A central role for epidermal segment border cells in the induction of muscle patterning in the *Drosophila* embryo

Talila Volk¹,* and K. VijayRaghavan²

¹Department of Molecular Genetics and Virology, The Weizmann Institute of Science, Rehovot 76100, Israel
²National Centre for Biological Sciences, TIFR, PO Box 1234, Indian Institute of Science Campus, Bangalore 560012, India

*Author for correspondence

SUMMARY

The correct patterning of muscles in the *Drosophila* embryo depends on the migration of developing muscles over the ectoderm and on the attachment of these muscles to specific attachment sites. We investigate the mechanisms that are involved in this process and describe experiments that allow a genetic dissection of the role of the ectoderm in muscle migration and attachment.

We show that cells along the segmental border in the ectoderm are used by the developing muscles to reach their attachment sites. These segment border cells are recognized by dissociated myotubes in single suspensions in culture. Thus, developing muscles have properties that allow the specific recognition of the segment border cells and migrate to attach to these cells. The segment border cells are absent in the mutant *wingless* and *naked*. In these mutants, the muscles are severely disorganized. We show that this is not a mere consequence of disruption of the epidermis, since, in the mutant *patched*, where segmental patterning is affected, the segment border cells are present near their normal position; the muscles in this mutant are relatively organized. Similarly, in the mutant *lines* where ectopic segment border cells are present, the observed muscle derangement correlates well with the ectopic attachment sites that are present.

Finally, we have analyzed mutants at the *stripe* locus and have shown that lethal alleles disrupt muscle organization during embryogenesis. Enhancer-trap alleles of *stripe* that we have analyzed show reporter gene expression in the segment border cells.

Our results indicate a role for the segment border cells in guidance of migrating muscle fibers to their attachment sites.

Key words: *Drosophila*, muscle patterning, muscle attachments, segment polarity genes, *wingless*, *patched*, *naked*, *lines*, *stripe*

INTRODUCTION

During *Drosophila* embryogenesis, a set of somatic muscles that forms a complex pattern on the inner surface of the larval epidermis is constructed. Each abdominal hemisegment from the second (A2) to the seventh (A7) has an equivalent set of 30 muscles. This precise patterning of the somatic muscles requires their correct attachment to the epidermis. Each muscle is attached to the epidermis by structures called apodemes. These are ectoderm derived, and are placed at regular intervals along each segment border. The intricate pattern of somatic musculature is completed by 13 hours after egg laying (Campos-Ortega and Hartenstein, 1985; Crossley, 1978).

Initial signs for patterning of the somatic mesoderm are evident shortly before germ band shortening, when the ventrolateral portion of the mesoderm becomes segmentally organized. During germ band shortening, somatic mesodermal cells migrate to their final positions within each segment and begin to fuse to founder cells; at the end of this process, myotubes are grouped together to form dorsal, pleural and ventral muscle precursor clusters. Three processes occur subsequently: fusion of additional mesodermal cells to the nascent myotubes, elongation of the fused cells and the directional migration of these myotubes towards their future attachment. During this second phase of migration, the myotube leading edge extends numerous filopodia. These filopodia are observed during the migration process, but not at a later stage when muscle attachment has been established (Bate, 1990, 1993).

The cellular and molecular mechanisms that direct the myotubes towards their attachment sites are not known. The clues that the filopodia on the leading edge of the myotube recognize on the epidermis, and the mechanism by which this recognition results with the correct migration of the muscle, are not characterized.

The ectoderm is thought to help maintain positional information for the correct migration and arrangement of the different types of myotubes (Bate, 1990). Experiments performed in other insects show that rotation of pieces of the ectoderm can induce changes in somatic muscle patterning (Williams and Caveney, 1980a,b). The patterning of the epidermis and cell fate determination in the ectoderm have already occurred by stage 12 (staging is according to Campos-
Ortega and Hartenstein, 1985). This is revealed by the pattern of expression of various segment polarity genes (Bejoove and Martinez-Arias, 1991). At this stage, the myotubes are yet to migrate from their initial clusters towards their final attachment sites on the epidermis. It is, therefore, suggestive that the epidermis at this time provides cues to guide these migrating myotubes. However, this hypothesis is yet to be proven.

The experiments of Williams and Caveney (1980a,b) suggest a polarity in the epidermal cues that direct a muscle towards its final attachment site on the epidermis. To examine whether such a polarity of cues exist on the Drosophila epidermis, and identify the cells involved, we have used antibody and reporter gene markers to study muscle attachment in mutants affecting segment polarity. The positions of cells that are candidates for guiding the developing muscle are altered or these cells are absent in the segment polarity mutants that we have tested. These mutants thus allow us to test the relevance of the position of segment border cells in the development of muscle attachment.

