Published September 1, 1996

Two Muscle-specific LIM Proteins in *Drosophila*

Beth E. Stronach, Sarah E. Siegrist, and Mary C. Beckerle

Department of Biology, University of Utah, Salt Lake City, Utah 84112

Abstract. The LIM domain defines a zinc-binding motif found in a growing number of eukaryotic proteins that regulate cell growth and differentiation during development. Members of the cysteine-rich protein (CRP) family of LIM proteins have been implicated in muscle differentiation in vertebrates. Here we report the identification and characterization of cDNA clones encoding two members of the CRP family in *Drosophila*, referred to as muscle LIM proteins (Mlp). *Mlp60A* encodes a protein with a single LIM domain linked to a glycine-rich region. *Mlp84B* encodes a protein with five tandem LIM-glycine modules. In the embryo, *Mlp* gene expression is spatially restricted to somatic, visceral, and pharyngeal muscles. Within the somatic musculature, *Mlp84B* transcripts are enriched at the terminal ends of muscle fibers, whereas *Mlp60A* transcripts are found throughout the muscle fibers. The distributions of the Mlp60A and Mlp84B proteins mirror their respective mRNA localizations, with Mlp84B enrichment occurring at sites of muscle attachment. Northern blot analysis revealed that *Mlp* gene expression is developmentally regulated, showing a biphasic pattern over the course of the *Drosophila* life cycle. Peaks of expression occur late in embryogenesis and during metamorphosis, when the musculature is differentiating. *Drosophila* Mlp60A and Mlp84B, like vertebrate members of the CRP family, have the ability to associate with the actin cytoskeleton when expressed in rat fibroblast cells. The temporal expression and spatial distribution of muscle LIM proteins in *Drosophila* are consistent with a role for Mlps in myogenesis, late in the differentiation pathway.

The LIM domain is a modular protein motif present in single or multiple copies in a wide variety of eukaryotic proteins that generally appear to regulate gene expression and cell differentiation during development (for review see Sadler et al., 1992; Sanchez-Garcia and Rabbitts, 1994; Dawid et al., 1995). The LIM motif is defined by a cysteine-rich consensus sequence, $CX_2CX_{16-23}HX_2CX_2CX_2CX_{16-21}CX_{2-3}(C,H,D)$ (Freyd et al., 1990; Karlsson et al., 1990; Sadler et al., 1992). Together the conserved Cys, His, and Asp residues coordinate two zinc atoms per LIM domain, giving rise to a double zinc finger (Michelsen et al., 1993, 1994; Kosa et al., 1994). The LIM domain has been shown to mediate specific protein–protein interactions and, in this way, may regulate protein activity and localization (Feuerstein et al., 1994; Schmeichel and Beckerle, 1994; Valge-Archer et al., 1994; Wu and Gill, 1994). Interestingly, recent structural studies have revealed that one of the two zinc-binding modules of the LIM domain displays a tertiary fold similar to DNA-binding domains in known transcription factors, raising the possibility that LIM domains might also be capable of interacting with nucleic acids (Perez-Alvarado et al., 1994).

The LIM motif was first identified in three developmentally regulated transcription factors, *Caenorhabditis elegans* Lin-11, rat Isl-1, and *C. elegans* Mec-3, from which the name LIM was derived (Way and Chalfie, 1988; Freyd et al., 1990; Karlsson et al., 1990). LIM domain proteins fall into two general categories: proteins in which LIM domains are associated with functional domains, such as homeodomains or kinase domains, and proteins that are comprised more or less exclusively of LIM domains. Notably, even LIM-only proteins, which lack obvious DNA-binding or catalytic sequences, have been implicated in the control of cell differentiation. For example, targeted disruption of the gene encoding rhombotin 2, a protooncogene product with two LIM domains, eliminates erythroid differentiation in mice (Warren et al., 1994). Likewise, experiments using a cell culture model system have revealed that the muscle LIM protein (MLP)[1], a member of the cysteine-rich protein (CRP) family of LIM-only proteins, is required for muscle differentiation (Arber et al., 1994).

Three evolutionarily conserved members of the CRP family, CRP1, CRP2, and MLP/CRP3, have been described (Liebhaber et al., 1990; Sadler et al., 1992; Weiskirchen and Bister, 1993; Arber et al., 1994; Crawford et al., 1994; Weiskirchen et al., 1995); all three family members are characterized by the presence of two copies of the LIM domain, each followed by a short glycine-rich region.

Address all correspondence to Mary C. Beckerle, Department of Biology, University of Utah, 201 S. Biology Bldg., Salt Lake City, UT 84112. Tel.: (801) 581-4485. Fax: (801) 581-4668.

1. *Abbreviations used in this paper*: CRP, cysteine-rich protein; GST, glutathione-*S*-transferase; MEF, myocyte enhancer factor; MLP (mlp), muscle LIM protein; nt, nucleotide; REF, rat embryo fibroblast.

Published September 1, 1996

CRPs exhibit tissue-specific distributions and temporally regulated expression during embryogenesis (Wang et al., 1992; Arber et al., 1994; Crawford et al., 1994). For example, in the developing chick, CRP1 is most prominent in tissues rich in smooth muscle, and expression levels increase dramatically during smooth muscle maturation (Crawford et al., 1994). In contrast, a dramatic reduction in the levels of transcripts encoding both CRP1 and CRP2 correlates with the transformation of fibroblast cells by both chemical carcinogens and viral oncogenes (Weiskirchen and Bister, 1993; Weiskirchen et al., 1995). CRPs are associated with elements of the actin cytoskeleton and can bind directly to another LIM protein called zyxin, which has been postulated to play a role in signal transduction at sites of membrane–substratum attachments enriched in integrin receptors (Sadler et al., 1992; Crawford et al., 1994). Collectively, the biochemical features, expression characteristics, and functional properties of the CRP family members lend credence to the hypothesis that CRPs are involved in promotion or maintenance of cell differentiation, particularly in muscle. However, the specific role(s) of CRPs in these developmental events is still unknown.

A number of discrete steps in muscle development have been defined in *Drosophila melanogaster*. As in vertebrates, myogenesis involves specification of mesoderm, commitment of cells to differentiate, and then expression of contractile proteins that mark terminal differentiation. Cell movements associated with gastrulation in *Drosophila* lead to the invagination and specification of cells that form the presumptive mesoderm (for review see Campos-Ortega and Hartenstein, 1985; Bate, 1993). These cells undergo several rounds of mitosis and ultimately become committed to differentiate into one of several major mesodermal derivatives: the somatic or body wall muscles, the visceral mesoderm or gut musculature, the cardiac mesoderm or dorsal vessel, and the fat body (Bate, 1993). In the somatic muscle lineage, fusion of myoblasts occurs midway through embryogenesis to produce syncytial myotubes (Bate, 1990). These newly formed myotubes migrate toward their proper attachment sites in the epidermis and make formal attachments to extracellular matrix via integrin receptors (Bogaert et al., 1987; Leptin et al., 1989). Integrins also link the visceral musculature to basal lamina surrounding the gut epithelium (Bogaert et al., 1987). Finally, completion of the terminal differentiation program in the striated body wall and gut muscles involves the assembly of functional myofibrils. Although many of the early events involved in the specification and subdivision of the mesoderm are fairly well understood, aside from the expression of structural components of the contractile machinery, relatively few regulatory genes have been described that act late in the differentiation program.

Based on the observation that a CRP family member appears to be required for terminal differentiation in vertebrate muscle development (Arber et al., 1994), we have undertaken a molecular genetic approach to study the role of CRPs using *Drosophila melanogaster* as a model system. Here we describe the identification and developmental expression of two genes, *Mlp60A* and *Mlp84B*, that encode muscle-specific LIM proteins related to vertebrate CRP family members. Our analysis has revealed striking conservation of sequence, timing of gene expression, tissue distribution of gene products, and subcellular localization among LIM proteins of vertebrates and invertebrates. *Mlp60A* and *Mlp84B* are both expressed during periods of significant cell differentiation during development of the fly. The restricted temporal and spatial expression of the muscle LIM proteins in *Drosophila* is consistent with a role in myogenesis, late in the muscle differentiation pathway.

Materials and Methods

Southern Genomic Blots

10 μg of genomic DNA, purchased from Promega Corp. (Madison, WI), was processed according to standard procedures (Sambrook et al., 1989) and transferred to Hybond N+ nylon membrane (Amersham Corp., Arlington Heights, IL) overnight in 20× SSC. Blots were subsequently hybridized and washed according to the manufacturer's protocol at 60°C (heterologous) or 65°C (homologous) with random-primed (Stratagene, La Jolla, CA) ^{32}P-labeled chicken *CSRP1*[2] DNA (Crawford et al., 1994), *Mlp60A* DNA, or *Mlp84B* DNA. The *CSRP1* probe consisted of a PCR-generated fragment, nucleotides (nt) 72–650 of the cDNA, corresponding to the coding region. *Mlp* probes were full-length cDNAs.

Library Screening

The same *CSRP1* DNA probe was used to screen an adult *Drosophila melanogaster* cDNA library (λEXLOX) derived from mRNA in bodies (Novagen Inc., Madison, WI). Phage plating and growth were carried out according to the manufacturer's protocols. Filter lifts and hybridization were performed essentially as for genomic blots except the final wash was more stringent. After plaque purification, we used the loxP/CRE recombination system to isolate plasmid DNA containing the clones of interest. In subsequent library screens, *Drosophila* clones identified in the first screen were used as probes to isolate more clones representing the gene.

Sequencing

Double-strand DNA was sequenced using the dideoxy chain termination method (Sanger et al., 1977) with Sequenase Version 2.0 (United States Biochemical Corp., Cleveland, OH) and α-^{35}S-dATP, or PCR Cycle Sequencing (GIBCO BRL, Gaithersburg, MD) with γ-^{33}P-ATP according to the manufacturer's directions. We sequenced a combination of full-length clones, restriction fragments subcloned into pBluescript (Stratagene), and deletion clones generated using ExoIII nuclease (New England Biolabs, Beverly, MA), using primers against vector sequences and specific internal primers. Both strands were sequenced in entirety. Sequence comparisons were generated using the GAP program within the GCG sequence analysis software package (version 7; Genetics Computer Group, Madison, WI) based on the algorithms derived from Needleman and Wunch (1970).

In Situ Hybridization to Polytene Chromosomes

Drosophila larval salivary gland dissection and squashes, as well as pretreatment of chromosomes on the slides before hybridization, were essentially as described (Pardue, 1986) but without heat or RNAse treatment. Double-strand DNA was random primer labeled using the Genius system (Boehringer Mannheim Biochemicals, Indianapolis, IN). Probes were as follows: #20 clone for *Mlp60A*, #21 clone for *Mlp84B*. Hybridization was carried out at 62°C overnight in 5× SSC, 1× Denhardt's reagent, 5 mM $MgCl_2$, 0.5% Genius blocking reagent. Washes, processing, and detection were performed essentially according to the Genius detection protocol, but with anti-digoxigenin antibody diluted 1:500, and reacted with chromosomes 2 h at room temperature. Finally, the chromosomes were stained briefly in aceto-orcein and observed with phase-contrast optics. Images of chromosomes were captured using a microscope (Axioskop; Carl Zeiss, Inc., Thornwood, NY) equipped with a video camera (DC-151 GB; Sony Corp., Park Ridge, NJ).

Northern Blots

Staged embryos, larvae, pupae, or adult females were used to isolate poly A$^+$ RNA using either of two procedures: (*a*) a combination of RNAgents

Published September 1, 1996

total RNA isolation kit (Promega Corp.) followed by poly A^+ selection using PolyATract mRNA Isolation System III (Promega Corp.) according to the supplier, or (*b*) total RNA was isolated using the hot phenol method (Jowett, 1986), and subsequent poly A^+ selection was carried out using oligo dT cellulose (Collaborative Research, Inc., Waltham, MA) according to the manufacturer. 5–6 μg mRNA from each developmental stage was electrophoresed through a denaturing formaldehyde gel in 1× MOPS buffer (Sambrook et al., 1989). After processing, the mRNA was transferred to Hybond N+ membrane (Amersham Corp.) overnight and subsequently hybridized with random primer ^{32}P-labeled probes as we had for the Southerns, but at higher stringency. The same blot was hybridized independently with each probe; after data were collected for each probe, the blot was stripped with boiling 0.5% SDS for 10 min and reused. LIM probes consisted of the entire coding regions of the cDNAs. *rp49* probe was a gift from A. Letsou (University of Utah, Salt Lake City, UT), containing *rp49* coding sequences cloned into pBR322.

Whole Mount In Situ Hybridization

Canton-S embryos were collected overnight on apple juice plates and dechorionated in 50% bleach. Embryo processing and hybridization were carried essentially as described by Tautz and Pfeifle (1989), with the following modifications for use with RNA probes. Hybridization solution consisted of 50% formamide, 5× SSC, 50 μg/ml heparin, 100 μg/ml yeast tRNA, 0.1% Tween-20, pH 4.5. Hybridization was carried out overnight at 65°C with digoxigenin-labeled riboprobes added to 0.25 ng/ml. Subsequent washes were performed at 65°C. Just before adding anti-digoxigenin antibody, embryos were blocked with 1× PBS, 0.1% Tween-20, and 1% blocking reagent supplied with Boehringer Mannheim nonradioactive detection kit. Probes were generated and labeled using the Boehringer Mannheim Genius RNA labeling kit according to the manufacturer. For detecting *Mlp60A* RNA, we used an antisense riboprobe corresponding to the 3′ untranslated region, nt 320–428, generated by digesting the full-length clone with NarI enzyme (New England Biolabs) and transcribing run-off RNA transcripts from the downstream SP6 promoter. To detect *Mlp84B* RNA, we used an antisense riboprobe corresponding to the last third of the #21 clone cDNA, nt 1071–1844, which was subcloned as an exonuclease deletion in pBluescript (Stratagene). This deletion, exo2b, was digested with an appropriate enzyme, and run-off transcripts were generated using the T3 promoter. Embryos were mounted in JB-4 resin (Polysciences, Inc., Warrington, PA) and photographed using differential interference contrast optics on a Zeiss Axiophot microscope.

