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Anterior-posterior subdivision and the diversification of the mesoderm in *Drosophila*

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SUMMARY

We have used a novel cell marker, in which the *twist* promoter directs the synthesis of the cell surface protein CD2 *(twi-CD2)* to examine the development of the mesoderm in the *Drosophila* embryo after gastrulation and to locate the progenitor cell populations for different mesodermal derivatives. We find that the early mesoderm in each segment is divided into a more anterior region with relatively low levels of *twist* and *twi-CD2* expression and a more posterior region where *twist* and *twi-CD2* expression are high. This subdivision coincides with regional assignments of cells to form different progenitors: dorsal anterior cells invaginate to form an internal layer from which the visceral mesoderm is derived. Ventral anterior cells form progenitors of mesodermal glial cells. Dorsal posterior cells form heart. Ventral *and* dorsal posterior cells form somatic muscles. We conclude that the metamerically repeated anterior-posterior subdivision of the mesoderm is an essential element in laying out the pattern of mesodermal progenitor cells and in distinguishing between an internal cell layer which will give rise to the progenitors of visceral muscles and an external layer which will generate the somatic muscles and the heart.

Key words: *Drosophila*, embryo, mesoderm, muscle, *twist*

INTRODUCTION

During early embryogenesis in *Drosophila*, the cells of the mesoderm are drawn from a population of cells that express *twist*. These *twist*-expressing cells occupy the mid ventral domain of the blastoderm stage embryo and, as well as the mesoderm, include cells of the endoderm and mesectoderm (for a review see Bate, 1993). The cells of the mesoderm invaginate through the ventral furrow at gastrulation to form an inner cell layer, which gives rise to many of the internal organs of the larval and adult fly, including the somatic and visceral muscles, the heart and the fat body. After invagination, and as the embryonic germ band extends, mesodermal cells spread dorsally to form a rather uniform monolayer of cells coating the inner face of the ectoderm. Thereafter, the mesoderm goes through a conspicuous, but as yet relatively undefined phase of rearrangement, which culminates in the segregation of the progenitors of the different mesodermal derivatives prior to the retraction of the germ band. In this paper we investigate this important phase of mesodermal development, using a novel marker which allows us to identify different cell populations in the early mesoderm and the progenitors that arise from them.

After gastrulation, *twist* expression persists in the mesoderm, but is lost from the endoderm and mesectoderm (Thisse et al., 1988). *twist* is required for gastrulation (Simpson, 1983; Nüsslein-Volhard et al., 1984), and is probably a key factor in initiating mesodermal differentiation. It encodes a transcription factor (Thisse et al., 1988) which is required for the expression of other mesodermal genes, including *tinman* (Bodmer et al., 1990) and the α_{PS2} integrin subunit (Leptin, 1991). Mesodermal reorganisation in the extended germ band embryo is accompanied by a decrease in *twist* expression, during which the level of *twist* protein is reduced in most cells but persists at a high level in others (Thisse et al., 1988). By the onset of germ band retraction, as larval myoblasts assemble and fuse to form somatic muscles, *twist* expression persists in only a small population of cells in every segment and by stage 13 it is confined to the progenitors of the adult muscles, some cells associated with the alary muscles, and the visceral mesoderm of the fore and hind gut (Bate et al., 1991).

Although Twist is a useful marker for mesodermal cells, like other nuclear proteins, it reveals nothing of cell shape and cannot be used effectively to analyse the changing relationships among mesodermal cells which follow gastrulation and accompany the segregation of the different derivatives. In this paper we describe the results of an analysis of the behaviour of the early embryonic mesoderm in *Drosophila* using a strain of flies that carry a genetic construct in which the *twist* promoter directs the synthesis of the cell surface protein CD2 *(twi-CD2)* (Dunin-Borkowski and Brown, 1995). In embryos carrying the construct, CD2 is expressed on the surfaces of mesodermal cells and can be detected using an antibody

against the CD2 protein. Because CD2 perdures, we can continue to follow many cells even after they have lost expression of endogenous Twist protein. In this way we can document the behaviour of the mesodermal cells following invagination into the interior of the embryo and prior to and during the appearance of different mesodermal derivatives. Recent evidence suggests that inductive signals from the ectoderm are important determinants of mesodermal pattern (Staehling-Hampton et al., 1994; Baker and Schubiger, 1995; Frasch, 1995; Baylies et al., 1995). Bearing in mind that these signals are likely to influence the regional differentiation of the mesoderm, we have used *twi-CD2* together with the expression of genes such as *engrailed* (*en*) and *wingless* (*wg*) to relate the developing spatial organisation in the mesoderm with that of the adjacent ectoderm.

Using these methods, we have followed the development of the embryonic mesoderm from gastrulation onwards. We have charted the origin and differentiation of the visceral muscles, the somatic muscles, the heart and their respective progenitors. We find that each of these different derivatives is produced by a different and distinct subset of the mesoderm in every segment. An important conclusion from this work is that the characteristic subdivision of the mesoderm into internal and external derivatives has its origin in a metamerically repeated variation in the behaviour of mesodermal cells along the anterior posterior axis of the early embryo.

MATERIALS AND METHODS

Fly stocks

Two different chimaeric genes, which had been inserted into the germline by P-element mediated transformation, were used for observing mesodermal development: *twi-CD2* (Dunin-Borkowski and Brown, 1995) and *en-lacZ* (Hama et al., 1990). A strain containing both chimaeric genes allowed us to relate mesodermal development to the position of *en*-expressing cells in the ectoderm. We also used a modified form of the *twi-CD2* construct (*twi-CD2**). The *twi-CD2** construct also contains the upstream regulatory sequences of the *twist* gene but lacks the downstream regulatory sequence of *twist*. One strain was found with one of three copies of *twi-CD2** inserted into *wg*. This was confirmed by the failure of this strain to complement the wg^{cx4} allele. In this line, CD2 is expressed in the ectodermal pattern of *wg* as well as in the mesoderm.

Whole-mount staining of embryos

Embryos were staged according to Campos Ortega and Hartenstein (1985) and stained as described by Dunin-Borkowski and Brown (1995). The primary antibodies used were: anti-CD2 OX-34 monoclonal antibody ascites (1: 5,000-10,000, Williams et al., 1987; a kind gift from A. Williams, available from Serotec), anti-Twist rabbit serum (1: 5000, Roth et al., 1989; a kind gift from S. Roth) and anti-β-galactosidase rabbit serum (1: 40000, Amersham).

Most stainings were done using horseradish peroxidase- (HRP) conjugated secondary antibodies. For labelling with two antibodies we first used a biotinylated secondary antibody and streptavidin-HRP as provided in the Vectastain Elite ABC Kit (Vector Laboratories). This was followed by an alkaline phosphatase-conjugated secondary antibody and stained with the Alkaline Phosphatase Substrate Kit 1 (red substrate: Vector Laboratories).

To double label for Twist and *engrailed* we also first antibody-stained embryos using the anti-Twist rabbit serum, as described above, rinsed them, fixed again and performed in situ hybridization with a DIG-RNA *engrailed* probe following the procedure of Tautz and Pfeifle (1989).

Photographs of stained embryos were taken using a Zeiss Axiophot microscope and the negatives scanned with a Nikon Coolscan. The scanned images were assembled using Photoshop 3.0 and labels and drawings added in FreeHand 4.0.

