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Development of the indirect flight muscles of *Drosophila*

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Summary

We have followed the pupal development of the indirect flight muscles (IFMs) of *Drosophila melanogaster*. At the onset of metamorphosis larval muscles start to histolyze, with the exception of a specific set of thoracic muscles. Myoblasts surround these persisting larval muscles and begin the formation of one group of adult indirect flight muscles, the dorsal longitudinal muscles. We show that the other group of indirect flight muscles, the dorsoventral muscles, develops simultaneously but without the use of larval templates. By morphological criteria and by patterns of specific gene expression, our experiments define events in IFM development.

Key words: muscle, myogenesis, myosin, actin, twist, *Drosophila*.

Introduction

The thorax of the fruit fly, *Drosophila melanogaster*, contains a large number of muscles ordered in an essentially invariant pattern. The bulk of these are the indirect flight muscles (IFMs), which consist of two groups, the dorsal longitudinal muscles (DLMs) and the dorsoventral muscles (DVMs) (Miller, 1950; Crossley, 1978; Lawrence, 1982). In addition to their position in the thorax, the IFMs differ from the other muscles in their ultrastructure (Tiegs, 1955), physiology (Ikeda, 1981) and biochemistry (Fujita and Hotta, 1979). Classical studies by Tiegs (1955), in particular, and by others (see Crossley (1978) for a review) examined some aspects of formation of the DLMs and the use of larval templates for their development. These studies and similar ones in other insects (Crossley, 1978) have defined some of the basic elements in the development of flight muscles in dipterans. Experiments of Poodry and Schneiderman (1970) defined a population of cells within the imaginal discs which they called adepithelial cells and suggested that these cells might be the adult myoblasts. This view was then confirmed by the ultrastructural studies of Reed *et al.* (1975) who showed that the adepithelial cells do indeed align and fuse to form imaginal muscles. The study of genetically marked mosaic muscles suggests that, while myoblasts associated with each disc contribute to the formation of specific adult muscles during normal development (Lawrence, 1982), they can contribute to diverse groups of muscles when ectopically placed (Lawrence and Brower, 1982). Little is known about how myoblasts are sequestered to imaginal discs, how they then migrate, divide and differentiate to form the adult thoracic muscles, and how and when the differences between muscles that share the same group of ancestral myoblasts arise. Nonetheless there is evidence that the information required for patterning the muscles is not autonomous to the mesoderm but may also be derived from the nervous system and the epidermis (Bock, 1942; Haget, 1953; Williams and Caveney, 1980*a*,*b*; Nuesch, 1968; Lawrence and Johnston, 1986).

Several mutants exist that affect the development of the IFMs (Deak *et al.* 1982; Fleming *et al.* 1983; Costello and Wyman, 1986). It is important to have an understanding of how these muscles develop in the wild type, if a detailed developmental analysis of the mutants is to reveal the role of the wild-type genes in myogenesis. Therefore, we have begun a study of the development of the IFMs. Using antibodies against the *twist* gene product and muscle-specific reporter genes as markers, we have documented the early events that lead to the formation of the IFMs, beginning with the release of myoblasts from imaginal discs to the initiation of adult myofibrillar synthesis. In addition to charting the events during myogenesis, our results show that development of the closely related muscles, the DVMs and the DLMs, differs in at least one aspect. Finally, our results allow us to suggest possible mechanisms operating in flight muscle development.

Materials and methods

Fly stocks

All fly stocks were raised on standard *Drosophila* culture

medium at 25°C. The white prepupal period, which lasts for one hour (1 h) at this temperature, is taken as the initial time (0 h) for our studies. Thus, the error in staging is ±30 min. White prepupae were collected on moist tissue paper and aged to desired periods. Adults emerge after 100 h under our growth conditions. The Canton-S (CS) strain was used as wild type. Histochemical staining for β-galactosidase activity in myosin heavy chain (*MHC*)- and *Actin-lacZ* gene fusion transformants was used to reveal the developing muscles. The *MHC-lacZ* transformant contains the upstream regulatory regions of the muscle *MHC* gene (Bernstein *et al.* 1983; Rozek and Davidson, 1983) fused to the *E.coli lacZ* gene, and results in the expression of the enzyme β-galactosidase in all muscles of the embryo, larva and the adult. The strain that we used has the *MHC-lacZ* containing P-element inserted on the second chromosome (Hess *et al.* 1989) and was obtained from Dr Norbert Hess and Dr Sandy Bernstein at the San Diego State University, San Diego, California, USA. The *Actin(88F)-lacZ* transformant strain is an in-frame gene fusion of the IFM specific actin gene (Hiromi *et al.* 1986) to the *lacZ* gene. The transformant used by us is located on the first chromosome and will be henceforth referred to as the *Actin-LacZ* strain. Expression of the fusion gene in this strain is observed only in the IFMs and not in other muscles of the adult or larva. The *Actin-lacZ* strain was obtained from Professor Yoshiki Hotta of the University of Tokyo, Japan.

Tissue preparations

Pupae of desired ages were dissected in *Drosophila* Ringer (in $g l^{-1}$: 6.5 NaCl, 0.14 KCl, 0.2 $NaHCO_3$, 0.12 $CaCl_2$, 0.1 NaH_2PO_4). The animals were cut open along the ventral midline and pinned on Sylgard (Dow Corning Corp., USA). The insides were cleaned by gently blowing Ringer on the preparation with a fine glass pipette attached to a rubber mouth tube. Dissected animals were fixed in a drop of 4% paraformaldehyde for 5–10 min. The tissue was subsequently rinsed in Ringer for β-galactosidase histochemistry or phosphate-buffered saline (PBS, in $g l^{-1}$: 200 NaCl, 5.0 KCl, 5.0 KH_2PO_4, 27.8 $Na_2HPO_4.2H_2O$) for immunocytochemistry with *twist* antibody as desired.

Histochemistry

Developing muscles were stained in the *MHC-lacZ* and *Actin-lacZ* transformants using the chromogenic substrate, X-gal (Sigma, St Louis, MO, USA). Dissected tissue was treated as described above and incubated in staining solution (10 mM $NaH_2PO_4.H_2O/Na_2HPO_4.2H_2O$ (pH 7.2), 150 mM NaCl, 1.0 mM $MgCl_2.6H_2O$, 3.5 mM $K_4[Fe^{II}(CN)_6]$, 3.5 mM $K_3[Fe^{III}(CN)_6]$, 0.3% Triton X-100) at 37°C (Simon *et al.* 1985) for 15–30 min. The preparations were then washed in Ringer, dehydrated in an alcohol series, cleared in xylene and mounted in DPX (Sisco Research Labs, Bombay, India).

Immunocytochemistry

Antibody raised in rabbit against the *twist* protein was a generous gift from Dr Fabienne Perrin-Schmitt, CNRS, Strasbourg, France (Thisse *et al.* 1988). The antibody was used at a dilution of 1:500. Pupal tissue fixed for 0.5–1 h was washed thoroughly with PBS, blocked with 0.5% bovine serum albumin (BSA), and incubated in the diluted *twist* antibody for 36 h at 4°C. The tissue was then washed in PBT (0.3% Triton X-100 in PBS), for 1 h and incubated for 1 h in 0.2% goat serum. The preparations were then bathed in biotinylated anti-rabbit second antibody for 1 h, washed in PBT, and the second antibody coupled to an avidin–horseradish peroxidase (HRP) complex using the ABC Vectastain kit (Vector laboratories, USA) as described by the manufacturer. The bound peroxidase was revealed using 0.5% diaminobenzidene (DAB, Sigma, USA.), 0.09% H_2O_2 in PBS till the colour reaction developed. After the reaction was stopped (by washing in PBS), the tissue was dehydrated in an alcohol series, cleared in xylene and mounted in DPX.

Histology

Staged pupae were made permeable to fixative by snipping off the ends, fixed in 2% glutaraldehyde, dehydrated in an alcohol series and embedded in Araldite (Fluka, Switzerland). For light microscopy, 5 μm sections were cut, stained with 1:1 methylene blue–toluidine blue and mounted in DPX.

