

# Developmental Genetic Analysis of Troponin T Mutations in Striated and Nonstriated Muscle Cells of *Caenorhabditis elegans*

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**Abstract.** We have been investigating a set of genes, collectively called *mups*, that are essential to striated body wall muscle cell positioning in *Caenorhabditis elegans*. Here we report our detailed characterization of the *mup-2* locus, which encodes troponin T (TnT). Mutants for a heat-sensitive allele, called *mup-2(e2346ts)*, and for a putative null, called *mup-2(up1)*, are defective for embryonic body wall muscle cell contraction, sarcomere organization, and cell positioning. Characterizations of the heat-sensitive allele demonstrate that mutants are also defective for regulated muscle contraction in larval and adult body wall muscle, defective for function of the nonstriated oviduct myoepithelial sheath, and defective for epidermal morphogenesis. We cloned the *mup-2* locus and its corresponding cDNA. The

cDNA encodes a predicted 405–amino acid protein homologous to vertebrate and invertebrate TnT and includes an invertebrate-specific COOH-terminal tail. The *mup-2* mutations lie within these cDNA sequences: *mup-2(up1)* is a termination codon near NH<sub>2</sub> terminus (Glu94) and *mup-2(e2346ts)* is a termination codon in the COOH-terminal invertebrate-specific tail (Trp342). TnT is a muscle contractile protein that, in association with the thin filament proteins tropomyosin, troponin I and troponin C, regulates myosin–actin interaction in response to a rise in intracellular Ca<sup>2+</sup>. Our findings demonstrate multiple essential functions for TnT and provide a basis to investigate the in vivo functions and protein interactions of TnT in striated and nonstriated muscles.

**S**TUDIES of many organisms, including vertebrates, indicate that the stable attachment of muscle to the skeleton requires muscle sarcomere assembly, muscle contraction and extracellular matrix formation. *Caenorhabditis elegans* offers unique advantages for investigations of the cellular mechanisms influencing the establishment and maintenance of muscle attachment. *C. elegans* has a simple cellular anatomy of only 959 adult somatic cells and contains a small number of muscle types (for review see Waterston, 1988). The cell divisions and migrations giving rise to these muscle types have been fully described at the cellular level (Sulston et al., 1983). Since the spatial relationships of individual muscle cells are easily visualized and are invariant, the attachments of muscle cells can be precisely determined in wild-type and mutant worms. Given these attributes, it is possible to study specific gene mutations to

dissect the molecular and cellular mechanisms of muscle attachment and positioning, including the contributions of sarcomere assembly and muscle contractile function to these processes.

Three general classes of genes have been defined, based on their mutant phenotypes, that are required for body wall muscle positioning. One class is called *mua* (mutants are muscle cell attachment abnormal): genes in this class are required during larval and adult stages to maintain attachments of body wall muscle to the hypodermis (Plenefisch, J., and E. Hedgecock, personal communication; E.A. Bucher, unpublished data).

Two other classes of genes (*pat* and *mup*) have been identified that affect positioning of embryonic muscle. Embryonic muscle development is a dynamic process that occurs during morphogenesis, when a ball of cells elongates into a tube-shaped worm. Stages of morphogenesis are identified by referring to the worm length relative to egg length (e.g., threefold stage is three times the length of the egg). The first class of embryonic mutants, the *Pat* class (Paralyzed, arrested elongation at twofold), does not elongate past the twofold stage of development. Although there is a range of twofold arresting mutants, the body wall muscle of severe *Pat* mutants is paralyzed (Waterston, 1989; Barstead and Waterston, 1991; Williams and Water-

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ston, 1994). Mutants of many *pat* genes show displacements of muscle quadrants that appear to arise after embryonic arrest. The second class of embryonic mutants, the Mup class (Muscle cell positioning abnormal) are capable of elongation past twofold. Despite full elongation, arrested hatched Mup mutants show a characteristic kinked appearance under the light microscope and, except for minor movements of the head and tail, are unable to move (Goh and Bogaert, 1991; Gatewood, B., and E.A. Bucher, manuscript in preparation). Mutants of the *mup* genes show displacements of muscle quadrants that arise during the threefold stage. The analysis of *mua*, *mup*, and *pat* mutants demonstrates that, although all three classes of genes are essential for muscle positioning, each of their developmental phenotypes is distinctive.

The functional relationships among the *pat*, *mup*, and *mua* classes of genes are unknown: these classes of genes may function in mechanistically similar processes or, alternatively, they may function in distinct processes. The molecular cloning of genes in these classes will be an important step in elaborating and distinguishing the molecular functions of these genes during muscle development. All of the *pat* genes cloned to date are muscle-specific genes and include extracellular matrix components (Rogalski et al., 1993; Williams and Waterston, 1994), cell attachment proteins (Barstead and Waterston, 1991; Williams and Waterston, 1994), and contractile proteins (Kagawa et al., 1995; Venolia and Waterston, 1990; Waterston, 1989; Williams and Waterston, 1994; Kagawa, H., personal communication; Williams, B., personal communication), supporting their common functions in aspects of muscle assembly and attachment. In contrast to the *pat* genes, *mup-1* and *mup-4* (Goh and Bogaert, 1991; Gatewood, B., and E.A. Bucher, manuscript in preparation) and genes in the *mua* class (Plenefisch, J., and E. Hedgecock, personal communication; E.A. Bucher, unpublished data) are thought to be hypodermal (epidermal) components necessary for stable and/or appropriate muscle attachments to the extracellular matrix and exoskeleton during embryogenesis or larval development. However, prior to this report, none of the *mup* genes had been cloned.

Here we report the cloning of the *mup-2* gene locus and genetic and developmental studies of mutants for the *mup-2* gene. We determined that *mup-2* encodes the muscle contractile protein troponin T (TnT)<sup>1</sup>. TnT tethers troponin I (TnI) and troponin C (TnC) to tropomyosin (Tm) on actin filaments, and this trimeric troponin complex confers Ca<sup>2+</sup> sensitivity to striated muscle cell contraction (reviewed in Leavis and Gergely, 1984). Phenotypic analysis demonstrates that *mup-2* mutant embryos have impairments in body wall muscle contraction, body wall muscle cell sarcomere organization, and in body wall muscle cell positioning and hypodermal morphogenesis. The availability of a conditional, heat-sensitive allele allowed us also to determine that adult *mup-2* mutants have impairments in body wall muscle contraction and in function of the myoepithelial sheath cells of the hermaphrodite oviduct. The identification of *mup-2* as a TnT homolog was surprising for several reasons: (a) mutations in other

components of the Ca<sup>2+</sup>-regulatory system (TnC and Tm) cause a Pat rather than a Mup phenotype; (b) the oviductal sheath cells of the somatic gonad are nonstriated myoepithelial cells, in which an essential TnT function would not be predicted; (c) under certain growth conditions *mup-2* mutants have specific defects in hypodermal morphogenesis, a consequence not expected for a mutation of a muscle-specific contractile protein. These studies, therefore, establish the importance of TnT function in muscle cell organization and function as well as in muscle-hypodermal cell interactions. Our findings suggest specific hypotheses for the cellular basis of the *mup-2* phenotypes.

## Materials and Methods

### General Maintenance and Strains Used

Worm strains were maintained and standard crosses performed as described in Brenner (Brenner, 1974). The wild-type parent of all strains used was the *C. elegans* strain N2 and all experiments were done at 20°C unless otherwise stated. Alleles used were as follows: LGII: *tra-2(q122gf)*; LGIII: *daf-2(e1368ts)*; LGIV: *him-8(e1489)*; *dpy-20(e2017am)*; LGX: *lon-2(e678)*; *dpy-8(e130)*; *lev-9(x16)*; *mup-2(e2346ts)*; *mup-2(up1)*; *sup-7(st5)*; *unc-6(e78)*; *unc-97(su110)*; *unc-98(su130)*. Rearrangements used: *mnDp30* (Meneely and Herman, 1979); *stDf1* (Bolten et al., 1984).

### Screen to Isolate the *mup-2(up1)* Allele

Wild-type males were mutagenized with 50 mM EMS (ethyl methane sulfonate) for 4 h at 22–24°C and crossed to *mup-2(e2346ts) unc-6(e78)/mup-2(e2346ts) unc-6(e78)* hermaphrodites at 15°C. Self progeny were recognized by their Unc phenotype and ignored. Individual wild type F<sub>1</sub> hermaphrodites, of genotype either *+/+mup-2(e2346ts) unc-6(e78)* or *mup-2\* +/mup-2(e2346ts) unc-6(e78)*, where the \* denotes a new mutant allele, were picked and placed on individual plates. These hermaphrodites were allowed to self-fertilize and lay about 20 eggs; the hermaphrodites were then transferred to new plates at 15°C, and the progeny on the original plate were shifted to 25°C. (Because of the sterile defect, eggs were shifted: shifting the parent would result in immediate sterility.) If a new *mup-2* mutation had been induced in the parent (*mup-2\* +/mup-2[e2346ts] unc-6(e78)*), then the 15°C plate would have had wild-type (*mup-2\* +/mup-2[e2346ts] unc-6(e78)*), dead (*mup-2\* +/mup-2\* +*), and Unc (*mup-2[e2346ts] unc-6(e78)/mup-2[e2346ts] unc-6(e78)*) worms, while the 25°C plate would have had only dead worms (same genotypes). The new allele was recovered from the 15°C plate by picking wild-type worms (*mup-2\* +/mup-2[e2346ts]*) and crossing them to *lon-2(e678)* males. The new allele was named *mup-2(up1)*.

### Genetic Localization of the *mup-2* Gene

*mup-2* was localized to an interval on the X chromosome (LG X) by the following crosses. *mup-2(e2346ts)/0* males were mated to *dpy-8(e130)/stDf1; mnDp30* hermaphrodites at 25°C and 15°C. Since *mnDp30* is a free duplication that is retained at random through germline mitosis, two classes of hermaphrodites will arise from this cross. The first class will be *mup-2(e2346ts)/dpy-8(e130) stDf1* (deficiency but no duplication), while the second class will be *mup-2(e2346ts)/dpy-8(e130) stDf1; mnDp30* (has deficiency and duplication). If *mup-2* is within the deficiency, the first class will be Mup at 25°C, but the second class will not, and this is what was found. The second class was distinguished from self progeny by the segregation of Mups in their offspring at 25°C. A viable line of the genotype *mup-2(e2346ts); mnDp30* was recovered from these crosses.

*mup-2* was further mapped to a small region between *sup-7(st5)* and *lev-9(x16)* using standard three-factor crosses between: (a) *dpy-8(e130)* and *unc-6(e78)*; between (b) *sup-7(st5)* and *unc-6(e78)*; and between (c) *lev-9(x16)* and *unc-6(e78)*. For the second three-factor cross, Sup non-Unc recombinants were selected at 20°C since homozygous *sup-7* animals are not viable at 15°C and 25°C. *sup-7(st5)* was followed using *dpy-20(e2017am)* which is completely suppressed by *sup-7(st5)* and partially suppressed by *sup-7(st5)/+; dpy-20(e2017am)*; *sup-7(st5)* is wild type in length, while *dpy-20(e2017am)*; *sup-7(st5)/+* is a weak Dpy. For the third three-factor cross Lev Unc offspring of these recombinants were tested by

1. Abbreviations used in this paper: Tm, tropomyosin; TnC, troponin C; TnI, troponin I; TnT, troponin T; tsp, temperature-sensitive period.

transferring a gravid adult onto plates containing 1 mM levamisole (Sigma Chemical Co., St. Louis, MO). Lev offspring are resistant to levamisole, while *+lev-9(x19)unc-6(e78)+unc-6(e78)* or *+unc-6(e78)/+unc-6(e78)* animals are killed or grow very slowly in 1 mM levamisole.