We also demonstrate that cells along the segmental border function to regulate muscle migration and attachment. This is shown by analyzing muscle defects in an enhancer trap lethal allele of the gene sr. Viable allelic combination of sr affect the development of the adult indirect flight muscles (Costello and Weyman, 1986; de la Pompa et al., 1989). The availability of enhancer trap alleles of sr allowed the comparison of the phenotypes observed with the expression pattern of the gene. We show that, in the embryo, sr is expressed in the segment border cells and mutants at this locus affect muscle patterning.

Our experiments thus identify cells on the epidermis whose position is important for muscle patterning and demonstrate a function for these cells. These results allow us to propose specific mechanisms by which migrating myotubes are guided by specific cues presented by the segment border cells.

MATERIALS AND METHODS

Fly strains

The following fly strains were used: wg^{1L114}, ptc, nkd^{6F}, limp^{16}, sr. They were obtained from the Bloomington stock center. The sr^{B14.0} strain (obtained from A. Kolodkin and C. Goodman; Bellen et al., 1992) carries a P-element insertion at chromosomal position 90E where the stripe gene is mapped. sr^{B14.0} homozygous are viable and show very weak whorls on the dorsal thoracic bristles, a characteristic of some hypomorphic sr alleles. sr^{B14.0} fails to complement sr mutants. We have generated a large number of other sr alleles by ‘local hopping’ of the P element at B14.0. These sr alleles were isolated by screening for a dark thoracic stripe, characteristic of stronger alleles of sr, in trans heterozygous with a viable sr allele. The sr allele sr^{VJ54} (referred to as VJ54 in the text) was isolated in this screen and is homozygous lethal. It fails to complement lethal alleles of sr induced by EMS, for lethal phenotype. Flies that carry VJ54 chromosome in trans with a viable allele of sr show strong dorsal thoracic stripe and these flies do not have the dorsal longitudinal muscles (K. VijayRaghavan, unpublished observations).

In order to distinguish between mutant and wild-type embryos, the balance chromosomes used were marked with a fzt-lacZ P-element.

Immunological reagents

To visualize embryonic muscles, we used anti-MSP polyclonal antibodies that were previously described (Volk et al., 1990). The serum was usually diluted 1:500 for staining.

Anti-groovin monoclonal antibodies were raised in mice that were immunized with membrane proteins from 0-16 hours old embryos. Details on the method used for immunization, and for screening of hybridomas will be published elsewhere (Strumpf and Volk, unpublished data).

Secondary antibodies used included goat anti-guinea pig, and HRP or rhodamine-conjugated goat anti-mouse IgM, or anti-guinea pig IgG (Jackson).

Embryonic cultures

Primary embryonic cultures were obtained essentially as described (Volk et al., 1990). In brief, embryos (6-9 hours old) were dechorionated, washed extensively and dissociated into a single-cell suspension using a 7 ml PYREX homogenizer (Corning no 21). Following several washes with Schneider medium, the cells were seeded on laminin-coated coverslips. (Drosophila laminin was kindly provided by Drs L. and J. Fessler, UCLA). Cultures were maintained for one or two days. The cells were then washed with PBS, fixed and permeabilized using a mixture of 3% paraformaldehyde and 0.5% Triton X-100 for 20 minutes, and then immunofluorescently labeled.

Whole-mount embryonic staining

In addition to the HRP (see below) staining to determine expression of the appropriate markers, we routinely stained the embryos collected from the different mutant lines for β-galactosidase activity, using X-gal as a substrate. Embryos that did not stain with X-gal were identified as homozygous mutant embryos.

Staining was performed essentially as previously described (Ashburner et al., 1989). In brief, embryos were collected and incubated as indicated, dechorionated and fixed with a mixture of 3% paraformaldehyde and heptan. Following two washes with PBT (PBS containing 0.1% Triton X-100), embryos were incubated in the X-gal staining solution, until blue staining was visible (15-30 minutes, at 37°C), and then washed and devitellinized with a methanol-heptan mixture. Permeabilization was performed by incubation in PBT containing 10% BSA for 2-3 hours, and incubation with primary antibodies was usually performed for 16 hours at 4°C.

In situ hybridization

In situ hybridization was performed essentially as previously described (Tautz and Pfeiffle, 1989), except for X-gal staining that was performed before the devitellinization step. The probe used for in situ hybridization was a 1.1 kb RI fragment of nautilus, kindly obtained from Dr Michelson (Harvard University), labeled by random priming with DIG-dUTP (Boehringer, Mannheim).

