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Twist Is Required for Muscle Template Splitting during Adult *Drosophila* Myogenesis

Richard M. Cripps[1] and Eric N. Olson[2]

Department of Molecular Biology and Oncology, Hamon Center for Basic Cancer Research, University of Texas Southwestern Medical Center at Dallas, 5323 Harry Hines Boulevard, Dallas, Texas 75235-9148

The basic helix–loop–helix transcription factor Twist is required for normal development of larval and adult somatic muscles in *Drosophila.* Adult flies normally have six pairs of dorsal longitudinal indirect flight muscles (DLMs), whereas when Twist function is reduced, only three pairs of DLMs are formed. Although *twist* is expressed in precursors of adult muscles throughout the larval and early pupal stages, we demonstrate that Twist function is required only during the late larval stage for DLM patterning. In wild-type flies, this is just prior to the time when three pairs of persistent larval muscle fibers split longitudinally to form templates for the six pairs of DLMs. By examining sections at various times during pupal development, we found that splitting of the larval muscles does not occur in *twist* mutants, indicating that Twist function is required to induce major changes in the larval templates prior to differentiation. The function of Twist in larval muscle splitting is likely mediated by myocyte enhancer factor-2 (MEF2) since in *Mef2* hypomorphic mutants splitting is also reduced and *Mef2* expression is dependent upon Twist. Our findings define specific roles for Twist and MEF2 during pupal myogenesis and demonstrate that these transcription factors function in adult muscle precursor cells to regulate downstream factors controlling muscle cell splitting and morphogenesis.

Key Words: **Twist; MEF2; *Drosophila;* adult myogenesis; imaginal discs; patterning.**

INTRODUCTION

Many genes have been identified whose functions are either sufficient or required for skeletal muscle differentiation (for reviews see Ludolf and Konieczny, 1995; Molkentin and Olson, 1996; Yun and Wold, 1996). However, numerous questions remain concerning the roles that many of these genes play in directing specific processes of muscle development such as myoblast fusion, expression of structural genes, and muscle patterning and attachment. To understand fully the cell biological events that take place *in vivo* during these times requires a system where one can easily visualize developing muscles and analyze gene expression and function in these cells.

The dorsal longitudinal indirect flight muscles (DLMs) of *Drosophila* are such a system. These large fibers, found in the thorax of the adult fly, develop during the pupal stage when undifferentiated myoblasts from the wing imaginal discs migrate and surround three pairs of larval muscles, the larval oblique muscles (LOMs; Fernandes *et al.*, 1991). The LOMs, unlike surrounding body wall muscle fibers, persist through a period of muscle histolysis and are used as scaffolds upon which the DLMs develop. As the adult myoblasts fuse with the larval muscle templates, each muscle splits longitudinally to generate the final pattern of six pairs of fibers per thorax.

Remodeling of larval muscles into imaginal fibers during metamorphosis has been studied in detail in Diptera (Crossley, 1972; Fernandes *et al.*, 1991) and Lepidoptera (Cifuentes-Diaz, 1989), indicating that this process is a commonly used mechanism of muscle development. Furthermore, many, although not all, Dipteran species have six pairs of DLM fibers within the adult thorax (Tiegs, 1955). This conservation in DLM pattern suggests that the determination of fiber number is an important process during myogenesis, the control of which is not fully understood. In *Drosophila,* ablation of the LOMs during the larval stage causes a delay in development and results in wide variabilities in fiber size and number, demonstrating an essential

[1] Present Address: Department of Biology, University of New Mexico, Albuquerque, New Mexico 87131-1091.

[2] To whom correspondence should be addressed. Fax: (214) 648 1196. E-mail: eolson@hamon.swmed.edu.

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role for these muscles in regulating adult muscle patterning (Farrell *et al.,* 1996; Fernandes and Keshishian, 1996). A major goal is the elucidation of these patterning events and understanding of how these mechanisms are controlled during muscle development.

A number of transcription factors are known to function during the development of the DLMs. Early studies indicated that the *erect wing* (*ewg*) gene is required for adult myogenesis (Deak *et al.,* 1982; Fleming *et al.,* 1983). *ewg* has recently been shown to encode a transcription factor expressed in the myoblasts as they surround the LOM templates (DeSimone *et al.,* 1995), and EWG function is required for splitting of the larval templates and maintaining the resulting fibers (DeSimone *et al.,* 1995; Roy and VijayRaghavan, 1998). *Twist* encodes a basic helix–loop–helix transcription factor which is expressed in all adult muscle precursor cells throughout the larval and early pupal stages (Bate *et al.,* 1991), and whose expression declines during muscle differentiation (Fernandes *et al.,* 1991; Currie and Bate, 1991). The MADS-box transcription factor, MEF2, is also expressed in adult muscle precursor cells at the end of the third larval instar (Ranganayakulu *et al.,* 1995), and it is directly regulated by Twist (Cripps *et al.,* 1998). Twist and MEF2 functions are required for normal somatic myogenesis at the embryonic stage (Lilly *et al.,* 1995; Bour *et al.,* 1995; Ranganayakulu *et al.,* 1995; Baylies and Bate, 1996), and they also play important roles in adult muscle development (Ranganayakulu *et al.,* 1995; Cripps *et al.,* 1998). Finally, the homeodomain protein Cut also accumulates in the adult muscle precursor cells during the late larval stage (Blochlinger *et al.,* 1993), but the function of this protein in the mesoderm is unclear. Therefore, although some of the cellular aspects of myogenesis are understood in this system, and although we know of several transcription factors whose presence during this time is required, it is important to understand how each factor might control specific aspects of myogenesis at particular times during pupal development.

