Development 1993 Supplement, 149-161 (1993)
Printed in Great Britain © The Company of Biologists Limited 1993

A dual requirement for neurogenic genes in *Drosophila* myogenesis

Michael Bate*, Emma Rushton and Manfred Frasch[1]

Department of Zoology, Cambridge University, Downing Street, Cambridge CB2 3EJ, UK
[1]Brookdale Center for Molecular Biology, Mount Sinai School of Medicine, New York, NY 10029, USA

*Author for correspondence

SUMMARY

In wild-type embryos of *Drosophila melanogaster*, the formation of differentiated larval muscles is preceded by the segregation of small numbers of progenitor or founder cells in the embryonic mesoderm. The founder cells, characterised by the expression of genes encoding putative transcription factors such as *S59* or *vestigial*, fuse with neighbouring myoblasts to form syncytial precursors of individual muscles. Founder cell segregation is deranged in embryos mutant for any of the neurogenic genes: enlarged clusters of cells expressing *S59* or *vestigial* are detected at the sites where small numbers of founder cells segregate in the wild type. In addition, muscle differentiation is deranged in such embryos in a way that appears to be closely linked to the extent of epidermal disruption caused by the neurogenic phenotype: myoblast fusion is limited to regions of the mesoderm beneath the residual epidermis left by the hyperplasia of the nervous system, and late expression of *S59* and *vestigial* is lost from mesoderm not lying within the margins of the residual epidermis. Thus neurogenic gene functions appear to be required both for the normal segregation of founder cells and for muscle differentiation. It is not clear whether either of these requirements reflects an essential function for any or all of the neurogenic genes within the mesoderm itself.

Key words: *Drosophila*, myogenesis, embryo, neurogenic gene, *Notch*

INTRODUCTION

During embryogenesis *Drosophila* constructs a complex pattern of larval muscles beneath the newly forming epidermis. There are 30 muscles per hemisegment from A2 to A7, (comparable patterns in other segments) and each muscle has a highly specific set of properties: size, shape, orientation, attachment and innervation. Muscle formation requires that neighbouring cells fuse to form multinucleate muscle precursors, and that adjacent precursors segregate to form separate elements of the final pattern. Thus in some way, individual elements of the pattern are specified and segregate as groups of cells to form particular muscles. One model of this process suggests that for each muscle, a cell called a *founder cell* is specified in the mesoderm (Bate, 1990). A characteristic programme of differentiation is initiated in such a cell (or cells) and neighbouring cells are entrained to this programme as they fuse with the founder to form a syncytial muscle precursor. Indeed, it has been shown for one subset of the muscles, that myogenesis is associated with the appearance, at specific locations in the mesoderm, of cells that express the homeobox-containing gene, *S59*, and these cells have been described as founders (Dohrmann et al., 1990). Neighbouring cells fuse with these founders to form the precursors of a small number of *S59*-expressing muscles in each segment. More recently, a number of other putative transcription factors (such as *apterous* [Bourgouin et al., 1992] and *vestigial* [*vg*; Williams et al., 1991]) have been shown to be similarly expressed in subsets of the larval muscles and their apparent founder cells in the embryonic mesoderm (for review see Bate, 1993).

Virtually nothing is known of the actual mechanisms that underlie the events of myogenesis in the fly, but there is at least a formal similarity between these events and the process of neurogenesis in insect embryos (Bate, 1990 and see Campos-Ortega, 1993 for review). During embryonic neurogenesis, neuroblasts, the precursor cells of the central nervous system, are defined by their position in an epithelium and segregate from surrounding cells which will later differentiate to form the ventral epidermis. Each neuroblast now divides repeatedly to produce a specific family of cells, which is unique to its position in the neuroepithelium. During myogenesis, founder cells are defined in the developing mesoderm and then fuse with neighbouring cells to form a syncytium with highly specific properties, which segregates from its neighbours. In both cases specification and segregation are an integral part of pattern formation. Although the mechanisms by which neuroblasts are specified are not yet clear, it has been exhaustively demonstrated that the segregation of neuroblasts from epidermoblasts in the ectoderm of the embryonic fly depends on the function of the neurogenic group of genes that includes *Notch* (*N*), *Delta* (*Dl*), *big brain* (*bib*), *neuralised* (*neu*), *mastermind* (*mam*) and the *Enhancer of split* (*E[spl]*) complex. Loss of function in any of these genes disrupts the

segregation process and leads to an overproduction of neuroblasts at the expense of surrounding epithelial cells (for review see Campos Ortega, 1993). The products of the neurogenic genes are expressed in the embryonic mesoderm (Kidd et al., 1986; Knust et al., 1987; Johansen et al., 1989; Kopczynski and Muskavitch, 1989; Fehon et al., 1991; Haenlin et al, 1990; Rao et al., 1990; Smoller et al., 1990; Bettler et al., 1991; Kooh et al, 1993) and it has been shown that their functions are required for the normal development of a variety of mesodermal derivatives (Corbin et al., 1991; Ruohola et al., 1991; Hartenstein et al., 1992), although the precise nature of this requirement remains unclear. Most interestingly, mesodermal domains of expression of *nautilus* (Michelson et al., 1990), the fly homologue of vertebrate MyoD (Weintraub et al., 1991), are clearly enlarged in embryos mutant for any of the neurogenic genes (Corbin et al., 1991). We have used a variety of methods to analyse the requirement for these genes in *Drosophila* myogenesis. Here we show that mutations in the neurogenic genes lead to an expansion of the domains of *S59* and *vg* expression in the mesoderm and produce severe abnormalities in muscle patterning and muscle differentiation. These mutant phenotypes appear to reflect two distinct requirements for neurogenic gene functions during myogenesis: the first is for the proper segregation of founder cells, the second for the proper differentiation of syncytial muscle fibres. Whether these reflect an autonomous requirement for the function of any or all of the neurogenic genes in the mesoderm itself is, as yet, unclear.

MATERIALS AND METHODS

Eggs were collected from wild-type (Oregon-R) and mutant (for details, see below) strains of flies on agar/apple juice plates (Wieschaus and Nüsslein-Volhard, 1986) and kept at 25°C. Embryogenesis lasts 21 hours at 25°C and embryonic stages are given according to the scheme of Campos-Ortega and Hartenstein (1985).

The following strains of flies were used in this work: Dl^{FX3}; N^{55e11} (Lehmann et al., 1983); *Df(1)svr*, N^{55e11} (Brand and Campos Ortega, 1988), provided by J.A. Campos Ortega; bib^{1DO5}; mam^{1J113}; neu^{1F65} (Lehmann et al., 1983); $E(spl)^{BX22}$ (Preiss et al., 1988); $E(spl)^{8DO6}$ (*Df3RE(spl)1*; Jürgens et al., 1984) provided by D. A. Hartley. We are very grateful for the provision of these flies.

For immunocytochemistry, eggs were collected, dechorionated, fixed and devitellenised according to the methods of Wieschaus and Nüsslein Volhard (1986) and stained with antibodies using standard protocols and the Vectastain ABC Elite Kit from Vectalabs. The anti-vestigial antibody was generously provided by Sean Carroll.

Polarising and Nomarski microscopy of late embryonic muscles and cuticles follows the protocol given by Drysdale et al. (1993). In summary, late embryos are dechorionated and placed singly in saline on a slide beneath a coverslip. The saline layer is then sucked from beneath the coverslip with a tissue, so that the coverslip flattens the embryo, extruding the internal organs and leaving a flattened cuticle with attached muscles. This preparation is then viewed on the stage of a Nikon polarising microscope (to view the muscle birefringence between crossed polarised beams) before transfer to a Zeiss Axiophot microscope (to see the cuticle pattern with Nomarski optics). Photographs were taken on Kodak Technical Pan in both cases.

Embryos were dissected and stained with toluidine blue according to the protocol given by Truman and Bate (1988).

Muscle nomenclature is according to the scheme given in Bate (1993).

RESULTS

Wild-type expression of *S59*

In wild-type embryos, *S59* is expressed in developing muscles (Fig. 1), in the embryonic CNS and in a small sector of the midgut. Expression begins about 6-7 hours after egg laying, in a single cell in the ventral mesoderm of each hemisegment. This cell divides to produce a pair of *S59*-expressing progeny. At the end of stage 11 a second cluster of *S59*-expressing cells appears in the ventral mesoderm, 4 cells in each abdominal hemisegment, but larger clusters in the thorax. As the germ band retracts, a pair of more dorsal mesodermal cells begins to express *S59* in each abdominal hemisegment (Fig. 2). These cells are not present in the thorax, and there are special patterns posterior to A7, so that, in all, 7 pairs of more dorsal *S59*-expressing cells segregate out on each side as the germ band retracts. These early patterns of mesodermal expression are a prelude to muscle formation and the recruitment of additional *S59*-expressing nuclei as myoblasts fuse with the *S59*-expressing muscle founders during and after germ band retraction. The final pattern of *S59*-expressing muscles consists of 3 muscles in

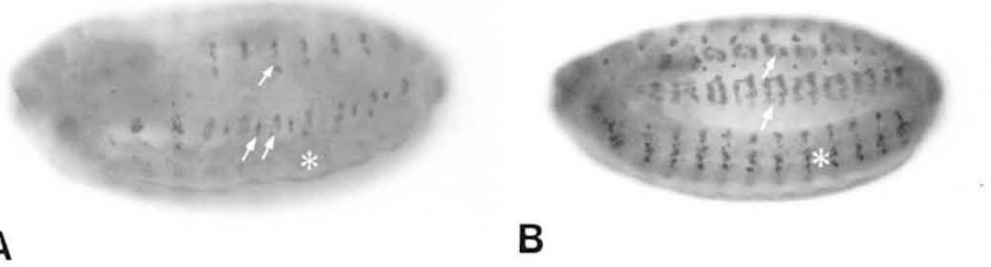


Fig. 1. Stage 14 embryos stained with anti-S59 (A) and anti-vestigial (B) antibodies. *S59* is expressed in three muscles in each abdominal segment (arrows in A3) as well as in the CNS (asterisk) and a region of the midgut (not in this plane of focus). *vestigial* is expressed in dorsal, lateral and ventral muscles (lateral and ventral muscles arrowed in A3) and in the CNS (asterisk).

