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Complex Patterns of Alternative Splicing Mediate the Spatial and Temporal Distribution of Perlecan/UNC-52 in *Caenorhabditis elegans*

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The *unc-52* gene encodes the nematode homologue of mammalian perlecan, the major heparan sulfate proteoglycan of the extracellular matrix. This is a large complex protein with regions similar to low-density lipoprotein receptors, laminin, and neural cell adhesion molecules (NCAMs). In this study, we extend our earlier work and demonstrate that a number of complex isoforms of this protein are expressed through alternative splicing. We identified three major classes of perlecan isoforms: a short form lacking the NCAM region and the C-terminal agrin-like region; a medium form containing the NCAM region, but still lacking the agrin-like region; and a newly identified long form that contains all five domains present in mammalian perlecan.

Using region-specific antibodies and *unc-52* mutants, we reveal a complex spatial and temporal expression pattern for these UNC-52 isoforms. As well, using a series of mutations affecting different regions and thus different isoforms of UNC-52, we demonstrate that the medium NCAM-containing isoforms are sufficient for myofilament lattice assembly in developing nematode body-wall muscle. Neither short isoforms nor isoforms containing the C-terminal agrin-like region are essential for sarcomere assembly or muscle cell attachment, and their role in development remains unclear.

INTRODUCTION

Basement membranes are specialized regions of extracellular matrix (ECM) that have important roles in many fundamental developmental and regenerative processes, including cell adhesion and migration, signal transduction, and even gene regulation (Martin and Timpl, 1987; Yurchenco and Schittny, 1990). Many of these processes are mediated by specific interactions between basement membrane components and transmembrane receptors such as integrin (Hynes, 1992). Basement membranes contain a large number of different components, including laminin, collagen, nidogen, and heparan sulfate proteoglycans (Yurchenco and O'Rear, 1994; Timpl and Brown, 1996). Homologues of these proteins have been identified in the nematode *Caenorhabditis elegans* (reviewed in Kramer, 1997), and mutations are associated with several of these components (Guo *et al.*, 1991; Ishii *et al.*, 1992; Rogalski *et al.*, 1993; Sibley *et al.*, 1993). This genetic approach is helping to reveal the function of these basement membrane proteins during morphogenesis. In this study, we focus on perlecan, the major basement membrane heparan sulfate proteoglycan, and its role in muscle development in *C. elegans*.

In *C. elegans*, a specialized basement membrane underlies the body-wall muscles and anchors the myofilament lattice through integrin-containing adhesion complexes (reviewed in Moerman and Fire, 1997). In adult animals, there are 95 body-wall muscle cells arranged in four quadrants, two dorsal and two ventral, beneath the hypodermis (reviewed in Waterston, 1988). Each quadrant runs the length of the animal and consists of a double row of spindle-shaped cells. Within each muscle cell, the thin and thick filaments of the myofilament lattice lie just beneath the plasma membrane facing the hypodermis. These filaments are anchored by a series of attachment structures to an underlying basement membrane (Francis and Waterston, 1985, 1991).

The actin-containing thin filaments are anchored by dense bodies, which are comparable to the Z-lines of vertebrate striated muscles (Waterston, 1988). Dense bodies are similar in composition to other cell-matrix adhesion complexes (Yamada and Geiger, 1997) and include vinculin (Barstead and Waterston, 1989, 1991b), talin (Moulder *et al.*, 1996), α-actinin (Barstead and Waterston, 1991b), UNC-97/PINCH (Hobert *et al.*, 1999), and integrin (Gettner *et al.*, 1995). The myosin-containing thick filaments, in turn, are anchored by

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M-lines (Waterston, 1988). The composition of M-lines in *C. elegans* is not well defined but includes integrin (Francis and Waterston, 1985; Gettner *et al.*, 1995), UNC-97/PINCH (Hobert *et al.*, 1999), and UNC-89 (Benian *et al.*, 1996). Integrins link both dense body and M-line components to the underlying basement membrane (Francis and Waterston, 1985; Gettner *et al.*, 1995). The interaction of integrin and basement membrane components is a key early event in the assembly of these attachment structures (reviewed in Moerman and Fire, 1997).

The *unc-52* gene encodes the nematode homologue of mammalian perlecan (Rogalski *et al.*, 1993), the major heparan sulfate proteoglycan of the ECM (Noonan *et al.*, 1991; Kallunki and Tryggvason, 1992; Murdoch *et al.*, 1992). Perlecan has five distinct domains, with similarity to the low-density lipoprotein receptor (domain II), laminin (domains III and V), and the neural cell adhesion molecule (NCAM) (domain IV). Perlecan has been implicated in a number of biological processes, including glomerular filtration (Farquhar, 1982), mitogenesis and angiogenesis (Aviezer *et al.*, 1994), and cell adhesion through interactions with focal adhesion complexes (Hayashi *et al.*, 1992; Chakravarti *et al.*, 1995). Biochemical studies indicate that perlecan binds both itself and other ECM components, including laminin, collagen, nidogen, and fibronectin (Laurie *et al.*, 1986; Yurchenco *et al.*, 1987; Heremans *et al.*, 1990; Battaglia *et al.*, 1992; Iozzo *et al.*, 1994). The multidomain structure of perlecan reflects the diverse functions proposed for this molecule.

In *C. elegans*, perlecan/UNC-52 is found in the basement membrane between the body-wall muscle cells and the hypodermis and is concentrated at muscle dense bodies and M-lines (Francis and Waterston, 1991; Rogalski *et al.*, 1993). The absence of UNC-52 blocks myofilament lattice assembly during embryogenesis, resulting in a paralyzed, arrested elongation at twofold (Pat) terminal phenotype (Williams and Waterston, 1994). The body-wall muscles in *unc-52(null)* mutants lack organized A- or I-bands, and morphological studies reveal that even the earliest stages of myofilament lattice assembly are defective (Rogalski *et al.*, 1993; Williams and Waterston, 1994).

Our previous studies have demonstrated that *unc-52* pre-mRNA gives rise to a number of distinct protein isoforms through regulated alternative splicing (Rogalski *et al.*, 1993, 1995; Lundquist *et al.*, 1996). These studies identified two major classes of UNC-52 isoforms: a short form (domains I–III) and a medium form (domains I–IV) (see Figure 1). In this study, an additional isoform is described, a long form (domains I–V), which is very similar to mammalian perlecan. Further isoform diversity is generated by alternative splicing within the various domains. For example, alternative splicing of exons 16, 17, and 18 gives rise to isoforms that vary in the number of NCAM-like immunoglobulin repeats within domain IV (Rogalski *et al.*, 1993, 1995). These exons each encode a single NCAM-repeat and are arranged such that single or multiple exons can be spliced from the pre-mRNA without disrupting the reading frame. Nonsense mutations in these alternatively spliced exons are not lethal, but instead result in progressive paralysis (Brenner, 1974; Gilchrist and Moerman, 1992; Rogalski *et al.*, 1993, 1995). The relatively mild phenotype reflects the fact that not all UNC-52 isoforms are eliminated in these mutants (Rogalski *et al.*, 1993, 1995). Removing the affected exons from the mRNA by eliminating the upstream splice acceptor site results in an almost wild-type animal (Rogalski *et al.*, 1995). Thus, individual NCAM repeats within this region can be removed without disrupting muscle assembly.

The *mec-8* gene encodes a putative RNA-binding protein that regulates some of the alternative splicing events within this region of *unc-52* (Lundquist *et al.*, 1996). *mec-8* mutations exhibit a synthetic lethal interaction with viable *unc-52* mutations; *mec-8; unc-52 (viable)* double mutants are paralyzed and arrest at the twofold stage of embryonic development (Lundquist and Herman, 1994). This synthetic lethal interaction results from the absence of *mec-8*-dependent splicing events. In a *mec-8(+)* background, splicing around affected exons permits the expression of some full-length products that are sufficient for embryogenesis to proceed normally (Lundquist *et al.*, 1996); however, in the absence of *mec-8*-function, these splicing events do not occur, preventing the expression of full-length products.

In this study, antibodies specific to domains III, IV, and V of UNC-52 were used to study the three major groups of protein products. We found both temporal and spatial differences in the localization of UNC-52 isoforms. In embryos, short (domains I–III) isoforms are associated with the pharyngeal and anal muscles, whereas domain IV-containing isoforms are associated with the body-wall muscles. In adults, domain IV-containing isoforms become more widely distributed and are detected in basement membranes adjacent to most contractile tissues. We identified Pat alleles that specifically eliminate domain IV-containing isoforms, indicating that these isoforms are essential for myofilament lattice assembly in the body-wall muscles. In addition, we show that it is possible to remove up to four consecutive NCAM repeats without adverse effects on muscle development. Our results suggest that the number of NCAM repeats within domain IV is not of primary importance, nor are specific repeats within this region essential. Using a similar approach, we examined the newly identified domain V of UNC-52. Surprisingly, a deletion that specifically removes this region has no adverse effects on myofilament assembly or stability.

MATERIALS AND METHODS

Nematode Strains and Culture Conditions

Nematodes were grown on NGM plates as described by Brenner (1974). Some strains were obtained from B. D. Williams (University of Illinois at Urbana-Champaign, Urbana, IL) and R. H. Waterston (Washington University, St. Louis, MO). Additional strains were provided by the Caenorhabditis Genetics Center. *C. elegans* strains used in this work include the wild-type strains N2; CB444, *unc-52(e444)*; CB669, *unc-52(e669)*; CB998, *unc-52(e998)*; CB1012, *unc-52 (e1012)*; CB1421, *unc-52(e1421)*; DM4001, *unc-52(st196::Tc1)*; RW6010, *unc-52(st549)/unc-52(st549)/mnDp34*; RW6011, *unc-52(st546)/unc-52(st546)/mnDp34*; and RW6013, *unc-52(st560)/unc-52(st560)/mnDp34*. The deletion allele *unc-52(gk3)* was provided by the *C. elegans* Reverse Genetics Core facility at the University of British Columbia.

PCR Amplification of Genomic DNA

Standard PCR reactions were performed essentially as described by Barstead *et al.* (1991). For long-range PCR, the standard PCR buffer and *Taq* polymerase were replaced with low-salt buffer and *Taq*Plus Polymerase (Stratagene, La Jolla, CA). All PCR mixtures were am-

plified in a Perkin Elmer-Cetus 480 thermocycler (Perkin-Elmer, Norwalk, CT) for 30 cycles consisting of 30 s at 95°C, 60 s at 53–57°C, and 1–5 min at 72°C, followed by a 5 min incubation at 72°C.

RT-PCR

C. elegans total RNA was generously provided by Eleanor Mathews (University of British Columbia). RT-PCR was performed essentially as described by Rogalski *et al.* (1993), except that 1 μg of RNA was used in each RT reaction.