For complementation tests, *mup-2(e2346ts)/0* males were mated to *unc-6(e78)*, *unc-97(su110)*, *unc-98(su130)* and *lev-9(x16)* hermaphrodites and the presence or absence of Mups among the offspring was scored at 25°C.

### RFLP Mapping of *mup-2*

Southern blots of genomic DNA from three different wild type *C. elegans* strains, N2, RC301 and TR403 were probed with different cosmids between *unc-6* and *sup-7* (provided by Coulson, A., and J. Sulston, MRC, Cambridge, UK) to search for RFLPs. Four RFLPs were detected by cosmids T15G10, F14G9, F22A3, and R06B12 (and called eP101, eP102, eP103, and eP104, respectively) in RC301. Southern blots of DNA of congeneric recombinant strains were probed with these RFLPs.

Recombinant congeneric strains were constructed using the strain RC301 between *mup-2* and *dpy-8*, 1.2 map units to the left of *mup-2*, and between *mup-2* and *unc-6*, 0.6 map units to the right of *mup-2*. The crosses were performed as follows. RC301 males were mated to N2 hermaphrodites carrying double mutations, *dpy-8(e130) mup-2(e2346ts)* and *mup-2(e2346ts) unc-6(e78)*, at 15°C. Wild-type hermaphrodites produced from the outcross were of the genotypes *dpy-8* (N2) *mup-2* (N2)/+ + (RC301) and *mup-2* (N2) *unc-6* (N2)/+ + (RC301). Recombinant self progeny (at 25°C) were isolated. Recombinant Dpy-non-Mup and Unc-non-Mup first isolated as *dpy-8(N2) mup-2(N2)/dpy-8(N2) + (RC301)* and *mup-2(N2) unc-6(N2)/+ (RC301) unc-6(N2)*, respectively, were allowed to self-fertilize for several generations until individual offspring no longer segregated Mups. Each of the lines established would therefore be homozygous for the N2-RC301 recombinant chromosomes with independent recombination events between *dpy-8* and *unc-6*. A total of 64 Dpy-non-Unc recombinant strains, and 56 Unc-non-Dpy recombinant strains were established and used as sources of DNA for RFLP mapping.

### Transformation Rescue

A heterozygous strain of genotype *+ mup-2(e2346ts) unc-6(e78)/lon-2(e678) + +* was used for transformation rescue because the *mup-2(e2346ts)* gonad defect makes the homozygous mutant unsuitable for injection. *lon-2* was included so that Uncs which are recombinants (which would segregate Lons) could be distinguished from homozygous rescued *Mup-2-Unc-6* animals. Cosmid R04A4, injected singly, or a mixture of cosmids that map just to the right of R04A4, were both fully able to rescue *mup-2(e2346ts)*.

Injections were done using a Leitz micro-manipulator fixed to a Nikon Diaphot inverted Nomarski microscope. Techniques were as described (Fire, 1986) except that the DNA was injected into the syncytial gonad instead of oocytes (Mello et al., 1991). A plasmid carrying a *rol-6* dominant mutation, pRF4 (about 200 µg/ml), as a positive control was co-injected with test DNA (200 µg/ml). Injected animals were allowed to recover for one day at 20°C and then shifted to 25°C. Transformed Rol and Unc-Rol animals were grown individually. Only inheritable Roller lines were checked for the presence of viable Uncs. The Uncs were grown at 25°C individually for several generations and also outcrossed to N2 males to check that they were homozygous *mup-2(e2346ts) unc-6(e78)*.

### Isolation and Analysis of *mup-2* Genomic and cDNA Sequences

Transformation rescue indicated that *mup-2* resides on cosmids K04G12 and R04A4. A worm λ2001 genomic library (provided by Coulson, A., and J. Sulston, MRC, Cambridge, UK) was screened with the cosmid K04G12 and genomic clone T36 was isolated. T36 was subcloned into vectors pEMBL8 or Bluescript KS<sup>+</sup>. T36 subclones were used as probes to screen  $1 \times 10^6$  cDNA clones of a *C. elegans* embryonic N2 cDNA library (Maruyama and Brenner, 1992) and three overlapping clones of similar length were isolated. The cDNA P23 was sequenced, the protein sequence translated, and the predicted protein sequences were used to search the EMBL SWISS PROT and NBRF-PIR databases. DNA and protein sequence analyses were carried out with the DNASTar program (DNASTar Inc.) and the McGene program.

### Sequencing *mup-2(e2346ts)* and *mup-2(up1)* cDNAs

RNA was extracted from worms by treatment with guanidinium thiocya-

nate (Sambrook et al., 1989) with the following additions and modifications. Worms were harvested from NGM-agar plates, allowed to swim in basal buffer for 30 min to eliminate bacteria from gut, washed with S buffer, and finally washed with 4% sucrose, 1 mM EDTA (recipes according to: Sulston and Hodgkin, 1988). Worms were homogenized by vortexing 5 min in the presence of siliconized sand and homogenization buffer containing 5 M guanidinium thiocyanate, 0.1 M Tris, pH 7.5, and 5% β-mercaptoethanol. Sodium lauryl sarcosinate was added to a final concentration of 2% following homogenization of worms.

cDNA was prepared from total RNA using Moloney murine leukemia virus reverse transcriptase (GIBCO BRL, Gaithersburg, MD). The reaction was performed according to instructions provided with the enzyme, with the exception that RNasin (Promega Biotec, Madison, WI) was added to a final concentration of 1 µg/µl in the reaction. Without purification, 5 µl of the reaction product was used for PCR in a total volume of 100 µl.

PCR was performed in a solution containing, in addition to primers and template, 50 mM KCl, 10 mM Tris, pH 8.3, at 20°C, 1.5 mM MgCl<sub>2</sub>, 0.075% Nonidet P-40, 20 µM each dNTP, 2.5 µg/100 µl AmpliTaq DNA polymerase (Perkin Elmer Cetus, Eden Prairie, MN). The concentration of dNTP was low in order to improve fidelity of polymerization. Single-stranded DNA was generated by an initial symmetric PCR, in which the concentration of both primers was 200 nM, followed by an asymmetric PCR, in which the concentration of one primer was 200 nM and the other 2 nM. Between the symmetric and asymmetric PCRs and between the asymmetric PCR and sequencing reaction, the PCR product was purified by ultrafiltration (Microcon-100; Amicon, Beverly, MA). One-twentieth of the product from the symmetric PCR was used as template in the asymmetric PCR.

For sequencing *mup-2(e2346ts)*, we used the Sequenase 2.0 kit (United States Biochemical, Cleveland, OH) and protocol, with the following exceptions. One-third of the asymmetric PCR product, following ultrafiltration and resuspension in sterile water, was combined with Sequenase reaction buffer and 1 µM sequencing primer. Although mainly single-stranded, the asymmetric PCR product performed best in the sequencing reaction when it had been heated with primer at 95–100°C for 5 min and then rapidly cooled by plunging in an ethanol-dry ice bath. The labeling and termination reactions were performed according to the kit's instructions. The sequence analysis was repeated on multiple samples derived from independent reverse transcriptions and polymerase chain reactions.

For sequencing *mup-2(up1)*, we performed the symmetric PCR with genomic DNA extracted from single embryos (Williams et al., 1992) and with the same concentrations listed here. The site of mutation was confirmed through sequencing of DNA from multiple embryos.

### Visualization of Worms

For staining with rhodamine-conjugated phalloidin (Sigma Chemical Co.), we fixed early L1 worms with -20°C acetone, let dry for around 30 min at 20°C, incubated for 30–60 min in phosphate buffered saline with 66 nM rhodamine-phalloidin for ~10 min before examination, and then mounted in glycerol.

For time lapse videotaping, embryos were mounted (Williams and Waterston, 1994) and followed using a model VE100 camera from DAGE-MTI (Michigan City, IN) and a time lapse video recorder (model BR-9000U) from JVC.

### Antibody Staining of Embryos

Embryos were stained either by the method of Goh and Bogaert (1991) or by the method of Barstead and Waterston (1991), yielding indistinguishable results. For staining myosin, we used monoclonal antibody DM5-6, (provided by D. Miller, Vanderbilt University, Nashville, TN) to *C. elegans* myosin heavy chain A. Both fluorescein (FITC)-conjugated or rhodamine (LRSC)-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) secondary antibodies (at a dilution of 0.01 mg/ml) were used for myosin staining. Some staining of embryos was performed with antibody Ne8/4c6.3 (from stocks at the MRC Laboratory of Molecular Biology, Cambridge, UK).

### Temperature-sensitive Period Determination of the *Mup* Phenotype

The temperature-sensitive period (tsp) was determined by shift-up (from 15° to 25°C) and shift-down (from 25° to 15°C) experiments. Each plate

was scored for the percentage of embryos hatching as Mups, or as wild-type larvae. Since each plate was incubated for different lengths of time at 15° or 25°C, the stages were normalized to the 25°C growth rate when plotting the tsp graph. The growth rate at 25°C is about twice that at 15°C for *mup-2(e2346ts)*, as is the case for wild-type embryos (developmental time at 25°C is = [1/2.1] developmental time at 16°C; Byerly et al., 1976).

In the shift-up experiment, batches of embryos of various stages laid by *mup-2(e2346ts)* adults that were grown at 15°C were transferred onto plates which had been equilibrated at 25°C. Embryos beyond the twofold stage were incubated at 15°C for various periods of time before they were shifted up. For the shift-down experiment, 2- to 16-cell stage embryos were obtained by bleaching gravid parents (grown at 15°C). These were quickly transferred to 25°C plates and shifted down to the permissive temperature (i.e., 15°C) at various stages. Embryos had to be obtained by bleaching because of the gonad defect at 25°C. As for the shift-up experiments, batches of twofold stage embryos were incubated at 25°C for various lengths of time before shifting down to 15°C.

### Temperature-sensitive Period of the Gonad Phenotype

The tsp was determined by shift-up (from 15° to 25°C) and shift-down (from 25° to 15°C) experiments. The larvae were staged using Nomarski optics by examination of gonads of individual worms. These worms were then transferred to individual plates at the appropriate temperature. Fertility was scored by looking for eggs or larvae after the worms had been adults for two or more days. For the shift-up experiments, adults raised at 15°C until adulthood and then shifted were scored as fertile if they had laid eggs prior to the shift.