Results

The larval and adult pattern of muscles and the markers for their study

At the end of the third instar, the *Drosophila* larva stops wandering and begins the process of metamorphosis into an adult. The elements of IFM formation are completed within 36 h of pupariation (the white prepupal stage, 0 h *A*fter *P*uparium *F*ormation (APF), marks the onset of metamorphosis). A schematic representation of major muscles of the adult thorax is shown in Fig. 1C: The DLMs, the DVMs and the jump muscle (the tergal depressor of the trochanter, TDT) are all shown, while other muscles in the thorax (see Miller, 1950 for a detailed description) have been omitted for clarity. These muscles develop in a programmed manner that begins with histolysis of most of the larval thoracic muscles. The pattern of larval mesothoracic muscles, shown in Fig. 1A was revealed by the activity of β-galactosidase in the *MHC-lacZ* transformant described in Materials and methods. The *MHC-lacZ* strain serves as a marker for the histolyzing larval muscles because of the perdurance of the β-galactosidase (Lawrence and Johnston, 1989) synthesized in the third instar and earlier. β-galactosidase staining in the *Actin-lacZ* transformant measures the activity of the regulatory element of the adult and IFM-specific actin gene (Hiromi *et al.* 1986). The *twist* gene, critical for development of the embryonic mesoderm, is also expressed in the adult myoblasts (Bate *et al.* 1991). We used antibodies against the *twist* protein to follow myoblasts during muscle development. By using antibody and histochemical staining, we were able to follow the metamorphosis of the larval pattern (Fig. 1A) into that of an adult (Fig. 1C).

Mesothoracic LOMs 1–3 do not undergo histolysis with most muscles of the larval thorax

The pattern of body wall muscles in the *Drosophila* larva is established in the late embryo and remains unchanged as the larva undergoes two molts before pupation (Bate, 1990). Soon after the onset of the white prepupal stage, larval muscles begin to histolyze. Histolysis proceeds in three distinct waves, the first wave resulting in the elimination of most of the muscles of the head and the thoracic segments but sparing most of the abdomen (Crossley, 1978). This first wave however, does not histolyze either the dilatory muscles

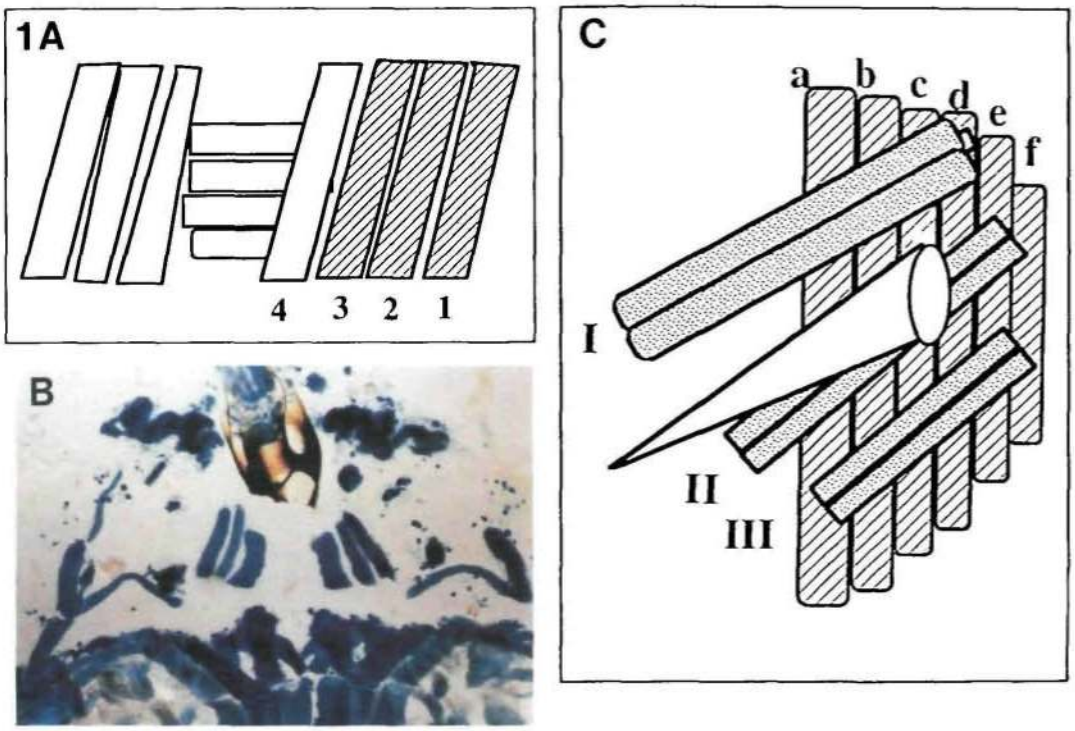

Fig. 1. The relationship of the LOMs in the second thoracic segment to the DLMs. (A) A schematic representation of the LOMs in the third instar larva, only one hemisegment is shown. The LOMs are numbered 1–4. (B) Histolysis spares the LOMs 1–3, stained here for β-galactosidase activity in an 8 h APF pupal dissection of the *MHC–lacZ* strain, both hemisegments are shown (Bar=50 μm). The LOMs are the three muscles in each hemisegment seen below the black larval mouth hooks. The intersegmental muscles that are used for head eversion have been snipped and are seen lateral to the three LOMs. (C) Schematic representation of the muscles in the adult thorax highlighting the major muscles. The three DVM bundles are marked I, II and III and stippled. The six DLM fibres are striped and the TDT is plain. Only one hemisegment is shown. Anterior is to the top, dorsal to the right. The relative sizes of the muscles are not to scale.

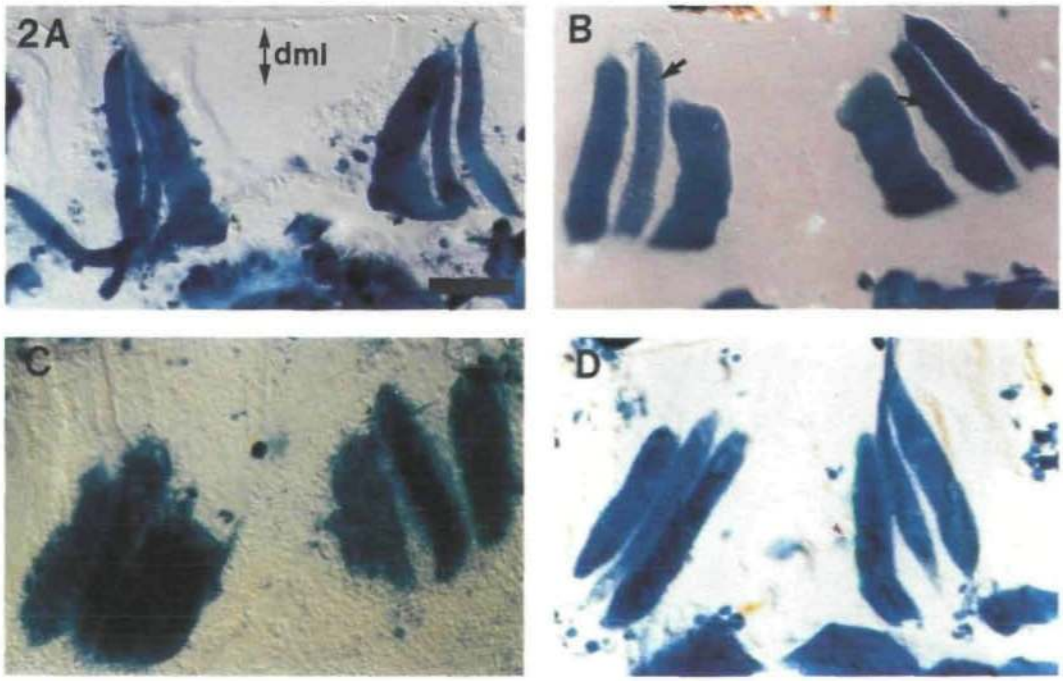

Fig. 2. Changes in LOM morphology: the formation of vacuoles and elongation. (A) A 6 h pupa. The LOMs spared by histolysis are shown. At this stage they still retain myofibrillar organization that can be seen in polarized light (not shown). (B) At 8 h, vacuoles are seen in the LOMs (arrows). (C) At 10 h the LOMs are now highly vacuolated. (D) At 12 h they are transformed into elongate structures ('pretemplates') that will soon start to split. Note that the *MHC–lacZ* gene activity does not indicate the presence of remnant larval muscles in the region adjacent to the LOMs where the DVMs will develop (compare with Fig. 4). β-galactosidase activity stained for by X-gal in a *MHC–lacZ* transformant. Bar=25 μm in all pictures. dml=dorsal midline.