Sequencing of cDNAs and PCR products

The yk48h9 cDNA was obtained as a λZapII clone from the *C. elegans* cDNA Project. The pBluescript plasmid containing the cDNA insert was excised following the protocol provided by Stratagene and transformed into *Escherichia coli* (XL1-Blue strain). This construct was designated DM#201. Plasmid DNA was prepared for sequencing using an alkaline lysis/polyethylene glycol precipitation procedure, and the clone was sequenced by the Nucleic Acid/Protein Service unit at the University of British Columbia. PCR and RT-PCR products were directly sequenced using the BRL dsDNA Cycle Sequencing System (Life Technologies, Gaithersburg, MD) as described by Rogalski *et al.* (1993). The *gk3* deletion was sequenced by the Nucleic Acid/Protein Service unit.

Generation of Polyclonal Antisera

The DH5α strain of *E. coli* was used for subcloning and protein expression. Fusion proteins from the DM#184 and DM#183 clones were used to generate the GM1 (domain III) and GM3 (domain IV) antisera, respectively. Construction of these clones was described previously (Rogalski *et al.*, 1995; Moerman *et al.*, 1996). We also generated a polyclonal serum, GM9, that recognizes domain V of UNC-52. This antiserum was raised against the DM#199 fusion protein (Ser 3250 to Gly 3332) and recognizes this fusion on Western blots; however, we were not able to obtain reproducible staining results in embryos or adult worms using this serum.

GST fusion proteins were purified as described by Smith and Johnson (1988). To generate polyclonal antisera, New Zealand White rabbits were injected subcutaneously with fusion protein emulsified in Freund's complete adjuvant (~0.5 mg protein per rabbit). Rabbits were boosted at ~4-wk intervals with fusion protein emulsified in Freund's incomplete adjuvant (~0.25 mg protein per rabbit), and blood samples were taken 12 d after injection. Immune response was monitored by Western blotting of fusion proteins and immunofluorescence staining as previously described (Moerman *et al.*, 1996). No staining was observed with preimmune sera or with the secondary antibodies by themselves. Staining was eliminated by preincubating the antisera with the specific fusion protein but not with control fusion proteins. In addition, no staining was observed in *unc-52(null)* mutant embryos.

Immunofluorescence Staining

Embryos were prepared and stained as previously described (Rogalski *et al.*, 1993). Larvae and adults were stained as described by Finney and Ruvkun (1990). For immunofluorescence staining, rabbit polyclonal sera were diluted as follows: GM1, 1:400; GM3, 1:1500–1:6000. The mouse monoclonal antibodies DM5.6 (Miller *et al.*, 1983) and MH3 (Francis and Waterston, 1991) were diluted 1:40 and 1:100, respectively. The secondary antibodies, FITC-labeled donkey anti-rabbit IgG F(ab')$_2$ and TRSC-labeled donkey anti-mouse IgG F(ab')$_2$ (Jackson ImmunoResearch Laboratories, West Grove, PA), were diluted 1:100–1:200. In some experiments, FITC-labeled phalloidin was used to visualize actin.

Microscopy

Confocal images were collected using the MRC 600 system (Bio-Rad Microsciences Division, Hercules, CA) attached to a Nikon Optiphot-2 compound microscope. Optical sections were collected at 0.2-μm intervals and combined using the "maximum projection" function. For publication, confocal images were arranged and annotated using Adobe Photoshop and printed on a Codonics NP-1600 printer. For polarized light microscopy, worms were viewed as described by Waterston *et al.* (1980).

Isolation of Deletion Revertants

To isolate Tc1 excision events from *unc-52(st196::Tc1)*, we first established a *mut-4(st700)* I; *unc-52(st196::Tc1)* II strain. Wild-type (N2) males were crossed to *dpy-5(e61)* I; *unc-52(st196::Tc1)* II hermaphrodites. Male progeny (*dpy-5/+; unc-52/+*) were then mated to *mut-4(st700)* I hermaphrodites, and F1 animals with the genotype *dpy-5/mut-4; unc-52/+* were identified by progeny testing. Unc non-Dpy progeny from these animals were picked singly onto new plates and allowed to self-cross. Unc animals that failed to segregate Dpy Unc progeny were expected to have the genotype *mut-4(st700); unc-52(st196::Tc1)*. Several independent lines that reverted at a high frequency ($>1 \times 10^{-3}$) were established and maintained by picking single Unc animals to new plates. Revertants were identified on the basis of their improved movement and larger body size compared with their Unc siblings. Revertants were maintained for several generations until a homozygous strain was established and then tested by PCR to determine whether they carried a detectable polymorphism in the region of interest.

Isolation of Lethal Tc1 Excision Events

To isolate lethal Tc1 excision events, N2 males were crossed to *unc-52(st196::Tc1); mut-4(st700)* hermaphrodites, and individual outcross progeny were transferred and brooded. These plates were scored for the presence of Unc progeny. If the Tc1 element did not excise, we expected to observe 25% Unc progeny; however, if the element excised and the excision site was repaired precisely, we expected to observe only wild-type progeny. Similarly, if the element excised and interrupted repair of the excision site resulted in a large deletion, we would not observe Unc progeny. Animals that failed to segregate Unc offspring were brooded, and plates were scored for the presence of Pat embryos. A single lethal allele, *ra112*, was obtained in this manner and was subsequently balanced with the free duplication *mnDp34* (Herman *et al.*, 1979).

Construction of mec-8; unc-52 *Double Mutants*

Previous work had established that *mec-8; unc-52(viable)* double mutants exhibit a synthetic lethal (Pat) phenotype (Lundquist and Herman, 1994). To construct *mec-8; unc-52(viable)* double mutants, N2 males were crossed to *unc-52(viable)* II hermaphrodites. Outcross males *(unc-52/+)* were then crossed to *mec-8(u74)* I hermaphrodites, and *mec-8/+; unc-52/+* animals were identified by progeny testing. From plates segregating Pat embryos, embryos were prepared and stained. To construct *mec-8; unc-52(revertant)* double mutants, spontaneous males from revertant strains were crossed to *mec-8(u74)* hermaphrodites, and *mec-8/+; unc-52/+* animals were identified by progeny testing. From plates that segregated Mec animals, single Mec hermaphrodites were transferred and brooded. The parental hermaphrodite was then tested by PCR for the deletion allele of *unc-52*. Strains carrying a deletion allele were retested for the wild-type *unc-52* allele to determine whether they were homozygous for the deletion. To confirm that these animals were homozygous for *mec-8*, they were stained with DiO (Molecular Probes, Eugene, OR) to evaluate the dye-filling defects (Herman and Hedgecock, 1990).

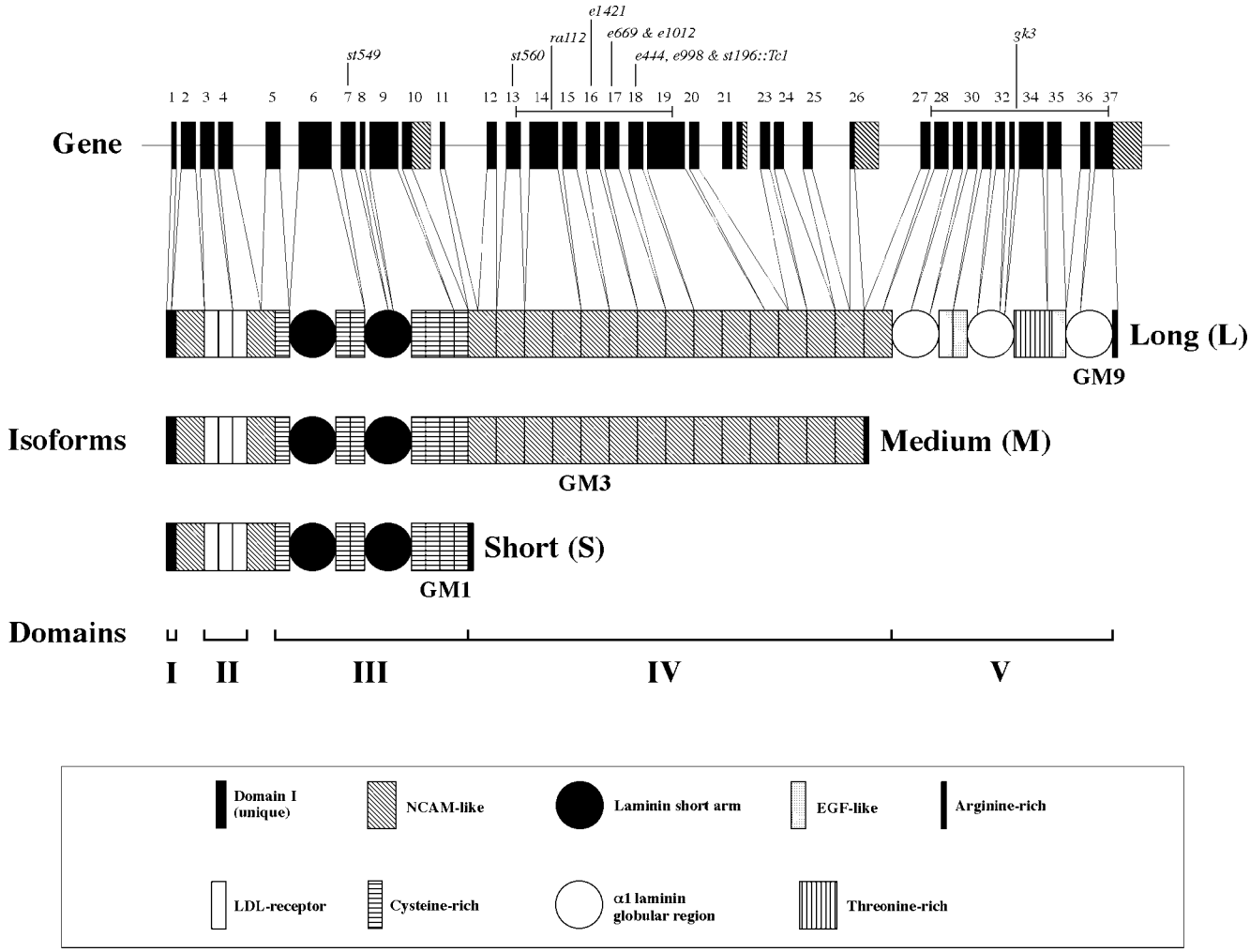

Figure 1. Structure of the *unc-52* gene and protein products. The *unc-52* gene consists of 37 exons and spans over 20 kb. Exons (boxes), introns (lines), and the three classes of protein products are shown. Mutant alleles used in this study are also indicated. The longest ORF encodes a protein of 3375 amino acids that is homologous to the mammalian heparan sulfate basement membrane proteoglycan perlecan. Like mammalian perlecan, this polypeptide can be divided into five domains (I–V). The first domain is unique, whereas the remaining four domains show similarity to the low-density lipoprotein receptor (domain II), α-laminin (domains III and V), and NCAM (domain IV). Additional isoforms are generated through alternative splicing of exons encoding alternative C termini, indicated on the gene as shaded regions. The various protein modules are indicated with shaded boxes or circles.

