

# Assembly of Body Wall Muscle and Muscle Cell Attachment Structures in *Caenorhabditis elegans*

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**Abstract.** *C. Elegans* has four muscle quadrants that are used for locomotion. Contraction is converted to locomotion because muscle cells are anchored to the cuticle (the outer covering of the worm) by a specialized basement membrane and hemidesmosome structures in the hypodermis (a cellular syncytium that covers the worm and secretes the cuticle). To study muscle assembly, we have used antibodies to determine the spatial and temporal distribution of muscle and attachment structure components in wild-type and mutant *C. elegans* embryos. Myofibrillar components are first observed diffusely distributed in the muscle cells, and are expressed in some dividing cells. Later, the components accumulate at the membrane adjacent to the hypodermis where the sarcomeres will form,

showing that the cells have become polarized. Assembly of muscle attachment structures is spatially and temporally coordinated with muscle assembly suggesting that important developmental signals may be passed between muscle and hypodermal cells. Analysis of embryos homozygous for mutations that affect muscle assembly show that muscle components closer to the membrane than the affected protein assemble quite well, while those further from the membrane do not. Our results suggest a model where lattice assembly is initiated at the membrane and the spatial organization of the structural elements of the muscle is dictated by membrane proximal events, not by the filament components themselves.

**M**ECHANISMS that govern the assembly of the highly ordered structures of striated muscle are not well understood. Early investigations emphasized the self assembly properties of thick and thin filament proteins in controlling filament length and association. However, recent evidence from both vertebrate and invertebrate systems have focused on the role of auxiliary proteins in dictating the structure. The vertebrate studies have used morphological studies of muscle cells in tissue culture and perturbation with drugs. Invertebrate studies with *Drosophila* and *Caenorhabditis* have centered on genetic dissection to define important components and to discern their roles.

In vertebrate studies molecular rulers have been put forward as regulators of thick and thin filament length. The size of nebulin is correlated with thin filament length in different types of vertebrate muscle (37, 39, 65). Furthermore, the sequence of nebulin contains repeats with a periodicity similar to that of the axial periodicity of thin filaments and individual repeats have been shown to bind actin (32, 33). Similarly, titin, a large molecule extending from the Z-disc to the M-line (22, 74), may regulate thick filament length (74). Titin has repeats that correspond to the periodicity of thick fila-

ments and the repeats localize to the region of the molecule that extends across the A-band (38, 40).

The placement of filaments within the lattice must also be precisely regulated. CapZ, a barbed end actin-capping protein (10, 11), and  $\alpha$ -actinin, an actin cross-linking protein (46, 54), are thought to be involved in positioning thin filaments, because both associate with nascent Z-discs before actin filaments become organized into the I-bands (57, 59). Furthermore, CapZ may orient the thin filaments in the lattice such that they always have their barbed ends at the Z-disc (12).

Titin, the proposed thick filament ruler, has also been implicated in the placement of thick filaments at the center of the sarcomere. This is based on the structure and localization of the molecule, as mentioned above, and on the observation that titin assembles at the Z-line before A-bands form (23, 59).

Microtubules may act as a scaffold for overall organization of the fibril. A cage of microtubules is observed surrounding developing fibrils in many systems (13, 19, 69). Colcemid, which blocks microtubule assembly, disrupts muscle assembly (64). In contrast, taxol treatment, which stabilizes microtubules allows for normal spatial organization of structural elements, except that thin filaments are replaced by microtubules (1). Furthermore, *Drosophila* mutants which accumulate very little if any  $\beta$ 3 tubulin, an isoform transiently expressed in developing muscle cells of *Drosophila*

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(35), show reduced viability and fertility, both of which could be affected by defects in muscle function (36). These results suggest that microtubules can specify the spatial organization of the fibril and hexagonal packing of filaments.

In addition to tubulin, directed mutant screens have identified over 100 genes from *D. melanogaster* (7) and about 50 genes from *C. elegans* (73, 75) that have been implicated in muscle assembly or function. Phenotypic studies of mutants carrying genetic lesions in some of these genes have clarified the *in vivo* roles of the wild-type gene products.

Thick filaments of *C. elegans* are composed of two myosin isoforms with differential localization within the filament: myosin A is localized at the central region of the thick filament, while myosin B is localized at the ends, where the filament tapers (47). Genetic analysis has shown that myosin A is uniquely required for the initiation of thick filament assembly (72), whereas paramyosin, a component of thick filament cores, is required for assembly of thick filaments of the correct length (45).

Thick and thin filaments in *D. melanogaster* and *C. elegans* can organize independently of one another (5, 6, 72, 75). In both *D. melanogaster* indirect flight muscle and *C. elegans* body wall muscle, the lateral alignment of thin filaments is not specified by their interaction with thick filaments, since mutants lacking thick filaments contain a near normal arrangement of thin filaments (6, 72). Similarly, in *D. melanogaster*, mutants lacking thin filaments can assemble thick filaments in a normal hexagonal array (6).

One possibility is that structures that anchor the filaments are important for their proper positioning. Vinculin is localized at the base of the nematode dense body, the structure that anchors the thin filaments. *C. elegans* mutants lacking detectable vinculin do not organize their thin filaments into I-bands (5). Thus, vinculin is clearly important for anchoring thin filaments, although the interaction may not be direct since vinculin is not thought to bind to actin (17). In contrast,  $\alpha$ -actinin, another dense body or Z-disc component, one that cross-links actin filaments, is dispensable for assembly of the indirect flight muscle of *D. melanogaster* (56).

Based on the observation that muscle assembly occurs near the membrane in primary cultures of muscle cells (15, 44, 59), the membrane is thought to play an important role in muscle assembly. This is also true in *C. elegans* where perlecan and  $\beta_1$  integrin (75), and in *D. melanogaster* where  $\beta_1$  integrin (50, 66, 76) are required for proper muscle assembly. However, whether these molecules act as nucleation sites for assembly and/or as indicators of spatial organization is not clear from these studies.

*C. elegans* offers several advantages for the study of muscle assembly *in vivo*. *C. elegans* embryology has been extensively characterized (62) allowing the identification of muscle cell precursors at early developmental times, as well as the accurate staging of fixed embryos. In addition to the many muscle-affecting mutants already identified (reviewed in 71, 73, 75), about 40 monoclonal antibodies which recognize muscle and muscle-associated molecules are available (20, 21, 47). These antibodies can be used to characterize muscle structure at various developmental times.

In this paper we describe the spatial and temporal distribution of structural components (see Table I) of the body wall muscle and of muscle attachment structures in wild-type and mutant embryos at various stages of development. The

results identify four stages of muscle assembly which are summarized in Table II. Furthermore, muscle assembly is spatially and temporally coordinated with the assembly of structures required for the transmission of force to the outside of the animal. These structures are assembled in part by the hypodermis, and therefore it is likely that muscle cells and hypodermal cells communicate during development. Parallel experiments determining the distribution of muscle components in mutants suggest that body wall muscle is assembled by the stepwise addition of components, beginning at the membrane. Furthermore, the spatial organization of the muscle is dictated by membrane proximal events.

## Materials and Methods

### Strains and General Maintenance of Stocks

Worms were grown on bacterial strain OP50 spread on NGM (nematode growth media) agar plates according to Brenner (8). The Bristol variety of the N2 strain of *C. elegans* is used as the wild-type strain, and the genotypes of the mutant strains used are as follows: *myo-3(st386)/sqt-3(e24)* (72), *unc-44(e362)deb-1(st555)/unc-5(e53)unc-24(e138)* (5), *pat-3(st552)/lqC1* (75), and *unc-52(st549)/mnDp34* (75).

The muscle affecting mutations (*myo-3(st386)*, *deb-1(st555)*, *pat-3(st552)*, and *unc-52(st549)*) are recessive lethals and therefore the strains were carried as balanced heterozygous strains. Recessive, closely linked markers in trans were used as balancers for this purpose, and alone have no effect on muscle structure. The balancer *mnDp34* (27) is a free duplication that covers the *unc-52* gene, and therefore adds a wild-type copy of the *unc-52* gene to the chromosomal copies. Embryos that do not receive the duplication during oocyte production or fertilization will only carry mutant alleles.

### Staging of Fixed Embryos

Two landmarks were used to determine the stage of fixed embryos, both of which take advantage of the invariant developmental program of *C. elegans* as documented by Sulston et al. (62). Between 290 and 340 min after the first cleavage, the dorsal hypodermal cells undergo characteristic changes in shape and position. The shape and position of each dorsal hypodermal cell at various developmental times is known (62), and can therefore be used as a marker for a given developmental stage. The position and shape of these cells was determined by staining embryos with monoclonal antibody MH27, which labels hypodermal cell boundaries (21).

The other developmental landmark used is the length of the embryo. The embryo elongates 3–4-fold between 350 min after the first cleavage and the time of hatching (62). Due to the constraints of the eggshell, as the embryo elongates it curls up, so that when it is twice its original length, the anterior and posterior ends touch. The length of the embryo is used to denote the developmental stage; the 1.5-fold stage occurs when the embryo is 1.5 times the length of the eggshell. The developmental time at which the embryo reaches certain lengths has been documented (62), and therefore, the length of the embryo can serve as a marker for different developmental stages.

The developmental time scale used in this paper is according to Sulston et al. (62), and the times stated refer to min after the first cleavage at 25°C.

