Drosophila myoblast city Encodes a Conserved Protein That Is Essential for Myoblast Fusion, Dorsal Closure, and Cytoskeletal Organization

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Abstract. The *Drosophila myoblast city* (*mbc*) locus was previously identified on the basis of a defect in myoblast fusion (Rushton et al., 1995. *Development [Camb.].* 121:1979–1988). We describe herein the isolation and characterization of the *mbc* gene. The *mbc* transcript and its encoded protein are expressed in a broad range of tissues, including somatic myoblasts, cardial cells, and visceral mesoderm. It is also expressed in the pole cells and in ectodermally derived tissues, including the epidermis. Consistent with this latter expression, *mbc* mutant embryos exhibit defects in dorsal closure and cytoskeletal organization in the migrating epidermis. Both the mesodermal and ectodermal defects are reminiscent of those induced by altered forms of Drac1 and suggest that *mbc* may function in the same pathway. MBC bears striking homology to human DOCK180, which interacts with the SH2-SH3 adapter protein Crk and may play a role in signal transduction from focal adhesions. Taken together, these results suggest the possibility that MBC is an intermediate in a signal transduction pathway from the *rho/rac* family of GTPases to events in the cytoskeleton and that this pathway may be used during myoblast fusion and dorsal closure.

In vertebrate organisms, a common feature of the myogenic differentiation program of all muscle fibers is the apparent recognition, adherence, and fusion between myoblasts that generate multinucleate syncitia (for review see Fischman, 1972; Wakelam, 1985). Since this process can occur in cultured cells, tissue culture systems have been invaluable in identifying regulators of myoblast fusion. Essential components include cell adhesion molecules, calcium and molecules that are regulated by it, metalloproteases, meltrins, lipids, and others (Yagami-Hiromasa et al., 1995; for reviews see Wakelam, 1985; Knudsen, 1991). Homologues to these vertebrate factors have not yet been shown to function in myogenesis in *Drosophila.* However, morphological studies have established that the differentiated muscle fibers of insects are also syncitial (Ball et al., 1985; Campos-Ortega and Hartenstein, 1985; Bate, 1990, 1993; Doberstein et al., 1997). Therefore, one might anticipate that similar molecules will control fundamental aspects of myoblast fusion in a variety of species. Moreover, the use of genetics to identify critical myogenic regulators and their relationship to other molecules in *Drosophila* seems likely to reveal parallel pathways in vertebrate organisms.

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While it seems unlikely that all aspects of myogenesis will be analogous between *Drosophila* and vertebrates, many appear to be conserved between these organisms. One example is the apparent conservation of myogenic regulatory molecules. Among these are *nautilus* (Michelson et al., 1990; Paterson et al., 1991), which, like its vertebrate counterparts (for review see Weintraub, 1993), can induce a somatic muscle differentiation program (Keller et al., 1997) and MEF2, an enhancer binding protein that is absolutely required for the induction of muscle-specific structural genes and myogenic differentiation (Lin et al., 1996; for review see Olson et al., 1995). Similarities are also apparent at the cellular level. For example, extensive proliferation of myosin-expressing myoblasts is not observed in either vertebrates (for review see Holtzer et al., 1975*a*) or *Drosophila* (Campos-Ortega and Hartenstein, 1985; Bate, 1993; Rushton et al., 1995). In addition, although fusion normally occurs before myosin expression in vertebrate cells, it is not an absolute prerequisite for expression of myosin in either system (Holtzer et al., 1975*a*, and references therein; see also Emerson and Beckner, 1975; Endo and Nadal-Ginard, 1987; Luo et al., 1994; Paululat et al., 1995; Rushton et al., 1995; Doberstein et al., 1997). Finally, recent studies have revealed striking similarities between the ultrastructure of fusing *Drosophila* myoblasts (Doberstein et al., 1997) and fusing vertebrate myoblasts (Engel et al., 1985; for review see Kalderon, 1980).

The above studies suggest several parallels between myogenesis in *Drosophila* and in vertebrate systems. However, little is actually known about the genes regulating myoblast fusion in *Drosophila* and their mechanism of action. Recently, several loss-of-function mutations that exhibit defects in myoblast fusion have been identified, including *rolling stone* (Paululat et al., 1995), *myoblast city* (*mbc*)[1] (Rushton et al., 1995), *blown fuse* (Doberstein et al., 1997), *sticks and stones* (Abmayr, S.M., M.R. Erickson, B.A. Bour, and M. Kulp. *J. Cell. Biochem.* 1994. 18D (Suppl.):474), and *singles bar* (Maeland, A.D., J.W. Bloor, and N.H. Brown. 1996. *Mol. Biol. Cell.* 7:39A). The protein coding sequence of *blown fuse*, the first of these genes to be identified, has not yet provided insight into its function. By comparison, examination of altered forms of the small *rho*-like GTPase, Drac1, has been useful in understanding myoblast fusion in *Drosophila* (Luo et al., 1994). *Drac1* is the *Drosophila* homologue of the vertebrate gene *rac1*, which has been shown to induce membrane ruffling through reorganization of the actin cytoskeleton (Ridley et al., 1992). Both dominant negative and constitutively active forms of Drac1 have been shown to cause defects in myoblast fusion (Luo et al., 1994). Interestingly, altered forms of Drac1 also disrupt the actin cytoskeleton in the epidermis, causing defects in cell migration and dorsal closure (Harden et al., 1995), and in apical regions of the wing imaginal disc (Eaton et al., 1995).

A role for the cytoskeleton in myoblast fusion in vertebrates has previously been shown using low concentrations of the inhibitor cytochalasin B, which interferes with the formation of actin filaments. In these studies, the fusion of myoblasts in culture was severely limited in the presence of cytochalasin B, and most myotubes contained only two nuclei (Sanger et al., 1971; Sanger and Holtzer, 1972). More recent studies have confirmed that both cytochalasin B and D inhibit myoblast fusion and correlate the lack of fusion with the disruption of actin filaments (Constantin et al., 1995). While the role of the cytoskeleton at this early stage of myoblast fusion remains unclear, it may be related to the formation of lipid-rich domains within the cell membrane. Just before fusion, for example, vertebrate myoblasts have been shown to undergo a topological change that results in the creation of protein-depleted, lipid-enriched membrane domains (Kalderon and Gilula, 1979; Fulton et al., 1981). These lipid-rich domains are believed to be associated with an increase in membrane fluidity (for review see Wakelam, 1985) and may create sites for membrane–membrane fusion. Thus, subcellular structures that organize these lipid-rich domains may be dependent on cytoskeletal rearrangements.

Herein we describe the isolation and characterization of the *mbc* gene. MBC is one of the first proteins identified in *Drosophila* that is essential for myoblast fusion. It is expressed in a broad range of tissues throughout embryonic development, including the presumptive musculature and epidermal cells involved in the process of dorsal closure. Consistent with its expression pattern, *mbc* mutant embryos exhibit defects in dorsal closure and cytoskeletal organization as well as myoblast fusion. These abnormalities are similar to those described above for the small GTPase Drac1, and suggest that (*a*) *mbc* functions in the same pathway as *Drac1* in the epidermis and (*b*) this pathway is used in the mesoderm for events leading to myoblast fusion. MBC has striking homology to DOCK180, a human gene that was identified on the basis of interaction with the small adapter protein Crk. DOCK180 may be involved in signal transduction from focal adhesions, and results reported herein are consistent with a similar function for MBC. Finally, open reading frames (ORFs) from several genome projects suggest that DOCK180 and MBC define a new gene family.

1. *Abbreviations used in this paper*: *mbc*, *myoblast city*; MHC, myosin heavy chain; *nau*, *nautilus*; nts, nucleotides; ORF, open reading frame.

Materials and Methods

Drosophila Stocks

All stocks were grown on standard cornmeal medium at 18 or 25°C, as necessary. Balancer and marked chromosomes are described in FlyBase (http://cbbridges.harvard.edu:7081). *Df(3R)mbc-30* has been described (Rushton et al., 1995). *Df(3R)mbc-15A* was created by treating males homozygous for *P{ry*$^+$*, lacZ}*A189.2F3 (Bloomington Stock Center) with 4,000 rads of γ-rays. Approximately 25,000 chromosomes were screened for the loss of the *ry*$^+$ marker. Three deficiencies, including *Df(3R)mbc-15A*, were recovered.

Df(3R)CA15 and *Df(3R)CA2* were obtained by imprecise excision of the homozygous lethal P-element insertion *l(3)04684* (Bloomington Stock Center, Bloomington, IN). Mobilization occurred in flies carrying *l(3)04684* over *Sb*, *Delta2-3 ry*, and excision events were recovered over MKRS or TM2. 677 excision events were analyzed. 330 imprecise excision events were identified by lack of complementation of *Df(3R)mbc-F5.3*/TM3 (see Fig. 1). These were subsequently reevaluated for lack of complementation of *l(3)95BCd* and *l(3)01152* and complementation of *mbc*^{S4}. Four deficiencies were obtained, two of which are shown in Fig. 1.

EMS mutageneses to obtain alleles of *mbc* have been described (Rushton et al., 1995). In similar screens, ~11,000 additional chromosomes were analyzed, and 14 new *mbc* alleles were obtained (Fig. 1). *mbc*$^{F5.3}$ was later found to be a small deletion. Additional mutations in this region identify other lethal complementation groups. A subset of these are shown in Fig. 1.

Enriched DNA and Southern Analysis

DNA enriched for the mutant chromosomes was obtained by mating heterozygous (*mbc*/+) males and females, and collecting the homozygous mutant embryos. Genomic DNA was prepared from unhatched embryos according to Jowett (1986).

Approximately 10 μg of DNA was digested with EcoRI and BamHI, separated on an 0.8% agarose gel, and blotted using the TurboBlotter Rapid Downward Transfer System (Schleicher and Schuell, Inc., Keene, NH). Blots were probed with various genomic fragments from region 95A-C and, as a control for loading, a 2-kb HindIII fragment that includes exons 2, 3a, and 3b from the gene encoding myosin heavy chain (MHC) (Wassenberg et al., 1987). All probes were labeled by random priming (Feinberg and Vogelstein, 1983).

