***Caenorhabditis elegans* UNC-98, a C₂H₂ Zn Finger Protein, Is a Novel Partner of UNC-97/PINCH in Muscle Adhesion Complexes[□]**

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To further understand the assembly and maintenance of the muscle contractile apparatus, we have identified a new protein, UNC-98, in the muscle of *Caenorhabditis elegans*. *unc-98* mutants display reduced motility and a characteristic defect in muscle structure. We show that the major defect in the mutant muscle is in the M-lines and dense bodies (Z-line analogs). Both functionally and compositionally, nematode M-lines and dense bodies are analogous to focal adhesions of nonmuscle cells. UNC-98 is a novel 310-residue polypeptide consisting of four C₂H₂ Zn fingers and several possible nuclear localization signal and nuclear export signal sequences. By use of UNC-98 antibodies and green fluorescent protein fusions (to full-length UNC-98 and UNC-98 fragments), we have shown that UNC-98 resides at M-lines, muscle cell nuclei, and possibly at dense bodies. Furthermore, we demonstrated that 1) the N-terminal 106 amino acids are both necessary and sufficient for nuclear localization, and 2) the C-terminal (fourth) Zn finger is required for localization to M-lines and dense bodies. UNC-98 interacts with UNC-97, a *C. elegans* homolog of PINCH. We propose that UNC-98 is both a structural component of muscle focal adhesions and a nuclear protein that influences gene expression.

INTRODUCTION

Despite increasing knowledge of their components, relatively little is known about how myofibrils are assembled, or how ordered myofibrils are maintained in the face of repeated muscle activity. Study of the model organism *Caenorhabditis elegans* is providing important insights into these questions (Waterston, 1988; Moerman and Fire, 1997). In the nematode, most of the muscle is located in the body wall and is used for locomotion. The nematode body wall muscle extends down the length of the worm in four quadrants and is clearly visible by polarized light microscopy. Unlike that of vertebrate muscle, this muscle is obliquely striated. In

addition, the myofibrils are not packed throughout the muscle cell, but are restricted to a single 1- to 2- μ m-thick zone closely apposed to the muscle cell membrane. Nevertheless, actin containing thin filaments are attached to Z-disk-like structures called dense bodies, and myosin-rich thick filaments are organized around M-lines. Most importantly, all the dense bodies and M-lines are anchored to the muscle cell membrane, which is attached to the hypodermis and cuticle, allowing the force of muscle contraction to transmit directly to the cuticle to create movement of the whole worm. Vertebrate striated muscle contains similar thin and thick filament attachment structures (Z-discs and M-lines, respectively), but only a few of them are attached to the muscle cell membrane. Specifically, a small fraction of Z-discs is anchored to the sarcolemma through costameres. Also, thin filaments of sarcomeres located at the ends of muscle cells, are anchored through attachment plaques at myotendinous junctions of skeletal muscle or the intercalated disks of cardiac muscle.

Thus, nematode muscle M-lines and dense bodies serve the function of analogous structures in vertebrate muscle in terms of attachment of thick and thin filaments. But, because

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of their membrane anchorage, they are also similar to vertebrate nonmuscle focal adhesions. This similarity extends to their protein compositions, as well. In many cultured adherent cells, focal adhesions (or focal contacts) are sites of cell attachment to the extracellular matrix where integrins and numerous (>30) associated proteins link the extracellular matrix to the actin cytoskeleton (for reviews, see Sastry and Burridge, 2000; Geiger and Bershadsky, 2001). These proteins include structural components such as talin, vinculin, and α -actinin, and numerous signaling molecules such as Src, focal adhesion kinase, and paxillin. Vertebrate focal adhesions, compared with vertebrate muscle Z-discs and M-lines, have few similarities in terms of protein composition. In Z-discs, although there are muscle-specific isoforms of actin, α -actinin, and filamin, most of the components are muscle and/or Z-disk specific (e.g., titin, nebulin, telethonin, myotilin, ALP, ZASP, and FATZ; Faulkner *et al.*, 2001).

Many of the proteins known to be components of *C. elegans* dense bodies and M-lines are orthologs of known components of vertebrate focal adhesions. In the extracellular matrix of *C. elegans* body wall muscle, concentrated underneath the dense bodies and M-lines is the nematode homolog of perlecan, UNC-52 (Rogalski *et al.*, 1993). Within the muscle cell membrane, localized at the bases of both dense bodies and M-lines are the integrins, including PAT-3- β -integrin (Williams and Waterston, 1994; Gettner *et al.*, 1995) and PAT-2- α -integrin (Williams, unpublished data). Traveling further internally, dense bodies and M-lines contain talin (Moulder *et al.*, 1996); UNC-97, a protein composed of five LIM domains (Hobert *et al.*, 1999); UNC-112, a conserved FERM domain-containing protein (Rogalski *et al.*, 2000); and PAT-4, which is integrin-linked kinase (Mackinnon *et al.*, 2002). Vinculin (*C. elegans* DEB-1; Barstead and Waterston, 1989; Barstead and Waterston, 1991) and α -actinin (Francis and Waterston, 1985) are found specifically in the dense bodies, whereas UNC-89 is found only in the M-lines (Benian *et al.*, 1996).

Loss-of-function or null mutations in many of the aforementioned proteins display the paralyzed arrested at two-fold stage ("Pat") embryonic lethal phenotype. The Pats comprise one of the two major phenotypic classes of muscle affecting mutations in the worm. In Pat animals, embryos do not move within the eggshell, elongation ceases at the two-fold stage, and death ensues around the time of hatching (Williams and Waterston, 1994). Mutations in the genes that encode the membrane-associated components of the muscle focal adhesions (*unc-52*, *unc-112*, *pat-3*, and *pat-4*) display the most severe Pat phenotype, in which neither thin filaments nor thick filaments are assembled into the myofibril lattice. Therefore, it is likely that perlecan (UNC-52) in the extracellular matrix and integrins in the muscle cell membrane form nucleating complexes that recruit other proteins to form M-lines and dense bodies, and this leads ultimately to the incorporation of thick and thin filaments into myofibrils. Antibody staining of wild-type embryos has provided additional evidence for this model (Hresko *et al.*, 1994). Moreover, recent studies indicate that integrin-linked kinase (PAT-4) and UNC-112 serve intermediary roles between the integrins and the muscle filaments, and act as adaptor proteins (Mackinnon *et al.*, 2002).

The second major phenotypic class of muscle-affecting mutations is called "Unc," which typically display unco-

ordinated or slow movement, or even paralysis. Genes in this class include *unc-54* (myosin heavy chain B; Epstein *et al.*, 1974), *unc-15* (paramyosin; Kagawa *et al.*, 1989), *unc-22* (twitchin; Benian *et al.*, 1989), and *unc-60* (ADF/cofilin; McKim *et al.*, 1994). For several genes, the loss-of-function phenotype is Unc, whereas the null phenotype is Pat. One example is *unc-97*. UNC-97 consists of five tandem LIM domains, and the use of an UNC-97::green fluorescent protein (GFP) fusion protein has shown it to be localized to muscle M-lines, dense bodies, and nuclei. A splice-site mutation of *unc-97* is Unc, whereas the RNAi phenotype is a Pat embryonic lethal (Hobert *et al.*, 1999). Moreover, a recently isolated null allele of *unc-97* is Pat (Cordes and Moerman, unpublished data). The original *unc-97* allele, *su110*, is a splice site mutation that is expected to result in a nearly complete UNC-97 except for the last ~15 amino acids of the last (10th) Zn finger. *su110* animals are limp, egg-laying defective, and slow moving to paralyzed. They have an interesting muscle phenotype observable by polarized light: if handled gently, they display nearly normal structure, but if pressure is applied, the myofibrils seem to collapse. Immunofluorescence microscopy by using antibodies against dense body components show that in *su110* mutants, some muscle cells have dense bodies that are fused into small aggregates or long strips. The ortholog of UNC-97 in mammals is called "PINCH." PINCH interacts with integrin-linked kinase and the adaptor protein Nck-2 and is part of a multicomponent complex that includes the integrins (for review, see Wu and Dedhar, 2001). Although the original PINCH, now called PINCH-1, was found to immunolocalize only to focal adhesions, a second PINCH protein in mammals, PINCH-2, has been shown recently to immunolocalize to both focal adhesions and nuclei (Zhang *et al.*, 2002).