RESULTS

A comparison between early patterns of *twist* and *twi-CD2* expression: the emergence of a modulated pattern of *twist* expression

We compared the staining patterns in early embryos using anti-Twist antibody and anti-CD2 (Fig. 1). Essentially the results are the same, revealing an invaginating population of mesodermal cells expressing Twist or CD2 at a high level, during and after gastrulation with the difference that the nuclear staining with the Twist antibody allows us to monitor patterns of cell division in the mesoderm (Fig. 1A), while CD2 expression reveals cell shapes (Fig. 1E). During stage 8 the invaginated cells divide as they move out of the ventral furrow and move laterally and dorsally forming a monolayer on the inner face of the ectoderm (not shown). At late stage 8, early stage 9, during the slow phase of germ band extension the cells divide again (Fig. 1A,E). Up to this point, both markers reveal that the cells are arranged rather uniformly on the inner surface of the embryo. During late stage 9, the pattern of *twist* and *twi-CD2* expression begins to show signs of a regular fluctuation along the anteroposterior axis. This modulation of expression develops gradually from stage 9, but by stage 10, when the mesodermal cells are undergoing a third division, it is pronounced throughout the postcephalic mesoderm (Figs 1A,B and 2). Using the CD2 expression pattern, we can divide the mesoderm in each segment into a more anterior region (A), with relatively weak expression and a posterior region (P), where expression is relatively high (Fig. 2C). The expression pattern resembles a sawtooth, in that levels of expression merge gradually between posterior A and anterior P but there is an abrupt change between posterior P and anterior A.

Another aspect of mesodermal pattern which becomes apparent during these stages is the conspicuous ordering of the dorsal mesoderm to form a series of crests and troughs along the anteroposterior axis of the embryo (Fig. 1F). The peak of each crest lies within the high CD2 domain, anterior to and separated by one cell from the sharp posterior border with the low CD2 domain (Fig. 2E).

Relation of the modulated pattern of CD2 expression to ectodermal markers

We recovered a strain of flies in which the *twi-CD2* construct is fortuitously inserted in the *wg* locus. In embryos from this strain, CD2 is expressed in *wg*-expressing cells of the ectoderm, presumably under the influence of *wg* regulatory elements. Using this CD2 insertion in *wg* we can map the periodic pattern of *twi-CD2* expression in the early mesoderm with reference to ectodermal landmarks. We confirmed this by comparing *twist* and *en* expression either by anti-Twist staining followed in situ hybidization to *en* mRNA, or antibody staining of embryos from a strain of flies carrying *en-LacZ* and *twi-CD2*, The sharp border between high and low CD2 expression domains lies along the stripe of ectodermal *en* expression overlapping it by about one cell (Fig. 2D). In agreement with this,

the peaks of the mesodermal crests, which lie two cells anterior to the boundary of high CD2 expression, are exactly aligned with the ectodermal stripe of *wg* expression (Figs 1F, 2E, 7B). Hence, in a stage 9/10 embryo, the sharp border between cells with high and low CD2 expression approximates a line one cell posterior to the parasegment boundary (Fig. 2E).

The visceral mesoderm and mesodermal cross bridges arise from cells of the low *twi-CD2* domain

At the onset of stage 10, it is possible to detect regular variations in the behaviour of mesodermal cells in the different domains of CD2 expression. The most obvious of these is a periodic fluctuation in the contacts between mesoderm and ectoderm. Cells in the posterior part of the high CD2 domain appear to adhere strongly to the ectoderm, whereas their immediate neighbours in the low CD2 domain tend to coalesce, lose contact with the ectoderm and move inwards (Fig. 3C,D). This inward movement of cells is the first sign of a large scale invagination of mesoderm in the regions where the tracheal pits are forming (although the formation of tracheal pits is not essential for this invagination to occur; unpublished observations). During stage 10, the cells of the low CD2 domain move inwards forming prominent clusters of cells at intervals along the anterior posterior axis (Fig. 3F,H). Probably all cells in the low domain, dorsal to the neurogenic region of the ectoderm, take part in this inward