Library Screens, Northern Blots, and DNA Sequencing

Cosmids containing genomic DNA in cytological region 95A-C were obtained from the European *Drosophila* Genome Project (EDGP). The P1 clone was isolated by the Berkeley *Drosophila* Genome Project (BDGP) and provided by A. Spradling. Bacteriophage lambda clones were isolated from a *Drosophila* genomic library in Charon 4 (Maniatis et al., 1978). Fragments were subcloned and analyzed using Southern blots of DNA enriched for the deficiency chromosomes (see above). All DNA between the distal breakpoint of *Df(3R)CA15* and the distal breakpoint of *Df(3R)mbc-15A* was recovered. Genomic fragments containing coding sequence were identified by probing Northern blots (Sambrook et al., 1989).

Subclones of genomic DNA that detected transcribed sequences were used to screen an embryonic 9–12-h cDNA library (Zinn et al., 1988), and several independent cDNA clones were obtained. These included Z5 (nucleotides [nts] 1–2854), Z1.2 (nts 597–1928), Z10b (nts 4184–7040), and

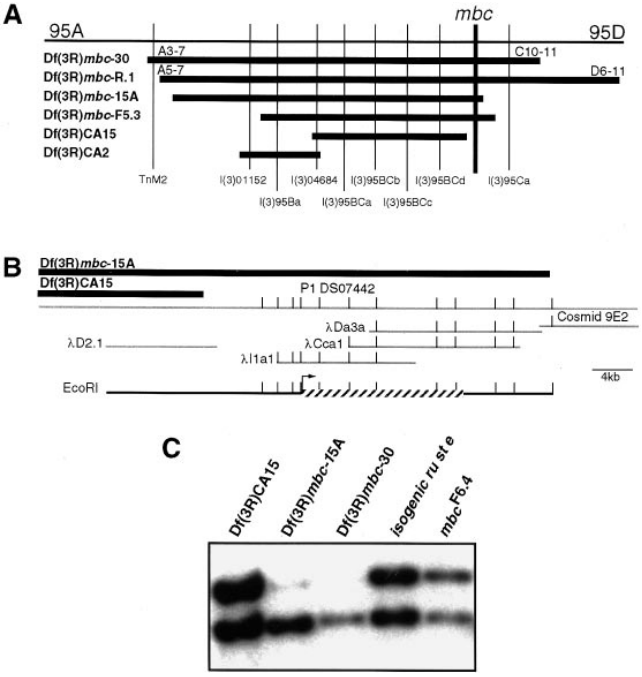


Figure 1. Genetic and molecular map of cytological region 95BC. (*A*) Deficiencies are represented by horizontal bars and lethal complementation groups by vertical lines. Groups *l(3)95BCa-d* have not been oriented with respect to each other. (*B*) Molecular map of the region between the distal breakpoints of *Df(3R)CA15* and *Df(3R)mbc-15A*, indicating P1, cosmid, and bacteriophage lambda clones (*gray lines*). *Eco*RI sites are indicated. The location of the *mbc* gene is indicated by a hatched bar, and the direction of transcription is marked by the arrow. (*C*) A Southern blot of *Eco*RI/*Bam*HI-digested DNA that was enriched for various deficiency chromosomes, including isogenic *ru st e* and $mbc^{F6.4}$ as controls. The test probe is a 2.8-kb *Eco*RI fragment from P1 clone DS07442 (*upper band*) while the control probe is a fragment from MHC (*lower band*).

Z1.1 and Z6 (nts 6366–7376). DNA fragments from these phages were subcloned into bacterial vectors and sequenced by the Penn State Nucleic Acid Facility. Coding sequence not covered by cDNA clones was obtained from cDNA primed from embryonic RNA and amplified by PCR. In all cases, *mbc* coding sequence was determined from both DNA strands.

Mutation Detection and Identification

Total RNA was prepared from *mbc*/isogenic *ru st e* adults. cDNA was synthesized with Superscript II (GIBCO BRL, Gaithersburg, MD) from various *mbc*-specific primers. Resulting cDNAs were used to localize mutations within the *mbc* transcript using the Non-Isotopic RNase Cleavage Assay (NIRCA) of the Mismatch Detect II_{TM} kit (Ambion, Inc., Austin, TX). The mutation in allele $mbc^{F6.4}$ was detected by a Southern blot of heterozygous mutant DNA digested with EcoRI and BamHI, as described above. The probe was a 6-kb genomic fragment corresponding to the middle of the transcript. Mutant sequences uncovered by either of these analyses were analyzed in DNA enriched for the *mbc* alleles. Appropriate regions were amplified by PCR and sequenced by the Penn State Nucleic Acid Facility.

Analysis of Maternal and Adult mRNA

Total RNA was prepared from unfertilized eggs, adult males, and adult females (Jowett, 1986). cDNA was synthesized from a primer in the 3′ untranslated region of the *mbc* gene, and the region between nucleotides 4563 and 5506 was amplified by PCR. Since this region of the *mbc* transcript spans an intron, contaminating genomic DNA does not give rise to a PCR product. This result was confirmed by amplification of a second region between nucleotides 622 and 1515 (data not shown). To ensure that approximately equal amounts of total RNA were present in each sample, a Northern blot of the original RNA was probed with *α1-tubulin* (Theurkauf et al., 1986) (data not shown).

Whole Mount Embryo Analysis

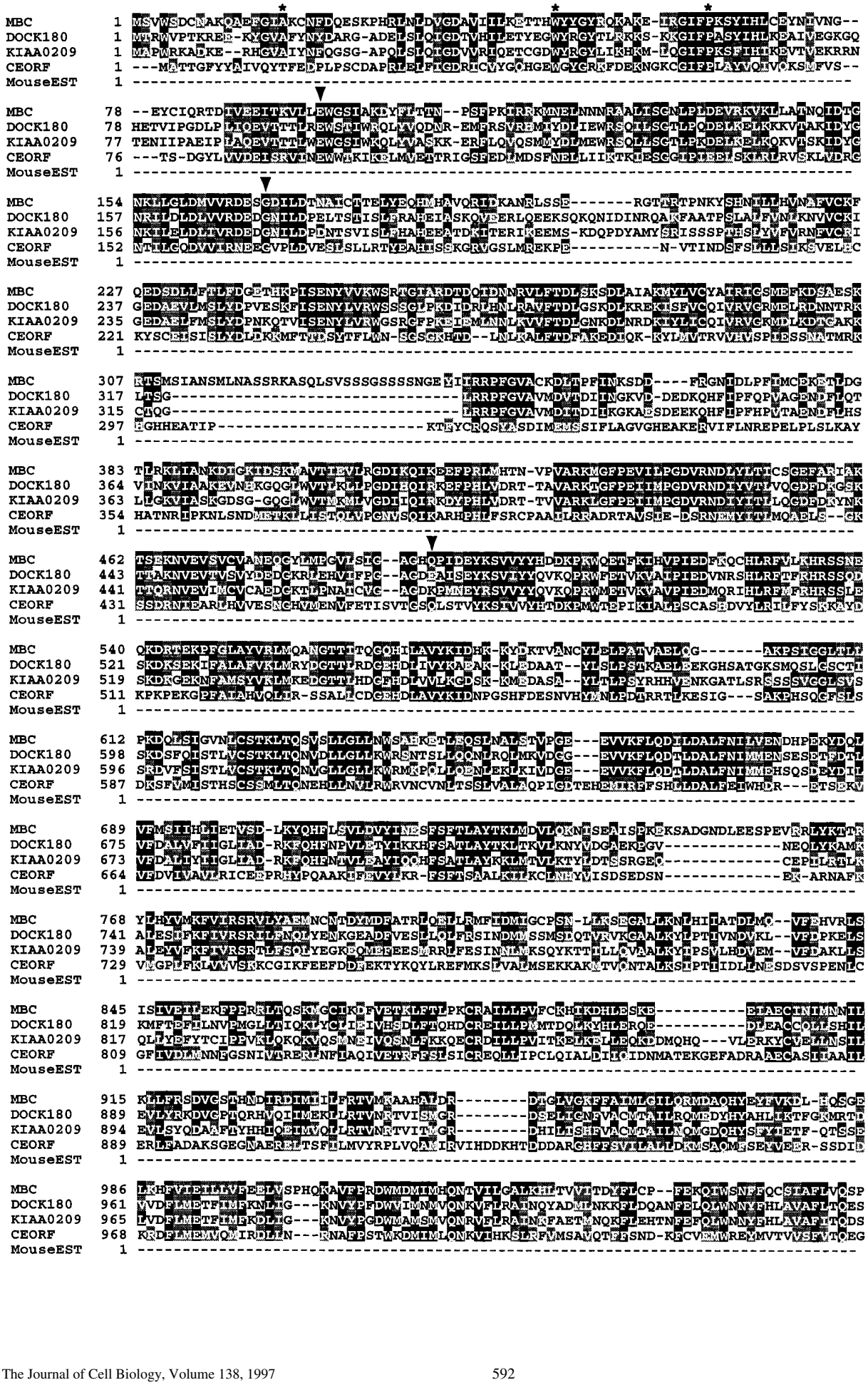
Embryos were collected on apple juice/agar plates for 0–6 h and aged as necessary. The embryonic expression pattern of *mbc* mRNA was determined as described (Tautz and Pfeifle, 1989; Michelson et al., 1990) using a digoxigenin-labeled cDNA fragment (nts 602–1922). *mbc*-encoded protein was analyzed using a polyclonal rat antiserum (Cocalico Biologicals, Reamstown, PA) that was directed against a 286–amino acid fusion protein. It included amino acids 1717–1970 from the COOH-terminal portion of MBC and was purified from inclusion bodies. Before use, the antiserum was affinity-purified against the original antigen coupled to Affigel 15 (BioRad Labs, Hercules, CA). For confocal studies, anti-MBC was used at a dilution of 1:50, rabbit anti-MEF2 was used at 1:1,000 (Bour et al., 1995), and monoclonal antiphosphotyrosine (Upstate Biotechnology, Inc., Lake Placid, NY) was used at a dilution of 1:100. For detection, fluorescein-conjugated goat anti–rabbit antiserum (Vector Laboratories, Burlingame, CA), fluorescein-conjugated goat anti–mouse antiserum (Rockland Inc., Gilbertsville, PA), and CY-3–conjugated goat anti–rat antiserum (Rockland Inc.) were used, as appropriate. All were preadsorbed overnight on 0–12-h embryos before use. Colorimetric immunohistochemistry used a monoclonal anti-MHC antibody (D. Keihart) at a dilution of 1:2,000 and an anti–Fasciclin III monoclonal supernatant (Patel et al., 1987) at a dilution of 1:10. These were detected with biotinylated antimouse antiserum and the Vectastain ABC kit (Vector Laboratories). Where necessary, balancer chromosomes were identified by β-galactosidase activity (Klambt et al., 1991) or by colorimetric immunohistochemistry using a mouse monoclonal anti–β-galactosidase antibody (Promega Corp., Madison, WI) at a dilution of 1:1,000. Staining with Texas red–conjugated phalloidin (Molecular Probes, Inc., Eugene, OR) was as described (Ashburner, 1989*b*). Four independent experiments were conducted. In two of these studies, wild-type and mutant embryos were treated in parallel throughout the entire analysis but kept in separate tubes. In two subsequent experiments, embryos from a wild-type stock and a balanced *mbc* mutant stock were pooled. As in the first two experiments, balancer-containing embryos from the mutant stock were identified using anti–β-galactosidase. An average of 30 unstained embryos were mounted and analyzed in each experiment. The results of all four experiments gave comparable results. In the phosphotyrosine experiments, a total of 30 embryos were analyzed in two independent experiments carried out in parallel in separate tubes.

