Ectoderm induces muscle-specific gene expression in Drosophila embryos

Rob Baker1,* and Gerold Schubiger2
1Department of Genetics and 2Department of Zoology, University of Washington, Seattle, WA 98195, USA
*Author for correspondence at present address: Department of Anatomy, University of Wisconsin, 1300 University Avenue, Madison WI 53706, USA

SUMMARY

We have inhibited normal cell-cell interactions between mesoderm and ectoderm in wild-type Drosophila embryos, and have assayed the consequences on muscle development. Although most cells in gastrulation-arrested embryos do not differentiate, they express latent germ layer-specific genes appropriate for their position. Mesoderm cells require proximity to ectoderm to express several muscle-specific genes. We show that ventral ectoderm induces mesoderm cells to express nautilus (a MyoD homologue) and to differentiate somatic myofibers, whereas dorsal ectoderm induces mesoderm cells to express visceral and cardiac muscle-specific genes. Our findings suggest that muscle determination in Drosophila is regulated by induction between germ layers during gastrulation.

Key words: Drosophila, induction, myogenesis, cell signalling

INTRODUCTION

Induction in amphibian embryos has been originally described by Spemann and Mangold (1924), and has since been studied in many species. In vertebrate embryos, cell-cell interactions between the mesoderm and ectoderm are required for the proper development of both germ layers. For instance, the mesoderm induces ectoderm to generate the neural plate during gastrulation (Nieuwkoop et al., 1985). Subsequent to this primary induction, the neuroectoderm controls somite organization (Nicolet, 1970; Lipton and Jacobsen, 1973). Studies on insect embryos have revealed similar inductive interactions between the mesoderm and ectoderm. Seidel first demonstrated that ectoderm induces muscle development in the underlying mesoderm, and that mesoderm is required for organization, but not histogenesis, of the ventral nerve cord (Seidel et al., 1940; Bock, 1942). Subsequent investigations have supported the paradigm that ectoderm, not mesoderm, behaves as the primary inducer of insect organogenesis (Kühn, 1971; Counce, 1973).

Upon cellularization of the Drosophila blastoderm, the mesoderm invaginates into the interior of the embryo and then spreads uniformly along the basal surface of the ectoderm (Leptin and Grunewald, 1990). Gastrulation therefore allows interactions between the ectoderm and mesoderm; however, it is not known to what extent such interactions regulate the expression of myogenic determinants within the mesoderm. During germ band extension, the mesoderm remains undifferentiated, but begins to differentially express muscle-specific genes (Michelson et al., 1990; Paterson et al., 1990; Azpiazu and Frasch, 1993; Bodmer, 1993). Meanwhile the mesoderm subdivides into three morphologically distinct sets of muscle progenitors: the somatopleura (presumptive somatic muscle), the splanchnopleura (presumptive visceral muscle) and cardiac muscle precursors (Campos-Ortega and Hartenstein, 1985; Bate, 1993).

Because no one has yet elucidated a blastoderm fate map for larval muscles, it is not known when muscle determination occurs. One possibility is that differential nuclear uptake of the dorsal morphogen within the presumptive mesoderm determines different myogenic fates, just as it specifies the fate of the presumptive ectoderm, mesectoderm, and mesoderm (Kosman et al., 1991; Ray et al., 1991; St. Johnston and Nüsslein-Volhard, 1992). Conversely, it is possible that muscle determination is regulated by inductive interactions between germ layers and/or between mesoderm cells themselves. For example, the adult male strap flight muscle acquires its identity by being innervated by specific neurons (Lawrence and Johnston, 1986). However, this is not a general mechanism for muscle determination; larval muscles differentiate normally in prospero mutant embryos in which muscle innervation does not occur (Broadie and Bate, 1993).

Here we demonstrate that specific regions of the ectoderm induce the expression of different myogenic genes in Drosophila embryos. We arrest gastrulation in wild-type embryos so that presumptive mesoderm cells remain on the ventral surface, preventing their interaction with ectoderm. The patterns of muscle morphogenesis and of muscle gene expression in these arrested embryos indicate that: (1) mesoderm must be near ectoderm cells to express muscle-specific genes; (2) hindgut and dorsal ectoderm independently induce visceral muscle development; (3) dorsal ectoderm induces heart development; (4) ventral ectoderm promotes somatic muscle development.

MATERIALS AND METHODS

Fly stocks, embryo collections, and developmental staging

Wild-type embryos were collected from the ‘Sevelen’ stock. Enhancer trap line A490.2M3 (Bellen et al., 1989), which expresses lacZ in the
midgut, was kindly provided by W. Gehring. For all genotypes, eggs were collected from females aged 3-10 days after eclosion. Overaged embryos were eliminated by precollecting eggs on food bottles for one hour, followed by three additional 20-minute precollections on agar plates flavored with cantaloupe juice and yeast pellets. Collections were made during 10-minute intervals.

