved.

The nematode *Caenorhabditis elegans* is particularly appropriate for the study of muscle, as genetic, biochemical, and morphological analyses of *C. elegans* muscle are all straightforward. More than 30 genes have been identified by mutations that affect muscle structure and function in *C. elegans* (reviewed by Waterston (1988)). Some of these genes encode structural components of the myofilaments, such as myosin heavy chains (Epstein et al., 1974; MacLeod et al., 1977, 1981; Waterston, 1989), actin (Landel et al. 1984), and paramyosin (Waterston et al., 1977; Kagawa et al., 1989). Mutations in other genes severely decrease motility with comparatively minor structural defects in the myofilament lattice; these mutations presumably disrupt the regulation or coordination of muscle contraction. For example, mutations in the gene *unc-22* result in an uncontrolled, almost incessant, twitching of the body-wall muscles, which suggests a regulatory defect (Brenner, 1974; Waterston et al., 1980). *unc-22* encodes a huge 750-kD protein, named twitchin, with a protein kinase domain and several copies of two motifs found in members of the immunoglobulin superfamily, myosin light chain kinase, and titin (Benian et al., 1989). Both genetic and molecular studies indicate that *unc-22* interacts with the *unc-54* myosin heavy chain (Moerman et al., 1982; Mori et al., 1988), which suggests that *unc-22* acts in conjunction with myosin at the end of the excitation-contraction coupling signal transduction pathway.

unc-93 is one of a set of five interacting genes (*unc-93, sup-9, sup-10, sup-11,* and *sup-18*) involved in muscle structure and function (Greenwald and Horvitz, 1980, 1982, 1986). *unc-93(e1500), unc-93(n200),* and *sup-10(n983)* are rare altered-function mutations that confer a distinctive abnormality in the regulation of *C. elegans* muscle contraction termed "rubber band" (Greenwald and Horvitz, 1980, 1986). When a rubber band mutant is touched on its head, the animal contracts and then quickly relaxes without moving backwards, whereas a wild-type worm simply moves backwards. Thus, rubber band mutants can contract their body wall muscles, but the regulation or coordination of the contraction is defective. These mutants are sluggish and flaccid, characteristics typical of *C. elegans* muscle mutants (Waterston et al., 1980), but have only minor structural defects in their body wall muscles (Greenwald and Horvitz, 1980; Waterston, 1988). Rubber band mutants are defective in egg laying, which indicates that there are abnormalities in the vulval and uterine muscles as well as in the body wall muscles (Greenwald and Horvitz, 1980, 1986). Genetic mosaic analysis has shown that *sup-10* functions within muscle cells (Herman, 1984).

The rubber band mutations do not eliminate gene activity, but rather produce abnormal protein products that disrupt muscle function (Greenwald and Horvitz, 1980, 1986). Loss-of-function, or null, mutations in *unc-93* or *sup-10* confer no visibly abnormal phenotype, presumably because these two genes are functionally redundant with another gene (or set of genes) that has sufficient overlap with *unc-93* and *sup-10* in regulating muscle contraction so as to maintain normal muscle function. Null mutations in *unc-93, sup-10, sup-9,* and *sup-18* were identified as recessive suppressors of the rubber band mutations (Greenwald and Horvitz, 1980, 1986). Further evidence that *unc-93* is likely to function in muscle is derived from this mutual suppression of *unc-93* and *sup-10* (Greenwald and Horvitz, 1980, 1986), which suggests that the two genes function in the same cell. Based upon previous detailed genetic analyses of these four genes, our model for their action is as follows. First, these genes function together to regulate muscle contraction, and their protein products interact with each other, possibly as a single protein complex. Second, the rubber band mutations produce aberrant gene products that cause defects in the regulation of muscle contraction. Third, the absence of the protein products of these genes does not adversely affect muscle contraction because these genes control a process that is functionally redundant with another process that regulates muscle contraction.

To understand how *unc-93* and the other functionally related genes regulate muscle contraction, we cloned *unc-93* using transposon tagging. In addition, we molecularly characterized the *unc-93* gene and identified the DNA sequence alterations in the rubber band mutations.

Materials and Methods

General Methods and Strains

General methods for the handling and culturing of *C. elegans* strains have been described by Brenner (1974). *C. elegans* was grown at 20°C, except in experiments with *daf-2(e1370ts)*, in which strains were maintained at 15°C and grown at 22.5° or 25°C to observe the Daf phenotype (Riddle et al., 1981; Swanson and Riddle, 1981). *C. elegans* variety Bristol strain N2 is the wild-type parent of all strains used in this work, except as described for the strains isolated in the *mut-2* genetic background (see below). The genetic markers used were as follows (unless otherwise noted, mutations are described by Hodgkin et al. [1988]): LGI: *unc-13(e51)*; LGII: *sup-9(n180)* and the new *sup-9* alleles identified in this work *(n1330, n1414, n1421, n1424, n1426, n1428, n1430, n1469)*; LGIII: *daf-2(e1370), unc-93(e1500, n200, e1500 n234, e1500 n243, e1500 n244, e1500 n246, e1500 n248, e1500 n254, e1500 n255)* and the new *unc-93* alleles identified in this work (*e1500 e2128* [M. Shen, personal communication]), *e1500 n1412, e1500 n1415, e1500 n1418, e1500 n1419, e1500 n1420, e1500 n1422, e1500 n1423, e1500 n1425, e1500 n1427, e1500 n1429, e1500 n1431, n1470, n1474, n1500, n1623* and *n1624* [D. Parry, personal communication], *e1500 n1907* and *e1500 n1912* [J. Thomas, personal communication]), *dpy-17(e164), sup-18(n1010)* and the new *sup-18* allele identified in this work *(n1539)*; LGV: *him-5(e1490)*; LGX: *sup-10(n183, n983)* and the new *sup-10* alleles identified in this work *(e2127, e2130,* and *e2131* (M. Shen, personal communication) and *n1413* and *n983 n1468).*

Isolation of Gamma Ray-induced Alleles

We mutagenized *sup-10(n983)* L4 hermaphrodites on Petri plates with gamma rays using a dose of 7,500 rads from a ^{60}Co source, as previously described by Greenwald and Horvitz (1980), and screened the F2 progeny for wild-type revertants. In the first experiment, *unc-93(n1470)* and *unc-93(n1474)* were isolated from about 12,000 haploid genomes screened. In a similar gamma-ray mutagenesis of *sup-10(n983)* animals, *unc-93(n1623)*, and *unc-93(n1624)* were isolated (D. Parry, personal communication). *unc-93(e1500 n1907)* and *unc-93(e1500 n1912)* were isolated as wild-type revertants among the F2 progeny of gamma ray-irradiated *unc-93(e1500)* hermaphrodites (J. Thomas, personal communication).

Isolation and Characterization of Putative Transposon-insertion Alleles

To isolate transposon-insertion alleles of *unc-93*, we constructed strains containing *unc-93(e1500)* or *sup-10(n983)* in a *mut-2* mutator background. These constructions were done using MT2879, a three-times backcrossed *mut-2(r459)* strain derived from TR674 (Finney, 1987; Collins et al., 1987). Because the *mut-2* activity in this strain is known to map very close to *unc-13* on LGI and because the males are sufficiently healthy to mate, this strain allows easy genetic manipulation of the *mut-2* activity (Finney, 1987). *mut-2* males were crossed with *unc-13; unc-93(e1500)* hermaphrodites, F1 cross-progeny of genotype *mut-2 +/+ unc-13; +/unc-93* were picked, and F2 Unc-93 non-Unc-13 progeny that did not segregate Unc-13 progeny (genotype *mut-2 +; unc-93*) were identified and used to establish a strain. This strain was screened for phenotypically wild-type revertants. The same strategy was used to construct a strain of genotype *mut-2(r459); sup-10(n983)*. One attempt to construct these strains yielded the mutation *sup-9(n1330)* as a preexisting mutation in a four times backcrossed *mut-2(r459)* strain. We obtained a total of 26 additional suppressor mutations and showed that these mutations failed to complement alleles of known genes. Of the revertants, 18 were derived from the *mut-2; unc-93(e1500)* strain: *sup-9(n1414, n1421, n1424, n1426, n1428, n1430), unc-93(e1500 n1412, e1500 n1415, e1500 n1418, e1500 n1419, e1500 n1420, e1500 n1422, e1500 n1423, e1500 n1425, e1500 n1427, e1500 n1429, e1500 n1431)*, and *sup-10(n1413)*. Four revertants were derived from the *mut-2; sup-10(n983)* strain: *sup-9(n1469), unc-93(n1500), sup-18(n1539)*, and *sup-10(n983 n1468)*. Four revertants were isolated from a strain with *unc-93(e1500)* in a mutator background derived from the mutator strains TR403, a wild isolate containing mutator activity, and TR679, which contains the *mut-2(r459)* mutation (M. Shen, personal communication; Collins et al., 1987): *unc-93(e1500 e2128)* and *sup-10(e2127, e2130, e2131)*. Because a transposon insertion event can occur in any generation, but the insertion will be detected only when homozygous, we isolated only one revertant from among the F2 progeny of any given worm in an attempt to insure the independence of the mutations. Among the *unc-93* alleles, only *unc-93(e1500 n1418)* and *unc-93(e1500 n1425)* could possibly be re-isolates of the same mutational event.