Antibody Production, Western Blot Analysis, and Immunostaining

Mlp coding sequences were cloned into the pGEX-2T expression vector (Pharmacia, Uppsala, Sweden) and expressed in bacteria as fusion proteins with glutathione-*S*-transferase (GST) sequences. Fusion protein purification was performed according to standard procedures (Ausubel et al., 1994). Mlp60A was cleaved from GST using thrombin (Sigma Chemical Co., St. Louis, MO), whereas for Mlp84B, the intact GST fusion was used as an immunogen. Purified protein for immunizing rabbits was obtained by separation on a preparative SDS polyacrylamide gel, followed by electroelution of the protein and extensive dialysis against PBS. For characterization of resultant polyclonal antibodies, 16–24-h *Drosophila* Canton-S embryos were collected, washed, and homogenized in Laemmli sample buffer (Laemmli, 1970). SDS-PAGE was performed according to the method of Laemmli (1970) with modifications described previously (Schmeichel and Beckerle, 1994). Subsequent Western immunoblots were carried out as described (Beckerle, 1986) using ^{125}I-protein A to detect primary antibody binding. For Western blots, anti-Mlp60A and anti-Mlp84B antibodies were used at dilutions of 1:600 and 1:1500, respectively.

Immunostaining of whole mount embryos was carried out essentially as described (Patel, 1994) using antibodies presorbed against fixed, early-stage embryos. Antibodies to Mlp60A were used at 1:100, anti-Mlp84B antibodies were used at 1:200, and HRP-conjugated goat anti–rabbit secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA) were used at 1:500. Embryos were mounted in 70% glycerol and photographed using differential interference contrast optics on a Zeiss Axiophot microscope. A similar muscle pattern was observed in embryos using an independently generated anti-peptide antibody against Mlp60A sequences. Confocal immunofluorescence microscopy was performed using similar procedures, and antibody dilutions except embryos were fixed for 4 min in a fixative composed of 9 ml 37% formaldehyde and 1 ml 0.5 M EGTA, pH 8.0, plus an equal volume of heptane (Kiehart and Feghali, 1986). Anti-muscle myosin antibody was kindly provided by D. Kiehart (Duke University, Durham, NC) and diluted to 1:400. A Texas red–conjugated goat anti–rabbit secondary antibody (Cappel Laboratories, Durham, NC) was used at 1:200. Images were captured using the confocal system (MRC-600; Bio-Rad Laboratories, Cambridge, MA) attached to an optiphot microscope (Nikon Inc., Garden City, NY). Low magnification images (×20) represent 6.7-μm sections, and high magnification images (×40) represent 4.2-μm optical sections. Images were assembled and labeled using software (Adobe Photoshop; Adobe Systems, Inc., Mountain View, CA) and subsequently printed on a printer (XLS 8600 PS; Eastman-Kodak Co., Rochester, NY).

Heterologous Expression and Immunofluorescence

Expression vector construction involved amplifying Mlp coding regions from full-length cDNAs using PCR. Primers encoded BamHI (5′ end) or NotI (3′end) restriction sites, and Pfu polymerase (Stratagene) was used to minimize the likelihood of errors. Amplified fragments were digested and ligated into a pcDNA1/NEO vector (Invitrogen, San Diego, CA) that was modified (gift from D. Nix, University of Utah, Salt Lake City, UT) by inserting sequences encoding the FLAG epitope downstream from the NotI site. Ligation at that site would generate an in-frame Mlp fusion with FLAG. Triplicate PCR samples were used to generate three independent constructs for microinjection. Plasmid DNA was isolated using a polyethylene glycol precipitation procedure (Sambrook et al., 1989) and finally resuspended in PBS. REF52 cells were grown to 50–70% confluence on coverslips in growth medium and microinjected with plasmid DNA at 250 ng/μl. Cells were fixed 24 h later and processed for fluorescence microscopy with rhodamine-phalloidin (Molecular Probes, Inc., Eugene, OR), and indirect immunofluorescence (Beckerle, 1986) with anti-FLAG M2Ab primary antibody (IBI A Kodak Co., New Haven, CT) at 1:600 and FITC-conjugated goat anti–mouse secondary antibody (Jackson Immunoresearch Laboratories) at 1:500.

Results

Identification of CRP-related Sequences in Metazoans

Members of the CRP family are characterized by the presence of two LIM domains, each followed by a glycine-rich repeat with the sequence GPKG(Y/F)G(Y/F)G(M/Q)GAG. The presence of this glycine-rich repeat distinguishes CRP family members from other small LIM-only proteins such as rhombotin. In addition, CRP family members display a potential nuclear targeting signal (KKYGPK) that partially overlaps the glycine-rich repeat.

To determine whether sequences related to those specifying avian CRPs are present in other organisms, we used a cDNA encoding CRP1 (referred to as *CSRP1*[2]) to probe genomic Southern blots of DNA from chicken, fly, human, mouse, yeast, and frog. As can be seen in Fig. 1, cross-hybridizing genomic DNA fragments are detected in all metazoan species examined using a *CSRP1* probe. No specific hybridization is observed with yeast genomic DNA, although yeast are known to possess genes encoding LIM domain proteins (Muller et al., 1994). The cross-hybridizing band observed in yeast genomic DNA (Fig. 1, lane 5) corresponds to an intense band of repetitive DNA observable in the ethidium bromide–stained agarose gel (not shown).

2. Proteins of the vertebrate CRP family are designated CRP1, CRP2, and MLP/CRP3; the corresponding genes are designated with the symbols *CSRP1*, *CSRP2*, and *CSRP3*.

Published September 1, 1996

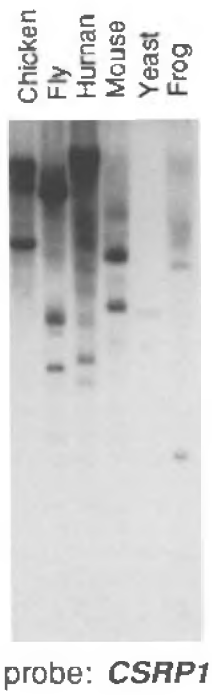


Figure 1. Metazoan species contain sequences related to avian *CSRP1*. *CSRP1* coding sequences were used to probe a genomic Southern blot of DNA isolated from various species. Each species, except for yeast, shows a unique set of cross-hybridizing fragments.

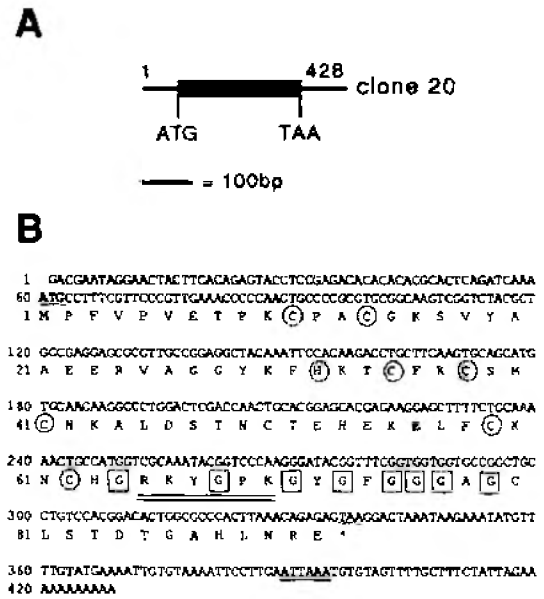


Figure 2. Nucleotide and predicted protein sequences encoded by *Drosophila Mlp60A* cDNA. (*A*) Schematic representation of cDNA clone #20 encoding Mlp60A. (*B*) Nucleotide and derived amino acid sequence of Mlp60A, a single LIM domain protein. Nucleotide and amino acid positions are indicated by numbers in the lefthand margin preceding each row. Translational start and stop codons and the polyadenylation signal (*single underline*). The conserved cysteine and histidine residues that define the LIM consensus (*circled*). Glycine residues that contribute to the glycine-rich region immediately after the LIM domain (*boxed*). A putative nuclear targeting signal that partially overlaps the glycine-rich region (*double underline*). These sequence data are available from EMBL/GenBank/DDBJ under the accession number X91244.

Molecular Cloning of cDNAs That Encode Drosophila Proteins Related to CRP

In an effort to identify and to characterize CRP family members in *Drosophila*, an adult *Drosophila* cDNA library was screened with an avian *CSRP1* probe. From 600,000 recombinants screened, two cross-hybridizing clones were isolated and characterized by sequence analysis (Figs. 2 and 3). The resulting cDNAs were shown to encode distinct, but closely related, proteins referred to initially as DmLIM-2 and DmLIM-3 (Stronach, B.E., T.B. Macalma, and M.C. Beckerle. 1994. 35th Annual *Drosophila* Research Conference. Chicago. 370*a*). Both DmLIM-2 and DmLIM-3 display features that are hallmarks of the CRP family, being comprised more or less exclusively of LIM-Gly repeats. The gene encoding DmLIM-2 was independently isolated by Arber and colleagues in a search for *Drosophila* sequences related to MLP/CRP3 (Arber et al., 1994). These authors have referred to this gene as *Mlp1*. We suggest renaming this gene, *Mlp60A*, to include information about the genomic location and to be consistent with standard *Drosophila* nomenclature (Flybase, 1994). Similarly, DmLIM-3 is hereafter referred to as muscle LIM protein 84B. *Mlp84B* corresponds to a novel gene sequence named for its relationship to *Mlp60A*, its tissue-specific expression, and its genomic location. Because the members of the CRP family have been most extensively characterized in birds, we use those sequences here for comparison with the *Drosophila* CRP family members. It should be noted that the avian CRPs are >90% identical to their counterparts in mouse and human (Weiskirchen et al., 1995).

Characterization of an Mlp60A cDNA

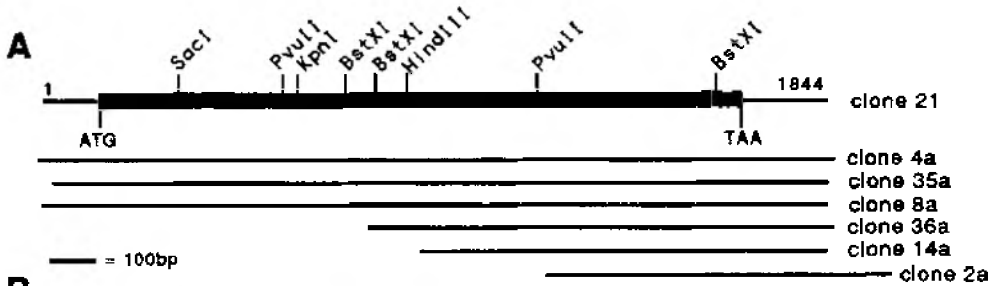
The nucleotide and deduced amino acid sequences of *Mlp60A* are shown in Fig. 2 *B*. The cDNA sequence is 428 nucleotides in length. Nine additional clones isolated and characterized in a subsequent screen provide only six additional nucleotides 5′ to what is presented in Fig. 2 *B* (not shown). The ATG (nt 60–63) is postulated to be the initiation codon since flanking sequences conform well to the consensus translation initiation site in *Drosophila*, cacaacCAaaATGgc (Cavener and Ray, 1991). A polyadenylation sequence, ATTAAA (Berget, 1984), precedes a 3′ poly A tail by 23 nucleotides.

The *Mlp60A* cDNA is predicted to encode a protein of 92 amino acids. The derived protein product is comprised of a single LIM domain linked to a glycine-rich repeat that closely resembles the glycine-rich sequence observed in CRP1 (Fig. 4, *A* and *C*). Like vertebrate CRPs, the LIM domain of Mlp60A exhibits the sequence $CX_2CX_{17}HX_2CX_2CX_{17}CX_2C$. In addition, the potential nuclear targeting signal is retained with one conservative lysine to arginine substitution. At the amino acid level, Mlp60A displays 52% identity and 62% similarity with CRP1 (Fig. 4 *B*). The greatest sequence similarity is achieved, however, when Mlp60A is aligned with the NH_2-terminal LIM domain of the CRP family member, MLP/CRP3 (Fig. 4 *B*); in this case, we observe 60% identity and 68% similarity.