### Immunological Techniques

Embryos were prepared for antibody staining in one of two ways. Most often, embryos were obtained by alkaline hypochlorite treatment of gravid adults as described by Sulston and Hodgkin (62). The embryos were then fixed in 3% paraformaldehyde (Electron Microscopy Sciences EM grade) in phosphate buffer (0.1 M sodium PO<sub>4</sub>, 0.1 mM EDTA, pH 7.0) for 10 min, washed three times in PBS (150 mM NaCl, 10 mM sodium PO<sub>4</sub>, pH 7.2), permeabilized for 10 min in -20°C MeOH, and washed three times in PBS and one time in wash buffer (PBS plus 0.5% Tween 20). Paraformaldehyde fixation was found to be incompatible with monoclonal antibody 4A1 (anti-tubulin), and therefore, embryos to be stained with 4A1 were freeze fractured by the method of Strome (60). Briefly, embryos obtained by cutting open gravid adults were sandwiched between a subbed slide (0.4 g gelatin, 0.04 g chromium potassium sulfate and 200 mg polylysine in 200 ml) and a coverslip in a small amount of M9. Excess M9 was removed

Table I. Summary of Antibodies

Antibody	Antigen	Gene	Adult Localization	Reference
MH25	UNK*	UNK*	base of m-lines and dense bodies; attachment plaques	20
MH24	vinculin	<i>deb-1</i>	base of dense bodies; attachment plaques	3, 20
MH35	$\alpha$ -actinin	UNK‡	throughout the dense body	4, 20
C4	actin	<i>act-1,2,3,4,5</i> §	thin filaments; cytoplasmic actin filaments	18, 42, 70
DM5.6	myosin A	<i>myo-3</i>	central region of thick filaments	47, 72
DM5.8	myosin B	<i>unc-54</i>	distal region of the thick filaments	47, 48
R6-2 (polyclonal)	myosin A and myosin B	<i>myo-3</i> <i>unc-54</i>	thick filaments	48, 72
MH42	UNK	UNK	M-line	71
MH46	300-380-Kd proteins	UNK	basement membrane; associates with hemidesmosomes	21
MH4	UNK	UNK	hemidesmosomes; intermediate filament subunit	21
MH5	UNK	UNK	hemidesmosomes	21
MH3	perlecan	<i>unc-52</i>	basement membrane	21, 55
4A1	tubulin	UNK	tubulin-containing structures	53
MH27	UNK	UNK	hypodermal cell boundaries: tight junctions	21

UNK, unknown

\* epitope dependent on  $\beta$ -integrin (75), the *pat-3* gene product (Gettner, S., C. Kenyon, L. Reichardt, J. Plenefisch, M. B. Buchner, and E. Hedgecock. 1992. *Mol. Biol. Cell.* 3:1088a).

‡ unknown if MH35 recognizes the product of the *am-1* gene which is one of the  $\alpha$ -actinin genes of *C. elegans*.

§ *act-5* is the fifth *C. elegans* conventional actin identified to date (Schriefer, L. A., J. A. Waddle, and R. H. Waterston, unpublished data).

with a piece of filter paper until contact was made between the embryo and the slide, and the embryo and the coverslip. The sample was frozen in liquid nitrogen for 10 min, removed, and the coverslip was pried off. The embryos were immediately fixed at 0°C in MeOH for 10 min, at 0°C in acetone for 2 min, and then air dried.

Fixed embryos were incubated in primary antibodies diluted in antibody dilution buffer (30% NGS, in wash buffer) for 1 h, followed by four 5-min washes in wash buffer. The primary antibodies were detected using either fluorescein or rhodamine conjugated goat anti-mouse or goat anti-rabbit IgG. All secondary antibodies were purchased from Chemicon and used at 10 ng/ml in antibody dilution buffer. After the final wash, embryos were resuspended in mounting medium (20 mM Tris pH 8, 0.2 M 1,4-diazabicyclo[2.2.2]octane (Sigma Chem. Co., St. Louis, MO), in 90% glycerol). Fluorescence was viewed using a Zeiss Universal fluorescence microscope equipped with epifluorescent illumination and selective filters. Photomicrographs were recorded on TMAX 400 film and the film was developed with TMAX developer according to the manufacturer (Kodak).

## Results

### Structure of Adult Body Wall Muscle

The body wall muscle cells of *C. elegans* adults are organized into four quadrants that are positioned adjacent to the hypodermis (61) which secretes the cuticle or outer covering of the worm. The hypodermis is partitioned into four sections that run parallel to the long axis of the worm; a dorsal, a ventral, and two lateral or seam hypodermal sections. The dorsal and ventral hypodermis is in turn divided into left and right sections by hypodermal ridges (61). Two body wall muscle quadrants are associated with the dorsal section, one on either side of the dorsal ridge, and two with the ventral; each quadrant consists of two rows of mononucleated cells (61). The cells are polarized in that the contractile apparatus is located adjacent to the membrane that contacts the hypo-

dermis, while other organelles, like the nucleus and the mitochondria, are situated closer to the interior of the worm.

The muscle is made up of repeated units with similarities to the sarcomeres of vertebrate-striated muscle (see Fig. 1). The contractile units of the body wall muscle are delineated on either end by a dense body, a structure analogous to the Z-line. Actin-containing thin filaments extend in either direction from the dense body, and interdigitate over part of their length with myosin-containing thick filaments. The filaments of each unit are oriented parallel to the long axis of the worm, but, unlike the sarcomeres of cross-striated muscle of vertebrates, the contractile units of the body wall muscle are offset from the adjacent unit by more than one  $1 \mu\text{m}$  (45). Therefore, the striations observed are oblique, oriented at a  $6^\circ$  angle to the long axis of the worm, and to the thick and thin filaments.

Thick and thin filaments are anchored to the membrane by the M-line and the dense body, respectively (20). Each M-line and dense body extends the entire depth of the lattice, anchoring all filaments directly to the membrane. At the ends of the cell, a half I-band ends the lattice, where thin filaments extend from the last A-band to a membrane associated, electron dense plaque, called an attachment plaque (20).

Body wall muscle cells are anchored to the cuticle of the worm in order for the force generated by contraction to be transduced into movement. Attachment is thought to be mediated by a specialized region of the basement membrane, and by hemidesmosome structures of the hypodermis (21). This region of the basement membrane adjacent to muscle cells is molecularly distinct from other regions (21). The hemidesmosome structures in the hypodermis are found adjacent to muscle cells and certain mechanosensory neurons (21).

Table II. Summary of Stages

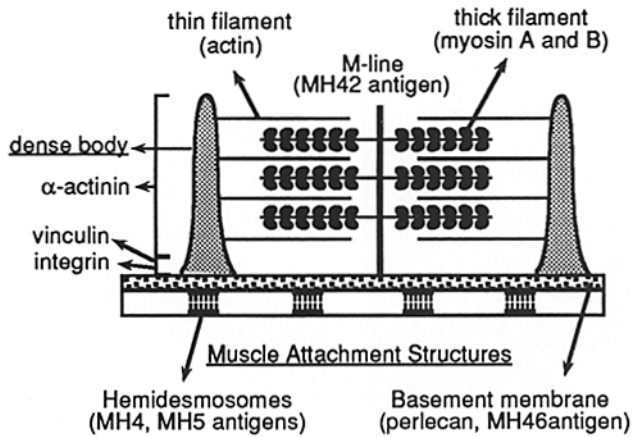
Muscle assembly stages	Developmental events	Lattice components	Basement membrane components	Hemidesmosome components
I. accumulation of structural components	<290 min; before dorsal hypodermal cells interdigitate		MH46 antigen diffusely distributed in dorsal and ventral hypodermal cells	MH4 and MH5 antigens diffusely distributed in dorsal and ventral hypodermal cells
	290 min; muscle cells adjacent to seam cells	vinculin, integrin and myosin A and B diffusely distributed in muscle cells		
II. polarization of muscle cells; basement membrane and hemidesmosome components accumulate in regions adjacent to muscle cells	310 min; interdigitation of dorsal hypodermal cells almost complete; muscle cells migrating onto dorsal and ventral hypodermis			MH4 and MH5 antigens organize in regions of dorsal and ventral hypodermis adjacent to muscle cells
	350 min; interdigitation of dorsal hypodermal cells is complete; muscle cells organized into quadrants adjacent to dorsal or ventral hypodermis	muscle components localize to membranes where adjacent muscle cells contact each other and the hypodermis; this is referred to as muscle cell polarization	MH46 antigen and perlecan localize to regions of contact between muscle cells	
III. muscle cells flatten against hypodermis; muscle, basement membrane, and hemidesmosome components are coextensive	420 min; 1.5-fold stage	MH42 first detected at this stage; breadth of region occupied by muscle components increases forming a band rather than a thin line	MH46 antigen and perlecan localized to regions adjacent to muscle components	MH4 and MH5 antigens localized to regions of the dorsal and ventral hypodermis adjacent to muscle components
IV. organization of sarcomeres and attachment structures	430 min; 1.75-fold stage	muscle components organized into sarcomeres		
	450 min; twofold stage		MH46 antigen and perlecan localized to regions adjacent to muscle; MH46 antigen appears to be associated with specific muscle structures	MH4 and MH5 antigens organized into hemidesmosomes
	>520 min; late threefold stage		MH46 antigen appears to be associated with hemidesmosomes	

Components of the lattice, basement membrane, and hemidesmosomes have been identified immunologically, and represent a potential molecular chain between the contractile apparatus and the hypodermis, and therefore to the cuticle. Integrin, a transmembrane receptor, is a component of the base of the dense bodies and M-lines (20), anchoring these structures to the basement membrane. The attachment may be mediated by perlecan, a basement membrane protein that is concentrated at dense bodies and M-lines (21). Another basement membrane component, the MH46 antigen, associates with hemidesmosomes, and therefore extends the chain to the hypodermis and cuticle (20). These connections would provide for closely spaced, periodic linkages between the lattice and the cuticle. A number of the components of the lattice and the basement membrane have also been identified

genetically (55, 71, 73, 75). The schematic in Fig. 1 summarizes some of the components and their localization in adult worms.

### Muscle and Hypodermal Cell Movements in the Embryo

*C. elegans* embryogenesis is invariant from one animal to the next (62), and from the time of the first cleavage to hatching takes ~800 min. The first half of embryogenesis is devoted to cell proliferation and gastrulation, while the second half is devoted to organogenesis. Soon after gastrulation, which occurs between 100 and 260 min, hypodermal and muscle cells undergo a characteristic set of movements that result in the formation of four muscle quadrants positioned adjacent



**Figure 1.** Schematic diagram illustrating the structural elements of a body wall muscle sarcomere. The diagram is not meant to indicate the relative size of the structures but is meant to depict the location of components defined by antibodies and the identified protein, where known.