We now report the existence of a new protein, UNC-98, that is likely to be part of the same protein complex as UNC-97 in nematode muscle. A loss-of-function mutation in the *unc-98* gene results in animals with reduced motility and abnormal muscle structure (Zengel and Epstein, 1980). By polarized light microscopy, *unc-98*(*su130*) has a less organized lattice, that is, fewer or less distinct A and I bands, and bright needle-like structures at the ends of the muscle cells. So far, this polarized light phenotype has been seen in only one other muscle Unc, *unc-96* (Zengel and Epstein, 1980). In their description of the electron microscopy (EM) appearance of *su130* muscle, Zengel and Epstein interpret the polarized light needles as being composed of thin filaments, and the overall structure of the muscle to have "an abnormal but definite pattern of A and I band organization, including distinct though irregular Z bodies" (dense bodies). Herein, we demonstrate that *unc-98* encodes a 310-residue polypeptide composed of four C2H2 Zn fingers and putative nuclear localization signal (NLS) and nuclear export signal (NES) sequences. Consistent with the mutant phenotype, UNC-98 is localized to M-lines and probably to dense bodies. Surprisingly, UNC-98 is also present in muscle cell nuclei. We hypothesize that UNC-98 is a structural component of muscle focal adhesions that is added at the final stages of assembly and may be involved in maintaining muscle structure.

MATERIALS AND METHODS

Strains and Genetics

The following strains were used: N2 wild-type strain, *unc-98(su130)*, *unc-98(sf19)*, deficiency *sfDf1*, *unc-97(su110)*, and OH122 [mgIs25 (integrated Ex[*unc-97::GFP*])]. For mapping *unc-98* we used TU899 [*stDp2* (X;II)/+ II; *uDf1* X], RW2552 [*stDp2*/+; *stDf6*], RW2551 [*stDp2*/+; *stDf5*], DSU130 [*dpy-7(e88) unc-98(su130)*] and RW6002[*stDp2*/+; *unc-18*]. To test for rescue of the *unc-98(su130)* by the deletion derivatives of UNC-98::GFP (constructs A and E described below), expressing (green) males from N2; *sfEx23[unc-98::GFP construct A]*, or N2; *sfEx25[unc-98::GFP construct E]* were mated with *dpy-7(e88) unc-98(su130)*. In the F2 generation, green Dpy hermaphrodites were examined by polarized light.

Polarized Light and Electron Microscopy

Polarized light microscopy was performed as described in Waterston *et al.* (1980). Transmission EM was performed essentially as described in Edens *et al.* (2001).

Motility Assays

Motility assays were performed by placing single, gravid adult hermaphrodites in 10 to 15 μ l of M9 buffer. Hermaphrodites were allowed to acclimate to the new environment for 30 s. Motility was then determined by counting the number of "beats" over a 1-min time interval. Normal, coordinated movement of the worm involves a forward or backward sinusoidal movement. This pattern of movement can be observed either on a solid surface, or in liquid, but in liquid the movement is much quicker. Thus, for the assay, a single beat was defined as one sine wave movement resulting in a complete swing of the anterior portion or "head" from left to right and left again. For all genotypes, $n = 50$.

F1 Noncomplementation Screen for New *unc-98* Alleles

Wild-type males were mutagenized by soaking in *N*-ethyl-*N*-nitrosourea at a concentration of 1.4 mM for 4 h (De Stasio *et al.*, 1997) and mated to *dpy-7(e88) unc-98(su130)* hermaphrodites. L4 non-Dpy cross progeny were picked singly, allowed to lay eggs, and then examined by polarized light microscopy for the *Unc-98* muscle phenotype. Non-Dpy F2 from a population of both Dpy and non-Dpy progeny were cloned to homozygote candidate new alleles.

Transgenic Animals and Rescue Analysis

Cosmid or DNA fragments (restriction fragments or polymerase chain reaction [PCR] products) were coinjected with the *rol-6* dominant transformation marker plasmid pRF4 (Mello and Fire, 1995). F1 rollers were cloned, and roller lines were established and then examined by polarized light microscopy for rescue of the *Unc-98* phenotype. The supplemental figure (on line) shows which segments of F08C6 were tested for rescue. Cosmid DNAs were prepared using Plasmid Maxi kit (QIAGEN, Valencia, CA). PCR products were generated using Advantage Genomic Polymerase Mix (BD Biosciences Clontech, Palo Alto, CA).

RNA Interference

RNA interference was carried out as described by Fire *et al.* (1998). A nearly complete cDNA for F08C6.7 was generated by reverse-transcription (RT)-PCR and cloned into pBluescript-SK by using the *HindIII* and *SstI* restriction sites. Sense and antisense RNA were produced using the T3 and T7 promoters of Bluescript and an RNA synthesis kit from Promega (Madison, WI). The RNAs were annealed and injected into the gonads of wild-type hermaphrodites.

unc-98::GFP Full-Length Translational Fusion Construct and Transgenic Lines

An ~7-kb long-range PCR fragment, including ~4 kb upstream of the predicted initiator methionine of F08C6.7 and ending at the penultimate predicted codon, was cloned, in-frame, into the promoterless GFP vector pPD95.77 (kindly provided by A. Fire, Carnegie Institute of Washington, Baltimore, MD) by using a *HindIII* site added at the 5' end and a *BamHI* site added at the 3' end. pPD95.77 includes the *unc-54* 3'-untranslated region (UTR) and lacks an NLS. The resulting construct is expected to use the *unc-98* promoter to drive expression of a fusion protein consisting of full-length UNC-98 protein and GFP at the C terminus. This fusion gene plasmid was injected into both wild-type and *unc-98* mutant animals at a concentration of ~44–88 ng/ μ l, either alone or together with *rol-6* DNA at ~80 ng/ μ l. Three stable roller/*unc-98::GFP*-expressing lines were obtained in the N2 background, and two such lines were obtained in the *unc-98(su130)* mutant background. Two *unc-98::GFP* expressing lines were identified by their green fluorescence in a wild-type background (i.e., no *rol-6* DNA had been used).

Generation of *unc-98::GFP* Deletion Derivatives to Map Regions Required for Nuclear versus Focal Adhesion Localization

Using a similar approach as outlined above, we generated four *unc-98::GFP* constructs (A–D) each having one less Zn finger from the C terminus, except that different 3' primers were used. Long-range PCR fragments were cloned into pPD95.77 between the *HindIII* and *BamHI* sites. We used site overlap extension (SOE) PCR (Warrens *et al.*, 1997) to generate an *unc-98::GFP* construct (E) in which the initiator methionine was followed directly by the four Zn finger regions (i.e., it has an in-frame deletion of residues 2–107). Transgenic lines were generated for each construct and identified by their green fluorescence (i.e., no additional transformation marker was used), with the exception of D, which was coinjected with *rol-6* DNA. We obtained six lines with A, one line with B, three lines with C, four lines with D, and one line with E.