To remove the additional unlinked Tc1 transposons present in the original isolate of *unc-93(e1500 n1415)* and thereby allow the detection of additional transposons by genomic Southern blot analysis, we backcrossed the original isolate of the *unc-93(e1500 n1415)* mutation to N2 worms. We crossed N2 males with *unc-93(e1500 n1415)* hermaphrodites, mated the cross-progeny males (genotype *unc-93(e1500 n1415)/+*) with *unc-93(e1500) dpy-17* hermaphrodites, picked Unc non-Dpy hermaphrodites (genotype *unc-93(e1500)/dpy-17/unc-93(e1500 n1415) +*), and, from the self progeny of these worms, picked phenotypically wild-type hermaphrodites (genotype *unc-93(e1500 n1415)* now backcrossed with Bristol strains two times). We generated males of this twice backcrossed *unc-93(e1500 n1415)* strain by heat shock treatment (Hodgkin, 1983), crossed these males to *daf-2 dpy-17* hermaphrodites, and mated the cross-progeny males (genotype *daf-2 + dpy-17/+ unc-93 +*) to *daf-2 dpy-17* hermaphrodites. (*daf-2* maps 4.2 map units to the left of *unc-93* and *dpy-17* map 3.4 units to the right of *unc-93*) (Edgley and Riddle, 1990). By repeating this cross a total of eight times, we constructed a strain containing *unc-93(e1500 n1415)* that is congenic with the Bristol N2 strain except in the region around *unc-93*. In the last cross, we picked phenotypically wild-type cross-progeny (genotype *daf-2 + dpy-17/+ unc-93 +*) hermaphrodites instead of males, and from their self progeny picked wild-type hermaphrodites that did not segregate any Daf or Dpy progeny (genotype *+ unc-93 +/+ unc-93 +*). We confirmed the genotype of these worms by crossing them with *unc-93(e1500); sup-10(n183)* males and observing only Unc cross-progeny males.

Molecular Biology

Standard techniques for molecular biology were used (Sambrook et al., 1989).

To clone the 6.7-kb Tcl-containing EcoRl fragment from the *unc-93(e1500 n1415)* strain, we purified EcoRl cut genomic DNA of approximately the desired size from an agarose gel, cloned the DNA into λNM1149, screened the resulting clones by hybridization with a Tcl probe (pTcl, construction described in Finney et al., 1988; Emmons, 1983) and identified a Tcl-positive clone with a 6.7-kb insert. We subcloned the 6.7 EcoRl insert in this phage, ϕ93-1, into pBS+ (Stratagene, La Jolla, CA) to construct p93-1. By cutting p93-1 with EcoRV, which cleaves very close to both ends of Tcl, and religating the DNA, we removed the Tcl DNA along with 183 bp (#2590-2773) of *unc-93* DNA to construct p93-2. We used p93-2 to probe the "JA N2" lambda library containing *C. elegans* wild-type genomic DNA (kindly provided by A. Coulson and J. Sulston) to isolate and characterize two overlapping phage clones, ϕ93-2 and ϕ93-3. These two phage clones were analyzed by A. Coulson and J. Sulston (personal communication) and placed on a contig, a set of overlapping DNA clones, that now spans ~10,000 kb of the physical map of LGIII (Coulson et al., 1986, 1988; and personal communication). To characterize the region of DNA we suspected to contain the *unc-93* gene, we subcloned a 6.3-kb BamHI fragment and the adjacent 3.5-kb EcoRl-BamHl fragment from ϕ93-2 into pBS+ to construct p93-3 and p93-11, respectively (restriction map shown in Fig. 7).

We used p93-2 to probe a mixed stage cDNA library (Kim and Horvitz, 1990) and isolated five positive clones from 165,000 clones screened. Two classes of positively hybridizing clones were isolated. Four clones are ~300 bp in length and hybridize to the 0.6-kb HindIII fragment shown on the left in Fig. 7. These clones are not likely to be related to *unc-93*, because they are derived from a region of DNA that is unchanged in *unc-93* mutants, except in complex rearrangements in which bona fide *unc-93* sequences are also rearranged (Fig. 7 and our unpublished data). One 2.6-kb clone is colocalized with the DNA polymorphisms observed in *unc-93* mutants and hybridizes to a transcript altered in animals carrying the Tcl insertion mutation *unc-93(e1500 n1415)* (Figs. 2 and 7). Thus, this cDNA is probably derived from the *unc-93* transcript. Based on Southern blot analysis and restriction enzyme mapping, this cDNA clone has an unrelated 400 bp of DNA at its 3′ end after the polyA tail of the *unc-93* cDNA, probably as a result of the ligation of two unrelated fragments during the construction of the cDNA library. The cDNA was subcloned as two separate EcoRl fragments into pBS+ to construct p93-5 and p93-6–the former contains the 3′ half of the cDNA clone and the latter contains the 5′ half (see Fig. 7 for the *unc-93* cDNA position).

RNA was isolated as described by Kim and Horvitz (1990). Staged animals were obtained as described by Meyer and Casson (1986) and Kim and Horvitz (1990). The L1 larvae were not fed before they were harvested, and the effect of starvation on *unc-93* RNA levels is not known. Northern blot analysis was performed as described by Meyer and Casson (1986) and modified by Miller et al. (1988).

The cDNA sequence is derived from clones p93-5 and p93-6, and the genomic sequence is derived from clones p93-3 and p93-11. For DNA sequencing, we constructed a series of nested deletions by the method of Henikoff (1984) and also used synthetic oligonucleotides derived from the *unc-93* sequence as primers. The sequencing reactions were done by the method of Sanger et al. (1977) with double-stranded DNA templates using the USB Sequenase kit (U.S. Biochemicals, Cleveland, OH) according to its instructions. All DNA sequences were determined for both strands, except for bases 1 to 41 and 5018 to 5055. The sequence of the *unc-93* cDNA and the genomic DNA are in full agreement, except at position 2963 (C in the genomic sequence and T in the cDNA sequence); because this cDNA sequence corresponds to a TAA stop codon in frame within the open reading frame, the T in the cDNA sequence is likely to be an artifact of the cloning process.

Primer extension, ribonuclease protection, and RNA polymerase chain reaction (PCR)[1] experiments were performed to determine the 5′ ends of the *unc-93* transcripts. For primer extension (Kingston, 1991), a ^{32}P end-labeled oligonucleotide (positions 542-513) was used as a primer with 50 μg of total RNA from L1 larvae or eggs. The sizes of the resulting DNA products were determined by comparison with the sizes of the products of DNA sequencing reactions in adjacent lanes on a 6% polyacrylamide/urea (sequencing) gel. For ribonuclease protection experiments, a ^{32}P-labeled anti-sense RNA probe from positions 549-283 was synthesized using the T3 promoter to express a SspI-digested deletion subclone of p93-11 missing DNA from positions 550-1336, according to the directions included with a RNA transcription kit (Stratagene, La Jolla, CA). The labeled probe (between 0.03 and 3 ng) was hybridized to 25 μg of total RNA from L1 larvae or eggs and then digested with ribonucleases A and T1 (Gilman, 1991). The sizes of the products were determined by comparison with the sizes of the products of DNA sequencing reactions and ^{32}P-labeled T3 RNA transcripts of known sizes on a 6% polyacrylamide/urea gel. Ribonuclease protection products that were present when yeast RNA was substituted for worm total RNA were considered to be unrelated to *unc-93* RNA. RNA PCR was carried out with the reagents in the GeneAmp Thermostable rTth Reverse Transcriptase RNA PCR kit (Perkin Elmer Cetus, Norwalk, CT) according to the instructions included. Reverse transcriptase reactions with 50 ng of polyA$^+$ egg RNA and an oligonucleotide primer were incubated at room temperature for 5 min, 60°C for 5 min and 70°C for 10 min. Reagents for PCR including the other oligonucleotide primer were added to these reactions and PCR was performed as follows: annealing at 55°C for 1 min, extension at 72°C for 1 min for the first 20 cycles and 2 min for the last 20 cycles, and denaturation for 1 min at 92°C, all for 40 cycles. With one oligonucleotide from exon 4 (positions 1313-1294) and the other in exon 1 upstream of the cDNA start site (positions 388-408), an ~630-bp fragment was generated from the larger *unc-93* transcript beginning at position 233. This fragment contained a single PvuII site as expected at position 700. In addition, a second round of PCR amplification with the same 5′ end primer and either of two primers from exon 2 (positions 660-644 and 729-713) yielded products of ~220 and 290 bp, as expected. RNA PCR with a primer from either SL1 (positions 2-22) (Krause and Hirsh, 1987) or SL2 (positions 1-22) (Huang and Hirsh, 1989) and a primer from exon 4 (positions 1313-1294 or 1293-1279) yielded multiple fragments, as is often observed for RNA PCR with SL1 and SL2 (M. Nonet, personal communication). A second round of PCR amplification with the same 5′ end primer and either of the two exon 2 primers yielded a single product for each reaction of the size expected for a *trans*-spliced leader added at position 497.