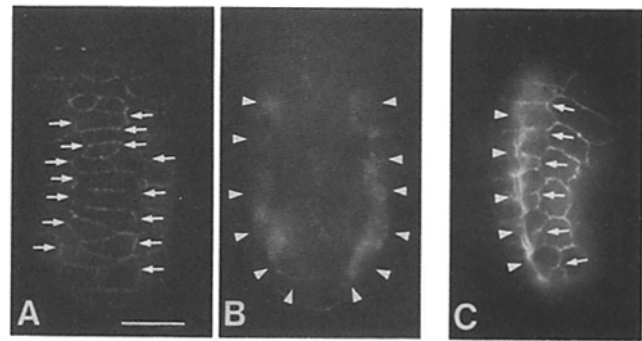
to dorsal and ventral hypodermis. At 250 min, the hypodermal cells are clustered in six rows (two each of dorsal, lateral or seam, and ventral hypodermal cells) forming a continuous sheet of cells covering the dorsal and lateral surfaces of the embryo, leaving the neuroblasts that occupy the ventral surface uncovered (62). In order, from the dorsal midline to the lateral edge of the embryo, is one row each of dorsal, seam, and ventral hypodermal cells. By ~290 min, the seam cells have migrated to the lateral surfaces of the embryo and the ventral cells have begun to enclose the ventral surface. At this stage, the muscle cells are organized in two lateral rows positioned at the midplane of the embryo, and are therefore probably adjacent to seam cells (Fig. 2, *A* and *B*). Over the next 20 min, the hypodermis extends ventrally, completely enclosing the embryo (62).

At 290 min, each dorsal hypodermal cell extends a process across the dorsal midline, and between the two opposing dorsal hypodermal cells, eventually contacting the opposite seam cell. By 340 min, a single row of rectangularly shaped hypodermal cells covers the dorsal surface, each cell straddling the dorsal midline (62). During this time (290–340 min), the muscle cells, which are initially in lateral rows adjacent to seam cells, migrate dorsally or ventrally away from the seam (see Fig. 13). By 340 min, the muscle cells are organized into four quadrants adjacent to dorsal and ventral hypodermis (Fig. 2 *C*).

The embryo begins to elongate at ~350 min, reaching more than three times its original length by hatching. As the embryo elongates, the muscle cells flatten against the hypodermis, and elongate to maintain continuity of the muscle cells in each quadrant. Muscle twitching is first detected at ~430 min and by 450 min the embryo rolls within the eggshell.

#### Muscle Development in Four Distinct Stages

Four stages of muscle assembly and the developmental time at which each occurs are summarized in Table II. The stages are compared with events occurring in the basement membrane and the hypodermis with respect to the development

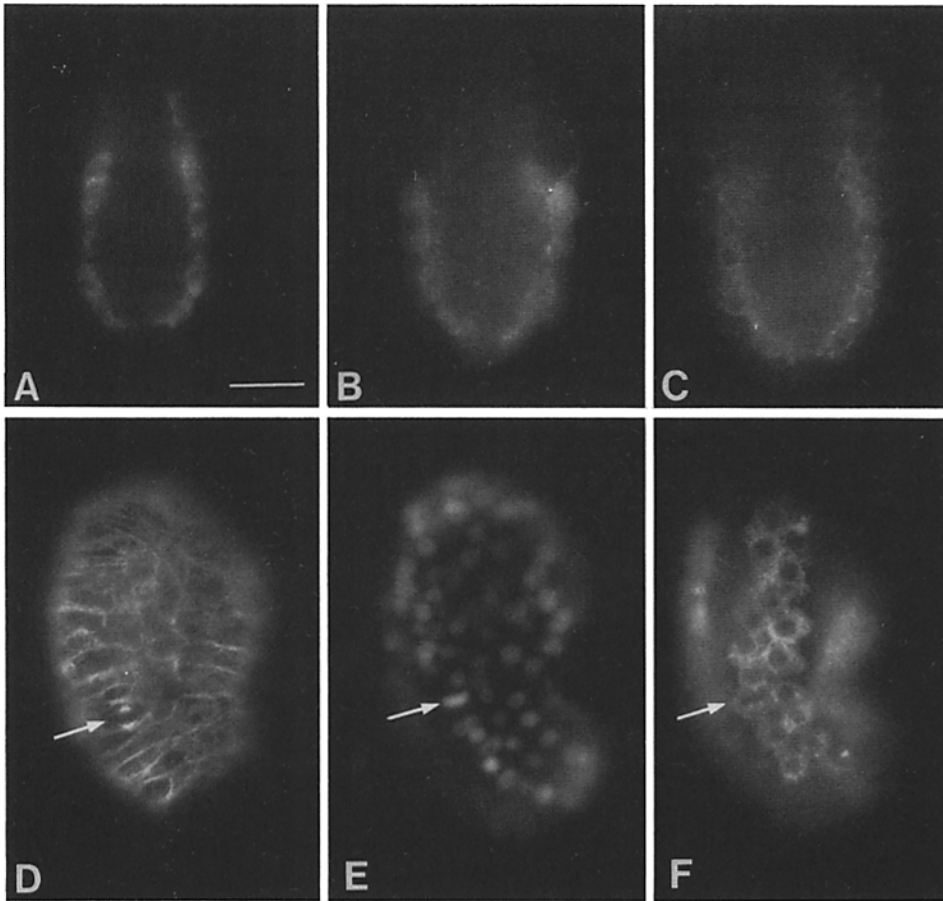


**Figure 2.** Position of hypodermal and muscle cells in 290- and 340-min embryos. Fluorescence micrographs of embryos labeled with MH27 to identify boundaries between hypodermal cells (*arrows*), and with DM5.6 to mark the position of muscle cells in the embryo. Embryos are oriented with their anterior ends toward the top of the page. Arrows indicate lateral boundaries of dorsal hypodermal cells, and arrowheads mark myosin positive staining. (*A* and *B*) Dorsal view of a 290-min embryo focused on the dorsal surface (*A*) or on the mid-plane (*B*) of the embryo. The two rows of muscle cells (*B*) are more lateral and more ventral than the dorsal hypodermal cells (*A*), and are therefore probably adjacent to seam cells. (*C*) Dorsolateral view of a 340-min embryo, showing the right row of seam cells and the right half of each dorsal hypodermal cell. One of the two dorsal muscle quadrants is in focus, and is clearly adjacent to the dorsal hypodermal cells. Bar, 10  $\mu$ M.

of muscle attachment structures (see below). Also listed in the table are key events in muscle and hypodermal cell movement, along with other general morphological changes that occur in the embryo. The times given in the table are based on the shape of dorsal hypodermal cells and on the length of the embryo as described by Sulston et al. (62; see Materials and Methods) and have not been measured directly.

Vinculin (Fig. 3 *B*), integrin (Fig. 3 *C*), and myosins A (Fig. 2 *B*; see also reference 16) and B (data not shown; see reference 25), can all be detected at 290 min, a stage when muscle cell precursors are in lateral rows adjacent to the seam cells. All these antigens appear widely distributed throughout the cytoplasm. Myosin A (Fig. 2 *A*), myosin B (data not shown), and integrin (Fig. 3 *C*) are restricted to muscle cell precursors, while vinculin (Fig. 3 *B*) is sometimes detected in non-muscle cells (compare Fig. 3, *A* and *B*). The non-muscle expression of vinculin appears to be in cells more exterior than the muscle cells, suggesting the presence of vinculin in hypodermal cells.

Thus, the stage at which muscle components are first detected is before all muscle cell precursors become post-mitotic, which does not occur until ~370 min; however, it should be noted that all the progeny of these cells will become body wall muscle (62). To confirm that the cells expressing muscle components are the same cells that are dividing, embryos were double labeled for myosin and tubulin. Multiple embryos were found which contain a muscle myosin-positive cell that also contains a mitotic spindle and chromosomes condensed at the metaphase plate (arrows in Fig. 3, *D–F*). Similar experiments using anti-integrin or anti-vinculin antibodies were not performed since this would have required direct labeling of the primary antibodies because MH25 (integrin), MH24 (vinculin), and 4A1 (tubulin) are mouse monoclonal antibodies. However, other double-



**Figure 3.** Fluorescence micrographs showing the early expression of muscle lattice components. (A-C) Dorsal view, focused on the mid-plane of 290-min embryos labeled with (A) R6-2 (myosin), (B) MH24 (vinculin), and (C) MH25 (integrin). The antigens accumulate in muscle cells while the muscle cells are in lateral rows adjacent to seam cells. A and B are of the same embryo. (D-F) Dorsolateral view of a 350-390-min embryo, viewed for (D) 4A1- (tubulin), (E) DAPI- (chromatin), or (C) R6-2- (myosin) dependent fluorescence. Arrow indicates a myosin-positive cell that is dividing, as indicated by the presence of a mitotic spindle (D) and condensed chromosomes (E) at the metaphase plate. Bar, 10  $\mu$ m.

labeling experiments show that muscle myosin-positive cells of embryos of various stages are also positive for integrin and vinculin. Therefore it is likely that all three components are expressed in dividing cells. Muscle-specific actin may also be expressed in presumptive muscle cells at this early stage; however, since the anti-actin monoclonal used (C4) (42) stains both muscle and non-muscle cells, the results cannot be interpreted. At this time, no *C. elegans* muscle-specific actin antibodies are available.

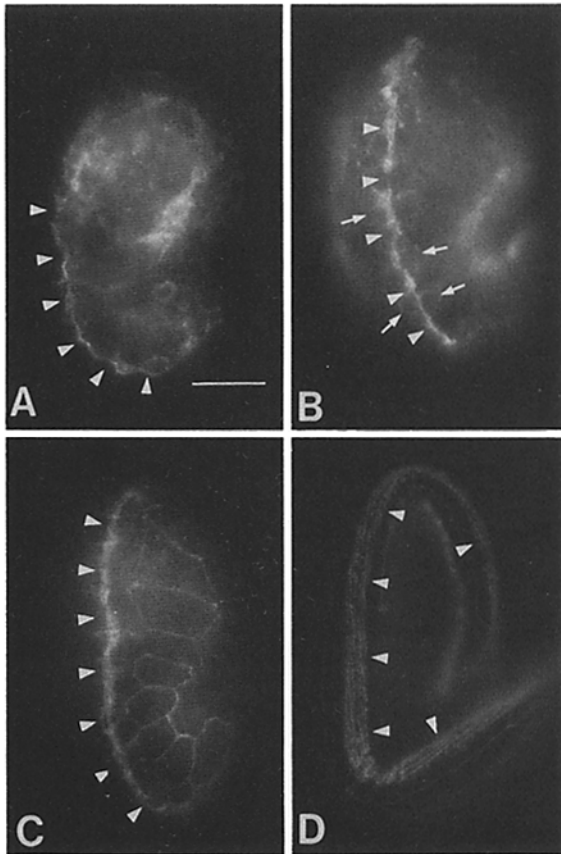
A significant change is evident by 350 min, when muscle components become localized near the hypodermis, showing that the muscle cells have become polarized. For example, integrin is localized at the membrane where adjacent muscle cells contact each other and the hypodermis (Fig. 4, A and B); embryos must be viewed from two different angles to confirm this localization. The lateral view of the embryo in Fig. 4 A shows that integrin (arrowheads) has accumulated at the membrane where muscle cells contact the hypodermis, and the dorsolateral view of a slightly older embryo in Fig. 4 B, shows integrin has accumulated at membranes where adjacent muscle cells contact each other. The integrin-dependent fluorescence in each micrograph appears as a continuous thin line along the length of the embryo. This specific subcellular distribution is also seen using antibodies against vinculin, actin, and myosin A or B (data not shown), which colocalize with integrin. The change in the distribution of muscle components from a diffuse distribution to a more localized distribution at the membrane is apparently a gradual change. Embryos of 310 min can be found that have some

of a given muscle component concentrated at this membrane, while some antigen is still diffuse in the cell.