Characterization of Mlp84B cDNAs

The nucleotide and deduced amino acid sequences of

Published September 1, 1996



B

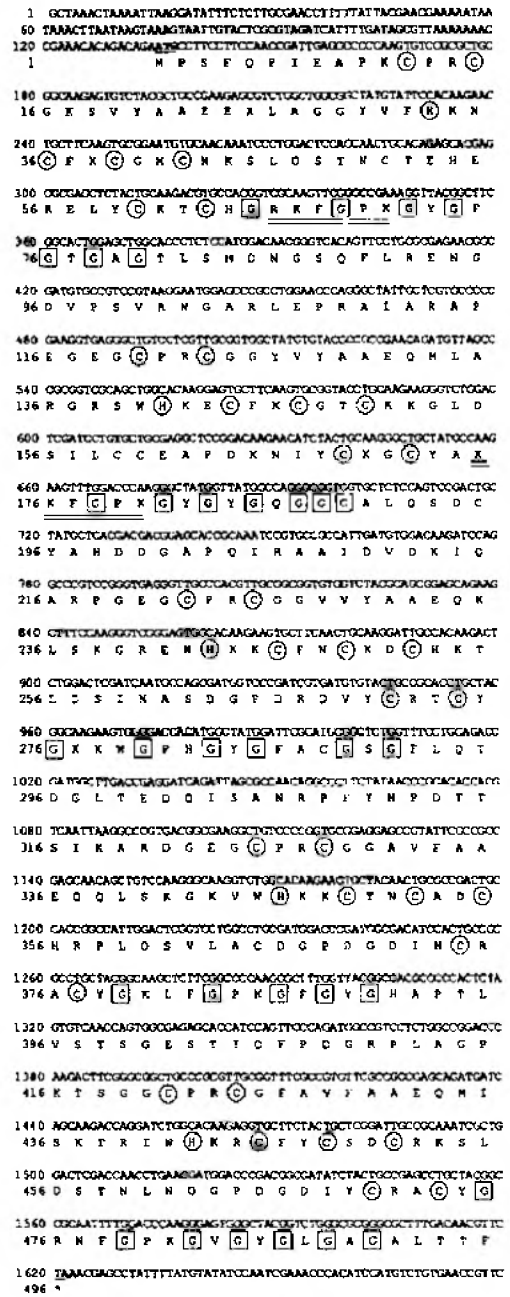


Figure 3. Nucleotide and predicted protein sequences encoded by *Drosophila Mlp84B* cDNAs. (*A*) Schematic representation of cDNA clones encoding Mlp84B. Clone #21 contains the entire open reading frame (*thickened line*). Several restriction enzyme sites, used in subcloning, are shown. Additional cDNAs encoding Mlp84B are indicated, with the lines representing their length and position relative to clone #21. (*B*) Nucleotide and derived amino acid sequence of Mlp84B, a five LIM domain protein. Nucleotide and amino acid positions are indicated by numbers in the left margin preceding each row. Translational start and stop codons (*underlined*). The conserved cysteine and histidine residues of the five LIM domains (*circled*). Glycine residues that comprise the glycine-rich regions following each LIM domain (*boxed*). Putative nuclear targeting signals found adjacent to the first and second LIM domains (*underlined twice*). These sequence data are available from EMBL/GenBank/DDBJ under the accession number X91245.

Published September 1, 1996

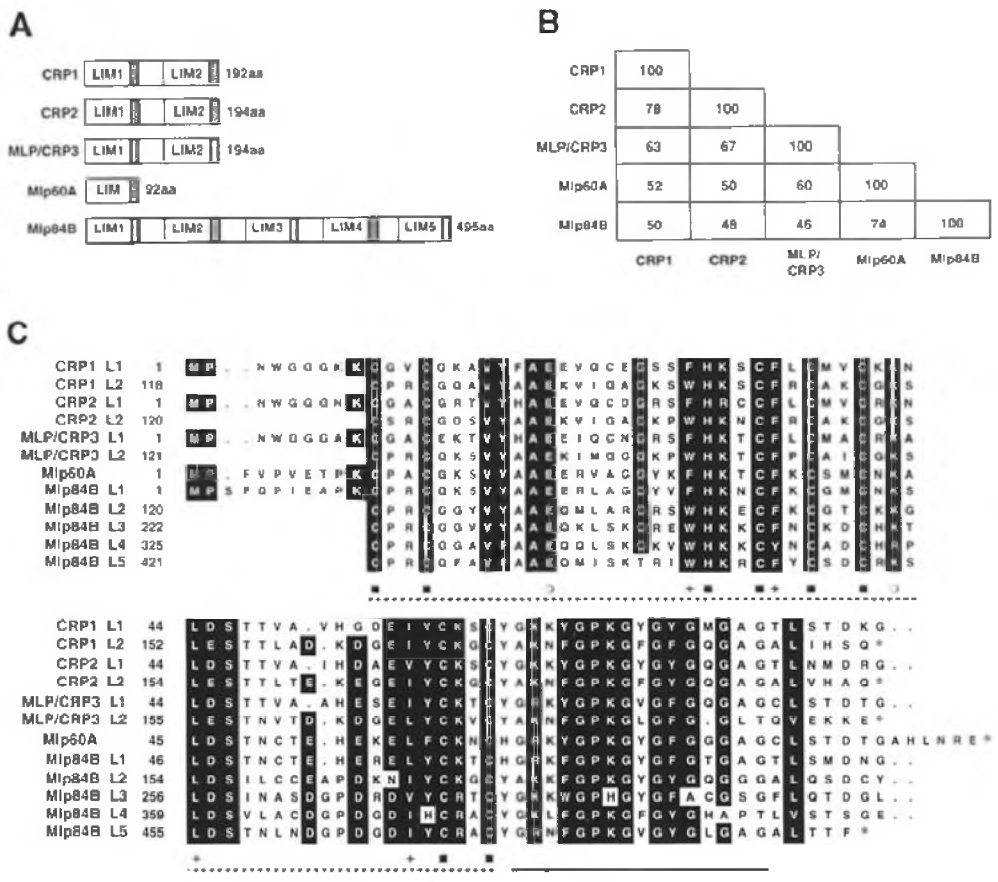


Figure 4. Drosophila muscle LIM proteins are closely related to vertebrate CRP family members. (*A*) Schematic representation of the vertebrate CRPs and the *Drosophila* Mlps showing LIM domain and glycine-rich (*stippled*) regions. Alignment of all the proteins at the amino terminus gives the highest sequence conservation. (*B*) Pairwise sequence comparisons among avian and *Drosophila* CRP family members. Numbers depict amino acid identity derived from analysis using the Genetics Computer Group GAP program with default parameters based on the algorithm of Needleman and Wunch (1970). (*C*) Amino acid alignment of all the individual LIM/glycine modules shown in *A*. The *L1–L5* designation after the protein names in the left margin defines which LIM domain of the protein is displayed in that row and is consistent with the nomenclature shown in *A*. Amino acid positions are numbered to the left of each row. Highlighted residues are identical or conserved in at least 90% of the domains, and the exceptions are not highlighted. The LIM region is marked by a dotted line below the last entry; similarly, the glycine-rich region is marked by a solid line. The cysteine and histidine residues that define the LIM domain are indicated by a shaded square at the bottom of a column. (*Open circles*) Conserved residues potentially involved in hydrogen bonding in the three-dimensional structure of a LIM domain (Perez-Alvarado et al., 1994). Similarly, crosses indicate conserved residues thought to contribute to a hydrophobic core (Perez-Alvarado et al., 1994). Avian CRP sequences are given in the following references (MLP/CRP3: Arber et al., 1994; CRP1: Crawford et al., 1994; CRP2: Weiskirchen et al., 1995).

Mlp84B are shown in Fig. 3 *B*. A cDNA clone (#21) containing the entire coding region is 1,844 nucleotides in length (Fig. 3 *A*). The ATG (nt 135–137) predicted to encode the initiator methionine is underlined. Sequences flanking the ATG conform well to the consensus translation start site for *Drosophila* (Cavener and Ray, 1991), and an in-frame stop codon is present 24 nucleotides upstream of the ATG. Although the cDNA clone #21 contains a stretch of nine adenine residues at the 3′ end, no standard polyadenylation signal is displayed. Additional clones encoding Mlp84B were characterized (Fig. 3 *A*). One clone, #2a, contains additional nucleotides beyond the end of clone #21 and includes a canonical polyadenylation site and subsequent poly A tail. Additional cDNA clones encoding Mlp84B extended sequences at the 5′ end by only four nucleotides (not shown), and some displayed polymorphisms that had no effect on the predicted protein sequence.

The *Mlp84B* cDNAs encode a protein of 495 amino acids with five copies of the LIM domain, each followed by a glycine-rich motif (Fig. 4 *A*). The five LIM-glycine cassettes in Mlp84B are separated by linker regions of variable length and composition. The first LIM domain of Mlp84B has the sequence $CX_2CX_{17}HX_2CX_2CX_2CX_{17}CX_2C$, which is exactly conserved with respect to avian CRPs. The following four LIM domains of Mlp84B display the consensus sequence, $CX_2CX_{17}HX_2CX_2CX_2CX_{18}CX_2C$, and, as indicated, have one additional residue in the second zinc finger of each LIM domain. The glycine-rich repeats after the LIM domains of Mlp84B are highly conserved in comparison to each other and to all CRP family members (Fig. 4 *C*). Partially overlapping with the glycine-rich motif after the first and second LIM domains of Mlp84B are putative nuclear localization signals like those found in CRP family members (Figs. 3 *B* and 4 *C*). These signals are not as well conserved within the glycine-rich motifs after the third, fourth, and fifth LIM domains of Mlp84B. Mlp84B shows 50% identity and 66% similarity with CRP1 at the amino acid level when CRP1 is aligned with the first two LIM domains of Mlp84B (Fig. 4 *B*). Mlp84B displays reduced similarity when compared with the other members of the CRP family, CRP2 or MLP/CRP3. *Drosophila* Mlp60A and Mlp84B are 74% identical and 83% similar at the amino acid level when Mlp60A is aligned with the first LIM domain of Mlp84B (Fig. 4 *B*).

Published September 1, 1996

In situ Localization of Mlp60A and Mlp84B to Polytene Chromosomes

The genomic locations of *Mlp60A* and *Mlp84B* have been mapped using in situ hybridization to the larval salivary gland polytene chromosomes. *Mlp60A*, encoding the single LIM protein, is detected within subdivisions 60A5-6; 60B11 on the distal tip of the right arm of chromosome 2 (Fig. 5 *A*). *Mlp84B*, coding for five LIM domains, is localized to subdivisions 84B3;84C2-6 near the centromere on the right arm of chromosome 3 (Fig. 5 *B*). Each gene appears to be unique, as the hybridization signal is seen at only a single site in the genome. This is consistent with the results of genomic Southern blotting, which reveal a simple pattern of restriction fragments hybridizing with cDNA probes derived from each gene (Fig. 5 *C*).

Expression of Mlp60A and Mlp84B during Drosophila Development

We have examined the expression of *Mlp60A* and *Mlp84B* by developmental Northern analysis (Fig. 6). Both *Mlp60A* and *Mlp84B* display a biphasic pattern of expression, with peaks late in embryogenesis and again during metamorphosis of the fly. The *Mlp60A* gene encodes a single abundant transcript of ~0.5 kb (Fig. 6 *A*). Transcripts are first detectable in 8–12-h embryos and peak strongly in 16–24-h embryos. A significant decrease in steady state RNA levels occurs during the larval stages. A second, less robust peak of expression is observed in pupae. *Mlp60A* transcripts persist in adults. The *Mlp84B* gene encodes a moderately abundant transcript of ~2.3 kb. *Mlp84B* RNA expression is strikingly similar to *Mlp60A* in its biphasic nature. Like *Mlp60A*, *Mlp84B* RNA is first detectable in 8–12-h embryos. Peak expression is observed in 16–24-h embryos. Transcript levels decline dramatically in larvae and elevate again during the larval to pupal transition. RNA levels are decreased, but still detectable, in adults. Neither *Mlp60A* nor *Mlp84B* mRNA is maternally inherited. The mRNA levels for each gene have been quantified using Phosphorimager analysis (Molecular Dynamics, Sunnyvale, CA), and the data were normalized with respect to the amount of mRNA loaded per lane (Fig. 6 *B*). The ribosomal protein gene, *rp49*, was used as a probe to assess the general quality and quantity of RNA loaded. Detailed analysis of the steady state levels of *rp49* transcripts revealed that *rp49* expression is not constant throughout development (Andres and Cherbas, 1992) but, rather, varies in a manner consistent with what we observed. The fluctuations in *rp49* transcript levels may reflect global changes in gene transcription during embryogenesis and metamorphosis. Although we detect the greatest amount of *rp49* transcripts at 16–24 h of embryogenesis, the increases in *Mlp60A* and *Mlp84B* expression are substantially greater than that observed for *rp49*.