We have recently identified a requirement for Twist in patterning the adult musculature (Cripps *et al.,* 1998). By reducing Twist function in the organism after embryogenesis, we observed a reduction in the number of DLM fibers in the adult thorax from six pairs in wild-type to three pairs in the mutant. There are three mechanisms that could potentially account for this phenotype: (1) Twist function may be required early to maintain the LOM scaffolds or to permit them to persist through the wave of muscle histolysis early during pupariation, such that in the *twist* mutants there is a reduction in the number of DLM templates that are formed. (2) When Twist function is reduced, the larval scaffolds might not split efficiently, so that the DLMs develop upon a reduced number of templates. (3) The LOMs persist through histolysis and split normally, but when Twist function is reduced, a proportion of the fibers subsequently degenerate.

To define more precisely the cellular events controlled by Twist during adult myogenesis, we carried out temperature-shift experiments in flies bearing conditional *twist* alleles. We found that the mutant phenotype results only when Twist function is reduced at the late larval stage. To determine what aspects of muscle development are controlled by Twist, we analyzed adult myogenesis at several subsequent timepoints during pupal development. When Twist function is reduced, there is little or no splitting of the LOM templates during the early pupal stage. Since we had previously shown a role for Twist in regulating *Mef2* expression in adult muscle precursor cells, we studied LOM splitting in hypomorphic *Mef2* mutants and also found larval splitting to be defective. Our findings therefore define an essential role for Twist, likely via MEF2, in patterning adult muscles, where Twist function is required in the invading imaginal myoblasts to signal specific cellular changes in the larval muscle templates.

MATERIALS AND METHODS

Fly Culture and Temperature Shifts

Gene and chromosome symbols are as described in Lindsley and Zimm (1992). Flies were grown on standard culture medium at the relevant temperatures. All experiments employing the *twist* temperature-sensitive mutant combination were performed as described previously (Cripps *et al.,* 1998). Briefly, the *twist* temperature-sensitive mutant is a heteroallelic combination of two different alleles, twi^{RY50} and twi^{V50}. Each of these mutants were maintained in a *y w* background and balanced over a *CyO* balancer carrying y^+ (Mardahl *et al.,* 1993). When these two strains are crossed to each other, the only y^- progeny are twi^{RY50}/twi^{V50} and are identifiable as first instar larvae by their light pigmentation. Flies laid eggs at 18 ± 1.0°C upon agar plates colored with red grape juice, and embryogenesis was allowed to proceed at this permissive temperature. After hatching, the *twist* mutant larvae were collected and subjected to the temperature shifts indicated. The restrictive temperature was 30 ± 1.0°C. All larvae collected were within 6 h of hatching to ensure an homogeneous population. There was usually a 1- to 2-day variability in the time of eclosion within any experimental set, and these variabilities are reflected in the slopes of the temperature shifts presented in Fig. 2. Controls used were either *y w* or *y w* carrying a *Mef2* promoter-*lacZ* construct (from −2884 to +521; Cripps *et al.,* 1998), to permit visualization of the adult muscle precursors.

The *Mef2* mutant studied was a heteroallelic combination of two lethal alleles, $Mef2^{65}$ and $Mef2^{113}$ (described in Ranganayakulu *et al.,* 1995). These were studied in the same manner as the *twist* mutants, using the y^+ marked *CyO* balancer chromosome. Experiments involving *Mef2* mutants were carried out at 25°C, using *y w* as a control.

All pupal ages given are hours after puparium formation (APF) at the appropriate temperature. To directly compare *twist* and *Mef2* mutant phenotypes we studied muscle development prior to LOM splitting (8 h at 30°C, 10 h at 25°C), just after splitting (18 h at 30°C, 23 h at 25°C), at a mid-pupal stage (40–45 h at 30°C, 51–58 h at 25°C), and at the adult stage.

Phenotypic Analyses

Adult muscle phenotypes were quantified by dissecting thoraces as described by Peckham *et al.* (1990), and counting the number of DLM fibers in each half thorax. Fiber numbers in pupal samples

were determined from sectioned material stained with hematoxylin and eosin (see below).

Samples were prepared for sectioning as described by Lyons *et al.* (1990). Briefly, pupae of the desired age and genotype were collected, and the anterior and posterior ends were removed using a scalpel. After fixation overnight in 4% (w/v) paraformaldehyde in PBS, samples were washed and embedded in agarose to permit easy handling. Samples were then dehydrated through an ethanol series and infiltrated with Paraplast embedding medium (Sigma). Sections of thickness 7 μm were cut using a Leica RM2025 rotary microtome.

After drying, sections were either stained with hematoxylin and eosin using standard procedures or prepared for antibody staining as follows: sections were deparaffinized in xylene and rehydrated through an ethanol series, and then subjected to "antigen rescue" using Antigen Retrieval Citra (BioGenex Corp., CA) according to the supplier's instructions. This treatment permits a reexposure of some antigens masked during fixation and is essential for good immunohistochemistry. After antigen rescue, sections were washed in 1× PBS, 0.1% Triton X-100 and incubated with primary and secondary antibodies. The primary antibodies used were anti-Twist (1:500, from B. Thisse and C. Thisse, University of Strasbourg, France) and anti-β-galactosidase (1:300, Promega).

Localized primary antibodies were detected using the Vectastain Elite staining kit (Vector Laboratories, CA) and diaminobenzidine color reaction. After detection, the samples were dehydrated through 70% ethanol, counterstained with eosin, and further dehydrated and mounted in Cytoseal 60 (Stephens Scientific, NJ).