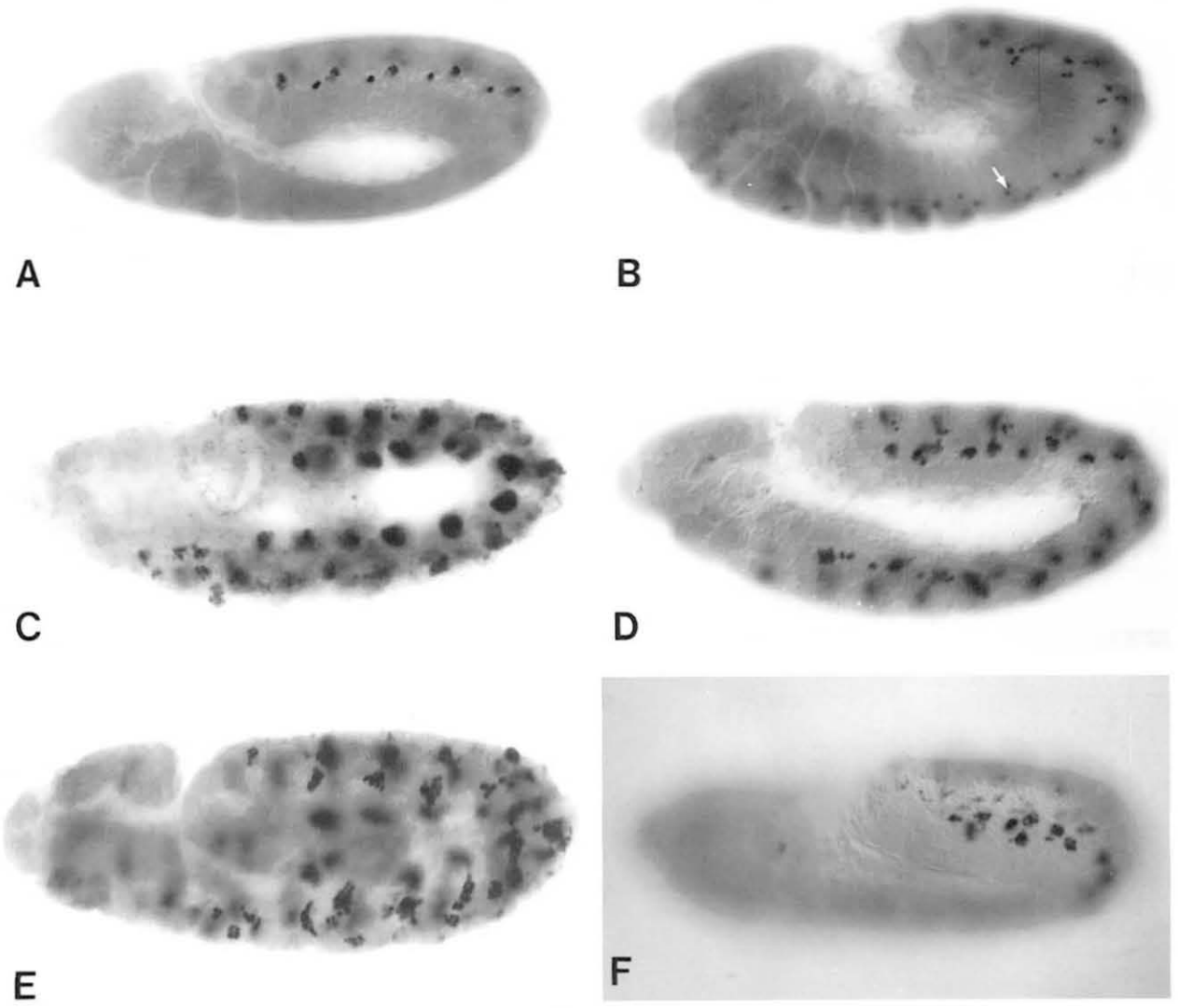


Fig. 2. Wild-type (A,B) and neurogenic mutant (C-F) embryos at late stage 11, early stage 12 (just prior to, or [B] at the onset of, germ band retraction) stained with anti-S59 antibody to reveal normal (A, B) and expanded (C-F) clusters of S59-expressing cells in the mesoderm. A shows the repeated pattern of two ventral cell clusters per abdominal hemisegment; B, the more dorsal pair of cells (arrowed in A2) which appears in each abdominal hemisegment (A1-7) as the germ band retracts. C-F show the expansion of clusters characteristic of embryos mutant for Dl^{FX3} (C), bib^{ID05} (D) neu^{IF65} (E) N^{55E11} (F).

each abdominal hemisegment (muscles VT1, VA2 [ventral] and muscle DT1 [dorsolateral]; nomenclature from Bate, 1993), with special patterns in the thorax and telson (Fig. 1). CNS expression begins shortly after the onset of expression in the mesoderm and evolves into a segmentally repeated pattern of presumed ganglion cells after germ band retraction. Cells in the midgut begin to express *S59* after the fusion of the anterior and posterior midgut primordia in 12- to 13-hour embryos. A detailed description of these expression patterns is given by Dohrmann et al. (1990).

S59 expression patterns in neurogenic mutant embryos

In embryos homozygous or hemizygous for mutations in any of the neurogenic genes we have looked at (*Dl, N, E(spl), bib, mam, neu*) we find a characteristic derangement of the normal pattern of mesodermal *S59* expression in the extended germ band (Fig. 2). At stage 11 we find that the expansion of *S59* expression in the CNS expected in a neurogenic mutant embryo is accompanied by the formation of enlarged clusters of *S59*-expressing cells in the ventral mesoderm. These clusters are located in the expected positions for wild type expression, but they are enlarged to a degree characteristic for the mutation involved and apparently closely correlated with the severity of the neurogenic phenotype (Fig. 2). In extreme cases, adjacent clusters coalesce, so that the exact number of cells in any cluster is difficult to resolve. As the germ band retracts, the more dorsal patches of mesodermal *S59* expression appear in the mutant embryos. As in the wild type these dorsal patches are

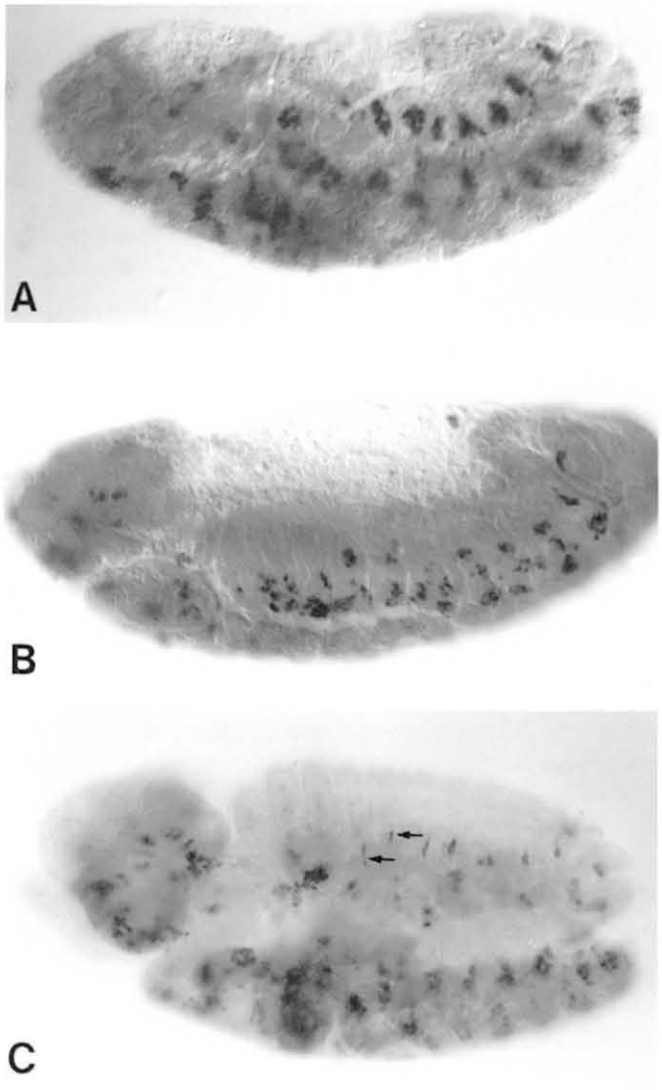


Fig. 3. Later patterns of *S59* expression revealed by antibody staining. (A) Dl^{FX3}, stage 14 embryo, showing the loss of ventral *S59* expression and the persistence of 7 dorsal clusters, corresponding to the dorsal clusters of A1-7 in the wild type embryo. In less extreme neurogenic phenotypes (B: bib^{ID05} stage 14), ventral *S59* expression persists, but only to the margins of the epidermis which remains despite the expansion of the CNS. In such embryos (C), relatively normal, strip-like patterns of *S59* expression may develop dorsally (arrowed in A1 and A2), following dorsal closure (stage 15), resembling the arrangement of *S59*-expressing nuclei in muscle DT1 (see figure 1A) in wild type embryos.

restricted to the abdominal segments where the precursor of muscle DT1 normally forms. In mutant embryos the patches are enlarged and, once again, the degree of derangement apparent in these more dorsal cells is characteristic of the mutation concerned (Fig. 3). Thus, as the germ band retracts, each of the mutant embryos shows a complex pattern of expanded *S59*-expressing cells dorsal to the developing CNS which itself shows a hyperplasic pattern of *S59* expression.