Results

Genetic Localization of mbc

myoblast city was originally identified in a genetic analysis of cytological region 95, on the right arm of the third chromosome (Rushton et al., 1995). Since this is the location of *nautilus* (*nau*), the *Drosophila* homologue of a conserved family of myogenic regulatory genes (Michelson et al., 1990; Paterson et al., 1991), it was of interest to examine genetic lesions in this region for defects in myogenesis. Alleles of *mbc* were revealed in this analysis since embryos mutant for *mbc* are characterized by an absence of differentiated muscle fibers and the presence of a correspondingly large number of unfused myoblasts (Rushton et al., 1995). Overlapping deficiencies and EMS-induced point mutations were therefore generated to refine the location of *mbc* and establish that it represents a novel gene, independent and separate from *nau.*

The current genetic map of this region is shown in Fig. 1. *Df(3R)mbc-30* has been described (Rushton et al., 1995). *Df(3R)mbc-15A* was generated by γ-irradiation of a homozygous viable P-element insertion in this region.


MBC
DOCK180
KIAA0209
CEORF
MouseEST

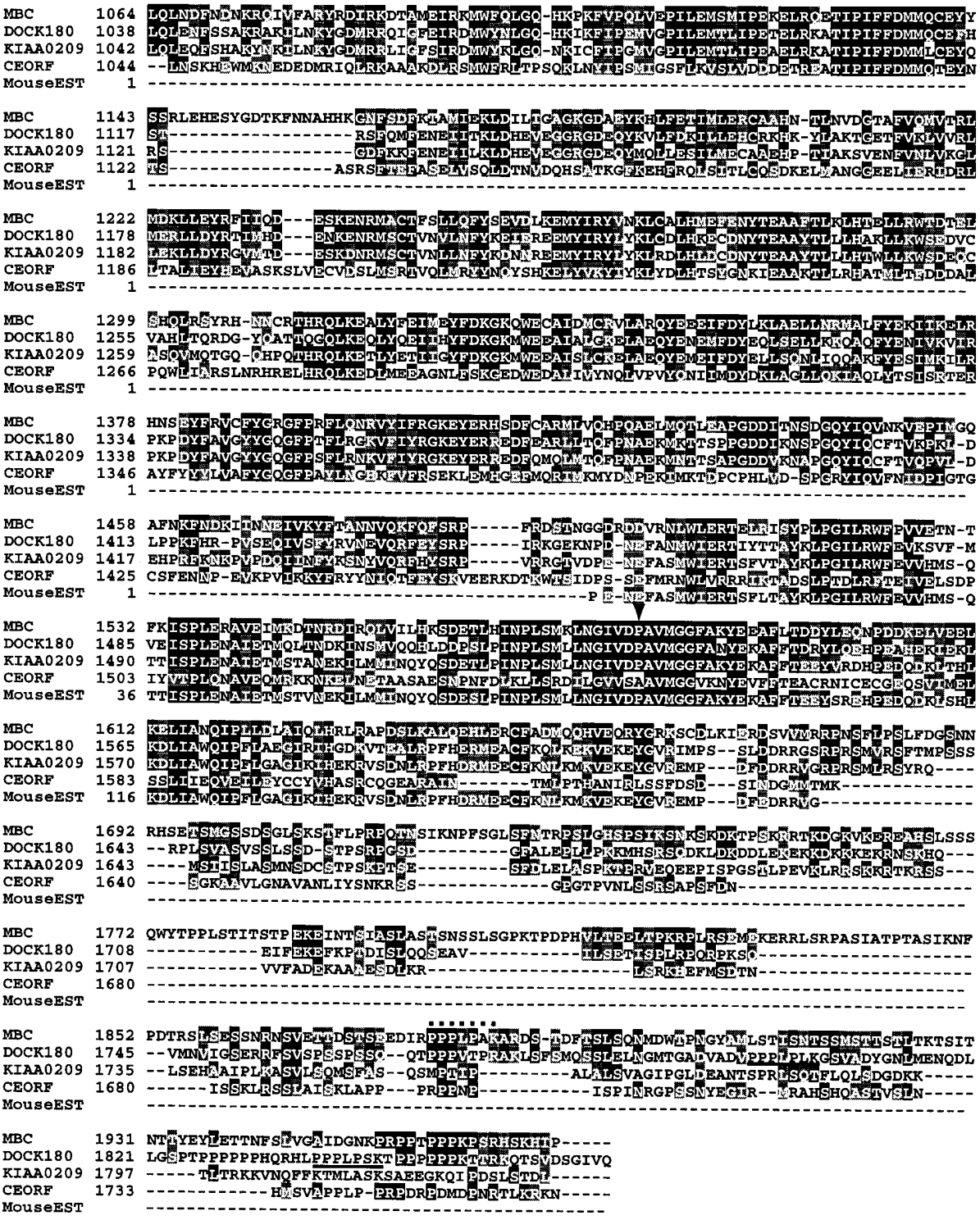


Figure 2. MBC sequence and alignment with human DOCK180 as well as related open reading frames. The alignment was done using Clustal W and presented using Boxshade. Black boxes indicate amino acid identity, while gray boxes indicate amino acid similarity to MBC. Arrowheads highlight mutations found in *mbc* alleles. The consensus Crk-binding sites (PPxLPxK) of DOCK180 are underlined. A potential Crk-binding site in MBC is noted with dots. Stars mark essential SH3 consensus residues (Musacchio et al., 1994). The GenBank/EMBL/DDBJ accession numbers are D86964 for the myeloblast-specific cDNA KIAA0209, Z81032 and Z81054 for the *C. elegans* ORFs, and AA110899 for the mouse expressed sequence tag. *mbc* sequence data are available from GenBank/EMBL/DDBJ under accession number AF007805.

Df(3R)CA15 and *Df(3R)CA2* were isolated by imprecise excision of *l(3)04684*, a homozygous-lethal P-element insertion. *Df(3R)CA15* deletes from the P-element insertion toward the distal end of the chromosome. In contrast, *Df(3R)CA2* deletes from the P-element toward the centromere. These deficiencies have been examined for the presence or absence of *nau* sequences by Southern analysis of DNA from embryos homozygous for the deficiencies. By comparison to *Df(3R)mbc-30* and *Df(3R)mbc-15A*, which completely remove *nau*, neither *Df(3R)CA15* nor *Df(3R)CA2* appear to remove any known *nau* sequences (data not shown). Consistent with this genetic map, recent results have established that *nau* is located in the region centromeric to the proximal breakpoint of *Df(3R)CA2* (Keller, C.A., and S.M. Abmayr, unpublished results). *Df(3R)mbc-F5.3* actually represents an EMS-induced deletion. It does not complement *mbc*, as shown in Fig. 1, but contains all known *nau* sequences (data not shown). Finally, EMS-induced point mutations reveal several additional complementation groups in this region. Other than the transposable element insert TnM2, only those groups distal to the *Df(3R)CA2* breakpoint are shown in Fig. 1.

These deficiencies refined the location of *mbc* to the region between the distal breakpoint of *Df(3R)CA15* and the distal breakpoint of *Df(3R)mbc-15A* and facilitated the cloning of the *mbc* gene. Of note, several attempts to isolate a P-element insertion in *mbc* were unsuccessful (data not shown). Therefore, a molecular walk through this region was initiated using DNA fragments isolated from P1 clones and cosmids that have been mapped to region 95BC. Fragments within this genetic interval were identified by Southern analysis of DNA from embryos homozygous for the various deficiency chromosomes. A representative example is shown in Fig. 1 *C*. The entire region between these deficiency breakpoints is diagrammed in Fig. 1 *B* and spans ~34 kb of DNA. As indicated, the organization of these fragments has been confirmed by isolation of bacteriophage lambda clones containing *Drosophila* genomic DNA.

