

# Vinculin Is Essential for Muscle Function in the Nematode

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**Abstract.** Actin filaments in the body wall muscle of the nematode *Caenorhabditis elegans* are attached to the sarcolemma through vinculin-containing structures called dense bodies, Z-line analogues. To investigate the in vivo function of vinculin, we executed a genetic screen designed to recover mutations in the region of the nematode vinculin gene, *deb-1*. According to four independent criteria, two of the isolated mutants were shown to be due to alterations in the *deb-1* gene. First, antibody staining showed that the mutants had reduced levels of vinculin. Second, the sequence of each mutant gene was altered from that of wild type, with one mu-

tation altering a conserved splice sequence and the other generating a premature amber stop codon. Third, the amber mutant was suppressed by the *sup-7* amber suppressor tRNA gene. Finally, injection of a cloned wild type copy of the gene rescued the mutant. Mutant animals lacking vinculin arrested development as L1 larvae. In such animals, embryonic elongation was interrupted at the twofold length, so that the mutants were shorter than wild type animals at the same stage. The mutants were paralyzed and had disorganized muscle, a phenotype consistent with the idea that vinculin is essential for muscle function in the nematode.

**T**HE formation and position of actin-membrane attachments is likely to be one of the principal mechanisms by which cells determine their shape and their direction of movement. Therefore, a large effort has been invested in identifying and characterizing the proteins responsible for these actin-membrane attachments. Vinculin, a 117-kD protein, is a major component of focal adhesions in cultured chicken cells (Geiger, 1979), as well as many other types of actin-membrane attachments in both muscle and nonmuscle cells (see Burridge et al., 1988; Otto, 1990 for reviews). In vitro, vinculin has been shown to interact with two other components of focal adhesions, talin (Otto, 1983; Burridge and Mangeat, 1984) and the actin binding protein  $\alpha$ -actinin (Wachsstock et al., 1987). It is not clear, however, whether the relatively low affinity binding to  $\alpha$ -actinin has any relevance to its in vivo function.

The amino acid sequence of vinculin was revealed through the cloning of cDNAs from chicken (Coutu and Craig, 1987; Price et al., 1987; Bendori et al., 1987) and from the nematode *Caenorhabditis elegans* (Barstead and Waterston, 1989). Coutu and Craig (1988) proposed (a) that the globular head of vinculin (Milam, 1985) encompassed the NH<sub>2</sub>-terminal two-thirds of the sequence, and (b) that the relatively basic tail region was separated from the head by a short proline-rich sequence and could be responsible for the observed interactions between vinculin and acidic phospholipids (Ito et al., 1983; Niggli et al., 1986; Coutu and Craig, 1988). By expressing portions of the cloned sequence in vitro, a talin binding sequence has been tentatively localized to amino acid residues 167–207 of the chicken protein (Bendori et al., 1989; Jones et al., 1989). These deleted vinculins also fail to localize to focal adhesions in transgenic cell lines,

suggesting that the talin interaction may be important, but since vinculin is known to self associate in vitro (Otto, 1983) such deletions may also be affecting vinculin-vinculin interactions.

Vinculin is a component of the dense bodies in the body wall muscle of the nematode *Caenorhabditis elegans* (Barstead and Waterston, 1989). These finger-like structures project from the basal sarcolemma in the highly ordered array associated with the regular structure of striated muscle (Waterston, 1988). Like the Z-discs of vertebrate striated muscle, the dense bodies bind actin filaments of opposite polarity. Their firm linkage to the sarcolemma, however, suggests that dense bodies are also analogous to focal adhesions. Moreover, in addition to vinculin, nematode dense bodies contain the actin binding protein  $\alpha$ -actinin (Francis and Waterston, 1985), further emphasizing their relationship to several of the types of actin-membrane attachments found in vertebrate cells. The dual nature of the nematode dense body may be similar to the actin-membrane attachments in nascent vertebrate myofibrils of developing striated muscle, which also have the characteristics of both focal adhesions and Z-discs (Dlugosz et al., 1984; Antin et al., 1986; Lin et al., 1989).

A mutational analysis of genes used for muscle assembly can lead to direct tests of the in vivo role of the affected protein as well as the identification of interacting proteins through the isolation of compensating mutations in other genes. With the cloning of the vinculin gene from *C. elegans* (Barstead and Waterston, 1989), it became possible, through the use of the physical map (Coulson et al., 1986, 1988), to find its position on the nematode genetic map. This allowed us to examine whether its position corresponded to any of

the 35 known muscle affecting genes, with particular attention to three genes known to affect thin filaments, *unc-52*, *unc-95*, *unc-97* (Brenner, 1974; Zengel and Epstein, 1980; R. Francis and R. H. Waterston, unpublished observations). None of the genes in this collection were located near the vinculin gene. Since vinculin was not the product of any known gene, we named the genetic unit producing vinculin *deb-1* (dense body-1), on chromosome IV. Given the potential value of a mutational analysis in dissecting vinculin function, we sought to recover mutations in the nematode *deb-1* gene. Here we report the successful recovery of *deb-1* mutants, describe their phenotypes, and determine the sequence changes. These mutants are nearly completely paralyzed, fail to organize normal muscle structure, and have reduced levels of vinculin. Although mutant animals may hatch, all arrest development in the first larval stage.

## Materials and Methods

### Screening for Essential Muscle Affecting Genes on Linkage Group IV

General methods of culture, handling, and genetics were used (Brenner, 1974). The following mutations were used: *unc* (uncoordinated); *fem* (feminization); *tra* (transformer); *sup* (suppressor); *dpy* (dumpy); linkage group (LG) II: *tra-2(q122)*; LG IV: *unc-44(e362)*, *unc-82(e1323)* *unc-24(e138)*, *fem-3(e1996)*, *dpy-20(e1282)*; LG X: *sup-7(st5)*. The genes *unc-24* and *unc-44* have no apparent effects on muscle organization and probably involve nervous system defects.

To facilitate a genetic screen to recover vinculin mutants we needed to develop criteria for recognizing such mutants. Previously we reported that (a) the nematode vinculin gene is present in a single copy, and (b) the gene mapped to a region of chromosome IV, near *unc-44*, that did not contain any previously known muscle affecting genes (Barstead and Waterston, 1989). Since the current set of known homozygous viable muscle affecting genes is likely to be nearly complete (Waterston, 1988), the absence of vinculin mutants from this set suggested that such mutations might be homozygous inviable.