Unexpectedly, at later stages, ventral *S59* expression (corresponding to muscles VT1 and VA2 in the wild type) disappears from the mesoderm of all the mutant embryos except for a few scattered cells and patches of persistent expression adjacent to the more ventral epidermis, which remains in embryos with a weaker neurogenic phenotype. The dorsal expression remains in more or less expanded clusters beneath the epidermis which occurs outside the domain of the expanded nervous system. That these clusters are indeed equivalent to the dorsal clusters of wild type embryos is shown by their segment specificity: they are only present in the abdomen where they form 7 distinct groups in each of the mutants, mirroring the segment specificity of muscle DT1 in the wild type (Fig. 3). Once again, the degree of aberration in these persistent clusters is closely correlated with the strength of the neurogenic phenotype. In the weaker alleles, dorsal cells migrate into the characteristic strip-like arrangement of differentiating muscle, as found in wild type embryos (Fig. 3). These muscle-like configurations only appear in late embryos (more than 13 hours AEL), but it is important to note that, in such cases, the muscles have formed, or appeared to form from a larger than normal cluster of *S59*-expressing cells in the retracting germ band (Fig. 2). Thus in the weaker mutants, there is an apparent correlation between the expansion of the nervous system and the degree of disruption to late phases of muscle differentiation. In such mutants the ventral mesoderm (close to the expanded nervous system) is deranged, whereas dorsal muscles (associated with apparently undisturbed epidermis) may form relatively normally. Dorsally and ventrally however, in such mutant embryos, the clusters of *S59*-expressing cells are expanded as the germ band shortens.

vestigial expression in neurogenic mutant embryos

We used a second marker, *vg*, to examine the effects of loss of neurogenic gene functions on a different subset of the forming muscles. *vg* encodes a novel nuclear protein (Williams et al., 1991), which, like *S59*, is expressed in a subset of the larval muscles, and also in the developing CNS (Fig. 1). *vg* too is expressed in small numbers of mesodermal cells prior to germ band retraction (Fig. 4) and these appear to act as muscle founder cells, recruiting neighbouring cells by fusion to form the syncytial precursors of the *vg*-expressing muscle subset. The sequence and pattern of mesodermal *vg* expression has yet to be described in detail (Bate et al., unpublished data), but it is clear that *vg*-expressing ventral longitudinal muscles are prefigured by a regular arrangement of small numbers of *vg*-expressing cells sitting just internal to the CNS in the late stage 11 embryo (Fig. 4). In neurogenic mutant embryos these cells are replaced by enlarged clusters of *vg*-expressing cells at equivalent positions overlying the enlarged CNS (Fig. 4). In mutant embryos with a strong neurogenic phenotype, ventral *vg* expression is lost as development proceeds. In weaker alleles, such as mam^{1113}, where there is still considerable epidermis formed ventrally, *vg* expression persists ventrally, and *vg*-expressing cells are clearly incorporated into longitudinally arranged muscle fibres (Fig. 5). Thus the normal segregation of the *vg*-expressing muscle founder cells, like their *S59*-expressing neighbours, requires neurogenic gene

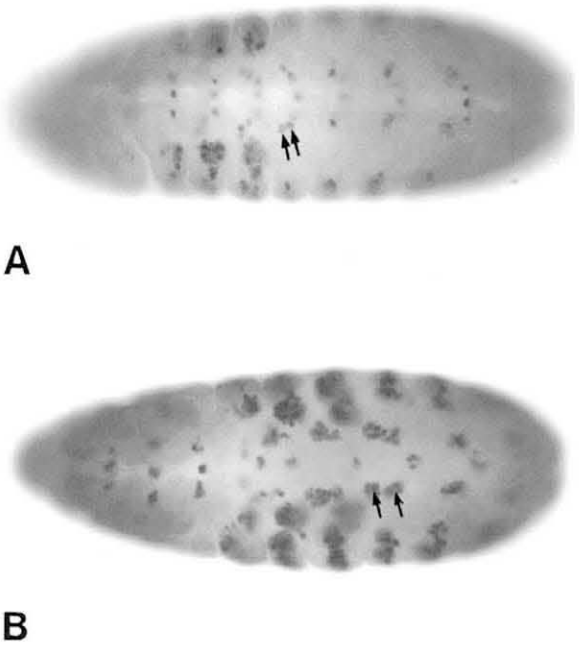


Fig. 4. Expression of *vg* in extended germ band embryos (late stage 11/early stage 12). (A) Wild type; (B) mam^{IJ113}. Both embryos viewed from the ventral surface of the thorax and anterior abdomen. The focus in both is internal to the CNS to show the *vg*-expressing nuclei of muscle founder cells in A (arrowed in A1) and clusters of *vg*-expressing cells at corresponding positions in the neurogenic mutant embryo (B, arrowed in A1). Cells on the midline are neural in both A and B.

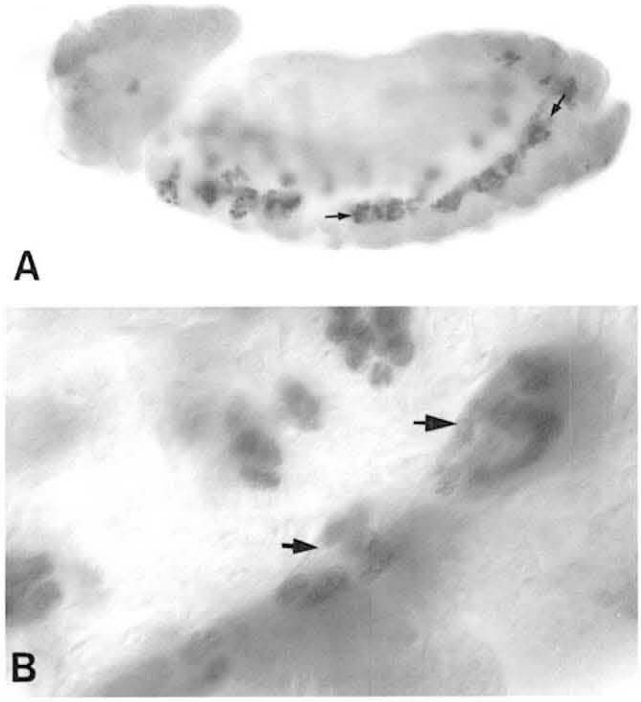


Fig. 5. *vg* expression persists ventrally in mutant embryos with weaker neurogenic phenotypes and *vg*-expressing nuclei may be incorporated into syncytial muscles. (A) Antibody staining shows *vg*-expressing cells ventrally (arrows) in a stage 14 mam^{IJ113} embryo. (B) At higher magnification these nuclei can be seen (arrows) to be incorporated in syncytial muscle fibres.

functions and continued *vg* expression and the formation of *vg*-expressing muscle fibres may require the presence of epidermis.

The phenotype of neurogenic mutants in polarised light

Late wild type embryos (just prior to hatching) show striking patterns of birefringence when flattened on a slide and viewed with a polarising microscope (Drysdale et al [1993]; see also Materials and methods and Fig. 6). The birefringence is largely caused by the highly oriented structure of the contractile proteins in the muscle myofibrils, together with a smaller contribution from crystals of uric acid in the Malpighian tubules, which are randomly extruded from the embryo as it flattens. Muscle birefringence can be used to assay both muscle differentiation and muscle patterning, and we have used it to look at the later development of muscles in neurogenic mutant embryos (Fig. 7). In each case we selected embryos that were at least 24 hours old, but whose internal tissues had not yet begun to degenerate, flattened

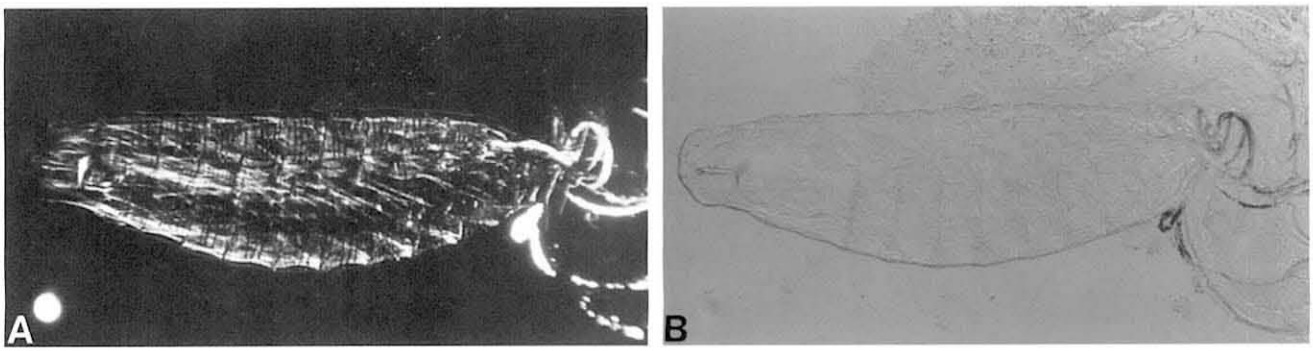


Fig. 6. Wild type embryo just prior to hatching flattened beneath a cover slip and viewed (A) between crossed beams of polarised light to reveal the birefringent pattern of muscle fibres and (B) with Nomarski optics to reveal the extent of the cuticle. Posteriorly in both, internal structures (other than the muscles) been extruded by the pressure of the cover slip.