As muscle cells polarize, perlecan, a muscle basement membrane proteoglycan, is first detected. Interestingly, it also localizes to membranes where adjacent muscle cells contact each other and the hypodermis (Fig. 5). The fact that perlecan is first detected at this late stage does not rule out the possibility that it accumulates earlier, but is not detected by the fixation and staining procedures used here.

In the third stage of assembly, muscle cells flatten against the hypodermis, and the breadth of the lattice increases, with evidence of alignment between cells (shown for integrin in Fig. 4 C). By the 1.5-fold stage (420 min) integrin staining is not detected as a thin line, but as a band that runs along the anterior-posterior axis of the embryo. Sometimes embryos are seen that have two antibody-positive bands associated with each quadrant, one associated with each row of muscle cells (data not shown). No obvious discontinuities are seen in the staining pattern shown in Fig. 4 C, suggesting alignment of components in adjacent cells. The M-line component recognized by MH42 is first detected at this stage and colocalizes with other lattice components at the membrane that contacts the hypodermis (data not shown).

Finally, the details of sarcomere organization become evident. At about the stage when the embryo starts twitching, structural elements of the muscle (i.e., dense bodies, I- and A- bands, and M-lines) can be detected using appropriate antibodies, and by the twofold stage (450 min), the structures can be detected along the entire length of each quadrant. Al-



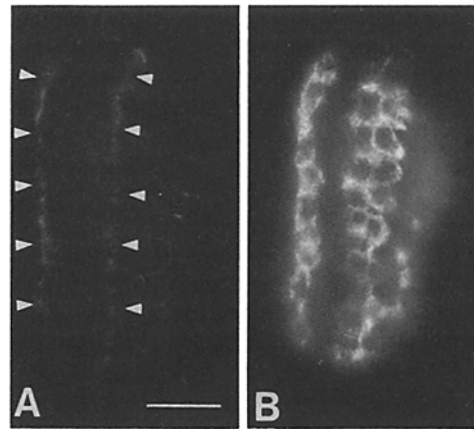
**Figure 4.** Localization of integrin (MH25) in embryos of various developmental stages. Fluorescence micrographs, each showing a single dorsal muscle quadrant, of embryos stained with MH25 and MH27. MH27-dependent fluorescence can be seen as a grid pattern in panels C and D. (A) Right lateral view of a 350-min embryo and (B) dorsolateral view of a 390-min embryo showing MH25-dependent fluorescence (arrowheads) at membranes of muscle cells that contact each other and the hypodermis. Muscle cell nuclei can be seen in B as negatively stained holes in the fluorescence pattern (arrows). (C) Dorsolateral view of a 420-min embryo. No obvious discontinuities in the MH25 staining pattern (arrowheads) are detected along the length of the quadrant. (D) Embryo of at least 520 min (threefold stage), showing the MH25 staining pattern (arrowheads) of lines and rows of dots that run obliquely to the long axis of the embryo. This pattern indicates that integrin is organized into dense bodies and M-lines. Bar, 10  $\mu$ m.

though actin can be detected along the length of the quadrant, the actin is not restricted to I-bands until the late threefold stage (>520 min). The structural elements can be easily resolved in threefold embryos (Figs. 4 D and 6).

The  $\alpha$ -actinin isoform recognized by MH35, and detected in adult body wall muscle (20) is not detected in embryos fixed by the method used here or any others used to date (Fig. 6 D). The method used allows penetration of antibodies up to the stage of cuticle deposition which begins at least 3 1/2 h after the muscle starts contracting (62).

#### **Development of Muscle Attachment Structures in Four Distinct Stages**

The close association between muscle cells and the hypodermis led us to investigate the temporal and spatial distribu-



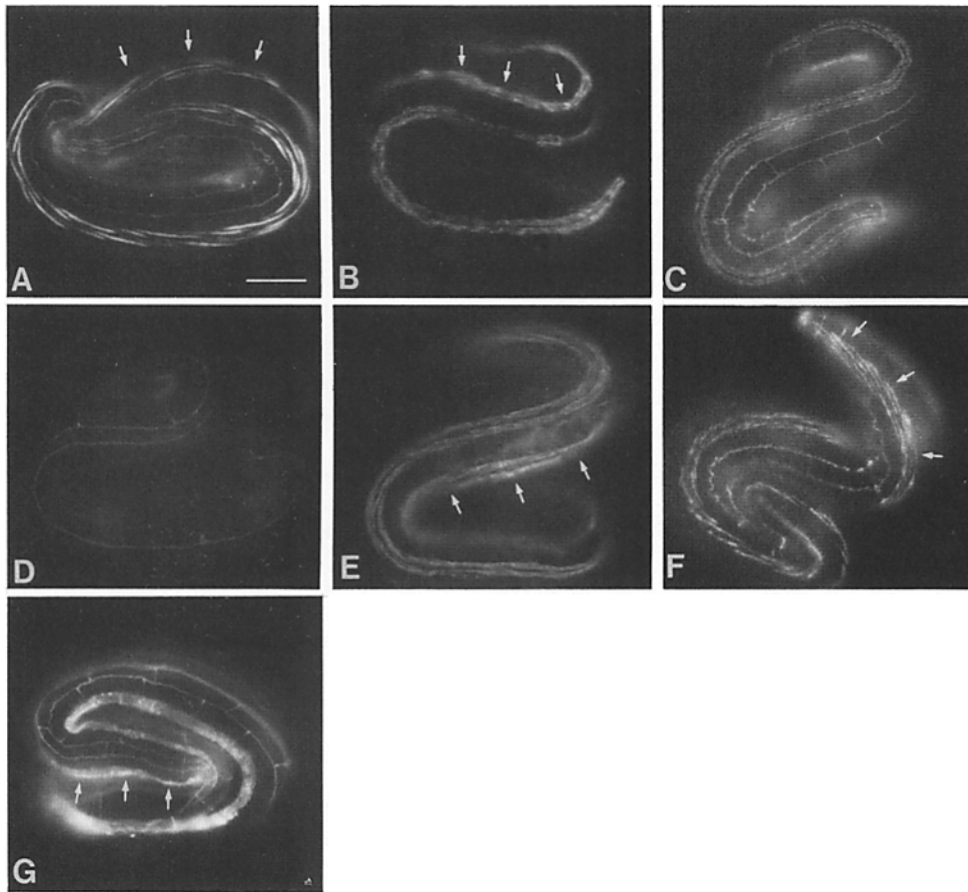
**Figure 5.** Localization of perlecan with respect to muscle cells in a 350-min embryo. Fluorescence micrograph of a dorsal view showing both dorsal muscle quadrants of an embryo labeled with (A) MH3 (perlecan; arrowheads) and (B) R6-2 (myosin). Comparison of A and B shows that perlecan is localized to regions of contact between muscle cells. MH27-dependent fluorescence is seen as fine lines that form a grid pattern in A. Bar, 10  $\mu$ m.

tion of hypodermal components. The hypodermal antigens recognized by MH4 and MH5, and the basement membrane antigen recognized by MH46 all associate with hemidesmosomes in adults (21). The antigens detected by these antibodies are first seen in embryos younger than 290 min. All three are expressed by the dorsal and ventral, but not seam, hypodermis at a stage when the muscle cells are adjacent to seam cells (shown for MH4 in Fig. 7 A). The MH5 and MH46 antigens are diffusely distributed in these cells, whereas the MH4 antigen localizes to the periphery of the cells.

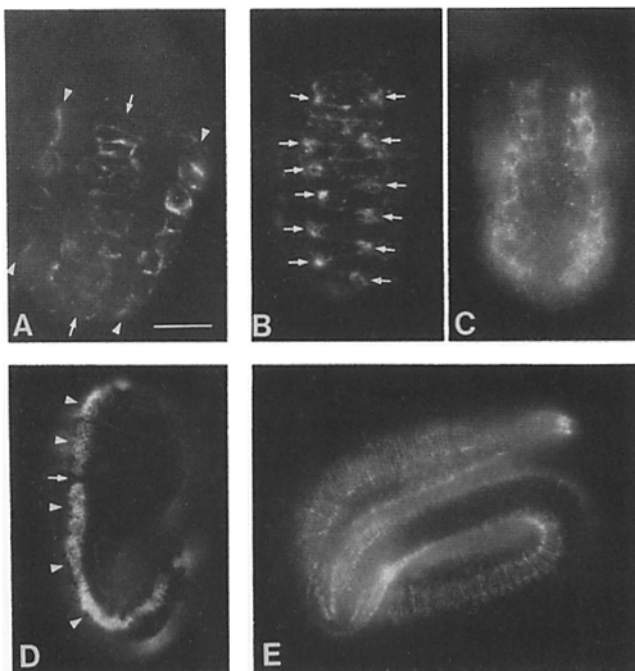
By 310 min, all three antigens accumulate in regions adjacent to muscle cells. By this time the muscle cells have begun to migrate off the seam cells and onto dorsal and ventral hypodermis. The MH4 and MH5 antigens in 310-min embryos colocalize in patches in regions of the dorsal and ventral hypodermis adjacent to the body wall muscle quadrants (shown for MH4 in Fig. 7, B and C). These MH4 (52) and MH5-positive patches are at opposite ends of adjacent dorsal hypodermal cells and are always associated with the end of the hypodermal cell that does not extend a process during interdigitation. The ventral hypodermal cells at this stage are in two rows, one on each side of the ventral midline. Patches of MH4 and MH5-positive material are seen in each cell and are adjacent to the ventral muscle quadrants (data not shown).