3A


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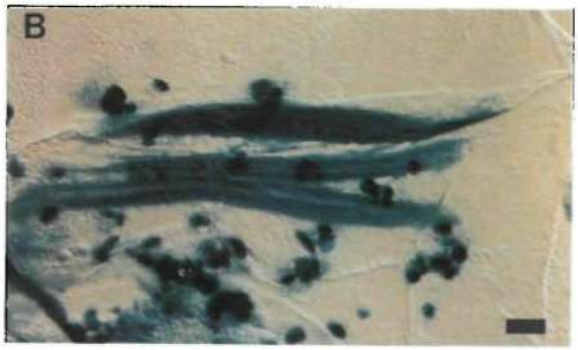
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4A


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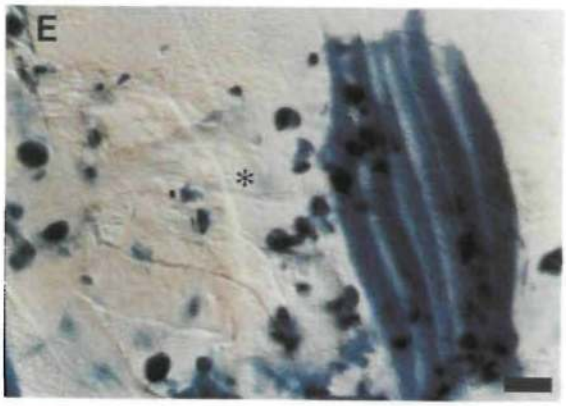
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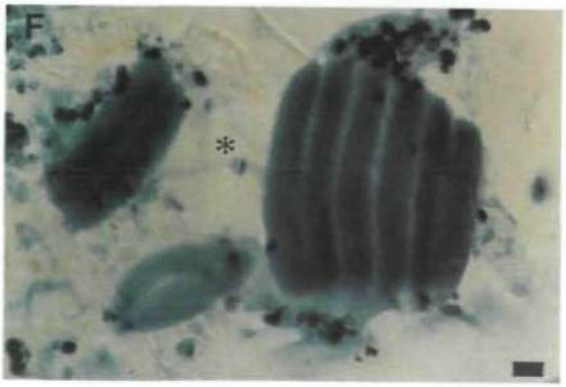
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Fig. 3. The splitting of the LOMs. The orientation of this figure is 90° with respect to the previous figures and with respect to Fig. 4. The dorsal midline runs horizontal at the top of each frame. β-galactosidase activity stained for with X-gal in a *MHC–lacZ* transformant. (A) Pretemplates just prior to splitting (13 h APF). (B) Splitting of the pretemplates is underway (14 h APF). However, the dorsalmost LOM (LOM 1, Fig. 1A) has not yet started splitting. (C) LOM 1 also starts to split (16 h APF). (D) Splitting is complete. The three pretemplates give rise to the 6 templates on which the DLMs form (20 h APF). Note that the *MHC–lacZ* staining does not show any sign of the developing DVMs. Compare this with the *Actin–lacZ* staining in Fig. 4. Only one hemisegment of the thorax is shown. Bar=10 μm in all pictures.

Fig. 4. Adult IFM-specific *Actin–lacZ* expression shows the presence of the developing IFMs (DVMs and DLMs). (A–D) Adult expression of the *MHC–lacZ* gene starts much after *Actin–lacZ* expression has begun (E–F). In this figure, the dorsal midline runs from the top to bottom at the right of each frame. Anterior is to the top. (A) 16 h APF. *Actin–lacZ* expression begins at 14 h APF and progressively become stronger. (B) 18 h APF, (C) 20 h APF and (D) 24 h APF. The developing DVMs (DVM I and II, Fig. 1C) are seen in addition to the six DLMs. DLMs I and II are respectively just above and below the asterisk in C. DVM III develops below the forming DLMs that are to the right of the asterisk in C but cannot be seen in these figures. (E) A 24 h *MHC–lacZ* pupa, stained for β-galactosidase activity. The IFMs, at this stage, do not express the adult *MHC–lacZ* gene, hence the DVMs (since they have no larval templates) are not histochemically detected even though they are developing in the region above below the asterisk and their unstained outline is visible. The DLMs can be seen due to the perdurance of the β-galactosidase activity expressed in the larval muscles. (F) 26 h APF. *MHC–lacZ* adult activity can be readily seen and both the DLMs and DVMs are observed. The asterisk (*) shows a nerve that lies between the developing DLMs and DVMs. Only one hemisegment is shown. Bar=10 μm in all pictures.

of the pharynx or 3 pairs of dorsal thoracic muscles (Robertson, 1936) known as the larval oblique muscles (LOMs, Costello and Wyman, 1986) which are later remodelled to form the DLMs (Crossley, 1978; Costello and Wyman, 1986). Hooper (1986) describes three layers of muscles in the larval body wall: the outer, the intermediate and the inner layers. Examination of the orientation of the muscles in the thorax during the third larval instar and the early stages of pupation allows us to allot the LOMs to the intermediate (LOM 1 and LOM 2) and external layer (LOM 3) of muscles (See Fig. 1A. Using Crossley's (1978) notation LOMs 1–4 are probably muscles 9, 10, 11 and 20, respectively). LOM 4 (Fig. 1A) degenerates in the first wave of histolysis. Anteriorly, LOMs 1–4 are inclined towards the dorsal midline, whereas the internal muscles are oriented away from it. The DLMs in the adult are mesothoracic in location and the suggestion that the LOMs involved in their development belong to the second thoracic segment of the larval bodywall is supported by the pattern of the histolyzing larval muscles. At the onset of histolysis, it is only the mesothoracic LOMs that retain their myofibrillar organization as seen in polarized light. By about 8 h APF, histolysis in the thorax is complete and the only persisting muscles are the mesothoracic LOMs and the group of intersegmental muscles that are used for head eversion. An 8 h pupal thorax, dissected open and stained for the activity of the *MHC–lacZ* gene, is shown in Fig. 1B.

The three LOMs that escape histolysis, change in shape, vacuolate and split into six

Selective histolysis of muscles is a feature seen in other Dipterans. Similar events have been investigated in the house fly *Musca domestica*, the blow fly *Calliphora* and in *Drosophila* itself (reviewed in Crossley, 1978). Adult development demands that there be dramatic changes in body shape during pupation. A cylindrical animal is to be remodelled into one with legs, wings and everted head. Eversion of the head and also of the imaginal discs is caused by modulating hydrostatic pressure inside the animal and the residual larval muscles are thought to be crucial for these events (Crossley, 1978). It is therefore pertinent that, by 6 h APF, while the LOMs are still birefringent and appear as intact muscles (Fig. 2A), disc eversion has occurred (Fig. 5A and Poodry, 1980). Shortly thereafter, at 8 h APF, the persistent LOMs exhibit a series of vacuoles (Fig. 2B). At this stage, the LOMs show a sharply reduced birefringence, seen under polarized light, when compared with the LOMs at 6 h APF (data not shown). At 10 h APF, the LOMs are highly vacuolated, and their shape is distorted (Fig. 2C) and within two hours (12 h APF) there is a drastic change in their morphology – they become elongated and vacuoles are still seen but perhaps to a lesser degree (Fig. 2D). In his study, Tiegs (1955) has described this process in *Drosophila* and our observations agree well with his findings that, while the LOMs lose their sarcomeric organization, the plasma membrane does not undergo phagocytosis. The most detailed analysis of events that are taking place at this time comes from electron microscopic studies in *Calliphora* by Crossley (1972) in which he demonstrates that the organization of the myofibrils is broken down inside the plasma membrane of the residual larval muscle. It is therefore relevant to note that, in our study, when animals containing the *MHC–lacZ* fusion gene are examined after histochemical staining for β-galactosidase activity, we do not observe the release of the enzyme from the LOMs. If the plasma membrane were damaged, the enzyme activity would not continue to be localized in the LOMs after the myofibres were broken down (Fig. 1B). The elongated LOMs now start the process of splitting into six. Fig. 3A shows the muscles just prior to the onset of splitting. The most dorsal LOM (LOM 1) is still vacuolated but the other two are not. At 14 h APF (Fig. 3B), the elongated LOMs, now no longer vacuolated, are in the process of splitting. Interestingly the most dorsal LOM lags behind and only begins to split at about 16 h APF (Fig. 3C). This delay in splitting of LOM 1 could be mediated by differences in innervation: the DLMs that

develop from LOM 1 (DLMs e and f, Fig. 1C) are innervated by branches of a single motoneuron while the other DLMs are each innervated by a separate motoneuron (Ikeda, 1981; Costello and Wyman, 1986). At 16 h APF the LOMs have an adult-like innervation, but at 10 h APF this pattern is not evident (J.F., unpublished observations). By 20 h APF, splitting is complete and the 3 LOMs in each hemisegment have given rise to 6 templates (Fig. 3D) which form the DLMs of the adult.