Since we knew the location of the wild type vinculin gene on chromosome IV (Barstead and Waterston, 1989), we designed a genetic screen to isolate mutations in essential genes in that region (see Fig. 1). Animals at the L4 larval stage homozygous for *unc-44(e362)IV*, a gene which does not affect muscle and which was estimated to be less than 1 map unit from *deb-1*, were treated with 50 mM ethane methane sulfonate for 4 h at 20°C and then mated with wild type N2 males. F1 progeny from this cross were either *unc-44/±* or *unc-44 lethal/+*. The F1 animals were transferred singly to 30-mm plates seeded with *E. coli* OP50 and allowed to reproduce. Those F1 animals with the genotype *unc-44 lethal/+* cannot give rise to animals with the *unc-44* phenotype except by recombination. Accordingly, the F2 broods were then examined for those lacking *Unc-44* animals, their absence indicating the presence of a new mutation in an essential gene closely linked to *unc-44*.

In *Caenorhabditis elegans*, complementation tests among mutations are typically done by crossing males carrying one mutation with hermaphrodites carrying a second mutation, and the cross-progeny are scored for the presence of the mutant phenotype. Since, however, the hermaphrodites can produce progeny without mating with a male, the crosses must be designed so that the self-progeny can be distinguished from the cross-progeny. For complementation tests among mutations in nonessential genes, cross-progeny are distinguished from self-progeny by the use of mutations in other marker genes or by the sex of the animals. For complementation tests among embryonic or early larval lethal mutations, however, it is not practical to use these criteria to distinguish self from cross-progeny, since at early stages there are no easily observable differences between the sexes and many marker phenotypes are not apparent until later in development. We therefore used a novel method to carry out complementation tests among lethal mutations. Using this method we eliminated the self-cross progeny by introducing the gene *tra-2(q122)* (Schedl and Kimble, 1988), which when present in one or two copies (*tra-2(q122)/tra-2(q122)* or *tra-2(q122)/+*) converts hermaphrodites into females that are not self-fertile but which do produce progeny when mated with a male.

To carry out complementation tests, *tra-2(q122)/tra-2(q122)* males were mated with strains carrying the lethal mutations to be tested. The self-sterile female progeny from these crosses were mated to males carrying a second mutation to be tested for complementation. The appearance of lethal progeny from this cross indicated the failure of the mutations to complement, suggesting they were in the same gene.

### Antibody Staining of Embryos

Embryos were released from gravid adults by treatment with basic hypochlorite and then washed four times with M9 buffer (Hodgkin et al., 1988). For antibody staining, the embryos were fixed 10 min in 3% formaldehyde (from paraformaldehyde), 0.1 M sodium phosphate, pH 7.0, 0.1 mM EDTA. After washing two times in PBS, pH 7.0, the embryos were treated with -20°C methanol for 10 min, washed two times with PBS, two times with PBS containing 0.5% Tween-20 (Sigma Chemical Company, St. Louis, MO), and once with antibody binding buffer, (PBS, pH 7.0, 0.5% Tween-20, 30% normal goat serum). A rabbit serum recognizing nematode myosin heavy chain (Francis, G. R., and R. H. Waterston, unpublished observations) and a mAb, MH24, recognizing nematode vinculin (Francis and Waterston, 1985) was incubated in binding buffer for 60 min. In one experiment, we used a mAb, DM5-6, which binds to nematode myosin heavy chain A (MHC A) (Miller et al., 1983), kindly provided by D. Miller (Duke University). The primary antibodies were removed by four washes with PBS/0.5% Tween-20 and the embryos were incubated with FITC-labeled goat antirabbit and rhodamine-labeled goat antimouse secondary antibodies (ICN Immunobiochemicals, Costa Mesa, CA). After washing, the embryos were mounted in glycerol and viewed as described (Francis and Waterston, 1985). Kodak T-Max 3200 film (Eastman Kodak Company, Rochester, NY), developed at 29°C at an ASA of 1600, was used to record the fluorescent images. For staining with rhodamine-phalloidin (Molecular Probes, Inc., Eugene, OR), hypochlorite-treated and washed embryos were fixed in 100% ethanol for 10 min at -20°C, and rehydrated with successive rinses with 70, 50, and 30% ethanol. The embryos were then incubated at room temperature in PBS, pH 7.0, and rhodamine-phalloidin at a concentration of 66 nM. Embryos were then mounted and viewed as described above.

### Heteroduplex Mismatch Analysis

DNA from the homozygous mutant animals was recovered using the polymerase chain reaction (PCR)<sup>1</sup> (Saiki et al., 1988). Six PCR fragments ranging in size from ~1,000–2,000 bp were required to cover all of the coding sequence of the gene. According to the sequence coordinates reported in Barstead and Waterston (1989), these fragments encompass the regions portrayed in Fig. 5 a and Table I. Approximately 100 mutant animals were picked into a drop of M9 buffer, washed in a drop of PCR lysis buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.2, 2.5 mM MgCl<sub>2</sub>, 0.45% NP-40, 0.45% Tween-20, 0.1 mg/ml gelatin) (Higuchi, 1989), and transferred in a small volume to a 0.5-ml microcentrifuge tube. The volume was brought to 0.1 ml with PCR lysis buffer, and Proteinase K (Boehringer Mannheim Biochemicals, Indianapolis, IN) was added to a concentration of 20 µg/ml. The animals were frozen at -70°C for 10 min, incubated under mineral oil at 60°C for 60 min, and finally heated to 95°C for 15 min to inactivate the protease. 10 µl of this solution was used in each of six PCR reactions to generate overlapping PCR fragments of 1–2 kb covering all of the coding sequence of the gene. Each primer pair was used at a concentration of 0.5 pmol/µl. 0.5 U of Taq polymerase (Perkin Elmer Cetus, Norwalk, CT) was used for each reaction. DNA was amplified 30 rounds in a DNA Thermocycler (Perkin Elmer Cetus, Norwalk, CT). Each of the six PCR fragments was purified on 0.7% agarose gels using GeneClean (Bio101, La Jolla, CA). The same reaction conditions were used to generate PCR fragments from wild type N2 DNA.