Identification of the mbc Gene

A single full-length transcript of ~7.5 kb was detected by Northern analysis throughout development using cloned DNA fragments within the 34-kb region described above. Although full-length clones were not obtained, several small overlapping cDNA clones provided most of the coding sequence of this transcript. The sequence of a small region not covered in the cDNA clones was obtained from embryonic mRNA by reverse transcriptase PCR amplification and sequencing. The embryonic transcript is ~7.4 kb, with a coding sequence of 5,910 nts. Untranslated regions at the 5′ and 3′ ends of the isolated cDNAs are 560 and 906 bp, respectively. While the genomic organization of *mbc* has not been analyzed completely, a minimum of eight introns have been identified in a genomic region that spans at least 16 kb. The cDNA sequence has been submitted to GenBank and is not reproduced herein. The deduced amino acid sequence is shown in Fig. 2.

To confirm that this transcript encodes the *mbc* gene, EMS-induced alleles of *mbc* were analyzed for sequence alterations. To date, 18 independent alleles of *mbc* have been generated. Four of these have been described previously (Rushton et al., 1995), while the remaining 14 were generated as part of this analysis. Southern analysis of all alleles was performed to reveal visible rearrangements induced by the chemical mutagen. This analysis uncovered a novel band in an EcoRI/BamHI double digest of DNA from $mbc^{F6.4}$ (data not shown). Sequence analysis confirmed that the BamHI site had been destroyed by a C to T transition. This missense mutation at amino acid 1579 of the coding sequence changes a proline to a leucine in a conserved region of the protein (Fig. 2, *arrowhead*).

Additional aberrations were uncovered using a procedure for detecting point mutations that is based on the ability of RNase A to cleave at single base pair mismatches. Several regions of the coding sequence were analyzed by this method and apparent alterations in the candidate sequence were found in three EMS-induced *mbc* alleles (data not shown). Direct sequencing of these alleles revealed that all were GC to AT transitions at single nucleotides, consistent with the most common form of EMS-induced mutations (Ashburner, 1989*a*). These changes resulted in nonsense mutations at amino acid 492 in $mbc^{F12.7}$ and at amino acid 97 in $mbc^{D11.2}$. By comparison, $mbc^{I6.6}$ is a missense mutation at amino acid 168 where a glycine has been replaced by a glutamic acid (Fig. 2).

Structural Homologues of MBC

The size of the *mbc*-encoded protein is 1,970 amino acids, with a predicted molecular mass of about 226 kD. Database homology comparisons using BLAST (Altschul et al., 1990) aligned the MBC protein with DOCK180, a human protein of 1,866 amino acids, with a predicted molecular mass of 215 kD (Hasegawa et al., 1996). DOCK180 was isolated on the basis of an interaction with Crk, a small adapter protein consisting mainly of SH2 and SH3 domains (Reichman et al., 1992; see Discussion). MBC and DOCK180 have significant homology throughout their entire length. In particular, DOCK180 contains a putative SH3 domain that proceeds from amino acids 11–71 and includes the three essential SH3 consensus residues (Musacchio et al., 1994). These three residues, along with several others within this domain, are identical in MBC. DOCK180 contains two copies of the Crk-binding consensus site PPxLPxK (Knudsen et al., 1994; Matsuda et al., 1996), while MBC has one exact and one slightly divergent copy of this consensus site (Fig. 2). By contrast, the putative ATP-binding site noted by Hasegawa et al. (1996) is not conserved. Several additional blocks of homology are present, notably a region in which 24 of 27 amino acids are identical (residues 1566–1592 of MBC). A lesion in the central proline of this block, identified above in $mbc^{F6.4}$, resulted in a mutant phenotype identical to that previously described (Rushton et al., 1995). This apparent loss-of-function phenotype is identical to that found in alleles with a severely truncated protein (e.g., $mbc^{D11.2}$; data not shown).

Subsequent BLAST searches also revealed two ORFs with extensive homology to MBC and DOCK180. The first ORF is from a human myeloid cell line, and the second is from the *Caenorhabditis elegans* genome project. The predicted myeloblast protein is highly homologous to

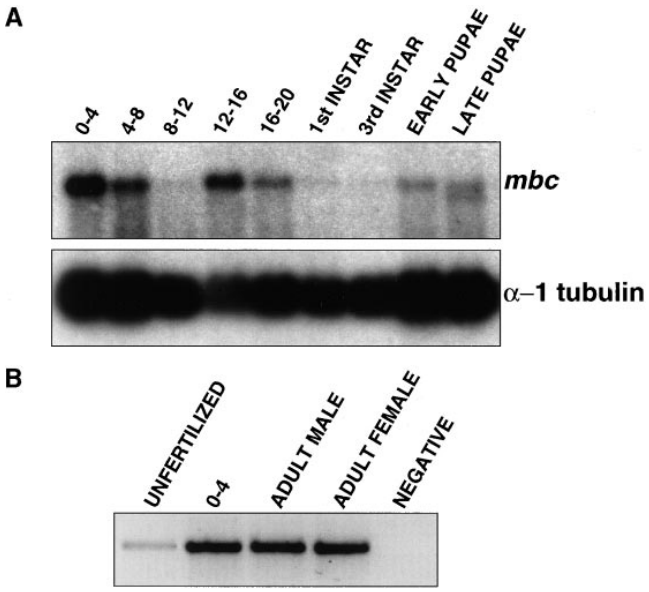


Figure 3. Temporal expression of the *mbc* transcript. (*A*) A Northern blot containing 4 μg of poly A^+ RNA from several stages of development. Probes included a 1.8-kb genomic EcoRI fragment that includes *mbc* coding sequence and a 400-bp Xba/HindIII fragment of *α1-tubulin* (Theurkauf et al., 1986) as a control for loading. (*B*) Reverse transcriptase PCR analysis of *mbc* RNA from unfertilized embryos. 0–4-h embryos, adult females, and adult males as detailed in Materials and Methods.

both MBC and DOCK180, while the predicted *C. elegans* protein is more divergent. Partial sequence from a mouse expressed sequence tag suggests the existence of a murine homologue as well.

Temporal Expression of mbc

Northern analysis revealed that *mbc* is expressed early in development, in embryos ~0–4 h after egg laying. *mbc* transcript levels remain relatively high during embryogenesis, with the possible exception of a decline from 8–12 h that may be, in part, an artifact of slightly degraded mRNA (Fig. 3 *A*). Expression was not evident during larval stages, but the transcript does reappear during pupation, suggesting a possible role in adult development. A form of *mbc* with slightly altered mobility appears late in metamorphosis. This transcript may reflect alternative splicing and is under further investigation (Fig. 3 *A*, lane *9*). PCR amplification of two different regions from the mRNA of unfertilized embryos revealed a small but detectable signal, and suggested that the transcript is maternally provided. Finally, the transcript was expressed in adult males and females, as evidenced by PCR analysis of cDNA (Fig. 3 *B*).

Spatial Expression Pattern of mbc mRNA and Protein during Embryogenesis

The earliest expression of the *mbc* transcript is in the pole

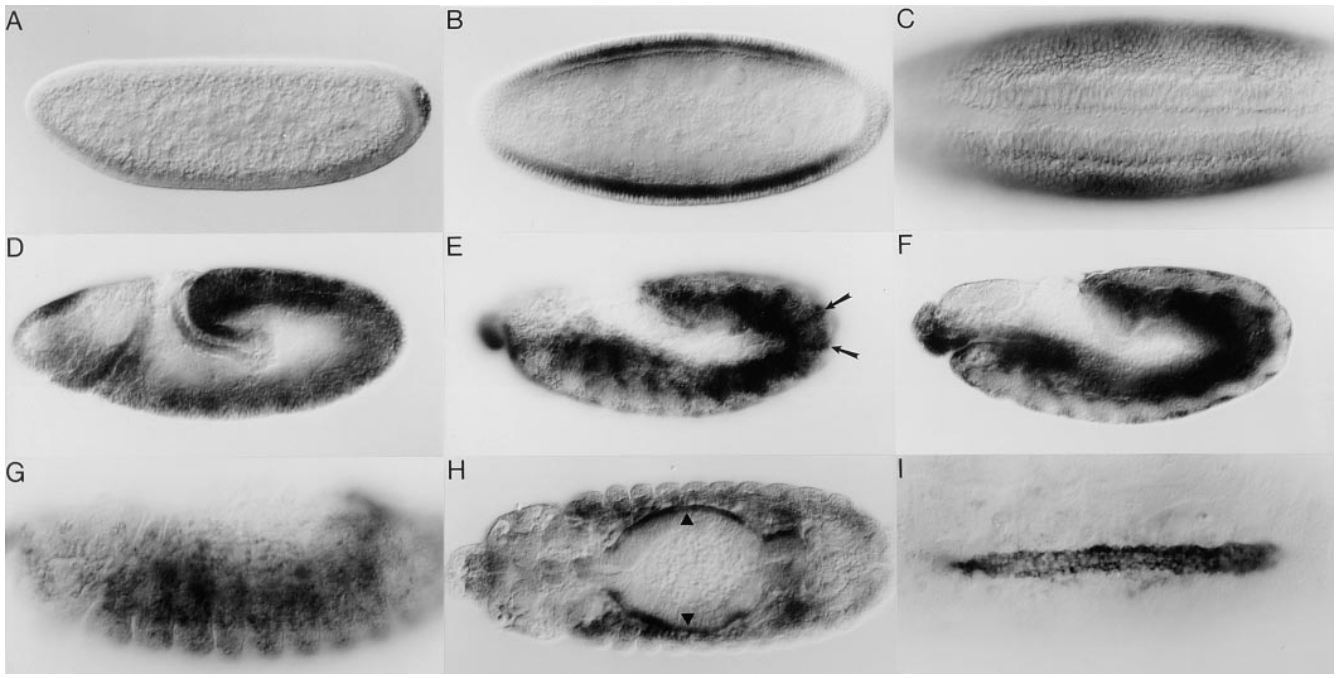


Figure 4. Spatial expression pattern of *mbc* mRNA in *wild-type* embryos. In all panels, anterior is to the left. In *A*, *D*, *E*, *F*, and *G*, dorsal is at the top. (*A*) Lateral view, early stage 4, before cellularization. (*B*) Dorsal view, stage 5. (*C*) Ventral view, stage 6; the invaginating ventral furrow is evident. (*D*) Lateral view, stage 9. (*E*) Lateral view of the ectoderm, late stage 12; arrows highlight ectodermal stripes. (*F*) Lateral view focusing on the mesoderm and endoderm of the same embryo as in *E.* (*G*) Lateral view, stage 14; focusing on mesodermal cells. (*H*) Dorsal view, stage 14; arrowheads indicate the visceral musculature. (*I*) Dorsal view; stage 16; expression is evident in the cardial and pericardial cells of the dorsal vessel.