Sections

Stained embryos were dehydrated, washed in ethanol and infiltrated with JB-4 embedding media (Polysciences, Inc, USA) according to the manufacturer’s instructions. Embryos were oriented in molds and the resin was allowed to harden in a desiccator. Sections (2-3 μm width) were obtained with a Sorvall MT2B microtome, mounted on slides and examined under a Zeiss Axioscope microscope.

RESULTS

The segmental groove cells specifically express the groovin marker, and are recognized by myotubes in culture

During development, myotubes migrate to the segment boundary and reach their attachment sites. Thus, cells that are close to or along the segment boundary could be involved in signalling the migrating myotubes, thus providing them with directional cues to reach their targets. We have isolated a mon-
Segment border cells induce muscle patterning

61

oclinal antibody to a surface protein that is expressed in the cells along the segmental groove. This marker, called groovin (Strumpf and Volk, unpublished data) allows the study of the development and function of the segment border cells in vivo and in vitro, and to test their roles as positional cues for the development of muscle attachment.

Whole-mount embryonic staining indicates that groovin is specifically localized to the surfaces of a row of cells located at the segmental groove, as shown in Fig. 1A,B. Additional staining is detected along the epithelial cells of the salivary glands, and on a group of cells in the lateral ectoderm region of each segment. Groovin is initially expressed at the end of stage 12 (staging is according to Campos Ortega and Hartenstein, 1985) in germ-band-retracted embryos. At stage 16, groovin is expressed by the ectodermal cells located at all muscle insertions, including both intrasegmental apodemes located at the ventral side of the embryo. Microscopic analysis of semi-thin sections of embryos stained with anti-groovin indicates that the protein is localized to the lateral and basal surfaces of cells located at the segmental groove (Fig. 1B).

To characterize further the ability of the myotubes to recognize groovin-expressing cells, we studied the formation of heterotypic aggregates between the two types of cells in primary embryonic cultures. Dissociated embryos at the end of gastrulation were cultured on laminin-coated coverslips as described (Volk et al., 1990). Formation of contacts between the myotubes and cells that were stained with the anti-groovin antibody was studied. The myotubes in the primary culture appear as multinucleated elongated cells, as shown in Fig. 1D. These myotubes form contacts exclusively with non-muscle cells that express groovin (Fig. 1C,D). Several multinucleated myotubes were often observed forming contacts with clusters of groovin-positive cells at both their ends (Fig. 1C,D). This observation suggests that under culture conditions the nascent myotubes specifically recognize and form contacts with cells from the segmental grooves, even in the absence of positional information available from other ectodermal cells.

Analysis of muscle patterning in segment polarity mutant embryos

At the end of germ band retraction, somatic muscle precursors begin to migrate towards their attachment sites from their initial clusters to form the repetitive final muscle pattern characteristic of each segment. This directional migration precedes the formation of stable contacts with the ectoderm, and may be controlled by specific interactions with the epidermis.
Fig. 2. Defects in muscle patterning visualized in nkd mutant embryos. Whole-mount staining with anti-groovin (A,E) or with anti-MSP-300 (B,F) of embryos at stage 14. Muscle organization visualized in semi-thin sections (2 μm thickness) of MSP-300-stained embryos (C,G). Notice that groovin staining along the segmental grooves is deleted in the nkd mutant embryo, and the myotubes are disorganized, but still attached to the basal surfaces of the ectoderm. Visualization of nau-positive muscle clusters in stage 12 embryos, by in situ hybridization with nau cDNA (D,H). The ventral and dorsal nau-positive clusters in the nkd embryo are almost normal, while the lateral precursors show weaker staining. A-D are wild-type embryos, and E-H are nkd mutant embryos. Bar indicates 10 μm.
We analyzed the contribution of the different parts of the epidermis within each segment to the induction of myotube directional migration and the formation of normal muscle patterning. To this end, we studied the extent of the disruption of muscle organization in embryos mutant for various segment polarity genes. Staining of these mutant embryos with anti-groovin antibodies helped us to estimate the degree of disorganization of the segment border cells. To ensure that the location of the initial muscle precursor clusters were not significantly altered, the mRNA expression of *nautilus* (*nau*) was analyzed in the different mutant embryos by in situ hybridization. *nautilus*, a gene related to the family of vertebrate myoD like genes, is expressed at the three somatic muscle precursors clusters (dorsal, ventral and pleural), prior to their migration (Michelson et al., 1990).