Here we express the age of experimental and control embryos in terms of real time after egg laying (AEL). The standard classification of embryos by 17 developmental stages (e.g. Campos-Ortega and Hartenstein, 1985) is based upon morphological characteristics that do not apply to gastrulation-arrested embryos; thus, we do not use a staging scheme to describe them.

**Embryo injections and video recordings**

Synchronously staged embryos were manually dechorionated, aligned, and injected as described in Baker et al. (1993). Dechorionated embryos were kept in humid chambers at room temperature (21.5-23.0°C) until 3 hours AEL, then they were deisecated for 5-7 minutes, covered with Halocarbon oil, and injected with 250 µg/ml cytochalasin D (Sigma) between 190 and 200 minutes AEL. For each injection slide, 3-5 control embryos were not injected, but otherwise processed identically. Embryos were incubated at 25°C after injection until they reached the desired age, then rinsed in heptane and fixed in 4% EM-grade paraformaldehyde (Electron Microscopy Sciences) in PBS. Embryos were manually devitellinized with tungsten needles under a PBS.

Time-lapse video recordings of live gastrulation-arrested embryos were made by placing injection slides on a Nikon microphot mounted with a MTI 65 camera hooked up to a Gyrr time-lapse video recorder. Embryos were observed under differential interference contrast (DIC) optics in order to distinguish the position of cortical nuclei.

**RNA and protein labeling**

We used digoxigenin-labeled DNA probes to detect RNA in whole embryos using the standard procedure (Ashburner, 1989). Probes were made and detected with the materials provided in the Genius kit (Boehringer-Mannheim).

For antibody labeling, we blocked embryos in PBTB (PBS plus 0.01% Triton X-100 and 3% BSA) for 1 hour, then incubated them with primary antibodies overnight at 4°C. Primary antibodies were diluted in PBTB in the following proportions: 1:1000 goat anti-horse-radish peroxidase (USB); 1:500 mouse anti-β-galactosidase (Promega); 1:500 rat anti-α-vinyl (S. Robinow); 1:200 rabbit anti-nautilus and 1:200 rabbit anti-twist (B. Paterson); 1:250 rabbit anti-β3-tubulin (R. Renkawitz-Pohl); 1:40 mouse anti-D4.1 (R. Fehon); 1:100 rabbit anti-vestigial (L. Maves). Embryos were rinsed in PBTB for an hour, then incubated with the appropriate biotin- or fluorochrome-conju-gated secondary antibodies (Jackson ImmunoResearch) diluted 1:200 in PBT for either 4 hours at 22°C or overnight at 4°C.

Biotinylated secondary antibodies were labeled by the standard immunolabeling procedure (Ashburner, 1989) and photographed with a Nikon microphot. Fluorescently labeled embryos were dehydrated in methanol and mounted in a 1:2 mixture of benzyl benzoate and benzyl alcohol (Sigma Immunochemicals), and imaged on a Bio-Rad MRC 600 confocal microscope. Photographic images were processed with Adobe Photoshop 2.5 (Adobe Systems Inc.) to normalize brightness and sharpen image resolution. Images were assembled and sized in Canvas 3.0.2 (Deneba Software), a program that we also used for generating scale bars, arrows, and text. All photographs were printed by a Tektronix Phaser IISDX dye sublimation printer.

**RESULTS**

**Morphology of gastrulation-arrested embryos**

We arrested gastrulation movements in *Drosophila* embryos to varying degrees by injecting them with cytochalasin D (CYT). CYT blocks actin polymerization, and can inhibit microfila
dent-dependent cell movements during a brief period of time prior to the onset of gastrulation; injection of CYT before 180 minutes after egg laying (AEL) inhibits cellularization, and later than 200 minutes AEL does not inhibit normal development (data not shown). The effectiveness of CYT varies between injected embryos, owing to variables inherent in our methods (e.g. the volume of CYT injected, the distribution of drug within the embryo, and the time of injection). Therefore, CYT-injected embryos may exhibit complete, partial, or no gastrulation movements, and accordingly can be classified into five distinct morphological groups (Fig. 1).

24% (106/435) of CYT-injected embryos, which we term *arrested embryos*, do not gastrulate and retain the general morphology of a cellular blastoderm (Fig. 2A-D). We have analyzed the development of arrested embryos in time-lapse video recordings. Because CYT inhibits cytokinesis but not DNA synthesis (Whittaker, 1979; unpublished observations), blastomeres enlarge with time, making it impossible for all cells to remain at the cortex. Within minutes after cellularization is completed, individual cells throughout the embryo