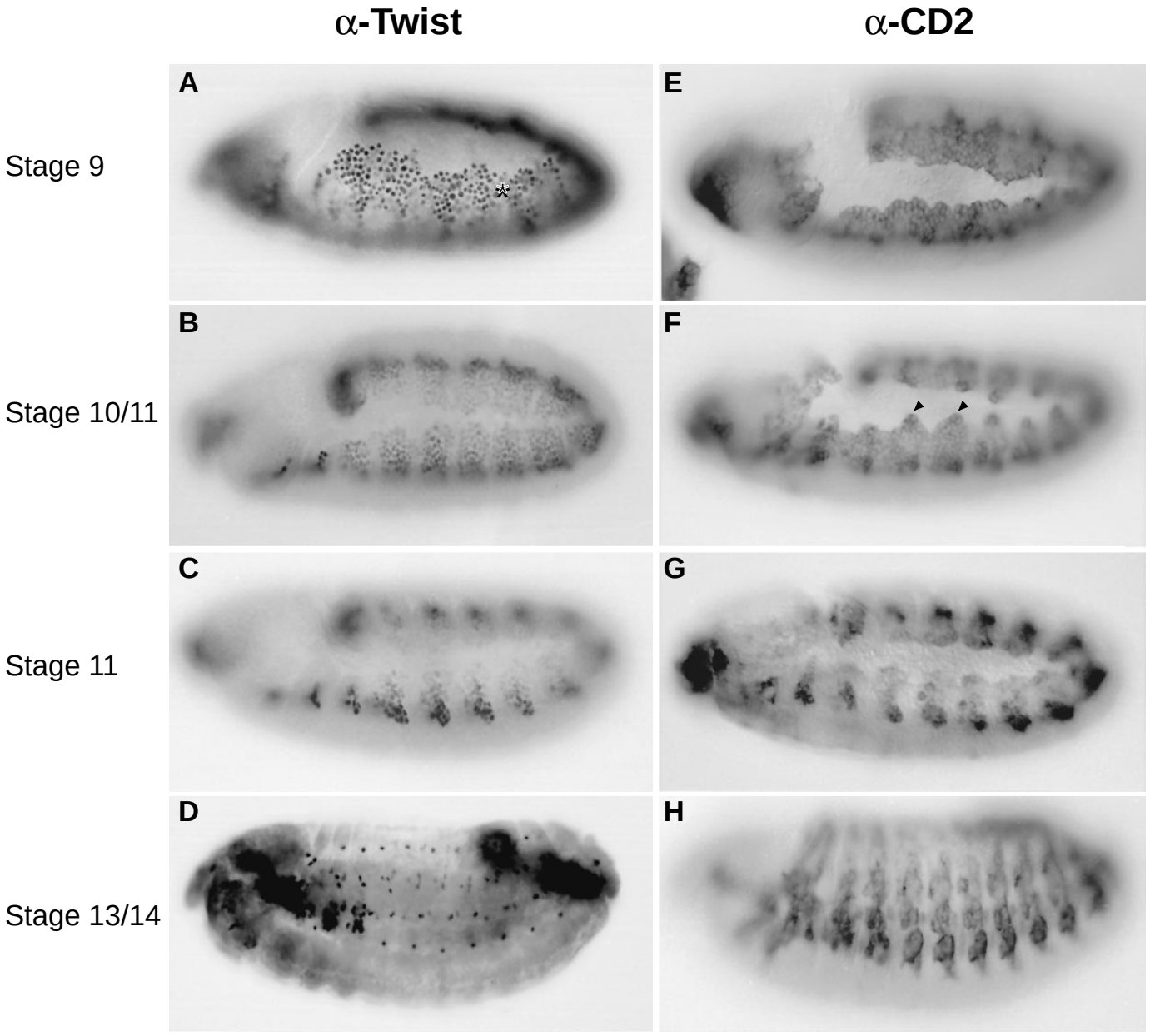

Fig. 1. The *twi-CD2* gene expresses CD2 in the same pattern as Twist but CD2 expression persists longer. Wild-type embryos stained with an antibody against Twist (A-D), and embryos carrying the *twi-CD2* gene stained with an antibody against CD2 (E-H) at the indicated stages of development. The regions of weaker staining of Twist (one is indicated by an asterisk in A) are cells in mitosis. These mesodermal cells are undergoing their second post blastoderm division. Two of the dorsal crests of the mesoderm are indicated by arrowheads in F.

movement, together with a few of their immediately posterior neighbours in the high CD2 domain.

The initial phase of mesodermal migration after gastrulation carries the dorsalmost cells of the mesoderm to within about two cells of the dorsal ectodermal margin, forming a slightly irregular dorsal edge to the mesoderm which is continuous along the anteroposterior axis during stage 8/9 (Fig. 1E). As cells now coalesce and move inwards in the low CD2 domain during late stage 9/early stage 10, the effect is to withdraw cells from the dorsal margin in a periodic fashion along the antero-posterior axis leaving behind the conspicuous mesodermal crests, which mark the high CD2 domain (Figs 1F, 3E,H). The peaks of the crests are located one to two cells from the dorsal margin of the epidermis, whereas the troughs are six to eight cells from it (not shown). Thus it is the cells that form the troughs which are actively moving ventrally, away from the dorsal edge of the epidermis. As these are among the cells of the low CD2 domain that are coalescing and invaginating it is likely that the formation of the troughs is a consequence of the aggregation and inward movement of these cells.

During stage 10 and early stage 11, the invaginated cell set in each segment spreads laterally, forming a conspicuous semicircular group of cells beneath each tracheal pit and coming into contact with the equivalent groups in neighbouring segments (Fig. 3F,H). Thus, dorsal to the forming nervous system the invaginated cells of the low CD2 domain spread laterally to form a continuous inner layer of the mesoderm (Fig. 3K,L). The invaginated cells appear to adhere as a group, spreading beneath their neighbours in the high CD2 domain and with a clear horizontal border distinguishing them from the cells of the dorsal crests (Fig. 3K). Ventrally each group is separated from the rest of the mesoderm by a crescent-shaped line of cells (Fig. 3G). As neighbouring groups come into contact, the crescents coalesce, forming a series of arches along the length of the embryo (Fig. 3K). This conspicuous line demarcates the furthest extent of the inwardly bulging cells, the ends of the arches being formed by the tucking back of each bulge towards the ectoderm at its junction with equivalent invaginations in neighbouring segments. These junctions between neighbouring invaginations approximate the position of the stripe of *wg* expression in the ectoderm (Fig. 4A). While the invaginated cells spread to form an inner layer, they also segregate into at least two distinct cell types: a well ordered file of cells at the margin of a less obviously structured cell set (Fig. 3G,K). Several markers allow us to follow the fate of the crescent-shaped line of cells and show that these are the progenitors of the midgut visceral mesoderm (data not shown; Patel et al., 1987; Azpiazu and Frasch, 1993). The fate of the more dorsal cell groups that the visceral mesoderm progeni-

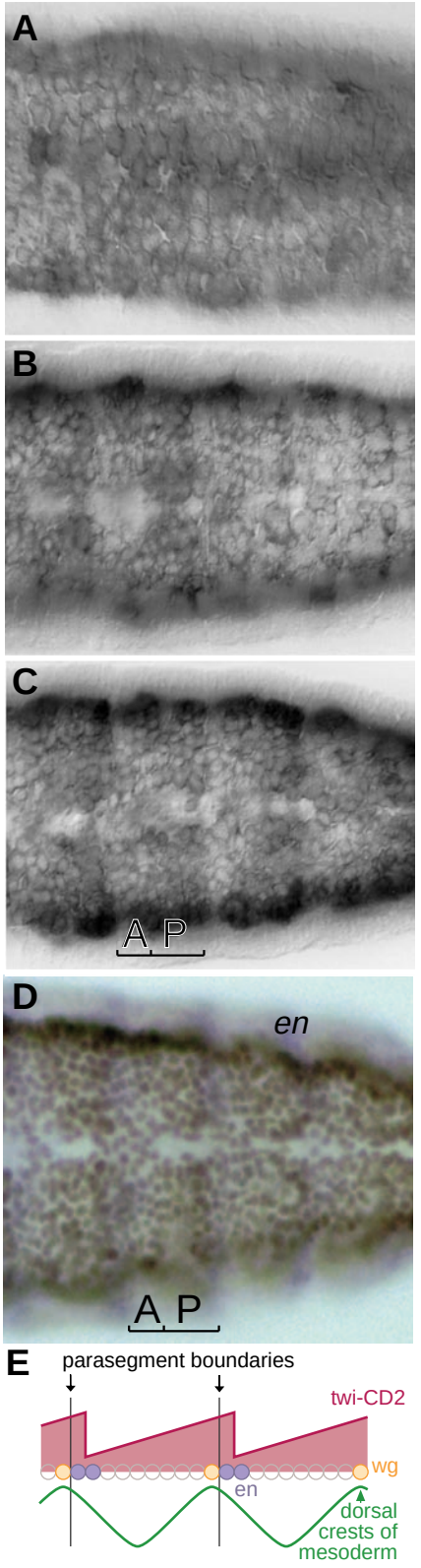

Fig. 2. The emergence of a modulated expression pattern of CD2 or Twist along the anterior-posterior axis subdivides the mesoderm in each segment into two groups. (A-C) Ventral views of four abdominal segments in progressively older embryos stained for CD2 at stages 9/10. (A) Initially, the staining for the *twi-CD2* marker is homogeneous throughout the mesoderm in each segment. Later the expression of CD2 becomes modulated along the anterior-posterior axis (B,C). Two groups of mesodermal cells can be distinguished in each segment: Anterior cells expressing CD2 weakly (group A) and Posterior cells expressing CD2 strongly (group P). (D) An embryo during early stage 10 stained with an antibody to Twist (brown) and a probe for *engrailed (en)* mRNA (blue) shows that the sharp boundary between the high and low domain of Twist expression lies one cell posterior to the parasegment border. (E) Summary diagram to show relative positions of parasegment border, sharp A/P boundary for CD2 expression and mesodermal crests (described later).