RESULTS

Defining the Temporal Requirement of Twist Function for Adult Myogenesis

Using a temperature-sensitive mutant of *twist* we demonstrated previously that a reduction in Twist function from the first larval instar through eclosion results in patterning defects in the dorsal longitudinal indirect flight muscles (DLMs; Cripps *et al.*, 1998). Wild-type thoraces have six DLM fibers per hemithorax, whereas when Twist function is reduced each hemithorax has an average of three fibers. These defects are apparent even at the mid-pupal stage (Figs. 1A and 1B), indicating that they arise early during the development of the muscles. In addition, defects are also observed in other skeletal muscles of the thorax at this stage (Figs. 1C and 1D). There are reductions in the numbers of dorsoventral indirect flight muscle fibers when compared to wild-type (arrows in Figs. 1C and 1D), and the tergal depressor of the trochanter, or "jump" muscle is often defective in size and location (arrowheads in Figs. 1C and 1D).

To define the role of Twist in adult myogenesis, we analyzed this phenotype in greater detail. We concentrated on the DLM phenotypes since these muscles are large and the defects are easily quantifiable as the total number of DLM fibers per hemithorax. Note that *twist* mutants raised at the permissive temperature also displayed slight differences in fiber number, indicating that even at the permissive temperature the mutant Twist is not fully functional (Cripps *et al.*, 1998; Figs. 2A and 2B).

Since *twist* is expressed in the adult muscle precursor cells from the late embryonic stage until the end of the first day of pupariation, we initially determined if the temperature-sensitive period for the adult muscle phenotype coincided with *twist* expression. Reduction of Twist function from the first larval instar through the middle of the pupal stage resulted in defects indistinguishable from those observed if the temperature shift was continued through eclosion (Fig. 2C). Conversely, maintenance of *twist* mutants at the permissive temperature through the middle of the pupal stage and subsequent shifting to the restrictive temperature resulted in defects indistinguishable from those observed if the mutants were maintained at the permissive temperature throughout development (Fig. 2D). These results confirm that the defects we observed were most likely due to a reduction in Twist function and not to some other gene affected by the temperature shifts. Furthermore, in the stock of *y w; twi^{V50}/CyO, y^{+}*, we occasionally observed adult twi^{V50} homozygous escapers, consistent with the observation that this allele is leaky (Thisse *et al.*, 1991); these homozygous *twist* mutants also showed a reduction in the number of DLM fibers per hemithorax (4.3 ± 0.4, $n = 9$).

To define when Twist function is most important during its period of expression, we carried out a number of intermediate temperature shifts (Figs. 2E–2G). Reductions in Twist function either during the larval stage until the middle of the third instar (Fig. 2E) or during the early mid-pupal stage (Fig. 2F) each had moderate effects upon DLM fiber number in the adult. More notably, reduction in Twist function for a single period at the late larval stage almost completely recapitulated the most severe phenotype observed (Fig. 2G). These results demonstrate that the most important time for Twist function in patterning the DLMs is at the late larval stage (shaded region in Fig. 2), since a single short temperature shift during this time has almost identical effects to the reduction of Twist function for the entire postembryonic period. The less severe effects observed in the other temperature shift experiments (Figs. 2E and 2F) are likely due to each of these regimes overlapping slightly with the defined temperature-sensitive period.

Twist Is Required for Adult Muscle Template Splitting

Since the most important time for Twist function is shortly before major developmental events take place in the DLM precursors, we reasoned that one or more of the processes of either larval muscle persistence through histolysis, larval muscle splitting, or DLM fiber maintenance might be affected in the *twist* mutants. We therefore studied pupal muscle development in paraffin sections of wild-type and mutant individuals to define which of these processes are affected in the mutants.

Because the persistent larval muscles are small at 8 h after puparium formation (APF; Fig. 3A and Shatoury, 1956), we confirmed our identification of these cells origi-