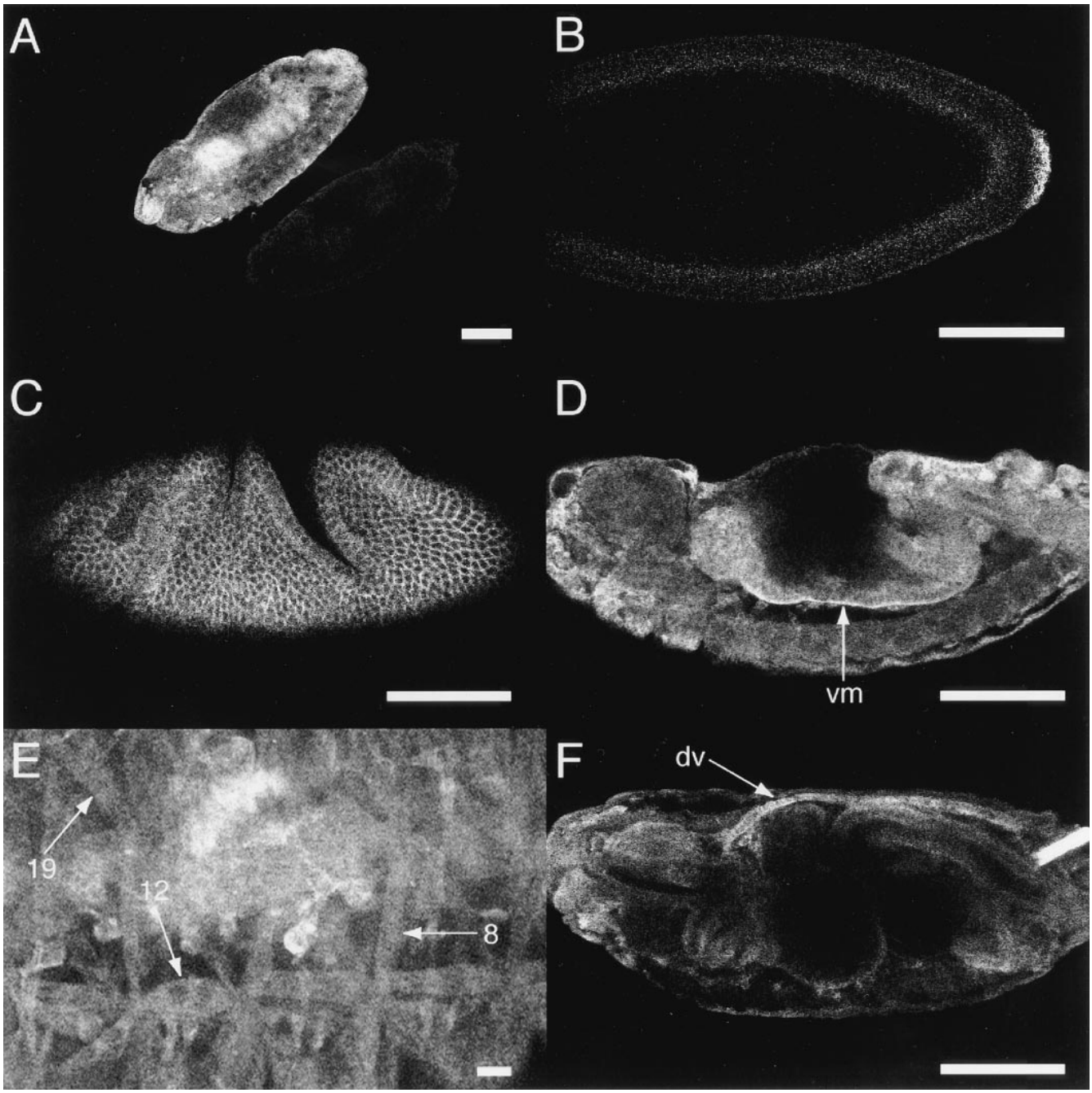


Figure 5. Spatial expression pattern of MBC in *wild-type* embryos. Anterior is to the left and dorsal to the top in all except *A*. (*A*) Stage 13 embryos from the progeny of *mbc*$^{D11.2}$/TM3 *lacZ-hg* stained immunohistochemically for MBC. The embryo to the top left expressed β-galactosidase and therefore carried TM3 *lacZ-hg*; the embryo to the bottom right (which is barely visible) did not express β-galactosidase (data not shown) and was therefore homozygous for *mbc*$^{D11.2}$. As anticipated, no MBC expression is visible in the homozygous mutant embryo, establishing specificity of the antiserum. (*B*) Wild-type; Lateral view, stage 5. (*C*) Wild-type; Lateral view, stage 8. (*D*) Wild-type; Lateral view, stage 14; arrow indicates the visceral musculature (*vm*). (*E* and *F*) Wild-type; Lateral views, stage 16; arrows in *E* highlight somatic muscles 8, 12, and 19, using the nomenclature of Crossley (1978). Arrow in *F* marks the dorsal vessel (*dv*). Bars: (*A–D* and *F*) 100 μm; (*E*) 10 μm.

cells (Fig. 4 *A*). It is later found in lateral portions of the embryo during cellularization (Fig. 4 *B*) but is not evident at the termini. Surprisingly, the ventral furrow, which will invaginate during gastrulation to form the mesoderm, shows no expression at this time (Fig. 4 *C*). At germband elongation, expression is still quite strong in the ectoderm (Fig. 4 *D*). By late stage 12, the mRNA appears to be decreasing in the ectoderm, leaving a pattern of stripes (Fig. 4 *E*, *arrows*). *mbc* is expressed in both the mesoderm and endoderm during stage 12 (Fig. 4 *F*). Expression decreases in both the epidermal layer and the somatic mesoderm during stage 14 (Fig. 4 *G*) but remains strong in the vis-

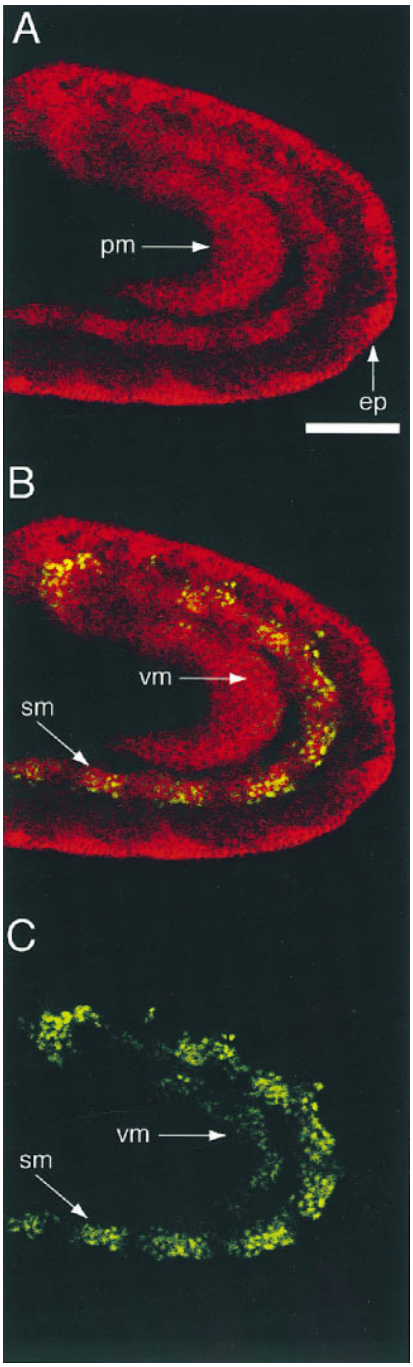


Figure 6. Colocalization of MBC with MEF2 in myoblasts. Using fluorescent immunohistochemistry and confocal microscopy, embryos were analyzed for expression of MBC (*red*) and MEF-2 (*green*). The posterior end of an embryo late in stage 12 that is oriented anterior to the left and dorsal to the top is shown. (*A*) MBC expression. Arrows indicate the posterior midgut primordium (*pm*) and the epidermis (*ep*). (*B*) A composite of *A* and *C*, illustrating colocalization in the somatic mesoderm (*sm*) and the visceral mesoderm (*vm*). (*C*) MEF-2 expression. Bar, 50 μm.

ceral musculature (Fig. 4 *H*, *arrowheads*). Examination of a stage 16 embryo revealed mRNA in both the cardial and pericardial cells of the dorsal vessel (Fig. 4 *I*). Of note, the *mbc* transcript is not observed in mature muscle fibers.

The expression pattern of MBC was analyzed by fluorescent immunohistochemistry and confocal microscopy using an antiserum directed against the COOH-terminal portion of the protein. Examination of embryos homozygous for $mbc^{D11.2}$ confirmed that the antiserum was specific (Fig. 5 *A*) since this allele encodes a severely truncated form of MBC that would not be detected. While slight temporal differences were evident between maximal levels of mRNA (stage 4; Fig. 4 *A*) and maximal levels of protein (stage 5; Fig. 5 *B*) in the pole cells, the expression of the protein essentially correlated with that of the mRNA. MBC appeared to be localized in the cytoplasm (Fig. 5 *C*), consistent with its human counterpart DOCK180. MBC is also present in the visceral musculature (Fig. 5 *D*, *arrow*) and the dorsal vessel (Fig. 5 *F*, *arrow*). Cross reactivity of the MBC antiserum was observed in the filtzkorper (Fig. 5 *F*) but does not correlate with the presence of transcript. Although mRNA was not evident in mature muscles, the protein could be detected at a low level (Fig. 5 *E*).