We chose one representative mutant from each of the four classes of segment polarity genes that affect different parts of the ectoderm within a given segment (Hooper and Scott, 1992). The *naked* mutant represents the first class, in which the anterior pattern elements are replaced with a naked cuticle (Jurgen et al., 1984). In *wingless* (*wg*) mutants, the ventral naked cuticle (posterior element) is deleted and is replaced by denticles (Nüsslein-Volhard and Wieschaus, 1980; Nüsslein-Volhard et al., 1984). *patched* (*ptc*) embryos represent a third class in which the central pattern elements are deleted and replaced with segment border pattern elements (Nüsslein-Volhard and Wieschaus, 1980). *lines* (*lin*) represents the fourth class, in which mutations cause the absence of the anterior part of each denticle band (Nüsslein-Volhard and Wieschaus, 1980; Nüsslein-Volhard et al., 1984). The degree of muscle patterning, and the structure of muscle attachment sites, was studied in embryos mutant for each of these segment polarity genes. Visualization of somatic muscles was carried out by staining the embryos with anti-MSP-300, a muscle-specific protein belonging to the spectrin superfamily (Volk, 1992).

**Muscle phenotype of *naked* mutant embryos**

The staining pattern of groovin along the cells of the segmental border in *naked* mutant embryos is severely altered. As shown in Fig. 2E, most of the staining along the segmental borders is abolished, while the staining at the salivary gland, and in the cell clusters at the lateral portion of the ectoderm, is not affected. The observation that the cells at the segmental border are not differentiated correctly in *nkd* mutant embryos is consistent with the cuticular phenotype seen at the end of embryogenesis in *naked* mutants. The muscle organization in *nkd* mutants, as visualized by MSP-300 staining, is severely affected. As shown in Fig. 2F, the entire organization of somatic muscles is disrupted, and myotubes are scattered along the ectoderm in a disordered configuration. The longitudinal myotubes, although fused and elongated, are scattered in the *nkd* mutant embryo, ignoring the attachment sites (Fig. 2G).
addition, the myotubes are more elongated, and contain larger numbers of nuclei as compared to the wild-type embryo (data not shown).

In situ hybridization to mRNA in whole-mount embryo using *nautilus* antisense as a probe, indicated that the level of organization of muscle precursor appears almost normal at the dorsal and ventral clusters, at stage 12. Staining of the lateral precursors is very weak, and in some cases is abolished as compared to the wild type (Fig. 2H). Since the organization of the *nau*-positive muscle clusters, at the dorsal and ventral aspects of the embryo, is not significantly altered, we conclude that the severe alterations in myotube arrangement observed in nkd mutants occurs subsequent to stage 12. These results suggest that correct epidermal patterning, regulated by the nkd+ product, is required for directing migrating myotubes. Groovin immunoreactivity is almost abolished in nkd mutants (Fig. 2E), suggesting that the segment border cells in these mutants have not differentiated correctly; the muscle phenotype of nkd mutants, therefore, suggests that the groovin-positive cells may be critical components in the guidance of migrating myotubes.

Muscle phenotype of *wingless* mutant embryos

Analysis of wg mutant embryos, stained with anti-groovin antibody, indicates that all staining along the segmental border is lost (Fig. 3A). Staining in the salivary glands is normal. This observation suggests that the cells at the segmental border do not differentiate correctly or are completely absent. The posterior part of the segment in wg mutant embryos is defective. This was shown by cuticle preparations (Nüsslein-Volhard and Wieschaus, 1980; Nüsslein-Volhard et al., 1984), as well as staining with antibodies to markers such as engrailed and patched (Dinardo et al., 1988; Lawrence and Johnston, 1989; Martinez-Arias et al., 1988; Rijsewijk et al., 1987; van der Heuvel et al., 1989). The somatic muscle organization in the wg mutant embryos is severely impaired, as shown in Fig. 3B. Myotubes are scattered along the ectoderm without any orientation. Often the myotubes are very elongated, as if they continue to fuse multiple myoblasts (Fig. 3C). Interestingly, muscle patterning in wg mutant embryos is reminiscent of nkd embryo patterning; both exhibiting significant reduction in the segment border cells.