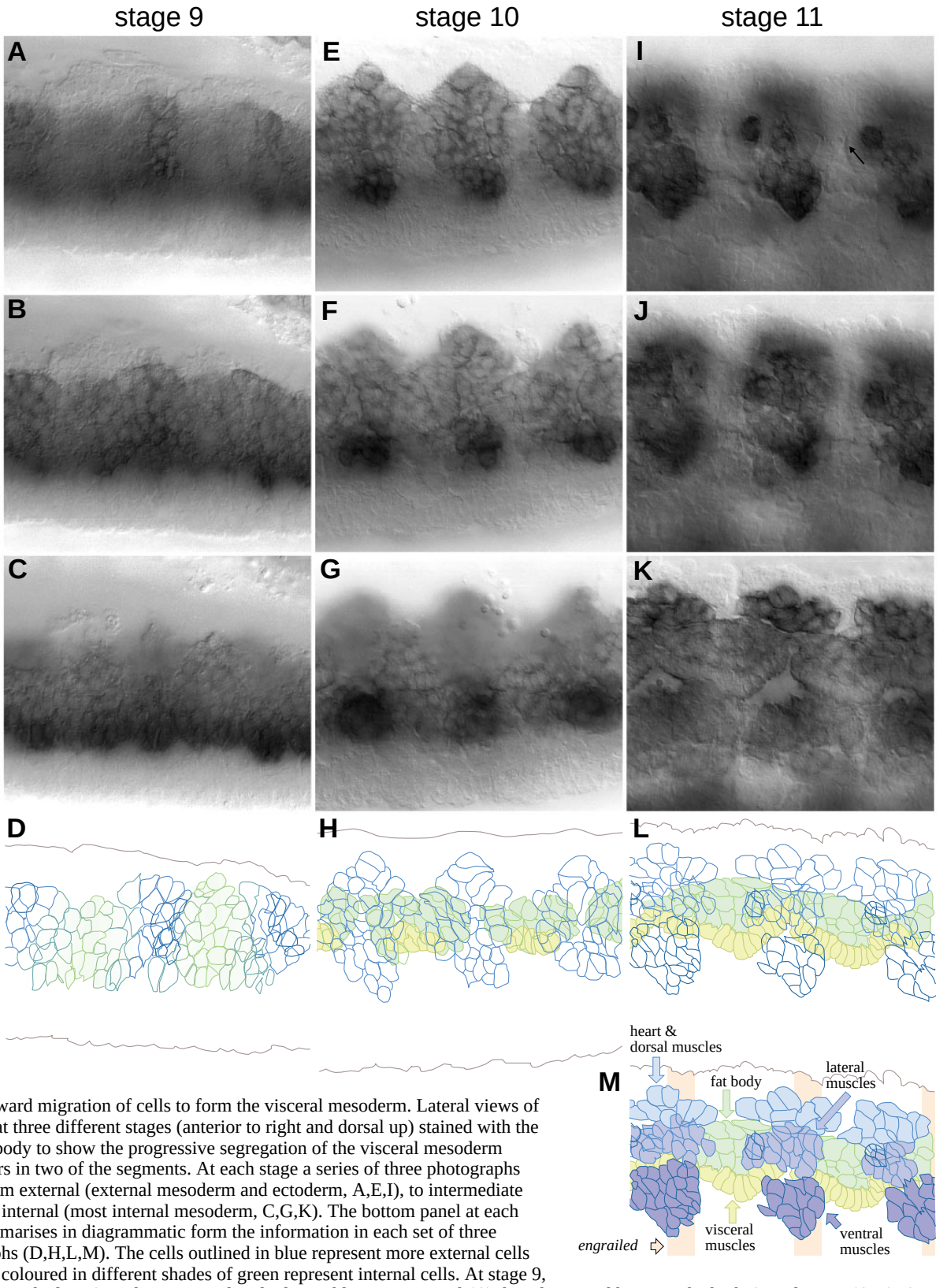

Fig. 3. Inward migration of cells to form the visceral mesoderm. Lateral views of embryos at three different stages (anterior to right and dorsal up) stained with the CD2 antibody to show the progressive segregation of the visceral mesoderm progenitors in two of the segments. At each stage a series of three photographs passes from external (external mesoderm and ectoderm, A,E,I), to intermediate (B,F,J) to internal (most internal mesoderm, C,G,K). The bottom panel at each stage summarises in diagrammatic form the information in each set of three photographs (D,H,L,M). The cells outlined in blue represent more external cells and those coloured in different shades of green represent internal cells. At stage 9, some cells in the low CD2 domain are already detectably more internal (C) than their neighbours in the high CD2 domain (A,B). At stage 10 the invaginated cells are conspicuous internal groups which are spreading laterally (F) and the file of visceral mesoderm progenitors has begun to segregate ventrally (G). At stage 11 the invaginated cells are internal to the tracheal pits (one is indicated by the arrow in I) and joined with their neighbours in adjacent segments, with the visceral mesoderm progenitors forming a conspicuous arched file of cells (K). In M the location of the different progenitor cell populations (including the probable fat body progenitors) is indicated with reference to ectodermal *en* expression. This fate map summarises additional evidence presented in the text and later figures.

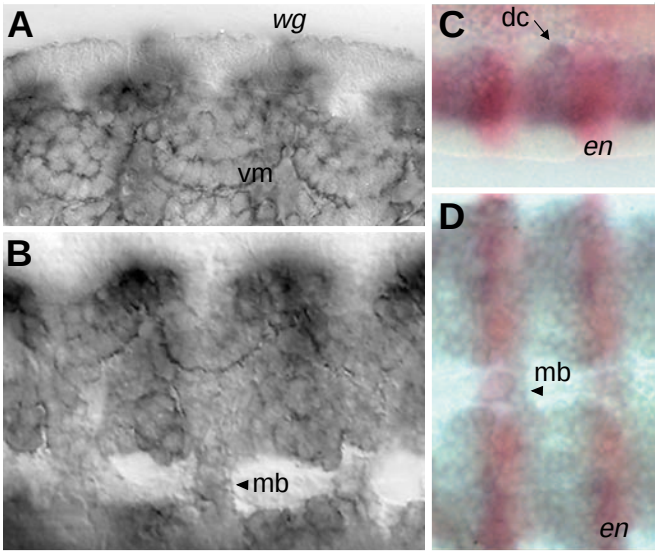

Fig. 4. Spatial organisation in the mesoderm relative to the epidermis.(A-B) Ventrolateral views of two stage 11 embryos carrying the *twi-CD2** construct, stained for CD2. In these embryos both the *wg*-expressing cells of the ectoderm and the mesodermal cells express CD2. The figure shows the relative positions of the visceral mesoderm progenitors (vm), the mesodermal cross bridges (mb, arrowhead), and ectodermal *wg*. (C-D) Stage 11 embryos carrying both the *twi-CD2* and the *en-lacZ* constructs stained for β-galactosidase (red) and CD2 (black) showing that the ectodermal stripe of *en* expression lies immediately posterior to the peak of the dorsal crest (dc, arrow) and coincides with the anterior edge of the mesodermal cross bridges (mb, arrowhead).