Fluorescent immunohistochemistry and confocal microscopy were used to confirm that MBC is present in myoblasts. For this analysis, the embryos were hybridized with antibodies to both MBC and MEF2. The *mef2* gene encodes a transcription factor that appears to be expressed throughout the mesoderm, including somatic muscle precursors and all muscle fibers (for review see Olson et al., 1995). Nuclei expressing MEF2 were visualized in a late stage 12 embryo in green (Fig. 6 *C*). By comparison, cytoplasmic expression of MBC was visualized in red (Fig. 6 *A*). As anticipated from the expression pattern of mRNA, MBC is present in ectodermal and endodermal germ layers. Of note, expression in the ectoderm is concentrated in the epidermal layer and appears to be absent from the underlying neuroectoderm. MBC is also clearly present in presumptive myoblasts, coincident with the MEF2-expressing nuclei (Fig. 6 *B*).

Examination of Mesodermal Derivatives in mbc Mutant Embryos

Given the broad expression pattern of *mbc*, it was of interest to examine *mbc* mutant embryos for defects in other tissues. For this purpose we used $mbc^{F12.7}$, since the protein is truncated at amino acid 492, and analyzed embryos that were genetically $mbc^{F12.7}$/*Df(3R)mbc-30.* These embryos exhibited the severe somatic muscle phenotype previously reported (Rushton et al., 1995) and shown in Fig. 7 *B*. By comparison, although the visceral musculature appeared to be present, as evidenced by myosin-staining cells, obvious defects in midgut constriction and orientation were observed in ~25% of the embryos (Fig. 7, *C* and *D*). However, these defects may be an indirect consequence of the lack of somatic muscles rather than a direct effect of the loss of MBC in either the visceral mesoderm or the endoderm. The overall structure of the heart, which expresses MBC late in development, appeared to be normal at this level of analysis (Fig. 7, *E* and *F*).

Examination of Dorsal Closure and Cytoskeletal Organization in the Epidermis

Although no epidermal defects had been reported in *mbc* mutant embryos (Rushton et al., 1995), the early expres-

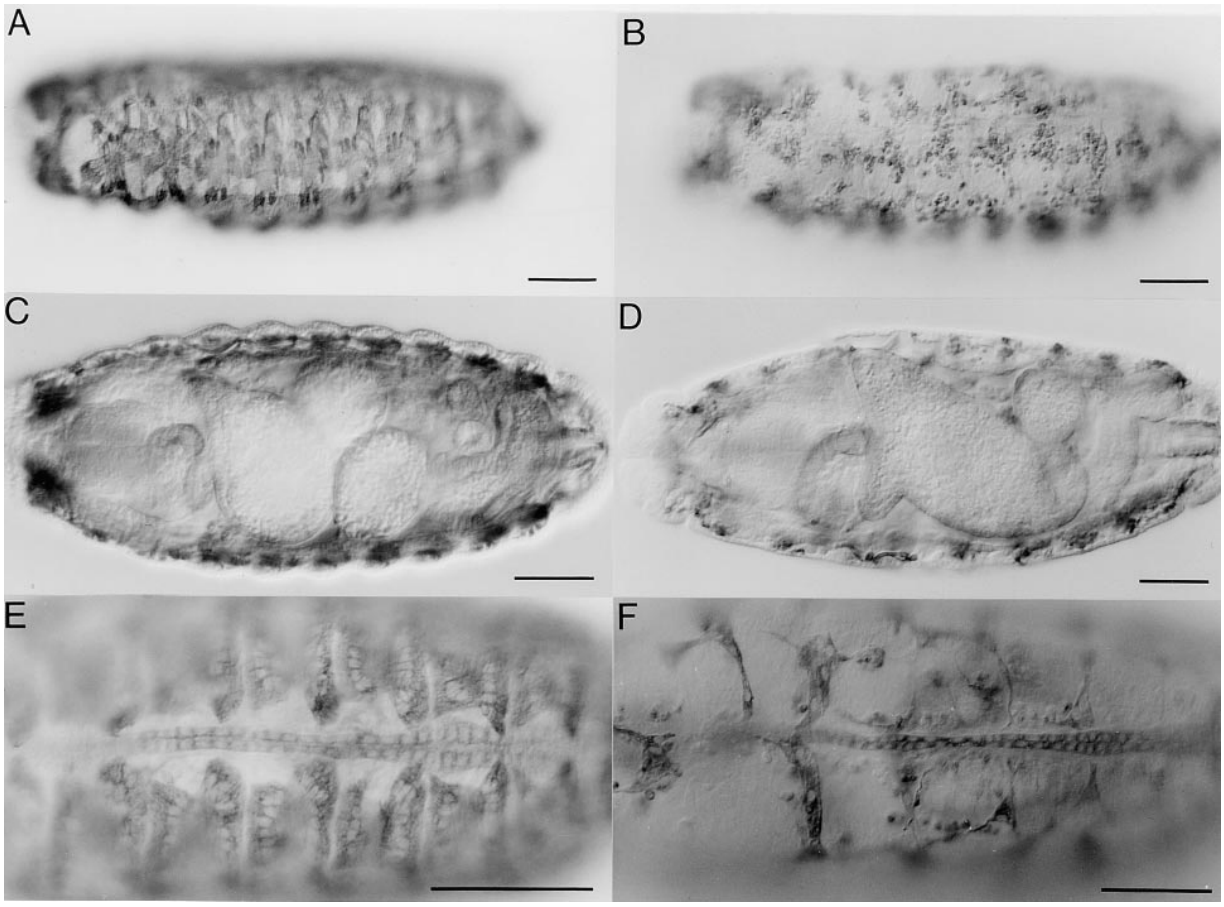


Figure 7. Analysis of mesodermal derivatives in *mbc* mutant embryos. Tissues were visualized with a monoclonal antibody to MHC. All embryos are oriented with anterior to the left. *A* and *B* are lateral views with dorsal to the top, *C* and *D* are ventral views, and *E* and *F* are dorsal views. *A*, *C*, and *E* are wild-type embryos; *B*, *D*, and *F* are $mbc^{F12.7}$/*Df(3R)mbc-30* transheterozygotes. (*A* and *B*) Somatic muscle pattern of stage 16 embryos. Defects in myoblast fusion, as previously described by Rushton et al. (1995), are evident in *B*. (*C* and *D*) Visceral musculature and gut formation in late stage 16 embryos. Note the midgut constrictions in *C* and the absence of these constrictions in *D*. (*E* and *F*) Dorsal vessel of stage 17 embryos. At this level, there are no obvious defects. Bars, 50 μm.

sion of *mbc* in the ectoderm, which persists in the epidermis into stage 14, led us to reexamine *mbc* mutant embryos for epidermal defects. Visualization of the epidermis with an antibody to Fasciclin III, a glycoprotein on the cell surface (Patel et al., 1987), revealed defects in dorsal closure in ~80% of the mutant embryos (Fig. 8, *E* and *F*). The extent of completion of dorsal closure varied from a relatively small opening surrounded by puckered misshapen cells (data not shown) to a very large opening (Fig. 8 *E*). In the normal course of dorsal closure in a wild-type embryo, the epidermal cells elongate as shown in Fig. 8 *B*, and the epithelium stretches over the entire circumference of the embryo (Young et al., 1993). In the early stages of dorsal closure in *mbc* mutant embryos, the cells along the leading edge of the epidermis appeared to be normal (data not shown). As dorsal closure neared completion, however, many cells along the leading edge ceased to be elongated, adopted a rounded shape, and expressed Fasciclin III abnormally along their migrating edge (Fig. 8, *B*, *D*, and *F*).

The cytoskeleton along the leading edge of the epidermis has been implicated in driving the process of dorsal closure (Young et al., 1993). We therefore used fluorescently conjugated phalloidin, which binds filamentous actin, to examine the *mbc* mutants for defects in cytoskeletal formation and organization. Both *wild-type* and *mbc* mutant embryos displayed some variability in the intensity and organization of staining, the range of which is shown in Fig. 9. As shown, the signal in *wild-type* embryos (Fig. 9 *A*, *a* and *c*) was always stronger than that in *mbc* mutant embryos (Fig. 9 *A*, *b* and *d*). While frequently more dramatic in cells along the migrating edge, this reduction in signal was also observed throughout the epidermis, consistent with the observed expression of *mbc.* In addition, it should be noted that ~20% of the *mbc* mutant embryos do not exhibit defects in dorsal closure (mentioned above). One might anticipate that these embryos would express relatively normal levels of filamentous actin and exhibit only mild cytoskeletal defects, such as that shown in Fig. 9 *A*, *b.* In summary, this analysis suggests that there is a modest but reproducible reduction in cytoskeletal organization in the epidermis of *mbc* mutant embryos. Unfortunately, examination of the cytoskeletal structure in muscle cells was complicated by the dynamic nature of *wild-type* muscle cells, making rigorous comparisons with comparable muscle cells in *mbc* mutant embryos difficult.