RESULTS

The unc-52 *Gene Encodes the Nematode Orthologue of Mammalian Perlecan*

The *unc-52* gene in *C. elegans* produces several large proteins that are homologous to perlecan, the mammalian basement membrane heparan sulfate proteoglycan. Our previous analysis of this gene identified 26 exons covering almost 15 kb of genomic DNA and revealed the presence of several alternatively spliced transcripts (Rogalski *et al.*, 1993, 1995). Two different polyadenylation sites located 8.5 kb apart are used to generate a number of large polypeptides containing domains I–IV or smaller polypeptides containing only domains I–III.

Recently, we discovered that the *unc-52* gene actually spans >20 kb of genomic DNA and consists of 37 exons (Figure 1). Eleven additional exons were identified downstream of exon 26 when the *C. elegans* Genome Consortium provided the sequence and annotation of cosmid C38C6. We confirmed the intron–exon boundaries in this region by sequencing a cDNA clone obtained from the *C. elegans* cDNA Project that extends from exon 27 to ~200 bp downstream of the putative stop codon in exon 37. In addition, cDNA fragments were generated by RT-PCR and sequenced to confirm that the newly identified exons are part of *unc-52* and to identify the splice sites used to join exons 26 and 27.

The longest potential ORF of the *unc-52* gene now encodes a 3375 amino acid protein consisting of a putative signal sequence and five distinct domains with a molecular weight of ~370 kDa (Figure 2). The newly identified exons encode sequences that are very similar to domain V of mouse and

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                        |Domain I                              |IgR1
   1 MKRSSTVLAA LLALLLVATN DAARHRKYRQ TYQDIDSDDD  DTSDVQITVF PSEKEVRDGR DVSFECRART SDNSVYPTVR  80
                                                                                |Domain II
  81 WARVGGPLPS SAHDSGGRLT INPVQLSDAG TYICVSDYNG  NTVEARATLS VVSYGPQEVS NGLRQAGQCM ADEKACGNNE 160
 161 CVKNDYVCDG EPDCRDRSDE ANCPAISRTC EPNEFKCNNN  KCVQKMWLCD GDDDCGDNSD ELNCNAKPSS SDCKPTEFQC 240
                                      |IgR2
 241 HDRRQCVPSS FHCDGTNDCH DGSDEVGCVQ PTVVDPPQTN  LQVPRGTTFS LTCKAVAVPE PYINWRLNWG PVCEPPRCLQ 320
                                                                        |Domain III
 321 TSEGGYGTLT IHDAQPVDQG AYTCEAINVK GRVLATPDCI  VRVVDDPRPQ PPQPPTAPPQ RASCDTRGAV TPYPNNYGTC 400
 401 ECKSQVTGPN CDQCKPGAFH LSEKSPEGCL KCFCFGVSND  CRSSGHYRTK DRLMFAGDAE GVTISDIEER TIDRNTPFSF 480
 481 FKTGYLTFDG TTDGVAKYWR LPQRFLGDKV TAYGGKMEFE  IEFSGSGHHS SEPMVVLKGN QNILVHRVRN QEHVLRSDSP 560
 561 VRITVETYET NYEQLNGAAA TREDLLMVLA DLDAFLIRAT  HVAHQTSTSL GDVSWEIAVD RYTPDGLALE VEQCVCPPGY 640
 641 LGTSCEDCAP GYERSGYGPY LGTCVPIQPR HQQCGPGAVA  PTAPAQGQCQ CKASVIGPNC DRCAPNSFGL APTNPQGCIP 720
 721 CFCSGVTQQC SASSYRRTSV SIDYARGDRD QLELTTSDSR  QPYSPQTRAE LSGQAIEFRS FEEARGQTLY WKLPEKFLGD 800
 801 KVTSYGGTLE YTFKFSGNGN SDQSADVILR GNDIALQYKH  REPFYADREN KVQIKIIETS WQRVDGQQAT REHLLMTLAD 880
 881 LDTLLIKSTY NDDCTDSQLL SANLEFAEPY GQGLTAAEVE  QCICPPGYVG TSCEDCAPGY SRTGGGLYLG LCEKCECNGH 960
 961 ASQCDKEYGY CLDCQHNTEG DQCERCKPGF VGDARRGTPN  DCQPEATRAP CHCNNHSPRG CDSFGRCLLC EHNTEGTHCE 1040
1041 RCKKGYYGDA TKGSPYDCTP CPCPGASDCY LDNEGQVACR  ICPAGLQGRL CNECAPGYTR SNKPAGRVCE PIGQVTNEDI 1120
            |Domain IV (IgR3 - IgR17)
1121 TFVQKPHEVL RVRIMEPKRQ IALPGDRVHW ICQVTGYTTE  KIHVEWTKVG EMSLPPNAKA YDGYLVLKGV EAENAGQYRC 1200
           GD FARNSPSQNS SGQRRHRRRR IRVRSRFYHH*
1201 TATTITQYAT DDALLTISKR ISGRPPQPVI DPPHLVVNEG  EPAAFRCWVP GIPDCQITWH REQLGGPLPH GVYQTGNALK 1280
1281 IPQSQLHHAG RYICSAANQY GTGQSPPAVL EVKKPVIPPK  VDPIRQTVDR DQPARFKCWV PGNSNVQLRW SRPGGAPLPS 1360
1361 GVQEQQGILH IPRASDQEVG QYVCTATDPS DNTPLQSEPV  QLNIRDPAPP QRGAAPQIDP PNQTVNVNDP AQFRCWVPGQ 1440
1441 PRAQLKWSRK DGRPLPNGIL ERDGFLRIDK SQLHDAGEYE  CTSTEPDGST QLSPPARLNV NQPQAIQPQV DPPVQTVNEG 1520
1521 EPSRIRCWVP GHPNIQLQFV KRGRRPLPAH ARFSQGNLEI  PRTLKSDEDE YICIATDPTT NRPVESNPAR VIVKSPIRPI 1600
1601 IDPAEQTVPE GSPFKIRCYV PGHPSVQLTF RRVSGQLNED  ADENNGLLAV QRAELTDEGD YICTARDPDT GAPIDSTPAT 1680
1681 VHVTNAAAPP QVEARPPQHP VITPQTQTIP EGDPARIQCT  VPGNPSAAQH LSFERVDGKG LPFGSSDDRG VLTIPSTQLQ 1760
1761 DAGEYVCLYS PENSPPVKTN PSTLNVTPEG TPPRPVATPP  LLSVAPGSPA RFNCVAHSDT PARIRWGFRE ENGPLPEHVN 1840
1841 QDGDDIVISE AGDRNVGEYV CSATNDFGTG VADPVRLEVT  EDQEPPTAVV EPRTWNGKPG ERHQFRCITT GSPTPKITWT 1920
1921 GPNGSPLPHD VTPLEPNILD FSNGRSELNG DYTCTASNPI  GEASDHGNVN IGPSLTVKTN PPGPKLIVTV GEPLQVKCEA 2000
2001 FGAPGDPEPE VEWLHDPGPE RGDLPDDFKP VTISEQFIRH  PNVGLGNAGV YTCKGSSAHA TATKNIYIEV VEPSRIATVS 2080
2081 ILGGSSQWFD QGEKGELICT ATGSSLVDRL EWEKVDDQLP  TDVEEHNEPG LLHFPSFKNS YAGEYRCNGY RNNEIIASAA 2160
2161 VHVHSSANAD DEPKVEIEPP RVRVVSQGDN IVLKCSVQGA  ENGEHFKWAL LRGGSLVRQL GTEPTLEITK ADPSNDFGVY 2240
2241 RCNVEDNNGL VIGSAFTAVS VGQQDKSHAQ IVKFDDKSDA  SFTCPIYSVP GSKVDWTYEN GDLPSKAVPN GNKIEIKEFD 2320
2321 DASAGTYVCK VSFDGNVVEG FVTAQMFVPD TIIQVLLEVS  SESPQIGDRA WFDCKVTGDP SAVISWTKEG NDDLPPNAQV 2400
2401 TGGRLLFTDL KEDNAGVYRC VAKTKAGPLQ TRTVLNVGSG  KQDQVTFTVA DSLPVVYTVG QPAYLSCIGK TETKPNQSVV 2480
                                                  RKRKHLGNR RGRRLRHRRR NAQNGPLSRK TRTTTKLFGSWF*
                                                                        |Domain V
2481 WTKEEGDLPS GSRVEQGVLM LPSVHRDDEG SYTCEIVKEE  NPVFSTVDLQ IDDFIPVIDG EPIELPPLSD EEIVNLDIEI 2560
2561 TLNTANPKGI IFETKRINSG DLLATPYDTI HHEAKITDYG  TVLYEFDIGN GRQIVETTNP INPNEWNVIK IKNDKNQVTI 2640
2641 QLNDESATIR QHTNPLPSLS TGVNRPVFIG GRHEPTNEAN  DFRGIISQVV LSGHNVGLGD ARIPSSVVKY DACASTNLCL 2720
2721 NGANCRNANN HHGFSCECAE EFHGEYCQWR SNSCHDESCN  TGICLDNEES WQCVCPLGTT GLRCEEKTEI PQPLGFTSDT 2800
2801 SFLAVKRPVK FESIKMKLRP QADSDEHILM YFASDYGSNT  KQYTSLSLIA NQVVLTVRRP DKEVQKIRSE TLEAGELIDV 2880
2881 AVRQAGNALV MTVDGNQVST IETDTLKPGT EIFIGGLPPG  LNSPDDVVEQ SFQGCVYEIL INSQDVDLQN LSSSGDISSC 2960
2961 EESQFPVEED DTTTTTTTEE PEAVIEEPTT EEPTTTEEPI  TEEPTEEPTT TEEPTTTEEP TTTTEEPTTT TTEEPYHIYE 3040
3041 TSRDDDPEII IPVETTTTST TTTSTTEEPE AEPALVLPTD  PVEENDVSDE EEEISTISTV SPDNGLDSDS DYSEGTLPPD 3120
3121 SSSEEIVVGD VYSTQEPNNI CANSTCGMNG QCVPRNMTHY  TCECKLYYDG PTCSLFKPIE HAARFDGDAF IELSSDEFPH 3200
3201 LTSEKDEIVA FKFKTEQQNG VLLWQGQRPT VQQMEDYISV  GIVNGHLHFS YELGGGAAHL ISEERVDDGK EHSVRFERKG 3280
3281 REGQMRIDNY REVDGRSTGI LAMLNVDGNI FVGGVPDISK  ATGGLFSNNF VGCIADVELN GVKLDLMATA IDGKNVKPCD 3360
3361 EWMHRKRWLY RRRVR*                                                                      3375
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Figure 2. Complete amino acid sequence of UNC-52/perlecan and isoforms. Domains are indicated, and the signal peptide is italicized and underlined. The alternative C termini of the short and medium length isoforms are indicted (bold), and the threonine-rich region in domain V is also shown (bold and underlined).

human perlecan, confirming our earlier conclusion that the UNC-52 proteins are the nematode orthologues of these mammalian proteoglycans (Rogalski *et al.*, 1993). Domain V of perlecan/UNC-52 consists of three globular regions interrupted by cysteine-rich repeats and is similar to the G domain of α1 laminin. Curiously, the nematode protein contains a region of ~180 amino acids that is not found in the mammalian proteins. This region is extremely rich in threonine (45/180) and serine (19/180) residues and also contains 12 repeats of the sequence EEP.