Wild type PCR fragments were labeled at their 5' ends by standard procedures (Maniatis, 1983) using T4 polynucleotide kinase (Pharmacia LKB Biotechnology, Piscataway, NJ) and  $\gamma^{32}$ P-ATP (Amersham International, Arlington Heights, IL). The labeled fragments were mixed with the corresponding unlabeled fragments from the mutant at molar ratios between 1:10 to 1:20 (labeled to unlabeled DNA) in a 1.5-ml microcentrifuge tube, boiled for 5 min, and hybridized under mineral oil at 65°C overnight in 0.3 M NaCl, 0.1 M Tris-HCl, pH 8, and 0.1 mM EDTA. Base pair mismatches between the wild type and mutant PCR fragments were detected as in Cotton et al. (1988) and Montandon et al. (1989). The chemicals used in this procedure were all obtained from Aldrich Chemical Company (Milwaukee,

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

**Table I. Sequence Coordinates of PCR Fragments**

Fragment	Coordinates
B	3921–5721
C	5500–6797
D	6501–7500
E	8791–10594
F	10342–11731
G	2000–3000

WI). The cleavage products from these reactions were electrophoresed on 5% acrylamide-urea denaturing gels. Gels were exposed to Kodak XAR-5 film (Eastman Kodak Company, Rochester, NY) with an intensifying screen.

Once a mismatched site was identified, DNA primers were made upstream of the site and the double-stranded PCR fragment was sequenced directly. The double-stranded DNA was prepared for sequencing using the protocol of Dubose and Hartl (1990), and sequenced using Sequenase (United States Biochemical Corp., Cleveland, OH).

### Transformation Rescue of the Vinculin Mutants

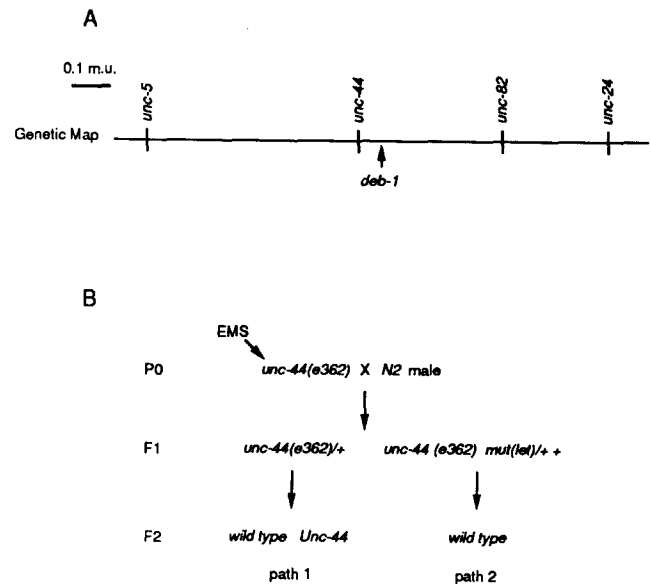
A lambda clone containing all of the sequenced region of the *deb-1* gene, plus an undetermined amount of flanking sequence, was mixed with a plasmid carrying the gene *rol-6(sul006)* (Kramer et al., 1990) in injection buffer (Fire, 1986) at concentrations of 10 and 100 ng/ $\mu$ l, respectively. Wild type N2 animals were injected with this mixture using the protocol of C. Mello (C. Mello, Harvard University, personal communication) and their progeny were examined for animals which rolled, indicating the presence of the marker *rol-6(sul006)* gene. These animals were tested by PCR to determine whether they also received a copy of the lambda clone carrying *deb-1* (data not shown). In all transgenic lines obtained, the transgene behaved as an extrachromosomal array, with ~50% of the progeny at each generation inheriting the transgene. Males with the genotype *unc-44(e362) deb-1(st555)/+ +* were crossed to hermaphrodites carrying the transgenic array to test for suppression of the *st555* mutation. The appearance of rolling Unc-44 progeny from outcross animals carrying the transgene and the *deb-1 unc-44* chromosome was taken as evidence of suppression. These rolling Unc-44 animals were picked and their progeny examined to rule out the possibility that they represented a recombination between *unc-44* and *deb-1*. All of the progeny from legitimately rescued animals were either Unc-44 rollers or arrested L1 lethals.

## Results

### Screening for Vinculin Mutants

Screens to recover mutations in essential genes in a defined genetic region are readily carried out in *C. elegans* (Herman, 1988); further criteria are required, however, to sort through the mutants to identify those which fall in a specific gene of interest. We decided that, at least in the initial stages, we would restrict our attention to those mutants that exhibited a phenotype like that of *myo-3* mutants (Waterston, 1989). Such mutants lack the myosin heavy chain form MHC A, are severely paralyzed, and arrest development at the L1 larval stage. Elongation of *myo-3* mutants is normal until they reach the twofold length, where they stop.

As described in Materials and Methods and illustrated in Fig. 1, we carried out a genetic screen to recover mutations in essential genes in the region of the nematode vinculin gene, *deb-1*, by looking for mutations closely linked to a gene, *unc-44*, known to be near *deb-1*. Out of ~1,000 F1 clones screened, three met the criteria of the screen; that is, they were linked to *unc-44*, they were paralyzed, and they arrested at the twofold embryonic length. These three isolates, *st554*, *st555*, and *st556* fell into three complementation