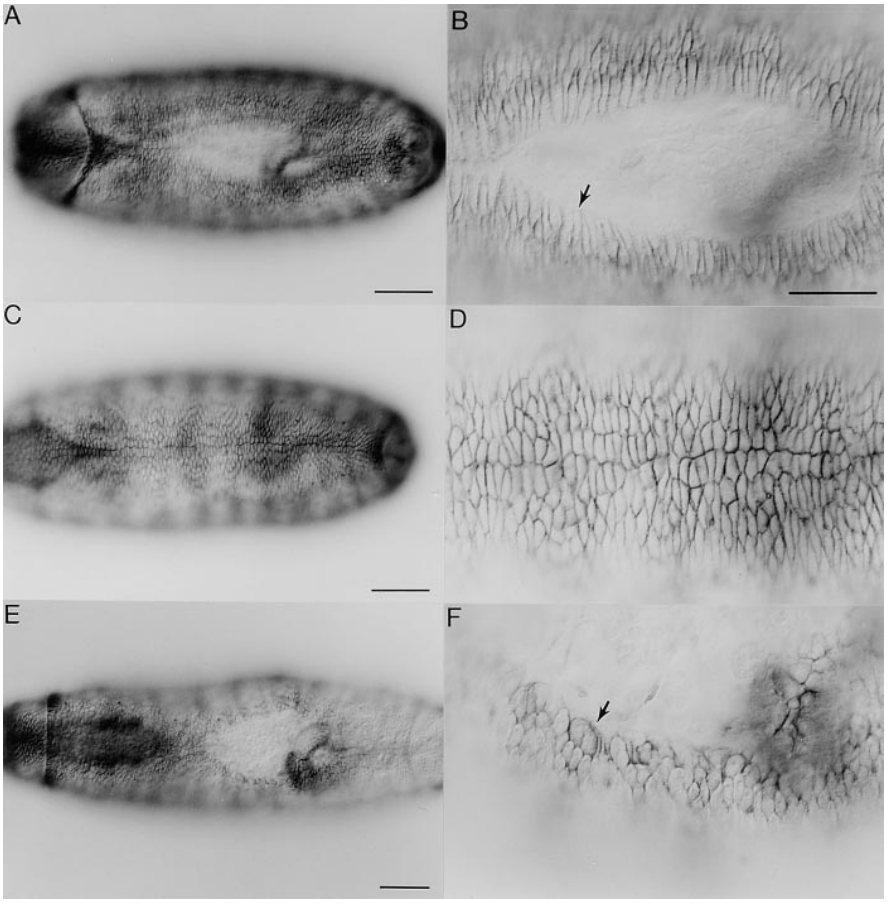


Figure 8. Defects in dorsal closure in *mbc* mutants revealed by staining with Fasciclin III. All panels are dorsal views with anterior to the left. *A–D* are wild-type embryos while *E* and *F* are $mbc^{F12.7}$/*Df(3R)mbc-30* transheterozygotes. *A* and *B* show a stage 15 embryo in the process of dorsal closure. Arrow in *B* denotes elongated cells at the leading edge. *C* and *D* show a stage 16 embryo that has completed dorsal closure. *E* and *F* show a stage 16 embryo that has a pronounced defect in dorsal closure. Arrow denotes cells that are misshapen and have an improper accumulation of Fasciclin III along the leading edge. Bars: (*A*, *C*, and *E*) 50 μm; (*B*, *D*, and *F*) 25 μm.

DOCK180, the apparent human homologue of *mbc*, may be involved in Crk-associated signal transduction from focal adhesions (Hasegawa et al., 1996). If *mbc* functions in a similar signal transduction pathway in *Drosophila*, we anticipated that it would be downstream of focal adhesions. Examination of putative focal adhesions in the epidermis of *mbc* mutant embryos was accomplished using a monoclonal antibody directed against phosphotyrosine, as previously described (Maher et al., 1985; Hanks et al., 1992; Harden et al., 1996). In contrast to the cytoskeletal defects described above, comparison of phosphotyrosine staining patterns in the epidermis of *wild-type* embryos and homozygous $mbc^{D11.2}$ embryos revealed no apparent difference during dorsal closure (Fig. 9 *B*, *a* and *b*). This observation is consistent with the possibility that MBC, like DOCK180, is downstream of phosphotyrosine-containing complexes in a signal transduction pathway that, in *Drosophila*, ultimately affects cell migration, dorsal closure, cytoskeletal organization, and myoblast fusion.

Discussion

The results reported here describe the cloning and characterization of *myoblast city*, a gene that was initially identified on the basis of a defect in myoblast fusion (Rushton et al., 1995). *mbc* encodes a novel *Drosophila* protein with a high degree of homology to the human Crk-associated protein, DOCK180 (Hasegawa et al., 1996). Consistent with that of its human counterpart, *mbc* expression is not restricted to the somatic mesoderm. Early in development, expression is observed in the pole cells and ectoderm but is absent from the mesodermal epithelium. Later in development, expression is most evident in the epidermis and mesoderm but is absent from neural tissues. The latest detectable expression is in mesodermal derivatives that include the heart and visceral musculature. Consistent with this pattern of expression, defects in myoblast fusion are accompanied by abnormalities in the midgut constrictions and in the ability of the epidermal cells to complete dorsal closure. These cells exhibit alterations in shape, migration, and deposition of Fasciclin III, as well as cytoskeletal organization. Previous studies have reported similar defects for Drac1 (Luo et al., 1994; Harden et al., 1995), the *Drosophila* homologue of the small GTPase *rac1*, and imply that *mbc* may function in the same pathway. Finally, ORFs identified from multiple genome sequencing projects may indicate that MBC and DOCK180 are members of a highly conserved gene family.

The Role of mbc in Ectodermally Derived Tissues

Early expression of *mbc* in the ectoderm and its persistence in the epidermis led us to examine *mbc* mutant embryos for epidermal defects. Using Fasciclin III as a marker, we observed that *mbc* mutant embryos were unable to complete the process of dorsal closure. Contractile filaments formed from actin and myosin are thought to provide the driving force for dorsal closure. Consistent with this suggestion, the absence of nonmuscle myosin in *zipper*

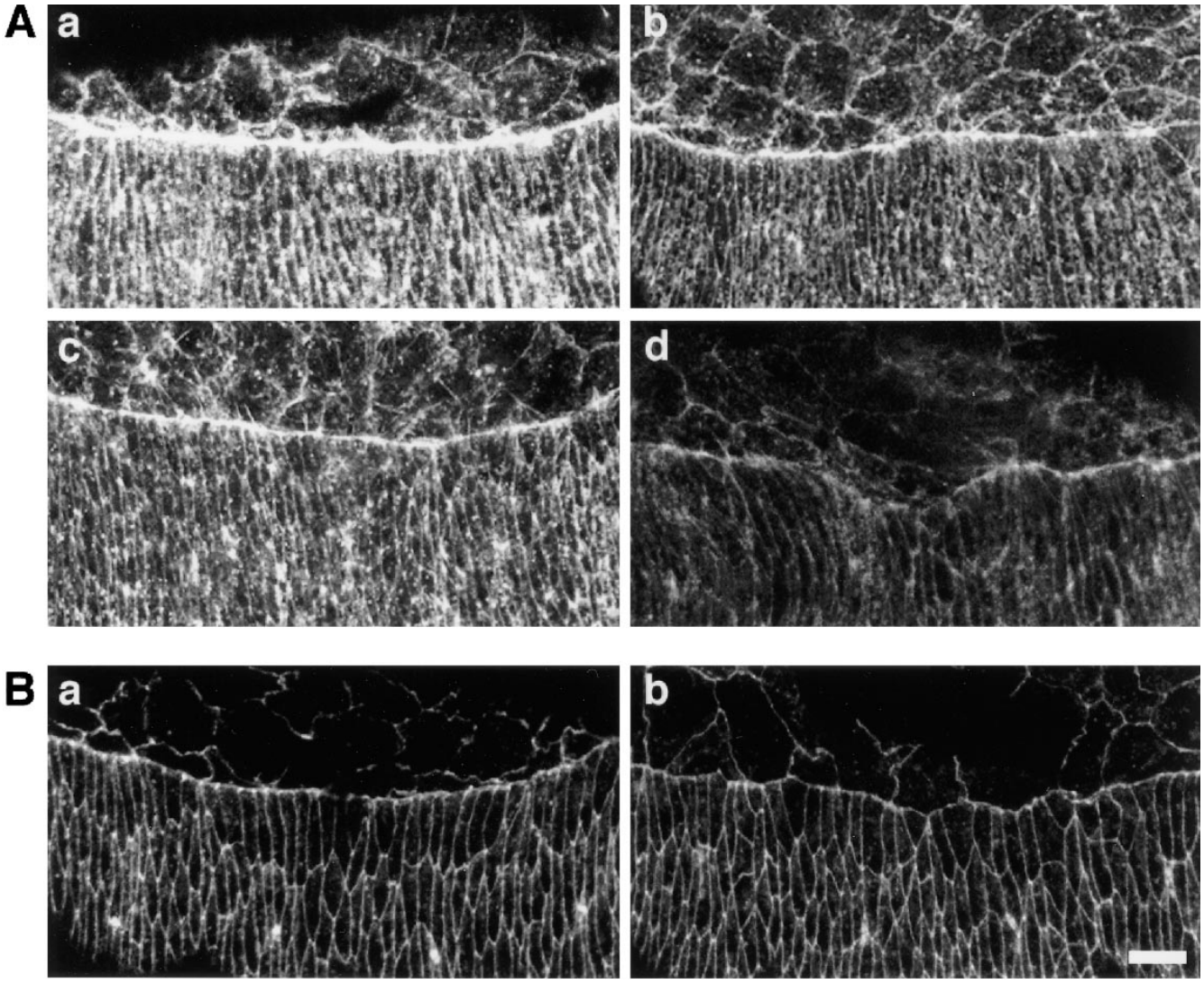


Figure 9. Accumulation of filamentous actin and phosphotyrosine in the epidermis of *mbc* mutant embryos. Confocal micrographs of embryos stained with Texas red–conjugated phalloidin (*A*) or an antiphosphotyrosine antibody (*B*). Anterior is to the left in all panels. *A*, *a* and *c*, and *B*, *a* show stage 14 wild-type embryos. *A*, *b* and *d*, and *B*, *b* show stage 14 $mbc^{D11.2}$ homozygous embryos. Bar, 10 μm.

mutant embryos is likely to be responsible for their failure to complete this process (Young et al., 1993). Similarly, overexpression of a form of Drac1 that disrupts both actin and nonmuscle myosin accumulation at the leading edge of the migrating epidermis also inhibits dorsal closure (Harden et al., 1995). Finally, the dorsal closure defects observed in *mbc* mutants are accompanied by reduced detection of filamentous actin. These results implicate *mbc* in cytoskeletal organization and dorsal closure and suggest that it may function in the same pathway as *Drac1*.