<table>
<thead>
<tr>
<th>Morphological Class</th>
<th>Frequency</th>
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<tbody>
<tr>
<td>A Acellular</td>
<td>3.4</td>
</tr>
<tr>
<td>B Arrested</td>
<td>24.4</td>
</tr>
<tr>
<td>C Invaginated</td>
<td>4.6</td>
</tr>
<tr>
<td>D Partially Gastrulated</td>
<td>61.4</td>
</tr>
<tr>
<td>E Unaffected</td>
<td>6.2</td>
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Fig. 1. 435 CYT-injected embryos, fixed between 6 and 10 hours AEL, were categorized into five different morphological classes. The percentage frequency for each class is given next to its schematic representation. (A) Acellular embryos fail to develop and show pycnotic nuclei and large yolk vesicles. (B) Arrested embryos complete cellularization but fail to undergo morphogenetic movements. (C) Invaginated embryos form a ventral furrow and internalize their presumptive mesoderm. However, germ band elongation and dorsal closure do not occur. (D) Partially gastrulated embryos exhibit normal morphogenesis in some regions. (E) Unaffected embryos appear to develop normally.
ingress into the yolk, causing the cortex to thicken from a monolayer to a multilayer of cells. However, cells do not shift their positions relative to the axes, so that limited contact between different germ layer primordia occurs in arrested embryos (indicated in Fig. 3B, D, and F). Cell enlargement and cortical thickening are most extensive in the lateral regions, or presumptive neuroectoderm; the smallest cells are situated at the dorsal midline, or amnioserosa primordium. Thus, the ultimate size of a cell appears to be proportional to the number of cell divisions it would normally undertake: neuroblasts divide upwards of eight times, while amnioserosa cells do not divide (Campos-Ortega and Hartenstein, 1985). As a consequence of nonuniform cell growth, arrested embryos progressively lose their blastoderm-like appearance with time.

About 5% (20/435) of CYT-injected embryos invaginate a ventral furrow but fail to extend the germ band; we term these invaginated embryos (Fig. 2E,F). Like control embryos, the mesoderm of invaginated embryos spreads dorsally beneath the ectoderm. Invaginated embryos therefore exhibit a greater degree of physical interactions between mesoderm and ectoderm than do arrested embryos, which accounts for striking differences in their myogenic development (see below).

**Germ layer-specific gene expression in arrested embryos**

Preliminary to investigating the role of induction between germ layers during muscle development, it was necessary to verify that the developmental commitment of the germ layers is not altered in gastrulation arrested embryos. Towards this end we probed arrested embryos for antigens uniquely expressed throughout either ectoderm, endoderm, or mesoderm.

**Ectoderm**

We have probed arrested embryos with antibodies against horse-radish peroxidase (HRP) and elav, which respectively label the cell membranes and nuclei of all neurons (Jan and Jan, 1982; Robinow and White, 1991). At 10 hours AEL, anti-HRP labels

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**Fig. 2.** Blastoderm-stage and CYT-injected embryos were fluorescently labeled with rhodamine-conjugated phalloidin to outline cell surfaces. Lateral (A,C,E) and parasagittal (B,D,F) optical sections are shown for each embryo; dorsal side is up, anterior is left. Scale bar, 50 µm. (A,B) 3.5 hour AEL control embryo during the final stages of cellularization. Embryos are injected with CYT at this stage to inhibit gastrulation. (C,D) A 6 hour AEL arrested embryo retains a blastoderm-like morphology. The larger cells in the ventrolateral region are neuroblasts (compare with Fig. 3B). (E,F) 6 hour AEL invaginated embryo has completed only the initial stages of morphogenesis. The invaginated mesoderm (ms) and partially involuted posterior midgut (pmg) are indicated in the interior of the embryo.
the brain, ventral nerve cord, and lateral sensory organs of control embryos (Fig. 3A). In arrested embryos fixed 9-12 hour AEL, anti-HRP labels two ventrolateral bands of cells, one on either side (Fig. 3B). Both neuronal bands extend roughly between 15% and 90% embryo length (EL), with an average width of 7 cells. The size and position of these bands match the domains of the neurogenic regions on the blastoderm fate map (Poulson, 1950; Campos-Ortega and Hartenstein, 1985). Arrested embryos labeled with anti-elav antibody show a similar lateral distribution of neuronal cells (Fig. 4D). We seldom observe neuronal cells within the dorsal ectoderm region, where the sensory neurons originate (Campos-Ortega and Hartenstein, 1985).

46 CYT-injected embryos were allowed to develop under oil for 30 hours, and analyzed for the presence of cuticle. One embryo generated a normal cuticle and 29 deposited varying amounts of disorganized cuticle consisting of either dorsal hairs only, or dorsal hairs and ventral denticles (data not shown). Because the method we used for visualizing cuticle destroys the soft tissues, we could not unambiguously determine whether or not a given cuticle was derived from an arrested or an invaginated embryo. Nevertheless, the determination of dorsal epidermis clearly requires neither normal gastrulation movements nor the presence of mesoderm, as is indicated by the presence of dorsal cuticle in ‘dorsalized’ mutant embryos (Nüsslein-Volhard et al., 1984).