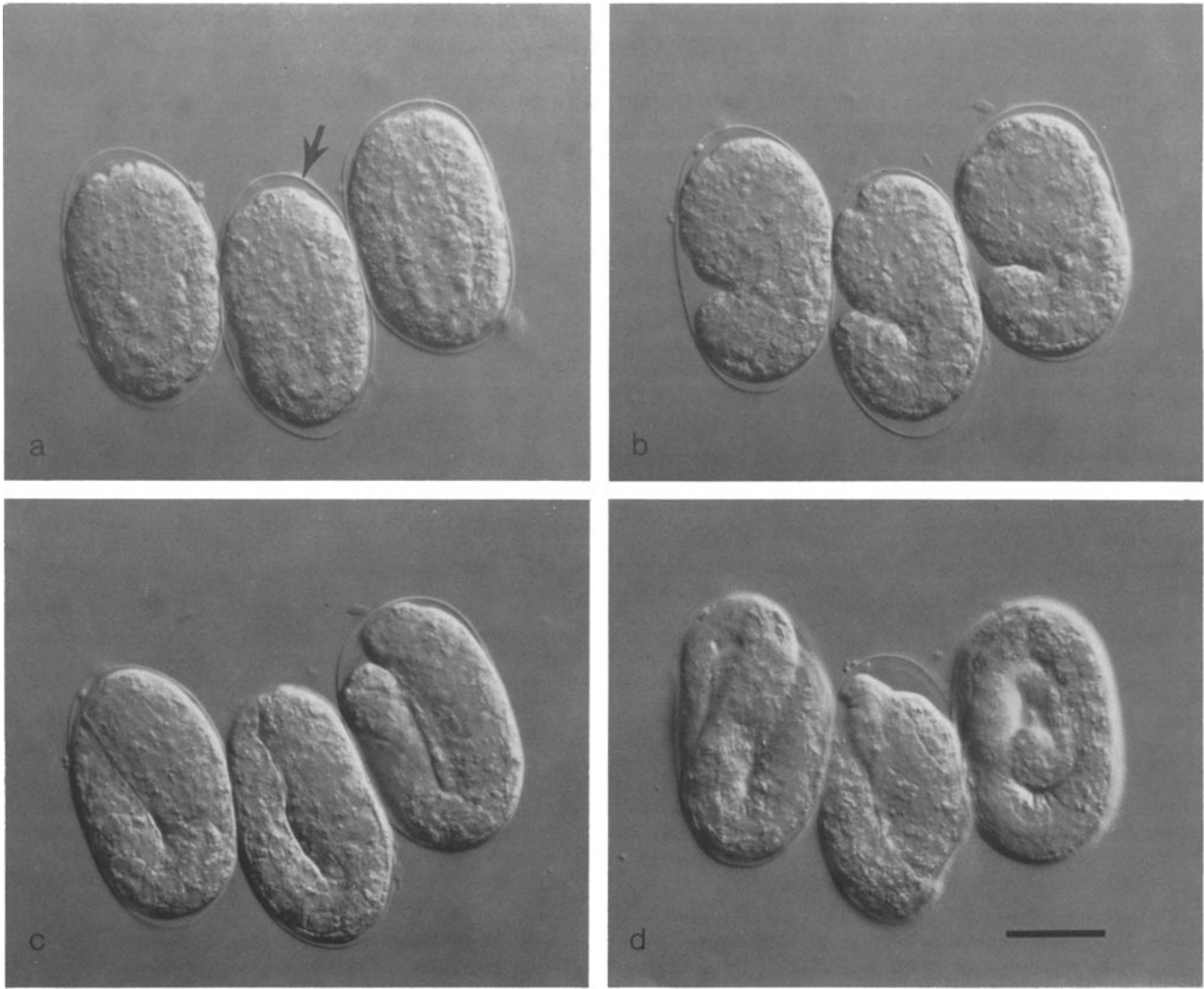


**Figure 1.** (A) Genetic position of the nematode *deb-1* gene as deduced from the physical map. The genetic and physical maps in this region of chromosome IV are well correlated (A. Coulson, MRC Laboratory of Molecular Biology, personal communication). The positioning of *deb-1* on the physical map (Barstead and Waterston, 1989) places it to the right of *unc-44* near the indicated position. (B) Scheme to recover mutations in essential genes in the region of *unc-44*. Mutagenized Unc-44 animals were mated to wild type N2 males. F1 hermaphrodites were cloned and their F2 self cross progeny were examined. If no lethal mutations were induced in the region of *unc-44(e362)*, then both wild type and Unc-44 progeny appeared in the F2 generation as is indicated in path 1 on the left. On the right, path 2 illustrates the results if a lethal mutation was induced in the region. In this case, F2 progeny which were homozygous for *unc-44(e362)* would also be homozygous for a mutation in an essential gene and consequently would not survive. The lack of Unc-44 progeny in the F2 generation, then, suggested that a lethal mutation was induced in the region of *unc-44*.

groups. Complementation tests were also done between each of these three isolates and the paralyzed, twofold lethal mutant *st385* obtained in a separate genome wide screen for mutants of this class (B. Williams and R. H. Waterston, unpublished results). The mutants *st385* and *st555* failed to complement and were likely to represent independent mutations in the same gene. The phenotype of the *st555/st385* trans-heterozygote resembled that of the *st385* or *st555* homozygotes; that is, it failed to elongate beyond twofold and died with little or no movement after hatching. To maintain these lethal mutants as heterozygous strains, a chromosome IV marked with two nearby genes, *unc-82(e1323)* and *unc-24(e138)*, was crossed into the mutants to produce strains with the genotype *unc-44(st555 or st385)/unc-82 unc-24*.

### Staining the Mutant Isolates with Antivinculin

To assess the mutant isolates for the accumulation of vinculin, we stained a population of embryos from each heterozygous strain with mAbs to nematode vinculin and with rabbit antibodies to nematode myosin as a positive control. We were able to recognize homozygous mutant animals in mixtures of nonlethal siblings even after fixation since the mutants exhibited a disproportionately elongated snout, which