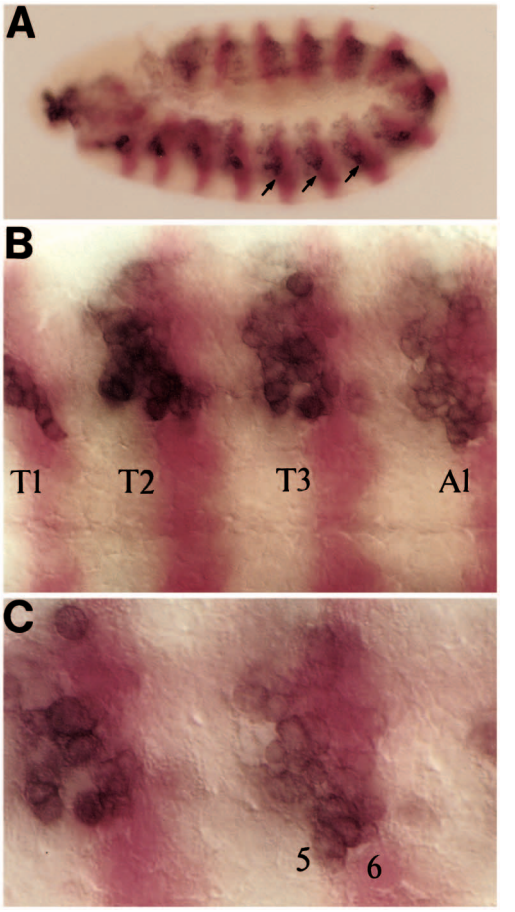

Fig. 5. Organisation of cells that give rise to the ventral external muscle precursors. (A-C) Stage 11 embryos carrying the *twi-CD2* and the *en-lacZ* constructs stained for β-galactosidase (red) and CD2 (black). (A) Lateral overview of the embryo at this stage. Cells with high levels of CD2 expression form conspicuous ventral prongs (arrows). (B) Higher magnification ventral view showing the organisation of these cells in the thoracic segments (T1, T2 and T3) and in the first abdominal one. (C) Higher magnification of B (T3 and A1) showing in A1 the insertion of the most ventral CD2-expressing cells between lateral neuroblasts of rows 5 and 6 of the developing central nervous system (marked in figure). Row 6 is *en*-expressing as shown by expression of β-galactosidase.

tors demarcate is less clear, but from our own observations and published data on the origin of the fat body (Hoshizaki et al., 1994) we conclude that they probably contribute to the fat body (Fig. 3M) and the gonadal mesoderm (Brookman et al., 1992).

More ventrally, over the forming nervous system, cells of the low CD2 domain form conspicuous mesodermal cross bridges (Fig. 4B) which span the ventral midline (Bate, 1993). Later in development, these cells will give rise to the mesodermal glial cells which lie on the dorsal surface of the central nervous system (Gorczyca et al., 1994). Since they occupy the region of low CD2 expression, the anterior margin of each cross bridge coincides, as expected, with the most posterior cells of the ectodermal stripe of *en* expression (Fig. 4D).

The somatic muscles are formed from cells of the high *twi-CD2* domain

The origins of the somatic muscle-forming cells in the mesodermal primordium have been unclear, although it has been suggested that somatic muscle is a characteristically ventral derivative of the mesoderm, which may require contact with the neurogenic ectoderm for its formation (Maggert et al., 1995). However, the S59-expressing founders of a subset of the muscles are known to arise within a domain of high *twist* expression and one of these cells, the founder of muscle DT1 (muscle nomenclature after Bate, 1993), is formed in the dorsolateral mesoderm, away from the neurogenic ectoderm (Dohrmann et al., 1990). Here we use CD2 to follow the fates of cells in the high CD2 domain and show that it is these cells that give rise to somatic muscles and that the progenitors of the somatic muscles are formed dorsally as well as ventrally in the mesoderm.

During stages 10/11, the pattern of expression in the high CD2 domain undergoes further changes (Figs 1F,G, 5). Cells over the central nervous system lose high levels of CD2, while immediately lateral and dorsal, the most posterior cells of the high domain form a region of strongly CD2-expressing cells, making contact ventrally with the ectoderm at the margins of the neurogenic region between neuroblast rows 5 and 6 (*wg*- and *en*-expressing respectively, Doe 1992; Fig. 5C). The mesodermal cells over the central nervous system will give rise to the internal ventral muscles (we use internal to include both the internal and intermediate muscles described by Bate, 1990), whereas, as we show here, the more lateral and dorsal cells with high CD2 expression will give rise to the precursors of external somatic muscles.

