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Fragile skeletal muscle attachments in dystrophic mutants of *Caenorhabditis elegans*: isolation and characterization of the *mua* genes

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SUMMARY

Over 30 *Caenorhabditis elegans* mutants were identified with normal muscle differentiation and initial locomotion followed by catastrophic detachment of skeletal muscles from the body wall. Reducing the strength of muscle contraction in these mutants with a myosin gene mutation suppresses muscle detachment. These dystrophic mutants identify a novel class of genes required for growth and maintenance of functional muscle attachments, not exceptional alleles of genes required for muscle differentiation and contractility. Nine new genes, named *mua*, and two previously published loci, *unc-23* and *vab-10*, cause fragile *mu*scle *a*ttachments. The primary sites of muscle detachment, including the plane of tissue separation, are characteristic for each gene. We suggest these genes identify feedback mechanisms whereby local strain regulates the extent of myofibril contraction and the placement of new muscle attachments in functioning muscles. Finally, we draw some comparisons to vertebrate skin fragility diseases and muscular dystrophies.

Key words: Mechanobullous skin disease, Skin, Muscular dystrophy, Nematode, Skeletal muscle, *Caenorhabditis elegans*

INTRODUCTION

Hereditary and acquired diseases that disrupt cell-cell and cell-matrix attachments, or associated cytoskeletal and extracellular matrix molecules, allow catastrophic separation of the tissue during normal use. Mechanobullous and related tissue fragility disorders of the skin identify structural elements in the mechanical paths from epidermis to dermis (see Fuchs and Cleveland, 1998). The primary sites of tissue separation in these diseases generally reflect the normal location of the affected molecule. Muscular dystrophy and similar myopathies with late onset and insidious progression have also been described as tissue fragility diseases (Law and Tidball, 1992; Brown and Lucy, 1993; Petrof et al., 1993). Whereas mechanobullous diseases identify major structural elements in the skin, the known muscular dystrophy gene products are apparently minor structural elements or regulatory proteins. Unlike cadherin- and integrin-based paths coupling the contractile sarcomeric cytoskeleton to neighboring muscle fibers or tendons, the dystrophin complex coupling the subsarcolemmal cytoskeleton to the basal lamina is not essential for myofibril formation or contraction (Berthier and Blaineau, 1997). In nematodes, mutations in dystrophin have no effect on muscle differentiation, but cause hypercontractility, i.e., rapid, exaggerated movement and hypersensitivity to acetylcholinesterase inhibitors (Bessou et al., 1998). Similarly, mutations in dystrophin and associated proteins have little effect on muscle differentiation and initial function in vertebrates (Campbell, 1995). However, the mutant fibers are mechanically compromised and damage easily with growth and normal use, culminating in muscular dystrophy.

Using *Caenorhabditis elegans*, we have developed a nematode model for hereditary tissue fragility with similarities to vertebrate muscular dystrophy and mechanobullous skin disease. Here we describe mutations in 11 genes, designated *mua* loci, causing progressive locomotor paralysis in juvenile animals. In these mutants, apparently healthy skeletal muscles spontaneously separate from the hypodermis and cuticle. The time of onset varies by gene and allele, and often by individual, but muscle detachment progresses irreversibly once begun. For each locus, detachment initiates at characteristic locations along the muscle. A closer examination reveals that tissue separation occurs at specific subcellular sites along the mechanical path from muscle to cuticle. Several *mua* loci are pleiotropic, affecting additional muscle types or non-muscle tissues.

By their dystrophic character, Mua mutants are phenotypically distinct from other *C. elegans* mutants affecting muscle development and function (Waterston et al., 1980; Williams and Waterston, 1994). By genetic recombination and complementation tests, the *mua* loci described here are distinct from the known genes affecting muscle differentiation and contractility. Moreover, for several loci, genetic tests suggest that the dystrophic mutants are amorphs, not exceptional

alleles. Based upon our studies, we propose that the *mua* loci represent a novel class of genes that are required for the mechanical integrity of skeletal muscle in active, growing animals.

MATERIALS AND METHODS

Strains and culture

Methods for routine culture and genetics of *C. elegans* are described in Epstein and Shakes (1995). Strains were grown at 20°C unless otherwise noted. Genetic nomenclature is described in Horvitz et al. (1979). Mutations used in this study, described in Riddle (1997), are listed below:

Chr. I: *ced-1(n1735), dpy-5(e61), lin-11(n382), unc-13(e1091), unc-54(e190)*

Chr. II: *dpy-10(e128), lin-4(e914), lin-23(e1883), let-22(m22), let-264(mn227), unc-4(e120), unc–52(e444), maDf4, mnC1, mnDf30, mnDf31, mnDf39, mnDf52, mnDf96*

Chr. III: *ced-7(n1892), dpy-1(e1) dpy-17(e164), dpy-18(e364, e499), emb-9(g70), nob-1(ct223, ct351) unc-25(e156), unc-32(e189), unc-36(e251), unc-45(s268), unc-69(e587), unc-93(e1500), ctDf2, ctDf3, eDf2, nDf40, nDf16, nDf40, qC1, sDp3, tDf7, tDf8, yDf10*

Chr. IV: *bli-6 (sc16), dpy-13(e184), dpy-20(e1282), unc-5(e53), unc-31(e928), mDf7, nDf19, sDf60*

Chr. V: *dpy-11(e224), him-5(e1490)*

Chr. X: *daf-12(rh84), dpy-6(e14), him-4(e1266), let-2(b246, mn153), lin-15(n765ts), lon-2(e678), mec-2(e1084), sdc-1(y66, y67), sup-10(n953), unc-3(e151), mnDf1, mnDf4, mnDf8, mnDf41, mnDp1*

Determination of sites and timing of muscle detachment

Five adult hermaphrodites were allowed to deposit eggs on freshly seeded plates for 3 hours and then removed. Alternatively, eggs were released from gravid adults into a drop of M9 buffer by incision and transferred to a seeded plate. At regular intervals, larvae that hatched from these eggs were examined by polarizing light microscopy to determine the extent of muscle detachment and by DIC microscopy to determine their developmental stage.

Immunofluorescence microscopy

Antibody staining protocols were as described in Francis and Waterston (1985). Mixed stage animals were harvested and resuspended in 50 μl of M9 buffer. 5 μl aliquots were spotted onto polylysine-coated slides and squashed beneath a coverslip. These sandwiches were immediately frozen on dry ice, after 10 minutes the coverslips were pried off, and the worms fixed in ice-cold methanol (4 minutes) followed by ice-cold acetone (2 minutes), and allowed to air dry. Primary antibody was directly spotted on the fixed specimens for 2 hours, washed in PBS, incubated with rabbit anti-mouse antibodies linked to FITC for 2 hours, washed in PBS and observed by epifluorescence on a Zeiss Axioskop.