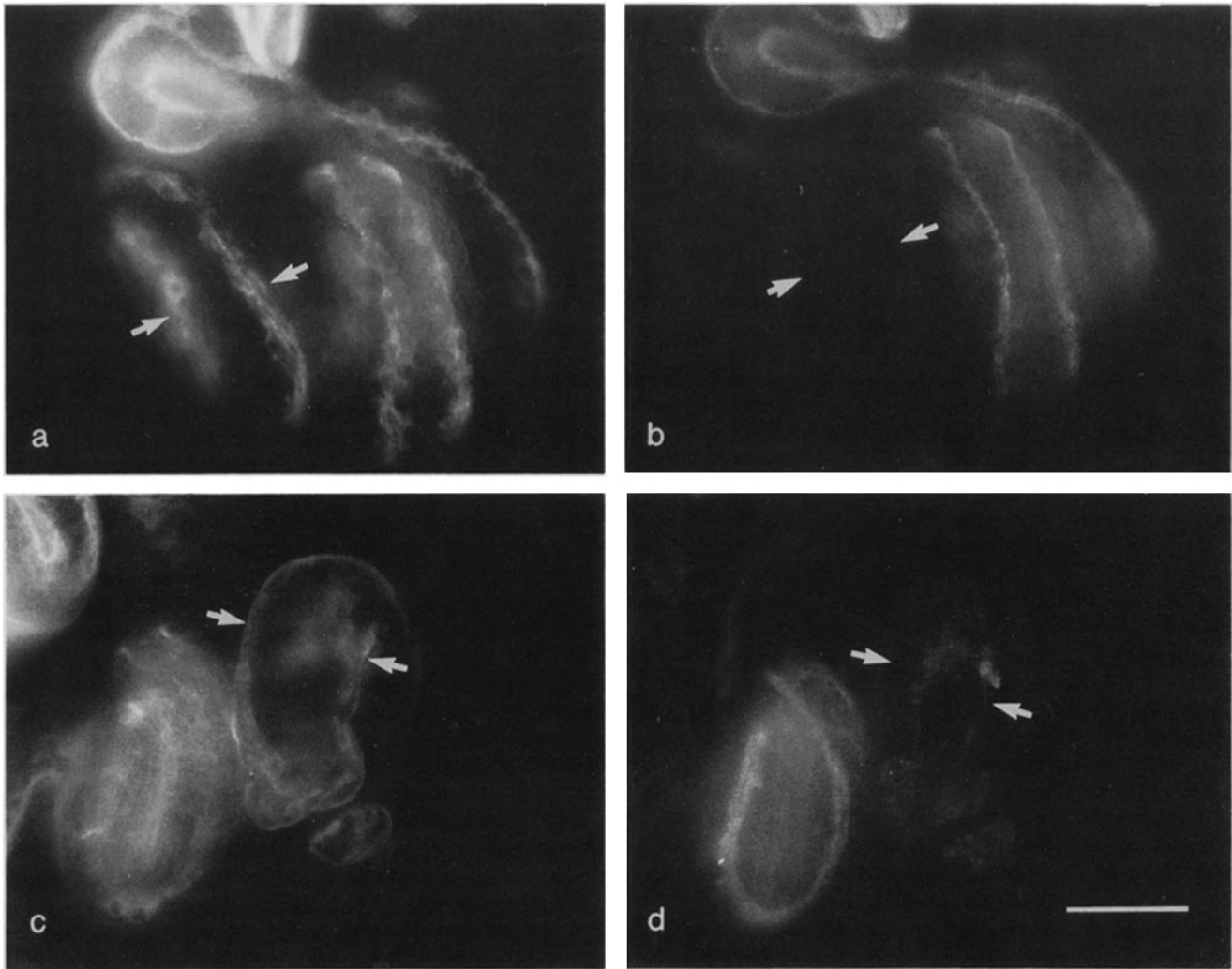
**Figure 2.** The development of mutant isolate *st555* embryo. At  $\sim 200$  min after fertilization (a) there was no detectable difference between mutant (arrow) and the two flanking wild type embryos. Although not apparent in these static images, the first difference could be detected at  $\sim 400$  min, when the embryos reached the 1 1/2-fold stage (b). At this stage the wild type embryos began to twitch, the first signs of muscle activity. By the twofold stage (c) they were vigorously rolling around inside the egg. The mutant embryo was completely paralyzed at these stages. At  $\sim 600$  min (d), as threefold embryos, the wild type animals continued to move. The mutant embryo remained paralyzed and had ceased to elongate, arresting at the twofold length. At this time morphological differences between wild type and arrested mutant twofold embryos became apparent. The snout of the mutant was disproportionately elongated and there were variably placed circumferential constrictions and indentations. Bar, 20  $\mu\text{m}$ .

was often bent, as well as circumferential constrictions and indentations in the surface of the embryo (Fig. 2 d). Two isolates emerged as good candidates for vinculin mutants. Homozygous *st555* animals did not stain with the antivinculin antibodies (Fig. 3 b), but stained strongly with the antimyosin antibody (Fig. 3 A). Homozygous *st385* animals stained weakly with antivinculin antibodies (Fig. 3 d). The mutants *st554* and *st556*, however, stained as strongly as wild type with both the antivinculin and antimyosin antibodies (not shown) and consequently were considered to be unlikely candidates for vinculin mutants.

#### **Phenotype of the Mutant Isolate *st555***

Since the *st555* animals appeared to lack all vinculin, we concentrated further analysis on this mutation. Less detailed

observation of *st385* animals suggested that they did not differ significantly in any of the phenotypes studied. In comparing *st555* embryos to wild type, the earliest phenotypic difference was apparent at  $\sim 400$  min after fertilization, which corresponds to the 1 1/2-fold stage of morphogenesis (Sulston et al., 1983). At this stage the wild type embryos began to twitch, showing the first signs of muscle activity, whereas mutant embryos remained immotile. As development proceeded and the wild type embryos elongated further to the twofold stage they began to roll vigorously around in the egg. By threefold, the wild type embryos actively crawled within the egg. Mutant embryos remained paralyzed throughout most of embryogenesis, suggesting that the body wall muscle was nonfunctional. The pharynx could be seen to pump, however, indicating that the pharyngeal muscle was



**Figure 3.** Staining of isolates *st555* and *st385* with antibodies to myosin and vinculin. Since the mutations were propagated in heterozygotes the populations contained both mutant and phenotypically wild type embryos. Several embryos from a population carrying the *st555* mutation are shown in *a* and *b*. *c* and *d* show three embryos from a population carrying the *st385* mutation. The embryos were double stained with antibodies to myosin and vinculin. Only the body wall muscles are visible. The arrows show the boundaries of a single arrested mutant. The indicated embryo in *a* and *b* is homozygous for *st555* and stains strongly with antibodies to myosin (*a*), but is completely negative for staining with antivinculin (*b*). *c* and *d* display an animal homozygous for *st385* that stains with antimyosin (*c*), and stains weakly with antivinculin (*d*). Bar, 20  $\mu$ m.