Endoderm
Embryos derived from enhancer trap line A490.2M3 express β-galactosidase (β-gal) within the midgut (Bellen et al., 1989; Fig. 3C). In arrested A490.2M3 embryos, β-gal is expressed at the anterior and posterior poles (Fig. 3D), corresponding to the blastoderm fate map positions of the presumptive midgut. The earliest we detect β-gal is 6 hours AEL in both control and arrested embryos, suggesting that the temporal program of midgut development does not require gastrulation.

Fig. 3. Control (A,C,E) and arrested (B,D,F) embryos were labeled for various germ layer-specific markers. All embryos are shown in lateral perspective, dorsal side up. (A,B) 10-hour AEL embryos labeled with anti-HRP antibody. Control embryo (A) shows neural signal in the brain, ventral nerve cord, and peripheral nervous system. The distribution of anti-HRP label in an arrested embryo (B) corresponds to the blastoderm fate map positions of the ventral neurogenic region and brain (compare with G). (C, D) 7-hour AEL embryos collected from enhancer trap line A490.2M3 and labeled with antibody against β-galactosidase (β-gal). This line expresses β-gal in the midgut of control embryos (C), and at the poles in arrested embryos (D). These polar regions correspond to the fate map position of the endodermal midgut primordia. (E,F) 12-hour AEL embryos labeled for mesoderm with antibody against β3-tubulin. Control embryo (E) shows label in macrophages and somatic muscle; heart and visceral muscle staining are not evident in this focal plane. Arrested embryo (F) expresses β3-tubulin in the midventral region, corresponding to the fate map position of the presumptive mesoderm. The dorsal signal may represent presumptive neurons in the brain that normally express this protein. Scale bar, 50 µm. (G) Blastoderm fate map of a wild-type embryo, indicating the approximate positions of the following tissue primordia: amnioserosa (as), anterior midgut (amg), dorsal ectoderm (de), foregut (fg), hindgut (hg), mesoderm (ms), posterior midgut (pmg), procephalic neurogenic region (pNR) and ventral neurogenic region (vNR). Illustration is based on fate maps constructed by Poulson (1950) and Campos-Ortega and Hartenstein (1985). 0% EL and 100% EL correspond to the posterior and anterior poles, respectively.
Mesoderm

We used antibodies against twist and β3-tubulin to identify mesoderm cells in arrested embryos. Mesoderm cells initially express twist before gastrulation begins (Thisse et al., 1988), and continue to do so until they differentiate (Bate et al., 1991). As mesoderm cells differentiate, they express β3-tubulin (Leiss et al., 1988; Fig. 3E). From 4 to 9 hours AEL, arrested embryos express twist protein in a midventral domain situated between 10% and 85% EL (Fig. 4A). From 10 to 14 hours AEL, β3-tubulin is expressed within this domain (Fig. 3F).

Up to 10% of β3-tubulin-labeled cells lie beneath the surface of ventral and dorsal ectoderm. We presume that these cells had individually migrated beneath the ectoderm before assuming the spindle-shaped morphology of somatic muscle precursors (Fig. 4B) or syncytial myofibers (Fig. 4C). However, the majority of presumptive mesoderm cells, particularly those within the midventral domain, do not differentiate. It has been shown, however, that cells committed to myogenic development express muscle myosin heavy chain (MHC), regardless of their ability to properly differentiate as muscle (Corbin et al., 1991; Bate et al., 1993). Thus, in order to identify myogenic cells in arrested embryos, we labeled them with anti-MHC antibody. We find that MHC is primarily expressed in two broad, longitudinal bands of mesoderm cells situated near the mesoderm/ectoderm boundary (Fig. 4D). Some of these cells extrude projections characteristic for somatic muscle precursors (Bate, 1990), although most are undifferentiated.

Muscle development and myogenic gene expression in arrested embryos

The three major larval myotypes (somatic, visceral, and cardiac...
muscle) become morphologically distinct several hours after gastrulation begins (Campos-Ortega and Hartenstein, 1985; Bate, 1993). Since most muscle cells do not differentiate in arrested embryos, we probed arrested embryos for myotype-specific markers.

Somatic muscle

Somatic muscle differentiation begins as muscle founder cells fuse with neighboring, ‘fusion-competent’ mesoderm cells, which results in the formation of syncytial myotubes (Bate, 1990). During germ band retraction (7.5-9.5 hours AEL), nau is expressed in a subset of somatic muscle founders of the thorax and abdomen (Michelson et al., 1990; Paterson et al., 1990; Fig. 5A). In 7.5-9.5 hour AEL arrested embryos, nau is expressed in two narrow, punctuated rows of cells near the ectoderm border (Fig. 5B). This pattern is similar to that of MHC expression (Fig. 4D), except that there are fewer labeled cells. The distribution of nau-expressing cells is variable between arrested embryos, each row consisting of 5-15 cells located between 10% and 70% EL. Double-labeling with anti-nau and anti-HRP antibodies indicates that although some muscle founder cells directly abut neurons, most are one or two cell diameters from the apparent ectoderm/mesoderm border.