Construction of an integrin β^{PAT-3}::GFP reporter

To study the effects of *mua* mutations on the localization of dense-body complexes, we examined the localization of integrin β^{PAT-3} as visualized with a GFP protein fusion. In this construct, the *A. victoria* GFP was fused in frame with β^{PAT-3} immediately upstream of the normal *pat-3* terminator codon. A 11 kb genomic fragment containing the entire *pat-3*-coding sequence plus about 5 kb 5′ UTR and about 1 kb 3′ UTR sequence excised from cosmid ZC504 with *Ava*I and cloned into the *Ava*I site in pNEB193 (New England Biolab). A *Not*I site was introduced immediately before the stop codon by PCR manipulation. A 1.3 kb terminal fragment containing the stop codon and a unique *Bal*I site 142 bp upstream of the stop codon was PCR amplified as two fragments. The 1st fragment terminates immediately before the stop codon and the 2nd fragment starts immediately before the stop codon. A *Not*I site was incorporated at the end of the 3′ and 5′ primers of the 1st and 2nd set primers, respectively. The two fragments were ligated at the *Not*I site. This fragment contains a *Not*I site immediately before the stop codon and was used to replace the original 1.3 kb terminal fragment of the *pat-3* genomic clone. GFP sequence containing the signal-enhancing S65C mutation was fused in frame to the end of the HA tag using overlapping PCR extension. A *Not*I site was introduced at both ends of this fusion gene by incorporating *Not*I sites into the primers. The HA::GFP fusion was inserted into the *pat-3 Not*I site, and the complete construct was reintroduced into *C. elegans* by germline transformation and shown to functionally rescue *pat-3(rh54)* mutants. When this construct was injected into *pat-3* null mutants, the animals were fully rescued by a heritable extrachromosomal array. Rescued animals had bright green fluorescence in specific patterns including all sites previously detected by immunofluorescence with MH25 anti-PAT-3 integrin antibodies. In particular, β^{PAT-3}::GFP is expressed continuously in muscle cells in larvae and adults. In body wall muscles, it is localized at Z-disc (dense body), M-line and dense plaque attachments. Vulval, uterine, anal depressor and sphincter muscles, but not pharynx, also express strongly. Muscle arms are also seen projecting to the nerve cords and ring. Hence, the fusion protein β^{PAT-3}::GFP is functional and behaves much like the wild-type protein.

RESULTS

Isolation and mapping of *mua* mutations

We screened for mutations causing progressive locomotor paralysis resulting from detachment of the skeletal muscles from the hypodermis. These mutants were named *mua*, mnemonic for fragile *mu*scle *a*ttachments (Table 1). From approximately 5000 F_1 colonies, 22 independent *mua* mutations were isolated following mutagenesis with EMS (ethylmethanesulfonate). Homozygous inviable mutants were recovered from heterozygous siblings. An additional 7 EMS alleles were generously provided by Dr Andrew Chisholm (University of California, Santa Cruz). Finally, *mua-3* alleles *rh222* and *rh236* were isolated following gamma-irradiation or arose spontaneously in the N2 Bristol / BO Bergerac hybrid NJ82, respectively. Each *mua* mutation proved recessive when outcrossed with wild-type males. Mutations on chromosome X were recognized by sex-linked transmission. Other mutations were genetically linked to specific autosomes and all were grouped by complementation.

Our mutant collection comprises alleles of nine new complementation groups, designated *mua-1* to *mua-10*, plus a previously described locus, *unc-23* (Waterston et al., 1980). In the course of this study, we also characterized *vab-10*, a locus whose Mua phenotype has not been previously described (Hodgkin, 1983). For convenience, we refer to all 11 genes collectively as *mua* loci. These genes map to all six chromosomes. Using reference alleles from each complementation group, these loci were mapped by a combination of 3-factor crosses and complementation tests with known chromosomal deficiencies or duplications (Fig. 1). Our mapping data, deposited with the Caenorhabditis Genetics Center, is available on-line through ACeDB (http://wormsrv1.sanger.ac.uk).

Several *mua* loci map near known genes affecting muscle differentiation and contractility. In these cases, complementation tests were used to establish that the mutations define distinct genes. In particular, *mua-3* ≠ *emb-9*, *mua-4* ≠ *let-*

Table 1. Muscle attachment (*mua*) genes

Gene	Chr.	Alleles	Viable	Primary Site
mua-1	II	*rh160*	yes	muscle-muscle
mua-2	III	*rh119, rh174*	yes	muscle-hypodermis
mua-3	III	class 1: *ar62, n2538, n2544, rh171, rh222, rh236, rh268*	no	muscle-hypodermis; intrahypodermal
		class 2: *rh169*	no	
		class 3: *rh195*	yes	
mua-4	III	*rh177, rh214*	no	muscle-hypodermis
mua-5	IV	*rh179, rh180, rh213, rh216, rh262*	no	muscle-hypodermis
mua-6	X	class 1: *rh225*		
		class 2: *rh85*	no	muscle-muscle
			yes	
mua-7	III	*n2542, n2543, rh217*	no	muscle-hypodermis
mua-9	II	*rh197*	no	intrahypodermal
mua-10	X	*rh267*	yes	muscle-hypodermis
unc-23	V	*e25, rh9, rh192, rh259*	yes	muscle-hypodermis
vab-10	I	*e678*	yes	muscle-hypodermis

805, *mua-5 ≠ deb-1* or *pat-8*, *mua-6 ≠ let-2* and *vab-10 ≠ mec-8*. Because *let-2* alleles exhibit a complex pattern of intragenic complementation, we tested the presumptive null allele *let-2(mn153)* against both alleles of *mua-6* (Meneely and Herman, 1981). Finally, the larval lethal mutation *let (ar62)* described in Bucher and Greenwald (1991) is an allele of *mua-3*.

Progressive locomotor paralysis in *mua* mutants

In general, skeletal muscle differentiation and contractility appear normal in *mua* embryos. Embryo elongation, from the early synchronous contractions of skeletal muscles through the final shape of newly hatched larva, appear normal (Hall and Hedgecock, 1991; Williams and Waterston 1994). The elongation and final shape of the pharyngeal myoepithelium also appear normal. In particular, pharyngeal muscle contractions leading to eggshell softening, are normal.

The posture, locomotion and feeding of newly hatched larvae are normal. During larval growth, many individuals develop impaired movement and abnormal body posture. Affected animals acquire permanent bends along portions of the body wall (Fig. 2). In severe cases, the animals become flaccid and paralyzed, and death may ensue.

Skeletal muscles detach from the hypodermis in *mua* mutants

We examined myofibril organization and hypodermal attachment of skeletal muscles in *mua* larvae by DIC and polarized light microscopy (Fig. 2). The musculature of newly hatched larvae are indistinguishable from wild type, consistent with their unimpaired movement. During subsequent growth, however, skeletal muscles