DNA sequence analysis was done using the DNA Inspector program (Textco, West Lebanon, NH), the DNA Strider program (Marck, 1988), and the University of Wisconsin GCG package (Devereux et al., 1984). We searched the Genpept(R) protein database (GenBank database, v67.0 and new sequences through 3/91) with the fasta program and the combined GenBank/EMBL DNA database (GenBank v68.0, EMBL v26.0) with the tfasta program. In addition, the BLAST program (available through NCBI) was used to search a combined database of PIR (v30.0), SwissProt (v19.0), GenPept (v69.0 and new sequences through 12/3/91) (Altschul et al., 1990). None of these searches yielded any proteins with significant sequence similarity to the unc-93 protein. No significant sequence similarity was found between DNA sequences in the GenBank/EMBL DNA database and the *unc-93* genomic DNA sequence using the fasta program. In addition, the sequences in intron 5 and intron 10, containing repeated sequences 3 and 2, respectively (see Fig. 3), were each used to search the GenBank/EMBL DNA database. For intron 10, the eight nucleotide repeat is similar to repeats of two and four nucleotides found in many other genes, but we do not know of any function for these repeats. Intron 5 did not show any significant sequence similarity to any DNA sequences. The naq/match program was used to search for *C. elegans* genes with multiple copies of the three repeated sequences. These sequences are found in several genes, but never tandemly repeated or even repeated within a 200-bp interval. For these short sequences, a single copy seems likely to be a random occurrence.

We used Southern blot analysis and PCR amplification (Saiki et al., 1988) to map the locations of *unc-93* mutations. PCR amplification reactions used the AmpliTaq polymerase (Perkin-Elmer Cetus, Norwalk, CT) according to the instructions supplied with the enzyme. Conditions for the PCR reactions were: annealing at 50°C for 1 min, extension at 72°C for 3 min for the first 20 cycles and 4 min for the last 10 cycles, and denaturation for 1 min at 92°C, all for 30 cycles. For the PCR amplification experiments, we used sets of primers that covered the entire sequenced region of *unc-93* except for 30 bp at the 5′ end, although all combinations of primers were not tested for each mutation. The *unc-93* primers were derived from the following sequences: positions 30 to 55, 388 to 408, 505 to 525, 514 to 533, 1293 to 1274, 1298 to 1279, 1313 to 1294, 2355 to 2379, 2413 to 2392, and 5055 to 5036. The two Tcl primers chosen were derived from sequences not in the inverted terminal repeats–positions 105 to 83 and 1396 to 1417 (Rosenzweig et al., 1983). DNA from all of the Tcl insertion alleles was PCR-amplified with primers from either end of Tcl and the PCR products were checked by digestion with EcoRV.

The DNA sequence alterations in strains containing the *unc-93(e1500 n234)* and *unc-93(n200)* mutations were determined from the products of PCR amplification. Ethyl methanesulfonate was used to generate the *e1500*

1. *Abbreviation used in this paper*: PCR, polymerase chain reaction.

and *n200* mutations and diethyl sulfate was used to generate *n234* (Greenwald and Horvitz, 1980). Genomic DNA was amplified with two sets of primers that spanned the *unc-93* region. The first set of primers was derived from positions 30 to 55 and 2413 to 2392; the second set of primers was derived from positions 2355 to 2379 and 5055 to 5036. The PCR reactions were done as described above. To avoid possible DNA sequence changes introduced by the Taq polymerase, we pooled the products of 10 separate PCR reactions for each of the PCR products. These pools were subcloned into pBS+, and at least four isolates of each of the PCR products were combined for use as templates in DNA sequencing reactions using oligonucleotide primers covering the *unc-93* region. We determined the DNA sequence of these subclones from position 196 to 5055, except for intron 14 from position 4264 to 4330. We ignored DNA sequence alterations that did not appear in all isolates of a particular subclone.

Results

Isolation of Transposon-insertion Alleles

We used the method of transposon tagging (Greenwald, 1985; Moerman et al., 1986) to clone the *unc-93* gene. *C. elegans* strains containing mutator mutations display elevated levels of transposition of the transposable elements Tc1, Tc3, Tc4, and Tc5 (Collins et al., 1987; Finney et al., 1988; Collins et al., 1989; Yuan et al., 1991; J. Collins and P. Anderson, personal communication). We constructed strains carrying either the *unc-93(e1500)* or the *sup-10(n983)* mutation in a mutator background. Because null alleles of *unc-93*, *sup-9*, *sup-10*, and *sup-18* can suppress the rubber band phenotype caused by these mutations, a worm carrying a transposon insertion in one of these four genes can be identified as a phenotypically wild-type revertant. In this way, 27 suppressor mutations that included alleles of all four genes were isolated: 13 alleles of *unc-93*, eight of *sup-9*, five of *sup-10*, and one of *sup-18* (Table I). Since *sup-18* mutations only partially suppress *e1500* but completely suppress *n983* (Greenwald and Horvitz, 1986), *sup-18* mutations would have been detected in these experiments only as suppressors of *n983*.

Identification of a Tc1 Insertion in the unc-93 Gene

These putative transposon-insertion mutations were isolated in mutator backgrounds, which have as many as several hundred copies of Tc1 (Emmons et al., 1983; Collins et al., 1987). To identify an insertion in *unc-93*, these mutations were backcrossed into the wild-type Bristol (N2) background (see Materials and Methods), which contains 30 copies of Tc1. We used Tc1 to probe a Southern blot containing genomic DNA from a 10 times backcrossed strain containing the *mut-2*–derived allele *unc-93(e1500 n1415)* and detected one extra Tc1-hybridizing band (data not shown). This Tc1 insertion mapped within about 0.8 map units of the *unc-93* locus, since 26 of 26 recombination events within an ∼7 map unit interval spanning *unc-93* failed to separate the Tc1 from the *unc-93* gene (Table II). Thus, this Tc1 inserted in or very near the *unc-93* gene. We cloned a 6.7-kb EcoR1 genomic fragment containing this Tc1 insertion to yield the plasmid p93-1. By removing the Tc1 element from p93-1, we constructed the plasmid p93-2, which contains the genomic DNA flanking this Tc1. When p93-2 was used to probe a Southern blot containing genomic DNA, the wild-type N2 strain contained a 5.1-kb EcoRI fragment, whereas *unc-93-(e1500 n1415)* contained the 6.7-kb EcoR1 fragment, as expected for a 1.6-kb Tc1 insertion into this fragment in the *unc-93(e1500 n1415)* strain (Fig. 1).

Identification of Polymorphisms Associated with unc-93 Alleles

To obtain clones with wild-type *unc-93* genomic DNA, we used p93-2 to probe a lambda library containing genomic DNA from the wild-type N2 strain. We isolated two overlapping phage clones containing genomic DNA from the region around *unc-93*, based on common restriction maps and genomic Southern blots (data not shown). To identify the site of the *unc-93* gene, we examined DNA from *unc-93* mutants generated by gamma rays for allele-specific polymorphisms. These types of alleles are likely to have polymorphisms detectable by Southern blot analysis (Graf and Chasin, 1982; Grosovsky et al., 1986; Moerman et al., 1986; Collins et al., 1987). Using Southern blot analysis, we identified five gamma ray–induced *unc-93* mutations that alter the wild-type 5.1-kb EcoR1 fragment, in addition to the Tc1 insertion in *unc-93(e1500 n1415)*; three of these changes are shown in

Table I. Genetic Screen Used to Isolate Putative Transposon-insertion Alleles

	Phenotype	Genotype*	Number of alleles‡
Initial strain	Unc	*unc-93(e1500)*	
Revertant strains	WT	*unc-93(0)*	12
	WT	*sup-9(0); unc-93(e1500)*	6
	WT	*unc-93(e1500); sup-10(0)*	4
Initial strain	Unc	*sup-10(n983)*	
Revertant strains	WT	*sup-10(0)*	1
	WT	*sup-9(0); sup-10(n983)*	1
	WT	*unc-93(0); sup-10(n983)*	1
	WT	*sup-18(0); sup-10(n983)*	1

We constructed strains with *unc-93(e1500)* or *sup-10(n983)* in a mutator background and isolated spontaneous non-Unc revertants, which proved to be of the genotypes indicated.
* Each strain also contains a mutator background (see Materials and Methods).
‡ One additional suppressor mutation, *sup-9(n1330)*, was isolated from the background of a *mut-2* parental strain (see Materials and Methods).

Table II. Mapping of the unc-93(e1500 n1415) Tc1 Insertion

Genotype of parent	Phenotype of recombinant	Genotype of homozygous recombinant	Tc1 present/total
daf-2 + dpy-17 / *+ unc-93 +*	Daf non-Dpy	*daf-2 unc-93*	6/6
		daf-2	0/8
	Dpy non-Daf	*unc-93 dpy-17*	3/3
		dpy-17	0/9

To map the Tc1 insertion in the ten-times backcrossed *unc-93(e1500 n1415)* strain, we identified recombination events in the interval between *daf-2* and *dpy-17*. The distances between *daf-2* and *unc-93* and between *unc-93* and *dpy-17* are 4.2 map units and 3.4 map units, respectively (Edgley and Riddle, 1990). We isolated Dpy non-Daf and Daf non-Dpy recombinants from the F1 progeny of *daf-2 + dpy-17/ + unc-93 +* heterozygotes. Animals homozygous for the recombinant chromosomes were picked in the F2 generation and tested for the presence of *unc-93(e1500 n1415)* by mating them with *sup-9(n180); unc-93 (e1500)* males or *unc-93(e1500); sup-10(n183)* males and observing either all Unc cross-progeny (F2 genotype *unc-93(e1500 n1415)*) or all non-Unc cross-progeny (F2 genotype *unc-93(+)*). The presence of the Tc1 insertion in the homozygous recombinants was determined by Southern blot analysis using pTc1 as a probe.