Muscle-specific Expression of Mlp60A and Mlp84B

We have analyzed the distributions of *Mlp60A* and *Mlp84B* transcripts during embryogenesis of the fly by in situ hybridization to whole mount embryos (Figs. 7 and 8). The results obtained using this technique were completely consistent with the timing of expression of *Mlp60A* and

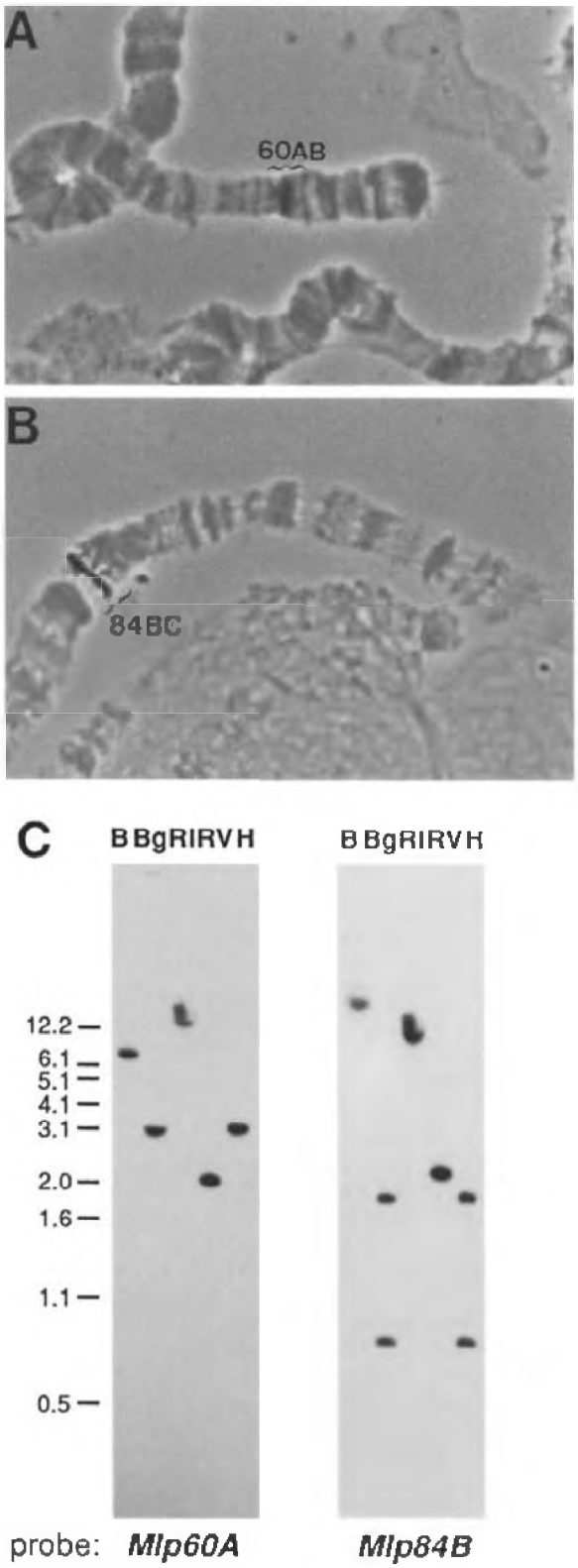


Figure 5. Cytological locations and genomic Southern blot analysis of *Drosophila Mlp60A* and *Mlp84B*. (*A*) *Mlp60A* maps to subdivision 60AB on the distal tip of the right arm of chromosome 2. (*B*) *Mlp84B* maps to subdivision 84BC on the right arm of chromosome 3. (*C*) Southern hybridization to *Drosophila* genomic DNA digested with five different restriction enzymes using either an *Mlp60A* or *Mlp84B* probe. Enzymes: *B*, BamHI; *Bg*, BglII; *RI*, EcoRI; *RV*, EcoRV; *H*, HindIII. Positions of molecular weight markers in kb (*left*).

Published September 1, 1996

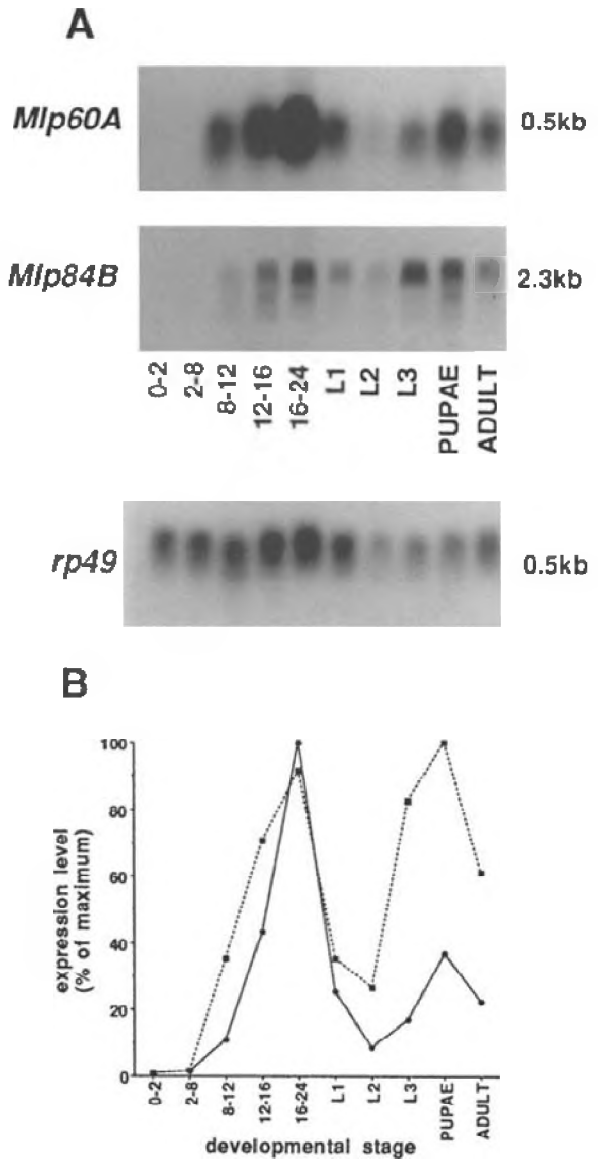


Figure 6. Drosophila Mlp gene expression is developmentally regulated throughout the *Drosophila* life cycle. (*A*) Developmental Northern blot analysis of poly A+ RNA from embryonic (numbered as hours of development at 25°C), larval, pupal, or adult stages. RNAs were hybridized with a ^{32}P-labeled *Mlp60A*, *Mlp84B*, or ribosomal protein, *rp49*, probe. Numbers to the right refer to the size of the hybridizing band. (*B*) Quantitation of Northern blot data. Expression levels are indicated as a percentage of maximum (100%), calculated individually for *Mlp60A* (*circles*) and *Mlp84B* (*squares*). The data have been normalized to account for the amount of RNA loaded per lane in micrograms.

Mlp84B revealed by Northern blot analysis (Fig. 6). Both *Mlp60A* and *Mlp84B* transcripts were observed in a subset of mesodermal derivatives of *Drosophila*. *Mlp60A* and *Mlp84B* genes are expressed in the somatic and visceral mesodermal lineages, but they are not expressed in cardiac mesoderm or the fat body.

In the developing somatic musculature, we begin to detect *Mlp60A* and *Mlp84B* mRNAs in stage 14 embryos, at ~10.5 h into embryogenesis (staging according to Campos-Ortega and Hartenstein, 1985). We observe the initial expression of both *Mlp60A* and *Mlp84B* weakly in the growing syncytial myotubes visualized as segmentally repeated groups of cells positioned dorsally, laterally, and ventrally within the embryo (Fig. 7, *A* and *B*). By this stage, the pattern of early muscle precursors that prefigures the mature pattern of somatic musculature is already complete (Bate, 1990). As development proceeds, nascent myotubes continue fusing with neighboring cells and migrate toward their proper attachment sites in the epidermis (Bate, 1990). During this time, the mRNA hybridization signals for *Mlp60A* and *Mlp84B* intensify (not shown), reflecting the increased mRNA levels observed by Northern analysis. Transcripts for both genes are observed in the completed pattern of larval somatic muscles in stage 16 embryos (Fig. 7, *C* and *D*). During stage 16, between 13 and 16 h of embryogenesis, terminal differentiation events including myofibrillogenesis, muscle fiber attachment to the body wall, and maturation of the myotendinous junction are taking place (Bate, 1993; Bernstein et al., 1993; Tepass and Hartenstein, 1994). Although Northern analysis shows that *Mlp60A* and *Mlp84B* expression persists throughout the rest of embryogenesis, deposition of the cuticle at stage 17 precludes whole mount in situ mRNA localization after this stage.

Although both *Mlp60A* and *Mlp84B* are coexpressed in the somatic muscles, their patterns of hybridization are distinct. *Mlp60A* mRNA appears to be distributed throughout mature myotubes, whereas *Mlp84B* mRNA hybridization is concentrated at the terminal portions of the myotubes near where they are making attachments to the epidermis. The difference in transcript distributions is easily visualized in the ventral-lateral longitudinal muscles, in which a significant proportion of *Mlp60A* staining is found in the middle of a muscle fiber between the segment boundaries (Fig. 7, *C* and *E*). This contrasts with the polarized distribution of *Mlp84B* transcripts seen as segmentally repeated double stripes with significant exclusion of signal in the middle of the segment (Fig. 7, compare *C* with *D*, and *E* with *F*). The difference in the distributions of *Mlp60A* and *Mlp84B* transcripts is also particularly striking in the large cephalic muscles located ventrally in a stage 16 embryo (Fig. 7, *G* and *H*).

Mlp60A and *Mlp84B* are also coexpressed in the visceral musculature surrounding the fore-, mid-, and hindgut of stage 14 and older embryos (Fig. 8). By the beginning of stage 14, the visceral muscles have already attached to the developing gut epithelia (Skaer, 1993; Tepass and Hartenstein, 1994). *Mlp* expression begins to be observed as the muscle cells spread and encircle the gut during stages 14 and 15 (Fig. 8, *A–D*). In addition to the presence in visceral mesoderm, both *Mlp60A* and *Mlp84B* are strongly expressed in pharyngeal muscle (Fig. 8, *E* and *F*). In contrast with what we observed in the somatic musculature, at this level of resolution, we do not detect a polarized distribution of *Mlp84B* transcripts in visceral or pharyngeal muscle.

Protein Distribution of Mlps in the Developing Musculature

To analyze the distributions of *Mlp* gene products during

Published September 1, 1996

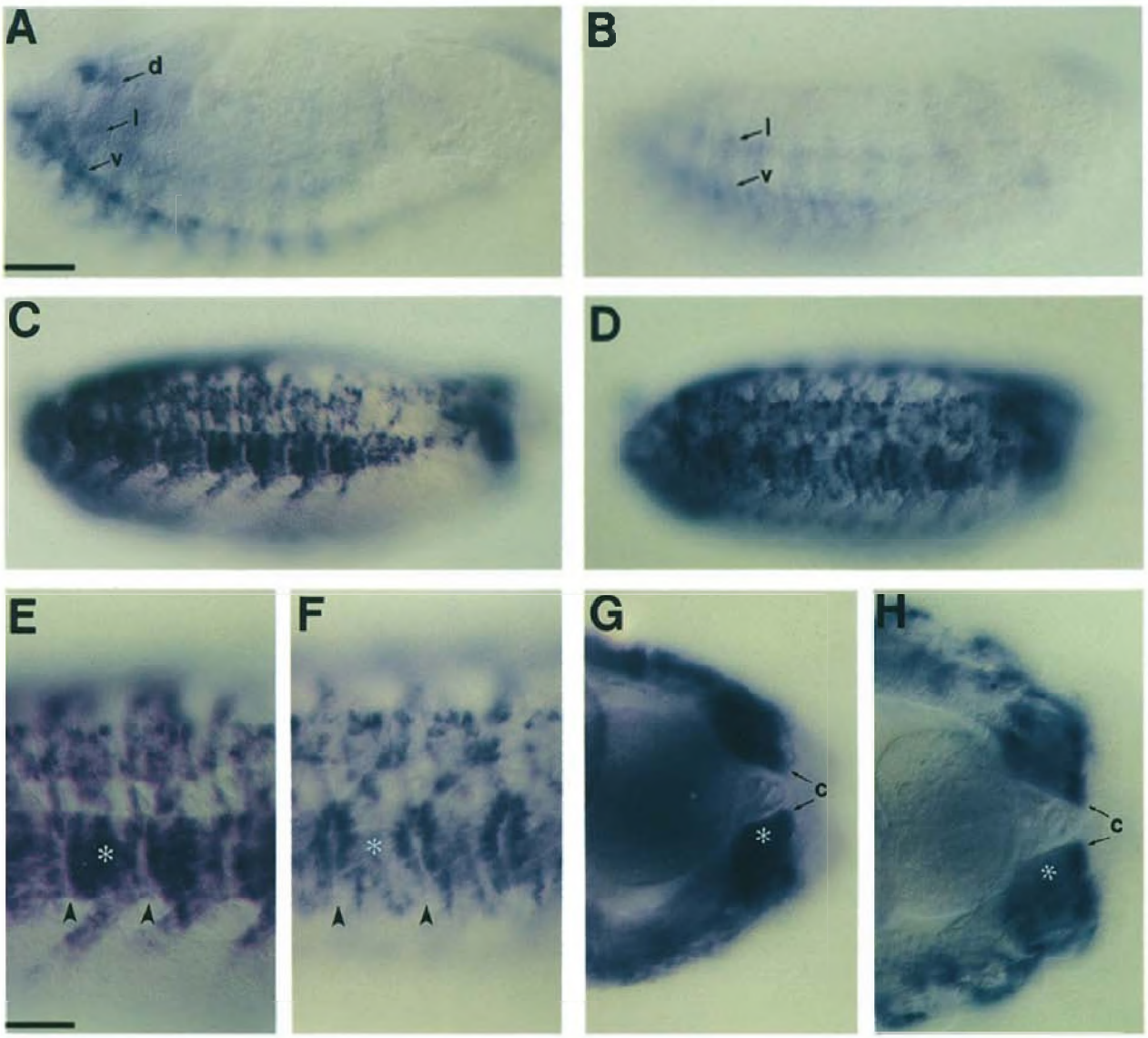


Figure 7. Drosophila Mlp genes are expressed in embryonic somatic muscles. (*A* and *B*) Stage 14 embryos hybridized with antisense *Mlp60A* (*A*) or *Mlp84B* (*B*) probes to reveal gene expression in developing syncytial myotubes positioned dorsally (*d*), laterally (*l*), and ventrally (*v*) within posterior segments. Dorsal groups are just out of focus in (*B*). (*C* and *D*) Stage 16 embryos showing staining of *Mlp60A* (*C*) or *Mlp84B* (*D*) in the completed pattern of larval musculature. (*E* and *F*) Higher magnification of embryos shown in *C* and *D* to reveal localization of *Mlp60A* (*E*) or *Mlp84B* (*F*) transcripts in muscles of two abdominal segments. (*Asterisk*) Ventral-lateral longitudinal muscles in one segment; (*arrowheads*) segment boundaries of one segment. Note that the majority of *Mlp60A* transcripts are found in the middle of the muscle fibers within each segment (*E*), whereas *Mlp84B* transcripts are largely excluded from the middle of the segment, being localized more prominently at the ends of muscle fibers near the segment boundaries (*F*). (*G* and *H*) High magnification of large cephalic muscles (*c*) positioned ventrally in stage 16 embryos, probed with either *Mlp60A* (*G*) or *Mlp84B* (*H*). Note the distinct patterns of mRNA localization relative to the asterisk; *Mlp60A* transcripts are found throughout the muscles, and *Mlp84B* transcripts localize near muscle attachment sites. In all frames, embryos are oriented with anterior to the right. (*A–F*) Lateral views of embryos with dorsal side up. (*G* and *H*) Ventral views of embryos. Bars: (*A–D*) 50 μm; (*E–H*) 25 μm.