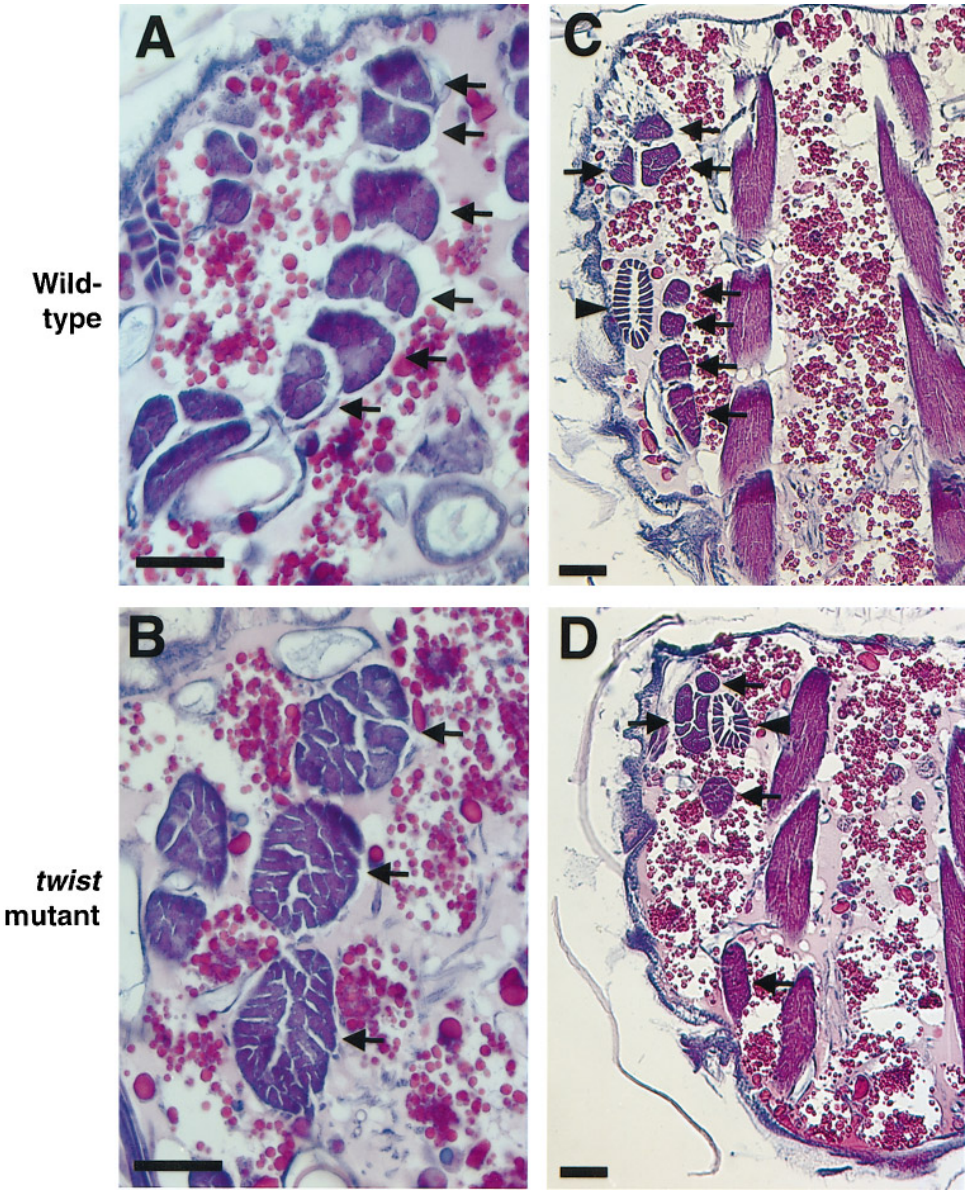

FIG. 1. Defects in thoracic muscle development at 40–45 h APF caused by reduction in Twist function. (A, B) Transverse sections through thorax; dorsal is to the top and the midline is to the right of the panel. (C, D) Horizontal sections through the thorax; anterior is to the top of the panel, and the midline is close to the right edge of the panel. (A) Wild-type flies have six DLM fibers per hemithorax (arrows), whereas when Twist function is reduced, there are only three fibers per hemithorax (B). (C) In wild-type there are seven dorsoventral indirect flight muscles (arrows) and a tubular muscle named the tergal depressor of the trochanter (TDT; arrowhead). (D) Twist mutants show slight reductions in the number of dorsoventral muscle fibers, and the TDT is located at an abnormal position in the thorax. Bar, 100 μm.

nally by localizing a number of epitopes in pupal thorax sections. *twist* is expressed only in the adult muscle precursor cells which invade the LOMs at this stage (Fernandes *et al.*, 1991). In sections, we detected Twist in large populations of cells surrounding eosinophilic tissue (arrows, Fig. 3B). In similar sections of pupae carrying a *Mef2–lacZ* transgene, which expresses *lacZ* in the adult muscle precursor cells (Cripps *et al.*, 1998), we detected β-galactosidase in a comparable population of cells (Fig. 3C). We interpret these data to indicate that the persistent larval muscles are the eosinophilic cells close to the dorsal thorax (arrows, Fig. 3A), and the adult muscle precursor cells stain strongly with hematoxylin and surround these larval muscles (asterisks, Fig. 3A). At later timepoints, the