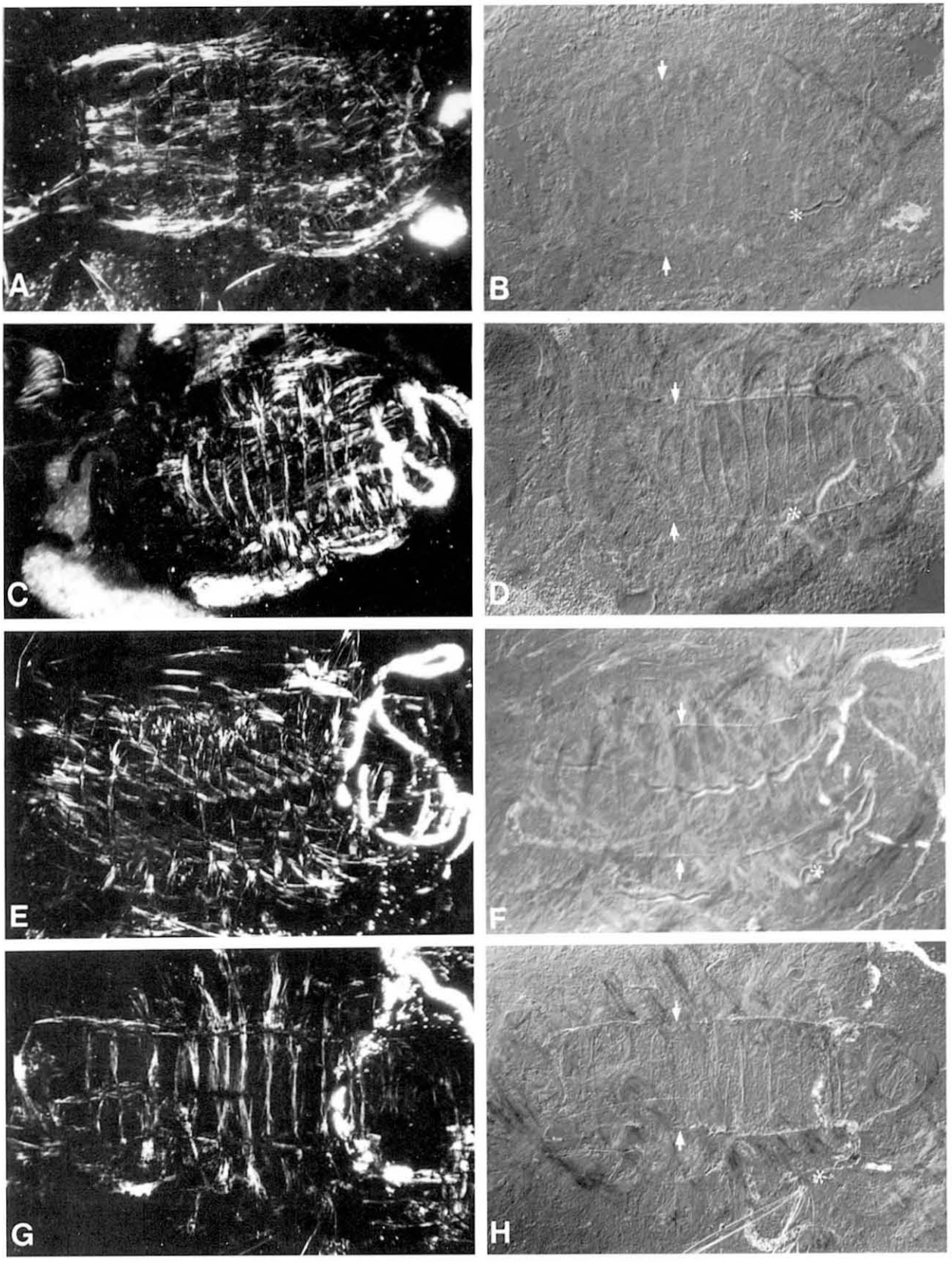
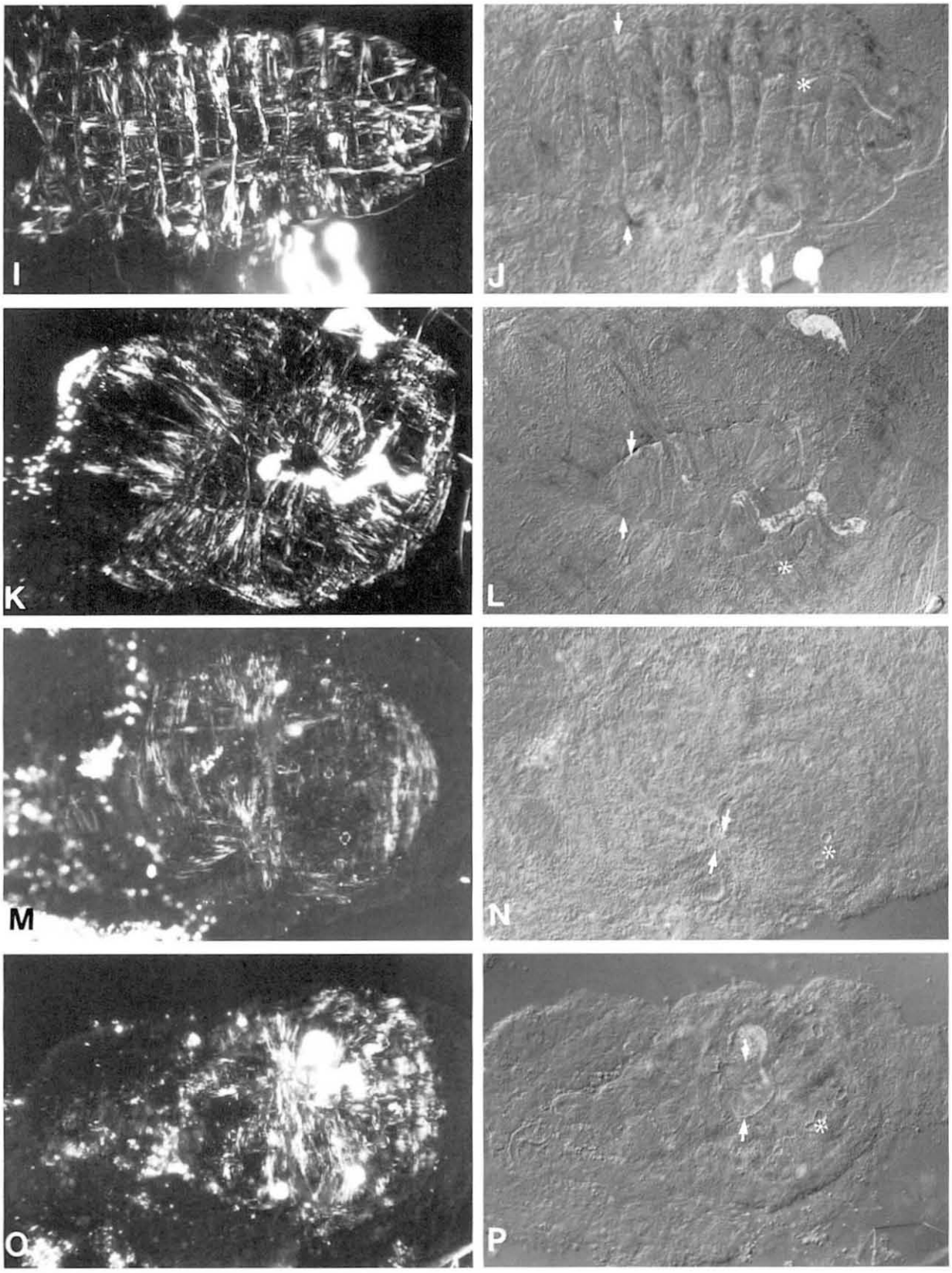


Fig. 7. As Fig. 6, using polarising and Nomarski optics to reveal muscle fibres and cuticle in different neurogenic mutant embryos. In each case, the same preparation is shown left and right, using the different techniques. The cuticle is shown on the right as a larger or



smaller shield of dorsal and dorsolateral structures. The ventral margins of this shield are arrowed. Asterisks indicate tracheae or tracheal fragments. (A,B) mam^{IJ113}. (C,D) *bib*. (E,F) $E(spl)^{BX22}$. (G,H) neu^{IF65}. (I,J) $Df(1)svr;N^{55e11}$. (K,L) N^{55e11}. (M,N) $E(spl)^{8DO6}$. (O,P) Dl^{FX3}. For further details see text.

them on slides and compared the phenotypes seen with polarised light (muscles, Malpighian tubules) with those seen with Nomarski optics in bright-field illumination (cuticle, tracheae). These preparations revealed a striking parallel between the degree of muscle differentiation and the extent of the differentiated cuticle formed in each of the mutant embryos. All the mutants have birefringence indicating that they succeed in differentiating myobrils with contractile proteins (Fig. 7). Weaker alleles (bib^{ID05}, mam^{IJ113}, $E(spl)^{BX22}$) also result in fused muscles in patterned arrangements, particularly in the more dorsal regions of the embryo, whereas stronger alleles result in few if any fused muscles, although the unfused, birefringent fibres may, nonetheless, have a patterned arrangement. In extreme cases (Dl^{FX3}, $E(spl)^{8DO6}$) the birefringence reveals a rather random pattern of fine fibres apparently radiating out from the small fragments of dorsal cuticle, which are still present in these embryos (Fig. 7).