Drosophila embryogenesis, we raised antibodies to Mlp60A and Mlp84B sequences that were expressed as fusion proteins in bacteria. Western blot analysis using rabbit anti-Mlp60A or rabbit anti-Mlp84B probes shows the specificity of the individual antibodies (Fig. 9). Anti-Mlp60A antibodies detect a single protein of ~9 kD in 16–24-h *Drosophila* embryonic lysates (Fig. 9 *B*). Anti-Mlp84B antibodies detect a single protein of ~53 kD in a duplicate lysate (Fig. 9 *C*). The preimmune sera harvested from both rabbits fail to show any reactivity with proteins in the 16–24-h embryo lysate (Fig. 9, *B* and *C*).

Immunocytochemical staining of embryos reveals that the distribution of muscle LIM proteins mimics the distribution of transcripts in various mesodermally derived tissues, including all somatic, visceral, and pharyngeal muscles. In these tissues, Mlp60A and Mlp84B are first observed in late stage 14 embryos. In the visceral musculature, although Mlp60A is seen reproducibly, the intensity of stain-

Published September 1, 1996

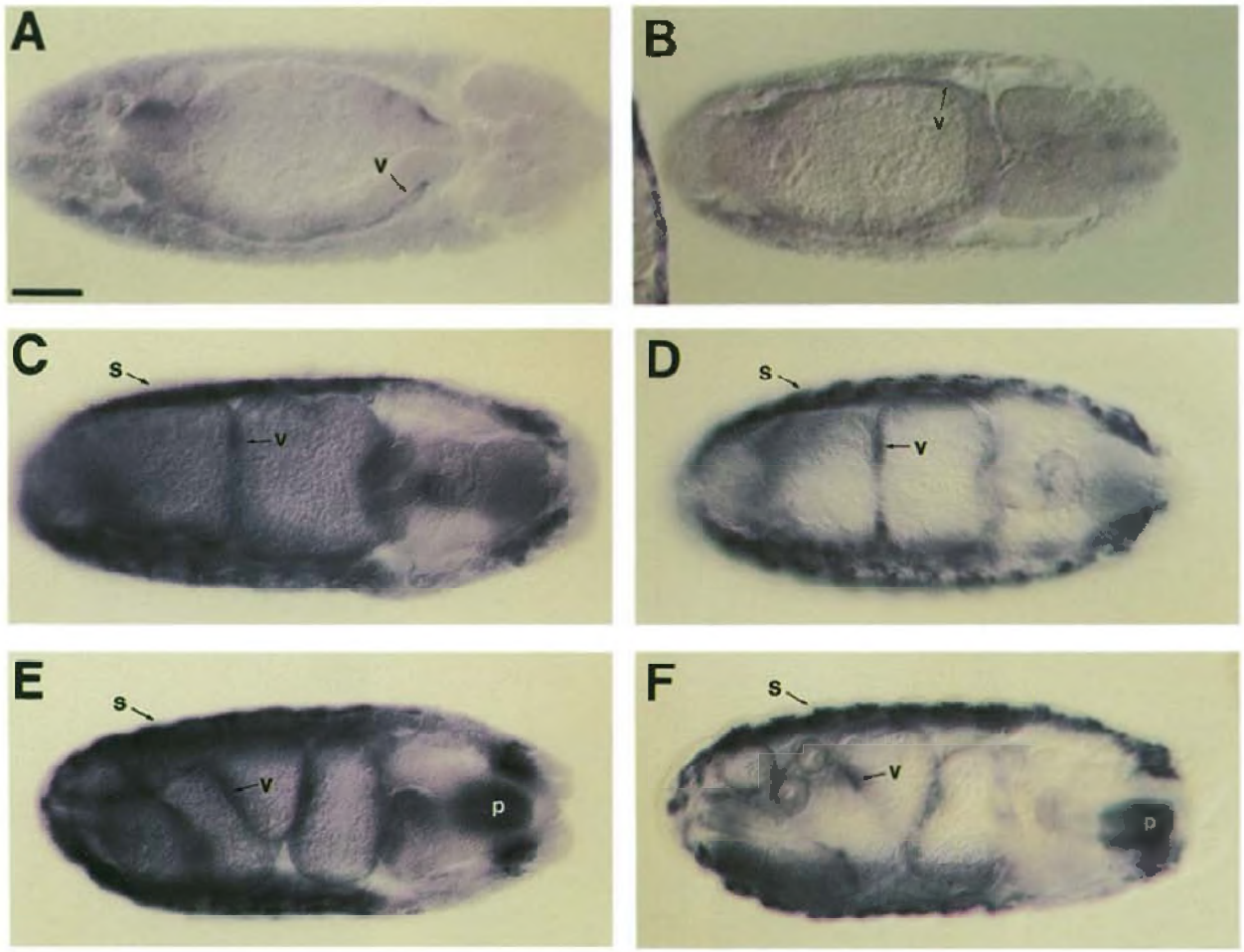


Figure 8. Drosophila Mlp60A and *Mlp84B* are expressed in embryonic visceral mesoderm. (*A* and *B*) Early stage 14 embryos showing *Mlp60A* (*A*) or *Mlp84B* (*B*) expression in the visceral mesodermal cells (*v*) surrounding the gut epithelium. (*C* and *D*) Later in stage 15 embryos, when the middle gut constriction has formed, *Mlp60A* (*C*) and *Mlp84B* (*D*) genes continue to be expressed in visceral mesoderm (*v*) attached to the developing fore-, mid-, and hindgut as well as in the somatic muscles (*s*) located laterally close to the body wall. (*E* and *F*) In late stage 16 embryos, *Mlp60A* (*E*) and *Mlp84B* (*F*) transcripts are seen in the visceral musculature (*v*), the pharynx (*p*), and the somatic muscles (*s*). In all frames, embryos are viewed ventrally and oriented with anterior to the right. Bar, 50 μm.

ing is never as robust as that seen for Mlp84B in the gut muscles and may reflect differences in the levels of protein expression in this tissue (not shown). When the mature pattern of somatic muscles is evident in stage 16 and older embryos, intense immunoreactivity is detected with both anti-Mlp60A and anti-Mlp84B antibodies. Both proteins are found throughout the myotubes (Fig. 10, *A* and *B*). Upon closer examination of the immunostained embryos, we discerned more intense staining for Mlp84B at the ends of muscle fibers at the point of attachment to the epidermis (Fig. 10, see *arrows* in *C* and *D*).

To further characterize the subcellular distributions of the *Drosophila* muscle LIM proteins, we used confocal microscopy to visualize embryos that were fluorescently labeled with anti-Mlp antibodies in parallel with an anti-muscle myosin antibody (Kiehart and Feghali, 1986) (Fig. 11). Examination of embryos by confocal optical sectioning allowed us to discern several prominent differences in protein distribution between the Mlps and myosin. First, the Mlps, although not enriched in muscle cell nuclei, do not show a significant nuclear exclusion as does myosin (Fig. 11, compare *A* with *B* and *C*). Second, Mlp84B, uniquely, becomes associated with the developing myotendinous junction, visualized as bright staining at the ends of myotubes (Fig. 11, see *arrowheads* in *B* and *C*). This enrichment at muscle attachment sites is largely absent before stage 16 (Fig. 12, *A* and *B*), when the midgut has constricted but is not yet convoluted. The redistribution of Mlp84B to the ends of muscle fibers after 14 h of development (Fig. 12, *C* and *D*) correlates with early signs of the development of functional myotendinous junctions, including somatic muscle attachment and visible muscle contractions. It appears then that the association of Mlp84B with the muscle attachment sites could serve as an early marker for the assembly of this junction. Finally, immunofluorescent detection of Mlps using confocal microscopy also revealed that both muscle LIM proteins appear to associate with linear cytoplasmic elements within the muscle cell syncytium, suggestive of the sarcomeric actin filament network (Figs. 11 and 12).

Published September 1, 1996

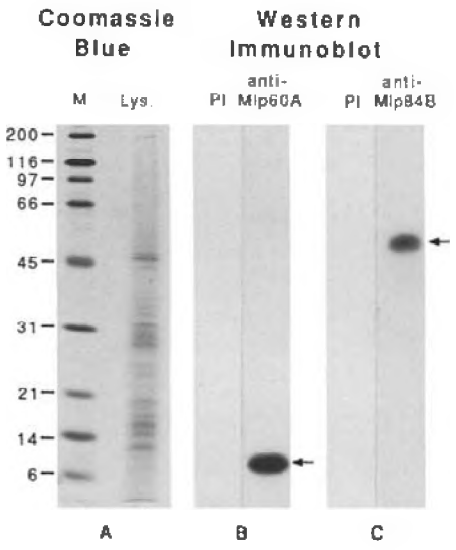


Figure 9. Characterization of anti-Mlp60A and anti-Mlp84B antibodies. (*A*) A Coomassie blue–stained 15% SDS polyacrylamide gel shows molecular mass markers (*M*) and 16–24-h *Drosophila* embryo lysate (*Lys.*). (*B*) Western immunoblot probed with anti-Mlp60A polyclonal antibodies. The antibody specifically recognizes a 9-kD polypeptide, which is not recognized by the preimmune serum (*PI*). (*C*) Western immunoblot probed with anti-Mlp84B antibodies that specifically recognize a 53-kD protein. This protein is not detected by the preimmune serum (*PI*).

Drosophila Mlp60A and Mlp84B Localize to the Cytoskeleton in Vertebrate Cells

Previous work has shown that CRP family members colocalize with the actin cytoskeleton in various cell types including muscle (Sadler et al., 1992; Arber et al., 1994; Crawford et al., 1994). Based on the extensive sequence conservation of the *Drosophila* LIM proteins with respect to their vertebrate counterparts and our observations regarding their subcellular distributions in *Drosophila* muscles, we were interested in evaluating the ability of the fly proteins to associate with the actin cytoskeleton. Therefore, we expressed FLAG epitope-tagged versions of the full-length *Drosophila* cDNAs under the control of a mammalian viral promoter in rat embryo fibroblast (REF) cells (REF52). When either *Drosophila* Mlp60A or Mlp84B is expressed in the REF52 cells, each shows significant colocalization with rhodamine-phalloidin–labeled actin bundles (Fig. 13), illustrating that the LIM-glycine repeats found in the fly proteins share with their vertebrate relatives the ability to associate with the actin cytoskeleton. The cytoskeletal staining observed with the anti-FLAG antibody can be attributed to the recognition of the expressed *Drosophila* sequences since no staining appears in untransfected cells (Fig. 13). Moreover, the localization of Mlp60A and Mlp84B to the actin cytoskeleton is specific since the majority of LIM-containing proteins do not associate with the cytoskeleton. Although we occasionally observe Mlp60A in cell nuclei, the physiological significance of this distribution is not clear. We never observed Mlp84B in the nuclei of REF52 cells.

Discussion

The CRP family of LIM domain proteins consists of at least three highly related isoforms: CRP1, CRP2, and MLP/CRP3 (Liebhaber et al., 1990; Arber et al., 1994; Crawford et al., 1994; Weiskirchen et al., 1995). To investigate the possible role of CRP proteins in differentiation during development, we have initiated a reverse genetic approach in *Drosophila melanogaster*. Here we have reported the identification and initial characterization of two LIM genes in the fly, *Mlp60A* and *Mlp84B*. These genes encode proteins that share many features with vertebrate members of the CRP family.

CRP Proteins Are Conserved in Drosophila

We have identified two new members of the CRP family in *Drosophila melanogaster*. Sequence analysis revealed a high degree of conservation within the LIM domains of both Mlp60A and Mlp84B in comparison with vertebrate CRPs. In addition to the identity and spacing of zinc-binding residues characteristic of the LIM motif consensus, many of the nonmetal coordinating residues are also conserved. In particular, residues that have been shown by nuclear magnetic resonance spectroscopic analysis of avian CRP1 (Perez-Alvarado et al., 1994) to be involved in hydrogen bonding and establishment of a hydrophobic protein core are highly conserved in the *Drosophila* muscle LIM proteins (Fig. 4 *C*); these residues are postulated to promote the proper overall fold of the LIM domain. The availability of the sequences of the *Drosophila* CRP family members has also pointed out a lack of conservation at some sites that were believed to be critical for establishing or maintaining the tertiary fold of the LIM domain of CRP1. These positions appear to accommodate more variability than previously thought, based on sequence comparisons of vertebrate proteins only. The overall sequence conservation, however, suggests that the global structural fold of the *Drosophila* Mlps is likely to be similar to their vertebrate counterparts and supports the notion that the proteins are functionally related.