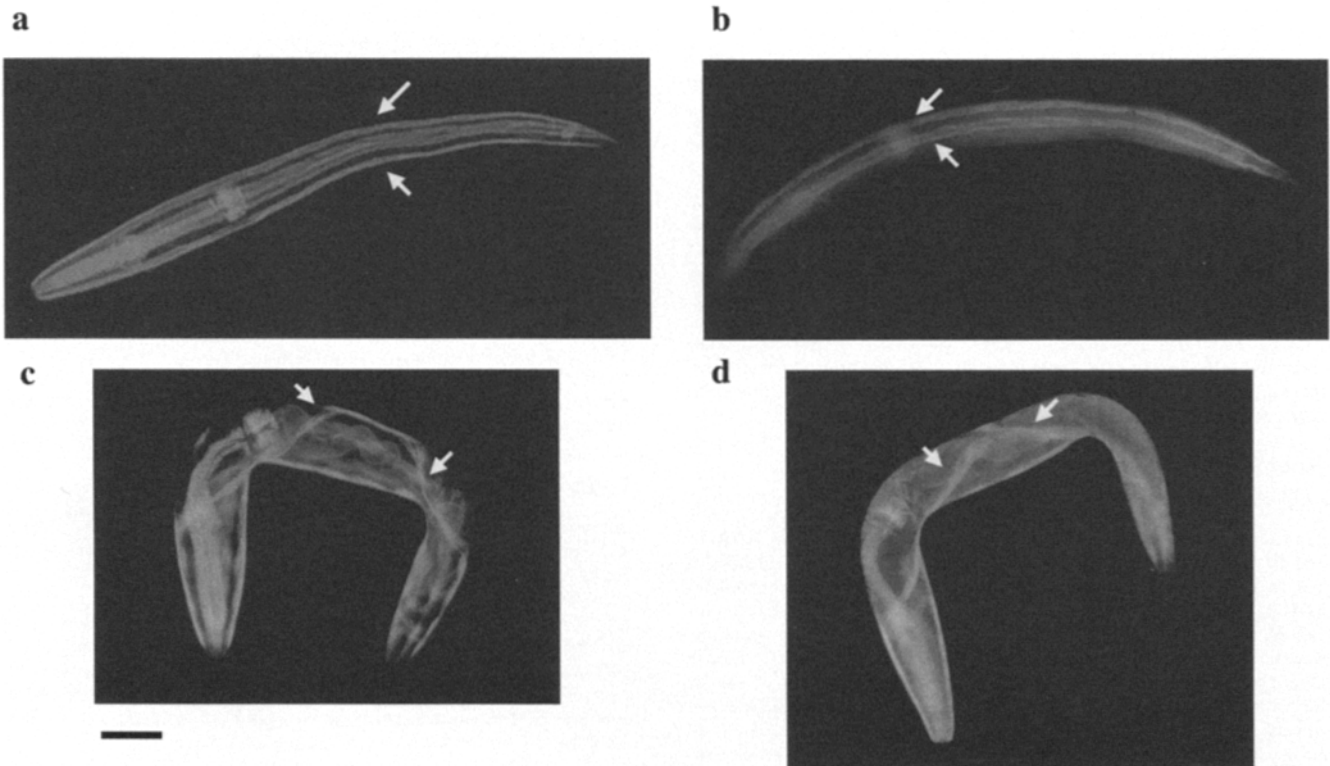
### Determination of *mup-2* Hatching Rates

*+mup-2(e2346ts)unc-6(e78)/lon-2(e678)++* hermaphrodites were placed individually on a plate at 25°C and allowed to self-fertilize. Hermaphrodites were transferred to a fresh plate daily until they were no longer fertile. 24 h after each hermaphrodite was removed, unhatched eggs and

Mups were scored (at 25°C, wild type eggs should hatch within 12 h). *+mup-2(up1)/lon-2(e678)+* hermaphrodites were used identically to the above protocol to examine *mup-2(up1)* homozygotes. Since *stDfl* segregates dead embryos, we set up the following crosses to test the *mup-2* hatching rate over *stDfl*. The strain *dpy-8(e130) stDfl(X); mnDp30(X:f)* hermaphrodites were crossed to *tra-2(q122gf)/tra-2(q122gf)* males (strain provided by Schedl, T., Washington University, St. Louis, MO). *tra-2(q122gf)* causes dominant feminization. *stDfl/+;tra-2(q122gf)/+* female cross progeny were identified and crossed to *mup-2(e2346ts);him-8(e1489)* males. This ensured absence of *stDfl* self progeny. The following genotypes would segregate: *stDfl/0* (males), *+/0* (males), *stDfl/mup-2* (females), *+/stDfl* (females). Dead embryos were scored as outlined above, however in this case 1/2 of dead embryos were assigned to the *stDfl/0* male genotype (*stDfl* embryos die in early embryonic stage). The remaining dead embryos were assigned to the *stDfl/mup-2(e2346ts)* genotype, including all obvious Mup embryos. We did not determine the hatching rates of *mup-2(up1)/stDfl* since the X-linked lethality makes these constructions technically difficult.

### Determination of Brood Sizes

Single hermaphrodites were placed individually on a plate and allowed to self-fertilize. The hermaphrodites were transferred to a fresh plate daily until the hermaphrodites were no longer fertile. The progeny were counted (Mups and non-Mups) several days after each hermaphrodite was removed to determine a total brood size for each hermaphrodite. During this analysis we discovered that many animals of the genotype *mup-2(e2346ts)/mup-2(up1)* were sterile (about 50%). We tested the number of sterile animals having the genotype *mup-2(e2346ts)/stDfl* by crossing *mup-2(e2346ts);him-8(e2489)* males to *stDfl/lon-2(e678)* hermaphrodites. We picked single progeny to separate plates and then asked if any cross progeny were sterile. Cross progeny of the genotype *mup-2(e2346ts)/lon-2(e678)* and self progeny were readily distinguished by their segregants. Since *mup-2(e2346ts)/lon-2(e678)* and *stDfl/lon-2(e678)* are always self fertile, we inferred that any sterile animals had the genotype *mup-*



**Figure 1.** *mup-2(e2346ts)* and *mup-2(up1)* larval arrest phenotype. The body wall muscle in wild-type worms lies along the length of the worm and is here visualized by phalloidin. (a) Wild-type muscle of an L1 larvae. The muscle position appears wild-type in the *mup-2(e2346ts)* raised at 15°C (b), whereas the *mup-2(e2346ts)* mutants raised at 25°C (c), or the *mup-2(up1)* mutants raised at any temperature (d), show the characteristic muscle displacement (arrows, c and d). The kinked body shape caused by the *e2346ts* and *up1* alleles is typical of the *mup* gene class. In each micrograph anterior is to the left and dorsal is toward the top of the page. Bar, 10  $\mu$ m.

2(*e2346ts*)/*stDf1*. We determined that 5/11 animals of this genotype were sterile, similar to our result of 50% sterility in *mup-2(e2346ts)/mup-2(up1)* heterozygotes. We did not determine the brood sizes of the remaining fertile *mup-2(e2346ts)/stDf1* since we did not know the genotypes until the phenotype of the progeny had been scored.

## Results

### Isolation and Terminal Phenotype of *mup-2* Alleles

We set out to identify mutants that had defective patterning of body wall muscle, but that had an otherwise wild type anatomy. The normal pattern of body wall muscle has been extensively described at the light microscope level (Sulston et al., 1983; Priess and Hirsh, 1986; Waterston, 1989; Hresko et al., 1994). Body wall muscle is the major striated muscle in the worm and is responsible for animal locomotion. In the adult, 95 individual body wall muscle cells lie in four quadrants, dorsal left/right and ventral left/right, that extend from the nose to the tail. In the embryo, 81 of these muscle cells are born and during morphogenesis establish their attachments to each other and to the hypodermis (epidermis); these attachments are maintained and elaborated during larval molts and sarcomere addition (Hresko et al., 1994). By the 1 1/2-fold to 1 3/4-fold stage of morphogenesis, muscle components are localized into recognizable sarcomeres, and attachment of muscle to the hypodermis is evidenced by embryo twitching. By the two-fold stage, the embryo demonstrates coordinated and vigorous muscle movement evidenced by active rolling in the eggshell. The muscle is then thought to become attached to the cuticle of the worm during the late threefold stage when the cuticle is synthesized. At hatching each muscle cell is comprised of two sarcomeres. Eight additional sarcomeres will be added during the four larval stages (L1–L4) that are punctuated by molts. The organization of the body wall muscle at the time of hatching is illustrated in Fig. 1 *a*.

We initially identified mutations with disruptions in muscle patterning through a screen for animals with a kinked body shape called Mup that is caused by muscle position defects (Goh and Bogaert, 1991). This screen yielded a single allele (*e2346ts*) of a gene we named *mup-2*. *mup-2(e2346ts)* is a conditional allele with a heat-sensitive period in embryogenesis. Embryos homozygous for *mup-2(e2346ts)*, and raised at the restrictive temperature (25°C), die soon after hatching as L1 larvae with the characteristic kinked Mup body shape (Fig. 1 *c*). These larvae are capable of moving their heads and tails but are unable to straighten their body. Some embryos do not hatch but upon gentle manipulation come out of the eggshell in the typical kinked form. In addition, *mup-2(e2346ts)* has a second heat-sensitive period in adulthood that results in sterility of homozygous hermaphrodites. Homozygous *mup-2(e2346ts)* males do not exhibit this adult temperature-sensitive period and are fully fertile.

Since *mup-2(e2346ts)* was the only existing allele, and temperature sensitive, we sought to ascertain whether the Mup phenotype was the true loss-of-function phenotype for this locus. Although *mup-2(e2346ts)* over a deficiency for the region resulted in Mup embryos at the restrictive temperature (see below), it was possible that a homozygous null allele may cause an earlier arrest resulting in

dead (non-Mup) embryos. Thus, we undertook an unbiased, noncomplementation screen that would detect alleles that resulted in either Mup or dead embryos (see Materials and Methods). Approximately 4,000 haploid genomes were screened and one new allele was isolated. This new allele, *mup-2(up1)*, is nonconditional and is Mup either when homozygous (Fig. 1 *d*) or when placed over *mup-2(e2346ts)* at the restrictive temperature (see below and Table I).

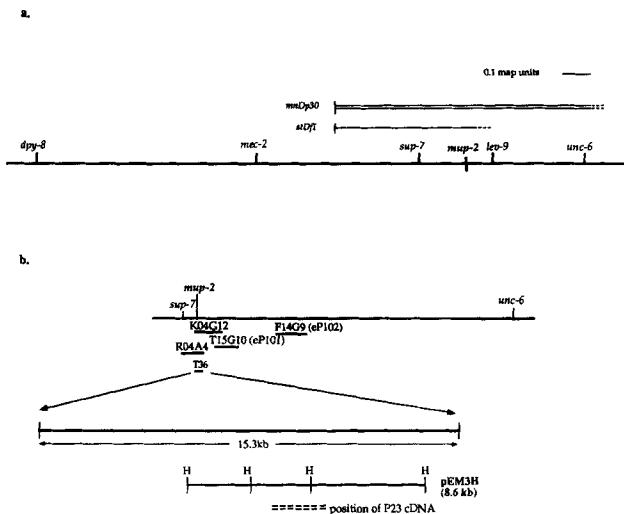
Since we were specifically interested in recovery of mutants that affected embryonic muscle development, we examined the body wall muscle cell organization in arrested L1 mutants using rhodamine-labeled phalloidin (Fig. 1). Phalloidin is a fungal toxin that binds filamentous actin and allows visualization of thin filaments in the muscles of fixed animals. We observed that homozygous *mup-2(e2346ts)* (grown at the restrictive temperature of 25°C) and *mup-2(up1)* arrested larvae have differentiated body wall muscle, but it is displaced from its normal position. Instead of the dorsal body wall muscle lying along the length of the worm, a set of dorsal muscles is displaced from the body wall at the two dorsal bends of the embryos. These displaced cells appear as a strip since the cells presumably are still attached to one another by their attachment plaques. Polarized light observation of living embryos shows the same muscle phenotype (data not shown). This defect appears to be of equal severity in the arrested *mup-2(e2346ts)* mutants raised at 25°C (Fig. 1 *c*) and in *mup-2(up1)* mutants raised at any temperature (*d*). Most *mup-2(e2346ts)* mutants raised at 15°C show a wild-type pattern of muscle position and structure (Fig. 1 *b*). The pharyngeal muscle appears to be normal, which is supported by the observation that the pharynx of hatched *mup-2* larvae pump and the embryos are capable of feeding. Thus, the terminal phenotype of *mup-2* embryos appears to result from the displacement of the dorsal body wall muscle quadrants from the hypodermis.