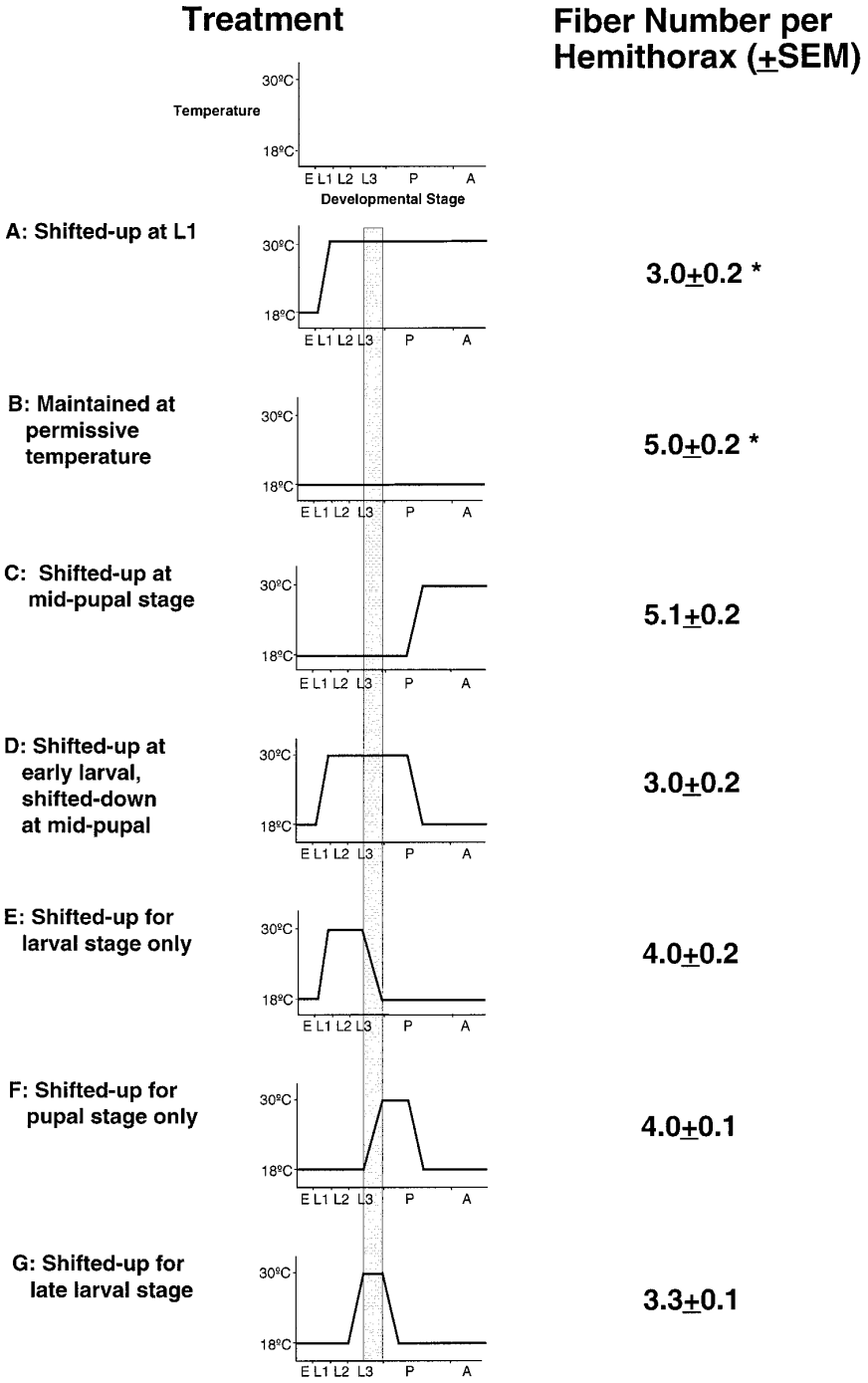

FIG. 2. Definition of the temperature sensitive period for *twist* in DLM development. First instar *twist* mutant larvae were harvested at the permissive temperature and subjected to the temperature regimes described on the left. After eclosion, the adults were dissected and the average number of DLM fibers per hemithorax was determined (right). At least 10 thoraces were analyzed for each sample. Note that there is a single developmental period (shaded) during which reduction in Twist function results in severe defects in adult DLM patterning. SEM, standard error of the mean. Developmental stages: E, embryonic; L1–3, larval instars; P, pupal; A, adult. *These data are from Cripps *et al.* (1998).

muscles and their precursors are sufficiently large to identify without the use of molecular analyses.

To study the numbers of fibers at different times during development, we analyzed pupal sections stained with hematoxylin and eosin and counted the numbers of fibers in each hemithorax. In wild-type pupae (Figs. 4A and 4B) the

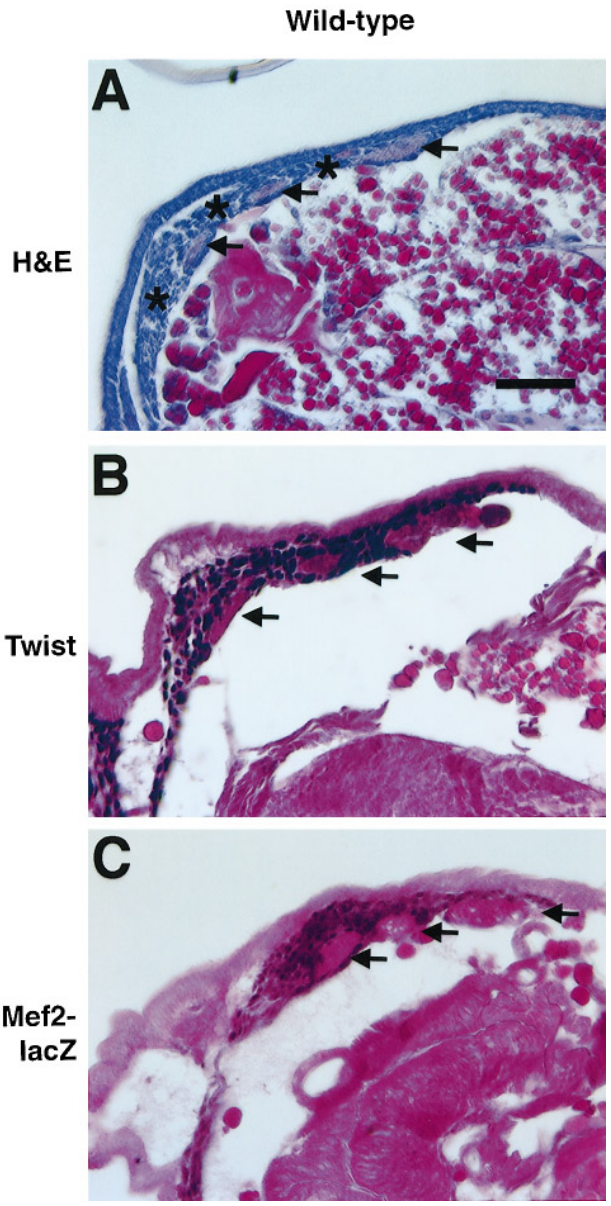

FIG. 3. Identification of persistent larval oblique muscles in wild-type pupal sections 8 h after puparium formation at 25°C. (A) Hematoxylin and eosin (H&E) staining; note the eosinophilic tissue (arrows) close to the dorsal thorax and the darkly stained cells surrounding this tissue (asterisks). (B) Anti-Twist immunohistochemistry identifies the darkly staining cells as the adult muscle precursors, surrounding the persistent LOMs (arrows). (C) β-Galactosidase accumulation in adult muscle precursors cells driven by a *Mef2–lacZ* construct, confirming the results from B above. In all panels, dorsal is to the top and the midline is to the right of the panel. Bar, 50 μm.

three persistent larval muscles in a single hemisegment can be seen at 8 h APF, surrounded by the adult muscle precursor cells (Fig. 4A). Over the next few hours, wild-type LOMs split longitudinally to generate the templates which will comprise the six mature DLM fibers per hemisegment (Fig. 4B; Fernandes *et al.*, 1991). After this time, there is no change in fiber number as the muscles grow.