The LOMs split and act as templates for the DLMs, but the DVMs develop de novo

Studies by Tiegs (1955) and Shatoury (1956) in *Drosophila*, and in other Diptera (see Crossley, 1978) all indicate that the DLMs develop on the residual LOMs. Most of these studies, however, do not commit themselves as to whether the DVMs develop on similar fibres. Shatoury (1956) claims that the 7 DVMs form on three pairs of larval muscles. By contrast, in *Phormia regina*, Beinbrech (1968) suggests that the DVMs arise from a *de novo* fusion of myoblasts, without a larval template. The number (6) and location (close to the dorsal midline) and orientation of the split LOMs all indicate that it is the DLMs that develop on these fibres and we can show that this is indeed the case simply by following these muscles through development. In Fig. 4, for example, the forming DLMs can be seen in relation to the DVMs. In whole mounts, these events can be readily followed to later stages of development, for example 30 h APF (Fig. 7D), by which time the muscles are one-third formed.

While perdurance of the β-galactosidase activity in larval muscles of the *MHC–lacZ* transformant allows us to observe the LOMs and their subsequent formation into 6 templates that will form the DLMs, it does not reveal any larval templates that will serve for formation of the DVMs. It could be that some persistent muscles do not stain with the *MHC–lacZ* marker but we find no evidence for such muscles when we use Nomarski optics to examine whole mounts. However, other experiments prove that the DVMs are developing at the same time as the DLMs, probably from a *de novo* fusion of myoblasts. The developing DVMs can be revealed by staining pupae of the *Actin–lacZ* (see Materials and methods) strain (Fig. 4A–D). A histochemical reaction for β-galactosidase activity in the developing DLMs and DVMs is first observed, at very low levels, at 14 h APF (data not shown) and increases by 16 h APF. At 18 h APF, the staining clearly shows the developing IFMs. These results should be compared with those from the *MHC–lacZ* strain (12–20 h APF, see Fig. 3). Here, intensity of staining decreases with time (requiring longer staining times for detection) supporting the interpretation that *MHC–lacZ* staining is due to perdurance of the β-galactosidase activity synthesized in the larval muscles. The adult expression of *MHC–lacZ* gene activity in the DLMs and DVMs seems to start well after the expression of the IFM-specific *Actin–lacZ* has begun (Fig. 4D–F). At 24 h APF, *Actin–lacZ* activity can be seen in the DLMs and DVMs, while adult expression of the *MHC–lacZ* gene is not yet detectable. Nonetheless the unstained DVMs can be seen with Nomarski optics in the *MHC–lacZ* strain (Fig. 4E). By about 26 h APF, *MHC–lacZ* expression appears strongly in both the DVMs and the DLMs, reflecting adult synthesis and the reaction times required for histochemical detection of this activity are now short. The different times of expression of the *MHC–lacZ* and the *Actin–lacZ* genes reflect native activity of the upstream regulatory elements of these genes. We do not know if protein synthesis from the native genes is also out of phase.

Myoblasts fuse with the splitting LOMs and also accumulate at distinct nearby sites where the DVMs and the TDT develop

Adepithelial cells, found between the folds of all imaginal discs, are the myoblasts that give rise to adult muscles (Poodry and Schneiderman, 1970; Reed *et al.* 1975). These myoblasts are recognized by antibodies raised against the *twist* protein (Bate *et al.* 1991) and we have used anti-*twist* antibodies to follow these cells as they assemble to form adult muscles. Just after pupariation, the discs begin to evert and simultaneously release myoblasts. The myoblasts that contribute to the IFMs are probably released from the wing disc. In dissections of pupae 6 h APF, the region of the wing disc that contains the adepithelial cells is seen to be positioned close to where these muscles will develop (Fig. 5A, B). At 8 h APF, the regions of the leg and wing discs that contribute to the thoracic exoskeleton have already fused (Fig. 5C, D). Myoblasts from the leg discs presumably take two paths, one group that will form the leg muscles (these are clearly seen in everting discs), the other migrating dorsally to contribute to muscles in the ventral thorax. The myoblasts are of different sizes and shapes: the larger ones (Fig. 5C) could be in mitosis and the differences in shape could be due to the cells being motile. However, it is also possible that the differences in size and shape are simply due to the high density of cells in the region and there are occasional groups of two or more myoblasts. Shatoury (1956) proposed that myoblasts initiate the splitting of the LOMs, but changes in innervation may also play a role in this process (see above) and splitting without myoblast involvement has been reported in other insects (Tiegs, 1955). By 14 h APF, the myoblasts have surrounded each splitting muscle (Fig. 6A) and by 16 h APF they are fusing with the splitting fibres (Fig. 6C, D). These myoblasts will contribute to the formation of the DLMs. In addition to the cells that fuse with the splitting LOMs, *twist* staining also reveals the accumulation of myoblasts in the region immediately adjacent to the developing DLMs (lateral and ventral thorax, Fig. 6B). This is the region spanned by the DVMs and the TDT in the adult thorax (Fig. 1C). The arrangement of the myoblasts in the ventral and lateral thorax shows a characteristic 'forked' pattern (Fig. 6B) that probably is indicative of the paths that they take while migrating to their final sites. By 20 h APF, myoblast nuclei can be seen inside the developing

