The \textit{mup-4} Locus in \textit{Caenorhabditis elegans} Is Essential for Hypodermal Integrity, Organismal Morphogenesis and Embryonic Body Wall Muscle Position

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ABSTRACT

\textit{mup-4} is a member of a set of genes essential for correct embryonic body wall muscle cell positions in \textit{Caenorhabditis elegans}. The \textit{mup-4} phenotype is variably expressed and three discrete arrest phenotypes arise during the phase of embryonic development when the worm elongates from a ball of cells to its worm shape (organismal morphogenesis). Mutants representing two of the phenotypic classes arrest without successful completion of elongation. Mutants of the third phenotypic class arrest after completion of elongation. Mutants that arrest after elongation display profound dorsal and ventral body wall muscle cell position abnormalities and a characteristic kinked body shape (the Mup phenotype) due to the muscle cell position abnormalities. Significantly, genetic mosaic analysis of \textit{mup-4} mutants demonstrates that \textit{mup-4} gene function is essential in the AB lineage, which generates most of the hypodermis (epidermis), a tissue with which muscle interacts. Consistent with the genetic mosaic data, phenotypic characterizations reveal that mutants have defects in hypodermal integrity and morphology. Our analyses support the conclusion that \textit{mup-4} is essential for hypodermal function and that this function is necessary for organismal morphogenesis and for the maintenance of body wall muscle position.

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Organismal morphogenesis is the correct development of muscle and hypodermis (the specialized epidermis that surrounds the worm and synthesizes the exoskeleton) \cite{PRIESS1986,WOOD1988,WIL1994}.

During organismal morphogenesis, muscle and hypodermis become positioned in a precise manner relative to one another. First, hypodermal cells migrate ventrally from a dorsal/lateral position to enclose the embryo. At this time, the 82 body wall muscle cells migrate to their correct positions (dorsal left and right and ventral left and right quadrants that extend from anterior to posterior) relative to the overlying hypodermis \cite{SULSTON1983}. After reaching these positions, the development and maintenance of the position of body wall muscle with respect to the hypodermis requires that the muscle cells establish precise attachments to the overlying extracellular matrix (ECM) and hypodermis, assemble functional sarcomeres, and establish structural linkages to the overlying cuticle (exoskeleton) during the threefold stage (see Figure 1; \textsc{Francis and Waterston} 1985, 1991; \textsc{Goh and Boeiling} 1991).

Direct interactions between hypodermis and muscle are important in establishing and maintaining body wall muscle position. In particular, studies demonstrate that the hypodermis expresses and organizes intermediate filaments and extracellular matrix structures, including hemidesmosomes, in regions directly overlying muscle
(FRANCIS and WATERSTON 1991; HRESKO et al. 1994). These structures ultimately are important for the structural linkage between muscle and the cuticle.

In addition to muscle forming attachments to the hypodermis, the functions of both of these tissues are necessary for organismal morphogenesis. Surprisingly, mutations of some muscle-specific genes, which affect muscle assembly and/or prevent muscle contraction, prevent embryos from elongating and mutants develop muscle position defects after arrest (WATERSTON 1989; BARSTEAD and WATERSTON 1991; WILLIAMS and WATERSTON 1994). Consequently, these genes are called pat for paralyzed and arrested elongation at twofold (WILLIAMS and WATERSTON 1994). In addition, mutations that affect normal cuticle synthesis and experimental manipulations that affect the cytoarchitecture of hypodermal cells or the integrity of the hypodermal sheet also affect elongation (PRIESS and HIRSH 1986).

Due to the interactions between muscle and hypodermis, it is expected that both hypodermal and muscle-specific genes will affect muscle attachment. Genes have also been identified that are hypothesized to function in the hypodermis for the establishment or maintenance of normal muscle position and at least one of these genes also affects organismal morphogenesis (this article). Mutations in several of these genes cause muscle position defects during late embryogenesis and have been called mup (E. HEDGECOCK, personal communication; GOH and BOGAERT 1991; MYERS et al. 1996). Mutations in several other of these genes cause larval muscle attachment defects and have been called mua (J. PLENEFISCH and E. HEDGECOCK, personal communication). Although many of the mup and mua classes of genes are proposed to function in the hypodermis, because of the intimate relationship of muscle and hypodermis, their mutant phenotypes alone are not sufficient to establish this. For example, the mup-2 gene has been shown to encode the muscle contractile protein tropomycin T (TnT) (GOH and BOGAERT 1991; MYERS et al. 1996) and all of the cloned pat genes encode muscle-specific genes (for examples see WATERSTON 1989; BARSTEAD and WATERSTON 1991; WILLIAMS and WATERSTON 1994). Thus, it is important to establish the cellular basis of these phenotypes since muscle position abnormalities could result from defects in any number of muscle or hypodermal cellular events.

Here we report our findings on mup-4, a zygotic gene that is essential for embryonic development. mup-4 was originally recovered in a genetic mosaic screen of embryonic lethals designed to recover cell-type specific genes that function in embryonic events such as morphogenesis (BUCHER and GREENWALD 1991). mup-4 mutants show profound muscle position defects, yet genetic mosaic analysis demonstrates that the essential requirement for mup-4 gene expression is in nonmuscle cells. Consistent with the cell lineage requirement, our detailed analyses of mup-4 mutants support the idea that mup-4 is required for normal hypodermal function. These genetic and phenotypic studies establish that mup-4 function is required for (1) hypodermal integrity, as evidenced by hypodermal defects; (2) organismal morphogenesis, as evidenced by failure of some mutants to complete elongation and likely caused by the defects in hypodermal integrity; and (3) maintenance of body wall muscle cell positions, as evidenced by the Mup phenotype, either by directly influencing the interactions between muscle and hypodermis or by indirectly affecting the maintenance of muscle cell positions by disruption of hypodermal cell functions.

MATERIALS AND METHODS

Strains: Strains were maintained and standard crosses were performed according to BRENNER (1974). The wild-type (WT) strain used was N2 Bristol. All experiments were done at 20°C.

The following alleles of mup-4 were used in this work: ar60, mg23, mg26, mg30, mg36, g2433, g2440, and g2574. mup-4 containing strains used in this study are listed in Table 1. The following recessive alleles were used: LG II, mup-1(e2439) GOH and BOGAERT 1991); LG III, cold-4(n1162) (ELLIS and HORTITZ 1986); sma-3(e491) (BRENNER 1974); mec-4(u82) (J. WAY and M. CHALFIE, personal communication); dpy-17(e164) (BRENNER 1974); lin-39(n709 ts) (CLARK et al. 1993); dpy-19(e1259) (HODGKIN 1983); unc-32(e189) (BRENNER 1974); unc-36(e251) (BRENNER 1974); gph-1(p66) (AUSTIN and KIMBLE 1987); mua-3(ar62) (BUCHER and GREENWALD 1991). The following dominant alleles were used: tra-2(q122af) I (SCHLEDL and KIMBLE 1988), which feminizes strains (DONIACH 1986), and lin-12(n302d) III, which has an egg laying defective phenotype (Egl) (GREENWALD et al. 1983). The following free duplications were used: dph-3(III; fJ) and dph-3(III; fJ) (HERMAN 1984). The following deficiencies were used: nd16 and nd20 (STGURDSON et al. 1984) and dph-172 and dph-173 (H. STEWART and D. BAILIE, personal communication). etII(III; IV) was used to balance the right half of LG III (ROSENBLUTH and BAILIE 1981; BARBAZUK et al. 1994).

Identification of mup-4 and alleles: mup-4(ar60) was identified previously (BUCHER and GREENWALD, 1991 and unpublished data). Briefly, the parental strain nd-1 unc-36 gph-1; qph-3 (STRAIN Jk830; AUSTIN and KIMBLE 1987; BUCHER and GREENWALD, 1991) was mutagenized and mup-4(ar60) was isolated as a lethal mutation complemented by the free duplication qph-3. The strain mup-4(ar60) nd-1 unc-36 gph-1; qph-3 (Buch and Greene, 1991) was used to complement mup-4(ar60) nd-1 unc-36 gph-1; qph-3 (WT) and mup-4(ar60) nd-1 unc-36 gph-1 (mutant embryos).