Two explanations may account for the observation that nau is expressed only near the border with the ectoderm: (1) ectoderm cells induce mesoderm to express nau, or (2) somatic muscle precursors originate from presumptive mesoderm cells bordering the ventral ectoderm. To distinguish between these alternatives we have analyzed nau expression in invaginated embryos, the class of CYT-injected embryos that complete ventral furrow formation (Fig. 2E,F). If ectoderm induces nau expression, we would expect that the greater the degree of mesoderm invagination, the more somatic muscle founder cells should appear. Conversely, if nau-expressing muscle founder cells are derived from a fixed number of progenitor cells, we would not expect different numbers of these founder cells between the two classes of CYT-injected embryos.

Invaginated embryos aged 7.5-9.5 hour AEL express nau in 11 transverse bands of cells beneath the ventral ectoderm (Fig. 5C). These bands are situated between 10% and 70% EL, and correspond to the fate map positions of the 11 thoracic and abdominal segments that normally express nau at this time. We
counted between 140 and 185 nau-expressing cells per invaginated embryo (n = 12 embryos), which is approximately one-quarter the number present in uninjected controls (Michelson et al., 1990; our own observations). To a large degree, this numerical difference must reflect the greater number of mesoderm and ectoderm cells in controls relative to CYT-injected embryos. Arrested and invaginated embryos, however, possess approximately the same number of cells, based on cell counts of α-tubulin-labeled injected embryos (data not shown). Therefore, the greater number of nau-expressing cells in invaginated embryos (140-185) relative to arrested embryos (10-30), correlates primarily to their degree of contact between ventral ectoderm and mesoderm. Moreover, the different patterns of nau-expressing cells in these two classes of

![Fig. 6](image).

Fig. 6. (A-D) 9- to 10-hour AEL embryos probed for visceral muscle markers. (A) Lateral perspective of a control embryo labeled for H2.0 RNA. Signals in visceral mesoderm (between asterisks) and around tracheal pits (arrows) are indicated. (B) Lateral perspective of an arrested embryo with H2.0 signal in posterior mesoderm, near the presumptive hindgut. Label in anterior mesoderm or in trachea progenitors is never detected in arrested embryos. (C) Horizontal perspective of a control embryo from enhancer trap line A490.2M3. β-gal is expressed in the visceral mesoderm of the midgut and in some neurons in head and peripheral nervous system. (D) Horizontal perspective of an invaginated embryo from line A490.2M3. β-gal is expressed in noncontiguous rows of visceral mesoderm beneath the dorsal epidermis. Note that posterior midgut cells (pmg) have migrated anteriorly. Anterior midgut (amg), hindgut (hg), and yolk (yk) are also indicated. (E-H) Embryos probed for tin RNA. (E) Lateral perspective of 6.0 hour AEL control embryo that expresses tin in visceral (v) and cardiac (c) muscle precursors in a segmentally reiterated pattern. Signal in the foregut (fg) is also evident. (F) Lateral perspective of a 5-hour AEL arrested embryo. tin RNA is detected in prospective mesoderm (m) and foregut ectoderm (fg). (G) Ventral perspective of 6.5-hour AEL arrested embryo shows tin signal in the prospective foregut. tin RNA can no longer be detected in the mesoderm. (H) Ventrolateral perspective of 6.5-hour AEL invaginated embryo; the line indicates the approximate position of the ventral midline. Expression is strong in the mesoderm cells beneath the dorsal ectoderm. The lower, out-of-focus row of tin-expressing cells lie on the far side of the embryo. Scale bars, 50 µm; all figures except D are shown to the same scale as A.
embryos (longitudinal rows versus transverse stripes) provides further indication that ectoderm cells induce nau expression in the mesoderm.

**Visceral muscle**

Although the visceral muscle of the hindgut and midgut develop independently and express different sets of genes (Bodmer, 1993; Tepass and Hartenstein, 1994), both express the homeobox gene H2.0 (Barad et al., 1988). H2.0 RNA is first detected about 5.5 hours AEL in mesoderm around the hindgut, and later (beginning 6.5 hours AEL) is expressed in the visceral muscle of the midgut (Fig. 6A). In arrested embryos aged between 5.5 and 10 hours AEL, H2.0 transcripts are detected only in a few presumptive mesoderm cells situated around 10% EL (Fig. 6B). These H2.0-expressing cells are positioned near the presumptive hindgut (compare with Fig. 3G), and begin to express H2.0 at the same time as the visceral mesoderm of the hindgut does in control embryos. During normal development, the visceral musculature of the midgut is derived from mesoderm cells situated throughout the metameric germ band (Azpiazu and Frasch, 1993), whereas the visceral muscle of the hindgut appears to be derived only from posterior mesoderm cells. Thus, the failure of the presumptive mesoderm to express H2.0 throughout most of the germ band suggests that hindgut-specific, but not midgut-specific, visceral muscle is determined in arrested embryos.