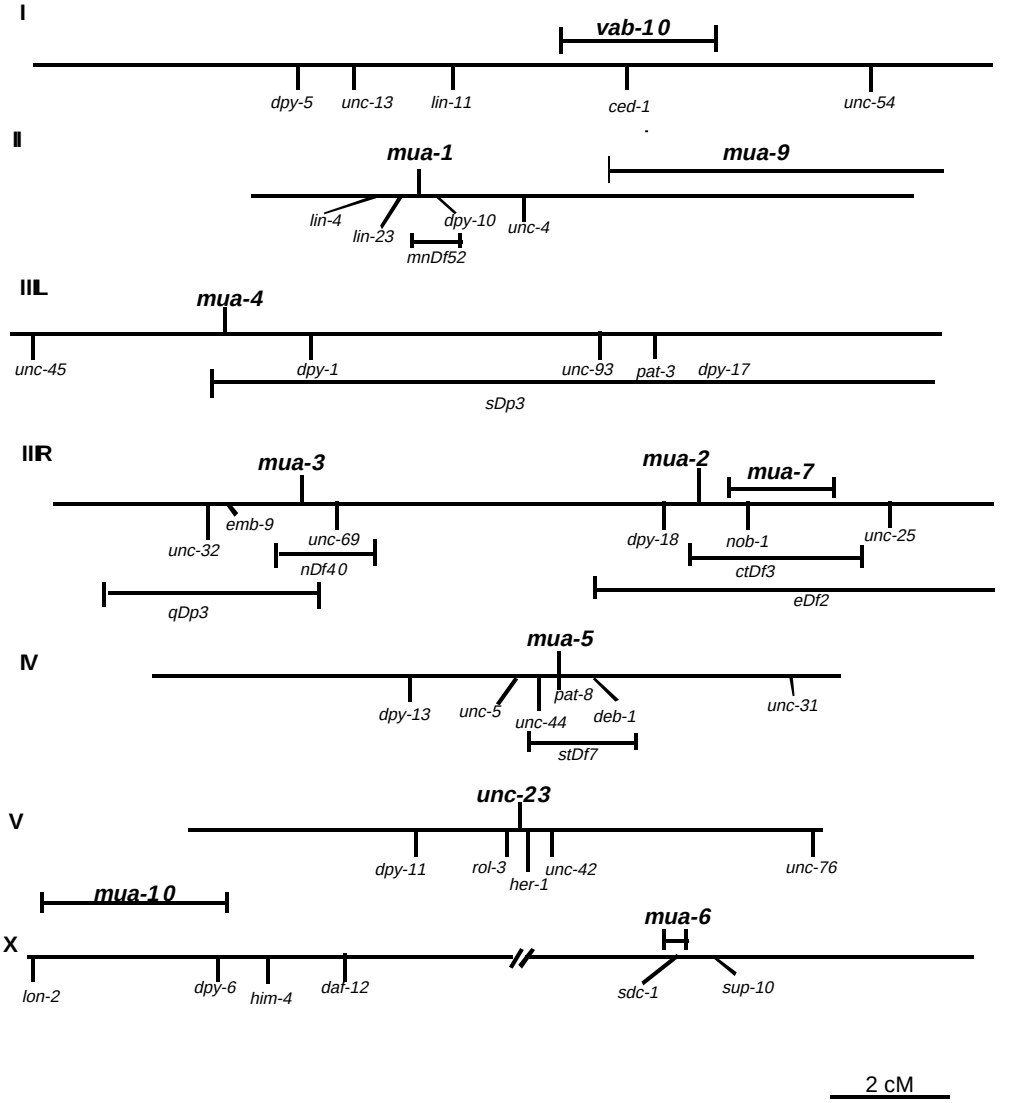

Fig. 1. Genetic map showing locations of *mua* loci.

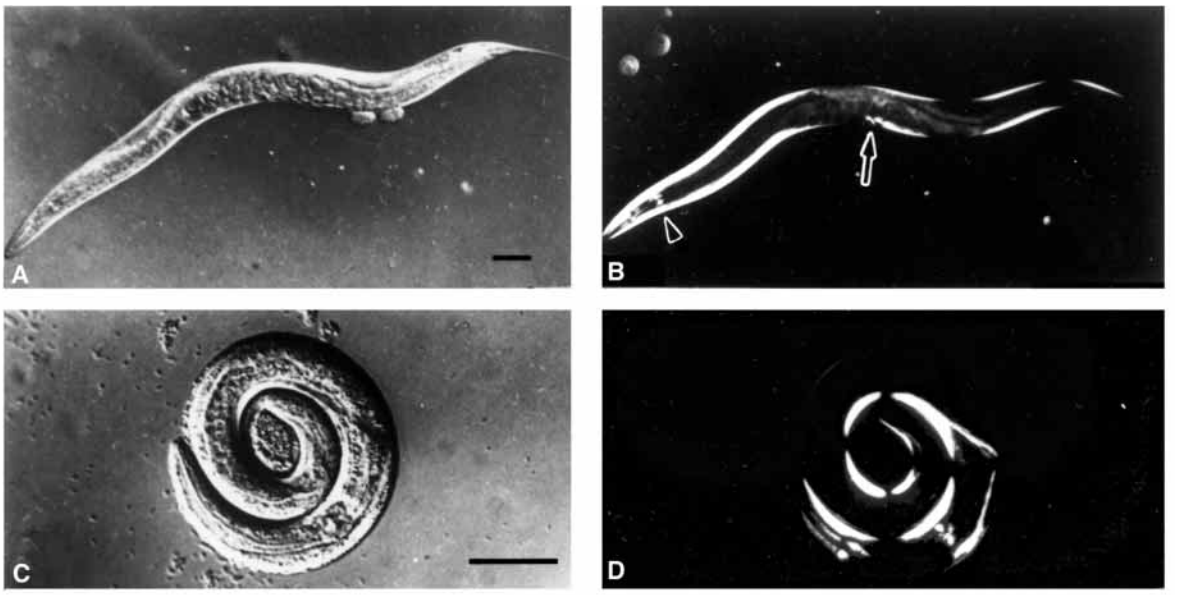

Fig. 2. Skeletal muscle detachment. (A,B) DIC and polarized light micrographs of wild-type adult hermaphrodite (N2) showing normal body posture and skeletal muscles. The four skeletal muscles appear as continuous birefringent bands arranged longitudinally along the dorsal and ventral hypodermis. The pharyngeal muscles are visible in head (arrowhead) and the vulval muscles visible on the ventral hypodermis in midbody (arrow). (C,D) *mua-5 (rh179)* L3 stage larva showing permanent coiled posture resulting from extensive detachment of the dorsal muscles from the hypodermis. Scale bars are 100 μm.

progressively detach from the hypodermis. The initial defects often appear in muscle regions that bear greater stress, i.e., the anteriormost muscle attachments in the head and the dorsal muscle attachments flanking the anal depressor. In some mutants, eventually the entire skeletal muscles separate from the hypodermis; such animals become completely paralyzed and generally die. The penetrance, onset and severity of muscle detachment are gene and allele specific (Table 1). The time course and severity of cellular defects parallel the posture and movement changes (Fig. 4).