The complementation tests to determine alleleism were as
follows. We tested the entire set of lethals recovered by Wightman (1992) for alleles that failed to complement lethals in our set (Bucher and Greenwald 1991). For the complementation tests, either dpv-17 map-4(mg30 or mg36) ncl-1 unc-36/++/+ or ced-4 dpv-17 map-4(mg23 or mg26) unc-36/++/+ males were mated to map-4(ar60) ncl-1 unc-36; glp-3. No Unc progeny segregated; therefore, the alleles fail to complement. Dr. David Ballie and colleagues tested our lethals against their set of lethals on LG III and identified that g2440, g2433, and g2574 failed to complement ar60. We confirmed this by mating dpv-17 map-4(qX) unc-32/++/+ males to dpv-17 map-4(mg36) ncl-1 unc-36; glp-3. No Dpy progeny segregated; therefore, the alleles fail to complement.

Analysis of stages of embryonic arrest: Stage of arrest was analyzed by placing 10 gravid hermaphrodites onto a plate to lay eggs for 12 hours and then recording the hatching rate. The progeny were scored at 1, 10, 20, and 34 hours after which all WT embryos should have hatched. The stages of unhatched eggs or arrested larvae were determined under the dissecting microscope.

Genetic strength of alleles: We examined the terminal phenotypes of mutants to determine if the alleles of map-4 were of different allelic strength. Mutants were examined by phal-loid staining to characterize the threefold Mup phenotype (see RESULTS). In addition, we quantitated the phenotypes of progeny segregating from independent parents of the genotypes. JK800: ncl-1 unc-36 glp-1; glp-3, GS234: map-4(ar60) ncl-1 unc-36 glp-1; glp-3, + map-4(ar60) ncl-1 unc-36 glp-1/sma-3 + unc-36 +, dpv-17 + map-4(mg36) ncl-1 unc-36/sma-3 + unc-36, ced-4 dpv-17 + map-4(mg26) unc-36/++ sma-3 + unc-36.

To generate map-4(ar60) ncl-1 unc-36 glp-1/nfd16 +, we mated map-4(ar60) ncl-1 unc-36 glp-1/++ + males to tra-2/++; nfd16/+. The use of tra-2/+; nfd16/+; feminized heterozygotes removed the complication in our analysis of possible nfd16 homozygous dead embryos that result from self-fertilization. A similar cross was done to generate dpv-17 map-4(mg36) ncl-1 unc-36/++ nfd16. Individual hermaphrodites were allowed to lay eggs overnight and the hermaphrodite was then removed. Progeny were allowed to develop for 2 days and then were scored as either dead (unhatched eggs) or larvae under the dissecting microscope. For quantitation of whole broods, the parent was transferred daily to fresh plates until all eggs were laid. The progeny were scored 2 days after removal of the parent.

Genetic mapping: map-4 was first mapped using genetic deficiencies nDf16 and nDf20. map-4(ar60) ncl-1 unc-36 glp-1/++ males were mated to nDf16/unc-36 dpv-17 nfd19 or to nDf20/unc-36 dpv-17 hermaphrodites. If the deficiencies complemented map-4(ar60), then viable Unc (unc-36) progeny would segregate.

We precisely mapped map-4 by three-factor mapping. We first mapped map-4 relative to ncl-1. dpv-17 map-4(mg36) ncl-1 unc-36/+++/dpv-17+++lin-12(d) males were mated to sma-3 hermaphrodites and WT progeny from the cross were cloned and picked to individual plates. lin-12(d) has a dominant Egl phenotype; therefore, cross progeny of the genotype dpv-17 lin-12(d)/+ sma-3 are Egl and only progeny from the parent dpv-17+ map-4(mg36) ncl-1 unc-36/+ sma-3+++ were examined for recombinants. Individual Unc recombinants were clonally picked and allowed to self-fertilize. The Sma Unc progeny were scored for the presence of the ncl-1 mutation, which is 0.8 map units (mu) rightward of sma-3 and 1.2 map units or centimorgan (cm) leftward of unc-36 (Hedgecock and Herman 1995). We recovered both Sma Ncl Unc and Sma Unc recombinants (Table 2). Since 10/60 recombinants included ncl-1, map-4 is left of unc-36 and ncl-1, and closely linked to ncl-1.

We used a similar strategy to map lin-39 relative to map-4. lin-39 is Egl as a homoygote. Egl Unc recombinants were picked and the progeny of the recombinants were scored for the presence of ncl-1. Since 8/44 recombinants were Egl Ncl Unc, map-4 is left of, and closely linked to, lin-39.

Mapping map-4 relative to mec-14 was accomplished by using the strain map-3 map-4(mg36) ncl-1 unc-36; sDp3. The strategy followed was similar to above in that + sma-3 map-4(mg36) ncl-1 unc-36/+/dpv-17+++/lin-12(d) males were mated to mec-14 hermaphrodites. Individual cross progeny were picked and only progeny from parents of the genotype sma-3 + map-4(mg36) ncl-1 unc-36/+ mec-14+++ were examined. Sma recombinants were scored for the presence of mec-14. mec-14 homozygotes are touch insensitive, which was scored by touching an eyelash hair on the worm body at the base of the pharynx (Chalfie and Sulston 1981). If recombination between sma-3 and mec-14 occurred, the Sma recombinant would have the genotype sma-3 + map-4(mg36) ncl-1 unc-36/sma-3 mec-14+++ and one third of the viable self-progeny from this parent would be Sma Mec. Twenty Sma progeny from each recombinant were scored for mec-14 touch insensitivity. Since we recovered both Sma Mec and Sma Non-Mec (5/37) recombinants, and we observed a lower than expected number of Sma Non-Mec recombinants, we concluded that map-4 is right of, and closely linked to, mec-14.

The chromosomal deficiencies, sDf127 and sDf135, were tested for breakpoints in the mec-14 to lin-39 interval. Individual Unc recombinants were obtained by allowing individual parents of the genotype dpv-17 sDf127 unc-32; sDp3 or dpv-17 sDf135 unc-32; sDp3 to lay eggs overnight. Parents were removed from the plate and eggs were allowed to develop for 24 hours. Homozygous deficiency progeny (dead eggs) were collected for PCR analysis (Williams et al. 1992). Primers were designed based on cosmid DNA sequences available from the C. elegans Genome Sequencing Project (Wilson et al. 1994). The sequence

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### Table 1

<table>
<thead>
<tr>
<th>Strain Name</th>
<th>Genotype of the strain</th>
</tr>
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<tr>
<td>GS234</td>
<td>map-4(ar60) ncl-1(e1865) unc-36(e251) glp-1(q46)III; dp3(III;f)</td>
</tr>
<tr>
<td>EE5</td>
<td>sma-3(e191) map-4(mg36) ncl-1(e1865) unc-36(e251)III; dp3(III;f)</td>
</tr>
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<td>BC414</td>
<td>dpv-17(e164) map-4(mg2433) unc-32(e189)III; dp3(III;f)</td>
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<td>BC4150</td>
<td>dpv-17(e164) map-4(mg2440) unc-32(e189)III; dp3(III;f)</td>
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<td>dpv-17(e164) map-4(mg30) ncl-1(e1865) unc-36(e251)III; dp3(III;f)</td>
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<tr>
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<td>ced-4(e1162) dpv-17(e164) map-4(mg23) unc-36(e251)III; dp3(III;f)</td>
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<td>NA0</td>
<td>ced-4(e1162) dpv-17(e164) map-4(mg26) unc-36(e251)III; dp3(III;f)</td>
</tr>
</tbody>
</table>

* Source: this article.
† Source: Dr. DAVID BALLIE (personal communication).
§ These strains do not have a strain name. Linked markers are described in MATERIALS AND METHODS.
sequences for primer pair 1 (Figure 2B) were derived from cosmid T20B12 (GenBank accession no. U10401; CATGGTGACGAAAAATTCGTAC and CATACCTGCGCCAACCCCT) and for primer pair 2 were derived from cosmid K07B8 (GenBank accession no. U10401; CCGCGATCGTTCGGCAAC and GTCCGATGGCTCCGAAAACG). The annealing temperature for primer pair 1 was 58°C and for primer pair 2 was 56°C and the amplified product for each primer pair was ∼1 kilobase (kb). Absence of amplification indicated that the deficiency deleted the chromosomal region that served as a template for PCR. In the absence of a PCR product, DNA was extracted and a genomic Southern blot was hybridized with a probe homologous to the deficiency. In the absence of a PCR product, DNA was extracted and a genomic Southern blot was hybridized with a probe homologous to the deficiency. In the absence of a PCR product, DNA was extracted and a genomic Southern blot was hybridized with a probe homologous to the deficiency.