On the basis of this new information, UNC-52 isoforms can be divided into three major groups (Figures 1 and 2). Short (S) isoforms contain the first three domains (I, II, and III), medium (M) isoforms contain the first four domains (I, II, III, and IV), and long (L) isoforms contain all five domains (I, II, III, IV, and V). Alternative splicing of exons 6, 16, 17, 18, 21, and 22 generates additional diversity within domains III and IV (Rogalski *et al.*, 1993, 1995).

Spatial and Temporal Differences in the Localization of UNC-52 Isoforms

Several studies have examined the localization of perlecan/UNC-52 in *C. elegans* (Francis and Waterston, 1991; Hresko *et al.*, 1994; Moerman *et al.*, 1996). In this study, we compare the distribution of UNC-52 isoforms using domain-specific antibodies. The polyclonal serum GM1 recognizes a region of domain III present in all UNC-52 isoforms (Figure 1) (Moerman *et al.*, 1996). The polyclonal serum GM3 recognizes a conserved region of domain IV (Figure 1) and has the same domain specificity as the mAbs MH2 and MH3 (Rogalski *et al.*, 1993, 1995). We double-labeled wild-type animals at different stages of development with these antisera and the mAb DM5.6, which recognizes the body-wall muscle myosin MHC A (Miller *et al.*, 1983). Our results demonstrate that there are spatial and temporal differences in the localization of UNC-52 isoforms.

Previous studies established that some UNC-52 isoforms are expressed in the body-wall muscles during embryogenesis and are localized to the underlying basement membrane (Rogalski *et al.*, 1993; Hresko *et al.*, 1994; Moerman *et al.*, 1996). In this study, we found that both GM1 and GM3 stain the body-wall muscles and are identical in this respect. Staining is first observed in comma stage embryos and is primarily found at regions of contact between adjacent muscle cells (Figure 3, A and B). Some intracellular staining of body-wall muscle cells is also observed, but neither sera stain the underlying hypodermis, suggesting that muscle cells are the primary source of UNC-52. Between the comma and 1.5-fold stages, staining spreads from regions of cell–cell contact over the basal face of each muscle cell, where the basement membrane is located (Figure 3, C and D).

In older embryos, dramatic differences in staining are observed with GM1 and GM3. Beginning at the 1.5-fold stage, GM1 stains the posterior end of the pharynx (Figure 3E), which is derived from the MS lineage (Sulston *et al.*, 1983). The anterior pharynx, which is derived from the AB lineage (Sulston *et al.*, 1983), begins to stain somewhat later. By the threefold stage, GM1 staining surrounds the pharynx from the anterior margin to the pharyngeal-intestinal valve (Figure 3G). GM1 also stains the anal sphincter and depressor muscles at this stage (Figure 3G). UNC-52 is specifically associated with contractile tissues in *C. elegans* and is not found in the basement membranes lining the pseudocoelom or surrounding the intestine (Table 1).

In contrast, GM3 only stains the body-wall muscles and does not stain the pharynx or anal muscles at any stage of embryonic development (Figure 3). On this basis, we conclude that M and/or L isoforms are restricted to the body-wall muscles during embryogenesis. GM1 staining of the pharynx and anal muscles must therefore be due to the presence of S isoforms in these tissues.

Both GM1 and GM3 stain the body-wall muscles in embryos, larvae, and adults. Briefly, in larvae and adults, these antisera stain the dense bodies, M-lines, and muscle cell margins, in addition to the basement membrane underlying the muscle quadrants. Both antisera also stain the basement membranes associated with the pharyngeal, anal, and sex-specific muscles in adult animals (Table 1). GM1 staining of the pharynx, body-wall muscles, and reproductive muscles is shown in Figure 4. We conclude that there are developmental changes in isoform localization because domain IV-containing isoforms are more widely distributed in adults than in embryos. For example, GM3 does not stain the pharynx or anal muscles in embryos but does stain these tissues in adults. These results are similar to those reported for the mAbs MH2 and MH3 (Table 1) (Francis and Waterston, 1991; Hresko *et al.*, 1994).

Other studies have shown that UNC-52 is found in the basement membrane surrounding the pharynx in larvae and adults (Francis and Waterston, 1991; Rogalski *et al.*, 1993). In this study, we also found that UNC-52 is concentrated over focal adhesion-like structures in the pharyngeal muscles. In animals prepared as described by Finney and Ruvkun (1990), GM1 staining surrounds the pharynx from the anterior margin to the pharyngeal–intestinal valve, and muscle cells within the terminal bulb stain with a punctate pattern (Figure 4A). This punctate staining pattern was not observed in animals prepared by freeze-fracture.

M Isoforms of UNC-52 Are Essential for Myofilament Lattice Assembly in the Body-Wall Muscles of C. elegans

The *st549* mutation introduces a stop codon into exon 7 of the *unc-52* gene and eliminates all UNC-52 protein isoforms (Rogalski *et al.*, 1995). We have shown that *unc-52(st549)* mutant embryos fail to stain with MH3, which recognizes M and L isoforms of UNC-52 (Rogalski *et al.*, 1993), and GM1, which recognizes all UNC-52 isoforms (Moerman *et al.*, 1996). In this study, we use these antibodies to examine the expression of UNC-52 isoforms in embryos homozygous for several additional lethal alleles of *unc-52*. These mutant embryos are phenotypically indistinguishable from *unc-52(st549)* embryos and fail to show body-wall muscle staining with GM1 or MH3; however, they do show pharyngeal staining with GM1, indicating expression of the S isoforms of UNC-52.

We double-labeled mutant embryos prepared from balanced stocks with GM1 and either DM5.6 or MH3. Four lethal alleles, *ra112*, *st546*, *st560*, and *st578*, lead to reduced anti-UNC-52 staining of the body-wall muscles relative to the pharynx or anal muscles. For example, in homozygous *st560* embryos, staining of the body-wall muscles with GM1

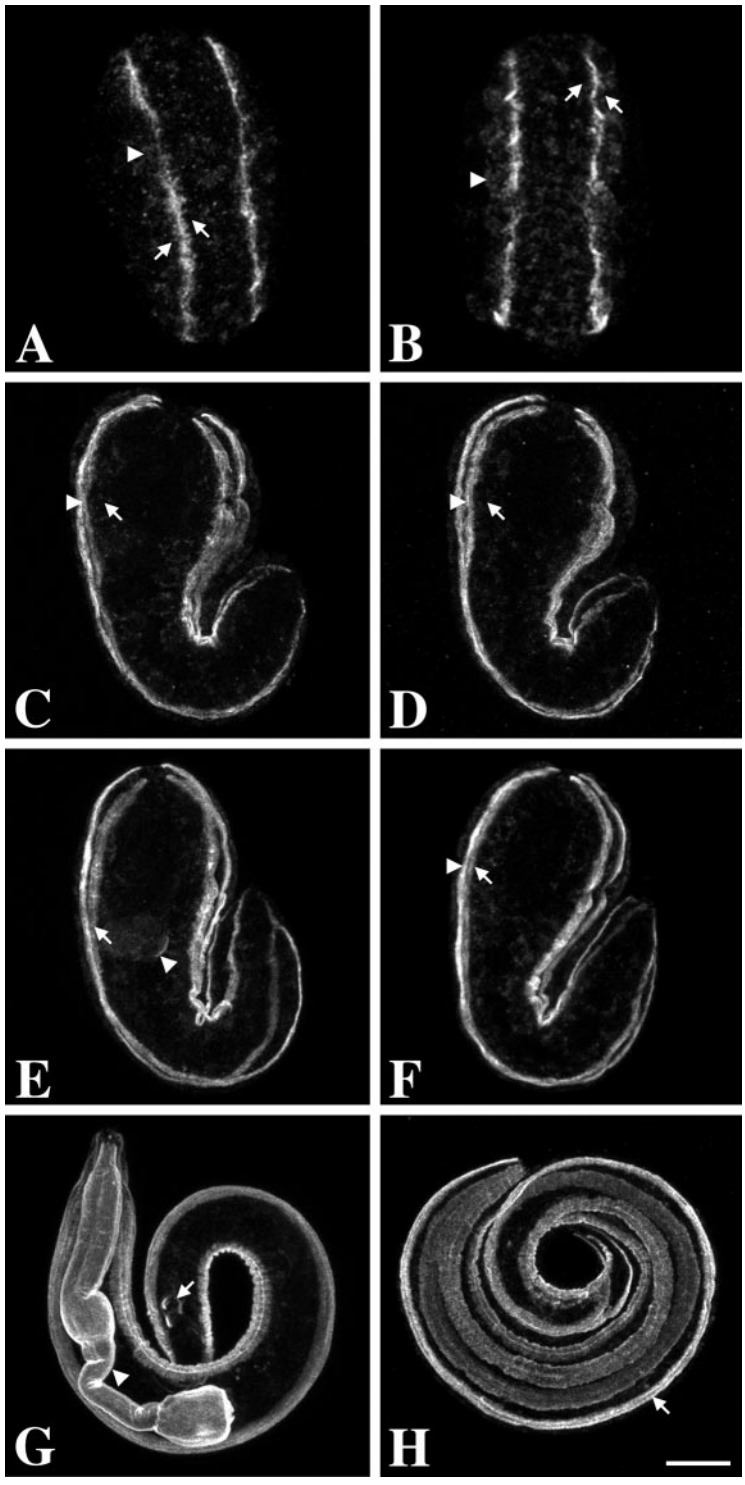

Figure 3. Localization of UNC-52 isoforms during embryonic development. Embryos were visualized by confocal microscopy. Wild-type embryos were labeled with GM1 (domain III; A, C, E, and G) and GM3 (domain IV; B, D, F, and H). A and B show the dorsal view of a comma-stage embryo (~350 min); these images are projections of the dorsal Z-sections. Arrows indicate positions of two adjacent muscle cells; the arrowhead indicates intracellular staining. Subsequent images are projections of the complete Z-series. C and D show the lateral view of late comma-stage embryo (350–400 min). Arrows indicate the position of a muscle cell, whereas the arrowheads indicate the basal face of the cell. E and F show the lateral view of a 1.5-fold embryo (~420 min). The arrow in both panels indicates the basal face of the muscle cells in a dorsal quadrant. Note the pharyngeal staining (arrowhead) in E. G and H show a threefold embryo (~800 min). Note that GM1 stains the pharynx (arrowhead in G), body-wall muscles, and anal muscles (arrow in G), whereas GM3 only stains the body-wall muscles. Bar, 10 μm.