The phenotype of the double mutant *Df(1)svr;N^{55e11}*

To explore the possibility that the mesodermal phenotypes we observe in neurogenic mutants are simply a secondary consequence of the enlargement of the central and peripheral nervous system and the concomitant reduction of the epidermis, we used the experimental approach adopted by Corbin et al. (1991) and looked at the phenotype of embryos that were doubly mutant for *N* and for a deficiency which removes the Achaete-Scute complex (AS-C). Embryos that are homozygous for deficiencies in AS-C fail to develop the proper complement of neuroblasts, and in severe cases, most of the CNS is missing (see Campos Ortega, 1993 for review). In addition, the PNS is reduced and, in the case of *Df(1)svr* (which removes the entire AS-C, together with the neighbouring genes *elav* and *vnd*, which are also required for CNS development [Garcia Bellido and Santamaria, 1978; Garcia-Bellido, 1979; Jimenez and Campos-Ortega, 1979, 1987, 1990; White, 1980; Campos et al., 1985]), all PNS neurons are missing, except for the chordotonal organs. In the double mutants there is a substantial rescue of the epidermal phenotype in lateral and dorsal regions although ventrally the epidermis, and the CNS are largely missing (Brand and Campos-Ortega, 1988; our observations: see Fig. 7). Following the experiments of Corbin et al. (1991) we reasoned that if the mesodermal phenotype, or some aspects of it, were secondary to the loss or derangement of epidermal cells inherent in the neurogenic phenotype, then these mesodermal effects, like the epidermal phenotype, should be ameliorated in the double mutant.

S59 expression

We find (M. B. and E. R., unpublished observations) that *S59* expression is deranged in the ventral mesoderm of *Df(1)svr* embryos, but dorsally, expression is normal, and muscle DT1 develops in its appropriate position. In the double mutants, we therefore concentrated our attention on

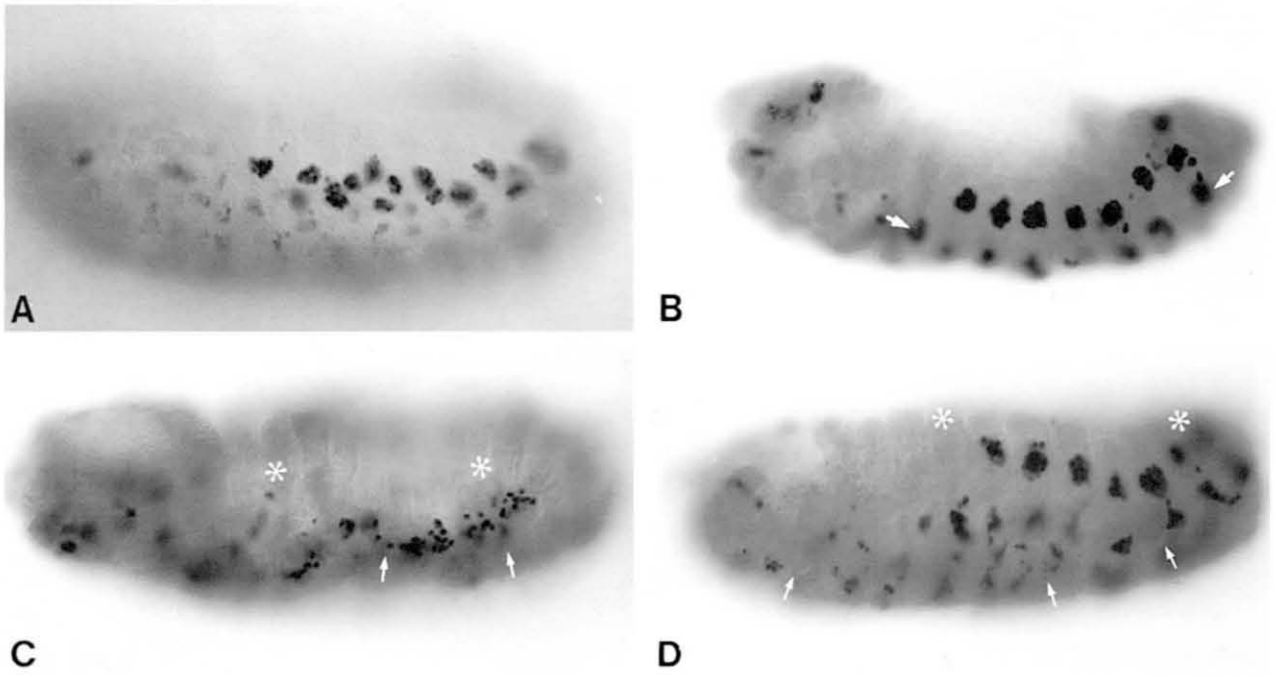


Fig. 8. *S59* expression revealed by antibody staining in N^{55E11} embryos (A,C) and in double mutant embryos $Df(1)svr;N^{55e11}$ (B,D). A and B are embryos just after germ band retraction, showing expanded dorsal and ventral *S59*-expressing cell clusters in A, and focussing in B on the seven enlarged dorsal clusters characteristic of the double mutant. Enlarged ventral clusters are also visible in B (arrows) but are not in this plane of focus. C and D are embryos after dorsal closure (stage 15) showing dorsal *S59*-expressing nuclei in A near the margin of the residual cuticle. Mesodermal expression of *S59* has disappeared from more ventral regions of the embryo, i.e. ventral to the margins of the cuticle. In the double mutant embryo (D), there is substantial rescue of the lateral epidermis and there is persistent mesodermal *S59* expression in these more ventral regions of the embryo, besides the persistent expression in the seven dorsal clusters of the abdomen. In C and D, asterisks indicate the dorsal midline and arrows show the ventral margins of the residual cuticle.

the dorsal expression pattern. In single mutant N^{55e11} embryos, we find expanded clusters of *S59*-expressing cells as the germ band retracts, and these persist in the shortened embryos, in a more or less scattered arrangement, with no sign of aggregation to form a normal muscle (Fig. 8). In the double mutants we also find expanded domains of *S59*-expressing cells dorsally, even though the enlarged anlage of the CNS is no longer present, and the extent of the epidermis appears to be far greater than in N^{55e11} (Figs 7, 8). These expanded clusters persist as the germ band retracts and form large, compact, conspicuous clusters in the shortened embryo. Interestingly, unlike the case of N^{55e11}, there is also some persistent ventral expression in the double mutant at late stages, and this appears to correlate closely with the expanded lateral epidermis present in these embryos. Clearly, the amelioration of the nervous system phenotype in the double mutants does not reduce the expansion of the dorsal *S59*-expressing clusters. On the other hand, the late loss of *S59* expression from ventral mesodermal cells characteristic of N^{55e11} mutants is partially rescued in the double mutant $Df(1)svr;N^{55e11}$ and this rescue appears to be related to the increased territory covered by epidermal cells in these embryos.

Birefringence

The single mutant, N^{55e11}, has a characteristic phenotype in polarised light (Fig. 7K,L), which fits into the series described above, in which increasing severity of the neurogenic phenotype correlates with apparent loss of myoblast fusion and muscle patterning. A shield of cuticle forms dorsally and, radiating out from this, there is a fan of birefringent fibres, with a few fibres crossing the shield itself. By contrast, in the double mutant, $Df(1)svr;N^{55e11}$ there is a substantial rescue of both the cuticular and the muscle phenotypes (Fig. 7I,J). The cuticle has expanded both in the anteroposterior and in the dorsoventral axes, although there is still no sign of ventral denticle belts. The muscle phenotype shows obvious signs of the strap-like structures typical of fused muscle fibres, and many of the fibres are oriented and attached in a clearly patterned fashion. It is only at the ventral margins of the cuticular shield that this organisation breaks down and here there are signs of disorganised and unfused muscle fibres. Thus the phenotype of the single and double mutant embryos seen in polarised light reveals a strong correlation between the strength of effects on the epidermis and nervous system and the degree of disorganisation in the late myogenic phenotype associated with muscle differentiation.