Antibody Staining and GFP Expression Analysis

The procedure for immunofluorescence localization of antigens in wild-type, *unc-98* mutant animals and *unc-98*-GFP translational fusion lines was carried out as described in Benian *et al.* (1996) except that the liquid nitrogen freeze-thaw step was increased to four times, and the worms were mounted using the ProLong Antifade kit (Molecular Probes). Mouse monoclonal antibodies used were as follows: MH13 (Waterston, 1988), anti-vinculin antibody MH24 (Francis and Waterston, 1985), anti- α -actinin antibody MH35 (Francis and Waterston, 1985), anti-actin clone C4, and anti-myosin myoA (Miller *et al.*, 1983). To visualize both MH13 and actin staining simultaneously in *su130* muscle (Figure 2, E–G), we used polyclonal antibodies AAN1 to a vertebrate actin (Cytoskeleton, Inc., Denver, CO) that had previously been shown to stain *C. elegans* actin (Ono *et al.*, 2003). Fluorescein isothiocyanate, tetramethylrhodamine B isothiocyanate, and Cy-3-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Most images were captured on Kodak Tmax 400 or Fuji Sensia 100 film, by using an Axioskop microscope (Carl Zeiss, Jena, Germany). Images were processed with Adobe Photoshop. The expression pattern and localization of UNC-98-GFP in transgenic animals was recorded from live animals that had been placed in M9 buffer containing 1 mM sodium azide. 4,6-Diamidino-2-phenylindole (DAPI) staining (1 h at 1 μ g/ml) was performed by carrying out the above-mentioned immunofluorescence staining procedure, but without addition of primary or secondary antibodies. Experiments using dual fluorophores to examine colocalization were acquired on an Axiovert microscope equipped with confocal capabilities (Carl Zeiss) and LSM software. Images were captured for the dual flu-

orophores as well as each fluorophores' channel individually. In addition, localization of UNC-98-GFP in muscle cell nuclei and the images of *su130* muscle stained with MH13 and anti-actin were acquired with a scientific-grade cooled charge-coupled device on a multiwavelength wide-field three-dimensional microscopy system. Samples were imaged in successive 0.25- μ m focal planes, and out-of-focus light was removed with a constrained iterative deconvolution algorithm (Weiner *et al.*, 1999).

Sequence Analysis

The DNA sequence of cosmid F08C6 was obtained from the WormBase Web site. We took into account both the predictions of exons in WormBase, and our own analysis by GeneMark.hmm (Shmeleva, Benian and Borodovsky, unpublished data). We incorporated the partial cDNA sequences available for this gene, generated by Y. Kohara and colleagues (exons 4–7 from yk275e5 and yk303a12). All other intron/exon boundaries were verified by determining the sequences of RT-PCR products. Only GeneMark.hmm predicted all the protein coding exons correctly. The 3' end of the *unc-98* mRNA was determined by finishing the sequence of clones yk275e5 and yk303a12 up to the polyA tail. The 5' end of the mRNA was determined by using a 5' rapid amplification of cDNA ends system kit (Invitrogen, Carlsbad, CA). The predicted UNC-98 polypeptide sequence was analyzed by BLAST searches for homologs and PFAM for protein domains, via their Web sites (www.ncbi.nlm.nih.gov/BLAST/ and www.sanger.ac.uk/Software/Pfam/search.html, respectively). We found the NLS and NES sequences by visual inspection and comparison to known consensus sequences.

Generation of Anti-UNC-98 Antibodies, Western Blots, and Immunofluorescence Microscopy

A cDNA encoding the complete UNC-98 amino acid sequence was amplified from a random primed cDNA library (kindly provided by R. Barstead, Oklahoma Medical Research Foundation, Oklahoma City, OK) by using primers with a *Bam*HI site added at the 5' end and a *Sal*I site added to the 3' end, and cloned into pBluescript, resulting in the plasmid pDM#461. After confirmation of the DNA sequence, the *Bam*HI/*Sal*I fragment from pDM#461 was cloned into pMAL-KK-1 (vector kindly provided by Dr. K. Kaibuchi, Nagoya University, Nagoya, Japan), to produce the plasmid pDM#489. After transformation into *Escherichia coli* BL21, the maltose binding protein-UNC-98 fusion protein was purified using amylose affinity resin (New England BioLabs, Beverly, MA). The MBP-UNC-98 was sent to Spring Valley Laboratories (Woodbine, MD) for generation of rabbit antibodies (called EU131). Antibodies were affinity purified by using an Affigel (Bio-Rad, Hercules, CA) column to which glutathione S-transferase (GST)-UNC-98 fusion protein had been covalently coupled. Immunoblot analysis of Laemmli total soluble proteins from wild-type, *unc-98* mutants, *unc-98(RNAi)* animals, and a line carrying an extrachromosomal array of *unc-98::GFP*, was performed as described in Benian *et al.* (1996) except that 10 or 12.5% acrylamide gels were run and transferred to nitrocellulose for 1 h. The protein concentrations of Laemmli extracts were determined by a filter paper dye-binding assay (Minamide and Bamberg, 1990), allowing us to load equal quantities of protein (~30 μ g) from wild-type and mutant strains. For the comparison of wild-type and *unc-98(RNAi)*, Laemmli extracts were prepared by the method of Hannak *et al.* (2002) from both 150 L4/young adult wild-type and *unc-98(RNAi)* animals. The affinity-purified anti-UNC-98 antibodies (EU131) were reacted against the blots at a 1:250 dilution. Affinity-purified rabbit antibodies to GFP were purchased from Chemicon International (Temecula, CA) and used at a 1:500 dilution. The affinity-purified anti-UNC-98 antibodies (EU131, at 1:500 dilution) were used in immunofluorescence localization on whole worms fixed with methanol/paraformaldehyde, as described above. Worms were costained with a rat anti-UNC-89 antibody called EU133 at a 1:500 dilution (Small, Flaherty, and Benian, our unpub-

lished data). These results are shown in Figure 8, A–J. Figure 8K shows results of anti-UNC-98 staining of frozen sections of wild-type animals (Benian *et al.*, 1996) fixed with *n*-heptane.

Screen for Interaction of UNC-98 with Known Components of Nematode Adherens Junctions

Full-length *unc-98* coding sequence contained in the *Bam*HI/*Sal*I fragment of pDM#461 was cloned into pGAD-C1 (James *et al.*, 1996) to make pDM#463 (UNC-98 full-length fusion with activator domain of GAL4). To make deletion derivatives, PCR-amplified fragments were similarly cloned into pGAD-C1. DNA sequencing was used to select clones that were free from PCR-induced mutations. pDM#429 (full-length UNC-97 fused with the DNA binding domain of GAL4) is described in MacKinnon *et al.* (2002). For making UNC-97 deletion derivatives, PCR-amplified fragments were cloned into pGBDU-C1 (Norman, Qadota, and Moerman, unpublished data). Two hybrid assays were performed as described in MacKinnon *et al.* (2002).

In Vitro Binding Assay

The MBP-UNC-98 fusion protein that was described above and used as immunogen to produce anti-UNC-98 antibodies was also used in these binding experiments. Similarly, to produce a GST-UNC-97 fusion protein, the *Bam*HI/*Bgl*II fragment of pDM#429 was cloned into pGEX-KK-1 (also provided by Dr. K. Kaibuchi). GST-UNC-97 and MBP-UNC-98 fusion proteins, and GST and MBP alone, were prepared from *E. coli*. Approximately 25 μ g each of either GST-UNC-97 or GST were incubated together with MBP-UNC-98 or MBP in binding buffer (20 mM NaCl, 0.1% Triton X-100, 10% glycerol) in a volume of 150 μ l for 3 h. Then ~50 μ l of glutathione-agarose affinity beads (Sigma-Aldrich, St. Louis, MO) were added and incubated for an additional 1 h, after which the beads were pelleted and washed five times with binding buffer. Incubations and washes were performed at 4°C. Bound proteins were extracted by heating to 95°C in 50 μ l of Laemmli sample buffer and separated on a 10% polyacrylamide SDS gel and stained with Coomassie Blue.

RESULTS

The *unc-98* Mutant Phenotype

In wild-type *C. elegans*, by polarized light microscopy, the spindle-shaped body wall muscle cells have a highly organized myofilament lattice in which bright A-bands alternate with dark I-bands (Figure 1A). The I-bands contain dots or dashes that correspond to dense bodies (seen at higher magnification). As first described by Zengel and Epstein (1980), *unc-98(su130)* has a less organized lattice and bright "needle-like" structures at the ends of the muscle cells (Figure 1B). As shown in Figure 2, B, D, and F, these needle-like structures do not stain with phalloidin, or antibodies to actin or myosin. However, as first noted by R. Francis and R. Waterston (Waterston, 1988), they do stain with monoclonal antibody MH13 (Figure 2E), which recognizes two intermediate filament proteins in the worm, IFA4 and IFB2 (Karabinos *et al.*, 2001). These monoclonals do not stain myofibrils in wild-type animals (our unpublished data). Costaining of *unc-98(su130)* with MH13 and anti-actin call into question the original interpretation of these accumulations of filaments as being thin filaments: needle-like structures are stained with MH13 but not anti-actin (Figure 2, E–G). Moreover, our myosin and phalloidin staining suggests that in *unc-98(su130)*, both A-bands and I-bands are disorganized as compared with wild-type (Figure 2, A–D).