is greatly reduced or absent (Figure 5E). Similarly, we observed no detectable staining of the body-wall muscles with MH3, indicating that isoforms with domain IV are greatly reduced or absent (Figure 5L). Within the body-wall muscle cells, myosin is not organized into ordered A-bands but instead forms large aggregates (Figure 5F) (Williams and Waterston, 1994). GM1 staining of the pharynx and anal muscles, however, appears to be unaffected (Figure 5E). Thus, S isoforms of UNC-52 are still expressed in these mutants. Similar results were observed with *ra112*, *st546*,

Table 1. Distribution of UNC-52/perlecan in embryos and adult hermaphrodites

Stage	Antisera	Domain	Body-wall muscle	Pharynx	Anal Muscles	Egg-laying Muscles	Gonad
Embryo	GM1	III	+	+	+	N/A	N/A
	GM3	IV	+	−	−	N/A	N/A
	MH3[a]	IV	+	−	−	N/A	N/A
Adult	GM1	III	+	+	+	+	+
	GM3	IV	+	+ (faintly)	+ (faintly)	+ (faintly)	+
	MH3[b]	IV	+	+ (faintly)	+ (faintly)	+ (faintly)	+

[a] Hresko *et al.*, 1994.

[b] Francis and Waterson, 1991.

and *st578* (our unpublished results). The lack of body-wall muscle staining with GM1 leads us to conclude that S isoforms are not present in body-wall muscles during embryogenesis. The body-wall muscle staining observed in wild-type embryos must, therefore, be due to M and/or L isoforms.

On the basis of these results, we predicted that the sequence changes in these alleles would be localized to the region encoding domain IV. To test this prediction, we amplified genomic DNA from homozygous mutant embryos using PCR and began sequencing downstream from exon 11, which encodes the first NCAM repeat in domain IV. We identified the nucleotide alterations in two of the *unc-52* alleles described above, and as expected, both were localized to the region encoding domain IV (Table 2). The *st560* mutation is a C to T transition in exon 13, which changes a glutamine residue (CAA) to an ochre stop codon. *ra112* has a 3283-bp out-of-frame deletion that extends from exon 13 to exon 19. Both mutations eliminate all UNC-52 isoforms with domain IV, demonstrating that these isoforms are essential for myofilament assembly in the body-wall muscles.

The *st560* and *ra112* mutations disrupt the expression of both M and L isoforms of UNC-52. We obtained a domain V-specific deletion from the *C. elegans* Reverse Genetics Core Facility to determine the effect of specifically eliminating the L isoforms. This deletion, *gk3*, is 2735 bp in length and removes most of the domain V-encoding region of *unc-52* (Figure 1). Animals homozygous for the *gk3* deletion are viable and essentially wild type in appearance. The only discernible phenotype is a slight difference in egg-laying behavior; *unc-52(gk3)* mutants tend to retain eggs within the gonad to a greater extent than wild-type animals. We emphasize that this defect is quite mild and resembles the behavior of wild-type animals under starved conditions. On the basis of this mild phenotype, we conclude that domain V is not essential, and M isoforms of UNC-52 are sufficient for myofilament lattice assembly.

UNC-52 Is Not Essential for Myofilament Lattice Assembly in the Pharyngeal Muscles of C. elegans

We have demonstrated that domain IV-containing isoforms of UNC-52 are essential for myofilament lattice assembly in the body-wall muscles. Because S isoforms of UNC-52 are restricted to the pharynx and anal muscles during embryonic development, we speculated that these isoforms might be required for myofilament assembly in these muscles. To test this prediction, we stained wild-type (N2) and *unc-52(st549)* embryos with fluorescently labeled phalloidin. Homozygous *st549* embryos do not express detectable levels of any UNC-52 isoforms, including those associated with the pharynx. Surprisingly, pharyngeal thin filaments did not appear to be disorganized in *unc-52(st549)* mutants.

In wild-type threefold embryos, phalloidin stains I-bands in the body-wall, anal, and pharyngeal muscles. By this stage, actin is organized into distinct half I-bands in the pharyngeal muscles. For example, in lateral views of M3 cells (procorpus), half I-bands can be seen extending inward from the basal and luminal faces of each cell (Figure 6A). The H-zone, which appears as a gap between half I-bands, can also be observed in appropriately oriented embryos. In cross sections, thin filaments radiate from a focal attachment site at the luminal face of each pharyngeal muscle cell to more broadly distributed attachment sites on the basal face (Figure 6B).

In *unc-52(st549)* embryos, the pharynx is compressed lengthwise and distorted in shape. The terminal bulb can be identified by its position, but the metacorpus cannot be readily distinguished from the procorpus and isthmus. These morphological defects are observed in all Pat mutants and probably result from failure to elongate; however, within the pharyngeal muscles, thin filaments are organized into well ordered half I-bands extending from both luminal and basal faces (Figure 6B). We examined the half I-bands in the five largest muscle layers (M3, M4, M5, M6, and M7 cells) and did not observe any disorganization. The apparent wild-type organization of the pharyngeal muscle thin filaments in *unc-52(null)* mutants is consistent with the observation of pharyngeal pumping in these animals (Williams and Waterston, 1994). We conclude that UNC-52 is not essential for myofilament lattice assembly in pharyngeal muscles.

Alternative Splicing within Domain IV Is Associated with Temporal and Spatial Differences in Isoform Expression

Alternative splicing of exons 16, 17, and 18 gives rise to isoforms that vary in the number of NCAM repeats within domain IV (Rogalski *et al.*, 1993, 1995). Mutations in these alternatively spliced exons eliminate a subset of M and L isoforms (Rogalski *et al.*, 1993, 1995). Five point mutations in this region have been sequenced; four are nonsense muta-

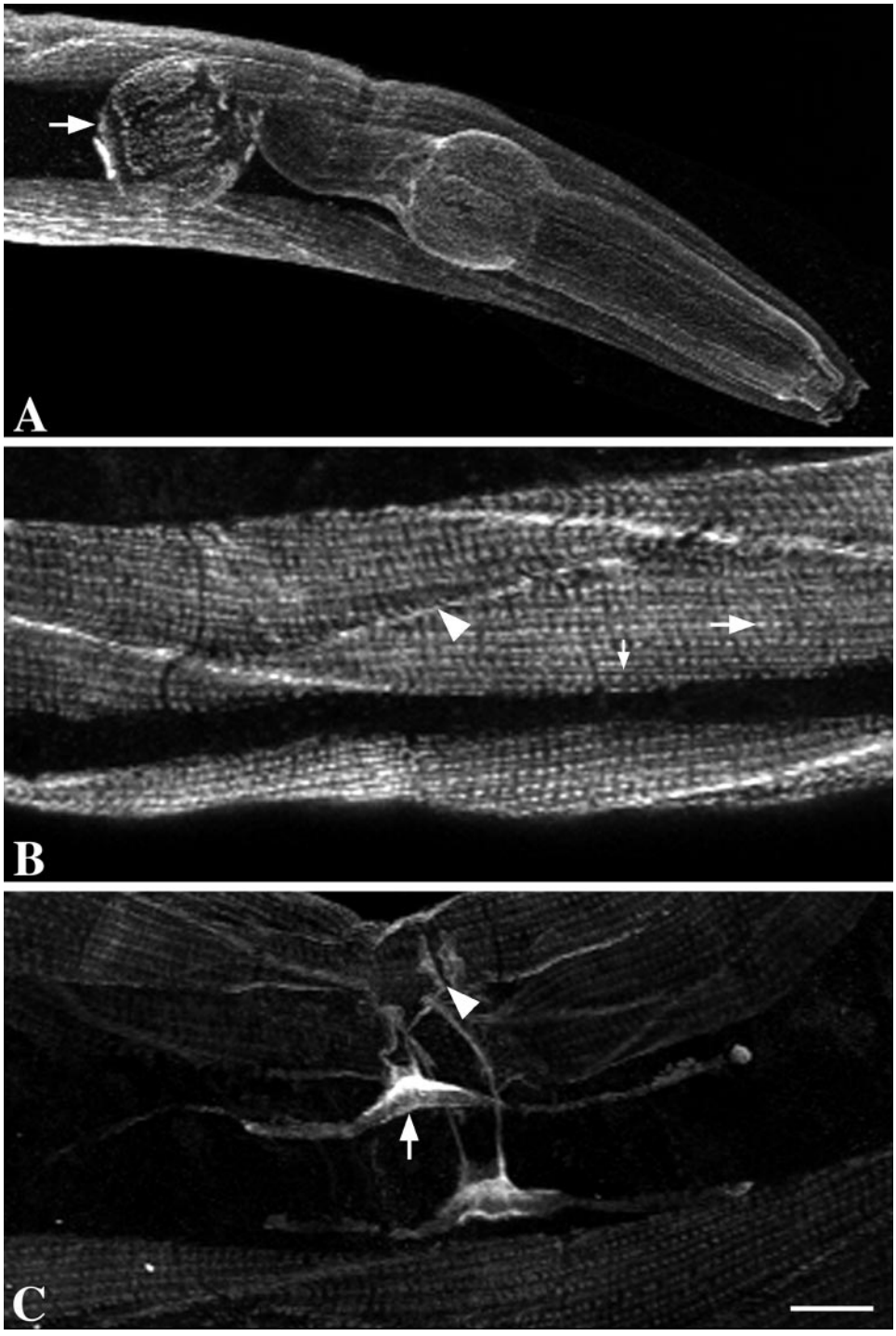

Figure 4. Immunolocalization of UNC-52 in adult hermaphrodites. Wild-type hermaphrodites were labeled with GM1. A shows the head from a young adult. The arrow indicates the terminal bulb of the pharynx. Note the punctate pattern over this region. B shows a section of the body-wall muscles from a young adult. The large arrow indicates a dense body, the small arrow indicates an M-line, and the arrowhead indicates the margin of a body-wall muscle cell. C shows the uterine region from an older adult (dorsal view). The arrow indicates the base of the uterine muscles, whereas the arrowhead indicates the vulva. Bar, 10 μm.

tions in either exon 17 (*e669* and *e1012*) or 18 (*e444* and *e998*), whereas the fifth (*e1421*) alters the splice donor site of exon 16 (Figure 1) (Rogalski *et al.*, 1995). Animals homozygous for these viable alleles develop normally as young larvae but become progressively paralyzed as they mature. This paralysis is caused by the gradual disruption of the myofilament lattice in the body-wall muscle cells posterior to the head (Mackenzie *et al.*, 1978b; Waterston *et al.*, 1980). We have shown that these mutations do not disrupt the accumulation of UNC-52 during early development (Rogalski *et al.*, 1995).