Tissue separation occurs at characteristic subcellular sites

The primary site of attachment failure was determined by DIC and FITC-phalloidin florescence microscopy. These sites appear characteristic for each gene (Table 1). Mutations in *mua-*

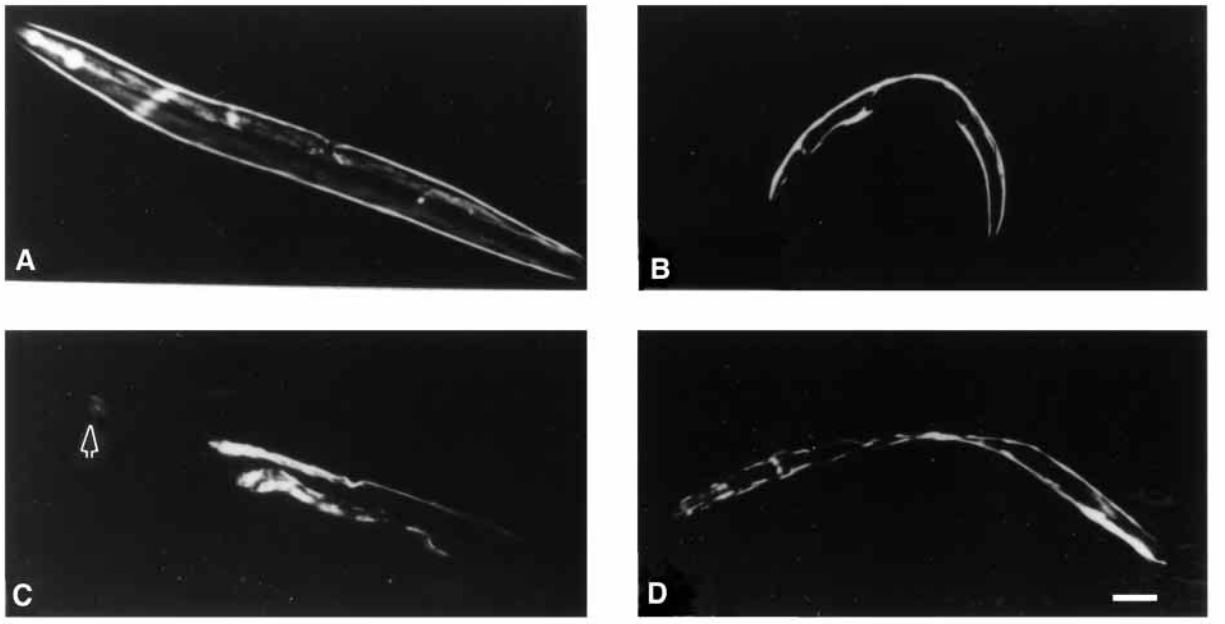

Fig. 3. Patterns of muscle detachment. (A) Adult wild-type. (B) *mua-1 (rh160)* L4 larva. Primary breaks occur between adjacent muscle cells. (C) *mua-2(rh119)* L4 larva. Primary breaks occur between muscle and hypodermis in the head. Muscle contraction has drawn muscles into the midbody. Location of pharynx, faintly visible in this micrograph, is indicated by arrowhead. (D) *mua-3(rh169)* L4 larva. Primary breaks occur within the hypodermis while muscles remain intact. Scale bar is 100 μm.

1 and *mua-6* cause separation both between adjacent muscle cells as well as between the muscle and hypodermis (Fig. 3B). Mutations in *mua-2*, *mua-5*, *mua-10*, *unc-23* and *vab-10* cause separation only between muscle and hypodermis (Fig. 3C). *mua-3* causes separation between muscle and hypodermis as well as rupture within the hypodermis itself, while *mua-9* appears to affect only intrahypodermal sites (Fig. 3D).

mua loci affect specific muscle types

We examined alleles of each *mua* locus for possible defects in other tissues, especially muscles. In general, pharyngeal muscles and feeding are normal. Moreover, hypodermal attachments of ALM/PLM mechanosensory neurons and uterus are normal. For six loci, *mua-1*, *mua-5*, *mua-7*, *mua-10*, *unc-23* and *vab-10*, we observed no primary defects in tissues other than the skeletal muscles. In *mua-1*, hermaphrodites often have vulval and uterine defects, resulting in egg retention and premature death due to uterine eversion. In rare non-Mua animals, however, these phenotypes were not observed, suggesting that any sex muscle defects are an indirect consequence of skeletal muscle detachment. No sex muscle defects were observed in other mutants; however, *mua-5* and *mua-7* arrest before maturation of the reproductive system. In *mua-10*, *unc-23* and *vab-10*, muscle detachment is restricted to the head. In *mua-2*, *mua-3* and *mua-6*, the anal depressor muscle frequently detaches from the dorsal hypodermis. In *mua-3* and *mua-9*, the hypodermis is especially fragile and ruptures concomitant with skeletal muscle detachment. In *mua-3*, there is no obvious hypodermal defect observed prior to the apparently simultaneous detachment of muscle and rupture within the hypodermis, whereas, in *mua-9*, the hypodermis appears to accumulate necrotic vesicles prior to rupture, suggesting that muscle detachment is a secondary consequence of hypodermal failure. In *mua-4*, cytokinesis is defective in both hypodermis and germline resulting in mutinucleate cells (Fig. 5).

Embryonically required structural proteins are normally present and arranged

The cellular architecture and arrangement of macromolecular structures (e.g. dense bodies, M lines) of muscles in all the *mua* mutants prior to detachment was normal as observed by DIC and polarized light microscopy. We examined the β^{PAT-3} integrin subunit in the adult musculature of all viable *mua* strains. (The β^{PAT-3} integrin is required for normal embryonic muscle assembly and is sensitive to perturbations of other proteins involved in this process, Hresko et al., 1994.) In all cases, no deviations from the wild type were observed (Fig. 6). We also examined the distribution of MH3, MH4, MH5, MH15 and MH44, antigens specific to muscle cells or the hypodermal attachment zone (Francis and Waterston, 1991) in *mua-1* and *mua-6* and F-actin by FITC-phalloidin staining in *mua-1*, *mua-2*, *mua-3* and *mua-6*. In all cases, no differences with wild type were seen. Finally, the ultrastructure of *mua-1(rh160)*, *mua-2(rh174)* and *mua-6(rh85)* L4 animals observed by transmission EM failed to show any obvious defects in the undetached muscles, surrounding matrix or hypodermal tissues.

unc-54 myosin mutation suppresses muscle detachment

To learn whether skeletal muscle contractions occurring during normal locomotion contribute to the failure of muscle attachments in *mua* mutants, we introduced a myosin heavy chain mutation, *unc-54(e190)*, which causes flaccid posture and