The MH46 antigen, a hypodermal basement membrane component, also becomes localized to regions adjacent to muscle cells, but unlike the MH4 and MH5 antigens, it remains dispersed until 350 min. In 350-min embryos, the MH46 antigen colocalizes with perlecan at the junction between adjacent muscle cells and the hypodermis (compare Fig. 8, A and B with Fig. 5, A and B).

By 420 min (1.5-fold stage), as the embryo elongates and the muscle cells flatten, all three hypodermal antigens, as well as perlecan, are coextensive with the developing muscle. That is, the regions occupied by the contractile apparatus, the basement membrane and hemidesmosome components have the same boundaries, although in different tissues.



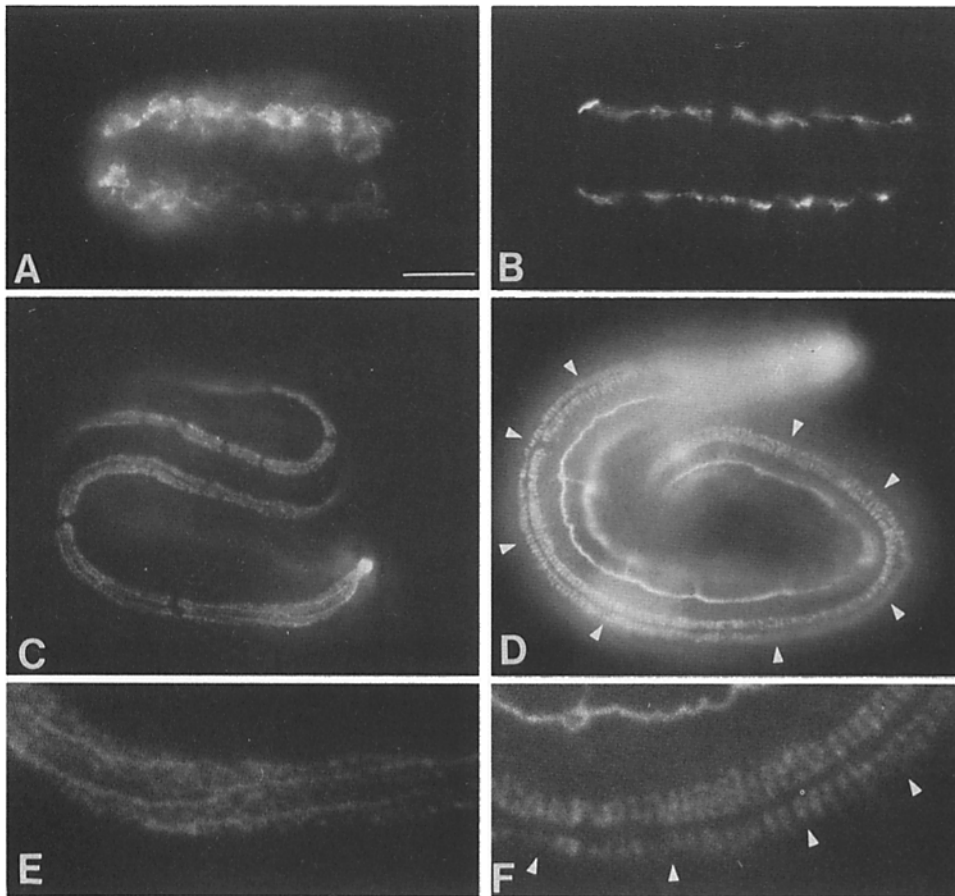
**Figure 6.** Localization of muscle components in embryos of at least 520 min (threefold stage). Embryos were labeled with MH27 (seen as fine lines that form a grid pattern in A, C, D, F, and G) and (A) DM5.6 (myosin A), (B) R6-2 (myosin), (C) MH24 (vinculin), (D) MH35 ( $\alpha$ -actinin), (E) C4 (actin), (F) MH42 (M-line component), and (G) MH3 (perlecan). Staining of at least one muscle quadrant is shown in each panel, and where applicable, arrows indicate staining of a second quadrant. Bar, 10  $\mu$ m.



**Figure 7.** Localization of the MH4 antigen in embryos of various developmental stages. Fluorescence micrographs of embryos labeled with MH27, MH4, and R6-2. Embryos shown in A-D are oriented with their anterior ends toward the top of the page. (A) Dorsal view, focused on the surface of a <290-min embryo showing MH4-dependent fluorescence in the rows of dorsal and ventral hy-

podermal cells. At this stage, all the hypodermal cells are clustered on the dorsal surface of the embryo. Anterior and posterior ends of the rows of dorsal (*arrows*) and ventral (*arrowheads*) hypodermal cells are indicated. (B and C) Dorsal view, focused on the surface (B), showing localization of MH4, or just below the surface (C), showing the position of muscle cells in a 310-min embryo. Comparison of A and B shows that the MH4 positive material is organized into patches, marked by arrows in B, in regions of the hypodermis adjacent to the muscle quadrants. (D) Lateral view of a 420-min embryo showing MH4-positive material (*arrowheads*) adjacent to one ventral muscle quadrant. Arrow indicates a break in the MH4 staining pattern associated with a region of the ventral hypodermis that contacts the H-shaped excretory cell. (E) Surface view of an embryo of at least 520 min showing MH4-positive material associated with one quadrant. The MH4 staining pattern is coincident with muscle cells, and consists of lines oriented perpendicular to the muscle lattice. Bar, 10  $\mu$ m.





**Figure 8.** Localization of the MH46 antigen in embryos at various developmental stages. Fluorescence micrographs of embryos labeled with MH46, MH27, and R6-2. (A and B) Dorsal view, with the anterior end towards the right, showing both dorsal muscle quadrants. Comparison of (A) R6-2 (myosin) and (B) MH46 (basement membrane component) staining in a 340-min embryo, shows that the MH46 antigen is localized to sites of contact between muscle cells. (C) Embryo of at least 520 min, with parts of 2 quadrants in focus. The MH46-dependent fluorescence is seen as rows of dots that run obliquely to the long axis of the worm. (D) A threefold stage embryo older than the embryo shown in C. The MH46-dependent fluorescence (arrowheads) is seen as lines that transverse the muscle cells, and are oriented perpendicular to the long axis of the worm. E and F are higher magnifications of regions of the embryos shown C and D, respectively. Arrowheads in F indicate the MH46-dependent fluorescence. Bars: (A–D) 10  $\mu$ m or (E and F) 33  $\mu$ m.

of MH5 appears punctate (data not shown), a difference that persists through adulthood (21).

Finally, the MH4 and MH5 antigens organize. This occurs by about the twofold stage (450 min), when the MH4 and MH5 staining pattern becomes similar to that seen in adults; i.e., a series of lines that run perpendicular to the muscle lattice in the region of the hypodermis adjacent to muscle cells (shown for MH4 in Fig. 7 E).

By contrast, the MH46 antigen does not appear to associate with hemidesmosomes until still later in development. At the twofold stage, and even into the threefold stage, the MH46 antigen is organized into dots aligned in rows that are oblique to the long axis of the embryo (Fig. 8, C and E). The pattern is reminiscent of the oblique striations of the body wall muscle, suggesting that the MH46 antigen is associated with a specific muscle structure. Remarkably, in older threefold embryos the MH46 staining pattern becomes similar to that detected by MH4 and MH5, as well as by MH46 in adults (compare Fig. 7 E with Fig. 8, D and F), and suggests an association with hemidesmosomes.

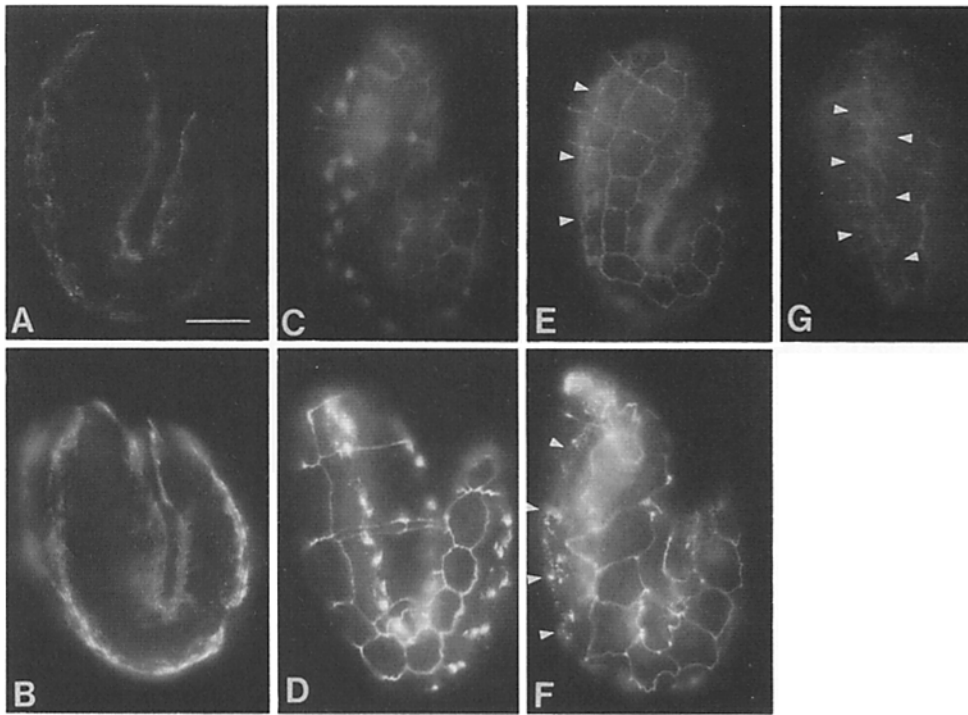
### **The Effect of *pat* Mutations on Body Wall Muscle Assembly**

The spatial and temporal distributions of muscle components in mutants with defects in muscle assembly were compared with those of wild type to determine when the assembly process was, and what components were, disrupted by the mutations. The proteins encoded by the genes to which the mutations map are known and represent components of dense

bodies, M-lines, thick filaments, and the basement membrane. Embryos homozygous for the mutations are negative for the antibody that recognizes the wild-type gene products and therefore the mutations may represent null alleles. We cannot rule out the possibility that an undetectable amount of the wild-type product or an undetectable truncated product is made (for a description of the mutant phenotype see reference 75). The distribution of components representing the major structural elements of the sarcomere, the dense body, M-line, A-band, and I-band was examined in embryos homozygous for each of the mutations.