Genetic constructions: To examine a subset of seam cells, we used a seam cell marker present in the strain JRB15 having the genotype N2: mup-4/+; pRF4::pRT1 (provided by J. Rothman). pRF4 is a dominant coinjection marker (rol-6 [su1006] [Mello et al. 1991]). pRT1 is a construct of 8 kb of C. elegans genomic DNA cloned into the BamHI site of pPD22.11, a lacZ expression vector (Fire et al. 1990). The reporter gene construct: marker array, pRF4::pRT1, was integrated into LG II (J. Rothman and P. Koelling, personal communication). The pRT1 genomic sequence drives lacZ expression in cell clones and allows to propagate for two generations. Mups were collected, half of which would also have inherited the seam cell transgene, and stained as described (Fire et al. 1990).

mup-4/+; mup-4/+ heterozygotes were generated by mating N2 males to mup-4/+ hermaphrodites. Male progeny of either the genotype +/+ or mup-4/+ were cloned mated to dpy-17 mup-4(g2440) unc-32; dpy-3 hermaphrodites and the parents of each cross were removed after 1 or 2 days to fresh plates. One half of these crosses were expected to segregate mup-1/+; mup-4(g2440)/+ progeny and this genotype would represent one half of the population. Progeny from 20 individual crosses were examined for Mup, Mua, or other phenotypes. We did not make the mup-1/+; mup-4/+ double homozygote to test epistasis since the most severe alleles have identical phenotypes. This double also could be used to test for functional redundancy if a novel phenotype arose; however, this genotype would represent only 1/4 of progeny from the mup-1(e2439)/+; mup-4(g2440)/+ parent and accurate quantitation of phenotypes within these highly pleiotropic populations was not feasible.

mup-4(g2440)+/+ mua-3 individuals were obtained by mating dpy-17 mup-4(g2440)+/+ unc-32/dpy-17+; lin-12+ males to feminized tra-2/+; rol-6 UNC-36 glp-1 mua-3/+; +/+ females. The parents were transferred to a fresh plate and progeny resulting from the mating were scored 2 and 3 days later. Although the individual genotypes could not be distinguished phenotypically, 1/4 of the progeny from this cross were dpy-17 mup-4+; +/+ unc-32+; +/+ rol-1 unc-36+; glp-1 mua-3. After 2 and 3 days, the phenotype of progeny resulting from the mating were scored for Mup, Mua, or other phenotypes. A total of 117 progeny were scored and all became WT adults.

mup-4(g2433) unc-3/mmp-4(g2433) unc-3 homzygotes were generated. First, dpy-17 mup-4++; +/+ unc-32+; +/+ rol-1 unc-36+; glp-1 mua-3 males were mated to dpy-17 unc-32 hermaphrodites. Dpy non-Unc-32 recombinant progeny were picked. The complete genotype of the recombinant chromosome was dpy-17 mup-4(g2433) unc-36 glp-1 mua-3. The genotype was confirmed by complementation tests to unc-36, mup-4(ar60), and mua-3. The presence of glp-1 was inferred because the recombination event occurred left of unc-36. rol-1 was not tested and could be present. This chromosome was balanced over the eT1 chromosome. The phenotype of the double homzygote was determined by examining segregants from the parent dpy-17 mup-4(g2433) unc-36 glp-1 mua-3/eT1. Individual hermaphrodites were allowed to lay eggs overnight and the hermaphrodite was removed from the plate. Eggs were allowed to develop for 24 hours and were scored for Mup, Mua, or other phenotypes.

Preparation and fixation of embryos for immunofluorescence: Similarly staged populations of mutant and WT embryos were obtained in either of the following ways. Starved NGM plates of mup-4(ar60) rol-6 unc-36; dbp-3, or N2 were chunked onto fresh NGM plates such that only dauer larvae recover. Alternatively, starved plates were incubated in the presence of 1% sodium dodecyl sulfate for 30 min to lyse all stages, except dauer larvae, which were then plated onto freshly seeded NGM plates. Dauer larvae synchronously recrude the larval life cycle. After these synchronized populations of hermaphrodites laid eggs for 1.5 to 2 days, eggs were collected in a wash of M9 (Sulston and Hodgkin 1988). Pure populations of embryos were obtained by treating the M9 wash to alkaline lysis treatment (25% bleach in 0.2 N NaOH), which lyses worms and larvae and enhances immunofluorescence staining. Embryos were fixed by either of two different methods: (1) 1–7 μl of concentrated worm embryos in M9 were placed onto a poly-L-lysine coated microscope slide (0.05% poly-L-lysine solution in H2O coated onto a glass slide
The *mup-4* Locus in *C. elegans*

A. 

```
| dp-37 | mec-14 | mec-4 | mec-14 pak-3 | ncl-1 | unc-26 | unc-32 | qDp3 |
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| sDp3 |
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| nDf16 |
```

```
| nDf20 |
```

```
| = s0.1 cM |
```