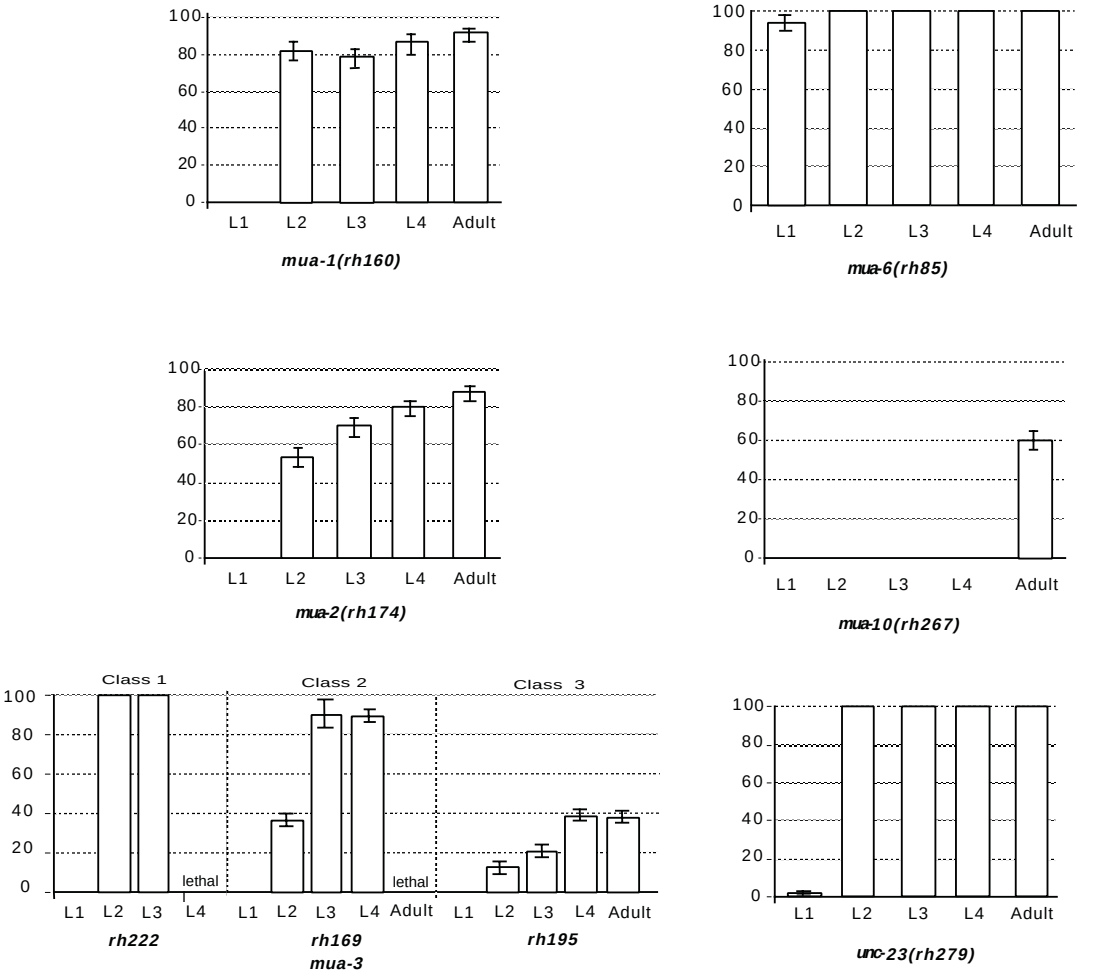

Fig. 4. Onset and penetrance of muscle detachment. The percentage of animals showing skeletal muscle detachment at various stages (see Methods). Approximately 100 animals were observed for each stage; error bars show the standard deviation calculated for sampling a binomial distribution.

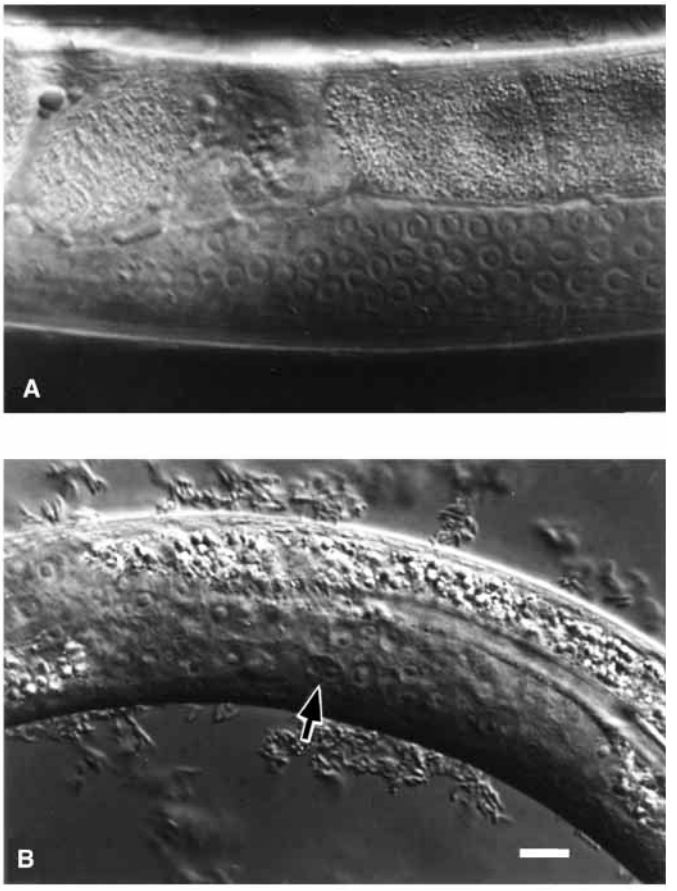

Fig. 5. *mua-4* cytokinesis defect. (A) DIC micrograph of N2 wild-type hermaphrodite showing developing germ cells. (B) *mua-4(rh177)* hermaphrodite showing abnormal germline including binucleate cells resulting from failed cytokinesis (arrow). Scale bar is 10 μm.

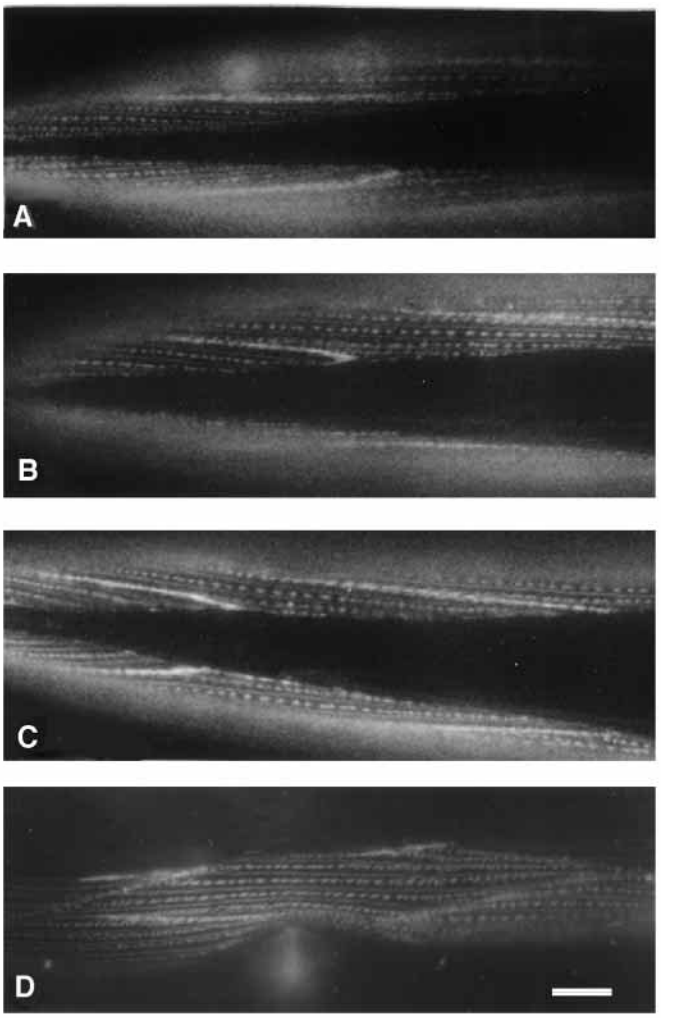

Fig. 6. β^{PAT-3} integrin localization in wild-type and *mua* muscle cells. (A) Animals of genotype *rhIs2*. These animals carry a chromosomally integrated copy of the β^{PAT-3}::GFP protein fusion construct, allowing visualization of integrin-containing attachment complexes, including the dense bodies, M-lines and regions of muscle/muscle apposition. (B-D) Micrographs showing localization of β^{PAT-3}::GFP protein in undetached muscles of *mua-2(rh174); rhIs2*, *mua-10(rh267); rhIs2* and *unc-23(rh292); rhIs2* animals, respectively. In all cases, the integrin-containing attachment structures are properly localized in normal patterns. Scale bar is 10 μm.

nearly paralyzed locomotion (Epstein et al., 1974). Under laboratory conditions, *unc-54* homozygotes feed and grow normally, becoming fertile adults. We examined the onset and progression of muscle detachment by polarized light microscopy in *unc-54* double mutants. For each *mua* mutant examined, the myosin mutation reduced the penetrance of muscle detachment (Table 2). In particular, this mutation completely suppresses detachment in *mua-1(rh160)* and *mua-10(rh267)*.