In the *twist* mutants, three persistent larval fibers are usually seen per hemithorax early during pupariation (Fig. 4C), indicating that these fibers form and escape histolysis in a wild-type manner. However, by 18 h APF at 30°C, there are only 3-4 fibers per hemithorax (Fig. 4D), indicating that they have not undergone efficient splitting. Although these fibers increase in size, there is little subsequent change in their number. As we have seen in the adult sections, total muscle volume is not significantly reduced in the *twist* mutant pupae compared to wild-type.

We counted the number of DLM fibers per hemithorax in wild-type and *twist* mutants and determined the average at each pupal stage analyzed (Fig. 5A). Clearly, there is little increase in the number of fibers in the *twist* mutants after splitting should have occurred, compared to the doubling seen in wild-type pupae. There is little subsequent change in the number of fibers in either wild-type pupae or the *twist* mutant pupae, indicating that the number of fibers determined around the time of splitting remains constant. Overall, these results demonstrate that Twist function is required for the splitting of the persistent LOMs into DLM pretemplates, and in this manner is required to regulate DLM fiber number.

MEF2 Function Is Required for Splitting

We have previously shown that one function of Twist in the adult muscle precursor cells at the end of the larval stage is to activate expression of *Mef2* (Cripps *et al.*, 1998). MEF2 function is also important in determining DLM fiber number since hypomorphic *Mef2* mutants which survive to the adult stage have reductions in the number of DLMs (Ranganayakulu *et al.*, 1995). It is therefore likely that the requirement for Twist in LOM splitting is via regulation of *Mef2* expression. We studied DLM development in $Mef2^{65}$/$Mef2^{113}$ mutant pupae to determine if LOM splitting is also defective when MEF2 function is reduced (Figs. 4E, 4F, and 5B). As with the *twist* mutant pupae, the escape of the three LOMs from histolysis proceeded normally in *Mef2* mutants (Fig. 4E). After the time at which splitting occurred in the wild-type controls there was only a small increase in fiber number in *Mef2* mutants (Fig. 4F), indicating a requirement for MEF2 function for splitting. Interestingly, the young DLM fibers are often smaller than controls at this stage of development, although the total muscle volume at eclosion is unchanged (Ranganayakulu *et al.*, 1995). This does not represent delayed splitting of the fibers since there is no significant change in fiber number from this stage to subsequent timepoints (Fig. 5B). These results demonstrate that both Twist and MEF2 function to control LOM splitting during the early pupal stage, most likely through a common molecular pathway.