B. 

```
| cosmid contig | cosmid gap | cosmid contig |
```

```
| mec-14 | mup-4 | lin-39 |
```

```
| sDf127 |
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| sDf135 |
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= PCR results |
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**Figure 2.**—Genetic map location of *mup-4*. (A) The genetic map depicts genes on LG III used in studies of *mup-4*. The free duplications, *qDp3* and *sDp3*, are indicated by wide solid lines. The deleted regions of LG III in the genetic deficiencies *nDf16* and *nDf20* are depicted by thin solid lines (EDGLEY and RIDDLE 1989). The precise breakpoints of the duplications and deficiencies have not been determined and dashed lines indicate that the break point lies somewhere in that region. Recombination mapping placed *mup-4* into a genetic interval of 0.02 cM between *mec-14* and *lin-39* on LG III (HEDGECOCK and HERMAN 1995). (B) *mup-4* was mapped relative to the deficiencies, *sDf127* and *sDf135*. The physical map of the region, consisting of two sequenced cosmid contigs and a gap between the two cosmid contigs, are shown relative to the genetic interval. The sequence from these contigs was used to design primers. The relative position of primer pair 1, which amplifies sequences from cosmid T20B12, and primer pair 2, which amplifies sequences from cosmid K07D8, for the PCR testing of *sDf127* and *sDf135* are shown (MATERIALS AND METHODS). The PCR results from homozygous deficiency individuals are indicated above the deficiencies as the presence (+) or absence (-) of PCR product.

and then baked on a heating plate at setting 7 for 10 min. A 24 X 24 mm glass microscope coverslip was placed on top of the drop. The embryos were freeze cracked by gently applying pressure to the coverslip while freezing on dry ice. The coverslip was "cracked" off and the slides were immediately placed into 0° methanol. The embryos were rehydrated through a methanol series of 75%, 50%, 25% methanol in H2O (CHEN et al. 1994). (2) The alternative protocol used to fix embryos was as described (BARSTEAD and WATERSTON 1991). Briefly, embryos were fixed in 3% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min and then permeabilized in a methanol gradient as described above. These methods gave identical results.

**Antibody staining of fixed embryos:** A blocking agent of 3% dry milk (Carnation) in PBS (pH 7.0) or 10% horse serum in PBS was applied to fixed/permeabilized embryos for 1 hr. Antibodies were diluted in blocking agent and applied to embryos for 2 hr at room temperature or overnight at 4°. Primary antibodies used in this work were kindly provided by H. KAGAWA (R224), R. WATERSTON (MH4, MH25, and MH27), and T. BOGAERT (Ne2/11b4.14; from stocks at MRC Laboratory of Molecular Biology, Cambridge, UK). The following primary antibodies were used: rabbit polyclonal R224 used at a 1:250 dilution (KAGAWA 1989); mouse monoclonal MH27 used at a 1:500 dilution (PRIESS and HIRSH 1986); monoclonal MH25 used at a 1:125 dilution (FRANCIS and WATERSTON 1991); mouse monoclonal MH4 used at a 1:125 dilution (FRANCIS and WATERSTON 1991); and mouse monoclonal Ne2/11b4.14 used at a 1:125 dilution (GOH and BOGAERT 1991). Antibody solutions were removed after incubation and
embryos were washed with PBS/0.01% Tween (Sigma) for 10 min for a total of four washes. Secondary antibodies used were either FITC-conjugated goat anti-rabbit or TRITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch laboratories, West Grove, PA), both used at a dilution of 1:1000. Secondary antibody dilutions were applied and subsequently followed with the wash procedure as described above. Embryos were mounted in 4% glycerol 10% Mowiol 4–88 (Calbiochem) 0.1 m Tris-HCl pH 8.5 and viewed on a Polyvar II (Reichert-Jung). T-Max-100 (Kodak) was used for all photomicroscopy and developed in HC-110 (dilution F; Kodak).

Time-lapse video microscopy: Embryos were mounted on 5% agar pads in M9 buffer and sealed with silicone grease (Williams and Waterston 1994). Embryos were followed with a video camera and controller (Hamamatsu C2400) and a time-lapse video recorder (NEC VC9507).

RESULTS

Genetic isolation and characterization of mup-4 alleles: The mutation ar60 was identified in a screen for zygotic lethals that are complemented by the free duplication dpl3 (III;f) (Bucher and Greenwald 1991). Analysis of ar60 was not described in this previous report. Seven additional alleles were recovered in independent screens for embryonic lethal mutations that are complemented by the free duplication, dpl3 (III;f) (Figure 2A; D. Baille, personal communication; Wightman 1992). All alleles fail to complement ar60 and each other (MATERIALS AND METHODS). These eight EMS-induced alleles arose at a frequency of ~1 in 500 haploid genomes. We named this gene mup-4 because most mutants exhibit muscle position defects and a kinked threefold/L1 shape (Figure 3) that is similar to the phenotype of other mutants in the Mup class (E. Hegdeck, personal communication; Goh and Bogaert 1991; Goh 1991; Myers et al. 1996). We will refer to this terminal threefold/L1 arrest phenotype as a threefold Mup mutant.

Mapping of mup-4: To determine if mup-4 represents a new muscle position effect gene, we genetically mapped mup-4. The map position of mup-4 was first delimited by complementation tests with ndf16 and ndf20, overlapping deficiencies that delete 0.85 and 0.63 mu, respectively (Figure 2A). ndf20 complemented, whereas ndf16 failed to complement, mup-4 (MATERIALS AND METHODS); therefore, mup-4 maps to a 0.3 mu region deleted only by ndf16. This map position is consistent with independent map data that positions mup-4 rightward of dpy-17 (D. Baille, personal communication).

To localize mup-4 precisely within the 0.3 mu interval, threefactor crosses were undertaken that required identifying viable recombinants in which mup-4 had been crossed off the chromosome (MATERIALS AND METHODS). We mapped relative to sma-3, mec-14, lin-39, ncl-1, and unc-36 (Table 2). The three-factor-mapping data place mup-4 between mec-14 and lin-39, an interval of ~0.02 mu (Figure 2A). No other known genes that affect muscle map to this interval. This genetic interval has been partially cloned into two sequenced cosmid contigs (200 and 60 kb; Figure 2B) that are separated by a gap that is bridged only by an unsequenced YAC (Wilson et al. 1994). The precise size of the gap between the contigs is unknown, but it must be at least 100 kb based on the size of two small YACs that also map completely inside the gap (R. Wilson, personal communication). Attempts to generate clones other than YACs to bridge this region have thus far been unsuccessful, although fosmid cloning of the region is now being attempted (S. Chissoe and R. Wilson, personal communication).

We then mapped mup-4 relative to two small deficiencies, sDf127 and sDf135, that complement mup-4 by PCR analysis of embryos homozygous for either deficiency: we asked whether we could PCR amplify specific genomic regions in the mec-14 to lin-39 interval (MATERIALS AND METHODS; Figure 2B; D. Baille, personal communication and our data). We found that the deficiencies delete sequences within the left, but not the right, cosmid contig. Thus, these data, in combination with three-factor mapping, demonstrate that mup-4 maps rightward of the deficiency breakpoints that lie within the cosmid contig gap and leftward of lin-39 (Figure 2B). Interestingly, the 5′ end of a predicted gene having similarity to another muscle position effect gene, mua-3, is located in the cosmid K07D8 that borders the right side of the contig gap (Genbank accession No. L16679; Figure 2B). This putative protein has been called MRP-1 for MUA related protein (J. Pleenfisch, personal communication). The 3′ end of the MRP-1 gene lies within the cosmid contig gap.

Genetic characterizations of mup-4 alleles: Genetic analyses demonstrate that the canonical allele, ar60, is recessive as are two other alleles, mg33 and mg26 (Table 3). In addition, analyses of ar60 and mg36 mutants show that ar60 and mg36 are variably expressed, since ~90% of the mutants arrest as threefold/L1 Mups, and the remaining 10% arrest earlier in embryogenesis appearing morphologically as either bean or twofold (Table 4). Although we have not determined if the other six alleles are variably expressed, all eight alleles cause an identical threefold Mup phenotype as assayed under the dissecting microscope and by phalloidin staining (data not shown). Since all eight alleles fail to complement, have the same mutant phenotype, and map to a similar genetic location, we conclude that all are alleles of the mup-4 gene. Since these alleles exist in different genetic backgrounds (Tables 3 and 4; MATERIALS AND METHODS), we conclude that neither the threefold mutant arrest phenotype nor the variable expressivity is the result of genetic markers in the background.
in trans to a deficiency. We compared the mutant phenotypes of mup-4(ar60)/nDf16 and mup-4(ar60)/mup-4(ar60) and of mg36/nDf16 and mg36/mg36 and found that all genotypes exhibit similar arrest phenotypes (Table 4). Thus, the ar60 and mg36 alleles are likely null. Indeed, we expect that all existing mup-4 alleles are greatly reduced in function and likely null since alleles of mup-4: (1) arise at high frequency in genetic screens; (2) exhibit the same Mup phenotype; and (3) were isolated in nonbiased screens for lethal mutations (i.e., the Mup phenotype was not selected for). All phenotypic studies presented below were undertaken on the canonical allele ar60.