Figure 1. *unc-98* mutants are defective in muscle structure and motility. (A–E) Polarized light microscopy of body wall muscle. Note the beautifully organized myofibrils in wild-type (N2) muscle (A). *unc-98(su130)* and *unc-98(sf19)* show disorganized myofibrils and bright needles at the ends of the muscle cells (B and C). Note the same appearance in the F1 progeny of animals injected with double-stranded RNA from F08C6.7 (D). Note the rescue of the *Unc-98* phenotype by an 8-kb fragment containing the F08C6.7 coding plus flanking sequences (E). The single needle seen in one of the muscle cells suggests either the animal is partially rescued, or is a genetic mosaic. Bar, 10 μ m. (F) Motility assays on adult worms of the indicated genotypes.

Figure 2. Further characterization of structural defects in *unc-98* mutant muscle. Localization of myosin and F-actin in the body wall muscle of wild type and *unc-98(su130)*, and MH13 epitopes in *unc-98(su130)*. Note that the banding of myosin staining in *unc-98(su130)* (B) does not follow the precise pattern seen in wild type (A). F-actin staining by phalloidin in *unc-98(su130)* (D) reveals an irregular banding pattern compared with wild type (C). This abnormality in I-bands is even more clear in the labeling with anti-actin antibodies (F). (E–G) represent the same muscle cells from *unc-98(su130)* costained with MH13 and anti-actin antibodies. Notice that the staining of *unc-98(su130)* muscle obtained with the MH13 monoclonal antibody (E) bears a strong resemblance to the appearance of *unc-98(su130)* by polarized light microscopy (Figure 1B). As shown in the composite image (G), the structures that probably correspond to the birefringent inclusions, react with MH13 but not with anti-actin. Bars, 5 μ m.

Figure 3. Electron microscopy of body wall muscle from wild-type (A) and *unc-98(sf19)* (B). These are cross sections of the myofilament lattice in which the filaments look like dots. Arrowheads denote dense bodies and arrows denote M-lines. Bar (A and B), 1 μ m.

To obtain further insight into *unc-98* function, we isolated additional *unc-98* mutant alleles. One allele, *sf19*, and one noncomplementing deficiency, *sfDf1* (see below), were recovered. As shown in Figure 1C, *unc-98(sf19)* homozygotes have the same polarized light phenotype as *unc-98(su130)*. Although not obviously slower when casually viewed moving along an agar surface, a quantitative motility assay demonstrates that both *su130* and *sf19* are slower than wild type, at a level of statistical significance (Figure 1F). In this assay, the average motility for wild-type is 95 beats/min versus 75 and 62 beats/min for *su130* and *sf19*, respectively ($p < 0.001$ against wild type, in a t test for independent samples). The motility of the trans-heterozygote, *sf19/sfDf1*, averages 50 beats/min, and these animals seem slower by casual observation. This motility defect is most obvious in liquid: whereas wild-type animals swim in a smooth sinusoidal manner, *sf19/sfDf1* animals swim in a jerky, flip-flop manner. Therefore, we conclude that *sf19* and *su130* are hypomorphic alleles of *unc-98*.

Although the EM appearance of *su130* was described by Zengel and Epstein (1980), we decided to perform EM on the new allele, *sf19*. As shown in Figure 3, A and B, we observe shorter, very irregular dense bodies, and shorter or even absent M-lines. (The severity of this disruption varies from one sarcomere to another). This EM also reveals that, as suggested by phalloidin and anti-myosin staining, both thin and thick filaments are poorly organized in *unc-98* mutants.

Molecular Cloning of the *unc-98* Gene

Previously, *unc-98* had been mapped near *dpy-7* (Zengel and Epstein, 1980). By use of deficiencies and duplications, and

later, by three-factor mapping, we placed *unc-98* to a narrow interval between cloned markers *dpy-7* and *unc-18*. This small region (~ 0.30 map units) on the left arm of the X chromosome is covered by five overlapping cosmids: F14G1, F08C6, C44E12, F26A10, and F27D9. Transgenic rescue of *unc-98* was obtained using F08C6, with two of four lines complementing the mutant phenotype. Restriction and PCR fragments, covering various regions and predicted genes within the cosmid, were then tested for transgenic rescue. The smallest fragment to rescue *unc-98* is a PCR-generated 8.1-kb fragment (Figure 1E) that includes only one (Wormbase and GeneMark.hmm) predicted gene, namely, F08C6.7. To determine whether there might be another unrecognized gene within this 8.1-kb region, a smaller 4.2-kb fragment (containing the 4 kb of sequence upstream of F08C6.7) was created for injection. This 4.2-kb fragment did not rescue leaving us to conclude that F08C6.7 is *unc-98*. As further confirmation that we had identified the *unc-98* sequence, we determined the RNAi phenotype of F08C6.7. As shown in Figure 1D, F1 progeny from wild-type hermaphrodites that had been injected with double-stranded RNA representing the nearly complete coding sequence for F08C6.7, have a polarized light appearance identical to *unc-98(su130)* and *unc-98(sf19)*.

To define the *unc-98* gene structure, we began with exon predictions displayed on Wormbase, and our own exon predictions made by GeneMark.hmm (Shmelyeva, Benian, and Borodovsky, unpublished data). The complete gene structure was determined by sequencing cDNAs, RT-PCR, and rapid amplification of cDNA ends products. Only GeneMark.hmm predicted all the protein coding exons correctly. Specifically, exon 2 (78 base pairs) was predicted by GeneMark.hmm but missed by WormBase, and GeneMark.hmm predicted the correct length of exon 1 as 24 base pairs (WormBase predicted exon 1 to be 18 base pairs longer at the 3' end). The *unc-98* mRNA is 1301 nucleotides (nt), with a 102 nt 5'-UTR and a 266-nt 3'-UTR and is encoded by seven exons (GenBank accession no. AF515600).

To further confirm our identification of the *unc-98* coding region and to gain insight into the mutant phenotype, we have determined the sequence alterations in the two homozygous viable *unc-98* mutant alleles. Both mutant alleles have G-to-A transitions in highly conserved splice acceptor sites. In *unc-98(sf19)* the mutation lies at the end of intron number 3, whereas in *su130* the mutation lies at the end of intron number 6 (Figure 4A). As shown below (Figures 7 and 8), either by Western blot or fluorescence microscopy, antibodies generated to UNC-98 fail to detect protein from these *unc-98* mutant animals. Thus, both alleles are loss-of-function. *sfDf1* is an ~ 40 -kb deletion (Figure 4B) that uncovers the first, second, and possibly the third exon of F08C6.7. *sfDf1* also uncovers at least four WormBase-predicted genes to the right of F08C6.7. *sfDf1* homozygotes are larval lethal. Because *sfDf1* homozygotes are not embryonic lethal, it is not likely that the null phenotype for *unc-98* is a Pat embryonic lethal.

UNC-98 Is 310 Residues and Contains 4 C2H2 Zn Fingers and Putative NLS and NES Sequences

The UNC-98 polypeptide is 310 amino acids long (Figure 5A) and has a calculated molecular mass of 35,371 Da. Computer algorithms predict only one type of domain,

Figure 4. Sequence alterations in two *unc-98* mutants and a noncomplementing deficiency. (A) *unc-98(su130)* and *unc-98(sf19)* are G-to-A transitions in the splice acceptor sites of introns 6 and 3, respectively, of F08C6.7. (B) *sfDf1* is an ~40-kb deletion that uncovers the first, second, and possibly the third exon of F08C6.7. *sfDf1* also uncovers at least four WormBase-predicted genes to the right of F08C6.7. These genes include F08C6.2 (cholinephosphate cytidyltransferase), F08C6.1 (thrombospondin), C44E12.1, and C44E12.3 (no homologies to proteins of known function).

namely, Zn fingers of the C2H2 class. UNC-98 contains three complete copies, and one "degenerate" copy of the C2H2 Zn finger (Figure 5, A and B). Studies on several Zn finger proteins have demonstrated that a Zn^{2+} ion is coordinated between a pair of β -strands and a single α -helix via a pair of cysteine and histidine residues (Lee *et al.*, 1989). We predict that the third (degenerate) Zn finger-like sequence in UNC-98 (residues 169–188) is not able to coordinate a Zn^{2+} ion because both cysteine and histidine pairs are too close together (each separated by only a single amino acid). This would, for example, cause the two histidines to have their side chains on opposite sides of the α -helix, not oriented to coordinate the Zn^{2+} ion.