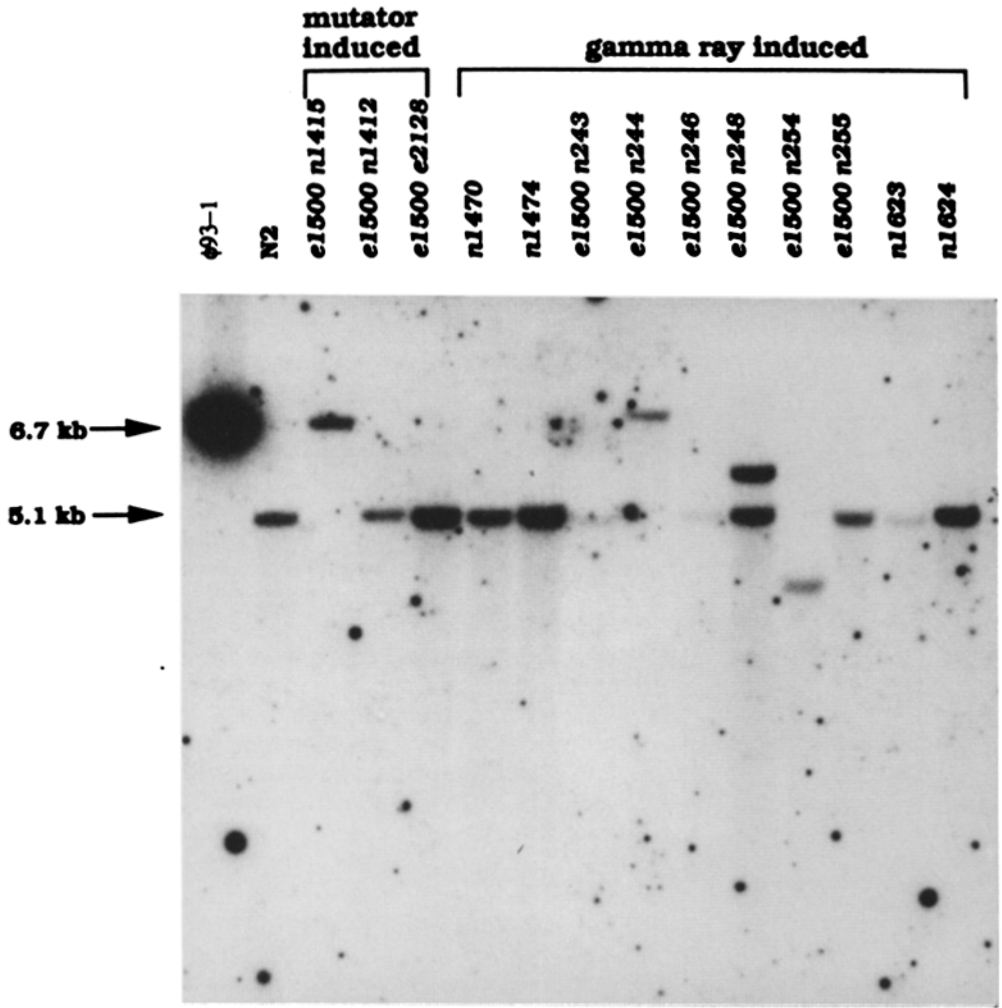


Figure 1. unc-93 Southern blot. EcoRI-digested DNA from the wild-type N2 and *unc-93* mutant strains and from the phage clone, ϕ93-1, was electrophoretically separated on an agarose gel, transferred to a nylon membrane, and probed with ^{32}P-labeled p93-2. The digested N2 DNA contains a 5.1-kb fragment and the *unc-93(el500 nl415)* DNA contains a 6.7-kb fragment, consistent with the insertion of a 1.6-kb Tcl element. The phage clone ϕ93-1 contains an insert of the 6.7-kb fragment from *el500 nl415.* Three other *unc-93* strains show polymorphisms: *el500 n244* has a 6.9-kb fragment, *el500 n248* has the wild-type 5.1-kb fragment as well as a 5.8-kb band–a pattern consistent with a tandem duplication–and *el500 n254* has a 4.3-kb fragment.

Fig. 1. (See below for detailed physical mapping of *unc-93* mutations.) These data suggest that part of the *unc-93* gene is contained in the 5.1-kb EcoRl fragment.

Identification and Characterization of the unc-93 Transcript

Using p93-2 to probe a mixed-stage cDNA library, we isolated one candidate *unc-93* cDNA clone from 165,000 clones screened. We used p93-6, a subclone containing the 5′ half of this cDNA, to probe a Southern blot containing wild-type genomic DNA and detected only DNA from the same genomic region detected by p93-2 (data not shown). Thus, the cDNA is derived from single-copy DNA in the *unc-93* region. This cDNA detects a single 2.2-kb transcript when used to probe a Northern blot of polyA$^+$ RNA from the wild type (Fig. 2). In animals carrying the Tcl insertion mutation *unc-93(el500 nl415)*, a transcript larger than the 3.5-kb ribosomal RNA band is detected instead (Fig. 2 *a*), providing further support that the 2.2-kb RNA is the *unc-93* transcript.

In a population of worms of mixed stages, the *unc-93* transcript is present at a level roughly 100- to 250-fold lower than that of the *unc-54* transcript, which encodes the major body wall myosin heavy chain (MacLeod et al., 1981) (Fig. 2 *a*). The low abundance of the RNA transcript suggests that the unc-93 protein is also present at low levels. The *unc-93* transcript accumulates at a higher level in L1 larvae than in eggs or in a mixed stage population consisting primarily of adults by weight (Fig. 2 *b*). This pattern of expression is consistent with the phenotype of *unc-93(el500)* and *unc-93(n200)* rubber band mutants, which we observed to be most uncoordinated as L1 larvae and to move progressively better as they grow older.

unc-93 cDNA and Genomic DNA Sequences

We determined the sequence of the *unc-93* cDNA and identified one long open reading frame (Fig. 3). In addition, we determined the sequence of 5055 bp of genomic DNA encompassing the *unc-93* cDNA (Fig. 3 *a*). A comparison of the two DNA sequences yields the exon/intron boundaries for the *unc-93* gene and defines 15 introns ranging in size from 45 to 605 bp. As the *unc-93* cDNA clone is 2168-bp long and the *unc-93* transcript is 2.2-kb long, this cDNA corresponds to very nearly all of the full-length mRNA transcript. The *unc-93* open reading frame ends in a TGA stop codon that is closely followed by an AATAAA polyadenylation signal (Proudfoot and Brownlee, 1976) and the site for the addition of a polyA tail.

To define the 5′ end of the *unc-93* transcript(s), we performed primer extension, ribonuclease protection, and RNA PCR experiments (see Materials and Methods). Primer extension from an oligonucleotide primer in exon 1 (positions 542-513) yielded two products: an approximately 310 nucleotide product that could correspond to a 2432-bp transcript beginning at position 233 and a 69 nucleotide product that could correspond to a 2191-bp transcript including a *trans*-spliced leader of 22 nucleotides added at position 496 (Figs. 3 and 4). Both SL1 and SL2 *trans*-spliced leaders are 22 nucleotides in length (Krause and Hirsh, 1987; Huang and Hirsh, 1989). The 3′ acceptor splice site at position 496 (TTTCAGA) matches the consensus for 3′ acceptor splice sites (TTTCAGY) in *C. elegans* (Emmons, 1988). We also identified two products in a ribonuclease protection experiment with an RNA probe derived from positions 549-283. An approximately 260 nucleotide fragment that resulted from protection of the entire *unc-93* portion of the probe corresponds to the larger transcript and an approximately 53 nucleotide fragment that would result from a splicing event at position 496 corresponds to the smaller *trans*-spliced transcript (data not shown). Position 233 is likely to be the 5′ end of the larger transcript because there are no potential 3′ acceptor splice sites (Emmons, 1988) between the end of the ribonuclease protection probe (position 283) and the predicted 5′ end of the larger transcript (position 233) (Fig. 3 *a*). RNA PCR experiments with a primer in exon 4 (positions 1313-1294) and a primer upstream of the *trans*-splice site acceptor (positions 388-410) yielded a product of about