The variable expressivity observed in the genetic studies could reflect thresholds of mup-4 zygotic expression/function at different developmental times. Alternatively, the variable expressivity could reflect a necessity of both a maternal and zygotic contribution of mup-4: the maternal product could supply the embryo until threefold; however, some oocytes may receive less maternal component, resulting in earlier arrest. In this study, we have specifically assessed the essential zygotic contribution and a potential maternal contribution has not been excluded.

WT muscle development: We examined embryonic body wall muscle in mup-4 mutants. We will first summarize WT muscle development (see also SULSTON et al. 1983; WATERSTON 1988; HRESKO et al. 1994). Aspects of WT and mutant muscle development can be followed using antibodies to muscle-specific proteins. At about 310 min after fertilization, the laterally located body wall muscles undergo migrations to form the two dorsal and two ventral quadrants of muscle, which are positioned underneath the hypodermis (bean stage; Figure 4). Muscle-specific proteins, such as myosin and actin (WATERSTON 1989; EPSTEIN et al. 1993; HRESKO et al. 1994; WILLIAMS and WATERSTON 1994) begin to accumulate during this stage. By 350 min, muscle cells are organized into quadrants and muscle-specific proteins become localized in the regions apposing the hypodermis (bean stage; Figures 4 and 5A). Also at this time, muscle proteins that make up muscle attachment structures, including the muscle proteins vinculin (BARSTEAD and WATERSTON 1989; BARSTEAD and WATERSTON 1991) and integrin (HRESKO et al. 1994; GETTNER et al. 1995), the muscle basement membrane component perlecan (ROGALSKI et al. 1993; HRESKO et al. 1994), and hypodermal proteins that make up hemidesmosomes (e.g., hypodermal intermediate filaments; HRESKO et al. 1994), are expressed and localized (Figures 1 and 4). At 430 min, the muscle is organized into thick filaments (1.75-fold stage; Figures 4 and 5C; HRESKO et al. 1994) and the embryo begins twitching. Also at this time, intermediate filaments are organized into hemidesmosomes in the dorsal and ventral hypodermal regions overlying body wall muscle (HRESKO et al. 1994). During mid-threefold, the cuticle exoskeleton is expressed and be-
The phenotype of embryos with two copies of the mup-4 allele revealed three different phenotypic classes. The threefold Mup phenotype is caused by muscle position defects arising during the threefold stage: Mutants that display the threefold Mup mutant phenotype must progress successfully through the bean, twofold, and threefold stages of elongation before subsequently arresting at threefold. Since muscle migration, initial muscle attachment, and sarcomere assembly occur prior to the threefold stage (Sulston et al., 1983; Hresko et al., 1994), we analyzed mixed embryonic populations of mutant mup-4 embryos for defects in these processes that could ultimately result in the threefold arrest class (~30% of the population should be mutant and most of these will develop the threefold Mup phenotype). Excepting the earlier bean and twofold arrest first class is morphologically bean stage mutants that have disorganized muscle (Figure 5B). The second class is morphologically twofold stage mutants that are grossly abnormal having a dorsal bulge (Figure 5D). In these mutants, individual body wall muscles still appear in dorsal/ventral quadrants, but the muscle quadrants do not extend into the nose and tail regions. The third class is morphologically threefold stage mutants (Figures 3B and 5F) in which the dorsal body wall muscle is mispositioned to the ventral side of the animal. Both dorsal and ventral muscle cell positions are affected since muscle cells are retracted from the nose and the tail (Table 5: Figures 3B and 5F).

We conclude that the three phenotypes represent terminal arrest phenotypes. (1) The bean, twofold, and threefold phenotypic classes were observed in all populations examined for immunofluorescence. This included synchronized populations, in which all embryos should have progressed through embryogenesis and hatched, as well as mixed stage populations. (2) The bean and twofold classes of embryos were observed at a very low frequency: this is consistent with our genetic studies in which ~10% of the mutants arrest at the bean or twofold stage (Table 4). (3) The bean and twofold classes of arrested embryos display abnormal enclosure of hypodermis and would thus be incapable of elongating to threefold (see below).

### TABLE 3

<table>
<thead>
<tr>
<th>Genotype of hermaphrodite</th>
<th>Phenotype of progeny</th>
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<tbody>
<tr>
<td></td>
<td>Unc</td>
</tr>
<tr>
<td>A. mup-4/+</td>
<td></td>
</tr>
<tr>
<td>+ mup-4(ar60) ncl-1 unc-36</td>
<td>470 (66.2)</td>
</tr>
<tr>
<td>sma-3 + + + unc-36</td>
<td>120 (73.2)</td>
</tr>
<tr>
<td>+ mup-4(mg23) ncl-1 unc-36</td>
<td>95 (66)</td>
</tr>
<tr>
<td>sma-3 + + + unc-36</td>
<td>261 (33.8)</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>B. mup-4/mup-4/+</td>
<td></td>
</tr>
<tr>
<td>ncl-1 unc-36 glp-1; qDp3</td>
<td>445 (39.6)</td>
</tr>
<tr>
<td>ncl-1 unc-36 glp-1; qDp3</td>
<td>261 (33.8)</td>
</tr>
</tbody>
</table>

The genetic behavior of three mup-4 alleles was tested. (A) Three mup-4 alleles, mup-4(ar60), mup-4(mg23), and mup-4(mg46), were placed in trans to a WT allele of mup-4. Parent hermaphrodites segregated either Mup, Uncs or Sma-Unc progeny. The phenotype of the progeny scored is indicated as a percentage of total viable animals (in parentheses). (B) The phenotype of embryos with two copies of the ar60 allele and one WT allele supplied by qDp3, was tested in progeny of the strain mup-4(ar60) ncl-1(e1865) unc-36(e251) glp-1(q46); qDp3. Free duplication loss was quantitated by determining the number of Mups and dead eggs. Progeny from the strain ncl-1(e1865) unc-36(e251) glp-1(q46); qDp3 provided a control for the rate of meiotic duplication inheritance based on the number of Unc-Glp progeny. This rate is represented as a percentage of total individuals scored (in parentheses).

comes structurally linked to the body wall muscle via the hypodermal intermediate filaments (Francis and Waterston 1991; Hresko et al. 1994). Because development is largely invariant, all these events will occur at essentially the same spatial and temporal sequence in every embryo.

### mup-4 terminal arrest phenotypes

Examination of mup-4(ar60) mutants by immunofluorescence microscopy revealed three different phenotypic classes. The first class is morphologically bean stage mutants that have disorganized muscle (Figure 5B). The second class is morphologically twofold stage mutants that are grossly abnormal having a dorsal bulge (Figure 5D). In these mutants, individual body wall muscles still appear in dorsal/ventral quadrants, but the muscle quadrants do not extend into the nose and tail regions. The third class is morphologically threefold stage mutants (Figures 3B and 5F) in which the dorsal body wall muscle is mispositioned to the ventral side of the animal. Both dorsal and ventral muscle cell positions are affected since muscle cells are retracted from the nose and the tail (Table 5: Figures 3B and 5F).

We conclude that the three phenotypes represent terminal arrest phenotypes. (1) The bean, twofold, and threefold phenotypic classes were observed in all populations examined for immunofluorescence. This included synchronized populations, in which all embryos should have progressed through embryogenesis and hatched, as well as mixed stage populations. (2) The bean and twofold classes of embryos were observed at a very low frequency: this is consistent with our genetic studies in which ~10% of the mutants arrest at the bean or twofold stage (Table 4). (3) The bean and twofold classes of arrested embryos display abnormal enclosure of hypodermis and would thus be incapable of elongating to threefold (see below).

### TABLE 4

<table>
<thead>
<tr>
<th>Mutant phenotype</th>
<th>Threefold/Mups</th>
<th>Bean/Twofold</th>
</tr>
</thead>
<tbody>
<tr>
<td>mup-4(ar60)</td>
<td>408 (91.6)</td>
<td>37 (8.3)</td>
</tr>
<tr>
<td>mup-4(ar60)/nDf16</td>
<td>66 (89.1)</td>
<td>7 (9.5)</td>
</tr>
<tr>
<td>mup-4(mg36)</td>
<td>97 (91.5)</td>
<td>9 (8.5)</td>
</tr>
<tr>
<td>mup-4(mg36)/nDf16</td>
<td>48 (87.3)</td>
<td>7 (12.7)</td>
</tr>
</tbody>
</table>

Mutant phenotypes of two different mup-4 alleles, ar60 and mg36, were compared to the phenotypes of ar60/nDf16 and mg36/nDf16. While most dead eggs arrest as three-fold Mups, a small percentage (indicated in parentheses) arrest as bean/twofold dead embryos. The bean/twofold arrest classes are grouped together because the mutant phenotype is difficult to distinguish under the dissecting microscope. We did not observe an increase in these arrest classes when these alleles were placed in trans to a deficiency.
classes, we never saw aberrant muscle cell positions, abnormal attachment positions (e.g., see GOH and BOGAERT 1991), or disorganized thick filaments prior to threefold (e.g., see MYERS et al. 1996). This inability to detect any abnormalities suggests that mutants that progress to the threefold stage appear grossly normal in their muscle development prior to threefold arrest.

To further assess muscle function up until threefold arrest, we compared WT and mutant embryonic muscle function by time-lapse video tape analysis. Analysis of 20 mutant embryos revealed that mutants twitch and roll within the eggshell and were indistinguishable from WT until about halfway through the threefold stage. At this time the mutant embryos slowed and within 30 min ceased movement, excluding residual movement of the tail (data not shown). These data are consistent with the immunofluorescence data and support that the threefold mutants initially have functional muscle and functional attachments between muscle and the hypodermis, but that muscle becomes mispositioned at some point after reaching the threefold stage.