To test this, we arrested embryos from the P[×acZ] stock A176.1M2 (Bellén et al., 1989). This line expresses β-gal in the visceral muscle of the midgut, but not of the hindgut (Fig. 6C). None of the arrested embryos from this stock (n = 87) express β-gal in the presumptive mesoderm. However, invaginated A176.1M2 embryos express β-gal in mesoderm cells situated beneath dorsal ectoderm (Fig. 6D). In some cases, posterior midgut cells had invaginated anteriorly along a track of visceral mesoderm (Fig. 6D), as they do in control embryos (Tepass and Hartenstein, 1994). These results suggest that visceral muscle of the midgut is induced by dorsal ectoderm.

**Cardiac muscle**

tinman (tin) encodes a homeobox protein required for the differentiation of both cardiac and visceral muscles (Bodmer, 1993; Azpiazu and Frasch, 1993). tin is initially transcribed throughout thoracic and abdominal mesoderm of blastoderm-stage embryos (3.5 hours AEL). During gastrulation, however, tin RNA disappears first in mesodermal cells beneath the ventral ectoderm (5 hours AEL), and later in visceral muscle precursors (6.5 hours AEL). During germ band retraction and dorsal closure (7-12 hours AEL), tin RNA is transcribed in cardiac muscle precursors. tin is also expressed in the foregut between 4 and 8 hours AEL (Fig. 6E).

We have assayed for tin RNA in arrested embryos at various times. Between 4.5 and 6 hours AEL, tin is expressed in presumptive foregut and in a strip of midventral presumptive mesoderm (Fig. 6F). Around 6 hours AEL, the tin signal dimin-

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**Fig. 7.** (A) 12-hour AEL Toll10B embryo labeled for MHC, shown in two different optical planes. MHC-expressing cells are generally undifferentiated. (B) Same embryo as in A, focused in deeper optical plane. MHC is depicted in green; the red counter stain is intended to highlight the yolk. Note that the yolk is clearly surrounded by gut (which is not labeled); in turn, the gut is surrounded by mesoderm. The arrow points to differentiated visceral muscle that adheres to the anterior edge of the gut. (C) 11-hour AEL wild-type embryo labeled for D4.1, shown in a midsagittal optical section. D4.1 is strongly expressed by ectoderm epithelia, including the foregut (fg), hindgut (hg), and epidermis (cells on the embryonic surface). The midgut (mg) weakly expresses this antigen, and mesoderm cells do not express it. (D) 11-hour AEL Toll10B embryo double labeled for D4.1 (red) and vestigial (green). Numerous epidermal cells occupy the apical surface. vestigial is normally expressed in a subset of somatic muscles and neurons (Williams et al., 1991). Arrow and arrowhead indicate syncytial vestigial-expressing cells with 3 and 4 nuclei, respectively, which may be syncytial muscle cells. Scale bar, 50 µm.
ishes in arrested embryos, and is undetectable in the presumptive mesoderm by 7 hours AEL, although foregut-specific signal remains strong (Fig. 6G). Conversely, mesodermal tin expression persists until 9 hours AEL in invaginated embryos. Between 7 and 9 hours AEL, tin RNA is present in bilateral, punctuated rows of cells lying beneath the dorsal ectoderm (Fig. 6H). In one collection of CYT-injected embryos, three invaginated embryos show a pattern of tin expression similar to the embryo in Fig. 6H, whereas 34 arrested embryos show tin signal only in the presumptive foregut. These observations indicate that proximity to the dorsal ectoderm is necessary for sustaining tin expression in the mesoderm after 7 hours AEL.

DISCUSSION

The role of dorsal and ventral ectoderm as inducers of somatic and splanchnic mesoderm, respectively, appears to be conserved between distantly related insect species. This paradigm was originally demonstrated by experiments on pre-gastrula stage Chrysopa (lacewing fly) embryos (Seidel et al., 1940; Bock, 1942). When the dorsal ectoderm of a given hemisegment is cauterized, the mesoderm interacts only with ventral ectoderm, and generates somatic muscle; these embryos do not differentiate heart or visceral muscle. If the ventral ectoderm is ablated instead, heart and visceral muscles develop beneath the dorsal ectoderm, and few somatic muscles differentiate.