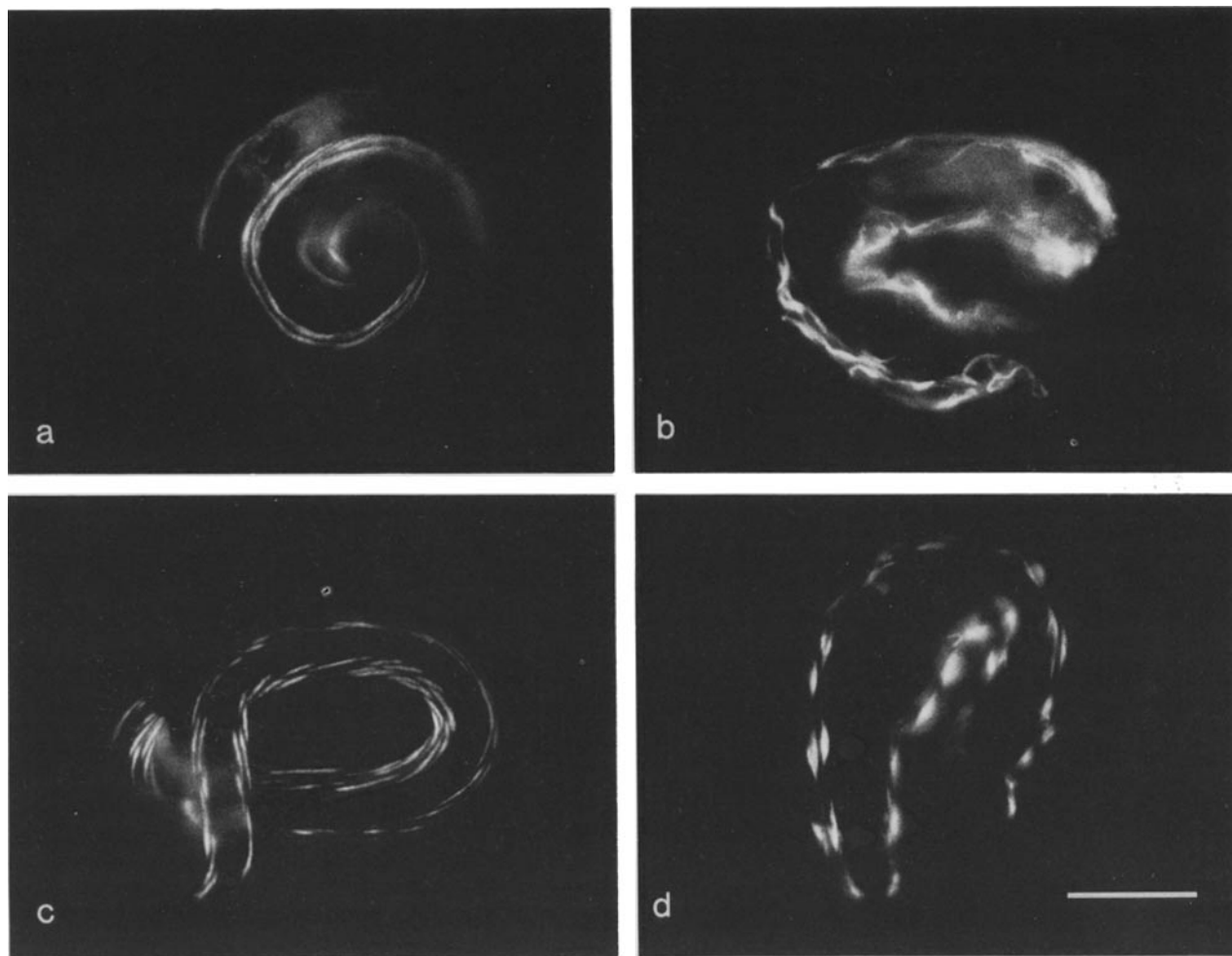
at least partially functional. Around the time of hatching, the mutant embryos twitched infrequently and weakly. Although these minor twitches became slightly more vigorous after hatching, they were never effective in generating movement. Initially, elongation of the embryos was normal (Figure 2, *a* and *b*), but, as in *myo-3* mutants, *st555* mutants ceased to elongate at about the twofold stage and hatched in this shape (Fig. 2, *c* and *d*). The mutants arrested and eventually died as L1 larvae.

To evaluate the effects of vinculin loss on the organization of the major myofilament proteins, actin and myosin, we stained populations of embryos with rhodamine-phalloidin (Fig. 4, *a* and *b*) and with antibodies to the myosin heavy chain isoform MHC A (Fig 4, *c* and *d*). Again, in these mixed populations, we used the abnormal morphological features described above for the older mutant embryos to distinguish them from wild type siblings. For purposes of comparison, we selected siblings of similar ages, which have

of course elongated to threefold or more. Both the actin and the myosin organization were abnormal in animals homozygous for the *st555* mutation, since neither of these sets of filaments became organized into discrete striations as was seen for the wild type embryos. The distribution of MHC A staining, which in wild type is organized as a pair of striations within each cell, was replaced in the mutant by a single centrally located patch. Actin staining of homozygous *st555* mutants was distributed widely in the cell, often without obvious proximity to the hypodermis.

#### **Genetic Mapping of *st555***

To examine the relationship of the *st555* isolate to other genetic markers, we first determined the map distance from *unc-44* (Fig. 1 *a*). We picked *unc-44* recombinants from a strain with the genotype *unc-44(e362) st555/+ +*. Out of 3,704 progeny tested, 14 had the Unc-44 phenotype, which



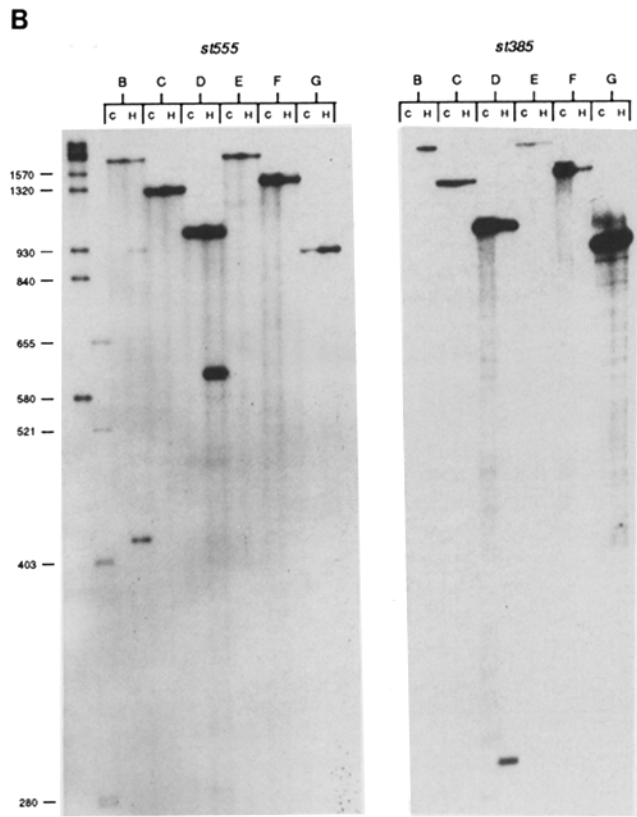
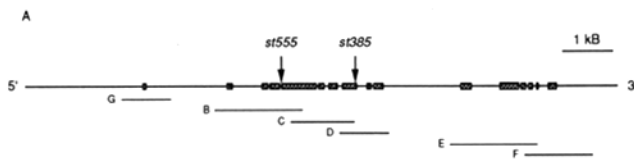
**Figure 4.** Staining of *st555* with rhodamine-phalloidin and antimyosin. Staining of wild type threefold embryos with rhodamine-phalloidin (a) or with antibodies to the nematode myosin heavy chain MHC A (c) shows that at this stage the muscle is well organized as striations are clearly apparent. Staining of arrested twofold embryos, which are chronologically similar to wild type threefold embryos, with rhodamine-phalloidin (b) or with anti-MHC A (d) shows that both actin and myosin fail to organize into discrete striations (see text). Bar, 20  $\mu$ m.

placed *st555* 0.6 map units from *unc-44*. To determine the map order we picked *unc-44* recombinants from a strain with the genotype *unc-44(e362) st555/unc-82(e1323) unc-24(e138)*. Of the *unc-44* recombinants scored, all picked up the *unc-82* marker, which placed *st555* to the right of *unc-44*. This genetic position was consistent with the known location of the vinculin gene on the physical map.