Cell fusion in neurogenic mutants

Although birefringence gives a good indication of the degree of myoblast fusion during myogenesis, it is not possible to say unequivocally from the birefringence alone, whether fusion has or has not occurred. To assess the degree of myoblast fusion in different neurogenic mutants we dissected wild type and mutant (recognisable by their expanded nervous systems) embryos 12-13 hours AEL (stage 16) and stained them with toluidine blue. In wild type embryos, preparations of this kind can be used to follow the sequence of muscle differentiation from the onset of myoblast fusion (Bate, 1990). Using this method we were able to find fused cells in the somatic mesoderm of all the neurogenic mutants, with the possible exception of $E(spl)^{8DO6}$. As with the phenotype of the birefringence, there is a clear correlation between the extent of the neurogenic phenotype, that is the expansion of the CNS and PNS, and the loss of epidermis, and the degree to which myoblast fusion occurs. Thus, in the weaker mutants there are well formed syncytial muscles in an orderly arrangement, inserted on the dorsal and lateral epidermis, as predicted from the observations using the polarising microscope, whereas in the strongest mutants, there is little apparent order in the mesoderm and cell fusion occurs rarely, to produce occasional bi- or trinucleate syncytia. In $E(spl)^{8DO6}$, we did not even find rare fusions, but this finding should be treated with caution - the embryos are difficult to dissect, and the number of fusion events that we see in other strong neurogenic mutants (Dl^{FX3}; N^{55e11}; neu^{1F65}) is small. We do not exclude the possibility of rare fusion occurring in $E(spl)^{8DO6}$. In those embryos such as *mam* and *bib* with large numbers of syncytial muscle fibres, relatively large well-formed muscles attach to the most dorsal regions of the epidermis. Ventrally, there is a tendency for the normal pattern of muscles to be replaced by large numbers of thinner, syncytial, spindle-shaped fibres arranged in parallel.

All the mutants, again with the possible exception of $E(spl)^{8DO6}$, show a striking phenotype in the relatively disorganised mesoderm where syncytial muscles fail to form. Concentrated in particular on the margins of the expanded nervous system abutting the edges of the epidermis, there are conspicuous clusters of prominent mesodermal cells, which in some cases appear to form rosettes about a central focus (Fig. 9). These cells are unfused and may represent a default state adopted when fusion fails to occur among muscle forming cells. This view is strengthened by observations in weaker mutants and in the double mutant $Df(1)svr;N^{55e11}$, where the unfused cells in these marginal clusters are often associated with cells which have fused to form bi- or trinucleate syncytia, but have not differentiated into well formed muscles (Fig. 9). Dorsally in the double mutant, the almost complete absence of fusion seen in N^{55e11} is rescued and syncytial muscles develop and insert on the expanded epidermis, again confirming our observations with the polarising microscope. Summarising, we find that the degree of myoblast fusion is closely related to the extent of the epidermal territory in a neurogenic mutant. Where cells fail to fuse, conspicuous clusters of mesodermal cells are formed, and if epidermal territories are expanded, cells in these clusters may be recruited to fusion.

DISCUSSION

The muscles of the *Drosophila* larva form a complex pattern of individual units each of which has a characteristic and distinctive set of properties. Muscles differ in, and can be recognised by, their position, insertion points, size, orientation and innervation. These properties are manifested by groups of myoblasts which, during and shortly after germ band retraction aggregate and fuse to form syncytial precursors for each of the differentiated muscles. Thus, the muscle pattern is formed by a mechanism which segregates

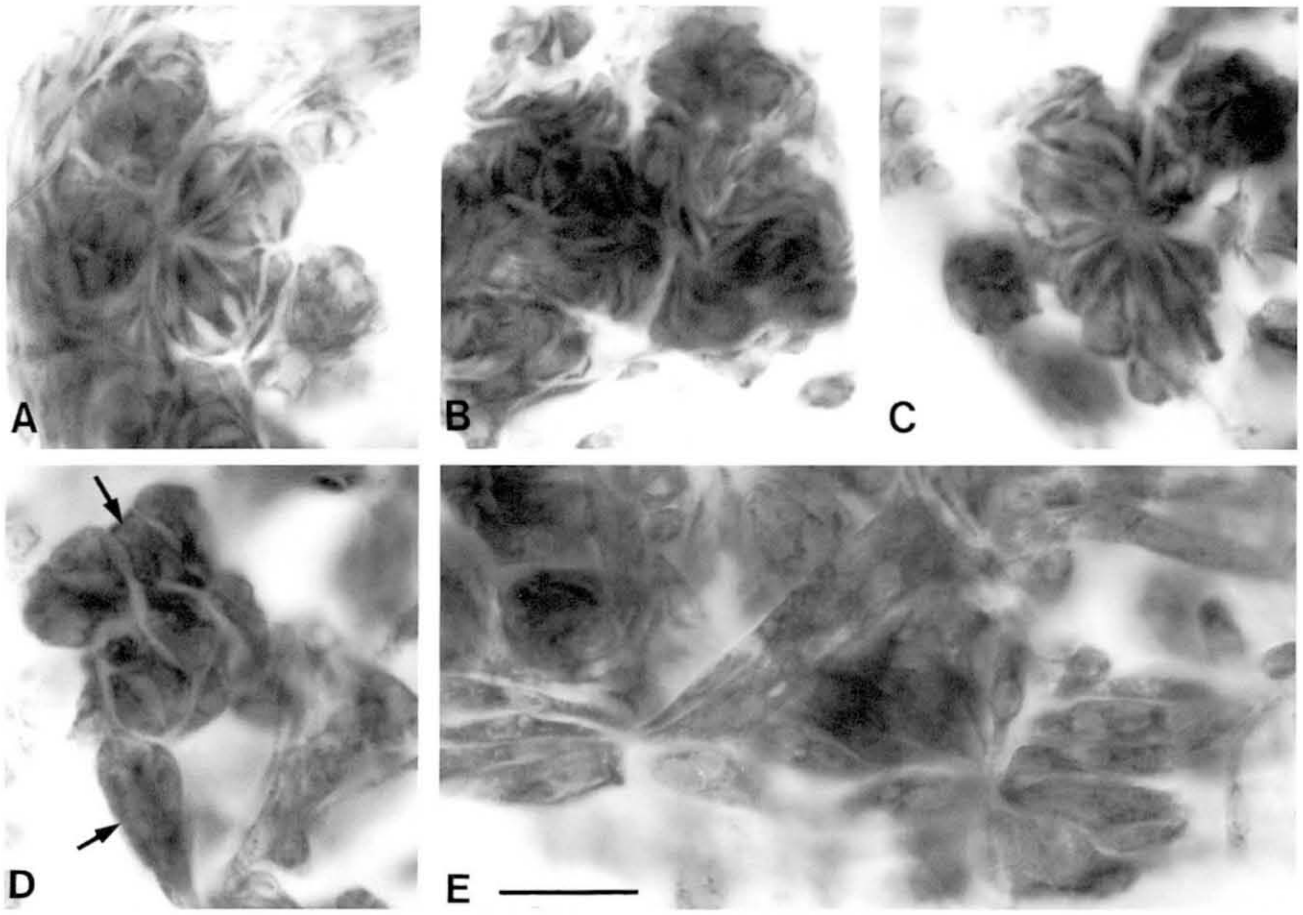


Fig. 9. Cells and syncytia in the mesoderm of neurogenic mutant embryos (stage 14) dissected and stained with toluidine blue. (A,B,C). Rosettes of unfused cells found over the enlarged CNS and abutting the ventral margins of the epidermis in N^{55E11} (A); neu^{1F65} (B); and Dl^{FX3} (C) embryos. D shows cases of cell fusion (arrowed) in a rosette-like structure over the margin of the expanded ventral epidermis in a double mutant $Df(1)svr;N^{55e11}$ embryo. E shows well formed syncytial muscle fibres over more dorsal regions of the epidermis in the same double mutant embryo. Scale bar, 15 μm.

mesodermal cells into separate and distinct muscle forming groups. There are two alternative ways in which the properties of the syncytial muscle precursors might be defined. Since cell fusion can lead to the entrainment of nuclei in a shared cytoplasm to common patterns of gene expression, the properties of the syncytium could be defined by the prior segregation of a set of founder cells, each of which would seed the formation of a different precursor by fusing with neighbouring cells. Alternatively, cells could be assigned in groups, the members of each group fusing to form a particular syncytial precursor. The founder cell hypothesis is consistent with the developmental history of the *S59*-expressing muscle subset. Expression begins in a small number of mesodermal cells with which surrounding myoblasts fuse to form the precursors of the three *S59*-expressing muscles in each segment. As they fuse, these myoblasts are recruited to *S59* expression. Although *S59* is the type example of such a "founder cell gene" there is an increasing catalogue of genes with similar expression patterns that define other subsets of the larval musculature and which are expressed in small numbers of putative founder cells prior to germ band retraction (for review, see Bate, 1993).