**mup-4 mutants express proteins associated with muscle attachment structures:** One possible explanation of the muscle position defects is that *mup-4* encodes a protein associated with muscle attachment structures (Figure 1) and loss of this protein leads to a more global defect in attachment structures. We examined *mup-4* threefold mutants for the expression and localization of several proteins that localize to attachment structures between muscle and hypodermis, which should be present during threefold. MH25 antibody recognizes the β-subunit of the integrin receptor, which is expressed by muscle and located at the base of muscle "lines, at dense bodies, and between muscle cells (Figure 1; FRANCIS and WATERSTON 1991; GETTNER et al. 1995). The MH4 antibody recognizes intermediate filaments, which are expressed by hypodermal cells. At the threefold stage the intermediate filaments are localized only in hypodermal regions directly overlying body wall muscle (FRANCIS and WATERSTON 1991). Analysis of embryonic populations demonstrates that these antigens are present in threefold Mup mutants (Figure 6B and D). Furthermore, the two proteins not only localize to attachment structures in *mup-4* mutants but appear to colocalize with the mispositioned body wall muscle (Figure 6A and C).
**Figure 5.**—Developmental comparison of body wall muscle cell position in WT and mup-4(ar60) mutant embryos by immunofluorescence analysis of the muscle thick filament protein paramyosin in body wall muscle. Fixed embryos from embryonic populations of GS234 (containing both WT and mutant animals) were stained with the antibody R224. Embryos were staged by morphological appearance. Anterior is left. Dorsal is up for all lateral views. (A) Dorsal view of a WT bean stage embryo (~290 min). Paramyosin is expressed at this stage (arrowheads), although muscle has not migrated to the dorsal or ventral quadrants. (B) Dorsal view of a mup-4 morphologically bean stage embryo. Body wall muscle cells are abnormally positioned. Several of the mispositioned cells are marked by arrows. (C) Lateral view of approximately a twofold embryo (430–450 min). Paramyosin appears organized into thick filaments (in contrast to the embryo in A). (D) Ventral view of a morphologically twofold mup-4 mutant. Muscle appears organized into quadrants (arrows); however, the body wall muscle appears displaced from the nose and tail regions (compare arrowheads in C and D) and the dorsal body morphology displays a "bulge" (open arrow). (E) Lateral views of a WT threefold embryo (after 450 min). Arrow indicates a muscle quadrant. (F) Threefold Mup embryo displaying the characteristic displaced dorsal body wall muscle (compare arrows in E and F and see Figure 3B). There is residual paramyosin staining on the dorsal side of the embryo; an open arrow indicates an individual muscle cell apparently still partially positioned on the dorsal side and crossing to the ventral side. Bar, 15 μm.
Mosaic analysis demonstrates an essential function for the mup-4 gene in the AB lineage. To determine which cells require mup-4 gene expression for organismal viability, mosaic analysis was undertaken as described (BUCHER and GREENWALD 1991; and Figure 7 legend). For instance, mup-4 could have essential functions within muscle cells that are required for normal muscle position (MYERS et al. 1996). Alternatively, the mup-4 gene could function in other cells, such as the hypodermis or neurons (GOH and BOGAERT 1991). These possibilities can be distinguished by genetic mosaic analysis (HERMAN 1989; BUCHER and GREENWALD 1991). To determine the cellular focus of mup-4 gene function, viable mosaic animals were recovered from a mup-4-containing strain, GS234, and compared to a control strain, JK830 (Figure 7 legend). As expected, all possible classes of mosaics were recovered from JK830 (AUSTIN and KIMBLE 1987; BUCHER and GREENWALD 1991; Figure 7A).

Certain classes of mosaics were recovered at essentially control frequencies from GS234, whereas other classes were not recovered due to the presence of mup-4(ar60) (which result in organismal death) (Figure 7B). Viable mosaics were identified in which mup-4 gene function was lost in the P1 blastomere and its descendants: thus, expression of mup-4 is not essential in P1 or its descendants. Since all but one body wall muscle is derived from P1, this demonstrates that mup-4 does not have an essential function in muscle. In contrast to the recovery of viable P1 mosaics lacking mup-4 gene function, no viable mosaics were ever recovered that represented loss of mup-4 function in the AB or ABp blastomere: thus, mup-4 is essential in the AB lineage. However, we were able to recover viable semi-Unc mosaics (also recovered due to the unc-36 marker) that are mutant for a subset of the AB lineage (ABpl or ABpr). Presumably these were viable due to loss of gene activity in a smaller proportion of cells that is not lethal (see DISCUSSION). We conclude that mup-4 function is essential in the AB lineage, which produces primarily hypodermal and neuronal cells, cell types that functionally interact with muscle.

We also recovered viable mosaics that lack mup-4 gene function in descendants of ABa and ABpl or ABpr that
do not exhibit a visible *unc-36* phenotype. Instead, these animals showed floppy head or tail regions and uncoordinated behavior similar to *mua-3* mutants (see below) and were often egg-laying defective with abnormal vulvae (defects often associated with hypodermal abnormalities). Rhodamine phalloidin staining of these “floppy” Unc animals revealed localized muscle position defects that are remarkably reminiscent of *mua-3* mutants (data not shown). Combined, these mosaic data demonstrate that *mup-4* gene function is required in the AB lineage and that loss of *mup-4* gene function in a small proportion of cells in the AB lineage causes defects in animal movement, muscle cell positions, and egg-laying abilities.

**Genetic interactions of *mup-4***: We made genetic double combinations between *mup-4* and either *mup-1* or *mua-3* alleles to test for possible genetic interactions with *mup-4*. Strong alleles of *mup-1* cause a threefold Mup phenotype similar to *mup-4* (Goh and Bogaert 1991). It has been proposed that *mup-1* is a hypodermally derived product based on the genetic suppression of *mup-1* by mutations in other hypodermal genes, such as *dpy-10*, which encodes a cuticle protein (Goh and Bogaert 1991). We generated heterozygous *mup-1(e2439)/+; mup-4(g2440)/+* animals and examined these double heterozygotes for Mup, Mua, or other visible phenotypes. All heterozygotes were WT.

*mua-3(ar62)* has a muscle attachment defect that occurs during the L1 to L2 molt (E. A. Bucher, unpublished observations; J. Plenefisch and E. Hedgecock, personal communication). Furthermore, mua-3(ar62) has been shown by mosaic analysis to be required in AB, similar to *mup-4* (Bucher and Greenwald 1991). We generated the double mutant, *mup-4(g2433) mua-3(ar62)/mup-4(g2433) mua-3(ar62)* and the resulting phenotype was Mup. This is consistent with the *mup-4* gene product acting earlier in development than *mua-
3. Furthermore, these two genes likely do not act redundantly during embryogenesis since a more severe phenotype was not produced. We also tested mup-4(g2440)+/+ mua-3(ar62) heterozygotes for possible intergenic noncomplementation by assaying for Mua or other obvious phenotypes. All heterozygotes were WT.

**WT hypodermal development:** We examined the hypodermis of mup-4 mutant embryos. Aspects of WT and mutant hypodermal development can be followed using a monoclonal antibody (mAb MH27) that localizes to adherens junctions of many cell types, including at hypodermal cell boundaries (Figure 8; Priess and Hirsh 1986; Waterston 1988; Podbielničcz and White 1994). The hypodermis initially resides as six rows of cells covering the dorsal/lateral surfaces of the embryo. After 200 min postfertilization, the ventral hypodermal cells migrate laterally to the ventral midline to enclose the embryo (Figures 4 and 8A). These migrations also position the lateral hypodermal seam cells at the sides of the embryo and dorsal hypodermal cells extend processes to interdigitate and connect to each seam cell. After enclosure, at about 250 min, the embryo begins to elongate, changing the shape of the embryo from a ball of cells to a tube-shaped worm (Figures 4 and 8C). The hypodermal cells continue to undergo shape changes and cellular fusions during elongation to generate the hypodermal syncitia (Figures 4 and 8E).

**Hypodermal cell specification and differentiation in mup-4 mutants:** We hypothesized that mup-4 gene activity is essential in the hypodermis since muscle is structurally connected to the hypodermis, genetic mosaic analysis is consistent with an essential requirement of the mup-4 gene in the hypodermis, and hypodermal defects occur in mup-4 mosaics. We considered whether defects in hypodermal cell specification, differentiation, or morphogenesis could cause the observed muscle position defects. To test if at least a subset of hypodermal cells were correctly specified, we used a transgenic enhancer trap marker expressed in seam cells (Materials and Methods). We observed no difference in the number of seam cells in mup-4 mutant embryos and WT embryos as assayed by the seam cell marker (data not shown).

To assess whether hypodermal cells were capable of differentiation, we tested for the expression and localization of several hypodermal specific antigens and structures including (1) a filamentous antigen in seam cells (Goht and Bogaert 1991), recognized by the antibody Ne2/b4.14 (data not shown); (2) a specialized cuticular structure, alae, visualized by Nomarski optics (data not shown); (3) the adherens junction protein, recognized by mAb MH27 (Figure 8; Priess and Hirsh 1986; Waterston 1988; Podbielničcz and White 1994); and (4) hypodermal intermediate filaments in regions where muscle apposes hypodermis in mutant embryos, recognized by mAb MH4 (Figure 6D). All of these markers that are characteristic of hypodermal specification and differentiation were observed in mup-4 mutants. Furthermore, the cellular localization of each of these markers in mutants is similar to WT.