Allelic comparisons, gene dosage and maternal effects

Seven loci, *mua-2*, *mua-3*, *mua-4*, *mua-5*, *mua-6*, *mua-7* and *unc-23*, have two or more independent alleles. Excepting *mua-3* and *mua-6*, discussed below, allelic comparisons revealed no significant differences in penetrance, onset or severity. Six loci, *mua-1*, *mua-2*, *mua-3*, *mua-5*, *mua-6* and *mua-7* map to regions where deficiency chromosomes are available (Fig. 1). With the two exceptions noted below, *mua/Df* hemizygotes are phenotypically indistinguishable from *mua/mua* homozygotes, consistent with a possible null phenotype. We examined *mua-1(rh160)*, *mua-2(rh174)*, *mua-3(rh195)*, *mua-6(rh85)*, *mua-10(rh267)*, *unc-23(rh299)* and *vab-10(e678)* for possible maternal effects. In all cases, self-progeny arising from homozygous and heterozygous hermaphrodites are phenotypically indistinguishable. Finally, cross-progeny of wild-type males and homozygous mutant hermaphrodites are phenotypically normal.

mua-3

These mutations form an allelic series: class 1 (*rh222*) < class 2 (*rh169*) < class 3 (*rh195*) < wild type (Table 1). Homozygous *mua-3(rh222)* larvae invariably become Mua by larval L2 stage, arresting in L3 (Fig. 4); six additional *mua-3* alleles have comparable phenotypes. Most *mua-3(rh169)* larvae remain non-Mua through L2 stage and do not arrest until L4. Neither class 1 nor class 2 alleles produce fertile adults. Finally, most *mua-3(rh195)* remain non-Mua and become fertile adults. The Mua phenotype in *rh195/rh222* heterozygotes is completely

Table 2. Suppression of muscle detachment in *unc-54* myosin mutants

Genotype	% Penetrance of Mua phenotype (*n*)*
mua-1(rh160)	92±3 (71)
mua-1; unc-54(e190)	0 (58)
mua-2 (rh174)	88±3 (85)
mua-2; unc-54	48±5 (88)
mua-3 (rh195)	37±4 (119)
mua-3; unc-54	13±3 (101)
mua-10(rh267)	60±6 (73)
mua-10; unc-54	0 (94)

*Percentage of animals showing muscle detachment±standard error. The number of animals examined (*n*) is given in parenthesis.

penetrant, consistent with *rh195* causing partial loss of function; *rh195/nDf40* hemizygotes are similar with slightly earlier onset.

mua-6

These mutations form an allelic series: class 1 (rh225) < class 2 (rh85) ≤ wild type (Table 1). Whereas *mua-6(rh225)* invariably arrest by stage L4, many *mua-6(rh85)* become fertile adults (Fig. 4). The Mua phenotype follows the series *rh225/rh225* ≈ *rh85/Df* ≈ *rh85/rh225* < *rh85/rh85*, suggesting that *rh85* is a partial loss of function and *rh225* is a possible null.

Homozygous extragenic mutations in the *smg* pathway, which mediates mRNA degradation (Hodgkin et al., 1989), efficiently suppress *mua-6(rh85)*. Either *smg-1(e1228)* or *smg(rh3010)* discovered as a spontaneous revertant, can suppress muscle detachment. In particular, 99% (*n*=508) of *rh85/rh85; rh3010/rh3010* hermaphrodites are non-Mua. Male progeny of matings between wild-type males and these hermaphrodites are all Mua. Characteristic of *smg* mutations, *rh3010* suppresses *dpy-5(e61)* homozygotes and enhances *e61/+* heterozygotes. Moreover, *smg(rh3010)* males have abnormal genitalia.

rev(rh3005) arose as a spontaneous heterozygous revertant of *mua-6(rh85)*. These non-Mua heterozygotes segregate true breeding Mua, non-Mua heterozygotes and inviable eggs, in 1:2:1 ratio. The viable male progeny from mating wild-type males to these hermaphrodites are all Mua. Moreover, non-Mua hermaphrodites from these crosses (*n*=34) invariably segregate *rh85* homozygotes, but no inviable eggs. A simple explanation for these results is that *rh3005* is an intragenic revertant complementing *rh85* but lethal in combination with either wild type or itself.

DISCUSSION

Fragile muscle attachments identify a new class of *C. elegans* genes

Some 40 genes required for skeletal muscle differentiation and function have been identified in *C. elegans* to date (Moerman and Fire, 1997). Mutations that disrupt myofibrillogenesis or muscle contractility prevent hypodermal elongation, resulting in a paralyzed larvae arrested at two-fold elongation, or a Pat phenotype (Williams and Waterston, 1994). Null alleles of various structural proteins in the mechanical pathways from basement membrane to myofilaments, e.g., collagen IV (*emb-9, let-2*), perlecan (*unc-52*), integrin (*pat-2, pat-3*), vinculin (*deb-1*) and myosin (*myo-3*), can confer a Pat phenotype. Null alleles of proteins required for neuromuscular transmission, coupling of excitation and contraction, and non-essential myofibrillar proteins cause a range of locomotion defects, recognized as uncoordinated larvae, or an Unc phenotype (Brenner 1974). The Pat and Unc phenotypes identify