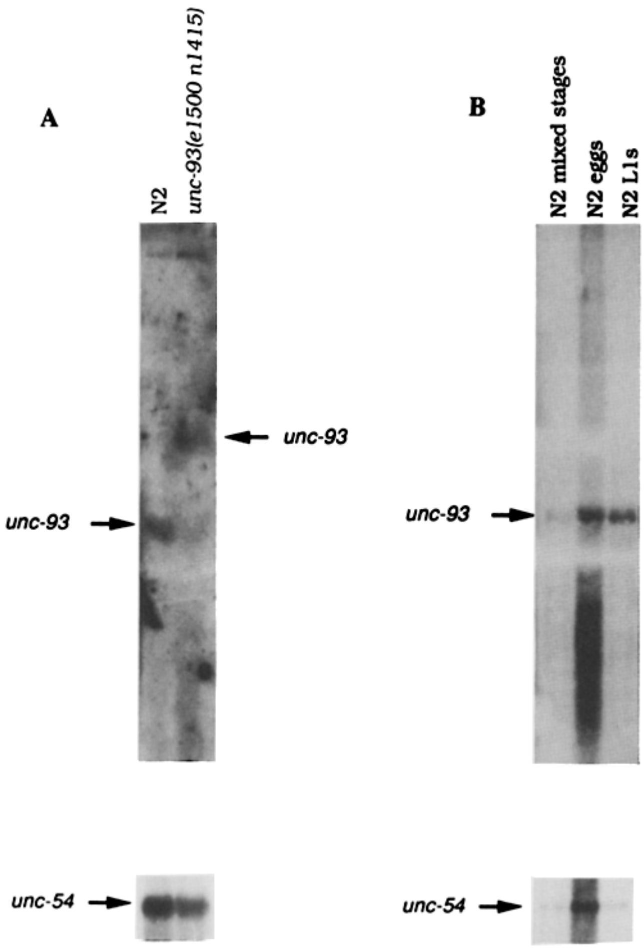
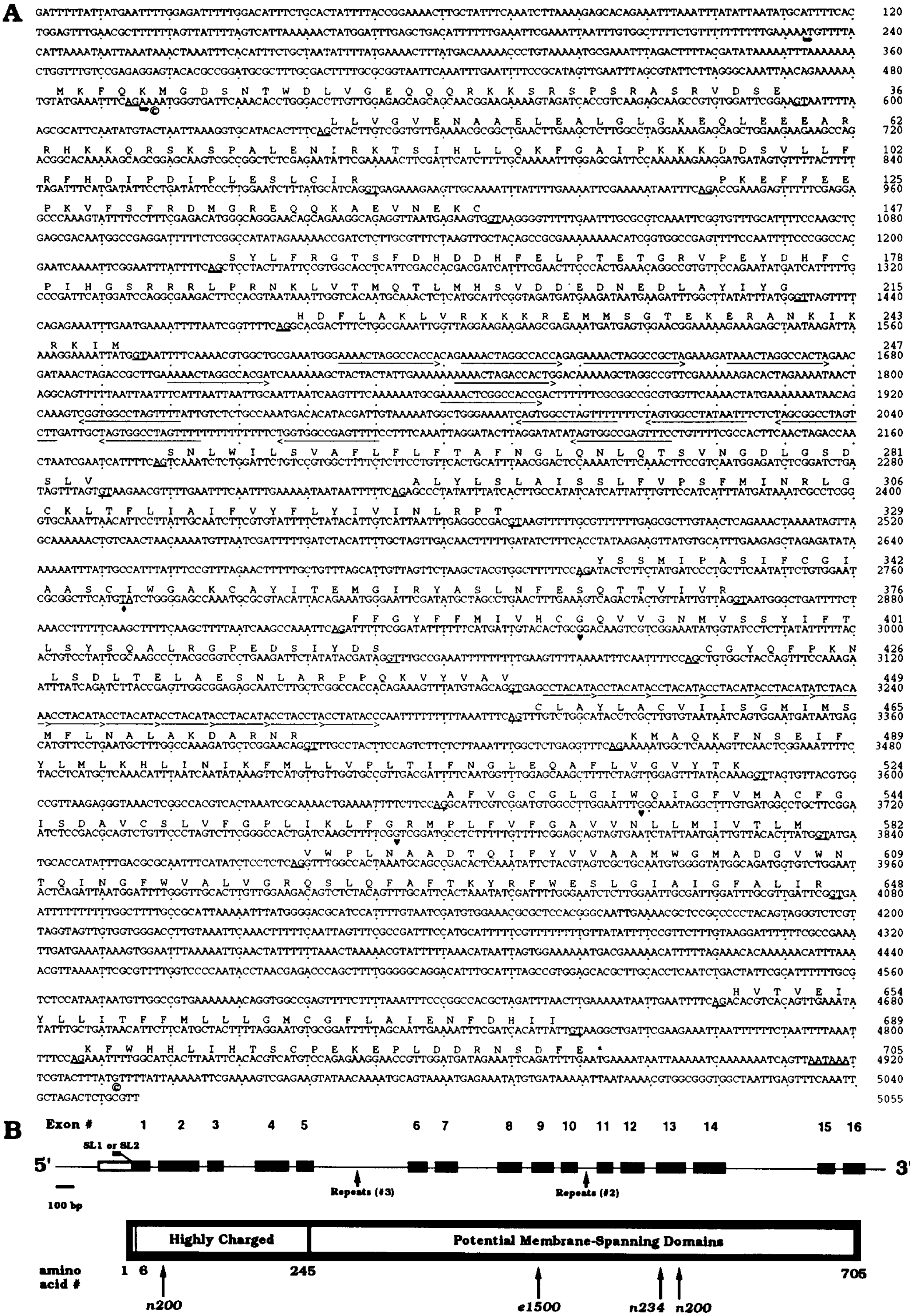


Figure 2. unc-93 Northern blot analysis. (*a*) Identification of the *unc-93* transcript. Each lane contains polyA+ RNA purified from ~1.7 mg of total RNA isolated from a mixed-stage culture of worms. A transcript of 2.2 kb is detected in the wild type (*N2*) lane. In the *unc-93(e1500 n1415)* lane, there is a transcript larger than the 3.5-kb ribosomal RNA band instead. This blot was probed first with ^{32}P-labeled p93-6 to detect the *unc-93* transcript and then with a ^{32}P-labeled 1.0-kb EcoRI-BamHI fragment from the *unc-54* plasmid pMRF (kindly provided by D. Hsu and A. Fire, personal communication). *unc-54* encodes the major body wall myosin heavy chain (MacLeod et al., 1981). Based on the relative intensities and lengths of exposure of the *unc-93* and *unc-54* bands, we estimate that the *unc-93* transcript is present at a level roughly 100- to 250-fold less than that of the *unc-54* transcript. (*b*) *unc-93* expression at different developmental stages. Northern blot analysis was performed essentially as in *a*, except that each lane contained polyA+ RNA purified from ~1 mg of total RNA. Because of variable yields in purification of polyA+ RNA, the amounts of RNA loaded in the different lanes might not be equal. Relative to the *unc-54* transcript, the *unc-93* transcript appears to be more abundant in RNA from L1 larvae than in RNA from eggs and in RNA from a mixed stage population. The 2.2-kb size of the *unc-93* transcript was determined by comparison with the sizes of ethidium bromide-stained ribosomal RNA bands of 1.75 and 3.5 kb (Files and Hirsh, 1981) in parallel lanes of total worm RNA and with a BRL 0.24-9.5 kb RNA ladder (Bethesda Research Laboratory, Gaithersburg, MD).

Figure 3. unc-93 sequence and structure. (*a*) *unc-93* nucleotide sequence and predicted amino acid sequence. The top numbers refer to amino acids, and the bottom numbers refer to nucleotides. The exon and intron boundaries are based on the comparisons between sequences of the *unc-93* cDNA and genomic DNA. The conserved GT and AG dinucleotides of splice junctions are underlined, as is the polyadenylation signal at position 4914 to 4919. The beginning of the cDNA (base 497) and the end of the cDNA (base 4932) are indicated by a © beneath the appropriate nucleotides. The 5′ end of the larger transcript (base 233) and the 3′ splice acceptor site for the *trans*-splice leaders (base 496) are indicated by a → beneath the appropriate nucleotide. A diamond beneath positions 2773 to 2774 indicates the Tc1 insertion site in *unc-93(e1500 n1415)* animals. A heart beneath positions 2960, 3689, 681, and 3773 indicates the locations of the *e1500*, *n234*, and *n200* (two changes) mutations, respectively. Repeated sequence 2 is indicated by arrows beneath the DNA sequences in intron 10 (positions 3190 to 3311), and repeated sequence 3 is similarly marked in intron 5 (positions 1575 to 2179). The orientations of repeated sequence 3 in intron 5 are indicated: an arrow to the right corresponds to the orientation shown in Fig. 5 *a*, and an arrow to the left corresponds to the inverse orientation. These sequence data are available from EMBL/GenBank/DDBJ under accession number X64415. (*b*) *unc-93* gene structure. The top line shows the exons as thick lines and the introns between them. The extended hollow line for exon 1 represents the larger transcript. The small thick line above the top line connected to exon 1 at position 496 represents the *trans*-spliced transcripts. The location of repeated sequence 2 in intron 10 and repeated sequence 3 in intron 5 is also shown. The bottom line shows the two regions of the unc-93 protein. Amino acid 1 is the NH_2 terminus of the protein encoded by the larger transcript and amino acid 6 is the NH_2 terminus of the protein encoded by the *trans*-spliced transcripts. The location in the protein of the mutations *e1500*, *n200*, and *n234* are indicated by arrows. *n200* has two arrows because two sequence changes were detected.


A
B
Exon #
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
SL1 or SL2
5'
3'
100 bp
Repeats (#3)
Repeats (#2)
Highly Charged
Potential Membrane-Spanning Domains
amino acid #
1 6 245 705
n200
e1500
n234
n200