The other notable features present in the UNC-98 sequence are three potential NLSs and two potential NESs

(Figure 5A). "Classical" NLS sequences are characterized by clusters of basic residues, but the definition of an NLS is vague because a diversity of sequences can act as a functional NLS (Dingwall and Laskey, 1991; Hodel *et al.*, 2001). Three potential NLSs in UNC-98 are as follows: K-E-A-R-K-E-R (residues 7–13), K-E-K-P-K-E-I-M-K (54–62), and K-V-S-K-K-R (205–210). Hodel *et al.* (2001), by measuring binding affinities between importin α and two known NLS sequences, have determined the relative importance of each residue in these NLSs via alanine scanning mutagenesis. This study suggests a basic core of an NLS with sequence K-(K/R)-X-(K/R). The first predicted NLS in UNC-98 conforms approximately to this consensus. The hydrophobic NES is ~10 residues long, is characterized by interspersed hydrophobic residues separated by one to three nonhydro-

Figure 5. Amino acid sequence and alignment of C2H2 Zn fingers in UNC-98. (A) In the UNC-98 amino acid sequence, predicted Zn finger domains and potential NLS and NES sequences are indicated. The blue rectangle denotes a predicted NES within a Zn finger. (B) An alignment to the consensus sequence for C2H2 class Zn fingers of three complete and one degenerate Zn finger in UNC-98. In the consensus sequence (Wolfe *et al.*, 2000), X represents any amino acid and Ψ is a hydrophobic residue.

phobic residues, and often fits the consensus sequence, L-X₂₋₃-(F,I,L,V,M)-X₂₋₃-L-X-(L,I) (Mattaj and Englmeier, 1998). In UNC-98, the first putative NES (residues 36–44), V-H-G-L-E-T-F-G-I, matches this consensus sequence, if hydrophobic residues substitute for leucines, as can be seen with other bone fide NESs (Powers, unpublished data). Interestingly, comparison to the predicted UNC-98 protein sequence from the closely related species *Caenorhabditis briggsae* (Borodovsky and Benian, unpublished data), reveals that both NES sequences, but only the first NLS sequence are well-conserved.

UNC-98::GFP Is localized to Muscle M-Lines, Dense Bodies, and the Nucleus

To determine where the *unc-98* gene is expressed and where the UNC-98 protein is localized, we made transgenic lines carrying *unc-98* with a GFP translational fusion (*unc-98::GFP*; Figure 6A). Because the 8.1-kb PCR fragment that rescues *unc-98* contains ~4 kb of sequence upstream of the initiator methionine, we used this 4-kb segment as a potential *unc-98* promoter. (The 5' end of this 4-kb segment is <1 kb from the initiator methionine of the next predicted gene, F08C6.2.) The construct was designed to express the full-length UNC-98 protein, with GFP fused to its C terminus. The vector (pPD95.77) did not contain a NLS sequence. Five independent lines in a wild-type background showed the same pattern of expression, although the level of expression varied somewhat between transgenic lines. Significantly, the same UNC-98::GFP was used to create two independent lines in an *unc-98(su130)* background, and both

Figure 6. Localization of the UNC-98 protein as determined by use of an *unc-98::GFP* translational fusion in transgenic animals. (A) Schematic representation of the construct used in five independent transgenic lines. Red boxes denote coding exons. Approximately 4 kb of sequence upstream of the 5' end of *unc-98* mRNA was used as a promoter. (B) Fluorescence micrograph of localization of the *unc-98::GFP* within a body wall muscle cell, suggesting that UNC-98 is located in dense bodies (seen as dashes) and M-lines (seen as lines composed of fine dots). Bar, 10 μm. (C and D) Fluorescence micrographs showing localization of *unc-98::GFP* to muscle cell nuclei. Transgenic animals carrying an *unc-98::GFP* array were stained with DAPI. The same area containing a portion of one muscle cell is shown separately imaged for the GFP signal (C) and the DAPI signal (D). Bar, 5 μm. (E) Deconvolved image of a muscle cell nucleus. Note the discontinuous localization pattern and exclusion from the nucleolus. Bar, 5 μm.

lines are fully rescued with respect to the muscle phenotype. In addition, the UNC-98::GFP showed that same pattern of expression and intracellular localization in both wild-type and *unc-98(su130)* backgrounds.

Expression was first observed in the developing embryo at the 1.5- to 2-fold stage, in which the UNC-98-GFP protein seemed localized to filamentous tracts, which generally follow myosin heavy chain A antibody staining, and therefore are interpreted to be located in myofibrils. Additionally, expression is seen in undefined puncta located along the filamentous tracts. In larvae and adults, expression was seen in body wall muscle, and in addition, anal depressor muscle and vulval muscles. As shown in Figure 6B, within adult body wall muscle, the UNC-98-GFP fusion localizes to dense bodies and M-lines. Within these muscle cells, we also see prominent localization of the UNC-98-GFP in the nucleus. As

shown in Figure 6, C and D, the UNC-98-GFP colocalizes with the DNA-binding dye DAPI. The signal is excluded from what seems to be the nucleolus and as shown under higher resolution in Figure 6E, the UNC-98-GFP signal is discontinuous. This nuclear localization is consistent with the presence of the NLS sequences noted above. During development, the nuclear localization of UNC-98::GFP begins at the late L2 to early L3 larval stages (our unpublished data).

Antibodies Generated to UNC-98 React with a Polypeptide of Expected Size from Wild Type, but not from *unc-98* Mutants

Although the data for intracellular location of UNC-98 protein determined with the use of a GFP translational fusion is highly suggestive, we wished to confirm these results by use of anti-UNC-98 antibodies. We also hoped that such antibodies could be used to determine whether the *unc-98* mutants produce UNC-98 protein. Thus, rabbit antibodies were generated to a bacterial fusion protein containing full-length UNC-98. After affinity purification, these antibodies (EU131) were used in immunoblot and immunofluorescent experiments. As shown in Figure 7A, EU131 reacts against two polypeptides in wild type, one of ~60 kDa (or a closely running doublet), the other of ~37 kDa. Of the two polypeptides reacting with EU131, the 37-kDa protein is closer in size to the molecular mass calculated for UNC-98 from the amino acid sequence (35.4 kDa). Significantly, this 37-kDa band cannot be detected in the *unc-98* mutants *su130* and *sf19* (Figure 7A). As shown in Figure 7B, the ~37-kDa band is also absent from the progeny of animals that had been injected with double-stranded RNA from *unc-98* (RNAi). Therefore, it is likely that the 37-kDa band is the product of *unc-98* gene, and the 60-kDa doublet are proteins that cross-react with UNC-98 antibodies and are the products of other genes. Interestingly, a BLAST search of the worm genome revealed two predicted proteins with strong homology to UNC-98, C34H3.2 (254 amino acids) and F35H8.3 (422 amino acids). It is possible that F35H8.3 encodes the 60-kDa, cross-reacting protein, because of its predicted molecular weight of 48.6-kDa, and because F35H8.3 protein contains seven C2H2 Zn fingers. Moreover, the two proteins are only similar within these Zn fingers.

Further evidence that the 37-kDa band is UNC-98 was provided by conducting a Western blot with an extract from one of the strains carrying an extrachromosomal array expressing an UNC-98-GFP fusion protein. As shown in Figure 7C, upon reaction with antibodies to GFP, a single polypeptide of ~64 kDa was detected. The measured size of this protein corresponds well with the sum of the measured (37-kDa) UNC-98 protein and GFP (27-kDa; Prasher *et al.*, 1992).