In contrast, these mutations have dramatic effects on the accumulation of UNC-52 isoforms associated with the body-wall muscles in older animals (Figure 7C), suggesting that there is an "adult-specific" subset of isoforms. In homozy-

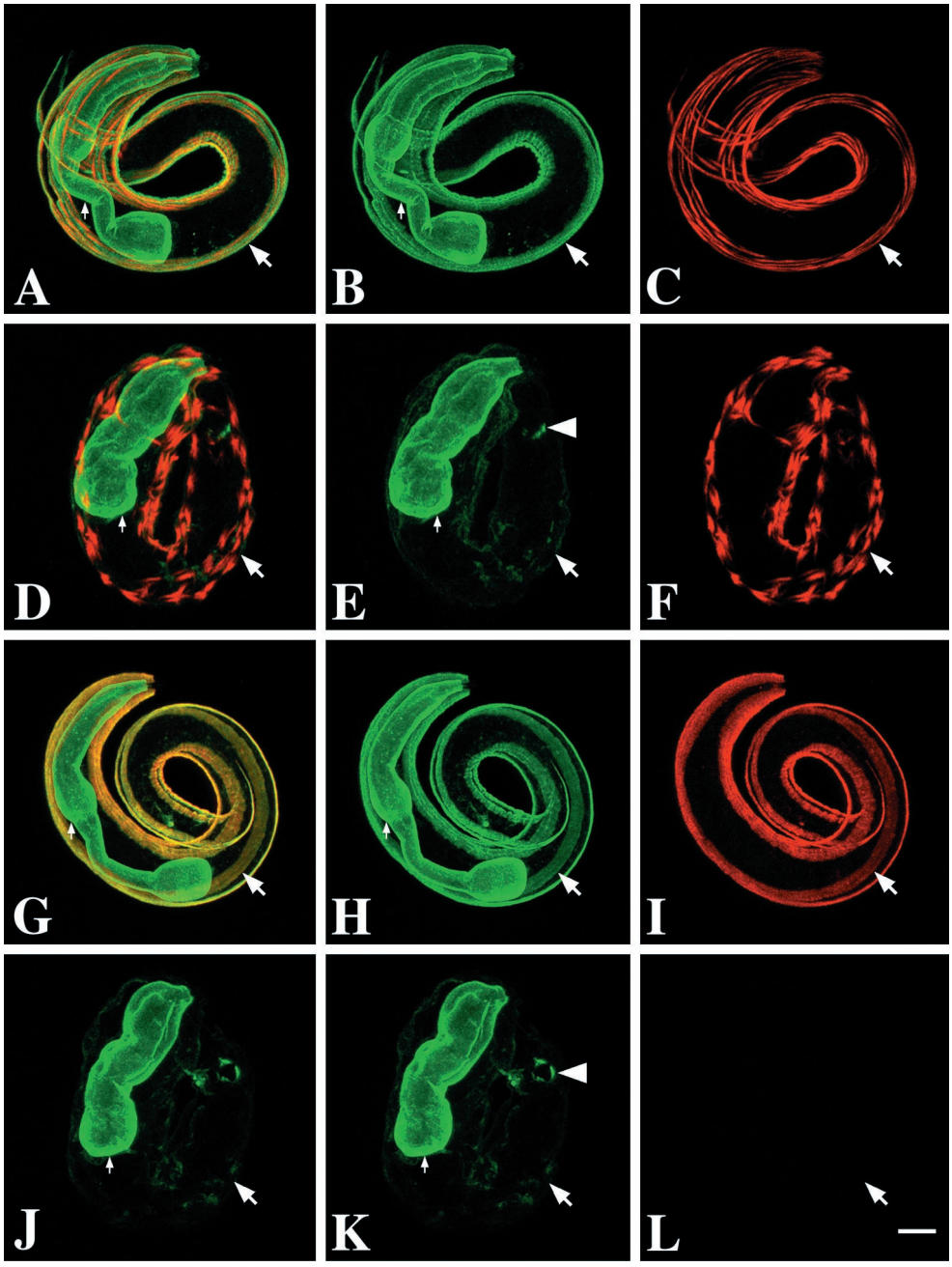

Figure 5. *unc-52(st560)* mutant embryos have tissue-specific staining defects and lack a subset of UNC-52 isoforms. Embryos were visualized by confocal microscopy. Embryos in A–F were double-labeled with GM1 (green), which recognizes all UNC-52 isoforms, and DM5.6 (red), which recognizes myosin heavy chain A (MHC A). Small arrows indicate the pharynx, and large arrows indicate a body-wall muscle quadrant. A and D show both channels simultaneously, whereas B, C, E, and F show single-channel images. A–C show a wild-type embryo, whereas D–F show an arrested *unc-52(st560)* mutant embryo. Note the reduced staining of body-wall muscles with GM1 and disorganization of MHC A in the mutant (E and F; compare with B and C). The pharynx and anal muscles (arrowhead in E) in the mutant, however, exhibit a wild-type staining pattern (compare B and E). Embryos in G–L were double-labeled with GM1 (green, FITC) and MH3 (red, TRSC), which recognizes an epitope in domain IV of UNC-52. G–I show a wild-type embryo; J–L show a *unc-52(st560)* mutant embryo. G and J show both channels simultaneously. Note the absence of MH3 staining in the mutant embryo in L. Although the *unc-52(st560)* mutant embryos shown are arrested at the twofold stage, they are comparable to threefold wild-type embryos. Bar, 10 μm.

gous *e444* hermaphrodites, for example, staining of the body-wall muscles appears to be normal until the L4 stage but is greatly reduced relative to wild-type animals in adults (Figure 7C). Within the body-wall muscles, myosin becomes highly disorganized and forms large aggregates (Figure 7D) (Waterston *et al.*, 1980). Curiously, staining of body-wall muscles in the head is not affected, even in older adults, and staining of the pharynx and uterine muscles also appears normal. We conclude that the *e444* mutation affects an adult-specific subset of UNC-52 isoforms associated with most of the body-wall muscles. Other viable alleles, including *e669*, *e998*, *e1012*, and *e1421*, behave in a similar manner.

Alternative splicing within this region is also associated with spatial differences in isoform expression. The gene *mec-8* encodes a putative RNA-binding protein that is required for a subset of these alternative splicing events (Lundquist *et al.*, 1996). We have shown that nonsense mutations in exon 18 block the expression of domain IV-containing isoforms in a *mec-8(−)* background (Lundquist *et al.*, 1996). We constructed additional *mec-8; unc-52* double mutants and found that some combinations do not eliminate all domain IV-containing isoforms. In *mec-8; unc-52(e669)* and *mec-8; unc-52(e1012)* double mutants, most of the body-wall muscles fail to stain with MH3; however, we did observe

Table 2. Alterations in domain IV of UNC-52

Allele	Alteration	Region	Phenotype
st560	CAA to TAA (Gln 1263 to Ochre)	Exon 13	Pat (lethal)
ra112	3283-bp deletion	Exon 13–19	Pat (lethal)
ra507	12-bp insertion	Exon 18	Wild type
ra511	566-bp deletion	Exon 18–19	Wild type
ra512	1206-bp deletion	Exon 15–18	Wild type
ra513	1293-bp deletion	Exon 15–18	Wild type
ra514	1457-bp deletion	Exon 16–18	Wild type
ra515	1519-bp deletion	Exon 15–18	Wild type
ra516	982-bp deletion	Exon 16–18	Wild type
ra517	1359-bp deletion	Exon 15–18	Wild type
ra518	1205-bp deletion	Exon 15–18	Wild type
ra519	1185-bp deletion	Exon 15–18	Wild type

strong staining of the anteriormost muscle cells in each of the body-wall muscle quadrants (Figure 8B). Both *e669* and *e1012* are point mutations that introduce translational stop codons into exon 17 (Rogalski *et al.*, 1995). Thus, in a *mec-8(−)* background, nonsense mutations in exon 17 greatly reduce or eliminate most domain IV-containing isoforms but allow expression of certain spatially restricted splice variants. These observations imply that certain splice variants are restricted to a subset of body-wall muscles.

Several NCAM Repeats within Domain IV Are Dispensable for Muscle Assembly

Previous studies suggested that single exons in the region of alternative splicing in domain IV are dispensable for protein function (Gilchrist and Moerman, 1992; Rogalski *et al.*, 1995). Here we have extended these studies and show that removing up to four exons (four NCAM repeats) does not affect viability or muscle assembly. The allele *unc-52(st196::Tc1)* has a Tc1 transposon insertion in exon 18, and homozygous animals exhibit the paralyzed phenotype typical of *unc-52(viable)* mutants. In a mutator background, the transposon excises at a high frequency ($>1 \times 10^{-3}$) to give phenotypically wild-type animals. Excision can be precise, where the wild-type sequence is restored, or imprecise, where deletions and other rearrangements occur (Kiff *et al.*, 1988; Moerman *et al.*, 1991). We isolated >120 independent revertants from a *mut-4; unc-52(st196::Tc1)* strain and asked whether any carried deletions or insertions in the region of interest.

Using primers flanking the Tc1 insertion, we amplified DNA fragments from homozygous revertants and identified 10 strains with DNA alterations (Table 2). One revertant has a 12-bp in-frame insertion, whereas the remaining nine contain deletions, ranging in size from 982 to 1519 bp. These deletions remove between two and four NCAM repeats without altering the reading frame (Table 2). We examined the body-wall muscles in these revertants using polarized light microscopy and found that these animals have normal muscle structure, including a wild-type number of sarcomeres in each muscle cell. These results suggest that the number of NCAM repeats is not of primary importance, nor are specific repeats within this interval essential.