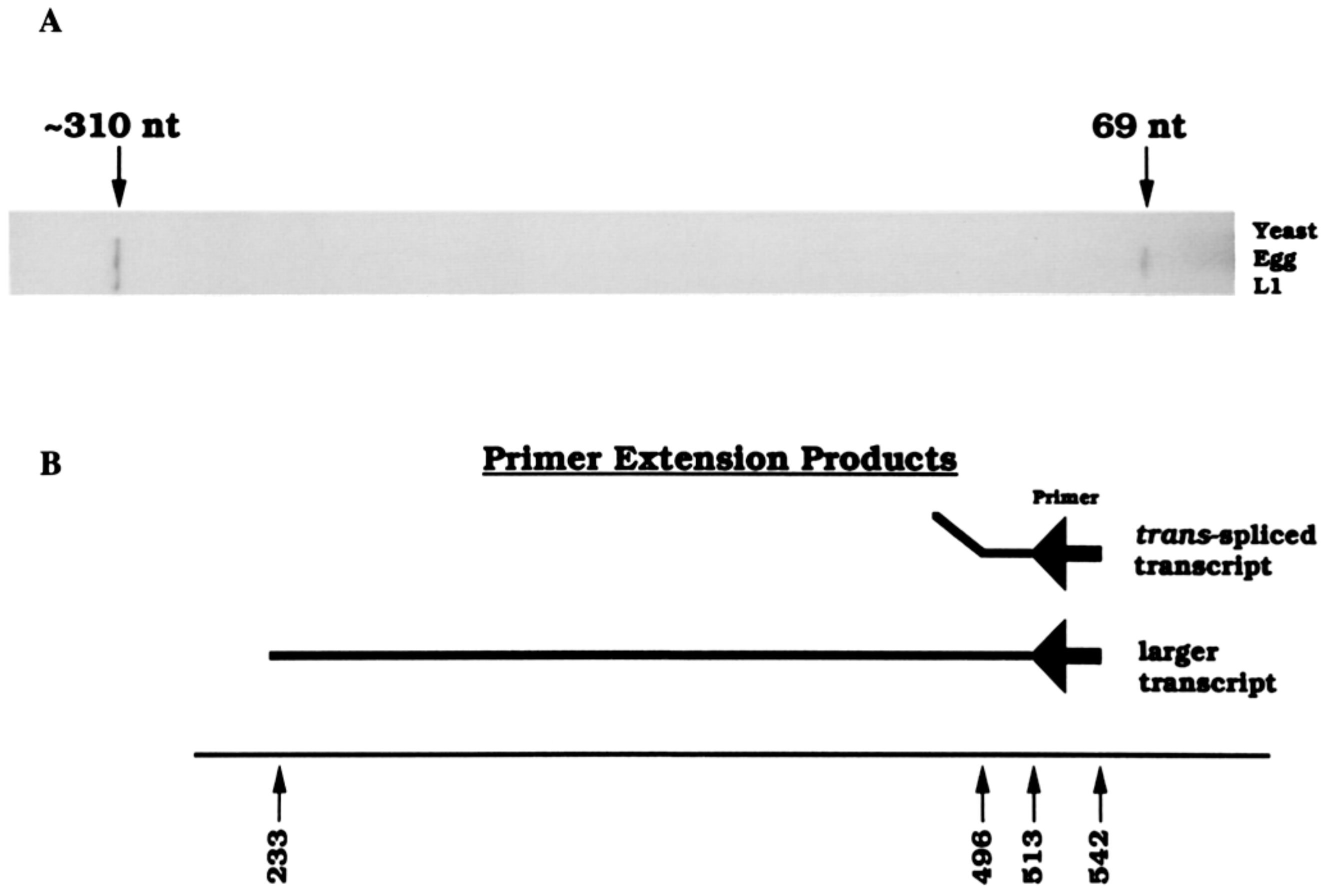


Figure 4. Primer extension from *unc-93* transcripts. (*a*) Identification of two 5′ ends of the *unc-93* transcripts. Each lane contains the products of a primer extension reaction using a ^{32}P end-labeled oligonucleotide (positions 542-513) as a primer. The template RNA in each lane is as follows: yeast RNA, a negative control, in the top lane, *C. elegans* egg RNA in the middle lane, and *C. elegans* L1 larval RNA in the bottom lane. At the bottom of the gel (right), the lanes narrowed causing the 69 nucleotide (*nt*) bands to run together. The sizes of the DNA products were determined by comparison with the sizes of the products of DNA sequencing reactions in adjacent lanes (data not shown). A ∼310-nt band and a 69-nt band are seen for both *C. elegans* samples, but not for the yeast sample. The presence of a 69-nt band in the egg and L1 larval RNA lanes is in agreement with the results of ribonuclease protection experiments in which we observed a protected band indicating the existence of the *trans*-spliced transcripts in eggs and L1 larvae (data not shown). (*b*) Diagram of primer extension products. The arrow at the right of the extension products represents the primer. The bottom line and the numbers below indicate positions in the DNA. The 5′ end of the larger transcript is at position 233 and the 3′ acceptor splice site is at position 496. The *trans*-spliced leader portion (22 nt) of the *trans*-spliced transcript is indicated by a diagonal line.

620 bp, the size expected for the larger transcript (data not shown). RNA PCR experiments with a primer in exon 2 and a primer containing the sequence of either the SL1 or SL2 *trans*-spliced leaders (Krause and Hirsh, 1987; Huang and Hirsh, 1989) yielded products consistent with the addition of each of the spliced leader sequences at position 496 (data not shown). These RNA PCR experiments suggest that both SL1 and SL2 can be *trans*-spliced to the *unc-93* transcript. The open reading frame of the larger transcript begins with an ATG at positions 484-486, which is preceded by a TAA stop codon at positions 469-471. The open reading frame in the *trans*-spliced smaller transcripts begins with an ATG at positions 499-501 and is missing the first five amino acids (Met-Lys-Phe-Gln-Lys) of the larger transcript.

The introns of *unc-93* contain three sets of repeated DNA sequences, as shown in Fig. 5 *a*. Two perfect copies of the first repeat, CAAGCTTTT, are present in tandem in intron 8 and one copy is present in both introns 12 and 13. Intron 10 is composed almost entirely of 12 direct, tandem imperfect repeats of the second sequence, CCTACATA (Figs. 3 and 5 *b*). The third sequence, AAAACTAGGCCACYR, is repeated imperfectly 14 times in intron 5. There are seven direct repeats of this sequence, separated by a variable number of nucleotides, followed by seven direct repeats in the opposite orientation, also separated by a variable number of nucleotides (Fig. 5 *c*). The DNA in intron 5 could form a stem-loop structure due to its palindromic nature. This sequence is also present once in each of introns 3, 12, and 14. None of these three sequences appears to be significantly repeated in any other *C. elegans* genes listed in the GenBank database (see Materials and Methods).

The codon usage bias for *unc-93* deviates from the consensus derived from *C. elegans* genes expressed at a high level (Emmons, 1988). For example, in highly expressed genes, the CCA proline codon is used 238 times and the other three proline codons are used a total of 14 times. By contrast, in *unc-93*, the CCA proline codon is used ten times and the other three proline codons are used a total of 14

A

Consensus Sequence	intron #	# of repeats
1) 5' CAAGCTTTT 3'	8 (2 repeats), 12, 13	4
2) 5' CCTACATA 3'	10	12
3) 5' AAAACTAGGCCACYR 3'	3, 5 (14 repeats), 12, 14	17

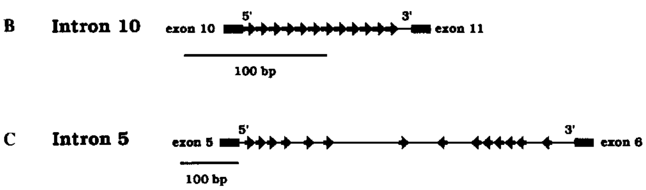


Figure 5. Repeated sequences in *unc-93* introns. (*a*) Three repeated DNA sequences were identified in the introns of the *unc-93* gene. Repeated sequence 1 is found four times with no mismatches. Repeated sequence 2 is found 11 times with one mismatch; the 12th copy has two mismatches. Repeated sequence 3 is found 17 times with three or fewer mismatches. (*b*) The arrangement of repeated sequence 2 in intron 10. (*c*) The arrangement of repeated sequence 3 in intron 5. See Fig. 3 legend for an explanation of the orientation of the arrows.

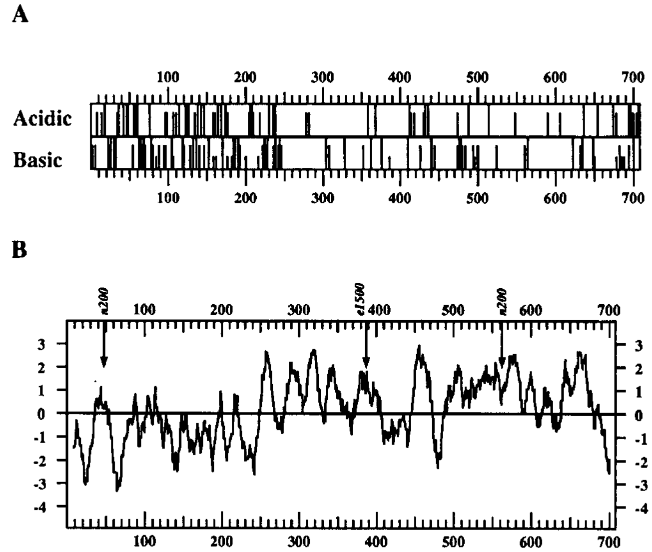


Figure 6. unc-93 protein features. (*a*) Acidic and basic residues. The top line shows the acidic amino acids: aspartic acid (*D*), intermediate bar; and glutamic acid (*E*), full bar. The bottom line shows the basic amino acids: histidine (*H*), small bar; lysine (*K*), intermediate bar; and arginine (*R*), full bar. Note the abundance of charged residues between amino acids 1 and 245. (*b*) Kyte and Doolittle (1982) hydrophobicity plot. Hydrophobic regions are assigned a positive value (above the line) and hydrophilic regions are assigned a negative value. A window of eleven residues is used here. We also used the methods of Rao and Argos (1986) and Klein, Kanehisa, and DeLisi (1985) to predict the location of membrane-spanning domains. All three of these methods predict the following five segments are membrane-spanning: amino acids 246-264, 309-326, 447-468, 566-584, and 654-673. There are five other candidate membrane-spanning regions predicted by only one or two of the methods. The locations of the rubber band mutations are indicated by arrows. The *e1500* mutation changes amino acid 388 from Gly to Arg, and the *n200* mutation changes amino acid 49 from Ala to Val and amino acid 562 from Gly to Val.