A glycine-rich region follows each LIM domain in all the CRP family members and serves to distinguish CRPs from other LIM-only proteins. Interestingly, the glycine-rich region is the most highly conserved feature of the *Drosophila* muscle LIM proteins in comparison with vertebrate CRPs. The consensus sequence, GPKG(F/Y)G(F/Y)GX-GAG, overlaps with a putative nuclear targeting sequence, KKYGPK, and displays a sequence that resembles an RNA-binding motif, (K/R)G(F/Y)(G/A)FVX(F/Y), found in many ribonucleoproteins (Burd and Dreyfuss, 1994). Although we do not yet understand the role of the glycine-rich repeats, the high degree of conservation among all the family members shows that this region has been restricted from changing over time and is therefore likely to be functionally significant.

The regions between the LIM-glycine modules of vertebrate CRPs and *Drosophila* Mlp84B exhibit substantial heterogeneity in both length and sequence. It is not clear whether this heterogeneity is an indication that the linker regions represent functionally inert spacers or that the sequence divergence reflects key functional differences among the family members.

Published September 1, 1996

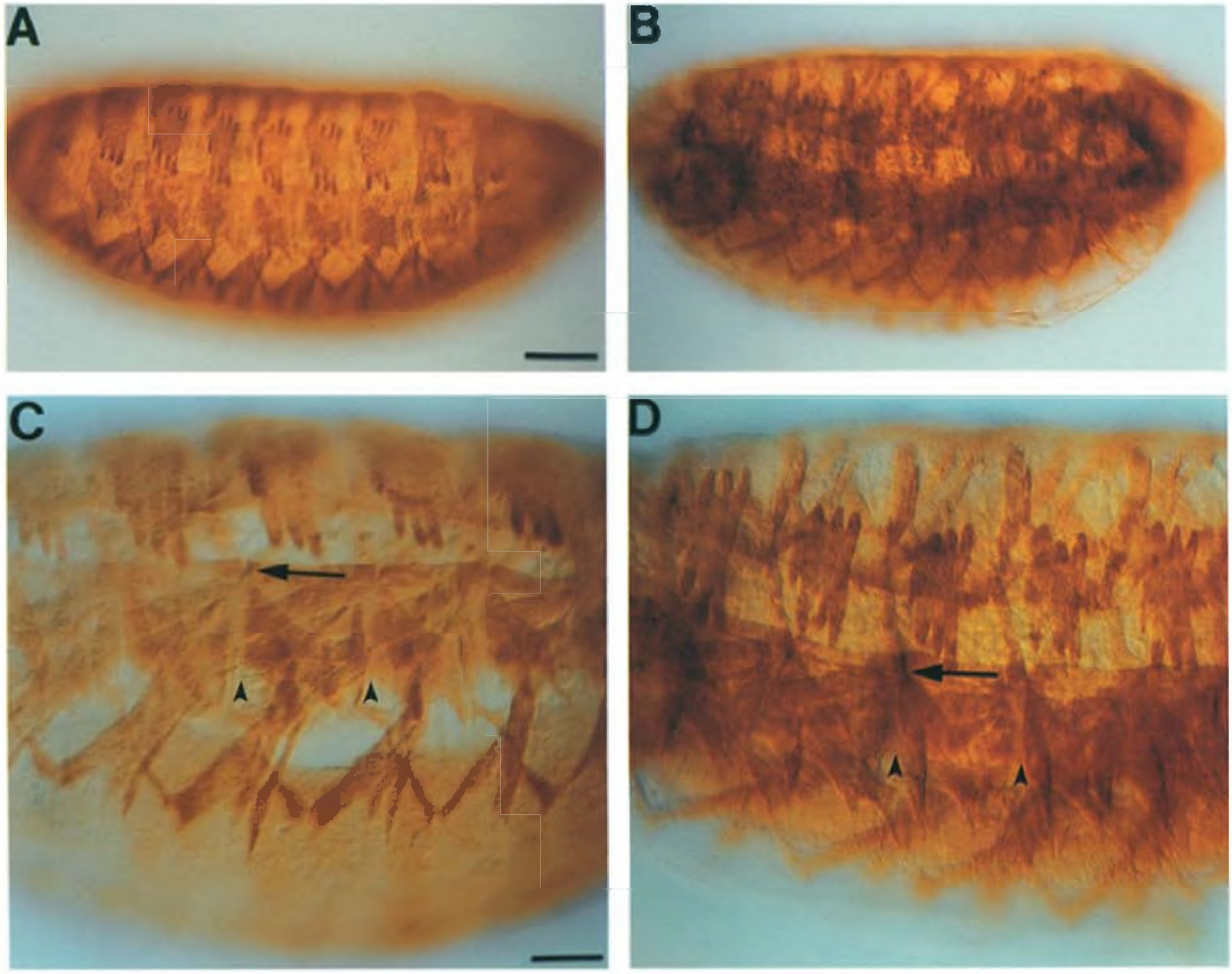


Figure 10. Immunostaining reveals muscle LIM protein localization in the differentiating somatic musculature. Mlp60A is detected in all somatic muscles of a stage 16 embryo (*A* and *C*). Mlp84B protein is also expressed in the mature pattern of somatic muscles (*B* and *D*). A higher magnification view of five segments reveals that Mlp84B (*D*) immunoreactivity is enhanced at the muscle attachment sites, while Mlp60A (*C*) is not. (*Arrows*) Ventral-lateral longitudinal muscle attachment sites that are coincident with the segment borders (*arrowheads*). In all panels, embryos are oriented with anterior to the right and dorsal up. Bars: (*A* and *B*) 50 μm; (*C* and *D*) 25 μm.

The general structures of the *Drosophila* Mlps are unique among the CRP family members because of the number of LIM-glycine modules. All vertebrate CRPs identified to date exhibit two LIM-glycine motifs, whereas *Drosophila* Mlp60A exhibits only one, and Mlp84B displays five complete LIM-glycine repeats. It is clear that a single LIM domain can act independently as a functional protein-binding unit (Schmeichel and Beckerle, 1994). If a single LIM domain is capable of mediating protein–protein interactions, then perhaps a protein like Mlp84B, with five repeats, could serve to dock five copies of the same protein or multiple proteins simultaneously. Recent work has highlighted the versatility and importance of modular protein-binding domains for protein function. Like LIM domains, the Src-homology domains (SH2 and SH3) may be found alone or in tandem with other functional domains within proteins. Even proteins comprised exclusively of SH2 and SH3 domains can function as adaptors that mediate the localized assembly of multimeric signaling complexes (for review see Pawson, 1994; Schlessinger, 1994). Thus, it seems plausible that the *Drosophila* Mlp84B protein, which displays five tandemly arrayed LIM domains, may act as a molecular scaffold that serves to juxtapose key signaling or structural components in a complex. Given the striking sequence similarity between the first LIM domain of Mlp84B and the only LIM domain of Mlp60A, it is possible that Mlp60A serves as a competitive inhibitor of Mlp84B function in muscle cells.

In fibroblasts and muscle cells, CRPs associate with the cellular actin cytoskeleton (Sadler et al., 1992; Arber et al., 1994; Crawford et al., 1994). We have shown that the *Drosophila* muscle LIM proteins retain the ability to associate with actin bundles when expressed in mammalian fibroblast cells. Since the regions of highest sequence conservation in the fly proteins correspond to the LIM-glycine repeats, it is likely that colocalization with actin is a conserved function that can be attributed to these regions. CRPs also interact with zyxin, a protein with LIM domains found at sites of cell adhesion where transmembrane signals are generated via integrin extracellular matrix recep-

Published September 1, 1996

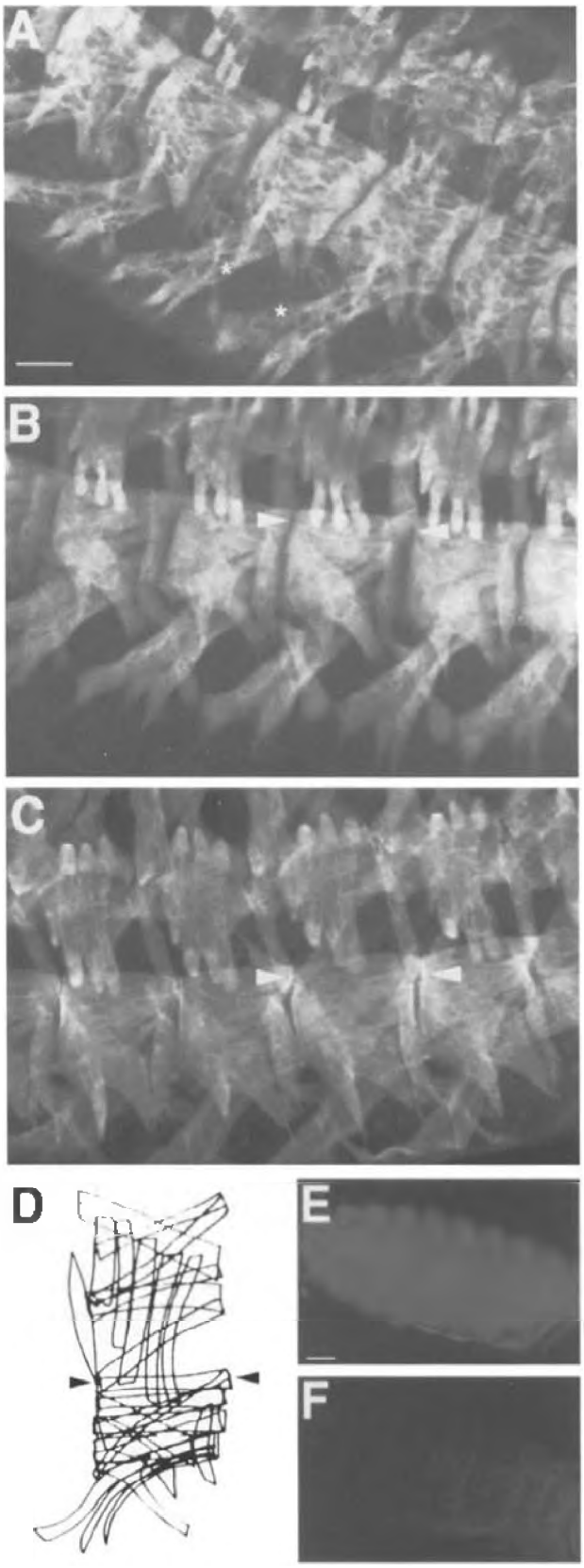


Figure 11. Confocal immunofluorescence microscopy shows the subcellular localization of Mlp60A and Mlp84B with respect to muscle myosin. (*A*) *Drosophila* muscle myosin protein is detected in the somatic muscles of a stage 16 embryo. Note the cytoplasmic expression and exclusion of myosin from muscle cell nuclei; a few nuclei are marked with an asterisk. In contrast, Mlp60A (*B*) and Mlp84B (*C*) are detected in both the cytoplasm and the nuclei of somatic muscle cells in stage 16 embryos. (*Arrowheads*) Enrichment of Mlp84B (*C*) at the myotendinous junction, as opposed to Mlp60A that is not enriched there (*arrowheads* in *B*). (*D*) Representation of the ventral and lateral muscles of one abdominal segment that can be observed in *A–C*. This panel has been adapted from Bate (1990). (*Arrowheads*) Ventral-lateral longitudinal muscle that corresponds to those similarly labeled in *B* and *C*. Preimmune sera from rabbits immunized with either Mlp60A (*E*) or Mlp84B (*F*) fail to stain embryos. In all panels, embryos are oriented with anterior to the right and dorsal up. Bars: (*A–C*) 20 μm; (*E* and *F*) 40 μm.

tors. We have identified a *Drosophila* gene that encodes a protein related to zyxin (Macalma, T.B., B.E. Stronach, and M.C. Beckerle, unpublished results), suggesting that the function of CRP–zyxin complexes in vertebrate cells may also be conserved in the fly.

Mlp Expression and Muscle Development

Like the vertebrate CRP family members, we have observed that the expression patterns of *Mlp60A* and *Mlp84B* are both spatially restricted in the fly embryo and developmentally regulated throughout the life cycle of *Drosophila*. *Mlp60A* and *Mlp84B* display tissue-specific gene expression in a subset of muscular tissues in the developing embryo. In particular, we observed *Mlp* gene products in somatic, visceral, and pharyngeal muscles late in embryogenesis.