The mutations most disruptive to muscle assembly, *unc-52(st549)* (55, 75) and *pat-3(st552)*, (75) map to genes that encode the membrane-associated molecules perlecan (55) and integrin (Gettner, S., C. Kenyon, L. Reichardt, J. Plenefisch, M. B. Buchner, and E. Hedgecock. 1992. *Mol. Biol. Cell.* 3:1088a). The body wall muscle cells of *unc-52(st549)* and *pat-3(st552)* embryos migrate to form quadrants, and attach to and spread on the basement membrane. This can easily be seen in mutant embryos stained for myosin (Fig. 9, A and B). Four quadrants can be detected, and the myosin-positive cells contact adjacent myosin-positive cells along the anterior-posterior axis. This could only occur if the cells flatten and elongate as the embryo elongates.

Although the muscle cells in *pat-3(st552)* and *unc-52(st549)* embryos migrate, attach and spread, the structural elements of the muscle are completely disrupted (75; Fig. 9, C–F). The MH42 antigen, an M-line protein, localizes to a region at the middle of each body wall muscle cell in the mutant embryos. Interestingly, this localization is similar to the local-



**Figure 9.** Localization of muscle lattice components in embryos homozygous for the mutation *unc-52(st549)* (A, C, E, and G) or *pat-3(st552)* (B, D, and F). Fluorescence micrographs showing embryos labeled for a muscle antigen and for the MH27 antigen. MH27-dependent fluorescence is seen as fine lines that form a grid pattern in the embryos shown in C–G. (A and B) Embryos labeled with R6-2 (myosin). No gaps are detected between muscle cells, although the myosin is not organized into A-bands. (C and D) Embryos labeled with MH42 (M-line component). The MH42 antigen appears to accumulate in the central region of each body wall muscle cell. (F) Embryo labeled with MH24 (vinculin). The MH24-positive material (arrowheads) does not appear to be organized into dense bodies (compare

with Fig. 6 C). (E and G) Embryos labeled with MH25 (integrin). (E) A 420–450-min embryo (1.75-fold stage), where the MH25 positive material (arrowheads) does not appear to be organized into M-lines or dense bodies. (G) A 420-min embryo in which the MH25-positive material (arrowheads) is not associated with the membranes where adjacent muscle cells contact each other and the hypodermis. Bar, 10  $\mu$ m.

ization of myosin A (75), but not of myosin B (Fig. 9, A and B) in these mutants, suggesting that the MH42 antigen is associated, even in mutant animals, with myosin A or a myosin A-associated protein.

Vinculin (MH24) and integrin (MH25) are both components of the base of the dense body (20). *pat-3(st552)* embryos show an MH24 staining pattern consistent with dense body disruption (Fig. 9 F). Likewise, *unc-52(st549)* embryos show an MH25 staining pattern consistent with dense body or M-line disruption (Fig. 9 E). Furthermore, the localization of integrin in *unc-52(st549)* embryos may be disrupted early, at the stage of muscle cell polarization. Embryos at the 1.5-fold stage (420 min) can be found in which integrin is not at the membranes where adjacent muscle cells contact each other and the hypodermis (Fig. 9 G).

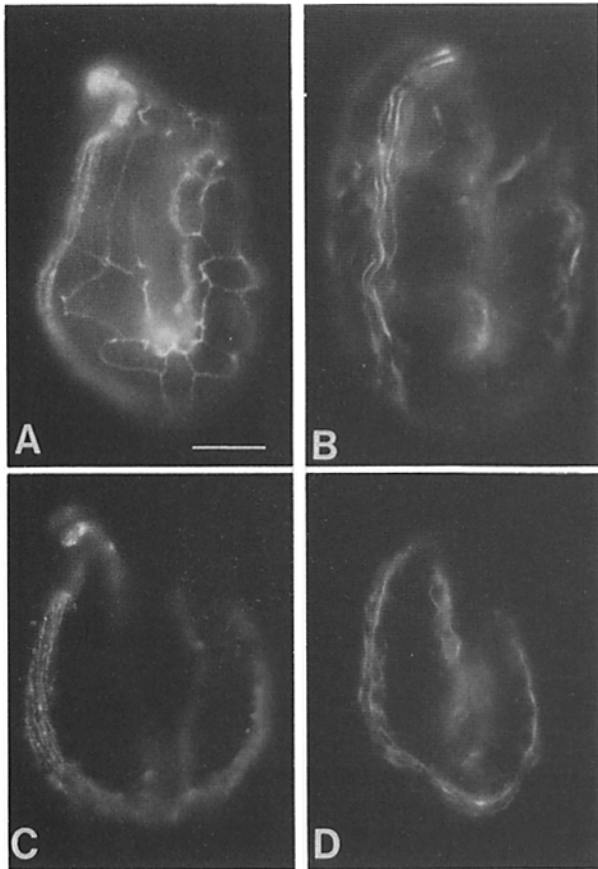
On the other hand, mutations that affect internal components of the lattice are less disruptive to muscle assembly. *deb-1(st555)* embryos, which are mutant for vinculin (5), have disrupted I-bands, but A-bands assemble quite well (5); *myo-3(st386)* embryos, which are mutant for myosin A (72), have disrupted A-bands, but I-bands assemble quite well (72). Furthermore, the localization of muscle components in *myo-3(st386)* and *deb-1(st555)* embryos shows that components closer to the membrane than the affected gene product are organized relatively well, whereas components further from the membrane are not. Integrin is associated with the base of the dense body, and is therefore closer to the membrane than vinculin. The integrin staining pattern seen in arrested *deb-1(st555)* embryos is not as well organized as in wild-type embryos (see Fig. 4 D), but lines and rows of dots that run obliquely to the long axis of the worm are detected

and appear to be spaced as in wild type (Fig. 10 A). This result is consistent with the organization of integrin into M-lines and dense bodies. However, actin filaments, which are anchored to the membrane by the dense body, and therefore further from the membrane than vinculin, are seen throughout the cell (compare Figs. 2 E and 10 B; see also 5). Likewise, at the M-line, integrin is located closer to the membrane than myosin A. The integrin staining pattern in *myo-3(st386)* embryo is consistent with the organization of integrin into M-lines as well as into dense bodies (Fig. 10 C). However, myosin B, which is further from the site at which the thick filaments are anchored to the membrane than is myosin A, is disorganized in *myo-3(st386)* embryos (Fig. 10 D).

#### **The Effect of *pat* Mutations on the Assembly of Attachment Structures**

Homozygous *unc-52(st549)* and *pat-3(st552)* embryos show an almost wild-type distribution of MH4 in late arrested animals, when considerable structural degradation might be expected (Fig. 11, A and D). Thin stripes, perpendicular to the anterior-posterior axis of the worm, are seen in regions of the hypodermis adjacent to muscle cells, and the spacing of these perpendicular stripes appears wild-type. The width of the region with respect to the circumference of the worm varies however, unlike the uniform width in wild-type embryos. Younger embryos with abnormal staining patterns were not seen.

The MH5 antigen in *unc-52(st549)* and *pat-3(st552)* embryos appears less organized than the MH4 antigen in that by the 1.75-fold stage (435 min) an abnormal pattern is de-



**Figure 10.** Localization of muscle components in embryos homozygous for *deb-1(st555)* (A and B) or *myo-3(st386)* (C and D). (A and C) Fluorescence micrographs of arrested embryos labeled with MH25 (integrin) and MH27. MH27-dependent fluorescence is visible only in A. The MH25 staining pattern is seen as lines and rows of dots that run obliquely to the long axis of the worm, similar to the pattern seen in wild-type (compare to Fig. 4 D). (B) Fluorescence micrograph of an embryo almost twofold in length (430–450-min) labeled with C4 (actin). Bundles or aggregates of actin filaments are seen all through the muscle cells, and are not organized into I-bands (compare to Fig. 6 E). (D) Fluorescence micrograph of a twofold embryo (450 min) labeled with DM5.8 (myosin B). Myosin B is seen all through the muscle cell and is not organized into A-bands. Bar, 10  $\mu$ m.

tected (Fig. 11, B and E). The staining pattern looks more diffuse and the zone where the antigen localizes is not of uniform width. Late arrested embryos show a near wild-type pattern of transverse stripes (Fig. 11, C and F), although in almost all cases the intensity of staining is reduced when compared to the staining seen in wild-type embryos.

Another component of muscle attachment structures, the basement membrane, is affected in *unc-52(st549)* and *pat-3(st552)* embryos, as shown by an altered MH46 staining pattern. By the 1.75-fold stage (compare Fig. 12, B and C with A) the MH46-positive zone is not of uniform width, and the dots of antigen are more randomly distributed than in wild-type embryos. Mutant embryos arrest elongation at the twofold stage, but development continues through hatching as assayed by pharyngeal development (75). However, in contrast to late wild-type threefold embryos, mutants with MH46-

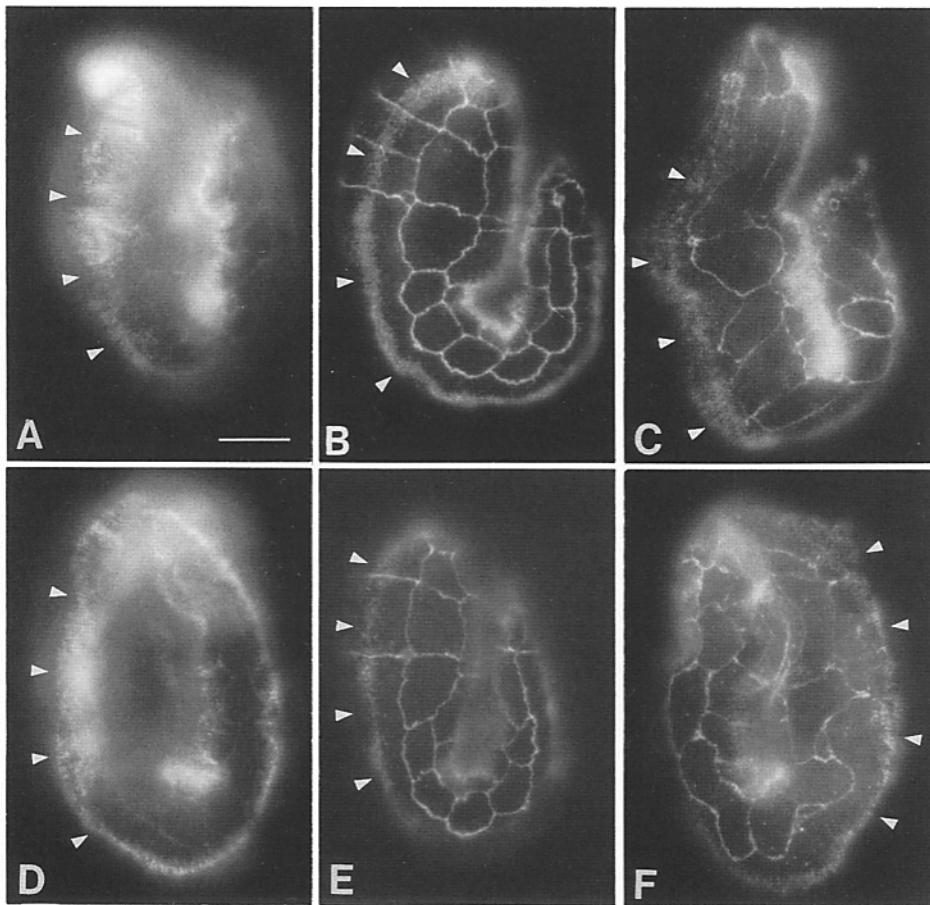
positive stripes traversing the muscle cells (Fig. 8, D and F) were never seen (Fig. 12 D).