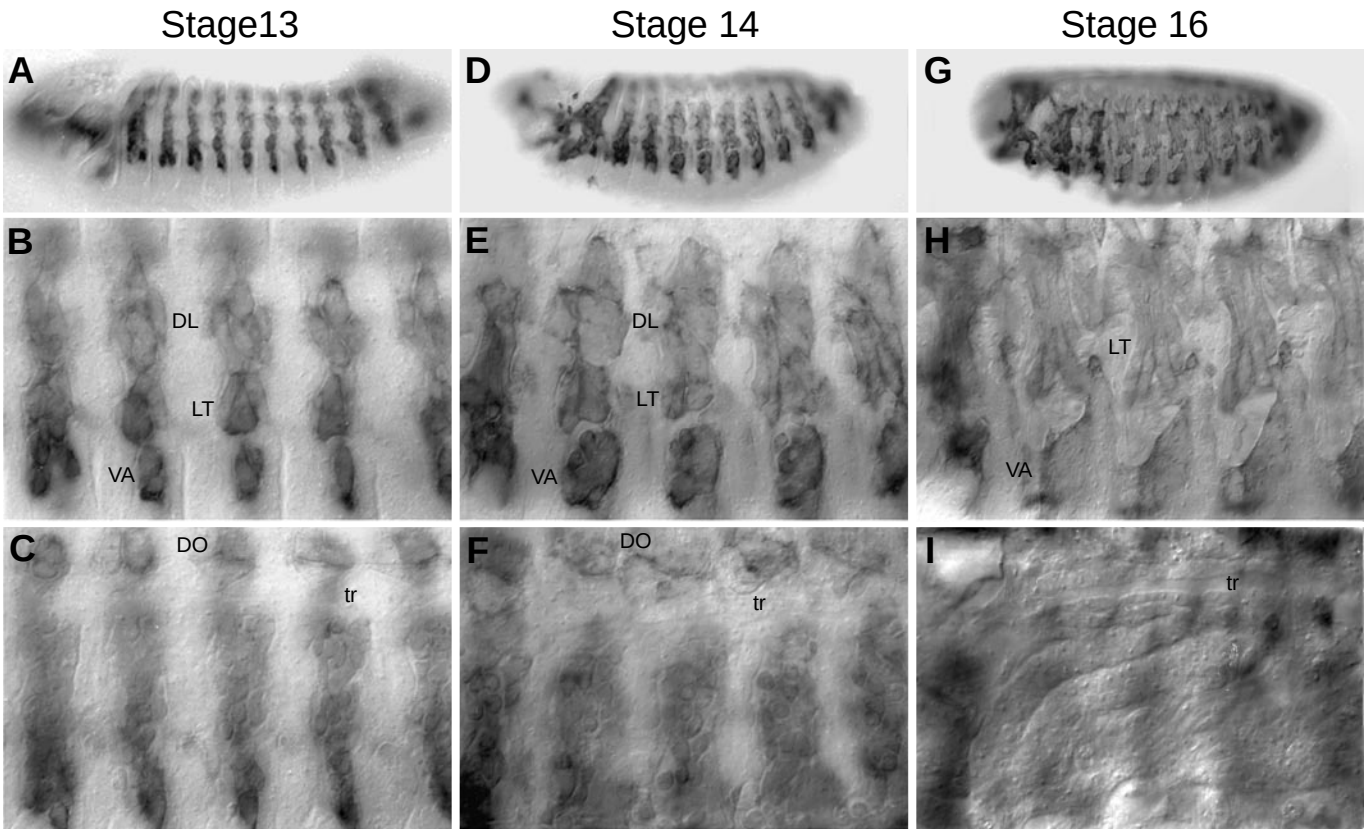

Fig. 6. External somatic muscles are formed from cells of the high CD2 domain. Embryos at three different stages stained with anti-CD2 antibody. The top three panels (A,D,G) show the embryos at a low magnification (20×). The panels below each of these show higher magnification images (65×) of the same embryos at two different focal planes: external top (B,E,H) and internal bottom (C,F,I). Internal views show region of myoblasts which fuse with progressively enlarging precursor cells visible in external views. Note the absence of such myoblasts in the stage 16 embryo (I), when fusion is complete. LT, lateral tranverse muscles; VA, ventral acute muscles; DL, dorsal lateral muscle group (individual precursors not identified); DO, dorsal oblique muscles and tr, lateral tracheal trunk. Note: although H and I are views of the same embryo at two different focal planes the photograph in I has been taken slightly more dorsally.

The close contact formed between mesoderm and ectoderm at the junction between NB rows 5 and 6 manifests itself in *twist* or CD2 stained preparations as a series of ventral prongs of expression (Fig. 5A). There appears to be a reorganisation of cells associated with the refinement of the high CD2 domain, such that some cells move outwards to form intimate contact with the overlying ectoderm. As a result of these movements the relation between the cells of the high CD2 domain and the stripe of ectodermal *en* expression becomes more complex. Thus, ventrally, the prongs of *twi-CD2*-expressing cells clearly extend further into the domain of *en* expression than their more dorsal neighbours (Fig. 5B). Initially, abdominal and thoracic patterns are fairly similar, each segment having a single ventral projection of strong *twi-CD2* expression. However, by the end of stage 11 there is a novel rearrangement of expression in the thorax, such that in T2 and T3, ventral *twi-CD2* expression consists of two prongs of cells, only the most posterior of which lies within the *en*-expressing domain (Fig. 5B). Later, the progeny of these two groups of cells will be separated by the anlagen of the leg discs and it seems likely that it is the formation of the leg primordium which leads to the subdivision of the CD2-expressing domain in T2 and T3. The pattern in T1 is unique (Fig. 5B), for which we have no explanation.

At the beginning of stage 12, as the germ band starts to retract, the posterior cells of the high CD2 domain, which will form external muscle precursors, undergo a further rearrangement, which appears to be associated with the formation of segment borders by the invagination of the ectoderm to form a furrow (not shown). The intersegmental furrow is aligned with the posterior margin of *en* expression in every segment. As the furrow forms, the high CD2 cells extend ventrally along the anterior margin of *en* expression in the ectoderm (not shown). During stage 13, the ventral prongs of expression become progressively more elongated and pointed and it begins to be possible to identify the precursors of individual muscles (Fig. 6A,B). These are large cells which lie closely apposed to the ectoderm. Internal to these large cells is a population of smaller myoblasts which is gradually depleted as cells fuse with the precursors of individual muscles (Fig. 6C,F,I). Thus, as the germ band retracts the cells of the high CD2 domain resolve into an external population of enlarging precursors and a less well defined, and declining population of myoblasts internal to them (Fig. 6).

The external muscle precursor cells recur at well defined

positions with reference to the ectoderm, most of them lying in a line along the anterior margin of the ectodermal stripe of *en*. We can divide this conspicuous grouping into the precursors of ventral, lateral and dorsolateral muscles (Fig. 6B,E,H). Within these groupings many of the precursors can be individually identified. The precursors that are clustered against the anterior margin of *en* expression are the progenitors of most, but not all, of the external muscles in the segment. The only external muscle that is not in this group is muscle VT1, which develops posterior to the *en* stripe, at the anterior margin of every segment from A2-A7 and which, for reasons which are not clear, does not stain strongly with *twi-CD2*, although it is derived from a Twist-expressing founder (Dohrmann et al., 1990).

It is not possible to follow the development of the most ventral cells of the high CD2 domain, which lie over the nervous system, because they lose CD2 expression. However, it is known that these most ventral cells give rise to the ventral internal muscles of the body wall (Bate, 1990).

There is an obvious gap between the precursors of the most dorsal muscles, which develop in close association with the progenitors of the heart (see below) and the progenitors of the more ventral muscles described above. These two groups are separated by a conspicuous landmark formed by the growth of the longitudinal tracheal trunk (Fig. 6C,F,I). This main branch of the tracheal system grows anteriorly and posteriorly along a line which divides the cells of the original high CD2 domain into a more dorsal grouping which includes the progenitors of the heart and the most dorsal muscles, and a more ventral grouping which includes the external muscle progenitors described above.

The dorsal crests give rise to heart progenitors

At the beginning of stage 10 the migrating mesodermal cells have begun to arrange themselves into an undulating series of crests and troughs at the dorsal margin of the ectoderm (Figs