In this paper we have shown that the normally limited domains of *S59* expression are expanded in the mesoderm of embryos mutant for the neurogenic genes that we have studied. A second marker, *vg*, shows that this effect is not restricted to those myoblasts that express *S59*, but probably represents a general requirement for neurogenic gene functions during the segregation of all muscle precursors. In addition, in the mutant embryos, we find that later phases in the sequence of myogenesis are deranged: there are defects in fusion and there is a premature loss of *S59* expression from regions of the mesoderm which initially express it. The relationship between the two phenotypes (expanded domains of *S59* expression and disrupted myogenesis) and the neurogenic phenotype is different. *S59*-expressing clusters are expanded dorsally and ventrally, so that the expansion of the domains of *S59* expression may be independent of both the expansion of the nervous system and the consequent loss of epidermis, both of which are most prominent ventrally. However, the disruption of muscle differentiation seems to be closely related to the enlargement of the nervous system. The degree of disruption of myogenesis closely follows the extent to which the epidermis is

reduced and the nervous system expanded and in the weaker mutants there is an obvious ventral to dorsal decline in the disruption to the muscles. A priori there is no simple reason for such a gradation in the phenotype unless the disruption to myogenesis is directly linked to the derangement of the ventral neurogenic region. In addition, when the nervous system phenotype is partially rescued (*Df(1)svr;N^{55e11}*) there is a dramatic improvement in myogenesis. By contrast, the double mutant embryos still have expanded domains of *S59* expression. Thus, the double mutants emphasise the relative independence of the expansion of *S59*-expressing clusters from the nervous system phenotype, as against the close relationship between the degree of expansion of the nervous system and the extent of disruption to myogenesis. One explanation for this finding would be that there is a direct requirement for neurogenic gene functions in the mesoderm for the proper segregation of *S59*-expressing cells, whereas the later effects on myogenesis are a secondary consequence of the disruptive effects of the neurogenic phenotype on the proper patterning and differentiation of the ectoderm.

Mesodermal requirement for neurogenic gene functions

All the neurogenic genes are expressed in the mesoderm during embryogenesis (Kidd et al., 1986; Knust et al., 1987; Johansen et al., 1989; Kopczynski and Muskavitch, 1989, Fehon et al., 1990; Haenlin et al., 1990; Rao et al., 1990; Smoller et al., 1990; Bettler et al., 1991; Kooh et al., 1993), but without further experiments it is impossible to say whether any or all of the effects we describe here are a direct consequence of loss of functions in the mesoderm itself. It could be, for example, that loss of neurogenic genes causes a general derangement of the ectoderm, which indirectly causes a disruption to the normal patterning of mesodermal cells immediately adjacent to it. Experiments in other insects have shown that the ectoderm is essential for normal mesodermal development. In particular Bock (Seidel et al., 1940) found that the differentiated cell type formed by any given sector of the mesoderm depends on the region of the ectoderm that it comes to underlie after migrating inwards at gastrulation. These experiments strongly suggest that there is an inductive role for the ectoderm in mesodermal patterning in insects. However, the partitioning of the mesoderm that Bock studied, i.e. its subdivision into different sectors (heart, fat body, visceral, somatic and gonadal mesoderm) is successfully achieved in many if not all the mutants that we looked at (Hartenstein et al., 1992) and it is not clear that the inductive effects that Bock inferred would be directly relevant for a process like the selection of mesodermal cells for *S59* expression. A simpler interpretation of the results we report here is that, during the development of the wild type embryo, domains of prospective gene expression are established in the somatic mesoderm from which the muscles will be formed. Within these domains, individual myoblasts are selected for expression by a mechanism which at the same time suppresses expression in other cells of the domain. In the neurogenic mutants this mechanism fails, so that large clusters of *S59* (or *vg*)-expressing cells are formed. The simplest model for this process would locate it in the mesoderm itself, but it would also be possible to formulate a mechanism whereby a normally restricted *S59*-activating signal passing from the ectoderm to the mesoderm would become more widespread, so leading to ectopic expression in the mutant embryos. On the basis of the results we report here, we cannot distinguish between these two alternatives, and it will be necessary to make an analysis of mosaic embryos in order to find the primary focus for the effect of neurogenic mutations on *S59* and *vg* expression.

A role for the ectoderm in myogenesis

The close connection between the strength of neurogenic phenotypes and the degree to which late myogenesis is disrupted suggests that there may be a role for ectodermal derivatives in the normal development of muscle in the fly. Poulson (1945) described the failure of muscle development in embryos deficient for *Notch*. On the basis of results of embryo culture experiments, Cross and Sang (1978) suggested that there might be a direct requirement for *Notch* in muscle development, although they also pointed out that they could not exclude a secondary effect of the deranged nervous system in their cultures. Our results show that the *Notch* phenotype in late myogenesis fits into a consistent series, which includes the other neurogenic loci and matches the degree of muscle disruption to the strength of the neurogenic phenotype. This is in agreement with the observations of Lehmann et al. (1983) who described mesodermal defects associated with the "strong" neurogenic phenotype and suggested that these could be "explained by taking neural and epidermal defects into account". In addition, in *Df(1)svr;N^{55e11}* embryos where the neurogenic phenotype is partially rescued by a complete loss of the AS-C, there is a clear rescue of disrupted muscle differentiation. This result strongly suggests a direct link between hyperplasia of the nervous system and defects in late phases of myogenesis, including myoblast fusion. We speculate that the epidermis may be essential for muscle assembly and that the effects of the hyperplastic nervous system on myogenesis depend on the associated loss of epidermis. Where epidermis is present, even in strong mutants, some myoblast fusions may occur, and in the less extreme phenotypes, well-formed muscles develop in dorsal regions away from the enlarged nervous system. In contrast, the formation of muscles is always deranged ventrally, and it is at the ventral margins of the residual epidermis that we find clusters of mesodermal cells in a novel arrangement, which may represent some intermediate state of differentiation when fusion is prevented. We do not know whether any of these clusters correspond to expanded domains of *S59* or *vg* expression, although it seems likely that included in this population are the ventral mesodermal cells that lose expression of *S59* or *vg* during later embryogenesis in the stronger mutants. Possibly the epidermis, or some product of epidermal cells, is a required substratum for fusion to occur (other mutations that disrupt the epidermis also lead to a reduction of myoblast fusion; M. Frasch, unpublished observations). We could envision, for example, an initial step in which a cell adheres to and flattens on the epidermis before neighbouring cells can fuse with it. Clusters of cells seen in toluidine blue stained embryos might then represent mesodermal cells attempting to adhere to the margin of the residual epidermis, or cells

with no competent partner with which to fuse. It is interesting in this context that Volk et al. (1990) report that, in serum free medium, cultured *Drosophila* myoblasts fail to adhere to and flatten on coverslips if laminin is not present. Adhesion and flattening are a prelude to the formation of multinucleate myotubes in their cultures. Prior to and during germ band retraction, laminin mRNAs are expressed in the mesoderm and the epidermis, but not at all in neurons (Montell and Goodman, 1989). Thus laminin might be substantially reduced in neurogenic mutant embryos, although we have as yet no direct observations to substantiate this idea. It is also interesting that, in *Drosophila*, syncytial muscle precursors always begin to form in close contact either with the CNS or the epidermis. It might be argued that, since the earliest signs of fusion occur not next to the epidermis, but in close proximity to the developing CNS (Bate, 1990), the epidermis itself cannot be essential for myogenesis. However, at the stage at which these first fusions occur, just before germ band retraction, the nervous system is still an integral part of the ventral embryonic epithelium, which, in a wild type embryo, includes both the precursors of the ventral epidermis and the CNS. Thus, the precursors of the epidermis are present both in the region of the forming CNS and outside it, and in a neurogenic mutant, where epidermal precursors would have been transformed into the neural pathway, they would be missing in the region of the expanded CNS. Although we suggest that there is a general phenotype for late myogenesis associated with loss of epidermis in neurogenic mutants, we do not exclude the possibility of a direct involvement for individual neurogenic genes in muscle differentiation which is masked by the derangements we describe here. In this respect, it may be significant that neu^{IF65} embryos seem to have a worse muscle phenotype than we would predict from the extent of the cuticle they produce.

During the normal sequence of muscle development, the onset of expression of genes like *S59* and *vg* in a limited set of mesodermal cells is followed by expression in additional cells as they are recruited to forming syncytia by fusion. In neurogenic mutant embryos, larger than normal clusters of mesodermal cells initiate the expression of *S59* and *vg* in a way which suggests that the early limitation of expression seen in the wild type requires the functions encoded by the neurogenic genes. The additionally expressing cells in the mutant embryos are likely to include those that would have been recruited to expression by fusion in the wild type. However, it is not clear what the consequences of precocious gene expression in these cells may be for the process of myogenesis itself. One model of myogenesis would suggest that each muscle is seeded by the definition of a single founder cell which recruits neighbouring cells by fusion. If early expression of *S59* or *vg* is in some way diagnostic for such founder cells, then in a neurogenic mutant embryo additional founder cells would be present in the expanded clusters of *S59*- or *vg*-expressing cells. Thus we might expect that if muscles formed in such embryos, they would be present as multiple copies, each muscle being founded by a separate cell. In weaker mutant embryos, such as *mam*, where *vg*-expressing cells are incorporated from expanded ventral clusters into ventral muscles, these muscles are indeed present as multiple copies. However, the issue is complicated by the fact that, more dorsally in such embryos, substantial muscles may be formed, apparently as single copies. As this example shows, although we find evidence of a dual requirement during muscle development for the functions encoded by the neurogenic genes, where and how these genes operate during myogenesis and the relationship between the two requirements we have demonstrated are, as yet, unresolved.