### Genetic Characterization of *mup-2* Alleles

It was important to ascertain whether *mup-2* was a new gene affecting muscle function or whether we had isolated additional (more severe) alleles of an already known locus. The absence of males at the restrictive temperature when *mup-2(e2346ts)* hermaphrodites were mated to N2 (wild type) placed *mup-2* on the *X* chromosome (*C. elegans* males are *X/0*). To further localize *mup-2* we undertook deficiency mapping (Materials and Methods; Fig. 2 *a*). *mup-2* was uncovered by the deficiency interval *stDf1* and covered by the duplication *mnDp30*. Standard three-factor crosses localized *mup-2* between *dpy-8* and *unc-6* on the genetic map (Edgley and Riddle, 1989). During the course of mapping *mup-2*, complementation tests were

Table I. Comparison of Hatching Rates of Mups

	% of Mups that hatch	SD	<i>n</i>
	%	%	
<i>mup-2(e2346ts)unc-6(e78)/</i> <i>mup-2(e2346ts)unc-6(e78)</i>	82	2	3
<i>mup-2(up1)/mup-2(e2346ts)</i>	51	8	5
<i>mup-2(e2346ts)/stDf1</i>	42	6	5
<i>mup-2(up1)/mup-2(up1)</i>	20	9	6



**Figure 2.** Localization of the *mup-2* gene. (a) Genetic map showing the position of *mup-2* on the X chromosome. *mup-2* is covered by duplication *mnDp30* and uncovered by the deficiency *stDfl*. (b) Physical map showing the position of *mup-2* relative to cosmids between *sup-7* and *unc-6*. Transformation rescue narrowed down the sequences capable of rescuing *mup-2* to the region of overlap between cosmids RO4A4 and K04G12. Phage T36 and its subclone pEM3H also rescued *mup-2* (*e2346ts*). The cDNA P23 maps to pEM3H as shown. H, HindIII.

carried out (Materials and Methods) between *mup-2(e2346ts)* and mutants of *unc-6*, *unc-97*, *unc-98*, and *lep-9*, which are genes in this region known to affect larval and/or adult muscle function (Edgley and Riddle, 1989). *mup-2* complemented each of these genes supporting that *mup-2* mutations define a new muscle-affecting locus in *C. elegans*.

To test whether *mup-2(e2346ts)* and *mup-2(up1)* are indeed loss-of-function alleles, and to determine the severity of the mutations (phenotype) relative to each other, we compared the embryonic phenotypes produced by each allele when homozygous as well as by *mup-2(e2346ts)* over a deficiency for the region (at 25°C). In all cases, Mup embryos were produced at the frequency expected for a recessive mutation. No additional or more severe phenotypes were seen, indicating that both alleles act genetically as strong loss-of-function or null alleles. While there was no difference between the two alleles in their embryonic phenotypes, the two alleles can be distinguished from each other by the percentage of Mup embryos capable of hatching (Table I). Homozygous *mup-2(e2346ts)* embryos hatch 82% of the time, contrasted to 22% of homozygous *mup-2(up1)* embryos. Embryos heterozygous for *mup-2(e2346ts)* and a deficiency of the region, or heterozygous for *mup-2(up1)*, hatched at an intermediate rate (42 or 50%, respectively). These hatching rates may reflect the muscle activity and hence the allelic strength. When used as a measure of the allelic severity, the hatching rate indicates that *mup-2(e2346ts)* likely retains some function since its phenotype is worse when heterozygous with a deficiency. Since fewer *mup-2(up1)* homozygotes hatch, this allele may be a true null. Furthermore, a student's *t* statistic of the difference between *mup-2(up1)/mup-2(e2346ts)* and *mup-2(e2346ts)/stDfl* hatching rates shows that the two

means are not statistically different, supporting that *mup-2(up1)* behaves genetically as a deficiency of the locus. Assessment of *mup-2(up1)/stDfl* was not practical (Materials and Methods).

To test further whether *mup-2(e2346ts)* is a loss of function, recessive allele or whether it is antimorphic, we generated the strain *mnDp30;mup-2(e2346ts)/mup-2(e2346ts)*. *mnDp30* is a free duplication that complements *mup-2* (see below); thus we could ask whether increased dosage of the *mup-2(e2356ts)* (i.e., 2:1 of mutant to wild type as compared to 1:1 in the heterozygous combination) revealed any antimorphic effects. We found that *mnDp30;mup-2(e2346ts)/mup-2(e2346ts)* hermaphrodites segregated wild-type and Mup progeny at 25°C demonstrating that one wild-type copy of *mup-2* is sufficient to complement the *mup-2(e2346ts)* and to maintain the strain. These data support that *mup-2(e2346ts)* is recessive.

### *mup-2* Encodes the Muscle Contractile Protein, Troponin T

We undertook to clone the *mup-2* locus because, in addition to the embryonic muscle phenotype described above, and in more detail below, *mup-2* has additional, discreet phenotypes that suggest functions in hypodermal development as well as in postembryonic muscle development. Since the description of these phenotypes is most interesting in the context of the identity of the protein, we will first present the cloning of the *mup-2* gene.

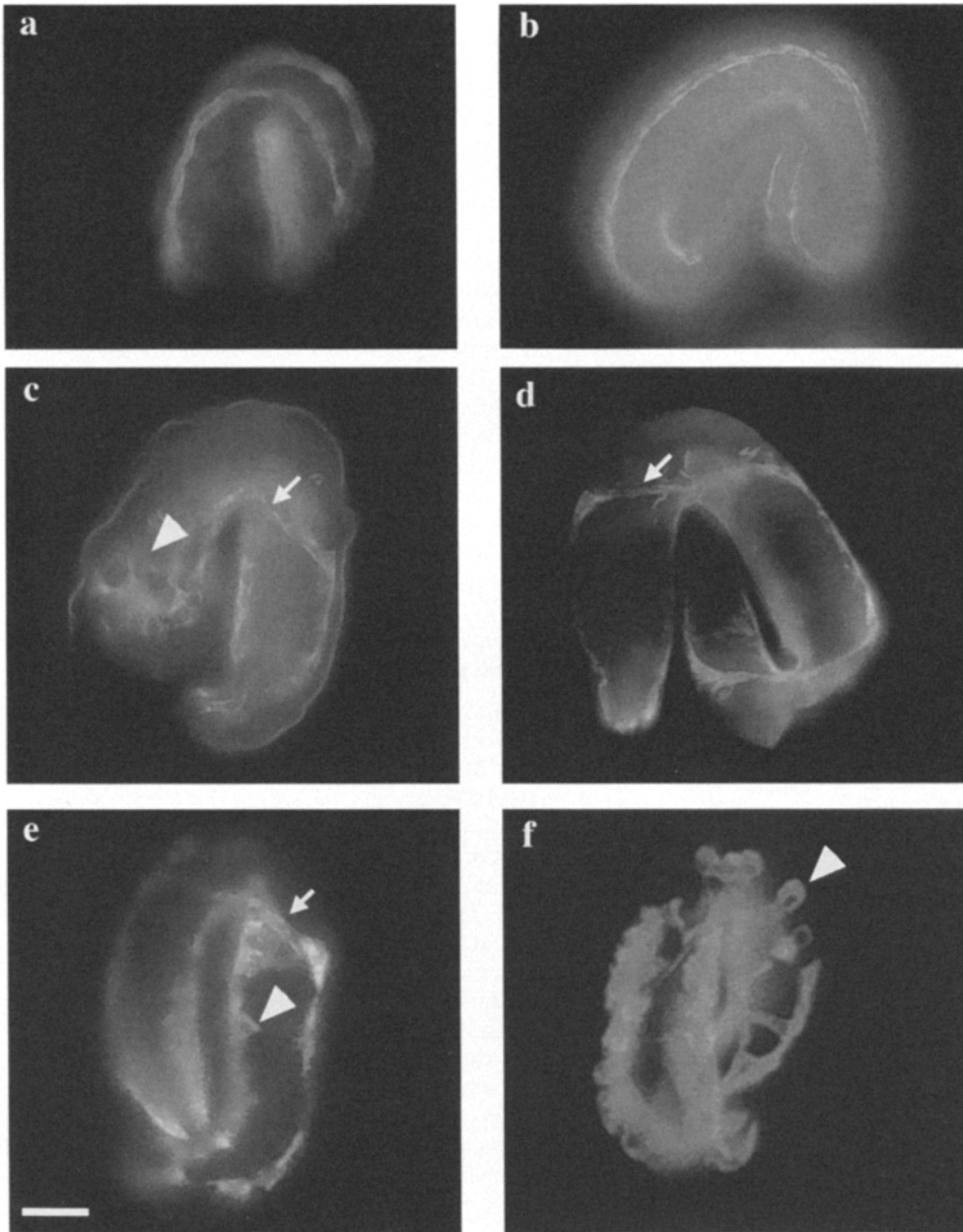
To clone *mup-2*, we first positioned *mup-2* on the physical map by identifying four RFLPs in the region and mapping *mup-2* relative to these RFLPs and to linked genetic markers (see Materials and Methods). These experiments localized *mup-2* to a small interval between *sup-7* and the RFLP eP102 on cosmid F14G9 (Fig. 2 b). *mup-2* was not separable from eP101. To test directly which cosmid(s) contained the *mup-2* sequences, cosmids between the gene *sup-7* and the eP102 RFLP were tested for their ability to rescue the *mup-2(e2346ts)* lethal and sterile phenotypes (Fig. 2 b, Materials and Methods). Smaller phage clones were subsequently recovered and an 8.6-kb subcloned fragment (pEM3H) of a phage clone (T36) was capable of rescue.

To determine the encoded sequence of *mup-2*, we screened an N2 (wild-type) embryonic cDNA library and isolated three overlapping cDNAs (Materials and Methods). The longest cDNA isolated, P23, was sequenced on both strands and appears to be full length or near full length (sequence data is available from Genbank/EMBL/DDBJ under accession number U44759). Conceptual translation of this cDNA yields a predicted protein product of 405 amino acids. The *mup-2(up1)* and *mup-2(e2346ts)* mutations reside within this transcription unit (see below), establishing that these sequences are the *mup-2* locus.

The *mup-2* predicted protein was found to be homologous to vertebrate and *Drosophila* isoforms of the muscle protein troponin T (Fig. 3), which is involved in the regulation of striated muscle contraction. Muscle contraction results from the MgATP-dependent interaction of myosin with actin (Cooke, 1986). Myosin-actin is regulated in muscle cells by the Ca<sup>2+</sup>-sensitivity conferred by regulatory proteins associated either with thin filaments or with







**Figure 4.** Developmental phenotype of *mup-2* embryos. Wild type (*a* and *b*) or *mup-2(up1)* (*c* and *d*) were stained with a mAb to myosin heavy chain B. *mup-2(e2346ts)* embryos (*e* and *f*) were stained with the mAb Ne.8/4c6.3, which recognizes an unknown thick filament antigen in muscle cells. Panels *a* and *c* are at twofold stage, *e* is 2 1/2-fold, *b*, *d*, and *f* are at the threefold stage. The body wall muscle is already displaced at the twofold stage in some *mup-2* embryos as shown in *c*. Another defect seen in some embryos comprises muscles that express muscle antigens, but lack organization of these antigens into discrete sarcomeres (*e*, *arrows*). In each panel the ventral aspect is toward the bottom of the page and anterior is to the left (although in threefold embryos, the head may be curled inward toward the middle of the animal). Bar, 10  $\mu$ m.