## Discussion

The observations presented in this paper define four stages of muscle assembly that can be detected in the body wall muscle cells of *C. elegans*. The stages are summarized in Table II and shown schematically in Fig. 13. With two clear exceptions, the observations are consistent with models depicting muscle assembly in vertebrates. Muscle components accumulate at the membrane before sarcomeres are detected, suggesting that membrane or membrane-associated molecules are important in templating or initiating the assembly process. This observation is consistent with observations made using primary cultures of cardiocytes (15, 44, 59, 68) or skeletal myocytes (2, 26), where myofibrils also appear to form at the membrane. A- and I-bands can assemble independently of one another in *C. elegans* (5, 72, 75), as well as in *D. melanogaster* (6) and cultured cells (1, 26, 29, 59, 64). Finally, actin is one of the last components to form fully striated sarcomeres in the body wall muscle. One of the late events in muscle assembly in vertebrate muscle cells in culture is the change from non-striated to striated actin bundles (26, 51), a process that may involve CapZ (57).

One clear difference between *C. elegans* and vertebrate muscle cell differentiation is the stage at which muscle specific proteins first accumulate in the cells. The only muscle protein known to be expressed in replicating vertebrate myoblasts is desmin (14, 28), whereas in *C. elegans* at least three, and probably other muscle proteins are expressed before all the cells become post-mitotic. This is shown for myosin in Fig. 3, D–F, where a dividing cell, as indicated by the presence of a mitotic spindle and condensed chromatin, is positive for muscle myosin. Although not directly demonstrated, vinculin and integrin are almost certainly expressed before the cells become post-mitotic, since both are expressed at about the same time and in what appear to be the same cells as myosin.

A second difference between muscle assembly in vertebrates and in *C. elegans* concerns the role of  $\alpha$ -actinin. Sarcomeric  $\alpha$ -actinin in cultured muscle cells is a component of NSMF (non-striated myofibrils) and of I-Z-I complexes, leading some investigators to propose that aggregates containing  $\alpha$ -actinin act as nucleating or organizing centers for thin filaments (59). Alternatively, others have proposed that  $\alpha$ -actinin may act to stabilize thin filaments at the Z-disc in cardiac muscle cells (63). The  $\alpha$ -actinin isoform present in the adult body wall muscle of *C. elegans* and recognized by MH35 is not detected in embryos, but is detected in L1 larvae (Frances, G. R., and R. H. Waterston, unpublished result). This result suggests that this isoform of  $\alpha$ -actinin is not required for the assembly of I-bands in body wall muscle cells. Similarly, *D. melanogaster* mutants lacking the  $\alpha$ -actinin isoform expressed in the indirect flight muscle have fairly well organized flight muscles, again suggesting  $\alpha$ -actinin is not required for assembly (56). Interestingly, neither the  $\alpha$ -actinin recognized by MH35, or the *D. melanogaster* larval isoform of  $\alpha$ -actinin are required for embryonic contractions, whereas the adult *D. melanogaster* muscle isoform is required for flight (24). Perhaps  $\alpha$ -actinin is required only



**Figure 11.** Localization of hypodermal hemidesmosome components in embryos homozygous for mutations *unc-52(st549)* (A–C) or *pat-3(st552)* (D–F). (A, C, D, and F) Fluorescence micrographs of arrested embryos double labeled with MH27 and MH4 (A and D) or MH5 (C and F). The MH4 and MH5 staining patterns (arrowheads) reveal a series of lines, perpendicular to the muscle lattice, in regions directly adjacent to the muscle cells. This pattern is similar to that detected in wild type. (B and E) Fluorescence micrograph of 1.75-fold (420–450 min) embryos labeled with MH27 and MH5. The MH5-dependent fluorescence (arrowheads) is detected as dots, randomly distributed in the region of the hypodermis adjacent to muscle cells. The grid pattern seen in the micrographs is due to MH27-dependent fluorescence. Bar, 10  $\mu$ m.

for stabilizing thin filaments within the I-band. Alternatively, *D. melanogaster* and *C. elegans* may express other  $\alpha$ -actinin isoforms that are responsible for organizing thin filaments during muscle formation. There is evidence for at least two  $\alpha$ -actinin genes in *C. elegans* (4), and multiple  $\alpha$ -actinin messages in flies (56).

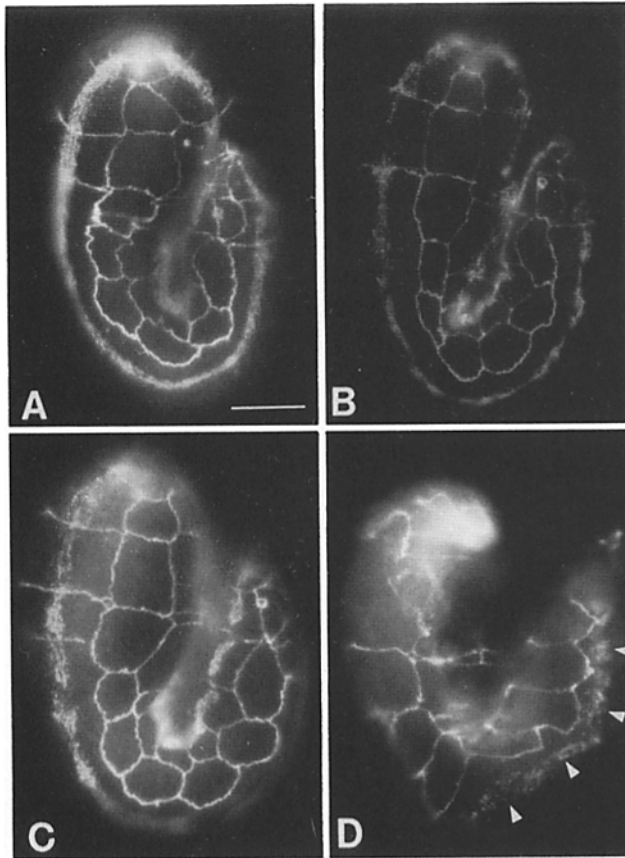
An early step in the assembly of *C. elegans* body wall muscle is the accumulation of muscle components at membranes where adjacent muscle cells contact each other and the hypodermis, which occurs at  $\sim$ 350 min. These components include proteins that will become part of the dense body, and thin and thick filaments (16). The resolution of the light microscope is not sufficient to allow us to decipher what assemblages are forming, but possibly filament assembly and muscle polarization occur simultaneously.

Genetic evidence presented here and in the accompanying paper (75) support the contention that membrane or membrane-associated molecules are important for body wall muscle assembly. Integrin is a transmembrane, heterodimeric receptor, consisting of an  $\alpha$  and a  $\beta$  subunit, which has been implicated in signal transduction (reviewed in 30, 34) and in assembly of multi-component structures (9). The *pat-3(st552)* mutation, a mutation in the *C. elegans*  $\beta_1$  integrin gene (Gettner, S., C. Kenyon, L. Reichardt, J. Plenefisch, M. B. Buchner, and E. Hedgecock. 1992. *Mol. Biol. Cell.* 3:1088a), has dramatic effects on the assembly of body wall muscle. The body wall muscle cell precursors of *pat-3(st552)* embryos migrate and align to form quadrants, and flatten against the hypodermis. However, the contractile units, at the

level of the dense bodies, the M-lines and the A- and I-bands are completely disrupted. The defect can be observed as early as 1.75-fold stage (430 min) when the structural elements of the muscle are first discernible in wild-type embryos. Therefore, it is likely that the disruptions are due to a failure to assemble the lattice, rather than due to secondary degradation of correctly assembled muscle.

The results presented concerning *pat-3(st552)* homozygotes are consistent with the observation that Z-discs do not form during embryogenesis of *Drosophila let(myospheroid)* (66), a null mutation in the  $\beta_1$  integrin gene (41). However, our results contradict those of Jaffredo et al., who suggest that  $\beta_1$  integrin is important for muscle cell migration in chick embryos (31). The reasons for the contradiction are not clear at this time. Possibly there are as yet unidentified  $\beta$  integrin genes of *C. elegans* that encode subunits required for migration, attachment, and flattening of muscle cells.