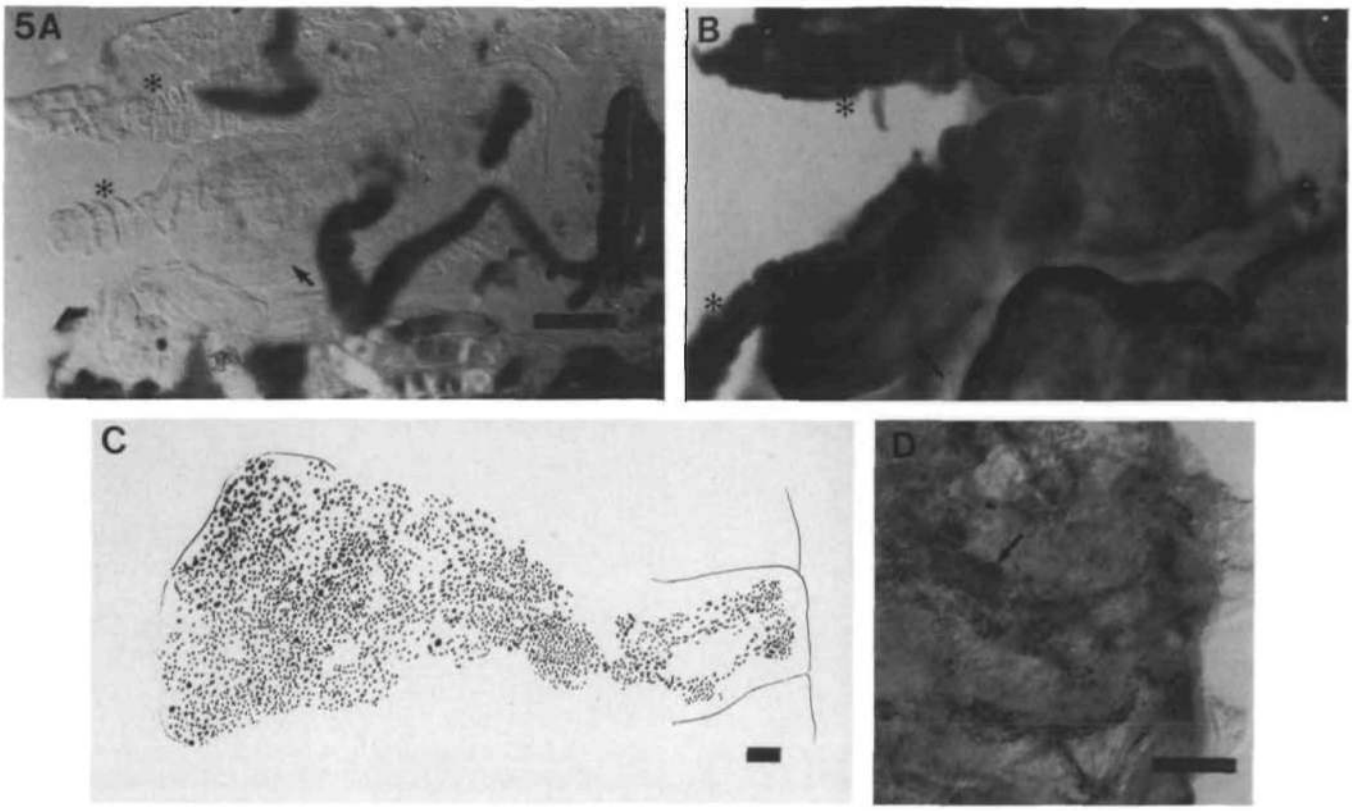

Fig. 5. Myoblasts, marked by the *twist* antibody, envelop the LOMs as early as 6 h APF and also accumulate in the ventral and lateral thorax. (A) 6 h pupa of the *MHC–lacZ* strain The everting wing disc (arrow) and the leg discs (asterisk, *) can be seen. The larval muscles stain blue and the LOMs are marked by a long arrow (B). 6 h pupa labelled with the *twist* antibody, with a large number of myoblasts (dots of antibody staining) surrounding the LOMs (not seen). These could be derived from the wing disc (arrow). Asterisks are adjacent to the everting leg discs. (C) Camera lucida drawing of *twist*-positive cells in a 6 h pupal thorax. Some of these cells are darker and larger than the rest. (D) *twist*-expressing cells in an 8 h pupa. The region that will give rise to the TDT is marked with an arrow. Bar=25 μm in all pictures. A, B show the left hemisegment; C, D the right hemisegment.

IFMs (both DLMs and DVMs) in plastic sections (Fig. 8A). Myoblasts that will give rise to the TDT can be seen as early as 8 h APF. These myoblasts can also be followed as they assemble to form the TDT (Figs 5D, 6B, 6E). Like the DVMs, there are no larval templates for the TDT.

The final steps in myogenesis: myoblast fusion and myofibre synthesis

At 24 h APF, well after 6 templates for the adult DLMs have already been established, *twist* antibody staining still reveals myoblasts around and between the developing fibres. Interestingly (Fig. 7A), there are intensely staining myoblasts at the base of a nerve that arrives at the DLMs, probably the posterior dorsal mesothoracic nerve (PDMN) that innervates the adult DLMs (Costello and Wyman, 1986). Possibly, myoblasts arriving to form the DLMs use this nerve as a path. At 30 h APF, myoblasts are still expressing *twist* (see Fig. 7C, D), even after the first myofibres have made an appearance (26 h APF). Most of the *twist*-positive activity, however, appears to be within the muscle, and interspersed between the myofibres. There is a marked shortening of the developing muscles at this stage. At 36 h APF, there is essentially no sign of *twist* expression and the early steps of adult flight muscle myogenesis are complete. The muscles now increase in size to complete the formation of the adult pattern as can be seen in later (48 h APF) sections shown in Fig. 8B and 8C.

Discussion

Formation of the adult pattern

The earliest events in the formation of the IFMs can be summarized as follows: adult myoblasts, derived from the embryonic mesoderm and associated with imaginal discs during larval life (Bate *et al.* 1991; Currie and Bate, 1991) begin to leave the everting discs as metamorphosis is initiated. These myoblasts, which are also called adepithelial cells due to their close association with the epithelium of imaginal discs (Poodry and Schneiderman, 1970), participate in the formation of adult muscle. With the onset of metamorphosis, larval bodywall muscles begin to histolyse. The histolysis, which at the onset removes most muscles of the anterior segments spares a set of muscles in the mesothorax, the

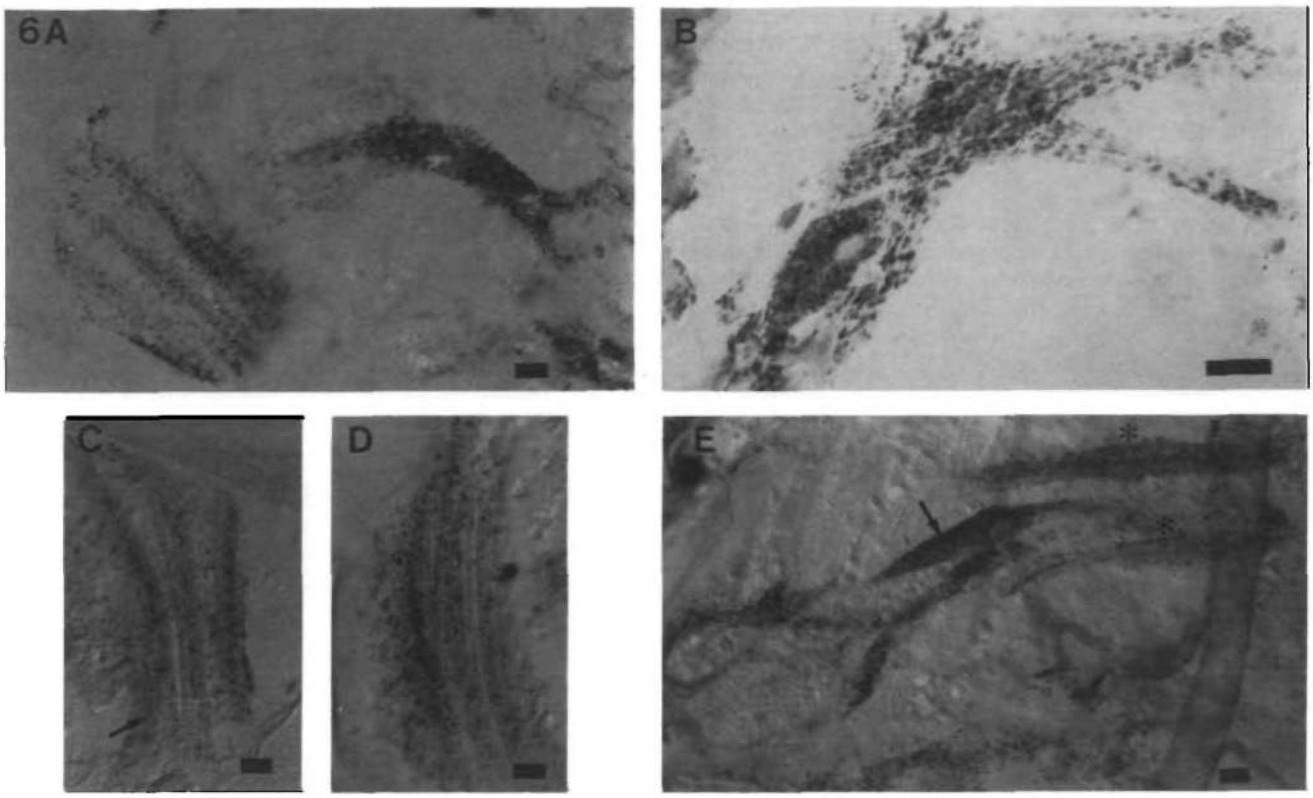

Fig. 6. The developing IFMs as seen by *twist* antibody staining to mark myoblasts. (A) A 14 h pupal thoracic hemisegment showing myoblasts enveloping each of the three pretemplates (left) two of which have started splitting (see Fig. 3B). The myoblasts also show a distinctive 'forked' organization in the ventral lateral thorax where the TDT and DVMs are forming (right). (B) Close up of the 'forked' pattern of organization of the myoblasts at this stage can be seen in the same animal as in A. The contralateral hemisegment is shown. These myoblasts will give rise to the TDT and DVMs. (C,D) 16 h pupal hemisegments of different pupae stained with the *twist* antibody. Arrow (in C) marks the DVM III that develops below the DLMs. Intensity of *twist* staining is greater around LOM1 (left of D), possibly an indication of the delayed splitting of this muscle. (E) 16 h. *twist* staining at the sites where the DVMs I and II develop (asterisk); the developing TDT (arrow) is seen in between the two DVM bundles. Bar=10 μm in all pictures.