#### **Examining the Mutants for DNA Sequence Changes**

With these preliminary indications that the *st385* and *st555* isolates might represent mutations in the vinculin gene, we decided to examine the vinculin gene from these mutants for DNA sequence changes. To recover DNA from the arrested mutant animals we adapted the PCR for use on small numbers of L1 larvae. Six PCR fragments ranging in size from  $\sim$ 1 or 2 kb were required to cover all of the coding sequence and much of the intronic sequence from the gene (Fig. 5 a). To detect the presence and position of possible base pair mismatches in mutant/wild type DNA heteroduplexes, we used the chemical cleavage method described by Cotton et al.

(1988). The tests for mismatched cytosines in wild type/*st555* or wild type/*st385* heteroduplexes (Fig. 5 b) indicated that there were three sites from *st555* and one site from *st385* that did not match the wild type sequence. DNA from the PCR fragments was directly sequenced using primers adjacent to the expected position of the mutation. The specific DNA sequence changes detected by this method are listed in Table II. For *st555* all three of the changes fall within introns; however, the G to A change at position 5325 alters a splice acceptor site immediately adjacent to the fifth exon. Since all known introns have a G residue at the intron-exon boundary of the acceptor site, and experimental evidence shows that this G is essential for normal splicing (for review see Padgett et al., 1986), this change almost certainly prevents splicing of the fourth intron, thereby inactivating the gene. The two other changes detected in *st555* fell in the middle of large introns and are probably not relevant to the mutant phenotype. Only a single C to T change at position 6796 was found for *st385*, and this change generated an in-frame UAG amber stop codon leading to the truncation of the protein at amino acid residue 565.



**Figure 5.** Heteroduplex mismatch analysis of *st555* and *st385*: The hydroxylamine reaction. (a) The shaded boxes indicate the exon sequences in the sequenced region of the *deb-1* gene. Six PCR fragments covering the indicated positions were required to cover all exons. The positions of the relevant DNA sequence changes are indicated for each isolate. (b) PCR fragments from wild type DNA were end-labeled and hybridized with wild type DNA for the controls (marked with a C above the gel track) and with the corresponding PCR fragments from animals homozygous for the *st555* or *st385* mutations (marked with an H above the gel track). The hybrids were then treated with hydroxylamine and piperidine to detect mismatched cytosines. For *st555*, two mismatches were detected in the B fragment and one mismatch was found in the D fragment. For *st385*, a single mismatch was found in the D fragment. The two leftmost lanes are molecular weight standards. Their sizes in base pairs are indicated on the left.

### Suppression of the *st385* Amber Mutant by the *sup-7* Amber Suppressor

The finding that the *st385* mutation generated an amber translational stop codon led us to try to suppress its effects by introducing the *sup-7* amber suppressor gene which encodes a mutated tRNA<sub>Trp</sub> that can read amber codons (Waterston, 1981; Wills et al., 1983). We constructed a strain with the genotype *unc-44(e362) deb-1(st385)/unc-24(e138) fem-3(e1996) dpy-20(e1282); sup-7(st5)*. In the absence of *sup-7*, viable Unc-44 animals should appear at a frequency of ~1 in 200 as a result of recombination. In the presence of the suppressor, adult Unc-44 animals made up ~25% of the progeny, indicating that the L1 arrest phenotype of *deb-1(st385)* was suppressed. Other phenotypes were incompletely suppressed. Although such animals were mo-

tile they did not move as well as *unc-44* homozygotes alone and their muscles were disorganized as assayed by polarized light microscopy (not shown). Furthermore, these animals were infertile, which prevented the establishment of a homozygous strain. Since *sup-7* by itself reduces fertility, the sterility of these strains may not be due solely to incomplete suppression of the vinculin mutation.

### Rescue of the Vinculin Mutant by Transformation with the Wild Type Gene

To test whether the mutations detected in the vinculin genes of *st555* and *st385* animals were solely responsible for the observed paralysis and failure to elongate, we reintroduced a cloned copy of the vinculin gene into the genome. To carry out the transformation, we injected a mixture of DNA containing the vinculin gene and the *rol-6(su1006)* gene into the gonads of wild type animals. The *rol-6* gene served as a dominant marker of transformation, so that roller progeny of injected animals were presumptive transformants. After allowing these animals to lay eggs, they were tested by PCR and found to carry the unselected copies of the vinculin gene (not shown). Several such cotransformants were obtained, all of which segregated rollers at a frequency of 30–50% in a manner that suggested that the transgenes were not integrated,

**Table II.** Sequence Changes in Vinculin Mutants

Isolate	Sequence change	Position
<i>st555</i>	G → A	4803
	G → A	5325
	G → A	6914
<i>st385</i>	C → T	6796



but were propagated as extrachromosomal arrays. One of these arrays, *stEx3*, was used to establish the strain RW9004.

To test whether a transgenic copy of the vinculin gene could rescue the mutant phenotype of *st555* and *st385*, the *stEx3* array was introduced by standard genetic crosses into a genetic background containing either *deb-1(st555) unc-44(e362)* or *deb-1(st385) unc-24(e138)*. Viable Unc-44 or Unc-24 progeny, as presumptive rescued animals, were selected and used to establish the stable lines, RW9005 and RW9007, respectively. The line RW9005 segregated progeny of two types: Unc-44 rollers, or paralyzed, arrested L1 animals. The genotype of RW9005, therefore, must have been *unc-44(e362) deb-1(st555); stEx3*. The line RW9007 segregated Unc-24 rollers and arrested L1 animals. The genotype of RW9007 must have been *deb-1(st385) unc-24(e138); stEx3*. This showed that a transgenic copy of the nematode vinculin gene was capable of rescuing the elongation defect and lethality of the *st555* and *st385* mutations.