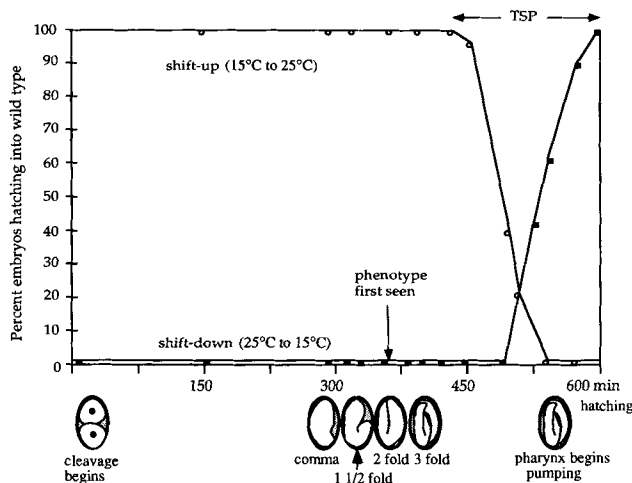
ament antigens. Embryos from either allele showed similar defects, the earliest of which appears at the twofold stage (Fig. 4 *c*). At this stage in wild type (Fig. 4 *a*), the muscle cells are aligned in the left and right dorsal and ventral quadrants, evidence sarcomeric distribution of most contractile proteins, and have made some attachments to the hypodermis which allow the embryos to move in their eggshell (Hresko et al., 1994). Twofold *mup-2* embryos can be found with muscle displaced from the body wall (*c*, *arrow*); however, most mutant twofold embryos appear wild type. It is during the threefold stage of embryogenesis that muscle displacement becomes prevalent (Fig. 4, *d* and *f*, *arrows*). In addition, examples can be found of occasional individual muscle cells displaced from their normal quadrants (data not shown).

The data in Figs. 1 and 4 also demonstrate that most muscle cells appear capable of assembling thick and thin

filaments in the *mup-2* mutants; however, some two- and threefold *mup-2* embryos show muscle cells that are expressing muscle proteins but do not appear to have stably assembled these proteins into sarcomeres (Fig. 4, *c*, *e*, and *f*, *arrowheads*). These cells appear in the micrographs as "round" cells since the muscle antigens appear localized around the nucleus rather than organized into sarcomeres. We cannot currently distinguish whether these disorganized sarcomeres are caused by defective assembly or by damage to the sarcomeres, and subsequent disassembly, due to defective muscle attachments.

To determine at what point during embryonic muscle development CeTnT-1 is required or when the effects due to the absence of CeTnT-1 become irreversible, we performed temperature shift-up (15° to 25°C) and shift-down (25° to 15°C) experiments using the *mup-2(e2346ts)* allele. These data are graphically represented in Fig. 5. The de-





**Figure 5.** Embryonic temperature sensitive period of *mup-2(e2346ts)*. Shift-up and shift-down experiments were carried out and the temperature sensitive period (TSP) was determined to be during the threefold stage. This is after movement normally begins in wild-type embryos. Below the graph are shown schematics of wild type embryonic development with key developmental stages highlighted. The times of development were normalized to development at 25°C. The shift-up line is offset for clarity. The number of animals scored for the shift-up experiment was as follows: 2–16 cell stage:  $n = 24$ ; gastrulating stage:  $n = 11$ ; bean/comma stage:  $n = 10$ ; 1 1/2-fold:  $n = 16$ ; twofold:  $n = 12$ ; twofold (1 h after twofold onset, i.e., +1 h):  $n = 12$ ; twofold (+2 h):  $n = 10$ ; twofold (+3 h):  $n = 10$ ; threefold (3 h after twofold onset, i.e., +3 h):  $n = 10$ ; threefold (+4 h):  $n = 11$ ; threefold (+5.5 h):  $n = 8$ ; threefold (+6 h):  $n = 10$ ; threefold (+7 h):  $n = 10$ ; threefold (+8 h):  $n = 9$ ; threefold (+8.67 h):  $n = 10$ . The number of animals scored for the shift-down experiment was as follows: 2–16 cell stage:  $n = 10$ ; gastrulating stage:  $n = 11$ ; bean/comma stage:  $n = 9$ ; 1 1/2-fold:  $n = 8$ ; twofold:  $n = 6$ ; twofold (.5 h after twofold onset, i.e., +.5 h):  $n = 9$ ; twofold (+1 h):  $n = 11$ ; threefold (+1.5 h):  $n = 9$ ; threefold (+2 h):  $n = 23$ ; threefold (+2.5 h):  $n = 10$ ; threefold (+3.25 h):  $n = 10$ ; threefold (+3.5 h):  $n = 10$ ; threefold (+4.25 h):  $n = 10$ ; L1 (4.5 h):  $n = 10$ .

velopment of the Mup phenotype in *mup-2(e2346ts)* embryos requires an elevated temperature only during the mid to late threefold stage: embryos shifted from 25° to 15°C during the early threefold stage hatch as wild type.

### *mup-2(e2346ts)* Embryos Raised at Intermediate Temperatures Develop Hypodermal Defects

Since the muscle and hypodermis interact during embryogenesis, we extended our analysis of *mup-2* mutants to determine if hypodermal development was also affected. Using an antibody to hypodermal adherens junctions (MH27; provided by R. Waterston, Washington University, St. Louis, MO) we examined *mup-2(e2346ts)* embryos raised at the restrictive temperature (25°C). In all cases, the hypodermal cells appeared wild type in arrested Mup L1 larvae (data not shown). *mup-2(e2346ts)* embryos raised at 15°C usually hatch as wild type L1 larvae and subsequently grow to adulthood having wild-type muscle and hypodermal morphology. In contrast, if we raised *mup-2(e2346ts)* embryos at intermediate temperatures (18°–23°C) we observed that many of these animals do not hatch as Mup

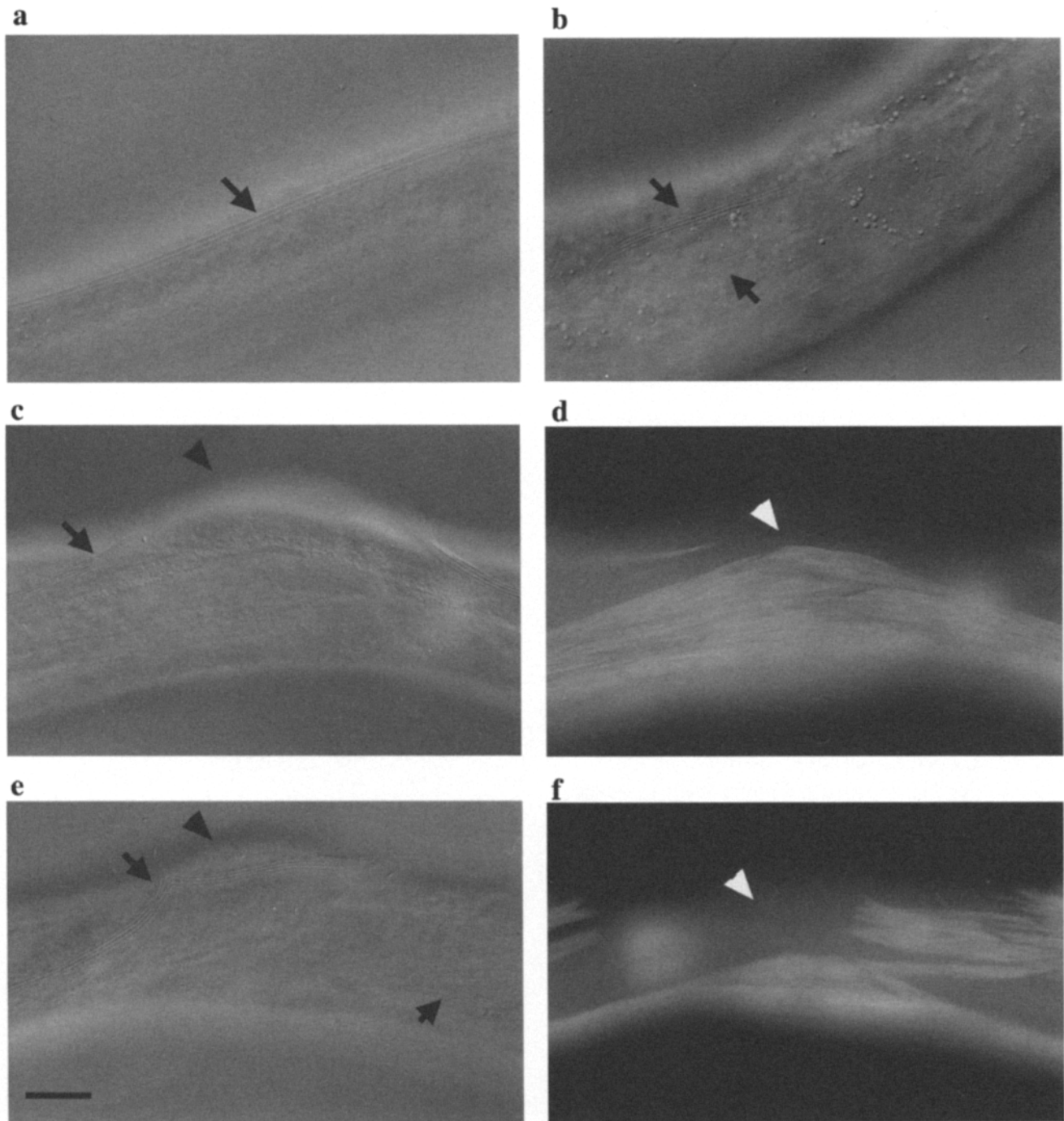
larvae: instead, these animals hatch and develop to adulthood and exhibit both hypodermal and muscle defects (Fig. 6). Obvious hypodermal morphological defects are seen under the dissecting microscope as localized bulges along the length of the worm (Fig. 6, *c* and *e*, arrowhead). When these worms are examined by phalloidin staining, it can be seen that these bulges usually correspond to places where the muscle is abnormally positioned and attached (Fig. 6, *d* and *f*, arrowhead). Nomarski observation of the hypodermis of adult mutants further reveals that the lateral hypodermal cells (seam cells) are affected since the lateral ridges (alae) that are secreted by the seam cells are bifurcated (Fig. 6, *b*, *c*, and *e*, arrows) as compared to the normally continuous alae in wild type (*a*, arrow). These adult seam cell abnormalities usually appear associated with muscle displacements.

### *CeTnT-1* Is Required for Coordinated Embryonic Muscle Contraction

The identity of *mup-2* as TnT, a protein known to be involved in the Ca<sup>2+</sup> regulation of contraction, raised the question of whether the body wall muscle of *mup-2* mutants are capable of normal contraction. The ability of mutant embryos to elongate past the twofold stage, in contrast to Pat mutants, suggested that they were capable of at least some contraction. Time-lapse video microscopy with Nomarski DIC optics was performed on developing wild-type, *mup-2(up1)*, and *mup-2(e2346ts)* embryos. Wild-type embryos begin muscle contraction by twitching (around the 1 3/4-fold stage), which then progresses to coordinated rolling at the twofold stage and continues until hatching. *mup-2* embryos begin twitching, but in contrast to wild type, they never develop coordinated rolling. Even *mup-2(e2346ts)* embryos raised at 15°C fail to demonstrate coordinated rolling in the eggshell.

### *CeTnT-1* Is Not Essential for Larval and Adult Body Wall Muscle Development, but Mutation Affects Coordinated Muscle Contraction

The above studies establish that *CeTnT-1* is essential during embryogenesis for organismal viability and muscle function. To examine whether *mup-2* is essential at other times of development for body wall muscle development, we raised *mup-2(e2346ts)* embryos at the permissive temperature (15°C) until hatching, and then shifted them to the restrictive temperature (25°C) at various times. Homozygous *mup-2(e2346ts)* worms, when shifted to the restrictive temperature after hatching, show qualitatively normal growth and movement on agar plates. When, however, *mup-2(e2346ts)* worms are placed in liquid (of temperatures 15–25°C), ~50% of the worms exhibit an unusual trembling instead of the normal swimming motion: the trembling appears as regionalized contractions of body wall muscle showing no obvious coordination along the length of the worm. In addition to incomplete penetration, the trembling phenotype is neither allele-specific nor stage-specific since both homozygous *mup-2(e2346ts)* and homozygous *mup-2(up1)* Mup larvae tremble in liquid. Thus, the trembling is not simply an unusual or antimorphic effect of the *mup-2(e2346ts)* allele, but rather is due to reduced or eliminated *CeTnT-1* function.