times. Similarly, to encode phenylalanine, the UUC codon is used 103 times and the UUU codon is used 18 times in highly expressed genes, but for *unc-93* the UUC codon is used 26 times and the UUU codon is used 28 times. Genes expressed at high levels have stronger codon usage bias than genes expressed at low levels in yeast and *C. elegans* (Bennetzen and Hall, 1982; C. Fields, personal communication). The weak bias in codon usage for *unc-93* supports the hypothesis that the unc-93 protein is expressed at a low level.

unc-93 Protein Sequence

There are two potential unc-93 proteins, which differ by five amino acids at their NH_2 termini, encoded by the different *unc-93* transcripts (see above). For discussion, we designate the NH_2-terminal methionine of the larger protein as amino acid 1 and the NH_2-terminal methionine of the smaller protein as amino acid 6. For simplicity, we discuss below only the larger protein, but all analyses have been done for both. The unc-93 protein has two distinct regions. The NH_2-terminal 245 amino acids are highly hydrophilic, consisting of 40% charged residues (Glu, Asp, Arg, His, and Lys) (Fig. 6 *a*). The COOH-terminal 460 amino acids are mostly hydrophobic and define five to ten potential membrane-spanning regions based upon hydrophobicity analysis (Fig. 6 *b*). The predicted protein has no signal–anchor sequence at its NH_2 terminus, so that the first internal membrane-spanning domain (probably amino acids 246-264) is likely to direct the insertion of the unc-93 protein into the membrane. The NH_2-terminal 245 amino acids are likely to be cytoplasmic according to the prediction scheme of Hartmann et al. (1989), which is based upon the difference in the charges of the 15 residues on each side of the first internal membrane-spanning domain, with the more positive portion facing the cytoplasm. This conclusion is also in agreement with the experiments of Parks and Lamb (1991), who showed that NH_2-terminal positively charged residues play a role in determining eukaryotic membrane protein topology. We have not found any significant sequence similarity between the unc-93 protein and the proteins in any of the databases that we searched. Thus, *unc-93* seems likely to encode a novel membrane-associated muscle protein.

Physical Mapping of unc-93 Mutations

To identify and map allele-specific polymorphisms, DNA from *unc-93* mutants was examined by Southern blot analysis using multiple restriction enzymes and probes. In addition, PCR was used with different primers derived from the sequence of the *unc-93* region to amplify DNA from all of these mutants. If a pair of primers yielded no DNA fragment or a DNA fragment different from that of the wild type in size, the region of DNA between the two primers was considered to be altered in this mutant. If no PCR product was generated by one set of primers and template DNA, another set of primers was used to show that the DNA template could be successfully amplified by PCR. The positions and orientations of the Tc1 insertions were determined by PCR using

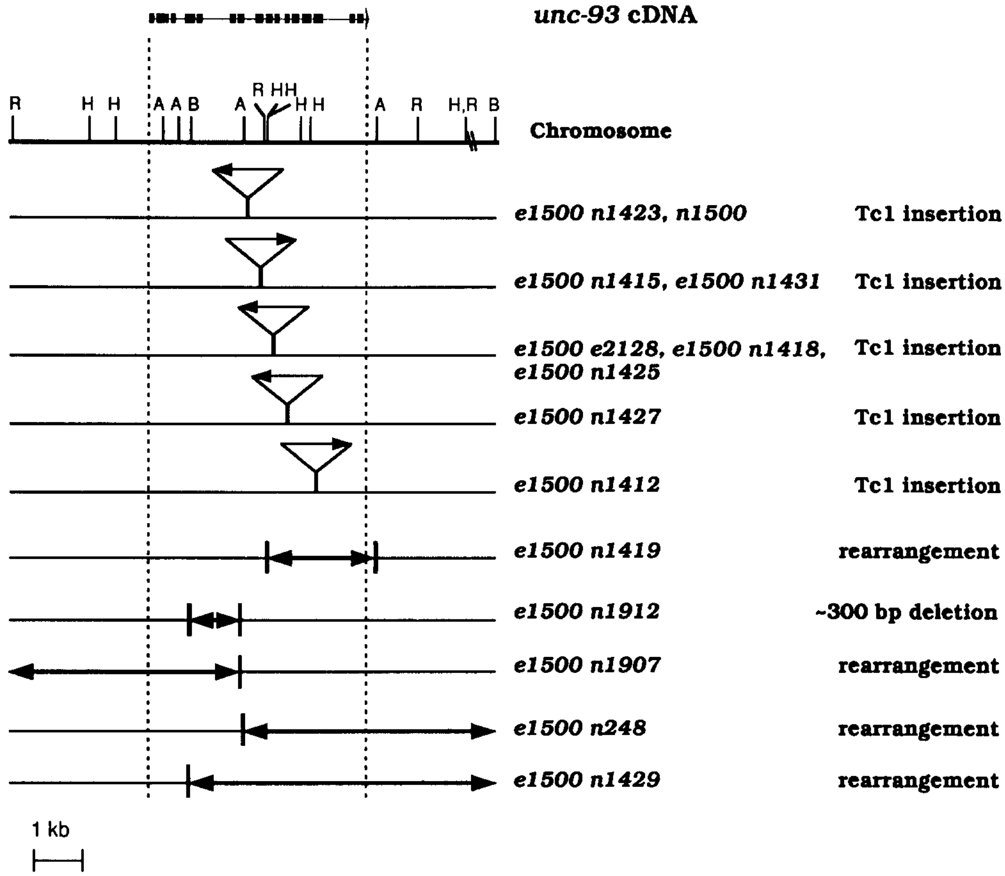


Figure 7. Co-localization of *unc-93* mutations and the *unc-93* cDNA. The location of the cDNA is based on a comparison of the sequences of the cDNA and the genomic DNA. For the cDNA, the boxes correspond to exons and the arrowhead indicates the direction of transcription. The location of each mutation has been determined by Southern blot analysis and PCR amplification with sets of primers from the *unc-93* region (see Materials and Methods). For the Tcl insertions, an arrow pointing to the right indicates that the Tcl DNA has inserted with its reading frame in the same direction as that of *unc-93*, and an arrow pointing to the left indicates the opposite orientation. The positions of the Tcl insertions are accurate to within ∼50 bp. For the other mutations, the double-arrowed region indicates the interval in which the mutation lies. Any DNA that is not indicated as changed in a given mutation was shown to be wild-type at the gross level of agarose gel electrophoresis. Complex rearrangements that were not localized precisely were found in three *unc-93* mutants: *e1500 n244, e1500 n254,* and *e1500 n255.* The p93-3 plasmid contains the only BamHl fragment shown, which is 6.3 kb, and the p93-11 plasmid contains the 3.5-kb EcoRl-BamHI fragment to the left of the 6.3-kb BamHl fragment. Additional EcoRl sites might be present to the right of the double break shown; the order of the rightmost HindIII and EcoRl sites has not been determined. (*A*) AvaI; (*B*) BamHI; (*H*) HindIII; (*R*) EcoRl.

pairs of primers, one from Tcl and the second from the flanking *unc-93* DNA (see Materials and Methods). Eleven of 13 mutator-derived *unc-93* alleles show polymorphisms, and nine of these are Tcl insertions (Fig. 7). There are five different insertion sites, within a resolution of about 50 bp. At each insertion site, only one orientation of Tcl insertion is found in these mutants. The other two mutator-derived alleles showing polymorphisms, *e1500 n1419* and *e1500 n1429*, are complex rearrangements. Six of twelve gamma ray-induced *unc-93* mutations show polymorphisms. Of the six gamma ray-induced mutations showing polymorphisms, five are complex rearrangements and one is an ∼300-bp deletion. The identification of 17 *unc-93* alleles that show alterations in this region confirms that we have cloned the *unc-93* locus. The colocalization of the cDNA and the DNA polymorphisms found in *unc-93* mutants provide strong evidence that this cDNA corresponds to the *unc-93* transcript (Fig. 7).

DNA Sequence Alterations in unc-93 Mutations

We determined the sequence of part of p93-1 and identified the site of the Tcl insertion in *unc-93(e1500 n1415)* between bases 2773 and 2774 in exon 8 (Fig. 3). This Tcl insertion caused a duplication of the TA dinucleotide on either side of the insertion, as has been observed for other Tcl insertions (Rosenzweig et al., 1983). The sequence of this insertion site (CATGTATCT) is similar to the consensus sequences for Tcl insertion sites—GA(G/T)(A/G)TA(T/C)(G/C)T and GA(T/G) ATATGT—derived by Eide and Anderson (1988) and Mori et al. (1988), respectively.

To identify the DNA sequence changes in presumptive *unc-93* point mutants, we used PCR to amplify DNA from these mutants, cloned the PCR products into a plasmid vector, and determined the sequence of the *unc-93* region (see Materials and Methods). The *e1500* rubber band mutation has a G→A transition at base pair 2960 that changes amino acid 388 from Gly to Arg (Fig. 3). The *n200* rubber band mutation has a C→T transition at base pair 681 that changes amino acid 49 from Ala to Val and a G→T transversion at base pair 3773 that changes amino acid 562 from Gly to Val (Fig. 3); we do not know whether one or both of these DNA changes is responsible for the phenotype caused by *n200.* The *n234* mutation has a G→A transition at base pair 3689 that changes amino acid 534 from Trp to an amber stop codon (Fig. 3). Because the *n234* mutation has been shown to be suppressed by mutations in the tRNA amber suppressor gene *sup-7* (Greenwald and Horvitz, 1980), this sequence change further confirms the identity between the *unc-93* genetic locus and the DNA that we have cloned. This mutation also suggests that the COOH-terminal 170 amino acids are required for *unc-93* function.