Analysis of the muscle precursor organization in wg mutants, as visualized in situ hybridization with a *nautilus* antisense RNA probe in whole-mount wg embryos, indicates that derangement of myotubes is already notable at stage 12 embryos. At this phase, the ventral muscle precursor clusters are missing, while the dorsal and lateral rows are present, suggesting that wg+ product is essential for gene expression in the ventral mesoderm. In line with this observation, ventral
myotubes are missing at later stages. However, it is important to emphasize that the remaining nau-positive muscle precursors, detected at stage 12 wg mutant embryos, are nevertheless arranged in a segmental pattern and the initial dorsal and lateral muscle clusters are observed. Therefore, a significant portion of the aberrations in muscle organization in this mutant may occur later than stage 12, namely, during directional migration of the myotubes.

**Muscle phenotype of patched mutant embryos**

The segment border region is duplicated in the ptc mutants and, in addition, the central part of the segment is not differentiated properly (Hooper and Scott, 1989; Nakano et al., 1989; Nüsslein-Volhard and Wieschaus, 1980; Nüsslein-Volhard et al., 1984). These alterations may result from the ectopic distribution of wg and en, described in these mutant embryos (Martinez-Arias et al., 1988). This description is supported by the staining of ptc embryos for groovin, in which an additional row of groovin-positive cells appears at the segmental border (Fig. 4A). Although a significant part of the central region of the segment is deleted, muscle derangement is less severe than that noticed in nkd and wg mutants (Fig. 4B,C). The pattern of longitudinal muscles and their attachment to the ectoderm is not significantly altered. Longitudinal myotubes attach to both rows of groovin-positive cells and, as a result, the attachment region is broadened (Fig. 4C). This observation implies that the initial directional migration of the myotubes towards their future attachment sites occurs, despite the disorganization of the central part of the segment exhibited by the ptc embryos. Duplication of the cells at the segmental border does not profoundly affect either myotube directional migration or attachment with the ectoderm.

The arrangement of muscle precursors in the ptc mutant embryos at stage 12, as indicated by in situ hybridization with a nautilus antisense RNA probe, is almost normal. Occasionally, a few of the clusters of the muscle precursors are not organized in a symmetric pattern, as found in wild-type embryos (Fig. 4D).

Thus, it appears that, although a significant portion of the central part of each segment is defective in ptc mutants, directional migration of the myotubes is not significantly altered. In addition, the structure of the muscle attachment sites in these mutants is basically normal.

**Muscle phenotype of lines mutant embryos**

*lines (lin)* represents a class of mutants in which a small portion of the anterior part of each denticle band is missing. Analysis of lin embryos, stained with anti-groovin antibody, revealed that, while at the ventral region of the embryos the staining for this protein is normal, the dorsal part of mutant embryo exhibits two rows of labeled cells (Fig. 5A). However, unlike the case of the ptc embryos, the two rows of groovin-positive
cells are separated by a row of groovin-negative cells. The remainder of the ectoderm is apparently normal.

Analysis of muscle organization in \textit{lin} mutant embryos was very informative, since the segment border cells are ectopically arranged within an almost normal ectoderm. In addition, the ventral side of the embryos, which exhibits subtle abnormal organization of the denticles seen at the end of embryogenesis, expresses groovin normally. This indicates that, although some cells apparently do not differentiate properly, the cells at the segmental border do differentiate correctly. The muscle patterning in the \textit{lin} embryos is normal at the ventral part of the embryo and the attachment sites are organized in a wild-type pattern. However, abnormalities are detected at the dorsal part of the embryo (Fig. 5B,C), at the same locations where groovin is ectopically expressed. Analysis of sections at the dorsal region of embryos labeled for both groovin and MSP-300 indicates that the myotubes attach to all the groovin-positive cells, creating a disorganized pattern at this region (Fig. 5C). Interestingly, the myotubes in this case are shorter, suggesting that attachment induces cessation of the fusion process. The organization of muscle precursors as revealed from \textit{nautilus} expression is normal (Fig. 5D). The ectopic segment border cells, therefore, do not affect muscle precursor clustering at stage 12 embryos; however, it directly affects the arrangement of myotubes in the vicinity of these cells.

In summary, our results are consistent with a novel mechanism through which the segment border cells are capable of inducing, the underlying myotubes to migrate and contact these cells. In addition, these results suggest that the formation of muscle-ectoderm attachment is related to the cessation of myotube fusion.

The \textit{stripe} gene is expressed at the segment border cells and affects muscle patterning

The defects in muscle patterning observed in mutants that either do not exhibit segment border cells, or express them ectopically, imply a central role for these cells in the directional migration of myotubes. To examine this hypothesis more directly, we analyzed the degree of muscle organization in embryos mutant for the \textit{stripe} gene, which is specifically expressed in the segment border cells.