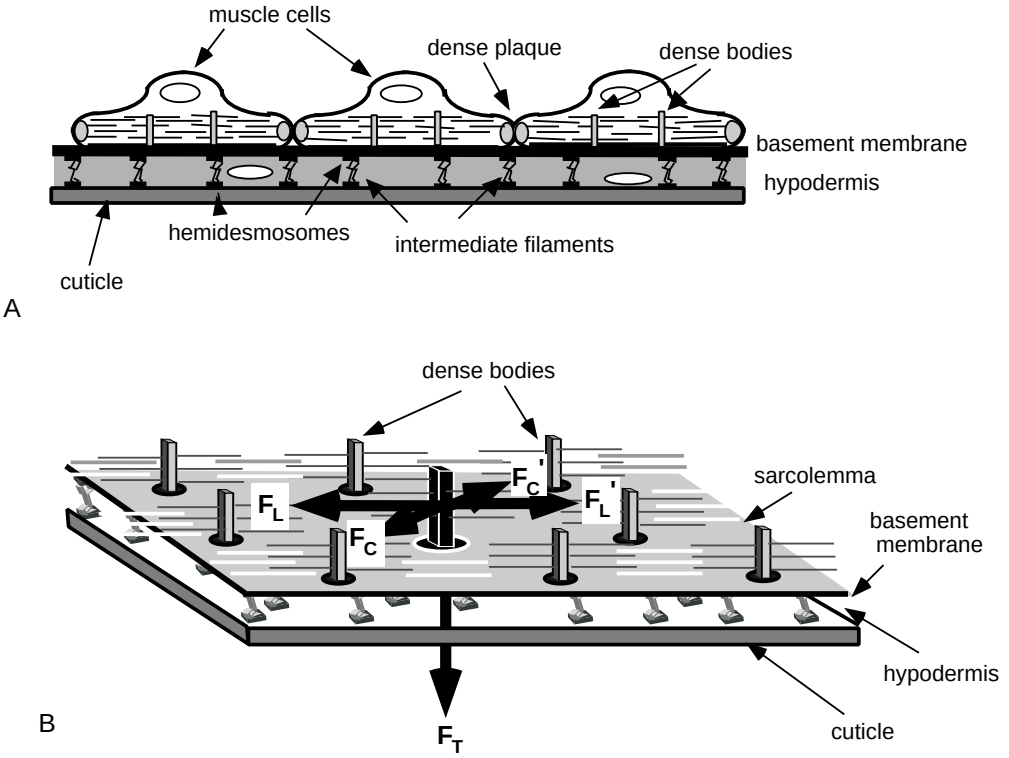

Fig. 7. Schematic diagrams identifying the major features of *Caenorhabditis elegans* body-wall muscle and the forces acting on a dense body. (A) Major features of body wall muscle and adjacent tissues. See text. (B) Forces of three different kinds act on a typical dense body: longitudinal forces of myofibril contraction, F_L and F_L', relayed along the sarcomeres, coupling forces, F_C and F_C', between adjacent myofibrils, and transverse forces, F_T, to the cuticle that do useful work against the hydrostatic skeleton. At any moment during locomotion, the opposing longitudinal forces on each face of a dense body are nearly in balance. Similarly, the coupling forces between myofibrils nearly cancel. Only the resultant of these four vectors, $F_L+F_L'+F_C+F_C'$, must be sustained by the transverse attachments to the cuticle.

partially overlapping sets of genes, i.e., several known genes with Pat null alleles also have Unc hypomorphic or antimorphic alleles.

The Mua phenotype, i.e., normal muscle differentiation, embryonic elongation and initial movement followed by progressive injury and detachment of functioning muscles, is qualitatively different from other *C. elegans* muscle mutants. Surprisingly, these dystrophic mutants identify a novel class of genes, not exceptional alleles of known *pat* or *unc* loci. Besides *unc-23* and *vab-10*, which confer typical Mua phenotypes, fragile muscle attachments have been described for mutations in two other genes. In *mup-1*, imperfectly segregated muscle cells bridging between dorsal and ventral skeletal muscles can detach from the hypodermis during larval growth (Bogaert and Goh, 1991; Moerman et al., 1996). Unlike *mua* mutants, the arrangement of hypodermal attachments and the mechanical forces on these cells are overtly abnormal. In certain *unc-52* hypomorphs, embryonic muscle positioning and myofibrillogenesis appear normal, but the muscles undergo progressive injury during larval growth (Mackenzie et al., 1978; Rogalski et al., 1993).

Muscle detachment in *mua* mutants is enhanced by muscle contraction

Tissues must withstand a wide range of mechanical forces and displacements occurring in the living animal, repairing occasional injuries. Tissues and cell junctions that are mechanically structured to withstand biologically normal levels of mechanical stress may fail under severe loads. In certain disease states, e.g. mechanobullous skin diseases or muscular dystrophies, normal levels of stress can result in mechanical failure (Epstein, 1992; Petroff et al., 1993; Figarella-Branger et al., 1997; Petrof, 1998).

As with some human muscular dystrophies, normal muscle use contributes to the failure of muscle attachments in *C. elegans* Mua mutants. In all four loci tested, reducing the frequency and strength of muscle contraction using a mutation in *unc-54*, the non-essential myosin heavy chain of skeletal muscles, suppressed muscle detachment (Table 2). For two loci, *mua-1* and *mua-10*, this suppression was essentially complete.

Rupture of muscle-hypodermal attachment begins at characteristic sites

In hereditary tissue fragility, the cellular and subcellular sites of tissue separation generally reflect the normal location of the affected protein. In skin fragility diseases, for example, abnormal structural components have been identified along the entire mechanical pathway from epidermis through dermis (Fuchs and Cleveland, 1998). Likewise, the initial sites of failure of each *mua* mutation are likely to identify specific cellular sites associated within the mechanical pathway between myofilament lattice and cuticle in *C. elegans.*

In nematodes, four skeletal muscles running longitudinally beneath the hypodermis mediate locomotion (Moerman and Fire, 1997). Ensheathed in basement membranes, these obliquely striated muscles are attached to the hypodermis along their entire lengths. During embryogenesis, muscle differentiation is polarized by contact with the hypodermis; myofilaments and their integrin-based anchorages assemble only where muscle cells and hypodermis come in contact across their fused basement membranes (Hresko et al., 1994). Coordinately, those regions of the hypodermis that directly overlie the skeletal muscles undergo cytoskeletal compaction. As a result of this reorganization, mature skeletal muscles are anchored firmly to the cuticle through an extremely thin, rigid sheet of hypodermal cytoplasm (White et al., 1976).

In nematode skeletal muscles, force and displacement of myofilament sliding are relayed both longitudinally along the myofibril and transversely across the hypodermis to the cuticle (Fig. 7). At I-bands, actin filaments from adjacent sarcomeres couple through dense bodies, homologous to the Z-line of vertebrate striated muscles, while intermediate filament proteins, presumably homologous to desmin, mechanically couple adjacent dense bodies. At the base of each dense body, a complex of integrin and vinculin couples the myofibril to the basement membrane. At half-I-bands, actin filaments from adjacent muscle cells couple together across dense attachment plaques. Although analogous to the attachment plaques of vertebrate cardiac muscle, dense plaques are integrin-based focal adhesions, not direct cell-cell junctions. Within the overlying hypodermis, a network of hemidesmosomes and compacted intermediate filaments completes the mechanical pathway from myofibril to cuticle.

The primary sites of muscle detachment appear characteristic for each *mua* locus. In *mua-2*, *mua-3*, *mua-5*, *mua-10*, *unc-23* and *vab-10*, the skeletal muscles remain more-or-less intact as they detach from the hypodermis suggesting these mutations affect components specifically required in the transverse pathway from muscle to cuticle. In *mua-1* and *mua-6*, individual muscle cells also separate from their neighbors suggesting these mutations affect components common to the longitudinal pathway from muscle to muscle and the transverse pathway to the cuticle. In *mua-3* and *mua-9*, the muscle detachment is associated with hypodermal cytolysis suggesting a primary defect in hypodermal hemidesmosomes or the associated intermediate filaments. Indeed, mosaic analysis of *mua-3* indicates its products are required in the hypodermis, not the skeletal muscles, for normal muscle attachment (Bucher and Greenwald, 1991). In dystrophic *unc-52* hypomorphs, myofibrils separate from the sarcolemma while the fused basement membranes between muscle and hypodermis remain intact (Rogalski et al., 1993). Here, the primary site of tissue separation only roughly approximates the affected protein, i.e., mutations in a basement membrane protein, perlecan, cause cytolysis within muscle cells.