LOMs, and these contribute to the formation of the adult DLMs. The LOMs are transformed into what we call the pretemplates (Fig. 3A). Myoblasts that initially surround the LOMs, now engulf each pretemplate as it splits, and give rise to the 6 DLM templates. We suggest that the LOMs have a similar function in myogenesis to that of the muscle pioneers in grasshopper embryos (Ho *et al.* 1983) because they span the region of the future DLMs. The adult DVMs, which, like the DLMs, are fibrillar indirect flight muscles, develop without any larval templates. Myoblasts migrate to the site where these muscles will develop, and fuse to form the muscle primordia. Shortly after splitting is completed, the IFM anlagen begin shortening. This coincides with the appearance of thickening within the muscles, which presumably are the first myofibres, and with the adult expression of *MHC–lacZ*. The muscles then elongate during the rest of pupal development, until they span the entire adult thorax.

The early events in IFM development, summarized above, take place during the first 36 h of pupal development, and fall into a distinct temporal sequence. During the first 8 h of development, the LOMs are clearly larval-like in appearance. In the next 4 h, they undergo a two-step transformation (vacuolation and elongation) into pretemplates, which subsequently undergo longitudinal cleavage. This splitting is complete by 20 h APF, after which the muscles increase in size. The adult-specific activity of the MHC promoter coincides with the onset of muscle shortening, at 26 h APF and myoblast incorporation continues even as the muscles begin to shorten. At 30 h APF, the IFMs are at their shortest and 6 h later they have begun growing out to span the adult thorax.

Despite their structural and functional similarity, the DLMs and the DVMs differ crucially in their development. The DLMs, as described above, are formed on transformed larval muscles. However, there is no obvious larval substratum for the DVMs. The lateral muscles found adjacent to the LOMs (see Fig. 1A) in the body wall of the third larval instar, would be good candidates for a larval substratum for the DVMs. However, in the early pupa, these muscles degenerate in the first wave of histolysis which spares the LOMs. That the DVMs do not have a larval substratum is substantiated by using the adult (IFM) specific *Actin–*

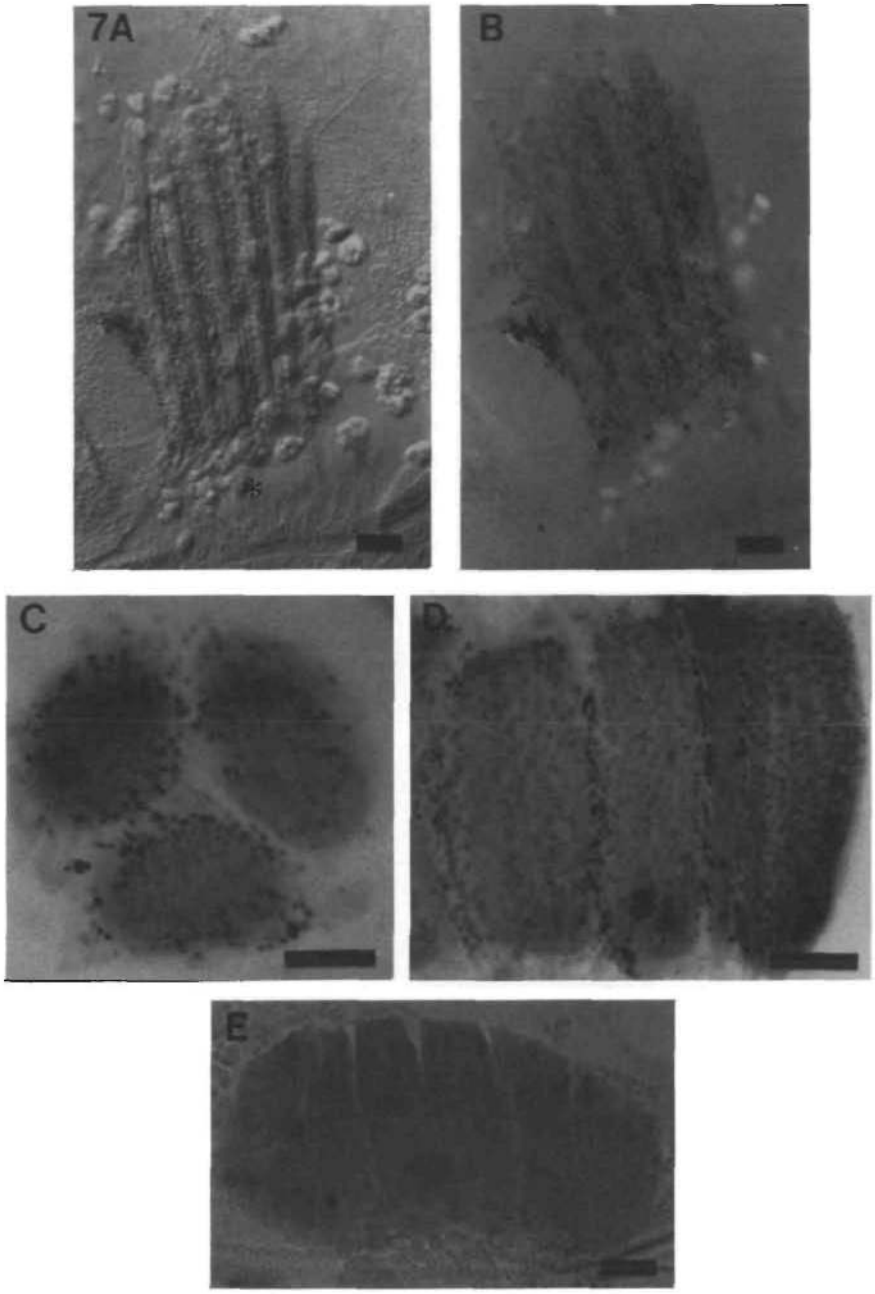

Fig. 7. The late expression of *twist*. (A,B) A 24 h pupal hemisegment labelled with the *twist* antibody at two slightly different planes of focus. A is in Nomarski optics and B in bright-field. (A) The outline of the LOMs that have split into six are clearly seen. Myoblasts can also be seen packed in the gaps between the six fibres. Some muscle attachment sites (asterisk) are also seen. (B) Myoblasts surrounding the split LOMs can be seen. Many myoblasts have already accumulated inside the LOMs and these have already started expressing the IFM *Actin–lacZ* gene, though *MHC–lacZ* expression in the adult muscles has not yet started (Fig. 4). (C) Close up of DVM I, 30 h APF. At this stage the preparation shows clearly the three component fibers of this DVM. *Twist*-positive cells (arrow points to some) still surround the muscles. (D) 30 h pupa showing 4 of the 6 DLMs. *Twist*-positive cells are seen in the gaps between the fibres while the signal has diminished greatly inside the developing DLMs. (E) At 36 h APF no *twist* expression is seen. Picture shows the DLMs. Bar=10 μm in all pictures.

lacZ transformant and the *MHC–lacZ* transformant, the latter being expressed in both larval and adult muscles. The developing DVMs can be seen with the *Actin–lacZ* transformant at a time (16–24 h APF) when they cannot be detected using the *MHC–lacZ* (see Fig. 3 and 4). *MHC–lacZ* can be detected in both DLMs and DVMs at a much later stage, indicating the onset of adult-specific promoter activity (Fig. 4F).