Viable allelic combinations of \textit{sr} mutants are affected in the development of the indirect flight muscles (Costello and Wyman, 1986). The defects seen suggest that muscle attachment to the epidermis is defective (see also Discussion). We generated stronger insertional alleles of \textit{sr} by ‘local hopping’ of the P-insertion (Cooley et al., 1988; Tower et al., 1993) in B14.0 (see also Materials and methods). Analysis of the reporter β-gal staining pattern in embryos of these strains revealed that the most prominent staining is located at the segment border cells. Embryos at stage 11 show broad β-gal segmental staining (Fig. 6A). From stage 12, towards the end of embryogenesis, the β-gal staining is prominent at the segment border cells, and at additional small clusters of cells in the ectoderm as well (Fig. 6B,C). Examination of sections of the β-gal-stained embryos revealed that the staining is restricted to the ectoderm and ectodermal derivatives (Fig. 6D). Muscle organization in one P-element (VJ-54) insertion line was severely impaired. All the myotubes were scattered and the muscle patterning was significantly disrupted (Fig. 7B,C). The myotubes ignore their attachment sites and form contacts either with other myotubes, or with cells of the visceral mesoderm (Fig. 7C). VJ54 fails to complement \textit{sr16}, a small
deficiency that covers the sr chromosomal region for this phenotype. These data suggest that the sr gene function is involved in the induction of normal directional migration of myotubes.

Interestingly, groovin expression by these mutants is only slightly disrupted. Staining with anti-groovin reveals a row of segment border cells, although the pattern of these cells is not completely normal (Fig. 7A). The clusters of nau-positive muscle precursors appear at their normal positions (Fig. 7D). Staining of embryos at stage 12 with anti-engrailed antibodies show normal stripes (not shown), indicating that sr is not a late segment polarity mutant. These data suggest that the sr gene function is carried out in embryos after stage 12 and is involved in the induction of normal myotube directional migration. Although the segment border cells are positive for groovin, they may be defective in maintaining the appropriate signals for myotube migration.

It was important to verify that, in P-insertion line VJ-54, the P-element hopped within the sr locus and did not, in any way, affect other genes. Subsequently, we performed the following analyses. (1) We found that the beta-galactosidase staining pattern of VJ-54 is identical to the sr pattern found in the other P-insertion lines, although it is weaker. This suggests that the P-element insertion in VJ-54 responds specifically to sr enhancer elements. (2) Southern blot analysis of genomic DNA from VJ-54 flies, probed with Bluescript plasmid sequences (which are included in the P-element insert), indicated that there is only a single P-element insertion in this line: its location differs from that of the parental B14 P-insertion (not shown). Thus, it appears that the P-element hopped from its original (B-14) site to a second location where it is also under the control of the sr enhancer. (3) This P-insertion line induced the severe muscle phenotype, observed in the mutant embryos, was deduced from a reversion of the P-insertion, following a digenic cross. Reversion of the severe muscle phenotype and embryonic lethality, seen in line VJ-54, could be induced; the revertant was homozygous viable. However, it exhibited the adult viable sr phenotype (a dark stripe in the thorax). All other excision lines that carried white eyes also exhibited the adult sr phenotype when crossed with viable sr alleles. These results suggest that imprecise excision at the initial B-14 location occurred in the generation of the VJ-54 line, but the second insertion in the sr locus induced the severe embryonic muscle phenotype.

In conclusion, the embryonic phenotype of the sr mutation primarily, and significantly, affects muscle patterning without affecting groovin expression. The expression of the β-gal reporter gene in the enhancer trap alleles of sr is mainly expressed at the segment border cells, supporting the hypothesis that these cells are crucial for induction of normal myotube migration. stripe may be involved in that signalling process.

DISCUSSION

The cellular and molecular mechanisms that instruct the
precise patterning of muscles are, as yet, unknown. The ectoderm has most likely a major role in this process (Williams and Caveney, 1980a,b). To dissect the cellular components that direct myotube migration towards their future attachment sites, we analyzed the degree of disruption of muscle patterning in embryos mutant for different segment polarity genes. Such mutant embryos exhibit deletions or alterations in the state of differentiation and arrangement of cells within each segment. Our observations suggest that the cells located at the segmental groove are of major importance for the induction of muscle patterning.