We are grateful to Alfonso Martinez Arias and Mary Baylies for many thought provoking discussions and much useful advice. This work is supported by a grant from the Wellcome Trust to M.B.

REFERENCES

Bate, M. (1990). The embryonic development of larval muscles in *Drosophila*. *Development* **110**, 791-804.

Bate M. (1993). The mesoderm and its derivatives. In *The Development of Drosophila melanogaster* (eds Bate M. and Martinez Arias A.) Cold Spring Harbor, NY: Cold Spring Harbor Press. **In press**

Beer, J., Technau, G. and Campos-Ortega, J. (1987). Lineage analysis of transplanted individual cells in embryos of *Drosophila melanogaster*. IV. Commitment and proliferative capabilities of individual mesodermal cells. *Roux' Arch. Dev. Biol.* **196**, 222-230.

Bettler, D., Schmid, A. and Yedvobnick, B. (1991). Early ventral expression of the *Drosophila* neurogenic locus, *mastermind*. *Dev. Biol.* **114**, 436-439.

Bourgouin C., Lundgren S.E. and Thomas J.B. (1992). *apterous* is a *Drosophila* LIM domain gene required for the development of a subset of embryonic muscles. *Neuron* **9**, 549-561.

Brand, M. and Campos-Ortega, J. A. (1988). Two groups of interrelated genes regulate early neurogenesis in *Drosophila melanogaster*. *Roux Arch. Dev. Biol.* **197**, 457-470.

Campos A. R., Grossman D. and White K. (1985). Mutant alleles at the locus *elav* in *Drosophila melanogaster* lead to nervous system defects: a developmental-genetic analysis. *J. Neurogenet.* **2**, 197-218.

Campos-Ortega, J. A. (1993). Early neurogenesis in *Drosophila melanogaster* In *The Development of* Drosophila melanogaster (eds Bate M. and Martinez Arias A.) Cold Spring Harbor, NY: Cold Spring Harbor Press. **In press**

Campos-Ortega, J. A. and Hartenstein V. (1985). *The Embryonic Development of Drosophila melanogaster* Berlin: Springer-Verlag.

Corbin V., Michelson, A. M., Abmayr, S. M., Neel, V., Alcamo, E., Maniatis, T. and Young, M. (1991). A role for the *Drosophila* neurogenic genes in mesoderm differentiation. *Cell* **67**, 311-323.

Cross D.P. and Sang J.H. (1978). Cell culture of individual *Drosophila* embryos. II. Culture of X-linked embryonic lethals. *J. Emb. exp. Morphol.* **45**, 173-187.

Dohrmann, C., Azpiazu, N. and Frasch, M. (1990). A new *Drosophila* homeo box gene is expressed in mesodermal precursor cells of distinct muscles during embryogenesis. *Genes Dev.* **4**, 2098-2111.

Drysdale R., Rushton E. and Bate M. (1993). Genes required for embryonic muscle development in *Drosophila melanogaster*: a survey of the X chromosome. *Roux's Arch. Dev. Biol.* **202**, 276-295.

Fehon R.G., Johansen K., Rebay I. and Artavanis-Tsakonas S. (1991). Complex cellular and subcellular regulation of Notch expression during embryonic and imaginal development of *Drosophila*: implications for *Notch* function. *J.Cell Biol.* **113**, 657-669.

Garcia-Bellido A. (1979). Genetic analysis of the *achaete scute* system of *Drosophila melanogaster*. *Genetics* **9**, 491-520.

Garcia-Bellido A. and Santamaria P. (1978). Developmental analysis of the *achaete scute* system of *Drosophila melanogaster*. *Genetics* **88**, 469-486

Hartenstein A.Y., Rugendorff A., Tepass U. and Hartenstein V. (1992). The function of the neurogenic genes during epithelial development in the *Drosophila* embryo. *Development* **116**, 1203-1219.

Haenlin, M., Kramatschek, B. and Campos-Ortega, J. A. (1990). The pattern of transcription of the neurogenic gene *Delta* of *Drosophila melanogaster*. *Development* **110**, 905-914.

Jimenez F. and Campos-Ortega J. A. (1979). A region of the *Drosophila* genome necessary for CNS development. *Nature* **282**, 310-312.

Jimenez F. and Campos-Ortega J. A. (1987). Genes in subdivision 1B of the *Drosophila melanogaster* X-chromosome and their influence on neural development. *J. Neurogenet.* **4**, 179-200.

Jimenez F. and Campos-Ortega J. A. (1990). Defective neuroblast commitment in mutants of the *achaete-scute* complex and adjacent genes of *D. melanogaster*. *Neuron* **5**, 8-89.

Johansen K. M., Fehon R. G. and Artavanis-Tsakonas S. (1989). The *Notch* gene product is a glycoprotein expressed on the cell surface of both epidermal and neuronal precursor cells during *Drosophila* development. *J. Cell Biol.* **109**, 2427-2440.

Jürgens G., Wieschaus E., Nüsslein-Volhard C. and Kluding M. (1984). Mutations affecting the pattern of larval cuticle in *Drosophila* II. Zygotic loci on the third chromosome.*Roux's Arch. Dev. Biol.* **193**, 283-295.

Kidd S., Kelley M. W. and Young M. W. (1986). Sequence of the *Notch* locus of *Drosophila*: relationship of the encoded protein to mammalian clotting and growth factors. *Mol. Cell. Biol.* **6**, 3094-3108.

Knust E.,Tietze K., and Campos-Ortega J. A. (1987). Molecular analysis of the neurogenic locus *Enhancer of split* of *Drosophila melanogaster*. *EMBO J.* **6**, 41-4123.

Kooh P. J., Fehon R.G. and Muskavitch M. A. T. (1993). Implications of dynamic patterns of *Delta* and *Notch* expression for cellular interactions during *Drosophila* development. *Development* **117**, 493-507.

Kopczynski C. C. and Muskavitch M. A. T. (1989). Complex spatiotemporal accumulation of alternative transcripts from the neurogenic gene *Delta* during *Drosophila* embryogenesis. *Development* **107**, 623-636.

Lehmann R., Jimenez F., Dietrich U. and Campos-Ortega J. A. (1983). On the phenotype and development of mutants of early neurogenesis in *Drosophila melanogaster*. *Roux's Arch. Dev. Biol.* **192**, 62-74.

Michelson A., Abmayr S., Bate M., Martinez Arias A. & Maniatis T. (1990). Expression of a *MyoD* family member prefigures muscle pattern in *Drosophila* embryos. *Genes Dev.* **4**, 2086-2097.

Montell D. J. and Goodman C. S. (1989). *Drosophila* laminin: sequence of B2 subunit and expression of all three subunits during embryogenesis. *J. Cell Biol.* **109**, 2441-2453.

Poulson D. F. (1945). Chromosomal control of embryogenesis in *Drosophila*. *Am. Naturalist* **79**, 340-363.

Preiss A., Hartley D. A., and Artavanis-Tsakonas S. (1988). The molecular genetics of Enhancer of split, a gene required for embryonic neural development in *Drosophila*. *EMBO J.* **7**, 3917-3927.

Rao, Y., Jan, L. Y. and Jan, Y. N. (1990). Similarity of the product of the *Drosophila* neurogenic gene *big brain* to transmembrane channel proteins. *Nature* **345**, 163-167.

Ruohola, H., Bremer, K. A., Baker, D., Swedlow, J. R., Jan, L. Y. and Jan, Y. N. (1991). Role of neurogenic genes in establishment of follicle cell fate and oocyte polarity during oogenesis in *Drosophila*. *Cell* **66**, 433-449.

Seidel F., Bock, E. and Krause, G. (1940). Die Organisation des Insekteneies. *Naturwiss.* **28**, 433-446.

Smoller D., Friedel C., Schmid A., Bettler D., Lam L. and Yedvobnick B. (1990). The *Drosophila* neurogenic gene *mastermind* encodes a nuclear protein unusually rich in amino acid homopolymers. *Genes Dev.* **4**, 1688-1700.

Truman J. W. and Bate M. (1988). Spatial and temporal patterns of neurogenesis in the central nervous system of *Drosophila melanogaster*. *Dev. Biol.* **125**, 145-157.

Volk T., Fessler, L. I. and Fessler J. H. (1990). A role for integrin in the formation of sarcomeric architecture. *Cell* **63**, 525-536.

Weintraub, H., Davis, R., Tapscott, S., Thayer, M., Krause, M., Benezra, R., Blackwell, T. K., Turner, D., Rupp, R., Hollenberg, S., Zhuang, Y. and Lassar, A. (1991). The *MyoD* gene family: nodal point during the specification of the muscle cell lineage. *Science* **251**, 761-766.

White K. (1980). Defective neural development in *Drosophila melanogaster* embryos deficient for the tip of the X-chromosome. *Dev. Biol.* **80**, 322-344.

Wieschaus E. and Nüsslein-Volhard, C. (1986). Looking at embryos. In *Drosophila a Practical Approach* (ed. D.B. Roberts). pp 199-227. Oxford, Washington D.C.: IRL Press.

Williams, J. A., Bell, J. B. and Carroll, S. B. (1991). Control of *Drosophila* wing and haltere development by the nuclear *vestigial* gene product. *Genes and Dev.* **5**, 2481-2495.