Anti-UNC-98 Antibodies Localize to Muscle M-Lines and Nuclei

These same anti-UNC-98 antibodies (EU131) were used to detect UNC-98 in the muscle of wild-type and *unc-98* mutant worms by using immunofluorescence microscopy. When used against wild-type embryos, these antibodies stain filamentous structures, presumably myofibrils, as early as the 1.5- to 2-fold stage. As shown in Figure 8, A–C, in wild-type adult animals fixed with methanol and paraformaldehyde, these antibodies stain the M-line region, colocalizing with UNC-89, a previously

Figure 7. By Western blotting, a UNC-98 polypeptide is detected in wild-type but not in *unc-98* mutants. (A) Total Laemmli-soluble proteins from wild-type (wt) and the *unc-98* mutant alleles *su130* and *sf19* were separated on a 10% denaturing polyacrylamide gel, transferred to membrane, and reacted with affinity-purified antibodies raised to a full-length UNC-98 fusion protein. Note the presence of a polypeptide of ~37 kDa present in wild-type (marked with an arrow), but not detected in the *unc-98* mutants. A second, presumably cross-reacting polypeptide of ~60 kDa is found in all three strains. Of the two polypeptides reacting with EU131, the 37-kDa protein is closer in size to the molecular mass calculated for UNC-98 from the deduced amino acid sequence (35,371). (B) Total Laemmli-soluble proteins from ~150 wild-type or ~150 *unc-98*(RNAi) adults were separated on the same gel as shown in A, transferred to membrane, and reacted with anti-UNC-98. Note the ~37-kDa protein present from wild-type (marked with an arrow), but not detectable from the RNAi animals. (C) Total Laemmli-soluble proteins from wild-type and a wild-type strain carrying an extrachromosomal array expressing full-length UNC-98-GFP fusion protein (*sfEx2[unc-98::GFP]*) were separated on a 12% denaturing polyacrylamide gel, transferred to membrane, and reacted with antibodies to GFP. Notice the presence of an ~64-kDa polypeptide from worms expressing the UNC-98-GFP fusion protein. The measured size of this protein corresponds well with the sum of the measured (37-kDa) UNC-98 protein and GFP (27-kDa). Positions of the molecular weight standards (1000×) are indicated on the left sides of A, B, and C.

described component of the M-line region (Benian *et al.*, 1996). EU131 staining was undetectable in the muscle of each of the *unc-98* mutants (Figure 8, D and G), consistent with the absence of the 37-kDa polypeptide on Western blots, and thus demonstrating the specificity of the antibodies for UNC-98 protein. In addition, UNC-89 staining revealed extensive disorganization of the M-line region in each of the *unc-98* mutants (Figure 8, E and H). This result correlates with the shortened and absent M-lines seen by EM in these mutants.

In wild-type muscle fixed with methanol and paraformaldehyde on whole worms, or alternatively, ethanol or methanol on worm frozen sections, anti-UNC-98 staining was only found at the M-line; no staining was observed at the dense bodies or in nuclei. However, when frozen sections were fixed with *n*-heptane, we saw staining of M-lines and, in addition, the muscle cell nuclei (Figure 8J). Thus, it would seem that there is indeed UNC-98 in the nucleus, but the epitopes detected by our antibodies are inactivated by the usual fixatives.

Figure 8. Immunofluorescence localization of UNC-98. (A–I) Localization in wild-type and in *unc-98* mutants. Worms were fixed using a combination of methanol and paraformaldehyde and costained with rabbit antibodies to UNC-98 (EU131; A, D, and G) and rat antibodies to UNC-89 (EU133; B, E, and H). Images of body wall muscle were obtained with a confocal microscope. Merged images are presented in C, F, and I. In wild-type (A–C), UNC-98 colocalizes with UNC-89, a known component of the M-line region (Benian *et al.*, 1996). Note the absence of UNC-98 staining in each *unc-98* mutant (D–F and G–I). Also note the disorganization of the M-line region in these *unc-98* mutant worms as revealed by staining with UNC-89 antibodies (E and H). Bar, 5 μ m. (J) Localization of UNC-98 in wild-type worms fixed with *n*-heptane. Note localization to the muscle cell nucleus (marked with an arrow) and M-lines in which structure was poorly maintained in this fixative. (K) Localization of UNC-98 in *unc-98*-rescued animals in which UNC-98 is expressed at higher than wild-type levels. *unc-98(su130)* animals carrying *rol-6*-marked transgenic arrays of the 8.1-kb *unc-98*-rescuing fragment were fixed in methanol/paraformaldehyde (as for A–I), and stained with anti-UNC-98 antibodies. Note localization to what seem to be M-lines, dense bodies, and the nucleus (marked with an arrow). Bar, 5 μ m.

However, given our UNC-98-GFP results, we expected to see antibody labeling of dense bodies, as well. One possibility is that UNC-98 really does reside in dense bodies, but at a lower than detectable concentration. To address this possibility, we used UNC-98 antibodies to stain animals genotypically *unc-98(su130)* that were rescued with a transgenic array carrying the 8.1-kb *unc-98*-rescuing fragment and *rol-6* marker DNA. Because extrachromosomal arrays contain multiple expressed copies of an introduced gene, these *unc-98*-rescued animals are likely to express higher than wild-type levels of UNC-98. As shown in Figure 8K, in these *unc-98*-rescued animals, anti-UNC-98 antibodies localize to all three structures labeled with UNC-98-GFP: M-lines, nuclei, and dense bodies.

Mapping of Regions Required for Nuclear Localization versus M-Line and Dense Body Localization

Curious as to how important it was to have all Zn fingers for proper localization of UNC-98, we generated four UNC-98::GFP constructs each missing one, two, three, or four Zn fingers (Figure 9A). Individual constructs were injected and the resulting transgenic lines were examined for

localization. Removal of as little as the C-terminal Zn finger (construct A) resulted in lack of proper localization to the M-line region and dense bodies. All four constructs (A–D) yielded the same result. A representative example of these constructs (construct B) is shown in Figure 9B (middle box). We conclude that the fourth Zn finger is necessary for localization of UNC-98 to these focal adhesion-like structures.

Significantly, even when all four Zn fingers were missing (construct D), nuclear localization could still be seen. This indicates that the N-terminal 110 residues of UNC-98 contain sufficient signals for nuclear localization. Indeed, two of the three predicted NLSs reside in the first 110 amino acids (Figure 5A). To obtain further evidence that the signals for nuclear localization reside in this N-terminal portion, we generated and tested transgenic animals expressing construct E: this protein begins with the normal initiator methionine, has an in-frame deletion of the next 106 residues, and then continues with the rest of the UNC-98 protein, including all four Zn fingers. As shown in Figure 9B (right-most box), this protein does not accumulate in the nucleus, but remarkably, localizes to M-lines and dense bodies. We conclude that the N-terminal 106 residues are both necessary and sufficient for nuclear localization, yet are not required for proper localization to M-lines and dense bodies. Addi-

Figure 9. Mapping regions of UNC-98 required for nuclear versus M-line and dense body localization. (A) Schematic representation of the six different UNC-98::GFP fusion proteins that were tested in transgenic animals. Localization results are shown in the first two columns to the right of the figure. Whether a construct can rescue the *Unc-98* polarized light muscle phenotype is indicated in the third column. The * indicates that construct A fails to rescue, and in fact, enhances the *Unc-98* phenotype (see Figure 10). f.l. denotes full-length UNC-98 protein. Constructs A–D remove successive Zn finger domains from the C terminus. Construct E begins with the normal initiator methionine and has an internal deletion of the N-terminal 106 amino acid residues. (B) Fluorescence microscopy of body wall muscle from transgenic animals expressing the f.l., construct B (representative of constructs A–D), and construct E. Arrows point out muscle cell nuclei. In construct B, note the nuclear localization but the lack of proper localization to M-lines and dense bodies. In construct E, note the lack of nuclear signal, but normal localization to M-lines and dense bodies. When placed in an *unc-98(su130)* mutant genetic background, construct E rescues the mutant phenotype, whereas construct A enhances the mutant phenotype. Bar, 10 μ m.

tionally, the ability to create a GFP construct that removes the nuclear localization strongly suggests that nuclear localization is not merely due to the presence of a GFP fusion or overexpression.