The formation of heterotypic aggregates between myotubes and groovin-positive cells in the culture system indicates that both cell types are able to recognize and contact each other. Since most of the embryonic cells in the primary culture are seeded as a single cell suspension, we assume that these aggregates were initiated by the migration of one cell type towards the other. Interestingly, this migration is not dependent upon the positional information provided by the intact segmental ectoderm; this process supports a mechanism by which the segment border cells provide positive signals to which myotubes respond. The specific interactions formed between the myotubes and the groovin-positive cells in vivo are not carried out simply because both cell types express specific integrin receptors on their surfaces. This conclusion is based on our previous analysis of muscle organization exhibited in the myospheroid, an integrin β-chain null mutant, in which neither integrin heterodimer is functional (Leptin et al., 1987, 1989; MacKrell et al., 1988; Wright, 1960). Detailed observation of myotube arrangement in these integrin mutant embryos indicated that correct muscle patterning is initially retained (T. Volk, unpublished observations). It is only later in development, when these muscles start to contract, that the myospheroid phenotype (rounded muscles) becomes apparent (Newman and Wright, 1981; Wright, 1960). Thus, directional migration of the myotubes towards their specific attachment sites precede, and is independent of, the formation of stable muscle attachments with the ectoderm.

**Cells at the segmental groove participate in the induction of muscle patterning**

The utilization of the anti-groovin antibody as a marker for the proper arrangement and differentiation state of the cells at the segmental grooves was essential for this study. Using this antibody, we could correlate the differentiation state of the cells at the segmental groove and the severity of the muscle phenotype. In both wg and nkd mutant embryos, there is no staining by anti-groovin antibody, suggesting that the cells at the segmental border are missing. These mutants show the most severe muscle disorganization. Myotubes are scattered along the inner faces of the ectoderm without any apparent pattern. Interestingly, the myotubes adhere to the ectoderm along their entire surface. This may suggest that the basal surfaces of the ectoderm between segmental borders are capable of maintaining adhesion of the myotubes, and is consistent with positive signals presented by the targeted attachment sites.

Since in both wg and nkd mutants additional domains of the ectoderm are defective, it was difficult to deduce which part of the ectoderm is crucial for the induction of normal myotube migration. To understand further the mechanism by which myotubes respond to cues set out by the different cells that compose a segment, we analyzed muscle organization in ptc embryos. In these mutant embryos, the segment border cells are present, although in a duplicated fashion. In addition, cells of the central part of the segment exhibit aberrant differentiation (Martinez-Arias et al., 1988).

The myotube directional migration in the ptc embryos was apparently less affected, despite the disorganization exhibited by the central part of the segment. The overall organization of the myotubes was less aberrant than that observed in nkd and wg mutants. Apparently, the duplication of the groovin-positive cells at the segmental border does not significantly affect the arrangement of myotubes around the segmental groove. This result raises the possibility that once most of the inductive signal/s are available, and are located at their normal position, a major portion of myotube directional migration is carried out normally.

Additional support for this conclusion is evident from the analysis of the lin mutant embryos. Here, groovin-positive cells are duplicated at certain locations along the ectoderm. The ectopic groovin expression, unlike that in ptc mutant embryos, is found along a second row of cells located 2-3 cells apart from the normal segmental border. Arrangement of the myotubes in the lin mutant embryos is abnormal only around the cells which ectopically express groovin. In these regions, the myotubes are shorter and attach to all the groovin-positive cells. This observation supports our suggestion that signalling occurs before formation of attachments, and the abnormal directional migration of the myotubes induced in the lin mutants results from the second signalling source visualized by the ectopic groovin-positive cells.

An additional interesting result concerns the number of nuclei present in each of the different mutant myotubes. Elongated myotubes, which contain a large number of nuclei, were observed in nkd and wg mutants. The myotubes in these embryos do not form the characteristic muscle attachments. This suggests that cessation of the fusion process is related to the formation of muscle-ectoderm attachments. The relatively short myotubes, which contain very few nuclei, observed in lin mutants support this hypothesis as well.

The ectoderm may influence the underlying mesodermal layer in three different fashions: direct cell interactions between the ectodermal and mesodermal cells lead to the activation of genes in the mesodermal cells; ectodermal genes such as ptc and wg may be active autonomously in the mesoderm, activating mesoderm-specific gene expression; a different mode of induction by the ectoderm may be carried out following differentiation of the ectoderm (stage 12 embryos). At this stage, cells at the ectoderm may specify positional signals that will direct the myotubes towards their specific attachment sites, enabling the development of the complex muscle pattern formation.