Although *Mlp60A* and *Mlp84B* are coexpressed within the somatic musculature, both the transcript and protein distributions are unique. Whereas *Mlp60A* mRNA is distributed throughout the muscle fibers, *Mlp84B* mRNA exhibits a polarized subcellular distribution, being localized at the ends of muscle fibers where they make attachments to the epidermis through the action of the PS integrins (Bogaert et al., 1987; Leptin et al., 1989). It is known that distribution of a specific mRNA can parallel the distribution of the cognate protein. For example, both *Drosophila crumbs* mRNA and protein are localized to the apical ends of polarized epithelial cells where Crumbs function is thought to be required (Tepass et al., 1990). Indeed, further analysis of the subcellular distribution of Mlp84B revealed an enrichment of protein at the muscle attachment sites. Thus, the polarized distribution of *Mlp84B* transcripts may serve as a source of localized protein, some of which remains associated with the attachment sites, while the rest is free to diffuse throughout the cytoplasm. The distinct subcellular distributions of Mlps in somatic muscle cells raise the intriguing possibility that Mlp84B functions within muscle cells at the attachment sites, or myotendinous junctions. This observation is consistent with the observation that vertebrate CRP family members interact with a constituent of integrin-rich junctional complexes (Sadler et al., 1992). In addition, all of the muscle tissues that express *Mlp* genes exhibit integrin-dependent attachment to extracellular matrix and highly ordered actin filament arrays (Crossley, 1978; Bogaert et al., 1987; Tepass and Hartenstein, 1994).

Of particular interest is the regulated entry of Mlp84B

Published September 1, 1996

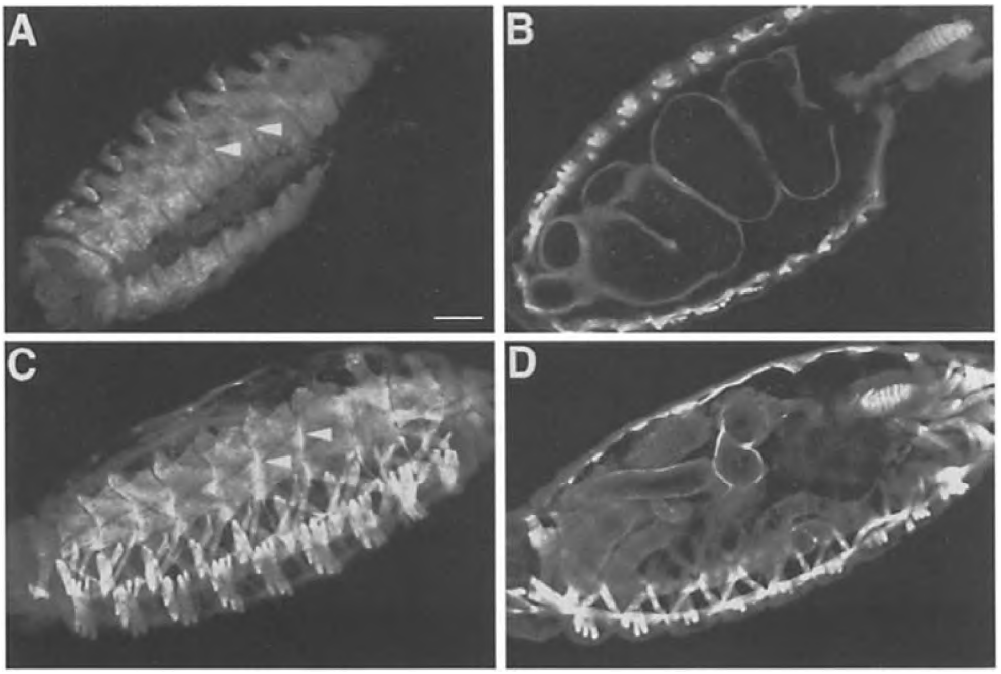


Figure 12 Mlp84B is an early marker of functional myotendinous junctions. (*Left panels*) Immunofluorescent detection of Mlp84B in dorsal body wall muscles. (*Right panels*) Deeper confocal sections of the same embryos to assess the midgut morphology and, therefore, the stage of development. (*A*) Mlp84B is not enriched at the muscle fiber termini (*arrowheads*) in an early stage 16 embryo, when the midgut (*B*) is constricted but not convoluted. This section also reveals that Mlp84B protein is expressed in the visceral and pharyngeal muscles. Note that Mlp84B protein begins to accumulate at the developing myotendinous junctions (*arrowheads* in *C*) of somatic muscles at ~14 h after egg lay, mid-stage 16, when the midgut becomes convoluted (*D*). In all panels, embryos are viewed dorso-laterally and oriented with anterior to the right. Bar, 40 μm.

protein into developing myotendinous junctions. Approximately 14 h after egg lay, which corresponds to a stage when the midgut of the embryo becomes convoluted, Mlp84B becomes strongly associated with the terminal junctions. It is not clear what molecular cues influence or stabilize this transit; however, it has been noted that this developmental time period roughly correlates with attachment of the somatic muscle fibers to the body wall and the first visible muscle contractions (Crossley, 1978; Bate, 1990; Abmayr et al., 1995). These observations suggest that Mlp84B might regulate or participate in the assembly of a functional mechanical link between the actin myofilament network and the extracellular matrix mediated through integrin receptors.

Analysis of the subcellular distributions of Mlps in the developing musculature also revealed localization of both Mlp60A and Mlp84B proteins in the nuclei of myotubes. Although Mlp60A and Mlp84B do display some nuclear localization, neither protein appears to be concentrated in cell nuclei. Given the small molecular mass of Mlp60A at 9 kD, the distribution in both cytoplasm and nuclei could be the result of passive equilibration between these subcellular compartments. The molecular mass of Mlp84B at 53 kD is, however, close to the predicted cutoff for free diffusion through nuclear pores, and its presence in nuclei may reflect an active transport process. It is clear that both Mlp protein sequences contain putative nuclear targeting information that overlaps with the highly conserved glycine-rich region. At this time we cannot distinguish between passive or active models for Mlp nuclear localization. It is worth noting, however, that we did not observe an exclusive nuclear localization of either Mlp60A or Mlp84B protein at any time during development in contrast with what has been reported for vertebrate MLP/CRP3 distribution in tissue-culture cells (Arber et al., 1994).

The muscle-specific expression patterns of *Mlp60A* and *Mlp84B* in *Drosophila*, coupled with the extensive sequence conservation with vertebrate MLP/CRP3, a protein clearly involved in muscle cell differentiation (Arber et al., 1994), suggest that *Mlps* function in myogenesis. Examination of the temporal expression of *Mlp* genes during embryogenesis has provided a context for considering their roles in myogenesis relative to other genes expressed in muscle. The expression of the *Mlp* genes is most coincident with those processes that occur late in the muscle differentiation program, after specification, proliferation, and subdivision of the mesoderm, but just before markers associated with overt differentiation, like the contractile proteins (Fig. 14). The onset of *Mlp* expression in both somatic and visceral mesoderm occurs between 10 and 11 h of development (stage 14). Both *Mlp60A* and *Mlp84B* levels continue to increase, peaking between 16 and 24 h of embryogenesis (stage 17). Events that specify and subdivide the mesoderm are completed by 7 h of development (Bate, 1993), long before the onset of *Mlp* expression. Similarly, determination of the final fates of mesodermal cells, influenced by positional cues in the embryo (Frasch, 1995; Maggert et al., 1995) and requiring the restricted expression of transcription factors (Bate, 1993; Bernstein et al.,

Published September 1, 1996

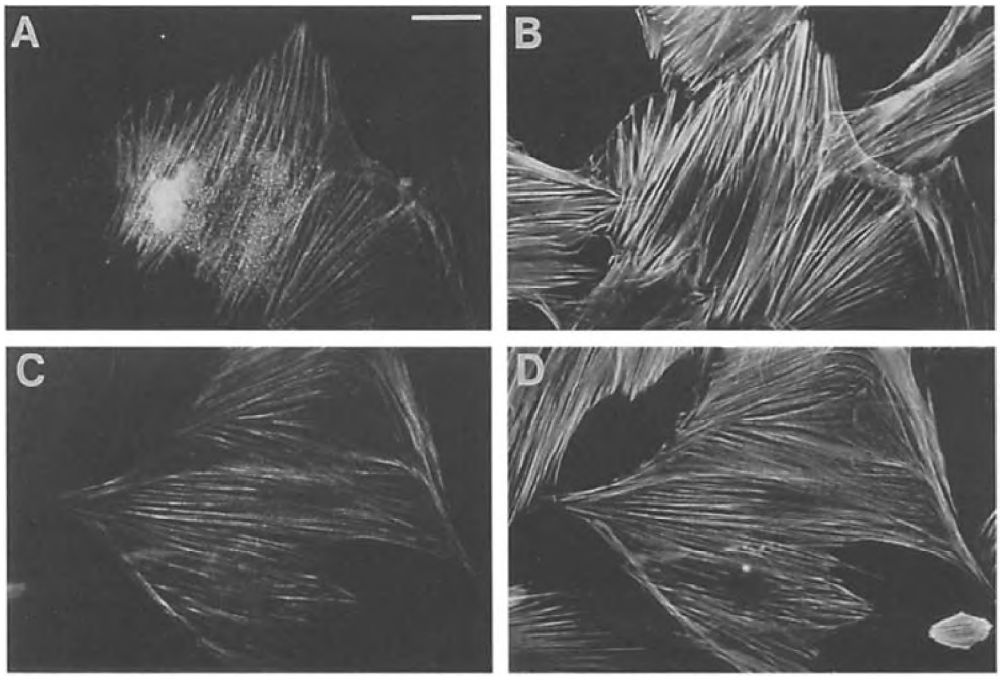


Figure 13. Drosophila muscle LIM proteins localize to the cytoskeleton in vertebrate cells. REF52 cells were transiently transfected with epitope-tagged Mlp60A (*A* and *B*) or Mlp84B (*C* and *D*) and were visualized using double-label immunofluorescence. An anti-FLAG mAb reveals transfected cells expressing Mlp60A (*A*), which localizes to the actin cytoskeleton. The same field of cells (*B*), containing both transfected and untransfected cells, has been stained with rhodamine-phalloidin to visualize filamentous actin. Note the colocalization of the expressed fly protein with actin filaments. Similarly, Mlp84B-FLAG (*C*) is distributed along actin stress fibers in transfected cells. Double labeling the same cells with rhodamine-phalloidin (*D*) confirms colocalization with the actin cytoskeleton. Note that expression of *Drosophila* Mlp sequences does not appear to adversely affect actin filament arrays. Bar, 30 μm.

1993), temporally precedes expression of *Mlp60A* and *Mlp84B* by a few hours, effectively precluding their involvement in commitment or patterning. Instead, the timing of *Mlp60A* and *Mlp84B* expression is concomitant with late events in myogenesis, such as cell migration, attachment, and cytoskeletal rearrangements. Although some cell fusion is still occurring in the somatic lineage during the time that the *Mlps* are expressed, we believe it is unlikely that *Mlp60A* or *Mlp84B* functions in this process because fusion begins at least 2 h before their expression, and, also, both genes are expressed in visceral and pharyngeal muscles that are mononucleate.

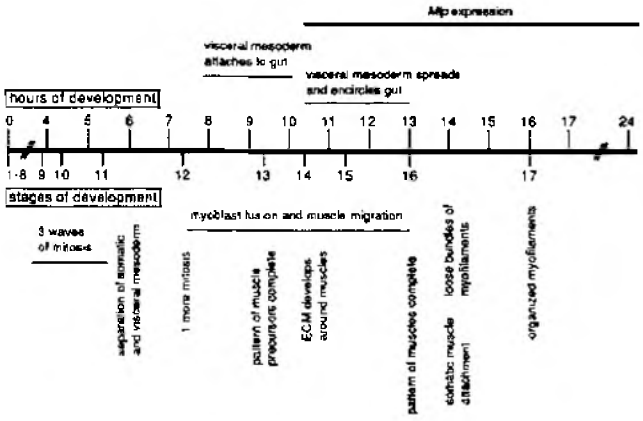


Figure 14. Developmental time line of myogenesis during *Drosophila* embryogenesis. Major myogenic events are noted with respect to developmental stages and hours of embryogenesis. *Mlp* expression coincides largely with late events in myogenesis, especially those involved with terminal muscle differentiation.

Other late myogenic events involve transcriptional upregulation of genes required for terminal differentiation, such as those that encode proteins of the contractile apparatus. Recent work has highlighted the importance of *Drosophila* myocyte enhancer factor (MEF) 2, a MADS box-containing transcription factor homologous to the vertebrate MEF proteins, in this process (Bour et al., 1995; Lilly et al., 1995). Although *Drosophila* MEF2 is expressed in the developing mesoderm from very early on, mutations in the gene exert their effects late in the myogenic program, after myoblasts have been specified. The phenotype manifests as a disrupted muscle pattern and loss of terminal differentiation markers, such as myosin heavy chain, with the implication that MEF2 may regulate expression of late structural genes. The *Mlp* genes are expressed just before the expression of myosin heavy chain but concomitant with the lethal phase of MEF2 mutants (Michelson et al., 1990). MEF2 may therefore be a reasonable candidate for participation in regulating or in interacting with the muscle LIM proteins, and we cannot rule out a role for the *Drosophila* muscle LIM proteins in transcriptional control. Compatible with this notion is the observation that Mips can be found in muscle cell nuclei, although the proteins do not appear to be concentrated there. Nonetheless, the hypothesis that *Drosophila* Mlps function late in myogenesis is consistent with studies showing that rat MLP/CRP3 is required for muscle differentiation subsequent to determination by the action of the MyoD family members (Arber et al., 1994). Likewise, expression of avian CRP1 protein coincides with the maturation of smooth muscle cells (Crawford et al., 1994).