As described earlier, a putative RNA-binding protein encoded by the *mec-8* gene regulates some of the alternative splicing events within this region of *unc-52* (Lundquist *et al.*, 1996). *mec-8* mutants exhibit a number of phenes, including mechanosensory and chemosensory defects, and a low-penetrance cold-sensitive lethality associated with muscle attachment defects (Lundquist and Herman, 1994). *mec-8* mutations also exhibit a synthetic lethal interaction with *unc-52(viable)* mutations; synthetic lethality results from the absence of *mec-8*-dependent splicing events. The deletions described in this study were isolated in a *mec-8(+)* background and remove part or all of the region in which *mec-8*-dependent splicing occurs. We tested two deletions, *ra515* and *ra516*, in combination with a putative null allele of *mec-8*, and found that these animals do not exhibit a synthetic lethal phenotype; however, they exhibit the full range of *mec-8* phenes, including the mechanosensory defects and cold-sensitive lethality.

DISCUSSION

Mammalian perlecan, a heparan sulfate proteoglycan, is an abundant component of most basement membranes. It is synthesized by a wide variety of cell types, including epithelial cells (Ohji *et al.*, 1994; van Det *et al.*, 1995), fibroblasts (Heremans *et al.*, 1989; Murdoch *et al.*, 1992), and myocytes (Murdoch *et al.*, 1993), and has been detected in all mammalian basement membranes surveyed to date (reviewed in Noonan and Hassell, 1993). In the nematode, we found that UNC-52 is localized to basement membranes associated with contractile tissues, including the pharyngeal, body-wall, and anal muscles. We did not detect UNC-52 in the basement membranes lining the pseudocoelom or surrounding the intestine. On this basis, we conclude that UNC-52 is not a general basement membrane component in *C. elegans* but is specifically associated with contractile tissues.

Recently, Graham *et al.* (1997) examined the distribution of the α1 and α2 collagen IV chains in *C. elegans*. The expression and localization of UNC-52 and collagen IV differ in several respects. First, collagen IV is more widely distributed than UNC-52 and is associated with the gonad and intestine at most developmental stages (Graham *et al.*, 1997). Second, collagen IV is expressed predominately in the body-wall muscles and is exported to basement membranes surrounding other tissues, including the pharynx and intestine (Graham *et al.*, 1997). In contrast, UNC-52 is expressed in a wider range of cell types, including pharyngeal and anal muscles, but does not diffuse beyond the expressing cells. Laser ablation studies established that UNC-52 acts cell autonomously and does not spread beyond the site of expression (Moerman *et al.*, 1996). We report here that UNC-52 isoforms exhibit spatial differences in localization. The inability of UNC-52 to diffuse or to be transported beyond the site of expression is probably important for establishing and maintaining these distinct spatial patterns.

A Subset of UNC-52 Isoforms Are Associated with Body-Wall Muscles during Embryogenesis and Are Required for Myofilament Lattice Assembly

In this study, we found that M isoforms of UNC-52 are essential for myofilament lattice assembly in the body-wall muscles. Myofilament lattice assembly in the nematode is remarkably similar to assembly of focal adhesions in mam-

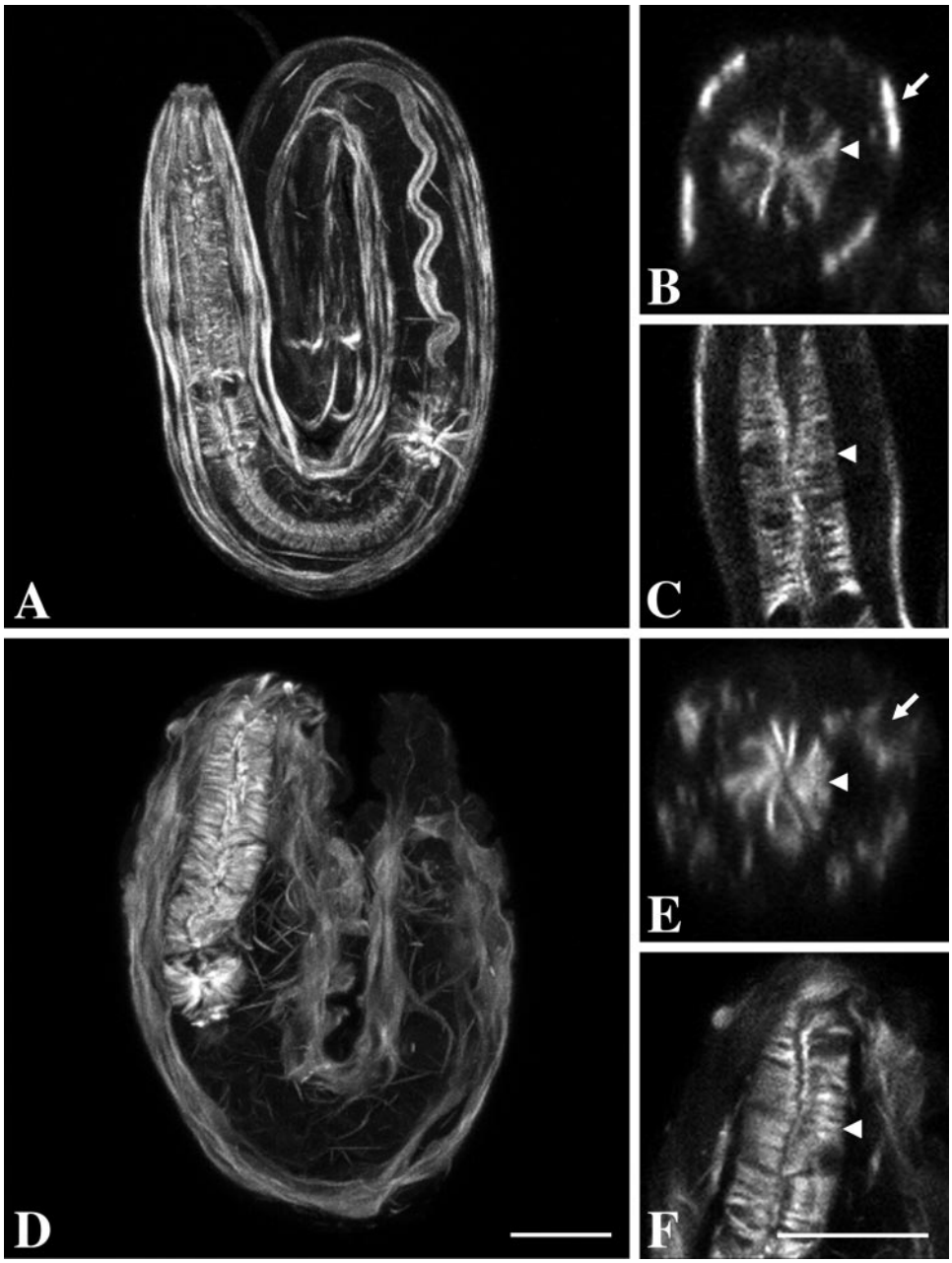

Figure 6. Phalloidin staining in wild-type and *unc-52(null)* embryos. Embryos were visualized by confocal microscopy. Wild-type (A–C) and *unc-52(st549)* (D–F) embryos were labeled with FITC-phalloidin. Images in A and D show staining from all focal planes. Computer-generated cross sections (B and E) and single focal plane images (C and F) are also shown. The arrowhead indicates the basal surface of the pharynx, whereas the arrow indicates the basal face of the body-wall muscles. Note the well organized thin filaments extending from the basal and apical faces of the pharynx in both wild-type and mutant embryos. Also note that actin in the body-wall muscles of the mutant is not associated with the basal cell membrane. Bar, 10 μm.

malian cell culture (Burridge *et al.*, 1988; Moerman and Fire, 1997). In both processes, integrin-ECM interactions are required to initiate assembly and stabilize existing adhesion complexes (Moerman and Fire, 1997; Yamada and Geiger, 1997). The localization of UNC-52 over the body-wall muscles and the effects of *unc-52(lethal)* mutations on myofilament assembly suggest that UNC-52 anchors the dense bodies and M-lines, perhaps through interactions with integrin. Whether UNC-52 plays an instructive role or simply an attachment role in assembly of integrin complexes at the muscle cell membrane is not clear; however, without a stable focal attachment structure at the muscle cell membrane, sarcomere units within muscle cells cannot be properly organized (Williams and Waterston, 1994; reviewed in Moerman and Fire, 1997).

Mammalian perlecan is widely expressed and has been detected in basement membranes of skeletal and cardiac myocytes (Murdoch *et al.*, 1993). A number of studies have demonstrated cell adhesive properties for various domains within perlecan (Hayashi *et al.*, 1992; Battaglia *et al.*, 1993; Chakravarti *et al.*, 1995), and integrin has been identified as a cell-surface mediator of this attachment (Hayashi *et al.*, 1992; Battaglia *et al.*, 1993). Two genes, *pat-3* (Gettner *et al.*, 1995) and *ina-1* (Baum and Garriga, 1997), have been shown

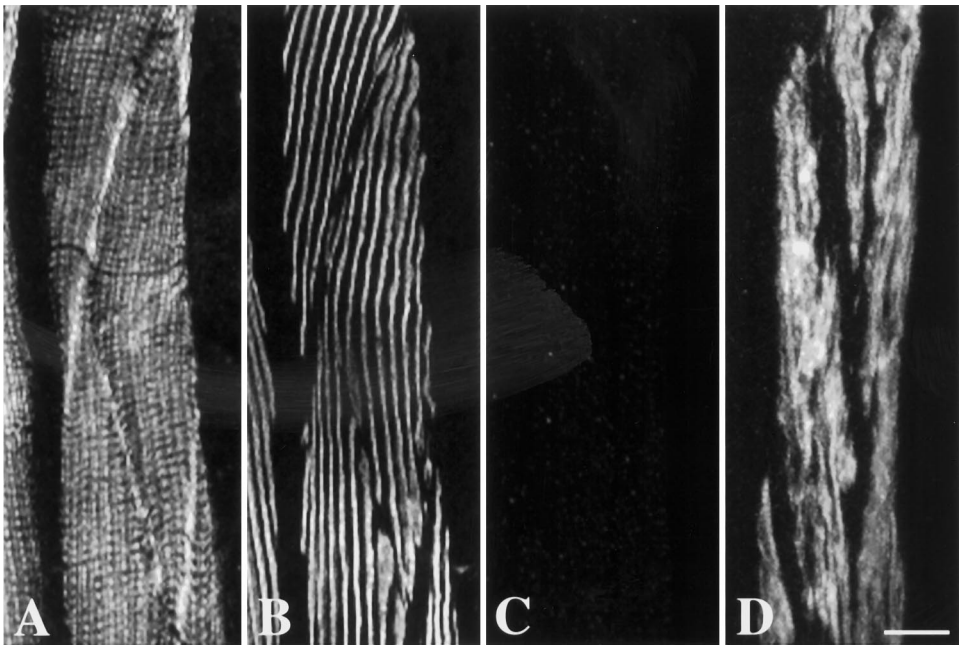

Figure 7. Immunolocalization of UNC-52 and myosin in *unc-52(viable)* mutants. Wild-type (A and B) and *unc-52(e444)* adults (C and D) were double-labeled with GM1 (UNC-52) and DM5.6 (MHC A). Note the reduced GM1 staining in C and disorganized myosin in D (compare with A and B). *unc-52(viable)* mutations disrupt the accumulation of UNC-52 and organization of myosin in late larval and adult animals. Bar, 10 μm.

to encode integrin subunits in *C. elegans*. The β-integrin encoded by the *pat-3* gene (βPAT-3) is expressed in various tissues, including the body-wall muscles, where it is localized at the transmembrane regions of dense bodies and M-lines (Francis and Waterston, 1985; Gettner *et al.*, 1995). In contrast, the α integrin encoded by the *ina-1* gene (αINA-1) is expressed most abundantly in the pharynx where it is concentrated beneath the basement membrane (Baum and Garriga, 1997). Thus, the body-wall and pharyngeal muscles synthesize distinct integrin heterodimers. An intriguing possibility is that different UNC-52 isoforms are capable of binding distinct integrin heterodimers. In the body-wall muscles, M isoforms colocalize with βPAT-3, whereas in the pharynx, S isoforms colocalize with αINA-1. These observations suggest that M and S isoforms may interact with different integrin complexes and function in distinct processes.