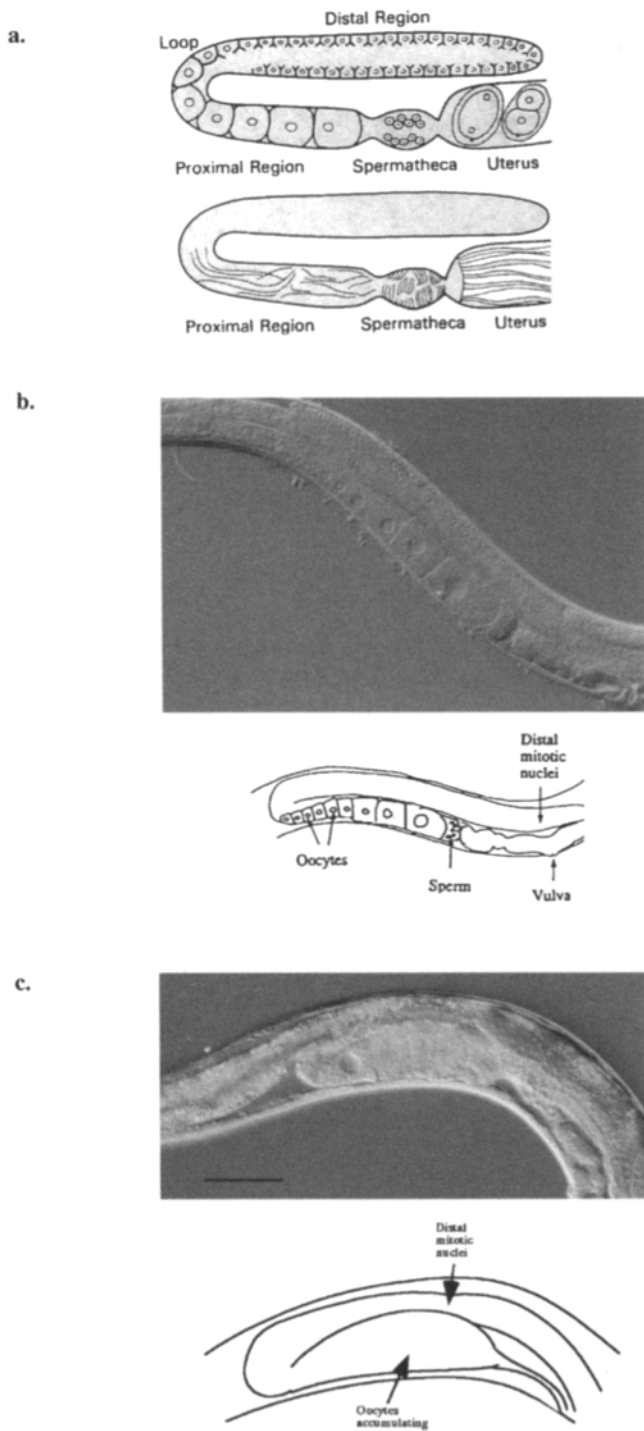


**Figure 6.** Hypodermal and muscle defects of *mup-2(e2346ts)* mutants raised at intermediate temperatures. Alae were visualized under Nomarski optics (*a-c* and *e*) and muscle visualized with rhodamine-phalloidin (*d* and *f*). *a* shows the typical appearance of alae on one side of a wild-type worm (*arrow*). *b* shows a bifurcated alae that has formed a ring-like structure (*arrows*). Defects in alae (*arrows* in *c* and *e*) and muscle (*d* and *f*) are shown which are correlated with a bulge (*arrowhead*) in the region. *c* and *d* are Nomarski and phalloidin views of the same animal as are *e* and *f*. Alae displacement appears dorsal to the muscle, consistent with a ventral displacement of the muscle (*arrow*) and dorsal displacement of the seam cells, which secrete the alae. A bifurcated alae is shown crossing to the other side of the worm (*arrow* in *e*). A break in the muscle is seen in the same region where the alae are abnormally positioned. All of the photographs show lateral views. Bar, 20  $\mu$ m.

### ***CeTnT-1* Is Required in Adult Stages for Hermaphrodite Gonad Function**

We also examined *mup-2(e2346ts)* animals shifted from 15° to 25°C after hatching to determine if other muscle types were affected. Pharyngeal and anal muscles ap-

peared uncompromised in function; however, as *mup-2(e2346ts)* homozygous hermaphrodites (but not males) grown at 25°C were sterile, this suggested a defect in hermaphrodite gonad function. The wild-type *C. elegans* gonad is schematized in Fig. 7 *a*. Fertilization normally oc-



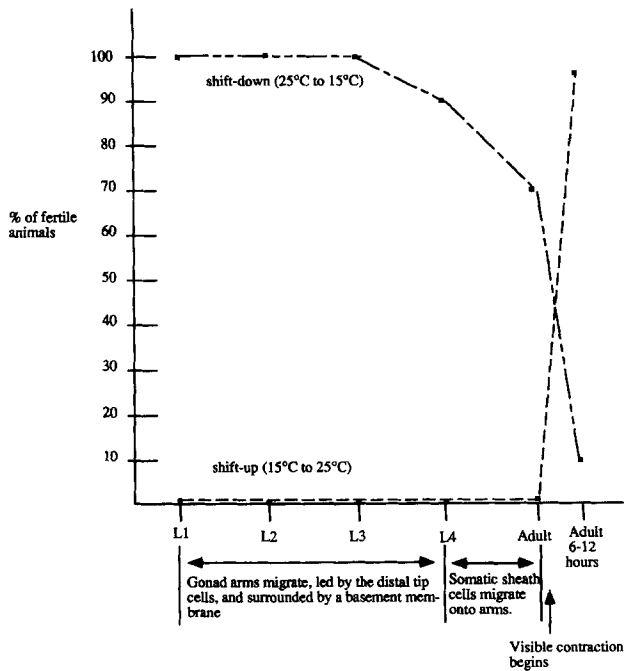
**Figure 7.** Sterility defect of *mup-2(e2346ts)* hermaphrodites. (a) Schematic of the wild-type gonad. The top schematic shows progression of germ-line cells through the gonad. Mitotic nuclei are in the distal region and oocytes begin to form the region of the somatic sheath which surrounds the gonad. The proximal region is myoepithelial and contains actin filaments as depicted by the lines in the drawing (adapted from Strome, 1986). (b) Lateral Nomarski image of a wild-type gonad showing normal progression of oocytes. A schematic is drawn underneath. (c) Lateral Nomarski image of a *mup-2(e2346ts)* gonad showing that the oviduct is greatly enlarged due to the accumulation of oocytes. A schematic is drawn underneath. Bar, 50  $\mu\text{m}$ .

cur when the oocytes enter the spermatheca. The ovotestes is surrounded by a somatic sheath which is myoepithelial and visibly contractile in the proximal region, a region which has been shown to contain both actin (Strome, 1986) and myosin (Ardizzi and Epstein, 1987). A highly coordinated sequence of contractions of this region is believed important for ovulation of oocytes into the spermatheca (McCarter, J., and T. Schedl, personal communication).

Gonads of *mup-2(e2346ts)* hermaphrodites shifted as larvae or adults to 25°C, and thus sterile, show an enlarged oviduct full of oocytes (Fig. 7 c). This suggests a failure of oocytes to progress into the spermatheca. In addition, sperm can be seen to leak out of their normal place in the spermatheca into both the gonad arm and the pseudocoelom (data not shown). These data suggest that TnT might be required for the proper contraction and/or integrity of the myoepithelial sheath surrounding the gonad.

To determine at what time *mup-2* function is required for fertility or when the effects due to the absence of TnT become irreversible, we used the *mup-2(e2346ts)* allele to carry out temperature shift experiments during the larval and adult stages when the gonad is developing. The temperature shift experiments assessed the fertility of *mup-2(e2346ts)* animals grown at 15°C and then either shifted from 15° to 25°C at different larval stages (shift up) or shifted as L1 larvae to 25°C and subsequently restored to 15°C at discrete time points (shift down). In the shift-down experiment, fertility was uncompromised only if the return to 15°C was prior to L4. Shifting of worms back to 15°C at L4 and later stages leads to a progressive decrease in the percent of fertile worms. Once *mup-2(e2346ts)* worms have been raised at 25°C until 12 h or longer after they have become adults, none are able to lay eggs and they all have the characteristic accumulation of oocytes in the oviduct. Thus, although some sterile worms arise when shifted as L4 larvae, these experiments demonstrate that CeTnT-1 is primarily required during adulthood when the oocytes are being fertilized and then laid (Fig. 8). In the reverse situation, *mup-2(e2346ts)* worms that are raised at 15°C until adulthood are able to lay eggs at 15°C, but quickly stop after they are shifted to 25°C, and show the characteristic accumulation of oocytes in the oviduct. The proximal region of this sheath becomes contractile just after the molt to adulthood when the oocytes begin to move into the spermatheca for fertilization. This timing coincides with the movement of oocytes from the oviduct into the spermatheca and uterus and is consistent with a proposed function of *mup-2* in the contraction of the oviduct myoepithelial sheath.

In addition to sterility at the restrictive temperature, *mup-2(e2346ts)* mutants also show a reduced brood size at the permissive temperature of 15°C. The average brood size (total number of progeny) of a self-fertilized wild-type (N2) hermaphrodite is ~300 progeny (Brenner, 1974). In contrast, the average brood size of the homozygous *mup-2(e2346ts)* hermaphrodite at 15°C is 96.5 (Table II). The average brood size of the *mup-2(e2346ts)/mup-2(up1)* hermaphrodite is the same as *mup-2(e2346ts)/mup-2(e2346ts)* hermaphrodites. Surprisingly, this reduced brood size is partially suppressed in the double mutant, *mup-2(e2346ts)/unc-6(e78)* (average brood = 155; see Discussion).



**Figure 8.** Effect of temperature shift on fertility of *mup-2(e2346ts)* hermaphrodites. Shift-up and shift-down experiments were carried out to determine the temperature-sensitive period of the sterility. The tsp was determined to be in the adult stage, soon after visible contraction begins. L1, L2, L3, and L4 are the worm larval stages. The development of the gonad during larval stages is delineated below the graph. The shift-up line is offset for clarity. The numbers of animals scored for each time point in the shift-up experiments are as follows:  $n = 7$  for L1;  $n = 9$  for L2;  $n = 6$  for L3;  $n = 10$  for L4;  $n = 10$  for Adult;  $n = 5$  for Adult 6–10 h (9 sterile). The numbers of animals scored for each time point in the shift-down experiments are as follows:  $n = 5$  for L1;  $n = 8$  for L2;  $n = 7$  for L3;  $n = 10$  for L4 (1 sterile);  $n = 10$  for Adult (3 sterile);  $n = 10$  for Adult 6–12 h (9 sterile).

Even though *mup-2(e2346ts)/mup-2(up1)* and *mup-2(e2346ts)/mup-2(e2346ts)* hermaphrodites have the same average brood size (brood sizes are based on the fertile hermaphrodites only), many of the *mup-2(e2346ts)/mup-2(up1)* hermaphrodites are completely sterile. A similar sterility is seen in *mup-2(e2346ts)/stDfl* hermaphrodites at 15°C (see Materials and Methods). No other hermaphrodites show this sterility at 15°C. The *mup-2(up1)* mutation again appears to have less function than the *mup-2(e2346ts)* allele in agreement with our earlier data and supports the conclusion that the *mup-2(up1)* allele behaves genetically like a deficiency for the region. The reduction in brood size at the permissive temperature of 15°C also demonstrates that the *mup-2(e2346ts)* allele is not completely wild type in its function at the permissive temperature. Thus, the gonad seems especially sensitive to a reduction in levels of TnT function since *mup-2(e2346ts)/mup-2(e2346ts)* embryos hatch at 15°C but grow up to have reduced brood sizes.