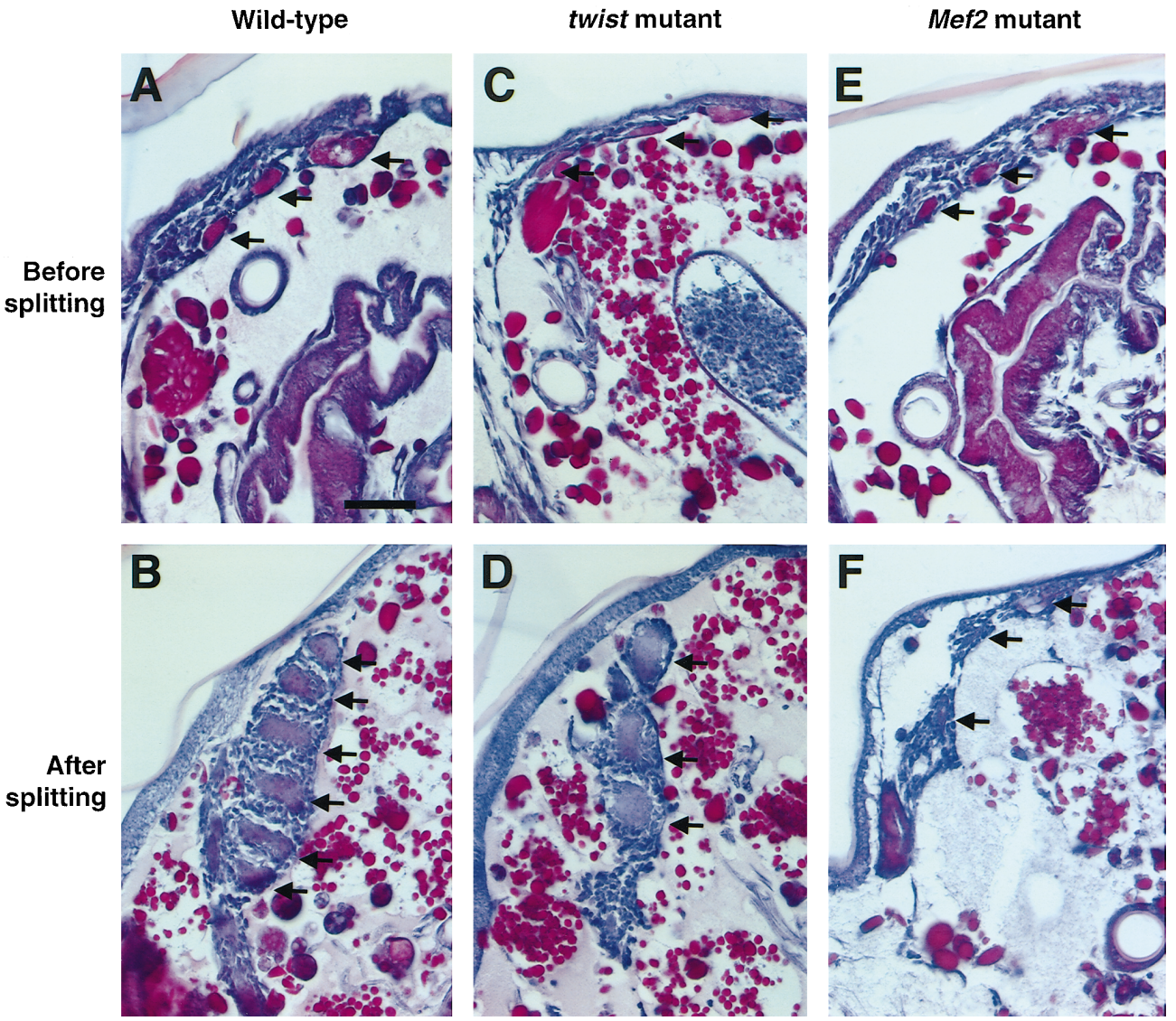

FIG. 4. Larval oblique muscle splitting does not occur when Twist or MEF2 function is reduced. (A, B) Wild-type control; (C, D) *twist* mutant; (E, F) *Mef2* mutant. The DLMs and their templates are indicated in each section by arrows. Note that in wild-type there are initially three persistent fibers per hemithorax (A), which subsequently split to form the final pattern of six fibers per hemithorax (B). In the *twist* mutants three templates are initially observed (C) and this number does not change through pupariation (D), demonstrating that longitudinal splitting of the LOMs does not occur. Similar results are found in the *Mef2* mutant samples (E, F). In all sections dorsal is to the top and the midline is to the right. Bar, 50 μm.

DISCUSSION

The unique developmental profile of the DLM fibers permits a detailed analysis of the many different cellular interactions that occur during myogenesis. During the early pupal stage, a subset of larval muscles persists through a wave of histolysis and dedifferentiates. These cells are invaded by a group of adult muscle precursor myoblasts, and the LOMs split longitudinally as fusion occurs between these distinct groups of cells. DLM development therefore involves interactions between two separate groups of muscle cells (the LOMs and the adult muscle precursors), as well as interactions of the developing muscles with the nervous system and with epidermal attachment sites. The sequences of cellular events that occur during many of these interactions have been described in detail (Shatoury, 1956; Fernandes *et al.*, 1991, 1996; Fernandes and Vijay-Raghavan, 1993), and it is now important to identify the

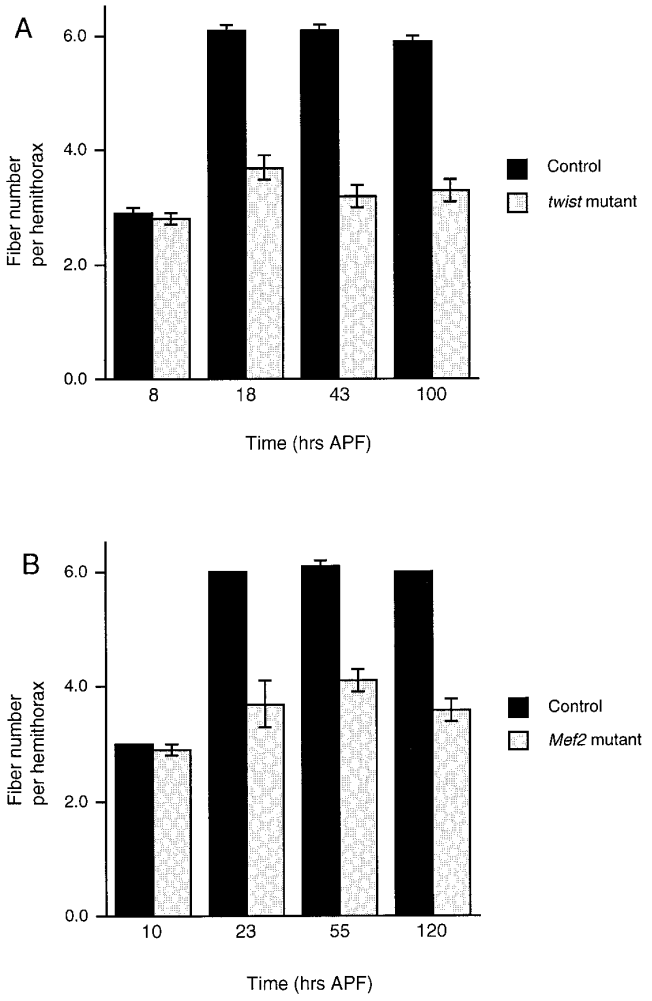

FIG. 5. (A) Summary of DLM template and fiber numbers observed through pupal development in: A, wild-type (filled bars) and *twist* mutants (shaded bars); B, wild-type (filled bars) and *Mef2* mutants (shaded bars). *X* axis, hours after puparium formation at 30°C (A) or 25°C (B). *Y* axis, average number of DLM fibers per hemithorax; error bars represent standard error of the mean. At least 10 hemithoraces were analyzed for each sample.

genes that control the different processes. Because many of the genes expressed in the adult muscles and their precursors have broad developmental expression patterns, they may control different processes at different times.

Here we used a conditional *twist* mutant to show that Twist function is required just prior to larval muscle splitting for normal DLM myogenesis, and that when Twist function is reduced, no splitting occurs. Twist also functions in aspects of adult myogenesis other than LOM splitting, since we also observe defects in the development of the dorsoventral muscles and the tergal depressor of the trochanter, neither of which develop upon larval templates (Fernandes *et al.*, 1991). These effects are similar to the requirement for Twist during embryogenesis in *Drosophila*, where high levels of Twist in somatic muscle precursors are required for somatic muscle differentiation (Baylies and Bate, 1996), although the defects observed in adult myogenesis are milder than those seen in embryonic muscle development (Cripps *et al.*, 1998).