Several observations suggest that UNC-52 interacts directly with βPAT-3 integrin. First, the distribution of UNC-52 in the basement membrane overlaps with that of βPAT-3 integrin at the plasma membrane. Second, *unc-52* and *pat-3* lethal mutants both exhibit a severe Pat phenotype and have similar defects in myofilament lattice assembly (Williams and Waterston, 1994). Third, βPAT-3 integrin is highly disorganized in *unc-52(lethal)* mutants (Hresko *et al.*, 1994), supporting the idea that UNC-52 is required to anchor integrin. In various extracellular and cell surface proteins, RGD (Arg-Gln-Asp) sequences mediate interactions with cell-surface integrins (Ruoslahti, 1996). Murine and nematode perlecan both contain RGD sequences in domain III,

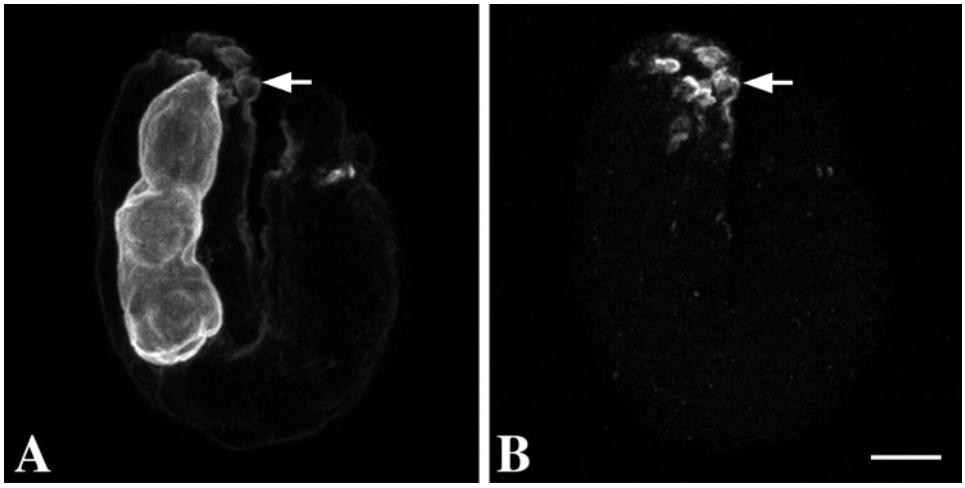

Figure 8. Expression of UNC-52 isoforms in *mec-8; unc-52* double mutants. A shows GM1 staining of a *mec-8(u74); unc-52(e1012)* double mutant. Note that pharyngeal staining appears to be normal, but body-wall muscle staining is greatly reduced, except over the anteriormost muscles. B shows MH3 staining of the same embryo. Note that body-wall muscle staining is restricted to the anteriormost muscle cells (indicated by arrows). Bar, 10 μm.

whereas nematode perlecan has an additional RGD in domain IV (Rogalski *et al.*, 1993). In contrast, human perlecan completely lacks this sequence (Kallunki and Tryggvason, 1992; Murdoch *et al.*, 1992). Murine perlecan has been shown to support integrin-mediated cell adhesion in an RGD-dependent manner (Hayashi *et al.*, 1992; Chakravarti *et al.*, 1995), although a recent study found that recombinant domain III from mammalian perlecan is not sufficient to bind integrin (Schulze *et al.*, 1996). Our results imply that domain IV is specifically required for myofilament lattice assembly in *C. elegans*. One of the NCAM repeats in domain IV, IgR12, has an RGD sequence that could mediate interaction with integrin; however, no direct evidence for an interaction between this domain and integrin has been demonstrated to date.

In contrast, domain V is not essential for integrin anchorage and myofilament lattice assembly. This domain is similar to the globular G-domain of α1 laminin and the C-terminal region of agrin (Noonan *et al.*, 1991; Kallunki and Tryggvason, 1992; Murdoch *et al.*, 1992; Patthy and Nikolics, 1994). The G-domain of α1 laminin is important for cell adhesion, including myoblast adhesion and neurite outgrowth, and has been shown to bind β-integrin (Sonnenberg *et al.*, 1990; Skubitz *et al.*, 1991; Yurchenco *et al.*, 1993). Consequently, domain V could interact directly with cell-surface components such as integrin. In the context of myofilament lattice assembly in *C. elegans*, such interactions are clearly dispensable; however, we do not exclude the possibility that domain V has a role in other developmental processes.

A Subset of UNC-52 Isoforms Are Associated with the Pharynx and Anal Muscles during Embryogenesis but Are Not Essential for Myofilament Assembly

In this study, we found that S isoforms of UNC-52 are associated with the pharynx and the anal sphincter and depressor muscles during embryogenesis; however, the role of these isoforms in pharyngeal or anal muscle development is not clear because we found no evidence of pharyngeal disruption in *unc-52(null)* mutants. Phalloidin staining established that the absence of UNC-52 has no discernible effect on the assembly of thin filaments in the pharynx. Similarly, preliminary studies suggest that the pharyngeal muscle-specific myosin, MHC C, assembles into well ordered A-bands in these mutants (Mullen, unpublished results).

Body-wall and pharyngeal muscle cells are known to express distinct sets of muscle proteins. For example, myosin heavy chains A and B are expressed in the body-wall muscles, whereas C and D are expressed in the pharyngeal muscles (MacKenzie *et al.*, 1978a; Ardizzi and Epstein, 1987). In this study, we describe the differential expression and localization of UNC-52 isoforms in the body-wall and pharyngeal muscles. Our results also emphasize the differences between body-wall and pharyngeal myofilament assembly. UNC-52 is clearly important for myofilament assembly in body-wall muscles but is dispensable for assembly in pharyngeal muscles. Similarly, vinculin, which is abundantly expressed in both body-wall and pharyngeal muscles (Francis and Waterston, 1985), is not essential for myofilament assembly in pharyngeal muscles (Mullen, unpublished results). The implication is that body-wall and pharyngeal muscles require different proteins for myofilament lattice assembly, even in cases where the same or similar proteins are expressed in both muscle types.

Evidence for a Temporal Shift between Early and Late UNC-52 Isoforms

Domain IV of perlecan is composed of Ig repeats similar to those of the NCAM. The number of NCAM repeats in this domain varies between species; mouse perlecan has 14 repeats, nematode perlecan has 15 repeats, and human perlecan has 22 repeats (Noonan *et al.*, 1991; Kallunki and Tryggvason, 1992; Murdoch *et al.*, 1992; Rogalski *et al.*, 1993). This repetitive structure is typical of ECM components and transmembrane proteins with large extracellular domains (reviewed in Vaugh and Bjorkman, 1996). In human perlecan, as in UNC-52, the NCAM repeats are encoded by multiple exons in a manner compatible with different combinatorial possibilities of expression (Kallunki and Tryggvason, 1992; Murdoch *et al.*, 1992; Cohen *et al.*, 1993). There are data indicating that alternative splicing occurs in domain IV of mouse perlecan, but, as yet, no data confirming splice variants of human perlecan (Noonan and Hassell, 1993; Iozzo *et al.*, 1994).

Alternative splicing between exons 15 and 19 regulates the number of NCAM repeats within domain IV of UNC-52 (Rogalski *et al.*, 1993, 1995) and is associated with both temporal and spatial differences in isoform expression. Our results imply that there is a transition or "shift" between early and late isoforms of UNC-52. This shift in isoform expression may be regulated through *mec-8*. Our observations on *mec-8; unc-52* double mutants also imply that alternative splicing within domain IV is associated with spatial differences in isoform expression within the body-wall muscles. Several recent studies suggest that body-wall muscles do not uniformly express the same set of genes. The *unc-129* gene, which encodes a member of the TGF-β family, is expressed in motor neurons and the dorsal body-wall muscles (Colavita *et al.*, 1998). In addition, the homeotic gene *mab-5* is expressed in a subset of the posterior body wall muscles (Wang *et al.*, 1993). These observations suggest that body-wall muscle cells in different locations express distinct sets of regulatory and structural genes (reviewed in Moerman and Fire, 1997). The spatially restricted expression of certain UNC-52 isoforms implies that they are specifically required by muscle cells in these locations. The distinct arrangement of muscle and hypodermal cells in the head of the animal may necessitate a specific UNC-52 isoform for myofilament assembly or cell adhesion.

Interestingly, alternative splicing within domain IV of UNC-52 is dispensable. Considering that these splicing events are highly regulated both temporally and spatially, this observation was surprising. A deletion identified in this study eliminates all variants except the 15–19 splice product and does not noticeably affect muscle development. This observation suggests that alternative splicing within this region is associated with fine modulation of function rather than large-scale changes in biophysical properties. In contrast, alternative splicing events that give rise to the three major groups of isoforms are likely to significantly change

the properties of these proteins, including their ability to interact with transmembrane receptors such as integrin.

Of particular importance in this study of perlecan function is our demonstration that a domain IV-containing isoform, not a domain V-containing isoform, is sufficient for myofilament organization and attachment. If perlecan/UNC-52 serves as an adhesive substrate for anchoring the myofilament lattice, then these results help to identify regions of UNC-52 that are critical for integrin anchorage and myofilament lattice assembly. In summary, this study demonstrates some of the functional complexity and structural plasticity of nematode perlecan. We suggest that the redundancy observed with perlecan may be an inherent property of large ECM proteins.

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