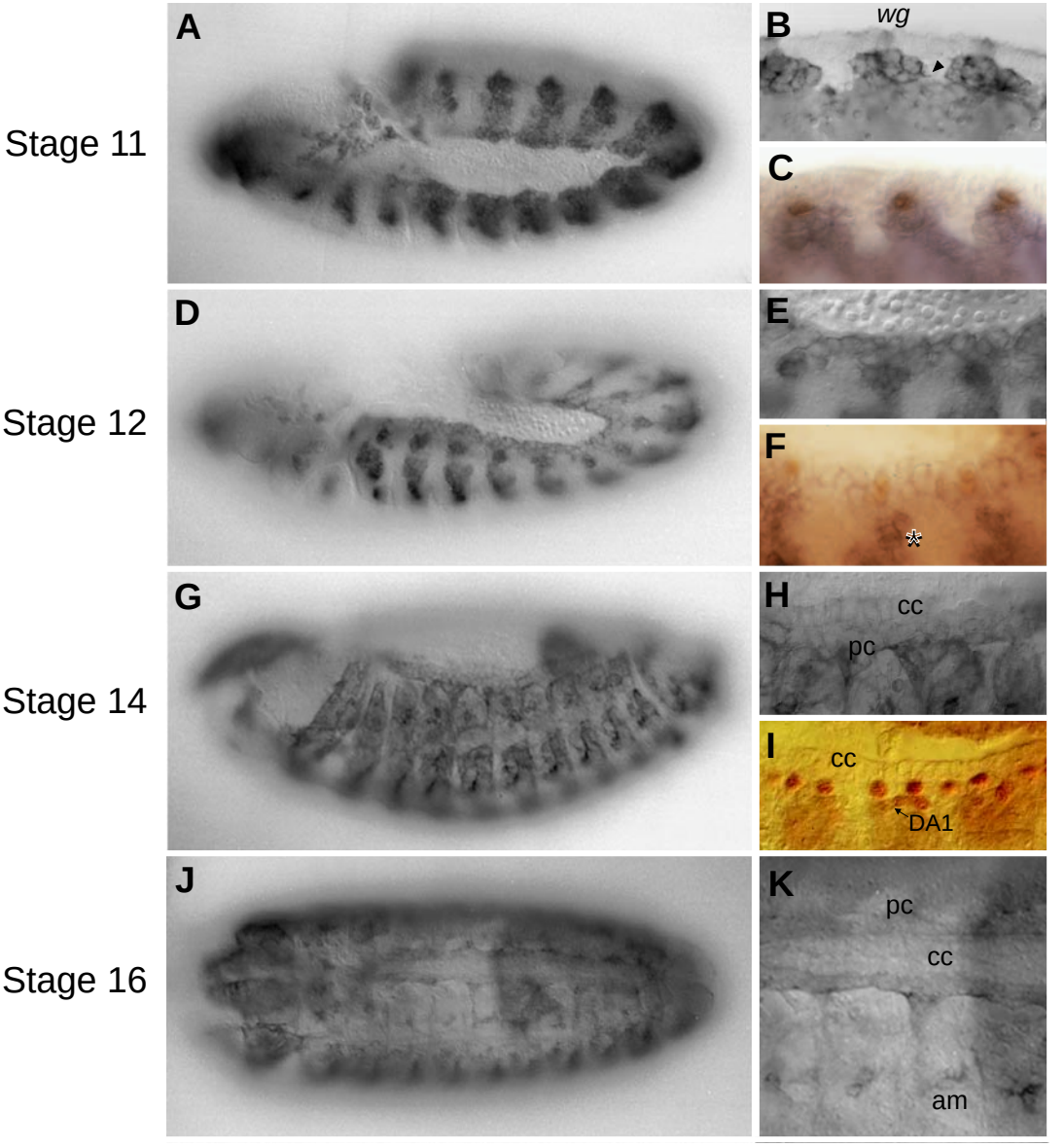

Fig. 7. The development of the heart and dorsal muscles. All embryos stained with antibody to CD2 (black); (C,F,I) embryos double stained with anti-CD2 and anti-eve (brown). (A-C) Embryos at stage 11. Dorsal crests have begun to spread laterally and form a series of conspicuous clusters in the most dorsal part of the high CD2 domain in each segment. (A) overview. (B) Embryo carrying *twi-CD2**; position of dorsal cluster shown with respect to wg-expressing cell in the ectoderm; filopodia (arrowhead) are extending between adjacent clusters. (C) Cells in the centre of each cluster are eve-positive. (D-F) Embryos at stage 12. Dorsal clusters have merged, to form a continuous cell set along the dorsal margin of the mesoderm. Precursors of dorsal external muscles are visible as darkly stained cells in F (asterisk). (F) Eve-positive cells persist at segmental intervals in the most dorsal mesoderm. (G-I) Embryos at stage 14. Pericardial cells (pc) and cardial cells (cc) have segregated at the dorsal margin and are visible in H and I. (I) The juxtaposition of Eve-expressing pericardial cells and the Eve-expressing precursor of the most dorsal internal muscle DA1. (J-K) By stage 16 the heart is complete beneath the epidermis which has closed dorsally. Cardial cells (cc) form a complete tube sheathed by pericardial cells (pc), with the triangular insertions of the alary muscles (am) at segmental intervals.

1F, 3E). By following the pattern of CD2 expression through to the completion of germ band retraction, it is possible to show that cells of the dorsal crests give rise to progenitors of the heart, including the central tube of cardial cells and the pericardial cells which lie on either side of them (Figs 3M, 7J,K). At the peak of each crest is a small number of cells which express *even-skipped* (*eve*; Fig. 7C). This mesodermal expression of Eve has been described previously and is followed by Eve expression in a small number of dorsal mesodermal cells (Fig. 7F,I), which are the precursors of the pericardial cells and a dorsal muscle (Frasch et al., 1987; Bate 1993).

As described above the dorsal crests originate by ventral movement of the cells in the troughs, associated with the inward movement of cells in the low CD2 domain in each segment. The effect of this movement is to give rise to a pattern of mesoderm cells which fluctuates between a line marking the future dorsal margins of the tracheal pits and the dorsal margin of the epidermis (Fig. 3E,I,M). The cells of the crests, i.e. those cells that lie between these two lines, show signs of coalescing as a group and during stage 11 this group begins to spread out laterally, with occasional filopodia visible, to fill the space left by the invaginated cells of the low CD2 domain (Figs 3K, 7A,B). By the onset of germ band retraction cells derived from adjoining groups have coalesced to form an irregular aggregation of cells aligned along the dorsal margin of the epidermis (Fig. 7D,E). There may be occasional breaks in the line, but by the completion of retraction, it is continuous. From this line, it is possible to follow the segregation of the cardioblasts, as a conspicuously well ordered file of cells at the dorsal most edge of the mesoderm, and slightly more ventral to them a more irregular set of cells, from which the pericardial cells will form (Fig. 7G-K).

Dorsal mesoderm gives rise to body wall muscles as well as heart progenitors

It has not previously been possible to show what the relationship is between progenitors of the most dorsal mesodermal derivative, the heart, and the progenitors of neighbouring dorsal muscles of the body wall or indeed whether dorsal mesodermal cells give rise to somatic muscles. By following CD2 expression we have been able to clarify this relationship and show that both cell types are formed from cells of the dorsal mesoderm.

As the germ band retracts, the precursors of the two most dorsal external muscles become apparent, as a pair of prominent, CD2-expressing cells dorsal to the main tracheal trunk (Figs 6C, 7F). These cells continue to expand and grow anteriorly and dorsally to form dorsal oblique muscles 1 and 2 (DO1, DO2), which lie closely apposed to the dorsal epidermis (not shown). The precursors of the dorsal internal muscles are less easy to identify, because like the ventral internal muscles, they do not have strong CD2 expression at this stage. Nonetheless, at stage 12, the enlarging precursor of the most dorsal muscle, dorsal acute 1 (DA1), is visible with Nomarski optics, lying between the conspicuously CD2-expressing precursor of muscle DO1 and the emerging pericardial cells (Fig. 7I). This identification is confirmed by the fact that the apparent founder of DA1 is an Eve-expressing cell, which appears in close proximity to the Eve-expressing pericardial cells (Frasch et al., 1987; Fig. 7I) and may well share a common lineage with these cells. Clearly therefore, the most dorsal muscles of the body wall arise from the dorsal mesoderm, in close association with the progenitors of the heart.

DISCUSSION

We have used a novel cell surface marker, CD2, to investigate the development of the mesoderm in *Drosophila* during the embryonic stages which follow gastrulation. Our aim has been to clarify the way in which the invaginated primordium of the mesoderm is subdivided into distinct groups of cells, which will give rise to the progenitors of the different mesodermal derivatives. We find that a metamerically repeated anterior-posterior subdivision of the mesoderm is a significant element in laying out the pattern of mesodermal progenitor cells and in distinguishing between an internal cell layer, which will give rise to the progenitors of visceral muscles, and an external layer, which will generate the somatic muscles and the heart.