In the present study, we characterized the influence of the ectoderm on the directional migration of myotubes carried out following their initial clustering. Formation of the correct muscle patterning was examined following the differentiation of the ectoderm. The naa expression pattern in stage 12 embryos indicated, in our study, the degree of derangement carried out in the different mutants at an early stage of myotube development. No major abnormalities were observed in
Segment border cells induce muscle patterning

...embryos mutant for ptc and lin, while in wg mutant embryos the ventral muscle precursor clusters were deleted and, in nkd mutants, the lateral clusters were not detected. The organization of the remaining clusters in both wg and nkd mutants, however, was not significantly altered. It was, therefore, assumed that the majority of the defects noted in the various mutant embryos indeed resulted from the effect of the ectoderm on myotube directional migration and elongation.

While most of the myotubes attach to the ectoderm at the segmental border sites, some of the pleural muscles do not attach to the ectoderm at these sites (these include pleural muscles 21, 22, 23, 24 and 18). These muscles may use other cues provided by either the ectoderm, or other myotubes for their arrangement. The ventral oblique muscles (15, 16, 17) are the only ones that cross the segmental border. These muscles send their filopodia towards the posterior segment and towards the midline. Here, the specific environment of the CNS may play a role in guidance. We noted, however, that groovin-positive cells at this region are located also across the segment border at the future attachment sites of these muscles and stain for β-galactosidase in the sr enhancer trap line. Thus, at the time when the ventral oblique muscles send their filopodia across the segment border, the cells that express both groovin and sr gene product are the cells at their targeted attachment sites.

There is not enough information available regarding the expression and possible autonomous action of the segment polarity genes in the mesoderm. It is possible that the observed defects in muscle organization in the various mutants resulted partly from a direct mesodermal function of the segment polarity genes. In addition, alterations in myoblast gene expression induced by the segment polarity genes before stage 12, could also result in muscle defects. Additional studies using early mesodermal markers are needed to analyze earlier effects of the ectoderm on mesoderm fate determination.

Taken together, this analysis suggests a central role for the cells at the segmental border in the induction of myotube directional migration and muscle patterning. Additional support for this conclusion comes from the analysis of the muscle phenotype in sr mutant embryos. The reporter gene expression pattern in B14.0 may reflect the correct expression pattern of the sr locus. First, the expression pattern seen is consistent with the depicted muscle attachment phenotype. All of the local hop alleles of sr that were isolated stain in the same manner as B14.0. The mutant Transabdominal (Tab) results from a chromosomal inversion of the AbdB gene into 90E, inducing mis-expression of this gene in the developing animal (Celinker and Lewis, 1993). Transabdominal mutants fail to complement sr, which results in the expression of the AbdB gene in presumptive attachment sites of the adult indirect flight muscles and in the attachment sites of the larval body wall muscles. This pattern of expression of AbdB in Tab animals is exactly the same as that of the reporter gene in V154 and B14.0 (Fernandes et al., unpublished data). The sr gene, is therefore primarily expressed at the segmental groove cells at the embryonic stage, and profoundly affects the formation of correct muscle patterning. The sr gene product may play an important role in the formation of the correct adult muscle patterning. The expression pattern of the reporter β-galactosidase gene in the B14.0 allele of sr in larvae is in clusters of cells in the notum of the third instar larval imaginal disc. These cells are the presumptive epidermal attachment cells of the adult indirect flight muscles (Fernandes, Celinker and VijayRaghavan, unpublished data); the adult sr phenotype is consistent with this expression pattern.

It is important to stress that the groovin-positive cells in the segmental border are present in the sr mutant embryos and, thus, their fate was not altered. Therefore, the sr gene may be directly involved in the signalling process.

In conclusion, the sr gene may exhibit parallel functions during embryonic and adult muscle development. In both systems, it is expressed in the muscle attachment sites and affects muscle organization. Dissecting the molecular nature of this gene is of major importance to the molecular understanding of muscle patterning.

In summary, this study demonstrated the central role of the cells at the segmental border in the maintenance of positional information needed to direct the nascent myotubes towards their attachment sites. A major effort is now needed to characterize the molecular mechanisms directly involved in these directional migration processes.

We thank B. Shilo for critical reading of the manuscript, E. Raz for fruitful discussions, M. Bate for inspiration and exchange of ideas, O. Lennert for the help in preparing the anti-groovin monoclonal antibody and K. Madhava for help with fly work. The nautilus eDNA was kindly obtained from Dr Michelson (Harvard University). The Drosophila laminin was a gift from Drs L. and J. Fessler (UCLA). This work was supported by an ICRF grant to T. Volk and by the Department of Biotechnology, Govt. of India.

REFERENCES


Hooper, J. E. and Scott, M. P. (1992). The molecular genetic basis of...


(Accepted 23 September 1993)