There are two distinct pathways of muscle formation in Diptera (Crossley, 1978). Muscles are either formed *de novo* by fusion of myoblasts, or alternatively, a residual myofibre derived from the delayed degeneration of a remnant larval muscle, serves as a scaffold for myoblast fusion. *De novo* muscle formation has been studied both in the *Drosophila* embryo (Bate, 1990) and pupa (Currie and Bate, 1991). Crossley (1972) reports in *Calliphora* that some larval muscles escape histolysis and are transformed into residual myofibres with which myoblasts fuse, releasing their nuclei into the fibre. In our study, we designate the transformed larval muscles as 'pretemplates', rather than 'residual myofibres', since it is the splitting of these pretemplates that gives rise to the 6 templates that will eventually become the 6 DLMs of the adult. For the formation of the IFMs in *Drosophila*, Shatoury (1956) describes the penetration of myoblasts into residual larval muscles, which are then converted into compact columns of myoblasts, the larval muscle attachments serving to anchor these columns. Shatoury's view is therefore one of invasion of larval muscle by myoblasts as opposed to fusion of myoblasts with a transformed larval muscle or a residual myofibre. Electron microscopic studies by

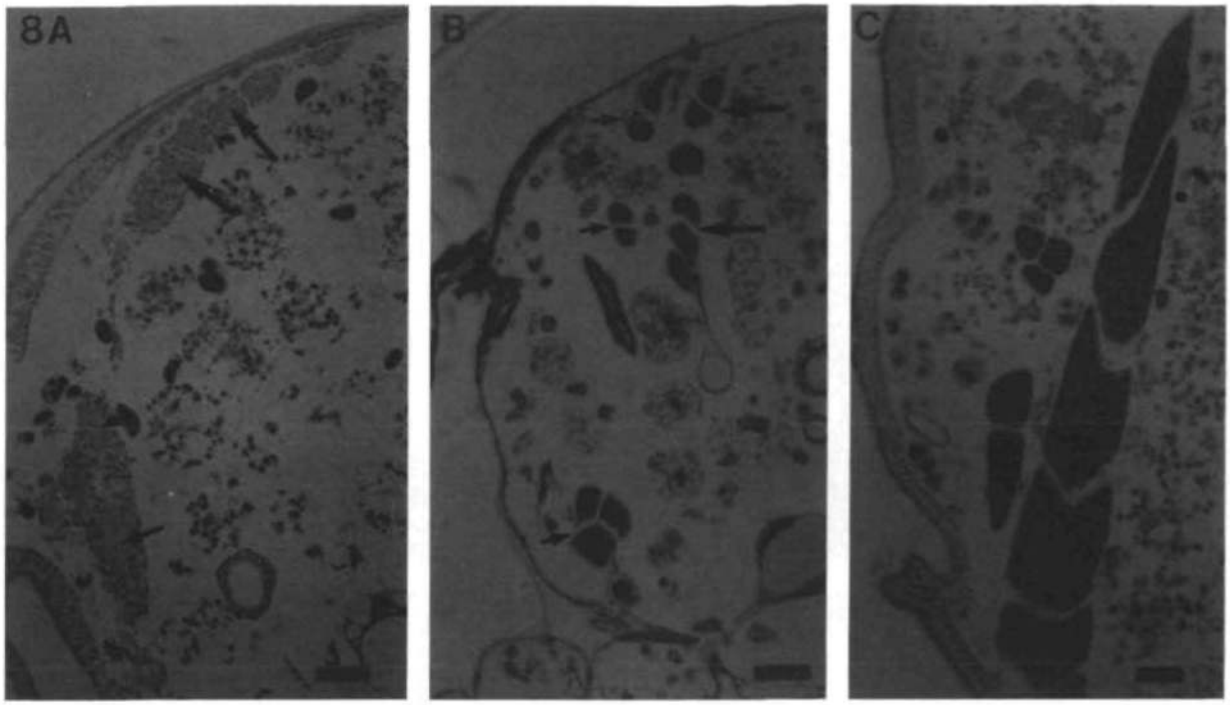

Fig. 8. Plastic sections of developing IFMs. (A) At 20 h, myoblast nuclei are seen inside the developing DLMs (long arrow) and DVMs (short arrow) and myoblasts still surround these structures. The other DVMs and the TDT are not present in this plane of section. Frontal section. (B) At 48 h IFM development is complete and the muscles continue to grow until emergence of the adult. DLMs (long arrow point to the region), DVMs (short arrow) and the TDT (asterisk) are shown. Frontal section. (C) A horizontal section, 48 h APF, shows the developed IFMs. Note the size of the 6 DLMs that go from the top to the bottom of the picture. The nuclei (light dots in the stained muscles) in the IFMs are arranged in characteristic rows. Bar=10 μm in all pictures.

Shafiq (1963), demonstrate fusion of myoblasts with one another. However, these studies are not clear about which muscle type was examined, namely, DLM or DVM. Development of DLMs in a Lepidopteran, *Pieris brassicae* has been shown to involve invasion of larval muscles by myoblasts (Cifuentes-Diaz, 1989). From our studies at the light microscopic level, it is not possible to demonstrate which of the modes of muscle formation are operative for the DLMs. However, the observation that *MHC–lacZ* activity does not diffuse from the LOMs, even when they are splitting, suggests that the membrane is intact, and hence points towards the fusion of myoblasts with the transformed larval muscle.

Origin of the IFM myoblasts

Histological studies have identified the adepithelial cells found in imaginal discs as the adult myoblasts (Poodry and Schneiderman, 1970; Reed *et al.* 1975). These myoblasts express *twist* and are segregated to imaginal disc precursors during embryonic development (Bate *et al.* 1991). Which of the imaginal discs is the source of myoblasts that make the IFMs? Although disc extirpation studies (Zalokar, 1947; Shatoury, 1956) implicate both the wing and the leg discs as possible sources, clonal analysis (Lawrence, 1982) suggests that IFM myoblasts are exclusively derived from the wing disc. Our studies show that, at 6 h APF, the evaginated wing disc is positioned directly over the region where the IFMs will develop, with some myoblasts clearly surrounding the LOMs and in our view it is likely that the IFMs are made from myoblasts leaving the wing disc. Myoblasts associated with the leg discs are seen to migrate ventrally into the everting leg, and dorsally into the thorax. Among the myoblasts that migrate dorsally, a group of closely packed cells which can be followed because of their characteristic appearance (Fig. 6B,D), are the likely precursors of the TDT. This view is also in agreement with the results of Lawrence's (1982) clonal analysis, which shows a separate origin for the IFMs and the TDT, despite their close physical proximity in the adult as well as during their pupal development.

Are myoblasts committed to form specific muscle types?

When myoblasts from the wing imaginal discs are transplanted into mature host larvae, they contribute to some muscles in the abdomen and thorax of the adult host (Lawrence and Brower, 1982). The results of this experiment imply that myoblasts in the wing disc are not restricted in their developmental potential, at least in the third larval instar. The clonal restrictions observed in adult muscle development (Lawrence, 1982; VijayRaghavan and Pinto, 1984) therefore suggest that there is a positional constraint imposed on adult myoblasts by their association with particular