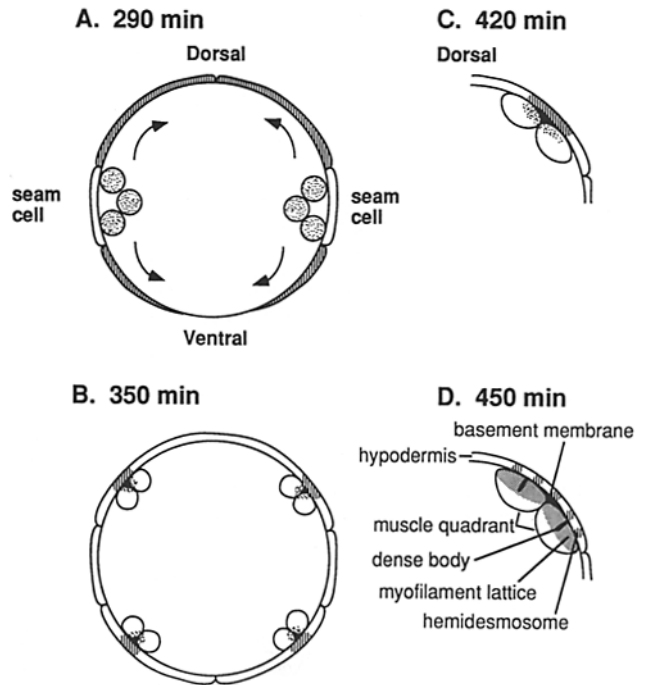
Another class of membrane-associated components that affect muscle cell differentiation are extracellular matrix proteins. It is clear that the composition of the extracellular matrix is important for muscle differentiation in vitro, although the molecules involved have not been fully defined. Mouse embryonic muscle cells will continue to divide when plated on laminin until fibronectin is removed from the media (67). *Drosophila* embryonic muscle cells in culture will not adhere to, or spread on, a number of known *Drosophila* extracellular matrix components, but will attach and undergo terminal differentiation when plated on laminin (66). Similarly, proper assembly of A- and I-bands (75) as well as the



**Figure 12.** Localization of the MH46 antigen in wild-type (A), *pat-3(st552)* (B), or *unc-52(st549)* homozygotes (C and D). (A–C) Fluorescence micrographs of 1.75-fold (420–450-min) embryos. MH46-dependent fluorescence in wild-type embryos (A) is observed as an ordered array of dots associated with muscle cells, and in the mutants (B and C) is observed as a random distribution of dots associated with muscle cells. (D) Fluorescence micrograph of an arrested *unc-52(st549)* homozygous embryo. Arrowheads indicate MH46-dependent fluorescence which is observed in a random distribution associated with muscle cells. The grid pattern seen in the micrographs is due to MH27-dependent fluorescence. Bar, 10  $\mu$ m.

assembly of the dense bodies and the M-lines (Fig. 8, C–G) is dependent on perlecan, a basement membrane proteoglycan, and thus identifies a specific basement membrane component necessary for muscle assembly.

In contrast, mutations in genes encoding internal components of the muscle do not completely disrupt lattice assembly, but instead selectively disrupt assembly of a subset of structures. Furthermore, only the distribution of components further from the membrane than the affected gene product is altered, while the distribution of components closer to the membrane is much less affected. This is true for both *myo-3(st386)* and *deb-1(st555)* which disrupt A- and I-bands, respectively, but do not affect the organization of integrin at the base of the M-line and the dense body. This suggests that body wall muscle assembly occurs by the stepwise addition of components, and is nucleated at the membrane or extracellular matrix. In addition, since A- and I-bands can assemble independently of one another (5, 72, 75), it is possible that M-lines plus A-bands, and dense bod-



**Figure 13.** Schematic diagram of muscle assembly in *C. elegans* depicting cross-sections of embryos at various developmental stages. The diagrams represent our view of body wall muscle assembly based on the data presented in this paper. (A) 290-min embryo. Hypodermal cells (2 dorsal, 2 lateral or seam, and 2 ventral) are shown as thin cells covering most of the embryo; only the ventral surface is exposed. Dorsal hypodermal cells reach only to the dorsal midline since they have not yet interdigitated. Hypodermal hemidesmosome components are present in dorsal and ventral hypodermal cells as indicated by the hatched region. Muscle cells (dots), are adjacent to seam cells and will migrate dorsally or ventrally to contact dorsal or ventral hypodermis. Some muscle cells will divide again before assuming their final position. Muscle cells (circles) are still rounded, but have become polarized. That is, myofibrillar components (dots) are localized to membranes where adjacent muscle cells contact each other and the hypodermis. Basement membrane components (black) are also localized to regions of contact between muscle cells. Hypodermal hemidesmosome components (hatched region) have become restricted to regions of the hypodermis adjacent to muscle cells. (C and D) One dorsal quadrant plus the surrounding region is used to illustrate events that occur in each of the four quadrants. (C) 420-min embryo. Muscle cells (ovals) are flattened, and the muscle (dots), basement membrane (black) and the hemidesmosome (hatched regions) components are coextensive. (D) 450-min embryo. Organization of the structural components of the muscle (stippled region) and the hypodermal hemidesmosomes (hatched regions of the hypodermis) is evident. See text for details of the model.

ies plus I-bands assemble as units, with the process in both cases beginning at the membrane. It is interesting to note that two membrane-associated components that are required for muscle assembly, perlecan and integrin, localize to the base of the dense body and M-line in adults. Perhaps these two molecules are part of the nucleation site for muscle assembly.

Strikingly, the pattern and spacing of the structural elements present in the body wall muscle of *myo-3(st386)* and *deb-1(st555)* embryos is quite well preserved. The spacing of

dense bodies and M-lines, as marked by anti-integrin antibodies, is not significantly different from that of wild type. This suggests the spacing of structural elements of the muscle are not imposed by the filamentous elements, but by events that occur at the membrane.

The spatial and temporal association of muscle and hemidesmosome assembly suggests that important developmental signals may be passed between muscle and hypodermal cells. Muscle cells may signal the recruitment of hemidesmosome components to regions of the hypodermis adjacent to muscle cells. The localization of the MH4 antigen in embryos in which the Cap or Cpp lineages are missing is consistent with this idea. Laser ablation of Cap or Cpp at the 28 cell stage results in embryos that are missing 16 muscle cells from the posterior of the left or the right side of the animal, respectively. The hypodermal cells of such embryos organize MH4 only in regions adjacent to muscle cells but not adjacent to regions lacking the ablated muscle cells (Shrimankar, P., and R. H. Waterston, personal communication). Hypodermal cells may in turn signal the recruitment of myofibrillar components to the membrane that contacts the hypodermis. This possibility is suggested by the observation that as muscle cells migrate onto dorsal or ventral hypodermis, muscle components associate with the membrane that contacts the hypodermis. It is interesting that the basement membrane protein recognized by MH46 is synthesized by dorsal and ventral hypodermis but associates with muscle cells and possibly with a specific structural element of the muscle during muscle assembly.

The contractile apparatus is linked to and assembles coordinately with hypodermal hemidesmosomes, but mutations that completely disrupt muscle assembly have little, if any, effect on the assembly and positioning of hemidesmosomes. The distribution of the MH4 and MH5 antigens in arrested *pat-3(st552)* and *unc-52(st549)* embryos, which have completely disrupted muscle structure, is similar to the distribution observed in wild-type embryos. This suggests that although signals passed between the muscle cells and the hypodermis may coordinate the assembly of muscle and the hemidesmosomes, the position of the hemidesmosomes in the hypodermis is not dictated by the position of structural elements in the muscle. We cannot rule out that the position of the structural elements of the muscle depends on the position of hemidesmosomes in the hypodermis, since no mutations are known that disrupt the assembly of hemidesmosomes.

Although *unc-52(st549)* and *pat-3(st552)* have no effect on the assembly of the hemidesmosomes, both alter the structure of the basement membrane as assayed by the distribution of the MH46 antigen. The *unc-52* gene product is a component of the same basement membrane as the MH46 antigen, and therefore it is not unexpected that a mutation in the *unc-52* gene might affect the organization of the MH46 antigen. The altered distribution of the MH46 antigen in *pat-3(st552)* mutants could be a secondary effect of disorganized muscle since the MH46 antigen appears to associate with muscle during part of embryogenesis.

### ***A Summary of Muscle Assembly***

The stages of *C. elegans* muscle assembly described below are diagrammed schematically in Fig. 13. Muscle components begin to accumulate while muscle cells are in two

lateral rows adjacent to the seam cells and before some muscle cells have undergone their terminal division. As the muscle cells migrate dorsally and ventrally to contact dorsal or ventral hypodermis, they encounter signals that direct the polarization of muscle cells. At about this time, hemidesmosome and basement membrane components are recruited to regions adjacent to muscle quadrants. The recruitment of hemidesmosome components is most likely specified by signals from the muscle cells.

Muscle cells flatten against the hypodermis, and the breadth of the developing lattice increases. At this stage, muscle, basement membrane and hemidesmosome components become coextensive and presumably early contacts are set up between structural elements of the muscle and the hemidesmosomes that help to keep the structures in register throughout elongation.

As sarcomeres assemble, events occurring at the membrane dictate the spatial deployment of structural elements along the fibril. The information that specifies spatial arrangement may involve integrin, which has been implicated in signal transduction in other systems, and perlecan. Once properly positioned, integrin and perlecan nucleate the assembly of dense bodies and the M-lines, which probably occurs by the stepwise addition of more internal components.

Actin becomes fully organized into I-bands late in development, at least 90 min after contractions are first observed. Even later,  $\alpha$ -actinin accumulates and assembles at the dense body.

Coordinate with muscle assembly is the organization of hypodermal components into hemidesmosomes. The mechanisms that specify the position and spacing of hemidesmosomes are unknown, but clearly perlecan is not required. Although links between the muscle and hemidesmosomes are likely to be set up early, possibly as early as 310 min, the connections may be reinforced later when the MH46 antigen, a basement membrane component that initially associates with muscle structures, associates with the hemidesmosomes.

A number of predictions of this model are directly testable. Muscle cell precursors can be killed early in development (62) to assess the influence of signals from muscle cells on the distribution of hemidesmosome and basement membrane components. Similar experiments can be done in which hypodermal cells are killed to assess their influence on the distribution of muscle components. Alternatively, the association between muscle cells, and dorsal or ventral hypodermal cells could be altered using mutations that change the fate of specific cells. In such mutants, muscle cells would be adjacent to cells which do not adopt the dorsal or ventral hypodermal cell fate, or dorsal and ventral hypodermal cells would be adjacent to cells which will not become muscle cells.

The isolation of mutations that alter the position of hemidesmosomes in the hypodermis would be useful in defining the molecules necessary for proper spacing of these structures. Furthermore, these mutations would be useful in determining if structural information, dictated by the spacing of hemidesmosomes, is transmitted to muscle cells and influences the spacing of structural elements of the muscle. Finally, many mutations that disrupt muscle have already been isolated (reviewed in 71, 73, 75). Using specific antibodies and the wild-type distributions described here, it should be

possible to more clearly define how, and when, muscle assembly is disrupted in the mutants.

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