**Hypodermal integrity and morphology in mup-4 mutants:** To assess hypodermal cell shapes and positions at different developmental stages in mup-4 mutants, we examined mutant embryonic populations for the pattern of staining with the adherens junction protein antibody. Similar to the three morphological classes of mutants exhibiting muscle defects (Figure 5), we observed three morphological classes of mup-4 mutants having hypodermal defects (Figure 8). The first class is morphologically bean stage mutants in which the hypodermis does not enclose the embryo: the cells appear in some regions as a row of two cells and in other regions as a clump of cells (instead of a sheet of six epithelial cells; Figure 8B). The second class is morphologically twofold mutants, which have abnormally shaped cells and hypodermal lesions from which cellular material extrudes in the dorsal neck region (Figure 8D). The third class is morphologically threefold mutants, which are fully enclosed by the hypodermal cells, but the cells are abnormally shaped (Figure 8F). The bean and twofold arrest class mutants were seen rarely, whereas the majority of mutants observed were of the threefold Mup phenotypic class. Because of the severity of the hypodermal defects (observed in synchronized as well as mixed populations), we conclude that the bean class and twofold class are terminal arrest phenotypes due to enclosure and hypodermal integrity defects and correspond to the early embryonic arrest class observed from our genetic studies (Tables 4 and 5) and in our studies of the muscle phenotype (Figure 5).

**The hypodermis of threefold mutant embryos appear normal until the midthreefold stage:** Mutants of the threefold class must proceed through the bean and twofold stages prior to threefold arrest. We were interested in whether mutants of the threefold arrest class show hypodermal defects prior to the threefold that ultimately result in the threefold phenotype. Excepting the rare bean or twofold arrest class mutants, when we examined mixed-staged, nonarrested populations the remaining bean and twofold embryos appeared WT. We saw no other hypodermal abnormalities in these embryonic populations between the twofold and threefold stages that would represent intermediate stages in the development of the threefold hypodermal abnormalities. Furthermore, staining with the paramyosin and the adherens junction antibodies revealed that there is a tight correlation of muscle position defects and abnormal hypodermal cell shapes in threefold embryos (data not shown). We conclude that the hypodermis in the threefold Mups appears WT until the time of muscle displacement and that the hypodermal defects in the threefold Mups are contemporaneous with muscle displacement.
WT

mup-4(ar60)

FIGURE 8.—Hypodermal cell patterns in WT and mup-4(ar60) mutants. Fluorescence micrographs of embryos that were labeled with monoclonal antibody MH27, which recognizes an antigen to the adherens junctions between hypodermal cells and pharyngeal cells. WT embryos and mup-4(ar60) mutant embryos have been placed into developmental stages based on gross morphology. Anterior is left. Dorsal is up for lateral views. (A) Dorsal view of a WT bean stage embryo (~290 min). At this stage, hypodermal cells are arranged into six cellular rows that enclose the embryo in one sheet. (B) Morphologically bean-stage mutant embryo. Mutant embryos display clumps (arrows) of hypodermal cells. (C) Lateral view of WT embryo. (D) Ventral view of twofold arrest class mutant. The arrow points to extruded dorsal hypodermis. The pharynx (demarcated by arrowheads) and intestine appear to be a length more characteristic of the threefold stage (compare arrowheads in D and E). (E) Lateral view of a WT threefold embryo. (F) Lateral view of threefold Mup mutant. The mutant exhibits abnormal hypodermal cellular shapes. Bar, 20 μm.
**TABLE 5**

Summary of *mup-4*(*art60*) mutant arrest classes

<table>
<thead>
<tr>
<th>Morphological stage of arrest</th>
<th>Muscle defects</th>
<th>Hypodermal defects</th>
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<tr>
<td>Bean</td>
<td>Muscle disorganized</td>
<td>Embryo not enclosed; patches of hypodermal cells</td>
</tr>
<tr>
<td>Twofold</td>
<td>Muscle in quadrants but retracted from nose and tail</td>
<td>Cellular material extruded dorsally</td>
</tr>
<tr>
<td>Threefold Mup</td>
<td>Dorsal body wall muscle detached to the ventral side; ventral muscle retracted from nose and tail</td>
<td>Cells abnormally shaped</td>
</tr>
</tbody>
</table>

Three arrest classes of *mup-4* mutants are depicted relative to the morphological arrest stages. Ten percent of all mutants arrest as the morphologically bean and twofold, and 90% arrest as threefold Mups (from Table 3). The mutant defects at arrest are summarized (from Figures 5 and 8).

**DISCUSSION**

*mup-4* is a new muscle-position-effect locus in *C. elegans*. At the time of embryonic arrest, most mutants have reached the threefold stage. During the threefold stage, the Mup phenotype is manifested as catastrophic displacement of dorsal muscles ventrally, and displacement of dorsal and ventral body wall muscles from the nose and the tail. Some of these mutants hatch and appear kinked in a croissant shape due to the muscle position defects. The threefold/L1 arrest of these mutants is likely a direct result of the catastrophic muscle defects, which are incompatible with feeding or digestion. In addition to the threefold/L1 arrest class, a small proportion of *mup-4* mutants arrest without successful elongation as morphologically bean or twofold (summarized in Table 5). All observed mutant classes display defects in hypodermal integrity and/or morphology and these defects support that *mup-4* could have functions in cellular events such as in hypodermal cell-cell interactions, cell-matrix interactions, and/or hypodermal cell cytoarchitecture. This is consistent with mosaic analysis, which shows that expression of the *mup-4* gene is essential within the AB blastomere that generates most of the hypodermis. Together, our analyses support that *mup-4* is important for hypodermal integrity which, when disrupted, leads to morphogenesis defects and, either directly or indirectly, to disruption of body wall muscle position.

The bean arrest class suggests that *mup-4* is essential for successful embryo enclosure by the hypodermis: Immunofluorescence studies demonstrate that morphologically bean stage mutants are not enclosed normally by the hypodermis and that elongation is abnormal. Hypodermal cells undergo dramatic changes that ultimately drive the process of enclosure and elongation. Studies have shown that successful embryo elongation relies on enclosure of the embryo by the hypodermis (Priess and Hirsh 1986; Williams-Masson et al. 1997; E. Williams-Masson, A. N. Malik, and J. Hardin, personal communication); maintenance of hypodermal integrity; functions of the embryonic sheath, the hypodermal cytoskeleton, and the cuticle (Priess and Hirsh 1986); and body wall muscle function (Waterston 1989; Williams and Waterston 1994). At the bean stage, six rows of hypodermal cells should surround the embryo. Enclosure is normally dependent on ventral hypodermal cells migrating to the ventral side of the embryo, led by two pairs of ventral cells, and subsequent stable adhesion of hypodermal cells at the ventral midline. This has been demonstrated in that ablation of the four leading cells inhibits enclosure (E. Williams-Masson, A. N. Malik and J. Hardin, personal communication). Despite the failure of these embryos to enclose, the cytoskeletal contractions that drive elongation proceed and cause hypodermal cells to contract into a ball of cells on the dorsal side (E. Williams-Masson, A. N. Malik and J. Hardin, personal communication; E. Williams-Masson et al., 1997). The *mup-4* bean arrest phenotype is reminiscent of these ablations (J. Hardin, personal communication): the hypodermal cells do not surround the embryo and cells appear in some regions as perhaps only two hypodermal cells across and in other regions as clumps of cells (Figure 8B). These defects could represent failure in either hypodermal migration or stable ventral hypodermal cell adhesion. In this regard, it is notable that some of the *mup-4* bean mutants assayed by MH27 displayed regions only two cells across (as opposed to a cohesive clump of cells or rows of six cells across). This is consistent with defects in adhesion of all hypodermal cells to one another that may be revealed after onset of cytoskeletal contractions. In any case, our observation of morphologically bean stage mutants suggests that *mup-4* is essential for successful enclosure or maintenance of enclosure by the hypodermis and that the enclosure defect likely causes elongation defects. Finally, the morphologically bean arrest class suggests that the *mup-4* gene must be expressed early during, and/or prior to, the phases of enclosure and elongation.

The twofold arrest class suggests that *mup-4* is essential for hypodermal integrity and embryo elongation: Immunofluorescence studies demonstrate that