Tissue specificity

The nematode contains several muscle types in addition to the body-wall muscles; the most distinctively different are the pharyngeal muscles, which are not only arranged in a radically different manner, but utilize unique myosins and transcription factors (Waterston, 1988; Okkema and Fire, 1994). None of the presently known *mua* mutations obviously affect pharyngeal muscle attachments. The pharynx is also unaffected by *pat* mutations that disrupt embryonic formation of the body-wall muscle attachment structures or initial myofilament lattice assembly, suggesting that the fundamental difference in the arrangement and composition extends to the genes involved in development and growth regulation of the different muscle

types. The prediction is that other, as yet unidentified genes play roles similar to the *mua* genes in the pharynx and other non-bodywall muscle groups. Finally, the recovery of the position specific phenotypes in *mua-10, vab-10* and *unc-23* suggests that attachments at the anterior tip of the worm have distinct or additional properties not found in muscles with more posterior attachments.

Possible mechanisms underlying dystrophic phenotype

Most known *C. elegans* genes required for muscle development appear strictly zygotic, i.e., maternal protein or mRNA, if expressed at all, have no significant contribution to the phenotype. Conceivably, maternal genes required for muscle cell attachment might present as Mua mutants. In this model, maternal stores of protein or mRNA made during oogenesis suffice for embryonic muscle differentiation and initial movement, but zygotic gene transcription is required to support muscle growth. Although an attractive conjecture, genetic tests of all seven *mua* loci with fertile alleles revealed no effect of maternal genotype on zygotic phenotype. For these cases at least, zygotic gene expression appears both necessary and sufficient for normal muscle attachment.

Some proteins required for muscle attachment could have distinct embryonic and larval isoforms, encoded either as alternative products of a single gene or as products of separate genes. In this model, mutations that selectively disrupt larval isoforms cause Mua phenotypes. At least one gene, *mua-3*, has a known homolog required for embryonic muscle attachment, and genetic tests indicate that the two genes are likely to have complementary functions in muscle cell attachment (E. Bucher, personal communication).

The growth and maintenance of functional muscles, while sharing much in common with embryonic muscle differentiation, entails several unique processes. For example, new myoblasts are incorporated into each skeletal muscle at the end of the first larval stage with remodeling of established muscle-muscle and muscle-hypodermal attachments. Similarly, a program of hypodermal cell division, cuticle synthesis and molting is repeated at each larval stage with suppression of skeletal and pharyngeal muscle contractions during cuticle deposition. The reason for this lethargus is not known but muscle attachments may be remodeled at this time. The position and timing of skeletal muscle detachment in *mua* mutants do not apparently correlate with sites of myoblast incorporation or a specific phase of the molt cycle.

Skeletal muscles increase nearly 5-fold in length during *C. elegans* larval growth. While the total number of muscle cells rises only from 81 to 95, the number of sarcomeres rises from 2 per cell at hatching to about 8-10 by the adult stage (Mackenzie et al., 1978). Importantly, the depth of each myofibril, and therefore the tension on each dense body attachment, increases roughly linearly with larval length. This differential scaling of myofibril volume versus attachment area suggests that some proteins required for muscle attachments in larger animals might be unimportant, if expressed at all, at earlier stages.

One possible role for the *mua* genes in active muscle may be to regulate the assembly or positioning of proteins to existing attachment complexes and/or to the sites of nascent new complexes to match growth. The genes responsible for this might be expected to include membrane-bound matrix receptors that form a direct or indirect link to the cellular cytoskeleton and attachment complexes. In addition, transcription factors are likely to be involved. Mutations that ablate this regulation could result in a Mua phenotype. Recently, the products of several *mua* loci have been identified by molecular cloning. Interestingly, these include an extracellular matrix receptor expressed on the surface of hypodermal cells and a presumptive transcription factor (our unpublished data), implicating the *mua* genes in executing regulatory roles.

Comparison with muscular dystrophy

Molecular cloning of the Duchenne muscular dystrophy gene identified a putative cytoskeletal protein, dystrophin, that was subsequently shown to link to a novel complex of proteins, including dystroglycan, sarcoglycans, sarcospan and syntrophins, coupling the basement membrane and subsarcolemmal cytoskeleton at costameres and myotendinous junctions (Campbell, 1995; Berthier and Blaineau, 1997). Unnecessary for muscle differentiation and contraction, this dystrophin-glycoprotein complex contributes to fiber integrity and resistance to injury from mechanical stresses associated with muscle use and skeletal growth (Brown and Lucy, 1993; Petrof, 1998). Mutations in various components, including laminin $\alpha 2$, sarcoglycans and dystrophin itself, cause congenital to late onset muscular dystrophies, depending upon the gene (Ozawa et al., 1998). Dystroglycan, a ubiquitous cell surface receptor for laminin and agrin, has other, essential roles including epithelial morphogenesis (Durbeej et al., 1998).

Other human muscular dystrophy genes encode extracellular (fukutin), membrane (caveolin-3, dysferlin) or intracellular (calpain-3, plectin) proteins that could interact functionally with the dystrophin-glycoprotein complex (Richard et al., 1995; Bashir et al., 1998; Kobayashi et al., 1998; Liu et al., 1998; Minetti et al., 1998). Reinforcing the view that muscular dystrophy is ultimately a tissue fragility disease, plectin mutations cause a combined syndrome of muscular dystrophy and epidermolysis bullosa in the skin (Andra et al., 1997; McLean et al., 1996). Finally, genes for Emery-Dreyfuss muscular dystrophy encode nuclear envelop components (emerin, lamin A/C) and an opthalmopharyngeal muscular dystrophy gene encodes an mRNA polyadenylation factor (PAB2) (Brais et al., 1998; Ellis et al., 1998; Bonne et al., 1999). Taken together, these results suggest muscular dystrophy identifies a regulatory mechanism important in the growth or maintenance of functioning muscle, not passive structural elements.

Dystroglycan, sarcoglycans, dystrophin and syntrophins all have *C. elegans* orthologs, suggesting the dystrophin-glycoprotein complex predates the metazoan radiation (our unpublished results). Loss-of-function mutations in the dystrophin/utrophin ortholog *dys-1*, which is normally expressed in skeletal and other muscles, cause a syndrome of locomotor hyperactivity, muscle hypercontraction and hypersensitivity to acetylcholine (Bessou et al., 1998). By inference, a similar defect in physiological regulation of muscle contraction likely underlies human muscular dystrophy with tissue injury an indirect consequence of chronic misregulation of contraction. FER-1, the *C. elegans* dysferlin ortholog, is expressed in spermatocytes where it is required for

membranous organelle fusion, but has no reported role in muscle (Achanzar and Ward, 1997). Finally, homologs of other human muscular dystrophy genes have been identified in *C. elegans* but little is known about their tissue expression or function, nor do they chromosomally map to known *mua* loci (Kobayashi et al., 1998; our unpublished results).

The Mua phenotype of *C. elegans* displays some parallels to the phenotypes associated with vertebrate muscular dystrophies. In particular, mutations in both sets of genes show a phenotypic onset relatively late in the life of the animal, with the initial assembly of the myofilament lattice, its attachments and anatomical organization of the musculature unaffected. The functionality of the musculature in affected individuals progressively decreases. These similarities appear not to be due, however, to molecular homology between the *mua* genes and the vertebrate dystrophy genes, although functional overlap in the roles played by the two groups remains a possibility. The *mua* genes thus appear to represent a genuinely novel group of genes responsible for cell attachment regulation in the musculoskeletal system in response to muscle growth and use.

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