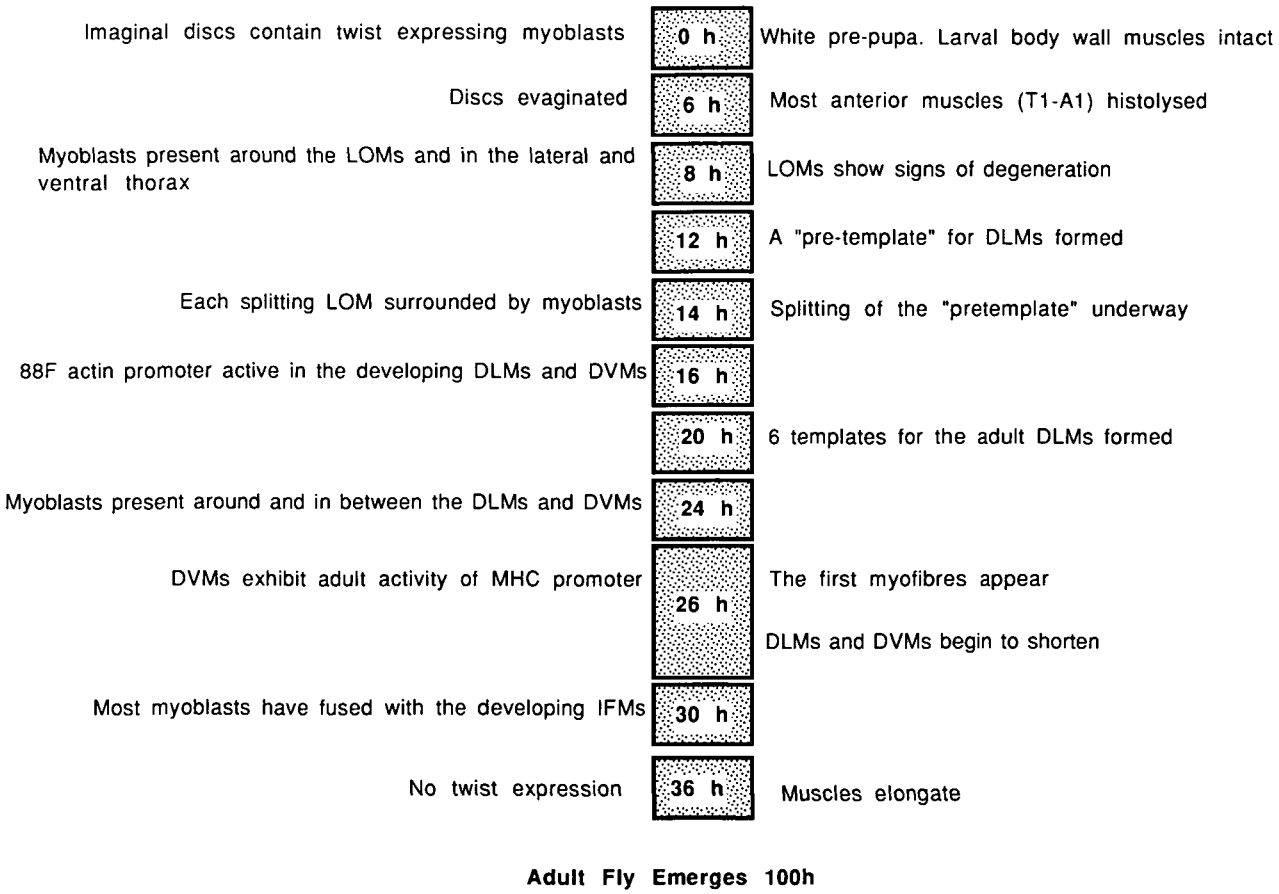

Fig. 9. Summary of events during IFM development, 0–36 h APF.

imaginal discs. It is evident that having left the disc, such a constraint would have to continue to operate on the myoblasts or they would not observe the restrictions demonstrated by Lawrence (1982). His analysis showed that clones of marked cells do not cross between the TDT and the IFMs. The TDT belongs to the ventral mesothoracic muscle set (mesothoracic leg disc) and the IFMs belong to the dorsal mesothoracic muscle set (wing disc). Nonetheless, during development (Fig. 6A,B) myoblasts that contribute to the IFMs are in close proximity to those that give rise to the TDT. If myoblasts are equivalent, what prevents those from the wing disc from contributing to the TDT? One possibility is that, as they leave their respective discs, the myoblasts follow strict nerve (Currie and Bate, 1991) epidermal, or tracheal pathways and that though the paths may at times be close, no mixing is allowed. Alternatively it may be that, unlike myoblasts from third larval instar discs, those in the pupa are committed, as they leave the disc, to form specific muscle types.

Lawrence's clonal analysis of the thoracic muscles showed that the IFMs and the direct flight muscles (DFMs), despite being structurally very dissimilar, are clonally related. If this is the case, there must be a mechanism that allows myoblasts arising from the wing disc to be distributed between these distinct sets of muscles. There could be distinct pathways that channel myoblasts to their appropriate destinations (these could be separated both spatially and temporally) and myoblasts could also be segregated after they reach the thorax. Possibly, the differences between the IFMs and the DFMs depend on such a prior segregation of myoblasts. The leg muscles and the muscles of the abdomen, both tubular muscles, develop later than the IFMs, whereas the TDT, which is also tubular, develops from precursors that are apparently segregating at the same time as those of the IFMs. We do not yet know whether the formation of the DFMs follows the pattern of the TDT, or of the other tubular muscles.

Myoblasts from the wing disc must also to be partitioned between the DLMs and the DVMs, and it may be that the presence of a larval substratum for the DLMs causes a segregation of some myoblasts to the LOMs. It is also posssible that among the population of myoblasts that leave the wing disc, there are founder cells that would recruit other myoblasts for the formation of the DLMs, the DVMs and the DFMs.

Role of nerves and the epidermis

In addition to a role in maintaining muscle (Costello and Wyman, 1986), nerves play an important part in their development. The formation of the male specific muscle in the 5th abdominal segment has been shown to be decisively influenced by the nerves that innervate it

(Lawrence, 1982). Adult abdominal muscle precursors are closely associated with nerves in the late embryo and the larva (Bate *et al.* 1991) and are distributed by the growing nerves to give the final abdominal pattern of muscles (Currie and Bate, 1991). It is likely that nerves are also involved in the development of the IFMs. As the wing disc evaginates, myoblasts are already found surrounding the LOMs, but it is not clear how they have reached the muscles. If nerves provide a pathway, adult sensory neurons cannot be involved because their axons leave the wing disc after it evaginates (Murray *et al.* 1984). It is possible therefore, that it is the larval nerves in the second thoracic segment that provide a route for myoblasts to reach the LOMs. Alternatively, there may be epidermal or tracheal pathways. Later-leaving myoblasts could use the developing adult innervation as pathways for distribution in the mesothorax.

Shatoury (1956) suggests that it is the myoblasts that bring about splitting of the LOMs. However, there are myoblasts around the larval substratum for at least 6 h before they surround each pretemplate as it splits. Thus, it is an additional factor, or another factor that initiates splitting of these muscles. The pattern of innervation of the LOMs is very distinct from that of the splitting LOMs (JF, unpublished observations) and it could be that it is the changing pattern of innervation that is decisive in initiating cleavage of the pretemplates. In this view, the rearrangement of LOM innervation would dictate the reordering of myoblasts on the larval templates. Thus myoblasts (because of an adhesive preference for nerves (Bate *et al.* 1991; Currie and Bate, 1991)) would move from a relatively disordered arrangement to an orderly one of six separate clusters, reflecting the new branching patterns of the adult motoneurons on the developing DLMs. This hypothesis has the additional virtue that it predicts the special characteristics (delayed cleavage) of LOM1 whose developing innervation is distinct from that of the other pre-templates (Ikeda, 1981). At about the same time that splitting of the pretemplates is initiated, a segregation of myoblasts between the DLMs and the DVMs also takes place. Here again, it might be that innervation is a decisive factor.

Despite the documented association of adult myoblasts with larval nerves (Bate *et al.* 1991; Currie and Bate, 1991), there are cases where myoblasts are clearly segregated in regions where there are no nerves. The mature wing disc is such a case (Bate *et al.* 1991), and we have shown that myoblasts leave the wing disc as it evaginates and presumably before an innervation has developed. In this case, it could well be that the epidermis provides cues for the distribution of the myoblasts. The adult dorsal thorax, which is spanned by the DLMs, is almost exclusively derived from the wing disc. Formation of the elongate pretemplates early in pupal development may depend on an active interaction between the forming muscles and the expanding adult epidermis, or may simply be a passive consequence of this expansion.

In this paper, we have provided a description of the early events of adult muscle development in the adult *Drosophila* thorax. We regard this work as an essential first step for a genetic and molecular analysis of the mechanisms of myogenesis and muscle patterning. Several genes have been identified, mutants in which affect the development of the adult IFMs (Deak *et al.* 1982; Fleming *et al.* 1983; Costello and Wyman, 1986). Our study of wild-type myogenesis is the basis for a detailed analysis of the developmental origin of adult phenotypes in IFM mutants and lays the groundwork for defining the wild-type function of these and other genes in adult muscle development.

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