morphologically twofold stage mutants also are not enclosed normally by the hypodermis and that elongation is abnormal. Immunofluorescence studies show that the twofold arrest mutants exhibit a dorsal “extrusion” (Figure 8D). Relevant to this, we have observed mutants in time-lapse video tape analyses that elongate to twofold, develop a lesion, and then arrest at twofold (B. K. Gatewood and E. A. Bucher, unpublished data). This dorsal “extrusion” (Figure 8D) and elongation phenotype is reminiscent of hypodermal cell ablation experiments of twofold embryos (Priess and Hirsh 1986). Thus, one possible explanation is that lesions arise between hypodermal cells in *mup-4* mutants similar to the insult caused by ablation. In addition, we also have observed mutants in time-lapse video tape analyses that elongate to threefold, develop a lesion, and then retract and arrest with a twofold morphology (B. K. Gatewood and E. A. Bucher, unpublished data). Indeed, the twofold class representatives we have observed by immunofluorescence (MH27 staining) may correspond most closely to these embryos that elongated and retracted: although the overall morphology of the twofold class of *mup-4* mutant embryos is twofold, the pharynx and intestine appear threefold (Figure 8D). For either of the two phenotypes observed (retraction of a fully elongated embryo or failure to elongate beyond twofold), a role of the embryonic sheath and/or cuticle, which are synthesized by the hypodermis, may be relevant. The embryonic sheath has been proposed to serve a role of the embryonic sheath and/or cuticle, which share a cellular environment with other nuclei within the AB lineage for organismal viability. Based upon this genetic requirement in AB, the hypodermal defects observed in *mup-4* mutants show hypodermal defects at the time of muscle detachment (Goh and Bogart 1991; Myers et al. 1996); therefore, muscle detachment per se does not cause defects in hypodermal cell shapes. Thus, the fact that *mup-4* mutants develop hypodermal defects coincident with muscle detachment strongly supports that *mup-4* mutants are defective for a function required for hypodermal morphology and integrity. This function is also required for maintenance of muscle cell position at threefold.

The essential requirement of *mup-4* gene function within the AB lineage: Genetic mosaic analysis demonstrates that the *mup-4* gene must be expressed in the AB lineage for organismal viability. Based upon this genetic requirement in AB, the hypodermal defects observed in *mup-4* mutants, and the fact that muscle cells interact with the hypodermis, we propose that this requirement in the AB lineage reflects a requirement for the expression of the *mup-4* gene in hypodermal cells. At the time of hatching, a total of 85 nuclei contribute to the hypodermis (excluding the rectum), most of which share a cellular environment with other nuclei since most of the hypodermal cells are multinucleate and generated by cell-to-cell fusions (Sulston et al. 1983; Podbielniicz and White 1994). In this regard, 72/85 hypodermal cell nuclei at hatching are descendants of the AB blastomere (Sulston et al. 1983) and the remaining 13 are from the C blastomere. All but one of the C-derived hypodermal nuclei contribute to the hyp7 syncytial hypodermal cell, which is comprised of 23 nuclei. Thus, if *mup-4* is expressed in all hypodermal cells, it is possible that it is also expressed in the C-derived hypodermal cells. However, due to the syncytial nature of hyp7, *mup-4* expression in C may not be observed as an essential requirement by genetic mosaic analysis. Furthermore, the syncytial nature of the hypodermis also may explain the ability to recover viable mosaics that have lost *mup-4* gene activity in a small portion of the AB lineage (e.g., ABpl or ABpr; also see: Bucher and Greenwald 1991). In contrast, nuclei de-
derived from the AB lineage contribute to all of the hypodermal cells and AB descendants are the sole contributors to most hypodermal cells. Thus, the essential requirement for mup-4 in AB and ABp, as well as the fact that the positions of muscle cells are affected in all regions of mup-4 mutants (Figures 3 and 5), are consistent with the hypothesis that most, or all, hypodermal cells require mup-4 gene function.

The cellular basis of muscle position defects and mup-4 gene function: In addition to having a function essential for hypodermal integrity, mup-4 also is essential for maintenance of muscle position at threefold. Major cellular changes occur at midthreefold that could be related to this timing of muscle displacement at midthreefold. (1) Body wall muscle is innervated during threefold, which may influence the force generated by muscle (Durbin 1987). (2) The cuticle is synthesized and attachments are established to the cuticle exoskeleton to transmit the force of body wall muscle contraction (Singh and Sulston 1978; Francis and Waterston 1991). (3) The hypodermis undergoes a dramatic rearrangement from the circumferentially organized actin filaments to a more disorganized arrangement (Pries and Hirsh 1986). With respect to these transitions, it is notable that time-lapse video microscopy of mup-4 and mup-2 (Myers et al., 1996) mutants demonstrates that the time of muscle displacement during threefold is remarkably similar, although the mutations affect different cellular events (see Introduction) and this similar timing may simply reflect the inability to sustain muscle positions and/or attachments in mutants for different genes when these numerous cellular changes occur.

Several muscle position-effect genes, including mup-1, mup-4, and mua-3, have been proposed to function in the hypodermis (J. Plenefisch and E. Hedgecock, personal communication; Goh and Bogaert 1991); however, specific aspects of their mutant phenotypes are different. For example, immunofluorescence analyses demonstrate that mup-1 and mup-4 mutants have an identical threefold Mup phenotype (B. K. Gatewood and E. A. Bucher, unpublished results). Despite this similarity at threefold, Goh and Bogaert (1991) observed abnormal muscle attachment processes during twofold and proposed that mup-1 is required to position muscle attachments. In contrast, we have not observed abnormal attachment processes in mup-4 twofold embryos. Furthermore, the mup-4 elongation and hypodermal defects have not been observed in mup-1 mutants. In another example, the mua-3 gene, like mup-4, has been demonstrated by mosaic analysis to function in the AB lineage (Bucher and Greenwald 1991). Despite this similarity in cellular focus, mua-3 mutants display muscle position defects that occur during larval stages and these most likely represent later requirements for the maintenance and elaboration of attachments during larval molting and sarcomere addition (Bucher and Greenwald 1991; E. A. Bucher, unpublished results; J. Plenefisch and E. Hedgecock, personal communication). Interestingly, viable mup-4 mosaics that have lost mup-4 function in a subset of AB cells have a phenotype similar to mua-3 (larval worms with localized muscle position defects; B. K. Gatewood and E. A. Bucher, unpublished results), although the muscle position defects in mup-4 mosaics may arise during embryogenesis rather than during larval molts.

Taken together, our data demonstrate that mup-4 is unique among those genes required for development or maintenance of embryonic muscle position. First, mup-4 mutants show defects in hypodermal integrity and organismal morphogenesis that are not observed in mutants for other mup genes. Second, we have established by our genetic mosaic analysis that the essential requirement for mup-4 gene expression is in nonmuscle cells, which has not been established for any other genes of the mup class. Finally, although the interdependence of muscle, nerve, and hypodermis is well established, our studies illustrate the importance of nonautonomous gene functions, in particular of the hypodermis, to the maintenance of muscle cell position. The mup-4 muscle position phenotype could be caused by a specific breakdown in muscle-hypodermal attachment structures, as has proposed for the mua-3 mutants, or could be an indirect consequence of the loss of hypodermal cell integrity. It is notable that the hypodermal intermediate filament protein that we assayed co-localizes to the mispositioned body wall muscle (Figure 6). This co-localization could reflect de novo expression of attachment structures in the hypodermis overlying the mispositioned muscle. Alternatively, this co-localization could reflect the displacement of the hypodermal cell membrane to the ventral regions, along with the muscle cell, due to a lesion occurring within the hypodermal cell itself (rather than a break between the muscle and hypodermal cells).

Ultrastructural analysis of mup-4 mutants and molecular cloning of the mup-4 gene will further elucidate the specific cellular defects and will examine the specific functions of mup-4 in hypodermal function and whether mup-4 mediates direct hypodermal-muscle cell interactions during development. In an effort to clone mup-4, we have mapped mup-4 to a small interval on LG III, which has been resistant to cosmid and YAC-end cloning efforts (S. Chissoe and R. Wilson, personal communication). At the boundary of this region lies the 5' end of a predicted transmembrane protein having extracellular epidermal growth factor-like and low-density lipoprotein-like motifs that appears to be a homologue of mua-3 (called MRP-1; J. Plenefisch, personal communication). We are currently testing the possibility that MRP-1 is mup-4. If mup-4 is indeed mup-1, this would most support a role of this novel protein in cell-
ECM interactions which could directly influence muscle-hypodermal cell-cell interactions.

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LITERATURE CITED


The mup-4 Locus in C. elegans


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