To determine whether the paralysis of the mutants was also corrected by the transgenic vinculin gene, we evaluated the movement of animals from RW9007. We have already observed that the mutation *unc-82(e1323)*, which by itself is known to affect muscle structure and to cause a subtle reduction in motility (Waterston, 1989), could be reliably detected in animals carrying *unc-24(e138)*. Since there were no detectable differences in the motility of the rescued animals from RW9007 and animals carrying *unc-24(e138)* by itself, the body wall muscle of the rescued animals must have been restored to near normal function. Furthermore, the rescued animals were of normal size, laid eggs, and defecated, indicating function of the pharyngeal, vulva, uterine, rectal, and intestinal muscle, all of which contain vinculin. These results confirmed that the transgenic copies of the vinculin gene on *stEx3* were capable of rescuing the elongation defect, paralysis, and lethality of the mutants.

## Discussion

A number of factors were important to our success in recovering vinculin mutants in the nematode. First, the cloning of the vinculin gene led to the finding that it was not a member of a closely related, redundant multi-gene family (Barstead and Waterston, 1989). Second, making use of the nearly complete physical map of the nematode genome (Coulson et al., 1986, 1988), we were able to locate the approximate position of the vinculin gene on the genetic map, and thereby eliminate the possibility that one of the known homozygous viable, muscle-affecting Unc mutations in the nematode was in the vinculin gene. The absence of vinculin mutants from this large class of Unc mutants suggested that mutations in the gene might be homozygous inviable. Third, the mutant phenotype was like that of another essential muscle gene, *myo-3*, allowing us to sharply focus our genetic screen. It is, in fact, likely that a relatively large class of muscle affecting genes have this phenotype (Williams, B., and R. H. Waterston, unpublished results). Finally, we were able to use antivinculin antibodies to screen candidate mutants rapidly and thereby identify those that did not produce normal levels of vinculin.

Our results show that the genetic changes responsible for the paralysis of the mutant embryos are those identified in the vinculin gene, thereby demonstrating that vinculin is es-

sential for normal body wall muscle function. The two mutations, *st555* and *st385*, were isolated independently, fail to complement, and map to a region 0.6 map units right of *unc-44*. The two mutations thus must be presumed to lie in the same gene, in a position consistent with the known location of the vinculin locus on the physical map. Since animals homozygous for *st385* or *st555* and animals that are *st385/st555* all result in the same constellation of phenotypes, and during extensive genetic mapping no partial or intermediate phenotypes were seen, it is highly unlikely that the paralysis, arrested elongation, and lethality are due to mutations at sites other than at this single locus.

That this single locus is the vinculin gene is shown by the following observations. (a) The vinculin genes from the mutants have an altered DNA sequence. The gene from the *st555* mutant has a G to A change in a splice acceptor site which is almost certain to prevent proper splicing of the message. The C to T change found in *st385* mutants leads to the introduction of a premature amber stop codon, resulting in the truncation of the protein at residue 565. Both of these mutations are associated with a reduction in the accumulation of vinculin as assayed by immunofluorescence. (b) The *sup-7* gene, encoding a mutant tRNA<sub>trp</sub> capable of translating amber stop codons, suppresses the paralysis, elongation defect, and lethality of *st385* mutants, again showing that these aspects of the mutant phenotype are due to this single mutation in the vinculin gene. (c) The rescue of the paralysis, elongation defect, and lethality of the *st555* and *st385* mutants by the introduction of transgenic copies of the wild type vinculin gene demonstrates that the changes in the mutated vinculin gene are solely responsible for all of these aspects of the mutant phenotype.

The reduction in the accumulation of vinculin in the mutants is accompanied by two additional phenotypic defects: (a) the almost complete paralysis of the embryo and (b) the failure of the embryo to complete elongation and, instead, arrest at the twofold embryonic length. In wild type animals, the early embryonic movements begin before innervation of the muscle (Durbin, 1987), so the early paralysis argues that the primary defect is within the muscle cell itself. Since vinculin is known to be a component of the muscle dense bodies and the muscle structure of the mutant is disorganized, we propose that the paralysis is directly related to a requirement for vinculin in the assembly of functional muscle. Ultrastructural examination of the developing muscle in the mutants will be necessary to evaluate its precise role.

The elongation of nematode embryos is likely to be due to circumferential tension developed by cytoskeletal elements of the hypodermis (Priess and Hirsh, 1986). So although Francis and Waterston (1985) did not detect vinculin in the hypodermis, the failure of vinculin mutants to complete elongation indicates that vinculin may be important for normal hypodermal function. Mutations affecting the myosin heavy chain MHC A, however, exhibit the same phenotype. Since MHC A is probably not present in the hypodermis (Ardizzi and Epstein, 1987), there is no obvious connection between the functions of these two proteins other than in muscle contraction. Our result suggests, rather, that muscle function per se may be necessary for some aspect of elongation.

Since the *st385* mutation generates an amber stop codon, the weak staining with antivinculin antibodies seen in this



mutant is probably due to the production of an unstable truncated protein. We can say with surety that this residual vinculin is not due to translation of a maternally encoded message since *st555* mutants show no such staining. The truncation occurs at amino acid 565, eighty residues downstream from the second of two internal repeats found in the nematode protein. As such, the mutant protein contains most of the head region of the molecule, including a putative talin binding site, but is missing 499 residues from the carboxy terminus. From present observations we cannot yet say whether the mutant protein is localized to membrane sites, but it is clearly insufficient, either in size or amount, for normal muscle function. Since animals heterozygous for *st385* are phenotypically wild type, this truncated protein does not significantly interfere with the function of the wild type protein.

The observation that the mutant animals have some motility late in embryogenesis or as L1 larvae suggests that the attachment of actin filaments to the membrane is not entirely dependent on vinculin. Clearly, for any movement to occur, we would expect that the actin filaments must be anchored. It is not uncommon for redundancies to exist in the cytoskeletal system. For example, actin filaments are anchored to red cell membranes by two distinct systems (Branton et al., 1981; Anderson and Marchesi, 1985). Recently, a direct interaction between  $\alpha$ -actinin and  $\beta$ -integrin has been reported (Otey et al., 1990), which may provide an alternative to the vinculin pathway of attachment. Finally, in the nematode pharynx two different mechanisms must exist for anchoring actin filaments to the membrane since vinculin is present in the attachments on the basal, but absent from the attachments on the luminal surface (Francis and Waterston, 1985). If the luminal attachments could substitute for the basal attachments, this might explain why vinculin mutants have at least a partially functional pharynx. This also suggests that genetic reversion of the null mutants reported here may lead to mutations that change the expression of proteins in alternative attachment pathways.

With the mutants in hand, multiple options are now open to us for further investigations of vinculin function. We expect that these approaches will provide complementary information to that obtained from *in vitro* studies, and will allow us to evaluate proposals as to the *in vivo* roles of vinculin.

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