Several of our UNC-98::GFP constructs were tested for their ability to rescue the *Unc-98* mutant phenotype. Remarkably, construct E, which fails to localize to the nucleus but localizes to M-lines and dense bodies, fully rescues the polarized light defects of *unc-98(su130)*. Construct A, which is missing the last Zn finger, localizes to nuclei but not properly to M-lines and dense bodies in a wild-type background. Furthermore, this construct when expressed in an *unc-98(su130)* background results in a dramatic enhancement of the muscle structural defect (Figure 10A), and a significant reduction in motility (Figure 10B). Apparently, high levels of an improperly assembled UNC-98 can impair myofibril structure and function beyond what is seen when UNC-98 is reduced in quantity or has low levels of improp-

erly assembled protein (as surmised to happen in the *unc-98* mutants *su130* or *sf19*).

UNC-98 Interacts with UNC-97, a *C. elegans* Homolog of Mammalian PINCH

We sought to determine whether UNC-98 interacts with any of the already known components of nematode dense bodies and M-lines. Using a two-hybrid construct of UNC-98, we screened a yeast two hybrid "bookshelf" of known cloned dense body components (PAT-3 cytoplasmic region, UNC-112, PAT-4, PAT-6, UNC-97, CeTalin, DEB-1, and ATN-1) and eight UNC-112-interacting molecules (Qadota and Moerman, our unpublished data). Because UNC-98 itself had high activity to activate the reporter transcription, we used an activator domain fusion of UNC-98, and DNA binding fusions of the other proteins in the two hybrid assay. From this screening, we identified three UNC-98-interacting mol-

Figure 10. An UNC-98 protein missing the C-terminal Zn finger enhances the phenotype of *unc-98(su130)*. Polarized light (A) and motility assays (B) on *dpy-7(e88) unc-98(su130)*; *sfEx23[unc-98::GFP construct A]*. Note that the body wall muscle of these animals displays a higher degree of disorganization and have worse motility compared with that of *dpy-7(e88) unc-98(su130)* alone.

ecules, UNC-97, HUM-6, and MEP-1. HUM-6 is a class VII unconventional myosin (Titus, unpublished data; Tuxworth *et al.*, 2001). MEP-1 is a conserved C2H2 Zn finger-containing protein that is a component of a putative chromatin-remodeling complex (Unhavaithaya *et al.*, 2002) and has also been implicated in posttranscriptional repression of *fem-3* mRNA and consequent normal switch from spermatogenesis to oogenesis in the worm (Belfiore *et al.*, 2002). The interaction of UNC-98 with HUM-6 and MEP-1 will be reported elsewhere.

We decided to focus on UNC-97 because this protein is also expressed in *C. elegans* muscle and shows a similar localization to UNC-98 (Hobert *et al.*, 1999). As shown in Figure 11, A and B, by using deletion derivatives of UNC-97 and UNC-98 in two-hybrid experiments, we were able to map the interaction sites on each protein. Thus, the first two LIM domains of UNC-97 are necessary and sufficient for binding to UNC-98. All four Zn fingers of UNC-98 are required for interaction with UNC-97. The fact that not all the LIM domains of UNC-97 showed interaction with UNC-98 suggests that the observed interaction is specific.

To obtain additional evidence for a possible interaction between UNC-98 and UNC-97, we performed an in vitro binding experiment (Figure 11C). We used bacterially expressed fusion proteins consisting of maltose binding protein fused to full-length UNC-98, and glutathione S-transferase fused to full-length UNC-97. As shown in Figure 11C, at least in vitro, MBP-UNC-98 can form a protein complex with GST-UNC-97, but not with GST alone. Furthermore, the complex formation between MBP-UNC-98 and GST-UNC-97 is not occurring through the MBP or GST moieties.

Genetic data are consistent with *unc-97* being required early and *unc-98* being required late in myofibril assembly. Moreover, these data do not rule out an in vivo interaction between UNC-98 and preassembled UNC-97. First, terminal phenotypes are different: the *unc-97* null phenotype is Pat embryonic lethal (Cordes and Moerman, unpublished data), whereas the likely null phenotype for *unc-98* is an adult with reduced motility (based on RNAi) or at least not Pat (based on *SfDf1* homozygotes). Second, UNC-97::GFP was found to be normally localized to M-lines, dense bodies, and nuclei, in *unc-98* loss-of-function mutant backgrounds. Third, an

unc-98 unc-97 "double" showed a phenotype no worse than *unc-97* alone: *unc-98* dsRNA was injected into *unc-97(su110)* animals. In the F1, although birefringent needles characteristic of *unc-98* were seen, there was no obvious worsening in terms of motility or viability compared to *unc-97(su110)* alone. Fourth, in *unc-97(su110)*, antibody staining showed that UNC-98 was localized normally to M-lines. Because the UNC-97 protein from *su110* retains the first two LIM domains (Hobert *et al.*, 1999), and our two-hybrid analysis shows that the first two LIM domains interact with UNC-98, this mutant would be expected to show normal UNC-98 localization.

DISCUSSION

We have shown that the *unc-98* gene encodes a 310 residue polypeptide composed of three or four C2H2 Zn finger domains, and several putative nuclear localization and export signal sequences. As described previously (Zengel and Epstein, 1980), and further corroborated by our studies, mutations in the *unc-98* gene result in homozygous viable adult worms that have a disorganized myofilament lattice and reduced motility. EM and immunofluorescence staining reveals that most of this disorganization is due to the disruption of the focal adhesion-like structures of nematode muscle, the M-lines, and dense bodies. We found UNC-98 protein localized to M-lines and possibly to dense bodies. We are uncertain about this latter localization because our GFP and antibody data are in conflict (see below). However, the *unc-98* mutant phenotype would suggest that UNC-98 affects dense bodies as well as M-lines. UNC-98 is likely to be a distal rather than a basal component of focal adhesions: 1) in *unc-98* mutants, by EM, dense bodies are present, but are short or look broken; 2) in *unc-98* mutants, α -actinin, a more distal component of dense bodies, stains in a more abnormal pattern, than does vinculin, which is located at the base of dense bodies (our unpublished data); and 3) the probable *unc-98* null is not embryonic lethal (see below), whereas null mutations in other, more basal components of these focal adhesion-like structures are embryonic lethal (Pat). This suggests that UNC-98 might be involved in the

Figure 11. UNC-98 interacts with UNC-97 (PINCH) by the yeast two-hybrid assay and in vitro. (A) Mapping of the binding site in UNC-97 for UNC-98. The UNC-97 protein is represented schematically as a long rectangle with amino acid number beneath. The yellow boxes represent the five LIM domains. The 11 rows of rectangles represent the portions of UNC-97 that were tested for binding to UNC-98. The blue colored rectangle represents the smallest fragment (containing the first two LIM domains) that showed binding to UNC-98. (B) Mapping of the binding site in UNC-98 for UNC-97. The UNC-98 protein is represented as a long rectangle with amino acid number beneath. The red and orange boxes denote the C2H2 Zn fingers, green boxes denote predicted NLS, and blue boxes denote predicted NES (one lies within the first Zn finger). The nine rows of rectangles represent the portions of UNC-98 that were assessed for binding to UNC-97. The blue colored rectangle represents the minimal region of UNC-98 (all four Zn fingers) required for binding to UNC-97. (C) We tested the ability of MBP-UNC-98 or

later steps of assembly of these structures or their stability or function, rather than their initial assembly during development.

By genetic criteria, the existing alleles of *unc-98* are not null: when either mutant allele is placed over a deficiency, the motility worsens. Moreover, we determined that both *su130* and *sf19* are single nucleotide mutations in intron splice acceptor sites (replacing AG with AA). In *C. elegans*, an "AA" sequence can be used as an inefficient 3' splice site (Aroian *et al.*, 1993) so that, at least some, properly spliced mRNA and full-length UNC-98 protein might be expected in our mutants. Nevertheless, two results are consistent with the existing mutations being loss-of-function: 1) the same polarized light phenotype was obtained by RNAi; and 2) by use of anti-UNC-98 antibodies, no UNC-98 protein was detectable in either mutant by fluorescence microscopy, or by immunoblot. Efforts are underway to obtain additional mutant alleles of *unc-98*.