#### Gonads of *mup-2(e2346ts)* Mutant Worms Show Defective Oviduct Contraction

To determine if the oviduct of *mup-2(e2346ts)* worms is

**Table II.** Average Brood Sizes of *mup-2* Hermaphrodites at 15°C

	Average brood size	SD	$n^*$
<i>mup-2(up1)/+</i>	303	36	5
<i>mup-2(e2346ts) unc-6(e78)/++</i>	299	64	5
<i>mup-2(e2346ts)/mup-2(e2346ts)</i>	96.5	57	7
<i>mup-2(e2346ts) unc-6(e78)/mup-2(e2346ts) unc-6(e78)</i>	155	40	13
<i>mup-2(e2346ts) unc-6(e78)/mup-2(e2346ts) unc-6(e78)</i>	101	58	5 <sup>†</sup>
<i>mup-2(up-1) +</i>			

\*The number of fertile parental hermaphrodites for which entire broods were determined.

<sup>†</sup>Many animals examined were sterile, similar to *mup-2(e2346ts)/stDfl* heterozygotes, and thus their brood size of zero was not included in the average.

capable of contraction at 25°C, we videotaped young adult worms that were anesthetized with 0.1% tricaine, 0.01% tetramisole (Kirby et al., 1990). This anesthetic inhibits body wall muscle movement, but not oviductal contractions. We found that the oviduct of these worms did not contract. Since the brood size is greatly reduced in *mup-2(e2346ts)* worms raised at 15°C we also videotaped anesthetized worms at 15°C. We found that the oviduct of these worms is capable of only weak contractions, very unlike the organized contractions in wild-type worms. Thus, CeTnT-1 is required for oviduct myoepithelial sheath function.

#### Discussion

This report describes the characterization of a new essential gene locus in *C. elegans*, *mup-2*, and our unexpected result that *mup-2* encodes Troponin T (CeTnT-1). TnT is a tropomyosin-binding protein that participates in  $Ca^{2+}$ -dependent, actin-based regulation of muscular contraction. The embryonic Mup phenotype of TnT represents a new phenotype in *C. elegans* for genes involved in the regulation of muscle contraction. Previous to this work, muscle genes, including structural genes, have been found to cause either abnormal movement (Uncs), or more recently, paralysis and arrested morphogenesis (Pats). Included among the more recently identified Pat mutations are the genes putatively encoding TnC and Tm (Williams and Waterston, 1994; Kagawa et al., 1995; Kagawa, H., and B. Williams, personal communication).

Our isolation and characterizations of a nonconditional allele, *mup-2(up1)*, and especially of a heat-sensitive allele, *mup-2(e2346ts)*, uniquely allowed us to ascertain multiple requirements for CeTnT-1 during development. During embryogenesis, CeTnT-1 is required for sarcomeric organization of body wall muscle and for stable attachment of these cells to the epidermis. That CeTnT-1 is required throughout development for normal regulation of body wall muscle contractions is demonstrated by the inability of either *mup-2(e2346ts)* or *mup-2(up1)* embryos to show coordinated rolling movement and the abnormal trembling behavior shown by mutants at any stage when placed in liquid media. In any case, except for the trembling behavior, it is curious that *mup-2(e2346ts)* larvae grown at restrictive temperatures are capable of apparently normal body wall muscle growth and attachment to the epidermis. The only other muscle group that obviously

requires CeTnT-1 is the oviduct of the worm, which is evidenced by hermaphrodite sterility and defective oviduct contractions.

The Mup (muscle position abnormal) phenotype of *mup-2*, as well as *mup-1* and *mup-4* mutants, involves dorsal displacement of muscle. We interpret the dorsal displacement in Mups as a consequence of the embryo orientation within the eggshell (ventral side inward and dorsal side outward). Thus, the greatest tension would be exerted dorsally, especially at the regions where the embryo bends. However, the *mup-2* phenotype, while giving the same kinked body shape, is actually distinctive from the Mup phenotype of either *mup-1* or *mup-4* that, in contrast to *mup-2*, are proposed to function in the hypodermis (Goh and Bogaert, 1991; Gatewood, B., and E.A. Bucher, manuscript in preparation): the muscle displacement in *mup-2* mutant embryos occurs almost exclusively at the bends of the embryo while there is total displacement of the dorsal body wall muscle quadrants toward the ventral surface in *mup-1* and *mup-4* mutant embryos (Goh and Bogaert, 1991; Gatewood, B., and E.A. Bucher, manuscript in preparation). Furthermore, while the muscle displacement in *mup-1* embryos may indeed be due to a problem with the attachment position of the muscle (Goh and Bogaert, 1991), it appears in *mup-2* embryos that the terminal phenotype is due to a displacement of the muscle (cell position). Our data suggest that this is not a defect in the attachment position of the muscle, but rather loss of attachments leading to mispositioned muscle. Embryonic attachment to the hypodermis appears to occur in two phases, the first being attachment of the muscle to the hypodermis prior to muscle twitching and the second occurring during the threefold stage when the muscle makes attachments to the newly secreted cuticle. The temperature shift experiments demonstrate an irreversible requirement for *mup-2* during the late threefold stages, which may reflect a requirement for CeTnT function for these final attachments to be stably made.

During embryogenesis the muscle forms and begins contraction, first as twitching, but evolving into coordinated contractions that result in the embryo rolling in its eggshell. In the absence of CeTnT-1 function, both actin and myosin are incorporated into sarcomeres in most cells and the cells twitch; however, even for *mup-2(e2346ts)* at 15°C, the twitching does not evolve into the coordinated contractions characteristic of rolling wild-type embryos. Although we detect occasional twofold embryos with disorganized sarcomeres and displaced muscle cells, the tsp within the threefold suggests that any displaced muscles in twofold embryos are either capable of repair during the down shift and/or that the localized muscle displacement does not cause a grossly Mup phenotype. Alternatively, the twofold defects may be very rare, and had they been represented in our temperature shift, they would have been Mup. In any case, we have never detected embryos that arrest prior to the threefold and thus we do not believe that these embryos are Pat. The *mup-2(e2346ts)* mutation truncates the invertebrate carboxy-terminal tail extension, which is of unknown function and is not thought to be involved in tethering Tn to the thin filament. At this point, however, we do not know at the restrictive temperature whether the *mup-2(e2346ts)* truncated protein is ren-

dered unstable in its association with the thin filament or, alternatively, whether it remains associated with the thin filament and is altered in its interactions with other thin filament proteins to cause the essentially null phenotype (as assayed by our genetic assays). In any case, since *mup-2(e2346ts)* is heat-sensitive, this demonstrates that the truncated protein is capable of being synthesized, assembled into thin filaments, and that essential, conserved domains of TnT can function in the absence of this carboxy-terminal tail under conditions of lowered temperatures.

These results prompt several questions. First, given the Pat and Unc phenotypes generally associated with mutations in contractile proteins, how could mutations in TnT cause the Mup phenotype? Second, why are larval and adult body wall muscle cells in mutants capable of apparently normal growth and attachment and why are most other muscle groups not obviously affected? Third, what are the implications of TnT functioning in nonstriated muscle cells? Fourth, given that TnT is a muscle-specific contractile protein, why under certain conditions does mutation of CeTnT-1 cause defects in hypodermal cells?

### Why Are CeTnT-1 Mutants Mup?

Three distinct, but not mutually exclusive, possibilities can be envisaged for why mutations of CeTnT-1 cause a Mup rather than a Pat phenotype. First there could be residual TnT activity in the mutant embryos. This residual activity could provide enough function for the muscles to contract initially allowing the embryos to proceed from the two- to threefold stage of embryogenesis and avoiding a Pat phenotype, but offering insufficient muscular function to ensure a wild-type phenotype.

Residual TnT functions could result from several mechanisms. One possibility is that neither of our *mup-2* alleles represent the true loss-of-function. The genetic data support that the *mup-2* alleles are both greatly reduced in function and the *mup-2(up1)* allele behaves genetically as a null. The fact that the *mup-2(up1)* mutation eliminates essentially all conserved functional domains of TnT if translated is also consistent with *mup-2(up1)* as null. These tests, however, do not rule out that maternal contributions of CeTnT-1 may provide residual activity. Current data support that contractile protein gene expression is primarily zygotic (e.g., Okkema et al., 1993; Chen et al., 1994; however also see Venolia and Waterston, 1990). A maternal contribution could be tested by mosaic analysis in which CeTnT-1 is removed from the germ line.

Another possible source of residual TnT function in CeTnT-1 mutants is expression of additional TnT isoforms that are partially redundant in function. These isoforms could be generated either by additional TnT genes in the genome or by alternative splicing of the *mup-2* gene transcript, similar to the TnT genes in *Drosophila* and vertebrates (e.g., Breitbart et al., 1985; Bucher et al., 1989; Fyrberg et al., 1990). We have examined the genomic organization of *mup-2* and our results demonstrate that the *mup-2* gene is not alternatively spliced (Allen, T. St. C., C.D. Myers, and E.A. Bucher, manuscript in preparation). There are two additional TnT isoform genes that we have identified in the sequenced databases generated by the *C. elegans* genome sequence consortium. We are now

analyzing the cDNAs, gene expression patterns, and mutations of these loci, to ascertain their relationship to CeTnT-1 functions.

A second possibility to explain why loss of CeTnT-1 is Mup is that TnT functions directly in sarcomeric and Z line attachment structures, which are essential to attachment of muscle to the hypodermis. The assembly of muscle filaments in muscle cells and the assembly of attachment proteins in the overlying hypodermis appear to be synchronous and may require communication between the two processes (Hresko et al., 1994). Thus attachment defects could arise due to essential contributions of TnT function in the assembly or stability of actin filaments. This possibility is consistent with phenotypes seen for TnT mutations of the *Drosophila upheld* locus (Fyrberg et al., 1990): in the mutation *up<sup>whu</sup>*, a missense mutation within the invertebrate tail (*Drosophila* residue Ser 311 is substituted to Phe), myofibril diameter is decreased. The data support that this reduction in diameter is possibly due to decreased TnT protein levels. Furthermore, severe reduction or loss of function alleles result in thin filaments that appear associated with Z discs, but that are not fully organized into myofibrils. Thus, if mutations in CeTnT-1 affect the assembly or stability of the actin filaments, they could interfere with communication between the muscle and hypodermis resulting in improper attachments. Consistent with this model, immunofluorescence staining of some *mup-2* embryos shows defects in sarcomeric organization which may reflect underlying assembly and attachment defects (Fig. 4, e, g, and f). It is possible that even a few improper attachments may be enough to change the stress on the muscle during contraction and result in the muscle displacement. Electron microscopy studies of *mup-2* mutants might be able to assess if defects in actin filament assembly are present. Consistent with this model, the *tsp* in late threefold corresponds to the time that actin becomes completely restricted to the I bands, and muscle makes its attachments to the cuticle (Hresko et al., 1994). In the absence of CeTnT-1 function, these final attachments to the exoskeleton might be defective, perhaps due to incompletely assembled or unstable actin filaments, and be insufficient to keep the muscle cells attached to the hypodermis during contraction. Also consistent with a direct function of TnT in attachment structures is a report of two hybrid studies that suggest a direct interaction between TnT and dystrophin, a muscle cytoskeletal protein (Pearlman et al., 1994); however, the *in vivo* relevance of these interactions has not been demonstrated.