The *Mlp* genes show a biphasic expression pattern, with a second peak of expression during metamorphosis, when additional myogenic events occur. The transition from a

larva to an adult fly requires massive changes in body and tissue morphology. Adult muscles are not simply remodeled from the existing larval musculature; rather, a second round of myogenesis occurs in preexisting groups of cells set aside during embryogenesis (Bate, 1993). We observed an increase in *Mlp* transcript levels during the transition from larval to pupal stages, at which time additional myogenic events take place, suggesting the Mlps are again required for proper differentiation. Interestingly, other myogenic genes, such as *MEF2* and *nautilus*, show biphasic expression patterns that presumably also reflect a requirement for their function in both rounds of myogenesis (Michelson et al., 1990; Nguyen et al., 1994).

In summary, *Drosophila* muscle LIM proteins display muscle-specific distributions and developmentally regulated gene expression with peak expression corresponding to times when the musculature is differentiating. *Mlp84B* transcripts and protein are enriched at muscle attachment sites in the embryo, and both Mlp60A and Mlp84B have the ability to associate with the actin cytoskeleton when expressed in vertebrate cells. Based on our observations, together with evidence about the physiological function of vertebrate CRP proteins, we postulate that Mlp60A and Mlp84B play a role in cell differentiation late in myogenesis. Examination of the phenotypic consequences of eliminating Mlp60A and Mlp84B function will be required to define the functional significance of these proteins in vivo.

We thank E. King, A. Letsou, K. Golic, J. Dickinson, D. Nix, D. Cimbora, K. Ahmad, and D. Kiehart for reagents and helpful discussions. We also acknowledge T. Macalma, J. Henderson, H. Louis, and P. Renfranz for technical assistance with sequencing and heterologous expression.

This work was supported by the National Institutes of Health (NIH) (GM50877) and an American Cancer Society faculty research award to M.C. Beckerle, as well as an NIH Genetics training grant T32GM07464 to B.E. Stronach. We also acknowledge B. Shackman and support from the Utah Regional Cancer Center (NIH Cancer Institute grant CA42014).

Received for publication 15 January 1996 and in revised form 24 April 1996.

References

Abmayr, S.M., M.S. Erickson, and B.A. Bour. 1995. Embryonic development of the larval body wall musculature of *Drosophila melanogaster*. *Trends Genet.* 11:153–159.

Andres, A.J., and P. Cherbas. 1992. Tissue-specific ecdysone responses: regulation of the *Drosophila* genes Eip28/29 and Eip40 during larval development. *Development (Camb.).* 116:865–876.

Arber, S., G. Halder, and P. Caroni. 1994. Muscle LIM protein, a novel essential regulator of myogenesis, promotes myogenic differentiation. *Cell.* 79: 221–231.

Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Siedman, J.A. Smith, and K. Struhl. 1994. Current Protocols in Molecular Biology. John Wiley & Sons, Boston, MA.

Bate, M. 1990. The embryonic development of larval muscles in *Drosophila*. *Development (Camb.).* 110:791–804.

Bate, M. 1993. The mesoderm and its derivatives. *In* The Development of *Drosophila melanogaster*. M. Bate and A. Martinez Arias, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 1013–1090.

Beckerle, M.C. 1986. Identification of a new protein localized at sites of cell–substrate adhesion. *J Cell Biol.* 103:1679–1687.

Berget, S.M. 1984. Are U4 small nuclear ribonucleoproteins involved in polyadenylation? *Nature (Lond.).* 309:179–182.

Bernstein, S.I., P.T. O'Donnell, and R.M. Cripps. 1993. Molecular genetic analysis of muscle development, structure, and function in *Drosophila*. *Int. Rev. Cytol.* 143:63–152.

Bogaert, T., N. Brown, and M. Wilcox. 1987. The *Drosophila* PS2 antigen is an invertebrate integrin that, like the fibronectin receptor, becomes localized to muscle attachments. *Cell.* 51:929–940.

Bour, B.A., M.A. O'Brien, W.L. Lockwood, E.S. Goldstein, R. Bodmer, P.H. Taghert, S.M. Abmayr, and H.T. Nguyen. 1995. *Drosophila* MEF2, a transcription factor that is essential for myogenesis. *Genes & Dev.* 9:730–741.

Burd, C.G., and G. Dreyfuss. 1994. Conserved structures and diversity of functions of RNA-binding proteins. *Science (Wash. DC).* 265:615–621.

Campos-Ortega, J.A., and V. Hartenstein. 1985. The Embryonic Development of *Drosophila melanogaster*. Springer-Verlag, Berlin, Germany. 227 pp.

Cavener, D.R., and S.C. Ray. 1991. Eukaryotic start and stop translation sites. *Nucleic Acids Res.* 19:3185–3192.

Crawford, A.W., J.D. Pino, and M.C. Beckerle. 1994. Biochemical and molecular characterization of the chicken cysteine-rich protein, a developmentally regulated LIM-domain protein that is associated with the actin cytoskeleton. *J. Cell Biol.* 124:117–127.

Crossley, A.C. 1978. The morphology and development of the *Drosophila* muscular system. *In* The Genetics and Biology of *Drosophila*. M. Ashburner and T.R.F. Wright, editors. Academic Press, London. 499–560.

Dawid, I.B., R. Toyama, and M. Taira. 1995. LIM domain proteins. *C.R. Acad. Sci. Ser. III. Sci. Vie.* 318:295–306.

Feuerstein, R., X. Wang, D. Song, N.E. Cooke, and S.A. Liebhaber. 1994. The LIM/double zinc-finger motif functions as a protein dimerization domain. *Proc. Natl. Acad. Sci. USA.* 91:10655–10659.

Flybase. 1994. The *Drosophila* genetic database. *Nucleic Acids Res.* 22:3456–3458.

Frasch, M. 1995. Induction of visceral and cardiac mesoderm by ectodermal Dpp in the early *Drosophila* embryo. *Nature (Lond.).* 374:464–467.

Freyd, G., S.K. Kim, and H.R. Horvitz. 1990. Novel cysteine-rich motif and homeodomain in the product of the *Caenorhabditis elegans* cell lineage gene *lin-11*. *Nature (Lond.).* 344:876–879.

Jowett, T. 1986. Preparation of nucleic acids. *In* Drosophila: A Practical Approach. D.B. Roberts, editor. IRL Press, Oxford, UK. 275–286.

Karlsson, O., S. Thor, T. Norberg, H. Ohlsson, and T. Edlund. 1990. Insulin enhancer binding protein *Isl-1* is a member of a novel class of proteins containing both a homeo- and a Cys-His domain. *Nature (Lond.).* 344:879–882.

Kiehart, D.P., and R. Feghali. 1986. Cytoplasmic myosin from *Drosophila melanogaster*. *J. Cell Biol.* 103:1517–1525.

Kosa, J.L., J.W. Michelsen, H.A. Louis, J.I. Olsen, D.R. Davis, M.C. Beckerle, and D.R. Winge. 1994. Common metal ion coordination in LIM domain proteins. *Biochemistry.* 33:468–477.

Laemmli, U.K. 1970. Cleavage of structual proteins during the assembly of the head of the bacteriophage T4. *Nature (Lond.).* 227:680–685.

Leptin, M., T. Bogaert, R. Lehmann, and M. Wilcox. 1989. The function of PS integrins during *Drosophila* embryogenesis. *Cell.* 56:401–408.

Liebhaber, S.A., J.G. Emery, M. Urbanek, X. Wang, and N. E. Cooke. 1990. Characterization of a human cDNA encoding a widely expressed and highly conserved cysteine-rich protein with an unusual zinc-finger motif. *Nucleic Acids Res.* 18:3871–3879.

Lilly, B., B. Zhao, G. Ranganayakulu, B.M. Paterson, R.A. Schulz, and E.N. Olson. 1995. Requirement of MADS domain transcription factor D-MEF2 for muscle formation in *Drosophila*. *Science (Wash. DC).* 267:688–693.

Maggert, K., M. Levine, and M. Frasch. 1995. The somatic-visceral subdivision of the embryonic mesoderm is initiated by dorsal gradient thresholds in *Drosophila*. *Development (Camb.).* 121:2107–2116.

Michelsen, J.W., K.L. Schmeichel, M.C. Beckerle, and D.R. Winge. 1993. The LIM motif defines a specific zinc-binding protein domain. *Proc. Natl. Acad. Sci. USA.* 90:4404–4408.

Michelsen, J.W., A.K. Sewell, H.A. Louis, J.I. Olsen, D.R. Davis, D.R. Winge, and M.C. Beckerle. 1994. Mutational analysis of the metal sites in an LIM domain. *J. Biol. Chem.* 269:11108–11113.

Michelson, A.M., S.M. Abmayr, M. Bate, A. Martinez Arias, and T. Maniatis. 1990. Expression of a MyoD family member prefigures muscle pattern in *Drosophila* embryos. *Genes & Dev.* 4:2086–2097.

Muller, L., G. Xu, R. Wells, C.P. Hollenberg, and W. Piepersberg. 1994. LRG1 is expressed during sporulation in *Saccharomyces cerevisiae* and contains motifs similar to LIM and rho/racGAP domains. *Nucleic Acids Res.* 22:3151–3154.

Needleman, S.B., and C.D. Wunch. 1970. A general method applicable to the search for similarities in the amino acid sequence of two proteins. *J. Mol. Biol.* 48:443–453.

Nguyen, H.T., R. Bodmer, S.M. Abmayr, J.C. McDermott, and N.A. Spoerel. 1994. D-mef2: a *Drosophila* mesoderm-specific MADS box-containing gene with a biphasic expression profile during embryogenesis. *Proc. Natl. Acad. Sci. USA.* 91:7520–7524.

Pardue, M.L. 1986. In situ hybridization to DNA of chromosomes and nuclei. *In* Drosophila: A Practical Approach. D.B. Roberts, editor. IRL Press, Oxford, UK. 111–136.

Patel, N.H. 1994. Imaging neuronal subsets and other cell types in whole-mount *Drosophila* embryos and larvae using antibody probes. *In Drosophila melanogaster*: Practical Uses in Cell and Molecular Biology. L.S.B. Goldstein and E.A. Fyrberg, editors. Academic Press, San Diego, CA. 446–487.

Pawson, T. 1994. SH2 and SH3 domains in signal transduction. *Adv. Cancer Res.* 64:87–110.

Perez-Alvarado, G.C., C. Miles, J.W. Michelsen, H.A. Louis, D.R. Winge, M.C. Beckerle, and M.F. Summers. 1994. Structure of the carboxy-terminal LIM domain from the cysteine-rich protein CRP. *Nat. Struct. Biol.* 1:388–398.

Sadler, I., A.W. Crawford, J.W. Michelsen, and M.C. Beckerle. 1992. Zyxin and cCRP: two interactive LIM domain proteins associated with the cytoskeleton. *J. Cell Biol.* 119:1573–1587.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 545 pp.

Sanchez-Garcia, I., and T.H. Rabbitts. 1994. The LIM domain: a new structural motif found in zinc-finger-like proteins. *Trends Genet.* 10:315–320.

Sanger, F., S. Nicklen, and A.R. Coulsen. 1977. DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* 74:5463–5467.

Schlessinger, J. 1994. SH2/SH3 signaling proteins. *Curr. Opin. Genet. Dev.* 4: 25–30.

Schmeichel, K.L., and M.C. Beckerle. 1994. The LIM domain is a modular protein-binding interface. *Cell.* 79:211–219.

Skaer, H. 1993. The alimentary canal. *In* The Development of *Drosophila melanogaster.* M. Bate and A. Martinez Arias, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 941–1012.

Tautz, D., and C. Pfeifle. 1989. A nonradioactive in situ hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals a translational control of segmentation gene hunchback. *Chromosoma (Berl.).* 98: 81–85.

Tepass, U., and V. Hartenstein. 1994. The development of cellular junctions in the *Drosophila* embryo. *Dev. Biol.* 161:563–596.

Tepass, U., C. Theres, and E. Knust. 1990. *crumbs* encodes an EGF-like protein expressed on apical membranes of *Drosophila* epithelial cells and required for organization of epithelia. *Cell.* 61:787–799.

Valge-Archer, V.E., H. Osada, A.J. Warren, A. Forster, J. Li, R. Baer, and T.H. Rabbitts. 1994. The LIM protein RBTN2 and the basic helix-loop-helix protein TAL1 are present in a complex in erythroid cells. *Proc. Natl. Acad. Sci. USA.* 91:8617–8621.

Wang, X., G. Lee, S.A. Liebhaber, and N.E. Cooke. 1992. Human cysteine-rich protein. A member of the LIM/double-finger family displaying coordinate serum induction with c-myc. *J. Biol. Chem.* 267:9176–9184.

Warren, A.J., W.H. Colledge, M.B. Carlton, M.J. Evans, A.J. Smith, and T.H. Rabbitts. 1994. The oncogenic cysteine-rich LIM domain protein rbtn2 is essential for erythroid development. *Cell.* 78:45–57.

Way, J.C., and M. Chalfie. 1988. *mec-3*, a homeobox-containing gene that specifies differentiation of the touch receptor neurons in *C. elegans*. *Cell.* 54:5–16.

Weiskirchen, R., and K. Bister. 1993. Suppression in transformed avian fibroblasts of a gene (crp) encoding a cysteine-rich protein containing LIM domains. *Oncogene.* 8:2317–2324.

Weiskirchen, R., J.D. Pino, T. Macalma, K. Bister, and M.C. Beckerle. 1995. The cysteine-rich protein family of highly related LIM domain proteins. *J. Biol. Chem.* 270:28946–28954.

Wu, R.Y., and G.N. Gill. 1994. LIM domain recognition of a tyrosine-containing tight turn. *J. Biol. Chem.* 269:25085–25090.