Discussion

We cloned the *C. elegans* muscle gene *unc-93* by transposon tagging. *unc-93* is likely to encode a novel membrane-associated protein involved in muscle contraction. The putative transposon-insertion alleles of *sup-9, sup-10*, and *sup-18* generated in this study should facilitate the cloning of these genes. The continued molecular characterization of this set of interacting genes should provide us with additional insight into mechanisms that regulate muscle contraction.

Two aspects of *unc-93* gene structure are striking. First, the *unc-93* gene has 15 introns and produces a 2.2-kb mRNA transcript, which is an unusually high density of introns for a *C. elegans* gene (Emmons, 1988). *C. elegans* genes expressed at high levels, such as those that encode actins (Edwards and Wood, 1983) or myosin heavy chains (MacLeod et al., 1981), have a much lower density of introns. Perhaps *C. elegans* genes expressed at low levels can tolerate a large number of introns and any resulting inefficiencies in RNA splicing. The second intriguing aspect of *unc-93* gene structure is the three repeated sequences found in its introns. Some introns have enhancer elements composed of repeated DNA sequences that regulate gene expression (Atchison, 1988), and it is possible that the *unc-93* repeated sequences regulate *unc-93* expression. However, no role can be assigned to these repeats at present. It is noteworthy that the exons encoding the two distinct regions of the unc-93 protein are separated by the largest intron of the *unc-93* gene. Intron 5, which contains 14 copies of repeated sequence 3 and could potentially form a stem-loop structure (Figs. 3 *b* and 5), might have joined the two distinct coding regions by a recombination event.

The rubber band phenotype caused by the altered-function *unc-93* alleles suggests a defect in the regulation or coordination of muscle contraction. The adult contains 95 mononucleate body wall muscle cells organized into four quadrants – two dorsal and two ventral (Sulston and Horvitz, 1977). Locomotion is achieved through the propagation of a wave of contraction and relaxation along the length of the worm, such that at a given time some of the dorsal muscle cells are contracted and the ventral muscle cells opposite them are relaxed, while adjacent dorsal cells are relaxed and the ventral cells opposite them are contracted (Chalfie and White, 1988). A defect in the muscle cells that disrupted the propagation of this wave of contraction and relaxation might result in a rubber band phenotype, in which both the anterior and posterior ends of the worm contract at the same time without any backwards movement. Such a defect might occur if the unc-93 protein were localized within the muscle cell membrane and if *unc-93* rubber band mutations disrupted communication among muscle cells, perhaps by affecting gap junctions, which are known to connect body wall muscle cells within a quadrant (White et al., 1986). The effect of rubber band mutations on the egg-laying muscles could be similarly explained, since these muscles are interconnected by gap junctions and presumably communicate with each other (White et al., 1986).

Alternatively, *unc-93* could regulate muscle contraction by functioning in the response of muscle cells to neuronal inputs in excitation-contraction coupling. Studies of excitation–contraction coupling in mammalian skeletal muscle have defined the steps of excitation–contraction coupling. The binding of a neurotransmitter by its receptor in the muscle cell membrane leads to an influx of sodium ions that triggers the depolarization of the muscle cell membrane (Shepherd, 1988). The dihydropyridine receptor, a calcium ion channel in the transverse tubules, acts as a voltage sensor to signal the ryanodine receptor (the calcium release channel) to release calcium ions from the sarcoplasmic reticulum into the cytoplasm (Catterall et al., 1988; Fill and Coronado, 1988; Jan and Jan, 1989). Calcium ions bind to troponin C in the thin filaments, which causes myosin to slide against actin to generate a contraction (Zot and Potter, 1987). The store of calcium ions in the sarcoplasmic reticulum is replenished by a calcium-dependent ATPase that pumps calcium ions from the cytoplasm back into the sarcoplasmic reticulum (MacLennan, 1970). Some of the details of excitation–contraction coupling differ in *C. elegans*, but the overall mechanism of muscle contraction is likely to be similar (Waterston, 1988). The contractile process in *C. elegans* is likely to be regulated via the release of calcium from the sarcoplasmic reticulum. However, *C. elegans* apparently does not have an equivalent to the transverse tubule system, possibly because the sarcoplasmic reticulum is in close proximity to the plasma membrane (Waterston, 1988). *C. elegans* uses both myosin and thin filament–linked calcium regulation of muscle contraction (Harris et al., 1977). For *C. elegans*, the details of the excitation–contraction coupling pathway between the acetylcholine receptor and the interaction of calcium ions with the thick and thin filaments in muscle cells are not yet known. Excitation–contraction coupling involves ion transport across both the muscle cell membrane and the membrane of the sarcoplasmic reticulum. Thus, *unc-93* might encode an ion transport protein or a protein that interacts with an ion transport protein localized to either of these membranes. If so, the rubber band phenotype could be caused by an ion channel with altered gating properties that disrupt muscle contraction. It is interesting to note that muscimol (Eldefrawi and Eldefrawi, 1987), a GABA agonist that seems likely to open $GABA_A$ chloride channels in body wall muscle (S. McIntire, E. Jorgensen, and H. R. Horvitz, manuscript in preparation), causes wild-type worms to behave like rubber band mutants (our unpublished data; E. Jorgensen, personal communication). This observation suggests that the rubber band mutant phenotype could be caused by a hyperpolarization of body wall muscle cells. Alternatively, *unc-93* could encode some other novel type of muscle membrane protein.

The rubber band mutation *e1500* changes amino acid 388 from Gly to Arg in a possible membrane-spanning domain (roughly amino acids 376 to 400) (Fig. 6 *b*). The altered-function of the unc-93 protein in *e1500* animals could be a result of the introduction of a charged amino acid in a hydrophobic region and/or the substitution of a bulky amino acid for the compact glycine. Because *n200* animals have two DNA changes in the *unc-93* gene, it is not possible to state whether both changes or only one of the two changes is responsible for the rubber band phenotype. In *n200* animals, the change of amino acid 49 from Ala to Val is in the highly charged NH_2-terminal putative cytoplasmic region and the change of amino acid 562 from Gly to Val probably affects the protein between two membrane-spanning domains. Both of the changes in *n200* animals and the change in *e1500* animals substitute a larger amino acid for a smaller one. In addition, because of its conformational flexibility, glycine (which is affected in both *n200* and *e1500* animals) is often used as a hinge between protein domains (Chou and Fasman, 1978). Thus, the *e1500* and *n200* mutations might directly or indirectly disrupt the configuration of *unc-93* membrane-spanning domains, thereby changing the interaction of these domains with each other or with other proteins.

Rare altered-function *unc-93* and *sup-10* alleles cause the rubber band phenotype and a disruption of muscle contraction (Greenwald and Horvitz, 1980, 1986). Because mutants that lack *unc-93*, *sup-9*, *sup-10*, or *sup-18* gene function display no visibly abnormal phenotype (Greenwald and Horvitz, 1980, 1986), there is likely to be one or more other proteins that can function in parallel to regulate the same aspect of muscle contraction. The functional redundancy for *unc-93* could reflect the ability of a single alternative gene or of a group of genes to replace *unc-93* function in *unc-93* null mutants. The *C. elegans* actin genes *act-1*, *act-2*, and *act-3* and collagen genes *rol-6* and *sqt-1* also have null alleles that result in a wild-type phenotype and altered-function alleles that result in visibly abnormal phenotypes (Waterston et al., 1984; Landel et al., 1984; Park and Horvitz, 1986; Kusch and Edgar, 1986). The function of each of these genes is redundant because it can be provided by other members of a homologous gene family (Landel et al., 1984; Kramer et al., 1988, 1990). Similarly, the functional redundancy of *unc-93* might be explained by an *unc-93* gene family, with an *unc-93* homolog able to function in place of *unc-93*. However, the functionally redundant protein is not likely to be an *unc-93* homolog because it can still properly regulate muscle contraction in the absence of *sup-9* or *sup-10*, whereas an *unc-93* homolog presumably would interact with the products of *sup-9* and *sup-10*. In the absence of *sup-9* or *sup-10* gene function, the *unc-93(e1500)* mutant protein does not disrupt the regulation of muscle contraction (Greenwald and Horvitz, 1980). This observation suggests that the *unc-93(+)* protein requires *sup-9* and *sup-10* proteins to function. Furthermore, genomic Southern blots probed at low stringency (e.g., 55°C, 0.75 M NaCl) with *unc-93* do not show any additional hybridizing bands (our unpublished data; M. Nadal-Vicens, personal communication). Thus, there is no evidence to support the existence of an *unc-93* gene family. Rather, we suggest that the functional redundancy of *unc-93* is due to a gene or set of genes unrelated by DNA sequence that can perform the same function. Based on the common phenotypes and suppression patterns observed among mutations in *unc-93*, *sup-9*, *sup-10*, and *sup-18*, these four genes are likely to act as a protein complex or in a common process in the membranes of muscle cells. The apparent functional redundancy of each of these genes could be due to proteins unrelated by sequence that act in parallel in a separate protein complex or pathway. In models in which the unc-93 protein interacts with gap junctions or ion channels, the rubber band mutations could be altering their gating properties to disrupt the regulation of muscle contraction even in the presence of a functionally redundant alternative protein complex or pathway.

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