Until recently, little was known about the spatial and temporal organisation of mesodermal development in *Drosophila*. Transplantation experiments in which single labelled cells were taken from the ventral furrow and placed ectopically in other, unlabelled embryos have indicated that the fates of mesodermal cells are not fixed at gastrulation (Beer et al., 1987). The fates adopted by the labelled clones of cells generated in these experiments appear to be linked to the position that the transplanted cell and its progeny occupy in the mesoderm after gastrulation, with visceral mesoderm cells in particular being associated predominantly with more dorsal transplants. Earlier experiments by Bock and Haget, using the embryos of other insects suggested that the ectoderm might be a source of inductive signals required for the normal patterning of the mesoderm (Seidel et al., 1940; Haget, 1953). Bock's experiments with lacewings showed that mesodermal cells in contact with dorsal ectoderm tended to form visceral muscles and heart, whereas contact with ventral ectoderm resulted in the formation of somatic, bodywall muscles. Recent work has confirmed and extended these ideas by providing evidence for inductive signals involved in mesodermal patterning in the fly and by identifying two of the signalling molecules concerned, Dpp (Staehling-Hampton et al., 1994; Frasch, 1995) and Wg (Baylies et al., 1995). Work by Baker and Schubiger (1995) shows that, in gastrulation arrested embryos, mesodermal cells express a variety of differentiated cell markers, depending on the region of the ectoderm that they come in contact with. As with Bock's experiments, their observations show that cells in contact with ventral ectoderm express a somatic muscle marker, the *MyoD* homologue *nau* (Michelson et al., 1990), whereas dorsal ectoderm provokes mesodermal cells to express other genes which are characteristic of visceral muscles and cardiac cells.

However, as we show here, the notion implicit in these experiments, that the partitioning of the mesodermal primordium into the progenitors of its different derivatives simply depends on a dorsoventral subdivision is wrong. In this view (e.g. Maggert et al., 1995) more dorsal cells form visceral muscles and heart, whereas more ventral cells form somatic muscles. However, by observing the behaviour of cells in the early mesoderm, we can show that the actual partitioning of the mesoderm into visceral and somatic muscle progenitors

depends on an anteroposterior subdivision of the mesoderm in each segment (Fig. 3M). More anterior cells form visceral mesoderm, more posterior cells form heart and muscles. Within these domains, it is certainly the more dorsal cells which form heart and visceral muscles, but dorsal as well as ventral cells give rise to somatic muscles.

Several lines of evidence support the conclusion that the full dorsoventral extent of the mesoderm (with the exception of the two most dorsal files of cells which produce the cardial cells and pericardial cells respectively) contributes to the formation of the somatic muscles. We see the progenitors of dorsal muscles arising in close association with the progenitors of the most dorsal mesodermal derivative, the heart. In addition, at least one of the myoblasts which contributes to the most dorsal internal muscle, DO1, expresses *eve*, and appears to share a common origin with the neighbouring, *eve*-expressing pericardial cells. It could be argued that cells from a more ventral muscle-forming domain of the mesoderm migrate dorsally to form the dorsal muscles, but there is no evidence for this. Except for the general invagination and dorsal migration of mesodermal cells at gastrulation, we see no sign of a dorsal migration of this kind, nor was there any evidence of migration in the mesodermal clones studied by Beer et al. (1987) apart from considerable anteroposterior dispersion within the visceral mesoderm. We might expect to see small movements of cells in the anteroposterior axis, because, as our observations confirm, continuous structures such as the heart, the visceral mesoderm and the fat body are initially formed as discrete, metamerically repeated clusters of cells (as also described by Azpiazu and Frasch, 1993, for the visceral mesoderm; Hoshizaki et al., 1994, for the fat body) which spread anteriorly and posteriorly and then coalesce to form connected cell groupings. From the point of view of understanding the origins of mesodermal pattern, an especially interesting feature of these clustered progenitors is that they are derived from particular levels in the anteroposterior axis of the mesoderm in each segment and not from its full extent. Our results emphasise the need for further study of cell lineages in the mesoderm, in order to clarify the relationships between the apparently distinct populations of progenitors that we identify. For example, we would like to know whether dorsal myoblasts and pericardial cells of the heart share a common progenitor, and if so, when these two cell types first become distinct.

Our conclusions follow from observing cells that express *twi-CD2*. CD2 expression mimics the expression of endogenous Twist protein, but perdures, with the result that we can follow the fates of cells which subsequently lose Twist expression as well as those that maintain it. Although this allows us to monitor large scale differences in cell behaviour within the mesoderm, such as the inward movement of cells in the more anterior domain of low *twi-CD2* expression in each segment, we do not claim that we can use *twi-CD2* to define the precise limits of the different populations of cells which we observe. In fact, what we provide here is a more detailed description of mesodermal cell behaviour during early embryogenesis, together with a rough fate map of the mesodermal primordium.

Fate maps of this kind give an indication of the spatial relationships between the progenitors of different differentiated cell types, that is they show which developmental pathways are followed by cells at different locations in normal embryonic development. What they cannot show, without further experiments, is the extent to which this partitioning of cells represents real restrictions on cell fate which assign cells in different locations to form different derivatives. Nonetheless, recent experiments show that at least one of the apparent subdivisions revealed by *twi-CD2* expression – in the anteroposterior axis in each segment – represents an actual limitation on cell fates in the early mesoderm, and that this restriction is an important element in the process of defining the progenitor pools for different mesodermal derivatives. The anterior region of relatively low CD2 expression corresponds (we cannot say how exactly) to an anterior domain within which *bap* expression is activated when cells are exposed to Dpp (Staehling-Hampton et al., 1994). The posterior domain of higher CD2 expression corresponds to a posterior stripe of cells which express *pox meso* in the absence of Dpp. Because *dpp* is normally expressed dorsally, *bap* expression is limited to a dorsal anterior quadrant of the mesoderm in each segment and it is these cells which, with CD2, we can follow as they invaginate and spread to form a continuous internal cell layer.

So far it is unclear how the anteroposterior subdivision that we observe in each segment of the mesoderm is achieved. We have mapped it with respect to the ectoderm and find that the sharp border between high and low domains of *twi-CD2* expression lies within the domain of *en* expression in the adjacent ectoderm. However, we speculate that the initial subdivision into an anterior and a posterior domain is an intrinsic property of the mesodermal primordium that is independent of the ectoderm. One reason for supposing that this is the case is provided by the observations of Staehling-Hampton et al. (1994). They showed that when Dpp is ectopically expressed in the invaginating ventral furrow, the response of mesodermal cells is discontinuous, in the form of a series of cell patches that express *bap*, separated by intervening cells that do not. Thus, even at this early stage, before mesodermal cells have invaginated over the ectoderm, there appear to be limited domains within the primordium that define the capacity of cells to respond to signals such as Dpp. Interestingly the *bap*-expressing domains which the experiment reveals are initially distributed in a pair rule fashion, which evolves, as the germ band extends, into a series of stripes corresponding to the low *twi-CD2* domain in every segment. The mesoderm as it invaginates inherits a reiterated pattern of pair rule gene expression which is initiated in the blastoderm stage embryo (for review see Pankratz and Jäckle, 1993) and it may be that it is the pair rule genes which are responsible for the early subdivision of the mesoderm into its anterior and posterior domains in each segment. Whatever the mechanism, we suggest that this subdivision is fundamental to the early patterning of the mesoderm. Together with inductive signals from the ectoderm, it provides a framework of spatial organisation which dictates the limits to the progenitor cell populations which we describe.

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