The distribution of cells that express nau, H2.0, and tin in gastrulation-arrested Drosophila embryos similarly suggests that dorsal and ventral ectoderm differentially induce undetermined mesoderm cells to make specific myotypes. This idea was previously suggested by the results of cell transplantation experiments of Beer et al. (1988). They removed dye-labeled mesoderm cells from the midventral furrow of early gastrulating embryos and individually transplanted them into cellular blastoderm-stage hosts, and monitored their subsequent development. In these experiments, clones of mesoderm cells periodically gave rise to mixed populations of somatic and visceral muscle, or of somatic muscle and fat. This result suggests that at the onset of gastrulation, muscle versus nonmuscle determination has not yet occurred. Furthermore, the types of muscles generated by these clones strongly correlated with the site of transplantation into the host, irrespective of their original

Fig. 8. Schematic representation of transverse sections of Drosophila embryos at different developmental stages. Embryos are oriented dorsal surface up; for simplification, the morphological contortions caused by germ band extension are not depicted. See text for details. (A) During stage 9 (4.5 hours AEL) the invaginated mesoderm spreads laterally and dorsally along the basal surface of the ectoderm. Mesoderm juxtaposed to the dorsal ectoderm are induced by dpp to maintain tin expression (blue arrows). Mesoderm juxtaposed to the ventral ectoderm are not induced in this manner, and eventually cease to express tin. (B) In stage 11 (6.0 hours AEL), some dorsal ectoderm (lateral epidermis) cells express an unidentified signal that induces ventral tin-expressing mesoderm cells to develop as midgut-specific visceral muscle (green). These cells soon separate from the lateral epidermis and discontinue to express tin. (C) By stage 12 (7.5 hours AEL) dpp-expressing cells in the dorsal ectoderm induce underlying mesoderm cells to maintain tin expression and become cardioblasts. Meanwhile, a subset of the somatopleura is induced (black arrows) by the ectoderm to develop as somatic muscle founders (red cells). These cells in turn laterally inhibit (red crossbars) their ‘fusion competent’ neighbors (yellow cells) from likewise becoming muscle founders via a Notch-dependent signal transduction pathway.
position within the donor embryo. For example, mesoderm cells transplanted into the dorsal ectoderm region of the host most frequently gave rise to visceral muscle, while those transplanted into the anterior midgut primordium predominantly gave rise to esophageal muscle. These observations, like those presented here, indicate that muscle determination is largely dependent on signals provided by the ectoderm.

In order to test our hypothesis by genetic means, we analyzed myogenesis in embryos derived from heterozygous

\textit{Toll}^{10B} females (henceforth called ‘\textit{Toll}^{10B}’ embryos’), which reportedly lack ectodermal derivatives (Erdélyi and Szabad, 1989). If inductive signals from the ectoderm induce myogenesis, then \textit{Toll}^{10B} embryos should neither express myogenic genes nor differentiate muscle. We observed, however, that at 12 hours AEL \textit{Toll}^{10B} embryos express MHC in a large number of cells, some of which are differentiated as muscle (Fig. 7A,B). This observation prompted us to assay these embryos for the presence of ectoderm. For this purpose we used an antibody against D4.1 (Fig. 7C), the product of the \textit{coracle} gene, which is normally expressed in the epidermis, hindgut, and foregut (Fehon et al., 1994). We found that all \textit{Toll}^{10B} embryos assayed express D4.1 throughout the apical surface beginning at 9 hours AEL (Fig. 7D), indicating a substantial presence of ectoderm in these embryos. Furthermore, roughly 30% of \textit{Toll}^{10B} embryos (17/56) deposit some larval cuticle (data not shown), which is secreted by the epidermis. The abnormal development of both ectoderm and mesoderm in these embryos is consistent with the hypothesis that ectoderm induces muscle development in the underlying mesoderm; however, further investigation is necessary to verify a causal relationship in this case.

### Signal peptides that induce mesoderm in vertebrates may induce muscle in Drosophila

Although somatic muscle founders normally abut ectodermal cells directly (Bate, 1990), in arrested embryos they generally lie 10 μm from the apparent ventral ectoderm boundary (Fig. 5B). Mesoderm cells most likely occupy the space between the naup-expressing cells and the neurogenic region; arrested embryos express the mesoderm-specific gene \textit{slit} in two rows of single cells along the ventral edge of the neurogenic ectoderm (unpublished observation). It therefore seems likely that ectoderm induces somatic muscle precursor development by a secreted signal with an effective range of two or three cell diameters.

One candidate for this signal is fibroblast growth factor (FGF), which induces mesoderm formation in vertebrate embryos (Kimmel et al., 1988). Although a homologue of FGF has yet to be discovered, \textit{Drosophila} embryos express a homologue of the FGF receptor, called DFR1, in the somatopleura preceding and during myoblast fusion (Shishido et al., 1993). DFR1 is expressed in large clusters of mesoderm cells in a pattern similar to the wild-type distribution of naup-expressing clusters. A deficiency of the 90D-E region, which includes the DFR1 locus, results in greatly reduced nau expression and somatic muscle differentiation (Shishido et al., 1993).