The requirement for Twist function relative to developmental changes observed during adult muscle development is illustrated in Fig. 6. The time difference between the requirement for Twist (late during the larval stage) and the first observable defects in muscle development (failure of LOM splitting during the early pupal stage) suggests that Twist does not directly initiate splitting, but rather that it controls some downstream factor that carries out this process. Consistent with this model, we have demonstrated previously that *Mef2* expression in the adult muscle precursor cells at the late larval stage is dependent upon Twist function (Cripps *et al.*, 1998). Here we show that *Mef2* hypomorphic mutants also fail to undergo LOM splitting, suggesting that *Mef2* is at least one of the Twist targets required to mediate splitting. Screening for mutations causing this phenotype might identify additional factors which control or carry out splitting. Recent data indicate that Notch is one of these factors (Anant *et al.*, 1998) since reduction in Notch function during the pupal stage reduces Twist accumulation and prevents LOM splitting.

It is interesting that Twist function in the adult muscle precursor cells is required for splitting to occur in the muscles to which the myoblasts fuse. Reductions in the adult muscle precursor cell population in the wing imaginal disc also result in a failure of the LOMs to split, presumably because invasion of LOMs by myoblasts never occurs (Roy and VijayRaghavan, 1998). Both these results indicate that some "signal" derives from the precursor cells and is received by the larval muscle templates. However, since the times of template splitting and of myoblast fusion overlap significantly (Fernandes *et al.*, 1991; see also Fig. 6), it is not clear if this "induction" is mediated by an intercellular diffusible signal, by cell–cell contact, or by release of the signal upon cell fusion. Presumably, splitting is mediated by cytoskeletal reorganization, and along these lines it is interesting to note that α-PS2 integrin is detected transiently in the adult muscle precursor cells just prior to fusion (Fernandes *et al.*, 1996). A role for this protein either in myoblast fusion or in signaling LOM splitting is also attractive in light of previous studies showing that α-PS2 is a direct transcriptional target for MEF2 (Ranganayakulu *et al.*, 1995).

Ablation of the LOMs during the larval stage, causing a reduction in the numbers of DLM templates, resulted in formation of varying numbers of muscle fibers by *de novo* myoblast fusion (Farrell *et al.*, 1996; Fernandes *et al.*, 1996). These results suggest that the templates are specifically involved in regulating fiber number. In this paper, we have also caused a reduction in the number of templates by preventing LOM splitting; however, this does not result in wildly varying numbers of DLM fibers. Perhaps the absence

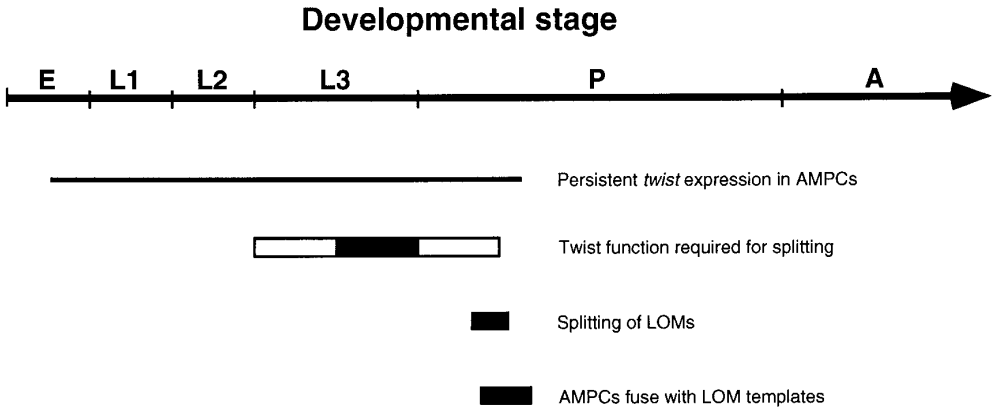

FIG. 6. Summary of *twist* expression and function in the DLM precursor cells during *Drosophila* development relative to morphological changes in the muscles. Data are from this manuscript, Bate *et al.* (1991), and Fernandes *et al.* (1991). AMPCs, adult muscle precursor cells. Developmental stages: E, embryonic; L1–3, larval instars; P, pupal; A, adult.

of Twist function in the muscle precursors renders the myoblasts incapable of *de novo* fusion, as occurs in wild-type muscles.

Both LOM ablation and reduction in Twist function result in alterations in DLM fiber number and yet the total muscle volume is not significantly altered. It has been suggested that muscle volume is regulated by the number of nuclei forming the muscles (Farrell *et al.*, 1996); if this is the case, then there are no major effects of Twist reduction upon myoblast proliferation. We addressed this question previously by showing that a reduction in *twist* function has no effect upon the precursor cell population at the end of the larval stage as visualized by expression of two markers (Cripps *et al.*, 1998). Although we have not studied precursor cell number during the pupal stage in these mutants, it is apparent from the sections presented in Fig. 4 that many myoblasts contact the LOMs in both *twist* and *Mef2* mutants, although we do occasionally see some reductions in cell number at these early stages. It is possible that reducing Twist function might have a minor effect on the number of myoblasts or their efficient migration from the wing disc to the site of muscle formation.

The dynamic series of events that takes place during adult myogenesis in the early pupal stage is reflected in dynamic patterns of gene expression during this time. Twist levels decline as the muscles differentiate (Fernandes *et al.*, 1991), and levels of several other proteins peak during the early pupal stage. There are also clearly a large number of genes yet to be identified which participate in the complex reorganization of the somatic musculature during the pupal stage, many of which also may function during embryonic muscle development, like *twist* and *Mef2*. A major challenge in the future will be to design and carry out systematic strategies to identify these genes, and to understand their functions at multiple stages of the life cycle.

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