A third possibility is that loss-of-function mutations in TnT cause a Mup phenotype due to a direct effect on the regulation of muscle contraction (i.e., through absent or impaired troponin/tropomyosin-conferred  $\text{Ca}^{2+}$  regulation). The proposed function for vertebrate TnT is to anchor TnI and TnC to tropomyosin and thus to the thin filament. This anchoring should be particularly important when the myoplasmic free calcium concentration is elevated, as during contraction, because at this time the association between TnI and actin is weak or nonexistent (Leavis and Gergely, 1984). In the absence of CeTnT-1, the tethering of TnI and TnC with the thin filament would be greatly compromised. We envisage that relaxation of the mutant muscle following a contraction would require

increased time to allow the Tn complex to re-establish inhibitory contacts on the thin filament. The hypothesized deficits would enhance either the duration of muscle contraction, or the peak force, and thereby lead to tearing of dorsal muscles from the body wall. That mutations in TnC and tropomyosin cause a Pat phenotype, rather than a Mup phenotype, is amenable to similar reasoning: instead of impairing the ability of muscles to relax, the putative mutations in TnC and tropomyosin would lock the thin filaments in the inhibited state and the muscle in the relaxed state.

This model of compromised regulation of contraction is consistent with the observation that *mup-2* mutant embryos show muscular contractions. The contractions occur without any apparent coordination over the embryos, and the embryos are unable to roll within the egg shell in the manner of wild-type embryos. Compromised regulation would also be consistent with the trembling phenotype seen in adult *mup-2* worms when placed in liquid solution. Wild type worms exhibit rapid contraction/relaxation cycles of the body wall musculature during swimming. In this regard, mutations of the *unc-22* gene, which encodes Twitchin (Benian et al., 1989), cause a twitching phenotype similar, although not identical to, the trembling phenotype of *mup-2*. Interestingly, Twitchin has been proposed to be involved in regulating contraction-relaxation times and recent studies of *Aplysia* muscle suggest that the physiological function of twitchin is to inhibit the rate of muscle relaxation (Probst et al., 1994).

The *tsp* of *mup-2* function in the late threefold stage of embryogenesis could be explained in this model as the time when the improper forces generated by unregulated muscle contraction finally result in the tearing off of the muscle. In this case the *tsp* reflects not the time of TnT requirement, but of irreversible damage (irreversible damage can also be invoked for the *tsp* in all of the above models). Temperature shifts from restrictive to permissive temperatures before this tearing happens would allow regulated contraction to be attained and the embryos could hatch as wild type instead of Mups. Since *mup-2(e2346ts)* embryos and larvae raised at permissive temperatures also show defective contraction, we propose that the contraction defects would be exacerbated at restrictive temperatures. Finally, it should be stressed that the explanations outlined above for defective contraction could also be invoked if another TnT gene is expressed in embryonic body wall muscle cells, and if loss of function of one of these genes (e.g., *mup-2*) is sufficient to compromise regulated contraction.

### **Why Do Mutations in CeTnT-1 Affect Only Specific Muscles?**

Since each body wall muscle cell grows from 2 sarcomeres to 10 during larval development it is surprising that the *mup-2(e2346ts)* larvae raised at the restrictive temperature are able to develop apparently normally, excepting subtle defects in contraction. One possibility is that the temperature sensitive mutation affects postembryonic functions differently than embryonic development (i.e., the *mup-2(e2346ts)* protein is capable of assembly into sarcomeres, but the *de novo* embryonic muscle assembly and embry-



onic rolling movements may be more demanding than adult function). It is also possible that the TnT synthesized and assembled during embryogenesis may be sufficient for muscle sarcomere growth and function during larval and adult stages. This is plausible since the *mup-2(e2346ts)* mutation truncates the COOH-terminal 62 residues. It may be that this invertebrate tail region has specific assembly functions in embryonic muscles. Alternatively, the ability for sarcomeric growth and muscle function may simply be due to the presence of additional TnT gene isoforms that are able to function in larval and adult body wall muscle. Similarly, additional TnT gene isoforms may be expressed in other muscle groups since, excepting the oviduct muscles, other muscle groups appear grossly normal and our *in situ* hybridization studies have not detected CeTnT-1 accumulation in other muscles (Allen, T.St.C., C.D. Myers, E.A. Bucher, manuscript in preparation).

### **Proposed Function for TnT in Nonstriated Muscles**

The sterility of *mup-2(e2346ts)* hermaphrodites was unexpected. Vertebrate TnT has been found exclusively in striated muscle whereas the myoepithelial sheath of the gonad is not obviously striated. The actin filaments in the myoepithelial sheath have been shown to resemble vertebrate smooth muscle in that they are not organized into repeating sarcomeres (Strome, 1986). However, the myosin heavy chain isoforms expressed in the gonad are the same as those expressed in the body wall muscle (Ardizzi and Epstein, 1987), and there is biochemical precedence for troponin function in the nonstriated muscles of the Ascidian (Endo and Obinata, 1981). It has been proposed that whether TnT is expressed in a cell relates to the isoform of Tm expressed, rather than to the gross organization of filaments (i.e., smooth vs. striated) (Meedel and Hastings, 1993). The *mup-2* mutant phenotype demonstrates that the contraction of this sheath is likely regulated by Ca<sup>2+</sup> under the control of the troponin/tropomyosin complex. That the sterility defect is caused by defective function of CeTnT-1 in the oviduct and not some other defect is further supported by time lapse video analysis demonstrating the compromised ability of oviduct muscles to contract. In addition, we have shown by *in situ* hybridization analysis that CeTnT-1 is expressed in the somatic sheath cells (Allen, T.St.C., C.D. Myers, E.A. Bucher, manuscript in preparation). Further studies of TnT function in the gonad should provide insight into TnT regulation in nonstriated muscle, perhaps representing an earlier evolutionary function for TnT, similar to Ascidian body wall muscle (Meedel and Hastings, 1993).

As was proposed for the body wall muscle, there are a number of possible explanations for why mutation of CeTnT-1 causes defective contraction of the myoepithelial sheath. Absence of CeTnT-1 function may eliminate contraction (effect on the assembly or stabilization of actin filaments) or may eliminate Tn/Tm thin filament regulation of contraction which, our data now support, is required for the progression of oocytes into the spermatheca. Analogous to the displacement of the body wall muscle, the *tsp* for the sterility defect could reflect irreversible damage caused by the stress of unregulated contraction or from the lack of stabilization of actin filaments. Indeed, the

leakage of sperm into the pseudocoelom suggests that the structural integrity of the sheath is compromised, although it is unclear whether this is a primary or secondary defect. Consistent with a myoepithelial structural problem for any of the above reasons, *unc-6*, which has been shown to encode a laminin/netrin-like protein present in the basement membrane (Goodman, 1994; Ishii et al., 1992), can partially suppress the reduced brood size of *mup-2(e2346ts)* hermaphrodite. This suppression may be due to an improvement in the structural integrity of the gonad.

### **Why Do Mutations of CeTnT-1 Cause Hypodermal Defects?**

The morphological defects of the hypodermis in *mup-2(e2346ts)* homozygous embryos grown at intermediate temperatures were surprising given the identity of *mup-2* as a muscle-specific protein. Interestingly, the mutants of the *pat* genes also have defects in hypodermal morphogenesis (Waterston, 1989; Barstead and Waterston, 1991; Williams and Waterston, 1994). Although this study further supports the close association between muscle and hypodermal development, the Mup and Pat defects are distinctly different. Pat mutants are unable to elongate past the twofold stage, a process that was initially thought to rely solely on hypodermal cytoskeletal functions (Priess and Hirsh, 1986). However, Pat mutants affect the ability of muscle to contract. Thus, the force of muscle contractions developed during the twofold stage, and absent in Pat mutants, may be important in reorganizing or stabilizing the hypodermal cytoskeleton or the force of muscle contraction may itself contribute to the movements of elongation.

In contrast, in the case of *mup-2(e2346ts)* embryos raised at intermediate temperatures, muscular force occurs, but localized defects in muscle and hypodermis are seen. The hypodermal defects are observed as localized bulges (Fig. 6, *c* and *e*) and abnormal alae (Fig. 6, *b*, *c*, and *e*). During normal wild-type development the muscle cells compress the hypodermal cytoplasm to a thin sheet where they attach to the hypodermis. As a result, the hypodermal nuclei and the bulk of cytoplasm are displaced to four epidermal ridges (dorsal, left and right lateral and ventral, complementary to the muscle quadrants; discussed in Hedgecock et al., 1987). The lateral cytoplasmic ridges coincide with the lateral seam cells which secrete alae in the first larval and adult stages along the length of the worm. One possibility for the hypodermal defects found in the *mup-2(e2346ts)* mutants raised at intermediate temperatures is that the muscle cells are at a threshold requirement for CeTnT-1 function. Consequently, most muscle cells have enough CeTnT-1 function to develop normally, but a few muscle cells do not. These muscle cells do not assemble and/or maintain normal attachments and thus become displaced during embryogenesis. Since these localized displacements are not catastrophic to animal viability these mutants are capable of development after hatching. The observed hypodermal bulges and alae defects observed in these mutants may result from lack of the usual compression of the dorsal hypodermal cytoplasm by muscle during larval development (due to abnormal or missing muscle attachments) and thus failure to compress the lat-

eral seam cell cytoplasm, which may function to maintain seam cell shape and position. Since alae are only secreted by seam cells, the abnormal alae observed in the adults must reflect abnormally positioned seam cells. The looped alae (Fig. 6 e) may reflect a broadening of a seam cell with the alae following the circumference of the cell. Similarly, bifurcated seam cells that cross dorsally may reflect a mispositioned seam cell due to lack of positional pressures (Fig. 6, a and e). In any case, these studies further emphasize the integrated nature of muscle cell development and force generation with skeletal morphogenesis, analogous to those seen for bone morphogenesis in vertebrates (e.g., Michelsson et al., 1994; O'Sullivan et al., 1994), although the basis for these mutual requirements remains unclear.

### Significance of the Discovery of TnT in *C. elegans*

Knowledge of TnT structure and function has been informed mainly by sequence comparisons among vertebrate skeletal, vertebrate cardiac, and *Drosophila* isoforms of TnT, as well as by biochemical studies with proteolytic fragments of TnT from fast twitch muscle of rabbit. Description of the isoform of TnT encoded by *mup-2* in *C. elegans* offers the opportunity to extend this knowledge of TnT structure and function. Tension development results from MgATP-dependent interaction of myosin (thick filaments) with actin (thin filaments) (Cooke, 1986). Tension development is regulated in muscle cells by the Ca<sup>2+</sup>-sensitivity conferred by regulatory proteins associated either with thin filaments or with thick filaments. Biochemical experiments with extracts of worms suggest that regulatory proteins are associated with both kinds of filament in *C. elegans* (Harris et al., 1977), and the results with *mup-2* confirm the existence of thin filament regulation based on Tn/Tm.

At the present time not much is known about the in vivo interactions of TnT with other sarcomeric proteins. This report, as well as reports of *Drosophila* (Fyrberg et al., 1990) and human TnT mutations (Thierfelder et al., 1994; Watkins et al., 1995) establish essential requirements for TnT in vivo. The *mup-2* mutations, including the *e2346ts* allele, will allow us to take advantage of *C. elegans* to explore these interactions. In particular the oviduct muscle is a sensitive assay of TnT function for which we can isolate extragenic suppressors of the sterile phenotype as well as exploit in vivo functional assays possible by transgenic analysis of CeTnT-1 constructs using the proven ability to rescue *mup-2* mutant defects in transgenic animals. These in vivo approaches are essential to our understanding of muscle contraction and the physical basis of human diseases such as Familial Hypertrophic Cardiomyopathy, which in some cases are caused by inherited mutations of human cardiac troponin T (Thierfelder et al., 1994; Watkins et al., 1995). In addition, the discovery of TnT function in the oviductal muscle provides a means to examine the function of Tn/Tm regulation in nonstriated muscle contraction and may provide insights into the evolution of this type of regulation of muscle contraction.

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