Our results also suggest that a signal from the dorsal ectoderm induces latent expression of \textit{tin}; only mesoderm cells that are juxtaposed to dorsal ectoderm continue to express \textit{tin} beyond 7 hours AEL (Fig. 6). Recent evidence strongly suggests that this inductive signal is the decapentaplegic (dpp) protein (Staehling-Hampton et al., 1994; M. Frasch, personal communication). dpp is a member of the TGF-β family of secreted growth factors that induce dorsal mesoderm in \textit{Xenopus} embryos (Green et al., 1990). Although dpp is expressed throughout the dorsal ectoderm during germ band extension, around 7 hours AEL it becomes restricted to two narrow stripes of ectoderm: one longitudinal row of dorsal cells that border the amnioserosa, and a row of ventrolateral cells bordering the neurogenic ectoderm (St. Johnston and Gelbart, 1987; Ray et al., 1991). It remains unclear why maintenance of dpp expression in the dorsal stripe, but not the ventrolateral stripe, induces latent dpp expression in the underlying mesoderm.

Another signal transduction pathway governed by the neurogenic genes (e.g. \textit{Notch} and \textit{Delta}) plays a critical role in \textit{Drosophila} myogenesis. Embryos lacking function for any of these genes exhibit developmental abnormalities in all germ layers (Poulson, 1945; Hartenstein et al., 1992). The most obvious defects are present in the ectoderm, which develops a grossly hypertrophied nervous system at the expense of ventral epidermis (Lehmann et al., 1983). These defects seem to be mirrored in the underlying mesoderm, which exhibits a hypertrophy of somatic muscle founder cells and a paucity of fused myotubes (Corbin et al., 1991; Bate et al., 1993). One important question is whether these muscle-specific defects are due to the loss of neurogenic gene function within the mesoderm itself, or whether the altered ectoderm fails to provide the appropriate signals for muscle development. To address this question, we have investigated the germ layer-specific requirements for Notch protein. In embryos that possess Notch function in the mesoderm but not in the ectoderm, only certain aspects of myogenic development are normalized relative to mutant embryos (manuscript in preparation). For instance, these embryos do not exhibit a hypertrophy of somatic muscle founders, and myoblast fusion occurs to a significant degree (Baker, Ph.D dissertation), suggesting that Notch is required in the mesoderm for muscle differentiation. However, the distribution of both muscle founders and fused myotubes is abnormal in these embryos, which suggests that Notch function in the ectoderm (and, by extension, normal ectodermal development) is required for proper muscle organization.

### A model for muscle induction in Drosophila embryos

On the basis of observations presented here and in previous reports, we suggest a working model for muscle induction in \textit{Drosophila} (summarized in Fig. 8). The mesoderm is initially pluripotent (Beer et al., 1988) until about 4.5 hours AEL (stage 9), when it forms a uniform layer of cells beneath the ectoderm (Fig. 8A). Shortly afterwards, the ventral mesoderm terminates \textit{tin} transcription, since its transcriptional maintenance requires a signal from the dorsal ectoderm (Fig. 6), which has been positively identified as dpp (Staehling-Hampton et al., 1994; M. Frasch, personal communication).

The subsequent divergence of splanchnic and cardiac precursors during stage 11 probably involves another inductive event (Fig. 8B). Around 6.5 hours AEL (stage 11), the ventral-most \textit{tin}-expressing cells ingress towards the yolk, cease to express \textit{tin}, and differentiate as visceral muscle. Meanwhile, the dorsal-most \textit{tin}-expressing cells maintain \textit{tin} expression.
and remain undifferentiated until the completion of dorsal closure, when they assemble into the heart. The proximate cause for this differential behavior of visceral and cardiac muscle does not appear to be the inactivation of tin transcription, since the splanchic muscle precursors still possess tin RNA after they segregate away from the lateral epidermis towards the gut (Bodmer, 1993).

Between 8 and 10 hours AEL (stage 12), prospective muscle founder cells are induced by short-range signals derived from the ectoderm, possibly an FGF homologue (Fig. 8C). DFR1, an FGF-receptor homologue, is expressed in a segmentally reiterated pattern within the mesoderm that prefigures the ventral, lateral, and dorsal somatic muscle clusters (Shishido et al., 1993), and may be necessary for founder cell determination. However, the final arrangement and number of founders are apparently regulated by cell-cell interactions between prospective muscle founder cells themselves (R. Baker, Ph.D dissertation). Thus, somatic muscle development probably involves at least three different inductive pathways: first, ectoderm induces somatopleura with the competence to generate muscle founders; second, potential muscle founders limit their own numbers by lateral inhibition; third, signaling between muscle founders and epidermis is necessary for the fusion of syncytial myotubes and for their arrangement and attachment to the epidermis (Bate, 1990; Volk and VijayRaghaven, 1994).

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