Recent studies have shown that *Drac1* is necessary for the presence of phosphotyrosine-containing complexes at the leading edge of the epidermis that have been suggested as focal adhesions (Harden et al., 1996). The *rho/rac* family of small GTPases has also been implicated in the formation of focal adhesions and in the organization of the actin cytoskeleton in vertebrates (Ridley and Hall, 1992, 1994; Ridley et al., 1992; Nobes and Hall, 1995; Chrzanowska-Wodnicka and Burridge, 1996; for reviews see Clark and Brugge, 1995; Richardson and Parsons, 1995; Takai et al., 1995). The loss of *mbc* does not appear to affect the formation of these phosphotyrosine-containing complexes, implying that *mbc* may function downstream of *Drac1*. This interpretation is consistent with one possible role of human DOCK180 in mediating a signal from focal adhesions to downstream effectors (Hasegawa et al., 1996). Specifically, DOCK180 was isolated on the basis of interaction with the small SH2-SH3 domain-containing adapter protein, Crk (Reichman et al., 1992). Studies addressing the roles of both c-Crk and its oncogenic counterpart v-Crk have suggested an involvement in signal transduction pathways that include receptor tyrosine kinases, *ras* and MAP kinase, and focal adhesions (Tanaka et al., 1993; Feller et al., 1994; Hempstead et al., 1994; Matsuda et al., 1994; Schaller and Parsons, 1994; Clark and Brugge, 1995; Richardson and Parsons, 1995; Hanks and Polte, 1997). Recently, several proteins have been identified on the basis of interaction with Crk and are likely to

be downstream effectors. Among these is the guanine nucleotide exchange factor C3G (Tanaka et al., 1994). Crk may be a critical mediator of signal transduction to events in the nucleus through these molecules. In addition, biochemical evidence has shown that v-Crk and c-Crk can interact with phosphorylated paxillin (Birge et al., 1993; Schaller and Parsons, 1995), one of the components of the focal adhesion (Turner et al., 1990).

It should be noted that although both *mbc* loss-of-function and dominant negative $Drac1^{N17}$ embryos exhibit similar defects in dorsal closure and cytoskeletal organization in the epidermis and fusion of myoblasts in the mesoderm (see below), $Drac1^{N17}$ also induces defects in the peripheral nervous system. In contrast, both the motor neurons and the peripheral nervous system of *mbc* mutant embryos appear to be normal (Rushton et al., 1995; Prokop et al., 1996; Erickson, M.R.S., and S.M. Abmayr, unpublished observation), consistent with the minimal level of MBC expressed in neural tissues. The simplest interpretation of this apparent inconsistency is that particular factors mediate different aspects of the *Drac1* signal transduction cascade. In support of this hypothesis, several different molecules have been identified on the basis of an interaction with vertebrate Crk (Feller et al., 1995, and references therein).

The Role of mbc in Myoblast Fusion

The most apparent mesodermal defect in embryos mutant for the *mbc* gene is an inability of myoblasts to fuse into muscle fibers, suggesting a role for *mbc* in the progression of cells from myoblasts to myotubes. This multistep process has been divided into several stages (Knudsen and Horwitz, 1977, 1978; for reviews see Bischoff, 1978; Wakelam, 1985) and includes the acquisition of fusion competence, a time-dependent behavior that may be related to withdrawal from the cell cycle (Bischoff and Holtzer, 1969; Yaffe, 1971; Holtzer et al., 1975*b*), myoblast adhesion, and plasma membrane union.

Several features of the *mbc*-encoded protein seem somewhat inconsistent with a role in either cell adhesion or membrane fusion itself. First, MBC does not have features reminiscent of cell adhesion molecules and appears to be present throughout the cytoplasm rather than membrane bound. Second, both MBC and its structural homologue, DOCK180 (Hasegawa et al., 1996), are expressed in a wide range of tissues that do not fuse. The potential conservation of MBC and DOCK180 in *C. elegans*, in which the muscle fibers remain mononucleate (Waterston, 1988), is also inconsistent with a direct role for *mbc* in the fusion process. An alternative possibility is that *mbc* functions in myoblast differentiation. As mentioned earlier, DOCK180 was identified and subsequently isolated on the basis of interaction with the adapter protein Crk (Reichman et al., 1992). Studies addressing the roles of both c-Crk and v-Crk have implicated these molecules in cell differentiation (Tanaka et al., 1993; Hempstead et al., 1994). Thus, *mbc* may be essential for a cytoskeleton-related step in differentiation through which, among other things, myoblasts become competent to fuse.

We favor the interpretation that the function of MBC in the mesoderm is analogous to its role in the epidermis and that it functions as an essential intermediate in a signal transduction cascade that also includes the small GTPase Drac1. This pathway could involve tyrosine phosphorylation of complexes that directly modulate events in the cytoskeleton through proteins that include MBC. Alternatively, MBC may function in signal transduction to the nucleus via the *ras* and MAP kinase pathway and may affect the cytoskeleton only indirectly. Interestingly, while vertebrate studies have not revealed a specific requirement for focal adhesions in myogenesis, they have implicated extracellular matrix components that stimulate focal adhesions, such as fibronectin, in myogenic differentiation (Chen, 1977; Furcht et al., 1978; Menko and Boettiger, 1987; Guan and Shalloway, 1992; Hanks et al., 1992; Enomoto et al., 1993). Additional studies in vertebrates support a role for the cytoskeleton in myoblast fusion. As previously described (see introduction), myoblast fusion is severely limited in the presence of cytochalasin B, an alkaloid that interferes with the assembly of actin filaments (Sanger et al., 1971; Sanger and Holtzer, 1972). While the role of the cytoskeleton in myoblast fusion remains unclear, it may be involved in the formation of lipid-rich domains within the cell membrane that create sites for membrane–membrane fusion. Alternatively, actin filaments may be required for the formation or organization of vesicles that have been observed under the plasma membrane just before fusion of both vertebrate and *Drosophila* myoblasts (Doberstein et al., 1997; for review see Kalderon, 1980). Interestingly, these vesicles are not observed in *mbc* mutant embryos, perhaps as a consequence of defects in the actin cytoskeleton (Doberstein et al., 1997).

Additional studies will be necessary to resolve the exact role of *mbc* in myoblast fusion. In particular, whereas focal adhesions in vertebrates are generally thought to mediate interactions between the cell and the extracellular matrix, no cell–matrix interactions have yet been identified in the *Drosophila* mesoderm (Tepass and Hartenstein, 1994). In addition, on the basis of examination of phosphotyrosine-containing complexes, our studies seem most consistent with a role for *mbc* downstream of Drac1 in the epidermis. By comparison, Doberstein et al. (1997) place *mbc* upstream of a constitutively active form of *Drac1*. As discussed by these authors, however, the analysis of *Drac1* is presently limited to targeted expression of altered forms of the protein and is problematic in the absence of a loss-of-function mutation. It may also reflect a second role for *Drac1* in myoblast fusion, not inconsistent with the suggestion that GTPases may act downstream of focal adhesions (Schaller and Parsons, 1994; Clark and Brugge, 1995; Hanks and Polte, 1997). One intriguing possibility consistent with our data and that of Doberstein et al. (1997) is an early requirement for activated Drac1, perhaps to facilitate recruitment of paired vesicles to the membrane via the cytoskeleton, followed by an equally important requirement for Drac1 inactivation later, before fusion. One final issue is that genetic studies have not yet revealed a role for integrin subunits, one of the major components of vertebrate focal adhesions, in myoblast fusion. The larval body wall muscles in embryos mutant for the major integrin subunits, β_{PS}, α_{PS1}, and α_{PS2}, do not appear to exhibit defects in fusion (Brown, 1994; Roote and Zusman, 1995; for reviews see Brown, 1993; Gotwals et al., 1994).

However, the number and alternatively spliced forms of integrins identified in *Drosophila* has continued to increase (Gotwals et al., 1994), and family members that play other roles in myogenesis may yet be isolated. Thus, greater knowledge of GTPases and integrins and the identification of *Drosophila* homologues to components of vertebrate focal adhesions are likely to refine our working model.

The Role of mbc in Other Tissues

Although *mbc* is quite highly expressed in the heart and the visceral musculature late in development, these tissues do not appear to be severely affected by the loss of *mbc.* The visceral musculature does appear to be somewhat defective, as evidenced by the absence of midgut constrictions in a low percentage of embryos, but the heart appears to be relatively normal. One interpretation of such behavior is that another gene, yet to be identified, serves a redundant role in these tissues. Another interpretation is that, while the level of expression observed in unfertilized eggs is quite low, adequate maternally derived MBC protein may be available to embryos lacking zygotic expression of a functional protein. This may be particularly true for the pole cells, which express relatively high levels of MBC early in development.

In summary, we have reported the cloning and characterization of *mbc*, a novel gene that is essential for events leading to myoblast fusion and dorsal closure. The striking conservation of this molecule with DOCK180, a human gene that may be a target of a signal transduction cascade activated through focal adhesions, suggests the involvement of a signaling cascade in myogenesis, perhaps through organization of the actin cytoskeleton. As a more detailed picture of DOCK180, focal adhesions, and the family of *rho/rac* like GTPases is revealed, our understanding of the precise role of *mbc* will grow. Thus, the further identification of common features and homologous genes in different developmental systems may allow us to take advantage of the benefits of each to address the function of a conserved pathway.

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