In wild-type animals expressing full-length UNC-98 fused to GFP, GFP signal was detected in body wall muscle M-lines, dense bodies, and nuclei. In support of these being the true in vivo locations for UNC-98 protein, the same construct was able to rescue the muscle structural defects in *unc-98* mutants, resulting in the same localization pattern. Antibodies raised to UNC-98 protein localized only to the M-line when animals were fixed with the usual fixatives (methanol/paraformaldehyde, ethanol, or methanol). However, when *n*-heptane was used as fixative, anti-UNC-98 labeling was seen at M-lines and in muscle cell nuclei. This suggests that there is indeed endogenous UNC-98 in nuclei, but the usual fixatives destroy enough epitopes so that the UNC-98 antigen cannot be detected. Further support for a nuclear, in addition to M-line location for UNC-98, is that when anti-UNC-98 antibodies were used to stain animals that were genotypically *unc-98* but rescued for the UNC-98 muscle structure defect, labeling was seen at M-lines, nuclei, and dense bodies. As these rescued animals carry extrachromosomal arrays with multiple expressed copies of wild-type *unc-98*, these animals are likely to express higher than wild-type levels of UNC-98 protein. Although it is possible that high-level expression might result in ectopic localization of UNC-98, the distinct pattern of dense body labeling seems more than coincidental. Thus, our interpretation is that in wild-type animals, UNC-98 resides at M-lines, and in muscle cell nuclei and dense bodies. However, there may be low concentrations of UNC-98 in nuclei and at dense bodies. This explanation seems plausible for the nuclear location, because UNC-98, with NLS and NES sequences, might be quickly shuttling in and out of the nucleus, never achieving a high nuclear concentration at any one time.

We determined some of the regions of UNC-98 that are required for nuclear versus focal adhesion localization. We examined the location of various deletion derivatives of

MBP alone to form a complex with GST-UNC-97 or GST alone, in vitro. The indicated proteins were incubated together and then either GST-UNC-97 or GST was pelleted after reaction with glutathione affinity beads. Bound proteins were extracted with Laemmli sample buffer, separated on SDS-PAGE, and stained with Coomassie Blue. Note that MBP-UNC-98, but not MBP was copelleted with GST-UNC-97 and not with GST. In the second lane from the left, the asterisks denote the GST-UNC-97 (~64 kDa) and MBP-UNC-98 (~77 kDa).

UNC-98 expressed as GFP fusion proteins in transgenic animals. Removal of just the C-terminal Zn finger (construct A) abolished UNC-98's proper assembly into M-lines and dense bodies, and not only failed to rescue but also even enhanced the *unc-98* loss-of-function phenotype. In contrast, a construct containing all four Zn fingers, but lacking the N-terminal 106 amino acids (construct E) assembled into M-lines and dense bodies, but no longer localized to nuclei. Therefore, we conclude that signals for nuclear localization reside in the N-terminal 106 residues, consistent with the presence of two of three predicted NLSs. It is significant that construct E can rescue the polarized light muscle structural defects of *unc-98(su130)*. This result supports, further, the necessity of the zinc fingers in UNC-98's role as a focal adhesion protein. Although these results deemphasize a structural role for the N-terminal region, this region may be necessary for yet another role not assayed by our current methods. Another explanation is that other genes/proteins can functionally compensate for lack of UNC-98's nuclear localization.

To our knowledge, UNC-98 is the first C2H2 (TFIIIA- or Kruppel-like) Zn finger domain containing protein that has been shown to be localized to discrete regions outside the nucleus. UNC-98 and UNC-97 (to which UNC-98 interacts) are also the only known components of *C. elegans* muscle to have a dual intracellular residence (to the nucleus and focal adhesion structures). This dual location is well-known for a number of Zn finger proteins found in vertebrate muscle and nonmuscle cells. Muscle LIM protein (MLP) is a LIM-only protein composed of two LIM (double Zn finger) domains and is expressed in striated muscle (Arber *et al.*, 1994). MLP is first detected during myotube formation and is first localized to nuclei, and later, to both nuclei and myofibrils, specifically in thin filaments and Z-lines (Arber *et al.*, 1994). Recently, a second mammalian muscle Zn finger protein, muscle-specific RING finger-1 (MURF-1), has been shown to have a dual myofibril and nuclear location (Arber *et al.*, 2002). By antibody staining, MURF-1 has multiple and variable locations in striated muscle including diffuse cytoplasm, the M-line, and the nucleus (at least in some cells).

In mammalian nonmuscle cells, a number of LIM family proteins have been found that are primarily located in focal adhesions. Further experimental evidence has shown that they can also accumulate in the nucleus. Nuclear localization has been found especially when nuclear export has been inhibited by, for example, deleting nuclear export sequences from the protein, or by treatment with leptomycin B. Thus, this is evidence that these proteins shuttle between the cytoplasm and the nucleus. These proteins include zyxin (Nix and Beckerle, 1997; Nix *et al.*, 2001), paxillin (Thomas *et al.*, 1999), lipoma preferred partner (Petit *et al.*, 2000), thyroid receptor interacting protein-6 (Wang *et al.*, 1999), and the paxillin-related protein Hic5 (Yang *et al.*, 2000). What role these proteins are actually performing, especially in the nucleus, is unknown, but it is thought that these proteins might communicate information from adhesion sites to the nucleus (Aplin and Juliano, 2001). It is interesting to note that nuclear MLP has been shown to interact with MyoD-E47 heterodimers, and this leads to enhancement of the DNA-binding activity of the MyoD-E47 complex (Kong *et al.*, 1997). In addition, MURF-1 was shown to interact with three nuclear proteins including glucocorticoid modulatory

element binding protein-1, a known transcriptional regulator (McElhinny *et al.*, 2002).

Several lines of evidence indicate that UNC-98 interacts directly with UNC-97 in nematode muscle: 1) the colocalization of each GFP-fusion protein to focal adhesions and nuclei; 2) the identification of subdomains of UNC-98 and UNC-97 that are necessary and sufficient for interaction in yeast two-hybrid assays; and 3) recombinant UNC-98 and UNC-97 form a protein complex, *in vitro*. The mammalian homologs of UNC-97 are called PINCH. In nonmuscle cells, PINCH is just one of nine different proteins that interact with integrin-linked kinase in focal adhesions (Wu and Dedhar, 2001). Besides PINCH, many of the proteins making up the mammalian integrin-linked kinase complex, have counterparts in *C. elegans*. These nematode homologs are also located at the dense bodies and M-lines and many have been mutationally defined. These homologs include, but are not limited to the integrins, UNC-97 (PINCH), and integrin linked kinase (PAT-4; Mackinnon *et al.*, 2002). Our results show that UNC-98 is yet another member of this complex in nematode muscle. BLAST searches have not revealed any obvious UNC-98 homologs, thus far, except from other nematode species, that are the same size as UNC-98 and contain exactly the same number of Zn fingers. However, we found many mammalian proteins matching with high scores (E values of $\sim 10^{-16}$) that contain various numbers of Zn fingers. It is certainly possible that a functional counterpart to UNC-98 exists in other animals, but is composed of different numbers of C2H2 Zn fingers.

The fascinating dual location of UNC-98, focal adhesion-like structures, and nuclei leads us to the following speculation. UNC-98 might function both as a muscle adhesion complex protein and as a transcription factor, or work together with transcription factors, to influence gene expression. Perhaps the genes that are regulated by UNC-98 encode muscle adhesions proteins or other myofibrillar proteins. UNC-98 might mediate communication between focal adhesion sites and the nucleus. What would be the physiological function of such communication? We can speculate that the "strength" of attachment of the muscle cell, via its dense bodies and M-lines, is reported by UNC-98 moving from the adhesion sites to the nucleus. UNC-98 might accomplish this "reporting" function by itself, or together with other factors (e.g., UNC-97). Consistent with the idea that nuclear UNC-98 itself is a transcription factor is that it contains three to four C2H2 Zn fingers, a domain that is found in many known transcription factors and capable of binding DNA directly. Currently, through microarray experiments comparing wild type and *unc-98* mutants, we hope to provide evidence that UNC-98 plays a role in influencing gene expression.

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